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Research brief

Characterization of *Leishmania* (*Leishmania*) *amazonensis* promastigotes resistant to pentamidine

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Index Descriptors and Abbreviations:
ABC, ATP-binding cassette
DAPI, 4′,6′-diamidino-2-phenylindole
kb, kilobase
Mb, megabase
PATH, potassium antimonyl tartrate trihydrate
PRP1, pentamidine resistance protein 1
Leishmania
Pentamidine
Drug resistance
ABC transporter

ABSTRACT

Pentamidine is a second-line agent used in the treatment of leishmaniasis and its mode of action and mechanism of resistance is not well understood. It was previously demonstrated that transfection of promastigotes and amastigotes with the ABC transporter *PRP1* gene confers resistance to pentamidine. To further clarify this point, we generated *Leishmania amazonensis* mutants resistant to pentamidine. Our results indicated that this ABC transporter is not associated with pentamidine resistance in lines generated by drug pressure through amplification or overexpression mechanisms of *PRP1* gene.

1. Introduction

Leishmania species are responsible for a spectrum of parasitic diseases known as leishmaniasis with important foci in tropical and subtropical areas of the world. Vertebrate hosts are infected with flagellated extracellular promastigotes of the parasite via the bite of a sandfly. These promastigotes forms rapidly differentiate into nonflagellated amastigotes within mononuclear phagocytes of the vertebrate host. The clinical manifestations of leishmaniasis depend on both parasite species and host genetic factors and immune response (Murray et al., 2005). The basic treatment consists of administration of the pentavalent antimonials sodium stibogluconate (Pentostam) and *N*-methylglucamine (Glucantime), although high toxicity and cases of drug resistance have been described (Murray et al., 2005). Second-line drugs like miltefosine, amphotericin B and pentamidine are less useful due to problems associated with either cost or toxicity (Guerin et al., 2002).

The ABC proteins are the largest family of transmembrane proteins being found in all living organisms (Higgins, 1992). They have two transmembrane domains and two conserved nucleotide-binding domains with three characteristic motifs: the Walker motifs A and B and motif C, just upstream of the Walker B site, a signature of the ABC transporters that distinguishes the ABC family members (Higgins, 1992). Eight different subfamilies (ABCA to ABCH) of these highly conserved proteins are present in *Leishmania*, and several of them were already characterized in *Leishmania* species (Leprohon et al., 2006). We have previously described the ABC transporter PRP1, recently named as ABCC7 (Leprohon et al., 2006), and involved in pentamidine resistance in the promastigote and amastigote forms of *L. major* (Coelho et al., 2003, 2007). PRP1/ABCC7 is located intracellularly in promastigotes and amastigotes and is associated with the tubulovesicular element that is partly responsible for the exocytic and endocytic pathways (Coelho et al., 2007, 2006; Ghedin et al., 2001). Regarding the intracellular localization of PRP1/ABCC7, we proposed that pentamidine would be exocytosed by the cell throughout its flagellar pocket (Coelho et al., 2006).

Pentamidine, an aromatic diamidine, enters promastigote and amastigote forms of *Leishmania* cells via a carrier-mediated process by which high-affinity diamidines are recognized (Basselin et al., 2002). Mitochondrion is an important target of pentamidine and this drug is involved in the binding and disintegration of kinetoplast DNA (Basselin et al., 2002; Croft and Coombs, 2003; Croft

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et al., 2006). Topoisomerases are enzymes involved in the modulation of nuclear and kinetoplast DNA (kDNA) topology (Cheesman, 2000) and pentamidine is likely to act as an inhibitor of topoisomerase I (Jean-Moreno et al., 2006). In pentamidine-resistant parasites, alterations were observed in the sequence of kDNA minicircles, especially in AT-rich regions, known to be pentamidine binding sites (Basselin et al., 1998). In the wild-types *L. mexicana* and *L. donovani*, pentamidine is accumulated in the mitochondrion, whereas in pentamidine-resistant parasites the drug remains free in the cytosol (Basselin et al., 2002; Mukherjee et al., 2006). Reduction in the activities of mitochondrial dehydrogenases and F_1F_0 ATPase could cause a collapse in the mitochondrial membrane potencial that is responsible for pentamidine resistance in these mutant parasites (Basselin et al., 2002; Mukherjee et al., 2006). Although a number of elegant biochemical analyses were performed in these studies, gene amplification was not evaluated in these pentamidine-resistant mutants. Gene amplification is frequently associated to drug resistance in *Leishmania* (Beverley, 1991; Borst and Ouellette, 1995), and the strategies used by the parasite to overcome drug pressure may differ considerably among species, as pointed out by Dias et al. (2007), demonstrating that SbIII and terbinafine can lead to H region amplification in *L. major*, but not in *L. braziliensis*.

