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Characterisation of major vault protein during the life cycle of the human parasite *Schistosoma mansoni*

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ABSTRACT

Vaults are ribonucleoproteins (13 MDa) highly conserved among lower and higher eukaryotes. Their association produces a complex composed of three proteins named Major Vault Protein (MVP), vault (PolyADP-ribose) polymerase (VPARP) and Telomerase-associated protein (TEP1), plus a small untranslated RNA. The exact function of this complex is unknown, although the biological role of vaults has been associated with multidrug resistance phenotypes and signal transduction pathways. Genomic analysis showed that model organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, do not possess genes encoding vaults. However, we have found that vault-related genes are present in the *Schistosoma mansoni* genome. These observations raised questions on the involvement of vaults in mechanisms of adaptation of the parasite in its mammalian host. Therefore, molecular characterisation of the putative Major Vault Protein performed using bioinformatics tools showed that this vault component is highly conserved in *S. mansoni*. The MVP expression level was quantified by qRT-PCR using total RNA from susceptible (LE) and resistant (LE-PZQ) adult worm lineages, cercariae and mechanically transformed schistosomula (MTS) cultured for 3.5, 24, 48 and 72 h *in vitro*. Our results suggest a stage-specific expression in all developmental stages analysed. Western blotting has shown up-regulation of *Sm*MVP in the MTS-3.5, 72 h and resistant adult worms, and similar levels in all other stages. Furthermore, *Sm*MVP was found differentially expressed in adult males and females from the susceptible lineage. Further studies should clarify whether *Sm*MVP is somehow linked to drug resistance in *S. mansoni*.

1. Introduction

Human schistosomiasis, a major parasitic disease that affects more than 200 million individuals in 76 tropical and subtropical countries, is the second most prevalent tropical disease worldwide, causing high morbidity and mortality. Currently, praziquantel (PZQ) is the drug of choice for the treatment of schistosomiasis due to its low toxicity and low cost [1,2]. Despite the advantages of PZQ, *Schistosoma mansoni* resistance to PZQ has been found in isolates from endemic areas and can also be induced in the laboratory [3].

The success of *S. mansoni* parasitism has a direct relationship with the parasite's adaptation to different environments and hosts during its life cycle. Therefore, genes involved with cellular differentiation are of particular importance to understanding parasite's biology. In this context, during a bioinformatic analysis using the transcriptome and genome databases, we have identified putative entries coding for a 100 kDa major vault protein (MVP), the predominant component of vaults which represents more than 70% of the vault complex mass, as well as the vault poly (ADP-Ribose) polymerase (VPARP - 193 kDa), a telomerase-associated protein 1 (TEP1 - 240 kDa) and a small untranslated RNA (vRNA, approximately 140 b).

Vault particles are identified as 13-MDa ribonucleoproteins, measuring approximately 70 nm × 40 nm × 40 nm [4]. They are abundant and extremely conserved across a large number of species [5]. Although the exact function of vault remains unknown, several studies have shown the potential involvement of this complex in multidrug resistance. MVP is identical to the human lung resistance protein and its expression is increased in tumours as well as in various multiple drug resistance models [6,7]. Vault is also involved in the regulation of PTEN (phosphatase and tensin homolog) and EGFR (epidermal growth factor)-induced MAPK pathway, the activation of COP1 (Constitutive Photomorphogenic 1) for the degradation of c-Jun, import and/or activation of nuclear estrogen receptors, in the activation of the expression of INF-γ and several other events of the immune

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response, especially in innate immunity [7,8]. Despite a huge amount of data from diverse species and systems, defining the precise functions of vaults is still highly complex and challenging.

This report shows the conservation as well the mRNA and protein levels of *Sm*MVP. We observed up-regulation of this protein during cercariae to early-schistosomula transition and in PZQ-resistant adult worms. In addition, increased expression of MVP in the adult male relative to the female was observed for the LE strain. Moreover, bioinformatics analysis provided support for the presence of other protein components of vaults in the *S. mansoni* genome. The presence of the vault complex in *S. mansoni* suggests a possible role during infection of the mammalian host.

2. Materials and methods

2.1. Ethics statement

All experiments involving animals were authorised by the Ethical Committee for Animal Care of Federal University of Ouro Preto (CEUA-UFOP protocol no. 2011/55). These were in accordance with national and international regulations accepted for laboratory animal use and care.

