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Research article

Investigation of single nucleotide polymorphisms in *MRPA* and *AQP-1* genes of *Leishmania donovani* as resistance markers in visceral leishmaniasis in Kenya

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Abstract: Visceral Leishmaniasis (VL) remains a major public health problem mainly affecting the poorest populations across Asia, Africa, Middle East, Europe, Southern and Central America. For seven-decade now, the first-line drug of choice for leishmaniasis has been pentavalent antimonials. However, the clinical value of these drugs is threatened by the emergence of drug-resistant parasites. Clinical resistance to sodium stibogluconate (pentostam) has been a challenge in the Indian subcontinent, raising concerns for the endemic countries in Africa. This study aimed to identify and describe Single Nucleotide Polymorphism (SNPs) in gene markers associated with drug resistance among the clinical samples. The study was an experimental laboratory investigation on Dry Blood Spots (DBS). DNA was extracted from 18 VL positive samples, and Internal Transcribed Spacer-1 Polymerase Chain Reaction confirmed the positivity. Two target resistance markers, aquaglyceroporin 1 (*AQP-1*) and the Multi-Drug Resistant Protein A (*MRPA*), were PCR-amplified

and resulting amplicons sequenced using the Sanger sequencing platform. Multiple sequence alignments were performed using ClustalW, and the phylogenetic tree was constructed in MegaX using the Maximum Likelihood method**.** A total of 84 SNPs in the *AQP-1* gene were identified from six clinical samples. Fifty-nine of the SNPs (70.2%) were non-synonymous, while 25 (29.8%) were synonymous. Among the non-synonymous SNPs, three (5.1%) were nonsense, and 56 (94.9%) were missense point mutations. Two missense SNPs A188T and E185A in S17608 reported to be associated with drug resistance phenotype were observed. The study describes the resistance associated with the pentostam uptake by *Leishmania donovani*.

Keywords: antimony sodium stibogluconate; visceral leishmaniasis; single nucleotide polymorphism; drug resistance; aquaglyceroporin

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Abbreviations: *AQP-1*: Aquaglyceroporin 1; CL: Cutaneous Leishmaniasis; DBS: Dry Blood Spot; *LdAQP-1: Leishmania donovani* Aquaglyceroporin 1; MCL: Mucocutaneous Leishmaniasis; *MRPA*: Multi-Drug Resistant Protein A; PCR: Polymerase Chain Reaction; SSG: Sodium Stibogluconate; VL: Visceral Leishmaniasis

1. Introduction

Leishmaniasis is a parasitic disease caused by the protozoan parasites of the genus *Leishmania,* which is transmitted by the bite of a female phlebotomine sandfly. Over 90 species of known sandflies and over 20 *Leishmania* species are pathogenic to humans [1]. The common species include: *L. donovani* complex with two species (*L. donovani* and *L. infantum*); *L. tropica; L. major; L. aethiopica,* and the subgenus Viannia with four main species (*L. (V.) braziliensis; L. (V.) guyanensis; L. (V.) panamensis* and *L. (V.) peruviana). L. Mexicana* complex with three main species (*L. Mexicana, L. amazonensis, L. venezuelensis). L. donovani* is prevalent in East Africa, the Indian continent, and the Middle East, while *L. infantum* is found in Central and Southern America, North Africa, and Europe [2].

Leishmaniasis is endemic in 98 countries across Asia, Africa, the Middle East, Europe, South and Central America, with an estimated one billion people worldwide at risk of contracting the disease [3]. The disease is manifested in three forms: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis (VL). Cutaneous leishmaniasis is the most common form of the disease in the Middle East, Central Asia, America, and the Mediterranean basin [1]. It is characterised by skin lesions, which in complicated cases becomes chronic, debilitating, and disfiguring leading to social stigma with concomitant economic losses [3]. Mucocutaneous leishmaniasis is a less common form of the disease occurring in Bolivia, Brazil, Ethiopia, and Peru. It is considered as a subset of CL and causes lesions in the nose, mouth, or throat mucous membranes. Visceral leishmaniasis is the fetal form, and if untreated, it results in fatality rates of over 95% [1]. More than 95% of the reported cases in 2018 were from Brazil, Kenya, South Sudan, Sudan, China, India, Ethiopia, Nepal, Somalia and Iraq [1]. Visceral leishmaniasis affects the internal organs, including the spleen, liver, and bone marrow and is characterised by fever, weight loss, anaemia, and hepatosplenomegaly [4].