Based on these observations, we would like to know if pentamidine is able to induce gene amplification as demonstrated in *L. amazonensis* resistant to vinblastine whose *MDR1* gene was found amplified (Gueiros-Filho et al. 1995). In the present study, pentamidine was utilized to generate *L. amazonensis* promastigotes exhibiting two different levels of chemoresistant phenotypes. For the first time in *Leishmania*, we analyzed the association between pentamidine-resistant mutants and gene amplification. Both lines so generated were analyzed at cellular and molecular levels hypothesizing that the ABC transporter PRP1 could be a possible candidate to confer resistance.

2. Materials and methods

2.1. Drugs, strains, and culture conditions

Pentamidine isethionate, berenil (diminazene aceturate), 4′,6′-diamidino-2-phenylindole (DAPI), verapamil, and the trivalent antimonial [Sb(III)] salt potassium antimonyl tartrate trihydrate were supplied by Sigma. Promastigotes of *Leishmania (Leishmania) amazonensis* (MHOM/BR/1973/M2269), a kind gift of Prof. J.J. Shaw, were cultured in M199 medium supplemented as described (Kapler et al., 1990). Wild-type promastigotes of *L. amazonensis* were adapted to survive in medium containing increasing concentrations of pentamidine, starting at the value corresponding to the 50% inhibitory concentration (IC_{50}) for wild-type parasites. The cultures were stabilized for five subcultures before increasing pentamidine level. Mutant PEN^r 5 was selected for pentamidine resistance until it was resistant to 5 μg/ml.

Resistance to the drugs was determined by measuring the IC_{50} and the fold-resistance ratio (ratio between IC_{50} values for the resistant and wild-type cells) as previously described (Coelho et al., 2003). For reversal of drug resistance, non-toxic concentrations of verapamil were used incubating parasites with different concentrations of pentamidine for 72 h. The inhibitory effect on growth was determined as described above.

2.2. DNA isolation, pulsed-field gel electrophoresis, field inversion gel electrophoresis, and Southern blot analysis

Genomic DNA was purified according to the protocol described (Medina-Acosta and Cross, 1993), digested and submitted to electrophoresis in 0.8% agarose gel. Southern blot analysis was performed as previously described (Sambrook et al., 1989).

For pulsed-field gel electrophoresis (PFGE), agarose blocks containing *Leishmania* cells were prepared as described (Grondin et al., 1993). After washing, the cells were resuspended in phosphate-buffered saline (PBS, pH 7.8) at 1×10^8 cells/ml and mixed with low melting point agarose (1%). Parasites were lysed in the presence of EDTA (0.5 M, pH 9.5), sodium lauryl sarcosyl (1%), and proteinase K (1 mg/ml). Chromosomes were analyzed by a Bio-Rad (Hercules, CA, USA) contour-clamped homogeneous electric field (CHEF) mapper for DNA (0.2–2.0 Mbp) separation (16 h, 14 °C) in agarose gel (1%) in 0.5× Tris-borate-EDTA (TBE) running buffer (45, 45, and 1 mM, respectively). Chromosomes were revealed by ethidium bromide staining. For field inversion gel electrophoresis (FIGE), chromosomes were prepared as described above, loaded onto a 1% agarose gel in 0.5× TBE and performed in a FIGE Mapper Electrophoresis System (Bio-Rad) at room temperature using a 0.1–0.8 s linear shape switch time ramp with 180 V forward voltage and 120 V reverse voltage over a period of 11 h. The gel was stained with ethidium bromide, photographed, and transferred onto a nylon filter.

2.3. Fluorescence microscopy

Promastigote parasites (mid-log-phase) were washed (PBS) and then incubated (5 min) with DAPI (10 μM) for 5 min. After washing (PBS), live parasites were immobilized in low melting point agarose in PBS, as previously described (Coelho et al., 2006), transferred to a microscope slide, and viewed by fluorescence microscopy with an automated PALM Robot, MicroBeam System (PALM, Bernried, Germany) using appropriate DAPI excitation/emission filters.