2.2. In silico analysis

The MVP was identified by mining the *S. mansoni* sequences at GeneDB (http://www.genedb.org/genedb/smansoni/) using Blastp and as queries known *Homo sapiens* and *Mus musculus* proteins. Reference proteins from other species were searched in NCBI database to obtain a full set of putative homologue proteins in order to compare with the *S. mansoni* proteins. The BLASTp algorithm, underpinned by Pfam (v26.0), allowed for the detection of conserved protein domains or motifs from *S. mansoni* sequences. The whole protein sequences were used to perform the alignments. Multiple sequence alignments were performed using the program ClustalX 2.0 with default settings [9]. Phylogenetic tree was inferred using the Neighbor-Joining method (NJ) and JTT model [10]. The bootstrap consensus tree inferred from 1000 replicates was used to represent the evolutionary history of the taxa analysed. Molecular phylogenetic analyses were conducted using MEGA 5 software [11,12].

2.3. Parasites

The resistance in this isolate (LE-PZQ) was induced *in vivo* when infected *Biomphalaria glabrata* snails were submitted to three treatments with PZQ, each treatment administered on 5 consecutive days, with 1 week interval, for selection of less susceptible parasites to PZQ. It was passaged through mice and treated 45 days after infection with 400 mg/kg PZQ [13]. The LE (susceptible strain to PZQ) and LE-PZQ (resistant isolate to PZQ) adult worms were obtained from by liver perfusion of mice after 50 days of infection, washed in RPMI 1640 (Sigma Chemical Co.), and were quick-frozen in liquid nitrogen and stored at −80 °C until use.

The *S. mansoni* parasite (LE strain) [14] was maintained by routine passage through *B. glabrata* snails and BALB/c mice. The infected snails were induced to shed cercariae under light exposure for 2 h and the cercariae were recovered by sedimentation on ice. The mechanically transformed schistosomula (MTS) were prepared as previously described [15]. Briefly, cercariae were recovered and washed in RPMI 1640 medium (Invitrogen, Sao Paulo, Brazil) before vortexing at maximum speed for 90 s and immediately cultured for 3.5 h at 37 °C in a 5% CO_2 incubator. Then, the recovered schistosomula were washed with RPMI 1640 until no tails were detected. For subsequent incubations, the parasites were maintained in M169 medium supplemented with 10% FBS (Fetal Bovine Serum), penicillin (100 μg/mL), streptomycin (100 μg/mL) and 5% of Schneider's medium [16] at 37 °C in a 5% CO_2 incubator for 3.5, 24, 48 and 72 h.

2.4. Expression analysis of MVP by qRT-PCR

Total RNA from cercariae, schistosomula, adult worms and eggs was obtained using a combination of Trizol reagent (Sigma, Belo Horizonte, Brazil) and chloroform for extraction and column-purified using the "SV total RNA Isolation System" (Promega, Belo Horizonte, Brazil). The preparation was treated with RNase-free DNase I in 3 different rounds with decreasing enzyme concentrations (RQ1 DNase; Promega). RNA was quantified using a spectrophotometer and an aliquot containing 1 μg of total RNA was reverse transcribed using an oligodT primer from the Thermoscript RT-PCR System (Invitrogen), as described by the manufacturer. The efficiency of DNAse I treatment was evaluated by PCR amplification of the cDNA reaction mix without the addition of the Thermoscript enzyme. *S. mansoni* specific primers were designed using the program GeneRunner®. The primers for MVP were forward 5′-GAATGGGTGACGAGGAGTAC-3′ and reverse 5′-AGTCTGAGTGCCGA GTTTGG-3′ (GenBank no. 8346893). Reverse-transcribed cDNA samples were used as templates for PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen) and 7300 Real Time PCR System (Applied Biosystems; Rio de Janeiro, Brazil). Specific primers for *S. mansoni* EIF4E were used as an endogenous control (GeneDB ID: Smp_001500) (forward 5′TGTTCCAACCACGGTCTCG3′, reverse 5′TCGCCTTCCAATGCT TAGG3′) [17]. The efficiency of each pair of primers was evaluated according to the protocol developed by Applied Biosystems (cDNA dilutions were 1:10, 1:100 and 1:1000) [18,19]. For all investigated transcripts, three biological replicates were performed and their gene expression was normalised against the EIF4E transcript according to the $2^{-\Delta Ct}$ method using Applied Biosystems 7300 software [20].