The World Health Organization, estimates VL global burden at 50,000 to 90,000 new infections annually, with one billion people being at risk of contracting the disease. Globally VL annual deaths are estimated to be between 20,000 and 30,000 [1]. In Africa, VL is endemic in the Northern and Eastern regions [2]. Africa reports about 4,000 deaths annually due to VL [5]. An estimated 50–60% of people treated for VL develop Post-Kala-azar Dermal leishmaniasis (PKDL) in East Africa [6].

Kenya is one of the African countries known to be endemic for both Cutaneous and Visceral Leishmaniasis [7], with approximately 2,500 cases of VL occurring annually and 6.8 million people at risk of contracting the disease [8]. The first VL case in Kenya was reported during the 1940s, and the first severe outbreak was reported in Kitui district in 1952 with 303 reported cases. The first outbreak of VL in Marsabit County was reported in 2014, with 136 confirmed cases [10]. Since the first outbreak, Marsabit has reported sporadic VL cases with reported cases in 2015 [11], 2016, and 2017 [9]. In 2019 and 2020, there was an outbreak of VL in Marsabit and Kitui, respectively (undocumented). Many leishmaniasis cases in Kenya go unreported or undiagnosed; hence, limited statistics to determine the actual number of cases in Kenya [7].

Pentavalent antimonials have been the drug of choice for more than six-decade now [12]. However, these drugs' clinical significance is under threat due to reports on drug-resistant parasite [13]. There is limited evidence of drug resistance in Africa; however, Sudan reports a 2.7% rate of clinical failure in patients treated with Sodium Stibogluconate (SSG), which correlated with *in-vitro* parasite resistance [14]. Induced resistance in *in-vitro* studies suggests the mechanism of resistance to be associated with the uptake, efflux, and sequestration of the drugs in the parasites [15–17]. Aquaglyceroporin 1 [18] and the ATP-binding cassette (ABC) transporter *MRPA* [17,19] are involved in drug uptake and sequestration, respectively. The other genes that are associated with parasite resistance are Promastigote Surface Antigen 2 (*PSA-2*) [20], ornithine decarboxylase [19], mitogen-activated protein kinase [21].

This study reports SNPs that are associated with pentostam drug resistance in the *AQP-1* gene of clinical samples in kenya

2. Materials and methods

The current study was approved by the Jomo Kenyatta University of Agriculture and Technology Institutional Ethics Review Committee (JKUAT-IERC) under Reference number JKU/IERC/02316/0027. The study was an experimental Laboratory investigation conducted on samples archived at −30 ℃ at Kenya Medical Research Institute (KEMRI), Nairobi. The study samples were collected from *Leishmania* endemic areas in Kenya, and the laboratory investigations were performed at KEMRI Laboratory, Nairobi.

2.1. DBS processing

DNA lysates were prepared using the MightyPrep reagent (TAKARA BIO INC, Kusatsu, Shiga Prefecture, Japan; Cat No: 9182) following the manufacturer's protocol with slight modifications. Briefly, one blood spot was cut and placed in 1.5 Eppendorf tubes, 100 μL of MightyPrep reagent was added to the tubes and incubated for 15 minutes on a 95 ℃ block while shaking 800 rpm.

2.2. Amplification of Leishmania positive samples

PCR was performed on the DBS lysates to amplify the Internal Transcribed Spacer (ITS) fragment to confirm leishmaniasis infection. The region between the Small Subunit (SSU) RNA and 5.8S RNA were amplified using the LITSR (ITS1-F) and L5.8S (ITS1-R) primers (Table 1). A total of 20 µL per PCR reaction was prepared; 2 µL of DBS lysate was added to 18 µL Thermo Scientific™ DreamTaq™ Hot Start Green PCR Master Mix (2x). The PCR assay was performed under the following conditions: Initial denaturation at 95 ℃ for 3 minutes, followed by 40 cycles of 95 ℃ for 30 seconds, 52 ℃ for 30 seconds and 72 ℃ for 60 seconds and a final extension at 72 ℃ for 15 minutes in the SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). Water as the negative control and the positive control (*L. donovani* obtained from Sudan with DNA concentration of 29.1 ng/µL) were used in the run. 5 µL aliquots of the PCR products were loaded on 2% agarose for electrophoresis analysis. The bands were visualised after staining with 2x GelRed™ for 1 hour on a shaker.