3. Results and discussion

Two cloned lines of *L. amazonensis* promastigotes resistant to pentamidine were used in the present study. One resistant line, PEN^r 0.5, was maintained in pentamidine (0.5 μg/ml) with no increase drug concentration for more than 6 months. The other line, PEN^r 5, was selected by increasing drug pressure up to 5 μg/ml. PEN^r 0.5 and PEN^r 5 promastigotes showed 9- and 22-fold resistance to pentamidine, respectively, when compared to the wild-type parasites (Table 1; Fig. 1A). Resistant promastigotes were checked for cross-resistance to berenil, DAPI, potassium antimonyl tartrate trihydrate [Sb(III)], and verapamil but no cross-resistance was observed, except for berenil and DAPI, two analogues of pentamidine (Table 1). Similarly, pentamidine-resistant mutants of *L. mexicana* and *L. donovani* are cross-resistant to other diamidines as propamidine, stilbamidine, berenil, and DAPI (Basselin et al., 2002; Mukherjee et al., 2006). Interestingly, even the PEN^r 0.5 mutant, maintained in low levels of pentamidine (0.5 μg/ml), was highly resistant to pentamidine about 10-fold higher, regarding 0.37 ± 0.06 μg/ml of pentamidine for the wild-type parasite (Table 1). On the other hand, *L. amazonensis* transfected with the pSNBR/8kb *Sma*I-A plasmid, which contains the *PRP1* gene (Coelho et al., 2003), exhibit about 7-fold resistance to pentamidine and cross-resistance to berenil, DAPI, and potassium antimonyl tartrate trihydrate (data not shown).

Growing rates in the log phase as expressed by population doubling time for PEN^r 0.5 and the wild-type parasites were similar, 6.4 and 6.0 h, respectively, whereas an about 3-fold increase was observed for PEN^r 5 (16.0 h) (Fig. 1B), as previously observed in *L. amazonensis* and *L. donovani* resistant to pentamidine (Basselin et al., 1997). Although in PEN^r 5 mutant a lower parasite density was observed in the stationary phase, no difference was detected

Table 1
Resistance to pentamidine and cross-resistance to berenil, DAPI, potassium antimonyl tartrate trihydrate (PATH), and verapamil of *L. amazonensis* wild-type (La WT) and the drug resistant parasites PEN^r 0.5 and PEN^r 5

Drugs	IC_{50} *			Fold resistance	
	La WT	PEN^r 0.5	PEN^r 5	PEN^r 0.5	PEN^r 5
Pentamidine[a]	0.37 ± 0.06	3.3 ± 1.25	8.2 ± 0.7	9	22
Berenil [b]	2.78 ± 0.22	65 ± 12.25	128.8 ± 21.1	23.4	46.3
DAPI [b]	0.44 ± 0.05	1.3 ± 0.31	3.15 ± 0.23	2.95	7.16
PATH [a]	58.1 ± 15.7	73 ± 12.4	67.6 ± 16.1	1.3	1.2
Verapamil [b]	58 ± 4.4	57 ± 11.04	42.4 ± 3.1	0.98	0.73

* IC_{50} mean values ± standard deviation of three independent experiments done in duplicate.
[a] Concentrations in μg/ml.
[b] Concentrations in μM.

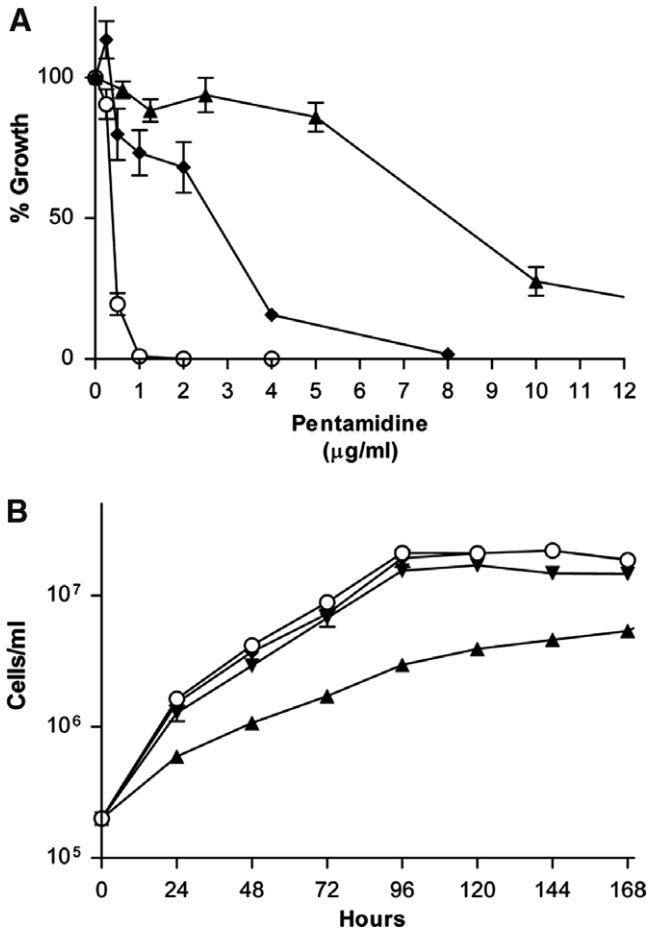

Fig. 1. Characterization of mutants resistant to pentamidine. (A) Growth curve of wild-type and resistant parasites in the presence of pentamidine. (B) Growth of promastigotes *in vitro*. The parasites were inoculated at 2×10^5/ml and parasite densities were measured. The mean of three independent experiments were calculated. Legend: (○): *L. amazonensis* (wild-type); (♦): PEN^r 0.5; (▲): PEN^r 5; (▼): PEN^r 5 without drug pressure for 6 months.