2.5. Detection of SmMVP by Western blotting

Determination of MVP expression was performed by Western blot using the monoclonal anti-human MVP MAB4141 antibody (Millipore, Sao Paulo, Brazil). Briefly, total protein extracts from cercariae, MTS at 3.5, 24, 48 and 72 h, and susceptible and PZQ-resistant adult worms were prepared by sonication in 25 mMTris–HCl pH 7.5, 1 mMDTT, 1 mM EDTA, and 10 μM of the following protease inhibitors: TLCK, TPCK, NEM, and PMSF. After centrifugation at 10,000 ×*g* for 15 min, the soluble protein concentration was determined by QuantiPro™ BCA Assay Kit (Sigma Aldrich, Sao Paulo, Brazil). Twenty micrograms of total soluble protein was separated by 10% SDS–PAGE [21,22]. The gel was transferred to a PVDF membrane at 25 V for 2 h at 4 °C. After 16 h incubation in blocking solution, the membrane was washed and incubated with primary anti-human MVP antibody at a 1:500 dilution. Alkaline phosphatase-conjugated anti-mouse IgG (Sigma) was used as the secondary antibody at 1:2500 and the reactivity visualised by NBT/BCIP Western blot detection reagents (Amresco, Sao Paulo, Brazil) as per the manufacturer's instructions. Differences in protein loading among the extracts present in the membrane were registered through densitometric analysis of the total protein content in each lane [23]. Relative expression levels of *Sm*MVP were obtained by densitometric analysis of the reactive bands, taking into account and correcting for the observed differences in total protein loading, using the Quantity one® (Bio-Rad, Sao Paulo, Brazil) software.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 (Irvine, CA, USA). Normality of the data was established using one-way analysis of variance (ANOVA). Tukey post-tests were used to investigate significant differential expression of transcripts throughout the investigated stages [24]. In all cases, the differences were considered significant when $p < 0.05$.

3. Results

3.1. In silico analysis

The *Smmvp* gene consists of 2613 bp extending from the first ATG codon at nucleotide 1846 to the TGA termination codon at nucleotide 11,600. The predicted protein, called *Sm*MVP, has 870 amino acids and a calculated theoretical molecular mass of 97.26 kDa. In the *S. mansoni* genome, the *Smmvp* gene is located in supercontig Smp_scaff000010 and contains eight exons and seven introns. A summary of the various sizes of the exons and introns, and the splicing sequences at the junctions of the intron–exon boundaries conform to the consensus splice signals, which occurs when a dinucleotide AG donor is at the 5′-end and a GT receptor is at the 3′ end, can be seen in Fig. 1.

An analysis of the MVP protein by the Pfam database indicates that MVP contains six conserved domains containing an average of 54 amino acids each, which are positioned in tandem with the first five domains being within the amino terminus (amino acids 28–89, 92–142, 143–195, 196–248 and 249–306) and the sixth domain located in the carboxy terminus of the peptide (amino acids 368–416) (Fig. 1). When the six Vault domains of *Sm*MVP are aligned, we noted several deletions and insertions, particularly when comparing the second and fifth domains. A protein database search for sequence similarity to the MVP protein demonstrated that it presents different degrees of similarity to a large number of previously identified vault proteins. The amino acid sequence alignment of the amino terminal portion (MVP repeat or Vault repeat - Family Vault) of the protein indicates that the MVP domains share high homology with MVP domains from other organisms (Fig. 2). The comparative analysis of amino acid sequence outside the Vault domain in the *Sm*MVP compared to that of other organisms revealed approximately 74% of similarity. Furthermore, a phylogenetic analysis revealed that *Sm*MVP is conserved among its orthologs proteins during evolution (Fig. 3). The MVP protein of the vault complex is most closely related to MVP of *Schistosoma japonicum* (AAX26375.2).

3.2. MVP mRNA expression and protein levels increase during cercariae to schistosomula transition

The gene expression profile of *Smmvp* was determined using qRT-PCR and mechanically transformed schistosomula (MTS) cultured for 3.5, 24, 48 and 72 h *in vitro* (Fig. 4A). The transcript levels of the *Sm*MVP gene were higher in the MTS-72 h, compared to the stages of other MTS and cercariae. To investigate whether the increase in MVP mRNA levels corresponds to an increase in protein expression, we performed western blot analysis. A single band of approximately 103 kDa was recognised by the anti-human antibody MAB4141 anti-MVP (Millipore) in each of the various developmental stages of *S. mansoni* analysed (Fig. 4B, C and D). We also observed an up-regulation of *Sm*MVP in MTS-3.5 and 72 h and similar levels in other stages.