2.3. PCR amplification of AQP-1 and MRPA genes

Visceral leishmaniasis positive samples were subjected to a second PCR to amplify *MRPA* and *AQP-1* genes associated with antimony resistance using specific primers (Table 1). A total of 20 µL per PCR reaction was set up as follows; 2 µL of DBS lysate was added to 18 µL Thermo Scientific™ DreamTaq™ Hot Start Green PCR Master Mix (2x). PCR reaction was performed in separate reactions at different conditions. The PCR program was set at initial denaturation at 95 ℃ for 3 minutes same for the two genes, followed by 40 cycles (*MRPA*) and 35 cycles (*AQP-1*) of 95 ℃ for 30 seconds, annealing at 56 ℃ (*MRPA*), 59 ℃ (*AQP-1*) for 30 seconds and 72 ℃ for 60 seconds and final extension at 72 ℃ for 15 minutes same for the two genes in the SimpliAmp™ Thermal Cycler (Thermo Fisher Scientific). The PCR products were then separated by electrophoresis on 2.0% agarose gel for 40 minutes at 100 V. The gel was stained with 2x GelRed for 1 hour and visualised using ultraviolet light.

Table 1. Primers for amplification of the ITS-1 region and the *AQP-1* and *MRPA* genes.

Gene	Protein	Forward and Reverse Primers [22,23]	Melting temp Tm	Amplicon size
ITS-1	Internal Transcribed Spacer 1	LITSR 5’CTGGATCATTTCCGATG3’ L5.8S 5’TGATACCACTTATCGCACTT3’	50 ℃ 54 ℃	311 bp
AQP-1	Aquaglyceroporin 1	5’CTGTGTCTTTGGTGCCTTTCC3’ 5’GCCTTTTGGGCGTCGTC3’	61 ℃ 57 ℃	129 bp
MRPA	Multidrug resistance protein A	5’CGAAAGTTGAGCAGGAGACG3’ 5’AATCCCCAAGCAGCCAGAC3’	60 ℃ 59 ℃	166 bp

2.4. Gel extraction and DNA purification

All the visible DNA bands were excised using a scalpel on an Ultra Slim Blue Light Transilluminator (Maestrogen). Then, the excised DNA bands were placed in sterile 1.5 ml

centrifuge tubes. Next, DNA was extracted and purified using the QIAquick® gel extraction kit from Qiagen under the manufacturers' instructions for subsequent DNA sequence analysis.

2.5. Sequencing and analysis of the AQP-1 and MRPA genes

Sanger sequencing was performed on the amplified target genes using forward and reverse primers (Macrogen, Amsterdam, The Netherlands). The paired-end reads for each gene were obtained from the chromatograms and assembled into respective consensus sequences using Bioedit software. Assemblies were queried in the National Center for Biotechnology Information (NCBI) nucleotide database using BLAST [24] basing on the blastn option with coverage and identity threshold of 95% to get the corresponding reference genes. Multiple sequence alignment was done using Clustalw [25], and SNPs obtained. For the *AQP-1* gene, the phylogenetic tree was constructed in MegaX [26] using the Maximum Likelihood method. The tree was drawn to scale with branch lengths measured in the number of substitutions per site before modifying it in Interactive Tree of Life (ITOL) [27].

3. Results

3.1. Amplification of VL positive samples

A total of Eighteen samples were PCR-amplified. As a result, bands of 311 bp of the ITS were obtained (Figure 1).