3.3. Expression analysis of MVP in susceptible and PZQ-resistant adult worms

The mRNA expression of the endogenous *Smmvp* gene was also investigated in adult female, male and paired susceptible (LE strain) and resistant (LE-PZQ isolate) worms by quantitative RT-PCR (Fig. 5A). The gene *Smmvp* was found to be more expressed in males relative to female worms in both susceptible and resistant lineages. Overall, *Smmvp* transcript levels are up-regulated in PZQ-resistant adult worms compared to its expression in susceptible worms. The *Sm*MVP protein levels were also evaluated in PZQ-resistant and susceptible adult worms by western blotting. In agreement with transcript abundance we observed higher protein levels of *Sm*MVP in males compared to females from susceptible adult worms. In addition, higher levels of *Sm*MVP from either paired or unpaired parasites were observed for PZQ-resistant worms (Fig. 5B and C).

4. Discussion

Vaults are ribonucleoprotein complexes highly conserved in eukaryotes, including several deuterostomes. Surprisingly, model organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana* and *Saccharomyces cerevisiae* do not possess the genes encoding for the components of this complex [8]. Recently, vault complexes have received considerable attention, especially for their size, which is about three times that of a ribosome, with a 13 MDa molecular weight but with a simpler molecular composition. Despite several reports on the expression of Vault proteins, the function of these complexes is still poorly understood. Due to their subcellular location in the cytoplasm and its association with the nuclear membrane and nuclear pore complex, it is believed that one of their functions is involved in the transport of protein and/or RNA between the cytoplasm and the nucleus [7].

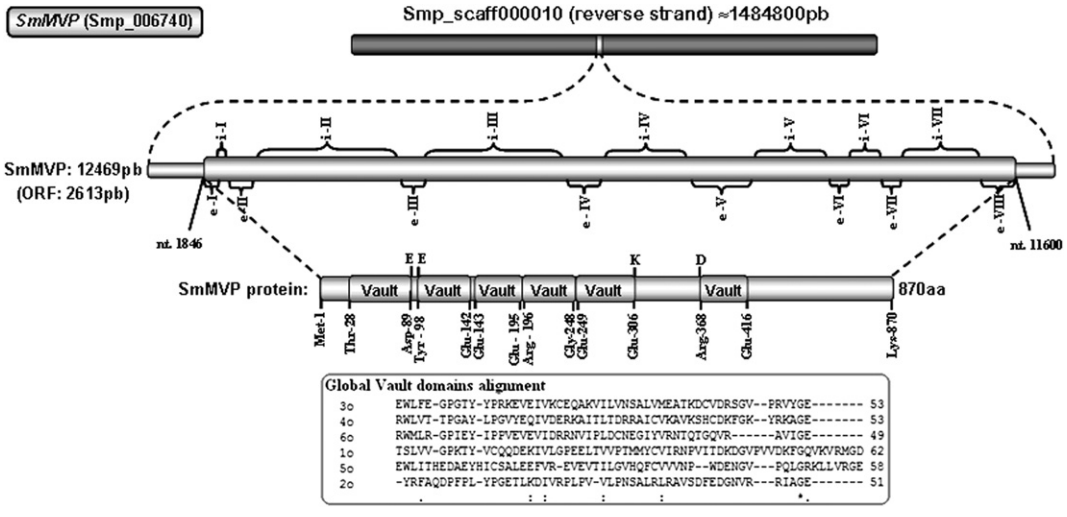

Fig. 1. Schematic representation of genomic and proteomic topologies in *Sm*MVP. Exons, introns and the first and last amino acids of each conserved domain are shown, with emphasis on the global alignment of the six domains conserved in the vault structure.