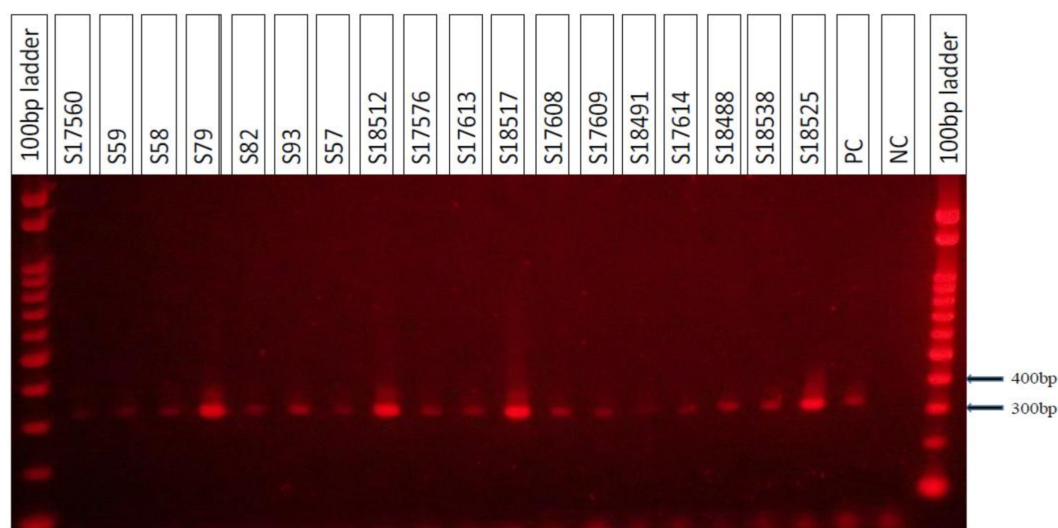

Figure 1. Gel image for PCR-amplified VL positive samples.

3.2. Amplification of AQP-1 and MRPA genes

Multi-Drug Resistance Protein A gene was amplified (166 bp) in all 18 samples, while *AQP-1* (129 bp) was only amplified in 12 samples (Table 2).

Table 2. PCR amplification in *MRPA* and *AQP-1* genes.

Sample ID	*MRPA*	*AQP-1*
057	+	+**S**
058	+	+
059	+	**NA**
079	+	+**S**
082	+	**NA**
093	+	+
17560	+	**NA**
17576	+	+**S**
17608	+	+**S**
17609	+	**NA**
17613	+	**NA**
17614	+	+
18488	+	+**S**
18491	+	+**S**
18512	+	**NA**
18517	+	+
18525	+	+
18538	+**S**	+

Notes: + indicates amplified; +**S** indicates amplified and SNPs present; NA indicates no amplification.

3.3. Single Nucleotide Polymorphism analysis in AQP-1 and MRPA genes

The NCBI nucleotide database query produced several likely hits, and two reference genes were obtained with accession numbers XM_003860882 and XM_003863045 for *MRPA* and *AQP-1* genes, respectively. In comparison with the reference genes, a total of 84 SNPs were identified in *AQP-1* gene translating to 130 point mutations in six samples. Fifty-nine (70.2%) of the SNPs were non-synonymous, while 25 (29.8%) were synonymous. Among the non-synonymous SNPs, 3 (5.1%) were nonsense, and 56 (94.9%) were missense point mutations. In the *AQP-11* gene, 59 (70.2%) of the SNPs were non-synonymous, while 25 (29.8%) were synonymous. Among the non-synonymous SNPs, 3 (5.1%) were nonsense, and 56 (94.9%) were missense. The missense SNPs were more prevalent in samples S57, S79, S17576, S17608 and were distributed among all six samples (Figure 2).

Figure 2. Heat map showing missense SNPs in *AQP-1* gene in all samples based on LDBPK310030 as the reference.

The *AQP-1* gene had two similar SNPs in the same positions, N137 and L181, in sample S18491 and sample S18488, however, sample S18488 had more than just the two SNPs at position L169, G168, and Y167. Many missense SNPs, including P183, E185, and A188, were only present in the *AQP-1* gene of sample S17608. No single missense SNP was found in all *AQP-1* genes of all samples (Supplementary Table S1).

Nonsense SNPs were observed at three positions in the *AQP-1* gene (Table 3). There was no nonsense SNP in S18491, one SNP in S17576 and S17608, and two SNPs in S57, S79 and S18488 in the *AQP-1* genes. No single nonsense SNP was found in the *AQP-1* genes of the six samples.

Table 3. Nonsense SNPs in the *AQP-11* gene in all samples.

	Samples						
SNPs	S57	S79	S17576	S17608	S18488	S18491	Amino acid change
T542A	+	+	+	-	+	-	L 181 Stop codon
C501A	-	+	-	-	+	-	Y 167 Stop codon
C501G	+	-	-	+	-	-	Y 167 Stop codon

Notes: +/- Indicates the presence/absence of a nonsense SNP in *AQP-1*; A: Adenine; T: Thymine; C: Cytosine; G: Guanine; L: Leucine; Y: Tyrosine.

The synonymous SNPs in the *AQP-1* gene were detected in all six samples (Figure 3). The *AQP-1* gene from the S17576 sample had the highest number of synonymous SNPs, while the *AQP-1* from S18488 had none (Supplementary Table S2). Comparing the *AQP-1* gene from six samples with the reference gene (accession number: XM_003863045) also showed insertions and deletions (Indels).

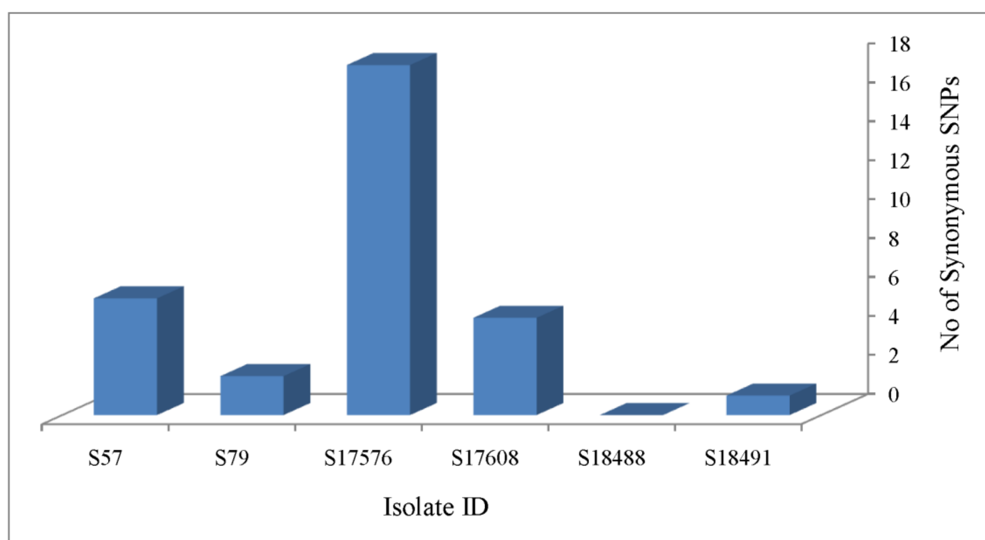

Figure 3. Distribution of the synonymous SNPs in *AQP-1* gene in six samples.

Only three non-synonymous SNPs were observed in the *MRPA* gene of S18491 sample, and they included P921, F975, and L972 (Supplementary Table S3).

3.4. Evolutionary relationship of the AQP-1 genes among samples

A total of 973 positions from *AQP-1* multiple sequence alignment were generated in the final data set involving seven nucleotide sequences samples (S57, S79, S17576, S17608, S18488, and S18491), Using LDBPK310030 as the reference gene, accession number XM_003863045. In addition, a maximum-likelihood phylogenetic tree was constructed based on SNPs in the *AQP-1* genes of all samples for evolutionary relationship (Figure 4).

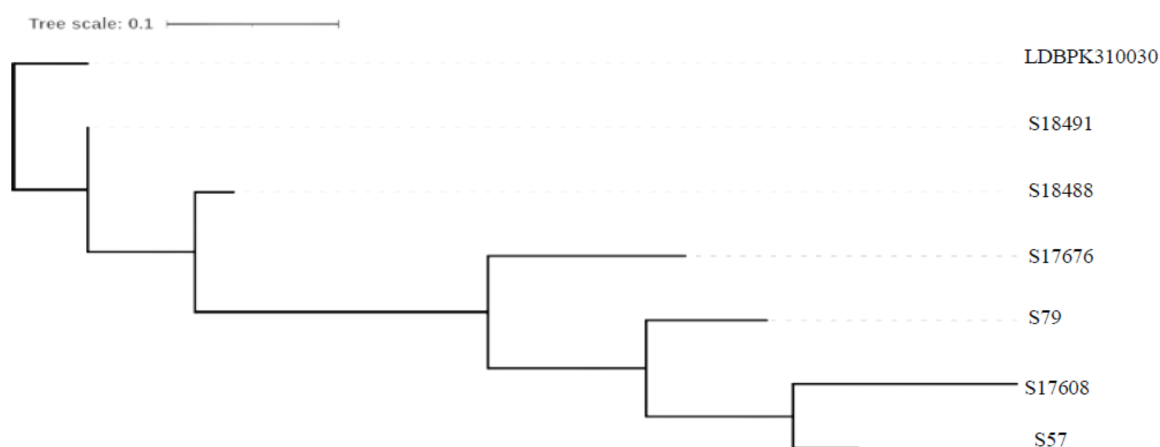

Figure 4. Maximum likelihood phylogenetic tree based on all SNPs in the *AQP-1* genes of the six samples.