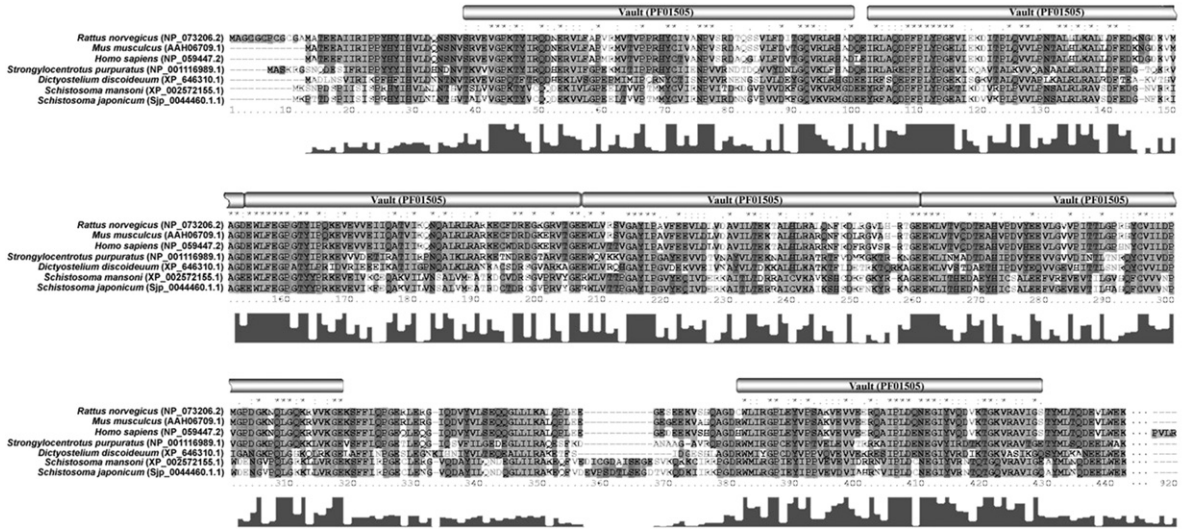

Fig. 2. CLUSTALX 2.0 multiple alignment of predicted amino acid sequences of *Sm*MVP and orthologs, showing the putative conserved domain: Vault (PF01505) obtained by using the Pfam algorithm.

Initially, we used the *S. mansoni* genome database to identify, by homology, ortholog sequences of MVP in rats, mice and humans. The predicted MVP in *S. mansoni* is a well conserved protein with characteristics equivalent to the MVP found in other organisms, such as conserved domains and similar molecular mass [25]. The structure of *Smmvp* gene appears to be quite complex. The eight predicted exons in *Sm*MVP are flanked by introns of varying sizes formed by a number of unusual bases. These data are corroborated by Berrimann et al. [25], who identified large introns with average size of 1692 pb in the 11,809 predicted genes of the *S. mansoni*. By analysing the exon/intron boundaries, we found that the processing of the transcriptional product follows the classical rule of splicing [26]. The 97.26 kDa calculated

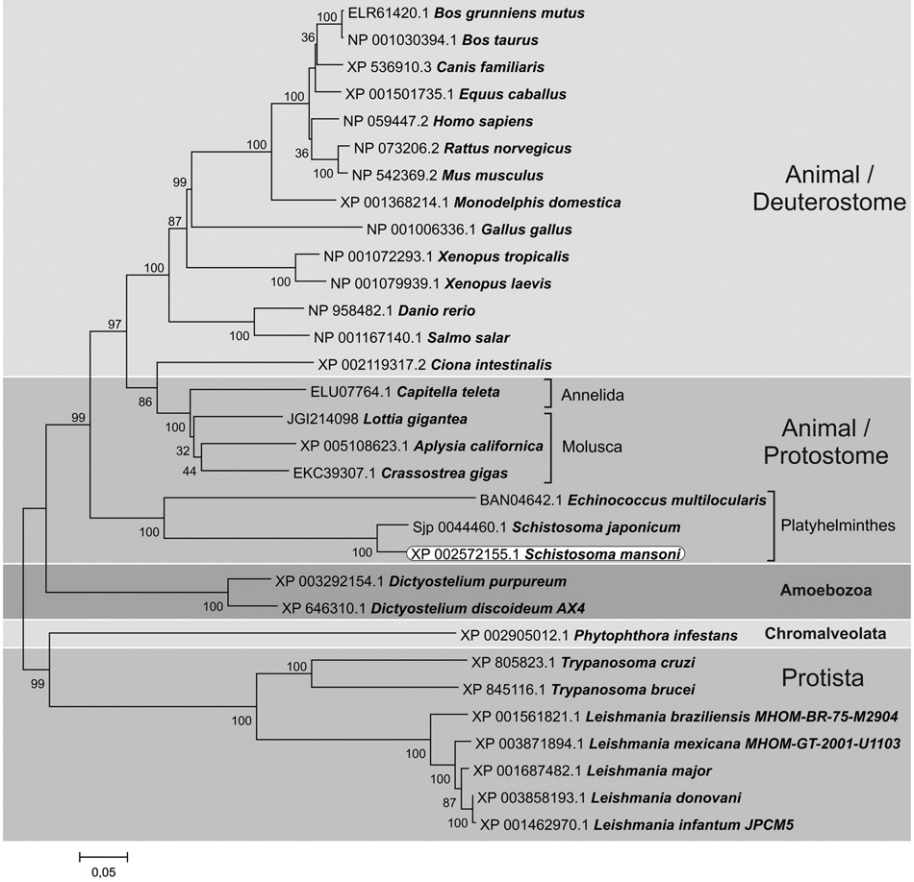

Fig. 3. Consensus phylogenetic tree based on amino acid sequences of vault complex. The tree construction and analysis of bootstrap were performed using ClustalX 2.0 and MEGA 5.0. For the consensus tree and reliability of the branches, phylogenetic bootstrapping with 1000 replicates for each sequence was used, and 50% was the minimum for considering the branch reliable.

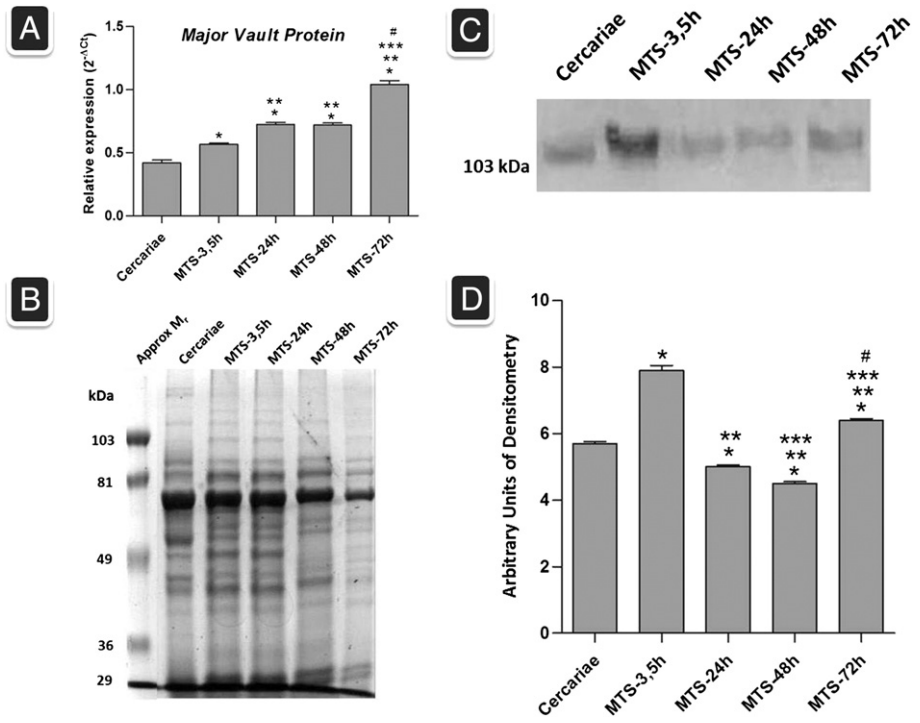

Fig. 4. Differential expressed of MVP throughout *S. mansoni* life cycle. A) The *Smmvp* mRNA expression levels were measured based on three replicates for each of the following stages: cercariae, MTS-3.5, 24, 48 and 72 h days using quantitative RT-PCR. Expression levels were calibrated according to the comparative $2^{-\Delta Ct}$ method, using the constitutively expressed *Sm*EIF4E as an endogenous control (one-way variance analysis followed by Tukey pairwise comparison $p < 0.05$). * Different from cercariae, ** different from MTS-3.5 h, *** different from MTS-24 h and # different from MTS-48 h. B) Twenty micrograms of crude extracts from cercariae, MTS-3.5, 24, 48 and 72 h was analysed by SDS/PAGE and stained with 10% Coomasie Blue. C) Detection of *Sm*MVP by Western blotting throughout *S. mansoni* life cycle. These extracts were divided into 10% SDS/PAGE and transferred to PVDF membrane followed by immunoblotting with human anti-human MVP at a dilution of 1:500. Subsequently, the membrane was incubated with anti-mouse IgG alkaline phosphatase-conjugated at a dilution of 1:2500 and revealed by NBT/BCIP solution. D) Densitometric analysis was performed using Gene Quantity software (Biorad). * Different from cercariae, ** different from MTS-3.5 h, *** different from MTS-24 h and # different from MTS-48 h.

molecular mass for *Sm*MVP is within the expected mass range of MVPs from several other organisms. According to the data reviewed above [27], the MVP of *Dictyostelium* (α and β), rat, and human were, respectively, 94 and 92 kDa [28], 99 kDa [29] and 100 kDa [30]. The arrangement of the six in tandem Vault domains of *Sm*MVP seems to be common to all investigated organisms, including human [31] and rat [32].