The pattern of branching in the phylogenetic tree reflected how the *AQP-1* gene evolved in the samples. The tree showed that the *AQP-1* genes from samples S57, S79, S17576, and S17608 are

closely related, forming a clade. *AQP-1* genes from S18488 and S18491 samples also have a close relationship with the reference gene, reflecting fewer missense SNPs than other samples (Figure 2).

4. Discussion

Treatment failure and SSG drug resistance in VL remains a public health concern in endemic countries. This study identifies and describes two SNPs A188T and E185A, in the *AQP-1* gene of clinical samples associated with drug resistance. Studies have shown that any point mutation in genes can confer drug resistance, therefore, strengthening the pathogen's tolerance level to therapeutics [28]. The greater the frequency and the higher the number of mutational changes, the greater the degree of resistance [28]. In the current study, an increased number of SNPs was observed in the *AQP-1* gene, as shown in the results. The increased number could be attributed to doctors administering SSG only and not adhering to the recommendations of SSG/PM [11], posing a high risk of developing drug resistance. The distance to the treatment centres in these endemic regions is far; this leads to non-compliance by patients and increases the speed at which the parasite becomes resistant, affecting the drug's effectiveness [11]. *Leishmania* drugs are expensive, rarely available, and mainly obtained as donations [11]. This unavailability of drugs can lead to sub-therapeutic drug concentration exposure where the sensitive lot succumbs and leads to the drug resistance spread [28].

Most of the observed SNPs in the current study caused amino acid change (non-synonymous) in the *AQP-1* gene. This gene belongs to the aquaporin (*AQP-1*) protein family and is involved in the transport of water, glycerol, and other small uncharged solutes [29]. After drug administration, *AQP-1* is responsible for the transportation of antimonite Sb III, an activated form of Pentostam or Glucantime into the *Leishmania* parasite [16]. Point mutations in the *AQP-1* gene could give rise to clinical drug resistance [16]. We observed two missense SNPs in *AQP-1* gene out of the 84 SNPs detected, to be associated with drug resistance phenotype [30,31]. At the position, A188 Alanine was replaced by Threonine (A188T). This change has been reported not to affect the 3D structure of the protein but shown to be susceptible to post-translational modification, which may affect the protein's function, thus altering the uptake of antimonials [31]. A study conducted in *Leishmania major AQP-11* showed a missense SNP at E152 (corresponding to E185 in the full-length sequence), which conferred Sb III resistance to laboratory mutants of the parasite [30]. This mutation corresponds to the observed SNP in our study at E185 (E185A) full-length coding sequence (945 bp) of *L. donovani.* The results, therefore, suggest resistance to Pentostam in the studied samples. The SNPs detected, which have not been reported previously, might also introduce new strains with different phenotypes.

There was limited information on the *MRPA* gene mutations associated with the drug-resistant phenotype at the time of the current study. We observed SNPs in one sample; however, no resistance has been previously reported due to this mutation. It has been noted that any point mutation in genes potentially involved in conferring resistance to drugs strengthens the pathogen's tolerance level to therapeutics [28]; therefore, *in-vitro* drug susceptibility studies are required to confirm the role of these mutations.

5. Conclusions/Recommendations

This study has generated information about SNPs that may facilitate the drug resistance phenotype among infecting *Leishmania* parasites in Kenya. The study is the first to report multiple SNPs in the *LdAQP-1* gene in clinical samples in Africa. It is also the first study in Kenya reporting SNPs that might be associated with the pentostam drug resistance phenotype. We recommend that further studies be performed to link the observed SNPs in this study that have not been previously studied to clinical drug resistance.

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Conflict of interest

The authors declare there is no conflict of interest.

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