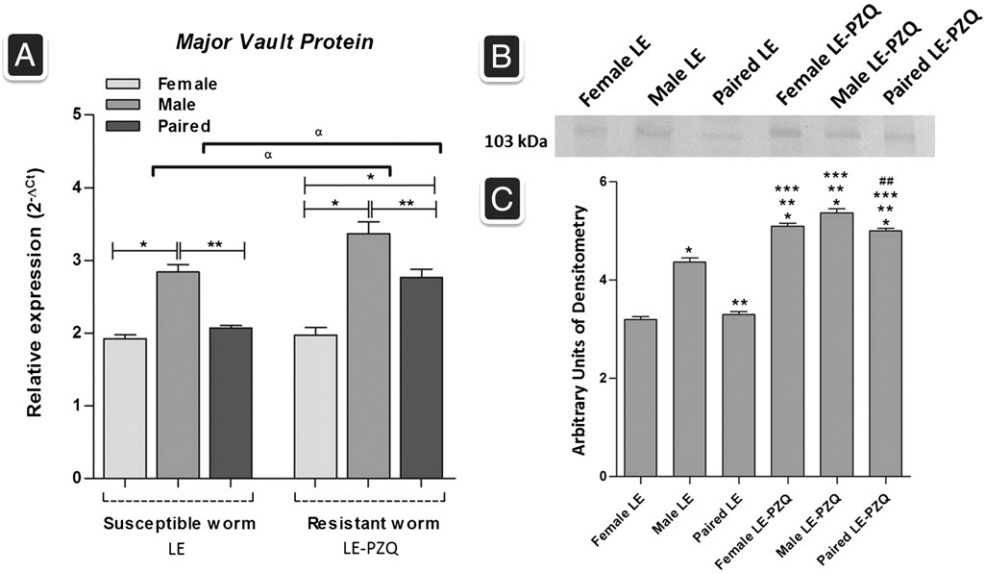

Fig. 5. MVP expression in susceptible adult worm (LE) and resistant adult worm (LP). A) The *Smmvp* mRNA expression levels were measured based on three replicates, for each of the following stages: susceptible (female, male and paired) and resistant (female, male and paired) adult worms using quantitative RT-PCR. Expression levels were calibrated according to the comparative $2^{-\Delta Ct}$ method, using the constitutively expressed *Sm*EIF4E as an endogenous control (*t* tests comparison $p < 0.05$). *different from female, **different from male and $^{\alpha}$different from susceptible adult worm. B) Detection of *Sm*MVP by Western blotting. Twenty micrograms of crude extracts from susceptible adult worm (LE) and resistance adult worm (LE-PZQ) was analysed by SDS/PAGE and stained with 10% Coomasie Blue. These extracts were divided into 10% SDS/PAGE and transferred to PVDF membrane followed by immunoblotting with human anti-MVP at a dilution of 1:500. Subsequently, the membrane was incubated with anti-IgG mouse alkaline phosphatase-conjugated at a dilution of 1:2500 and revealed by NBT/BCIP solution. C) Densitometric analysis was performed using Gene Quantity software (Biorad). *different from female LE, **different from male LE, ***different from paired LE, ##different from male LE-PZQ and $^{\alpha}$ different from LE (male and paired).

Overall, the amino acid sequence alignments of the six in tandem Vault domains of *Sm*MVP showed that these motifs are formed by amino acids 51–62, of which only one is kept in the same position in all six domains. There is a conserved substitution of amino acids in four positions and the sixth domain has the greatest number of deletions in comparison to the others. This variation between Vault domains, in particular the main structural domain of human MVP, was also previously observed [31]; seven repetitions of this domain in the primary structure of the human MVP are reported. The amino acid sequence comparison of the tandem repeats showed a variation of 48–60 amino acids, and the deletions and insertions are also shown. Similar results were reported for vaults isolated from rat liver where nine Vault domains were identified, also known as MVP domains, and a variation of 51–77 amino acids [32].

A comparative analysis of amino acids outside the Vault domain of other organisms revealed high conservation, suggesting its importance for the folding of the vault protein. In this context, Koslov et al. [31] showed that the region of association between the third and fourth domains of MVP vaults is relatively short and well conserved in various organisms. The global alignment of the conserved Vault domains and their *Sm*MVP orthologs revealed a large number of conserved amino acids and conserved substitutions in related positions. The *Sm*MVP showed only a deletion in the second Vault domain and an insertion of approximate 11 amino acids in the region between the fifth and sixth domains. The high identity homology of *Sm*MVP with respect to the MVP of *Strongylocentrotus purpuratus* (58%), *Dictyostelium discoideum* (MVPa and MVPb), *H. sapiens*, *R. norvegicus* and *M. musculus* (54%) showed high phylogenetic conservation. Kickhoefer and Rome [29] demonstrated an identity of 57% by primary sequence comparison of MVP in the rat and in the *D. discoideum*. Scheffer et al. [30] reported that the deduced amino acid sequence of human MVP shows 87.7% identity with the MVP of rats. The similarity observed in the global alignment between *Sm*MVP and its orthologs suggests its evolutionary conservation. These results are supported by bootstrap values found on the tree constructed using the amino acid sequences. Together, these results strongly evidence the existence of the *Smmvp* gene and of its protein product in *S. mansoni*. Database searches for the identification of other protein components of the Vault complex (VPARP and TEP1), in addition to candidates for the vRNA, were also conducted. *Sm*VPARP and *Sm*TEP1 were identified, however the script used to find candidate vRNAs in the genome of *S. mansoni* was inefficient (data not shown). These results are corroborated by others [33], who performed the homology-based annotation of ncRNAs present in the genomes of *S. mansoni* and *S. japonicum* that also failed to identify this type of RNA homology.

We next aimed to determine the expression profile of MVP by qRT-PCR, during the cercariae to schistosomula transition and adult worms. We have observed up-regulation of MVP expression in adult worm, MTS-3.5 h and MTS-72 h. The MVP constituent of Vaults has a differential profile of gene expression in cercariae and adult worms. Corroborating our findings, Han et al. [34] used genomic, transcriptome and proteomic information from the *S. mansoni* and *S. japonicum* to suggest differential expression levels cercariae and adult worms for cytoskeletal proteins (actin, tubulin, and myosin), chaperone proteins (HSPs), redox enzymes involved in processes (SOD/E-SOD), signalling proteins calcium (CaPB - calpain, calmodulin) and immunophilins, as well as genes and proteins associated with developmental stages and sex of the genus *Schistosoma*. In addition, there are few descriptions of gene expression patterns using early schistosomula [35]. The recent reports suggest that genes involved in diverse biological processes, especially tegument organisation and cell growth, cell adhesion and stress response, were differentially expressed.

To assess whether levels of transcripts for *Sm*MVP would reflect protein levels, western blotting analysis was performed. It was possible to demonstrate the presence of *Sm*MVP in both the soluble and insoluble fractions (data not shown). The results suggest different expression levels of *Sm*MVP at different stages of the life cycle of *S. mansoni*. Similarly, Kedersha et al. [36] demonstrated a basal expression of Vault in several cell types, as well as at different stages of development of *D. discoideum*. We noticed an increased expression of MVP in the adult male relative to adult female in the LE strain. This finding is in agreement with the expression levels for the multidrug resistance-associated protein 1 (*Sm*MRP1) previously reported [37]. Furthermore, we observed that both transcript and protein levels of the *Sm*MVP were up-regulated in PZQ-resistant adult worms; however further investigations are needed to ascertain whether this complex is involved in mechanisms of drug resistance exhibited by *S. mansoni*. The exact mechanisms leading to resistance of PZQ in *S. mansoni* remain to be elucidated.

In conclusion, we observed that the MVP present in *S. mansoni* contains domains conserved orthologs in the mouse, rat and human. Using human MVP antibodies, *Sm*MVP was increased in early schistosomula and in resistant adult worms. Together, these results indicate the presence of the Vault complex in *S. mansoni* and suggest that cellular processes regulated by vaults may be involved in the success of parasitism, drug resistance and maintenance of the infection in the mammalian host. Our studies have also opened up a range of perspectives regarding the role played by vault and/or its components in *S. mansoni* biology.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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