between PEN^r 0.5 and wild-type parasites (Fig. 1B). In *L. mexicana* amastigotes, it was previously shown that low levels of pentamidine resistance are rapidly lost once drug pressure is removed, whereas high pentamidine resistance remains stable (Sereno and Lemesre, 1997). Pentamidine resistance was stable in PEN^r 0.5 and PEN^r 5 parasites after 2 or 6 months without drug pressure, although IC_{50} values for the mutants had declined (data not shown). After 6 months without pentamidine, PEN^r 5 mutants exhibited a doubling time value (7.5 h) similar to that observed in wild-type parasites (6.0 h) (Fig. 1B).

Verapamil, a calcium channel blocker known to reverse multidrug resistance in *Plasmodium falciparum* and mammalian cells (Bitonti et al., 1988; Gottesman and Pastan, 1993), was described as a classical inhibitor of ABC transporters associated to efflux activity in *Leishmania* (Essodaigui et al., 1999). In *L. mexicana* pentamidine-resistant mutants, verapamil may reverse pentamidine resistance in promastigotes (Basselin et al., 2002), but not in axenic amastigotes (Sereno and Lemesre, 1997). Using non-toxic concentrations of verapamil, a dose-dependent reversion of pentamidine resistance was observed in resistant parasites when compared with those not treated with verapamil. The IC_{50} mean values for PEN^r 5 mutant resistant parasites that received pentamidine associated with 5, 10, or 20 μM of verapamil were 5.35 ± 0.77, 2.27 ± 0.75, and 0.41 ± 0.06, respectively. A similar effect was observed in PEN^r 0.5 mutants (data not shown).

Gene amplification has been frequently observed in *Leishmania* promastigotes mainly when drug resistance is induced *in vitro* (Beverley, 1991; Borst and Ouellette, 1995). However, no amplification was observed in a stepwise selected pentamidine-resistant line of *L. major* (PT-R20) (Ellenberger and Beverley, 1989). In order to test whether gene amplification is present in PEN^r 0.5 and PEN^r 5 mutants, the molecular karyotype of mutants and wild-type parasites were compared. After chromosome separation by PFGE analysis, no amplification was observed (Fig. 2A). In FIGE analysis, only the circular amplicon corresponding to the pSNBR/8kb *Sma*I-A plasmid (which contains the *PRP1* gene of *L. major*) (Coelho et al., 2003), was observed in *L. amazonensis* transfectants (Fig. 2B). We also looked for the presence of any restriction fragment amplified in the resistant lines through digestion of genomic DNA with different enzymes. Both, pentamidine-resistant mutants and *L. amazonensis* wild-type parasites displayed bands with comparable intensities (data not shown). Since overexpression of the ABC transporter PRP1 is involved in pentamidine resistance in transfectants of *L. major* (Coelho et al., 2003, 2007), we verified whether PRP1 overexpression is related to pentamidine resistance in our pentamidine-resistant mutants. Southern blot analysis using a specific probe of *L. major PRP1* gene indicated no amplification of *PRP1* gene in PEN^r 0.5 and PEN^r 5 lines (Fig. 2C and D). These data indicated that amplification of DNA, including that from *PRP1* gene, are not significant for parasites that turned resistant to pentamidine in *L. amazonensis*. Search for repetitive elements of DNA around *PRP1* gene of *L. major* showed absence of these elements (data not shown) and may thus explain the lack of DNA amplification for this *locus* in the parasite. This fact and the stability of the resistant phenotype for at least 6 months in absence of drug pressure suggest that stable mutations in one or more genes must be responsible for pentamidine resistance in this case. Mutations in *PRP1* gene could increase the activity of this ABC transporter conferring pentamidine resistance. MDR1 may also affect pentamidine accumulation inside the mitochondrion since its copy number has an inverse relationship to pentamidine resistance in *Leishmania* (Wong et al., 2007). Sequencing of *PRP1* and *MDR1* genes may indicate whether these ABC transporters are involved in pentamidine resistance in *Leishmania*.

Overexpression of genes not associated with gene amplification were already described in *Leishmania* mutants resistant to SbIII and methotrexate (Guimond et al., 2003; Marquis et al., 2005). Other interesting data showed that expression of some ABC transporters genes are modulated during the life cycle of *Leishmania* parasites, but no change was observed in *PRP1* (*ABCC7*) expression (Leprohon

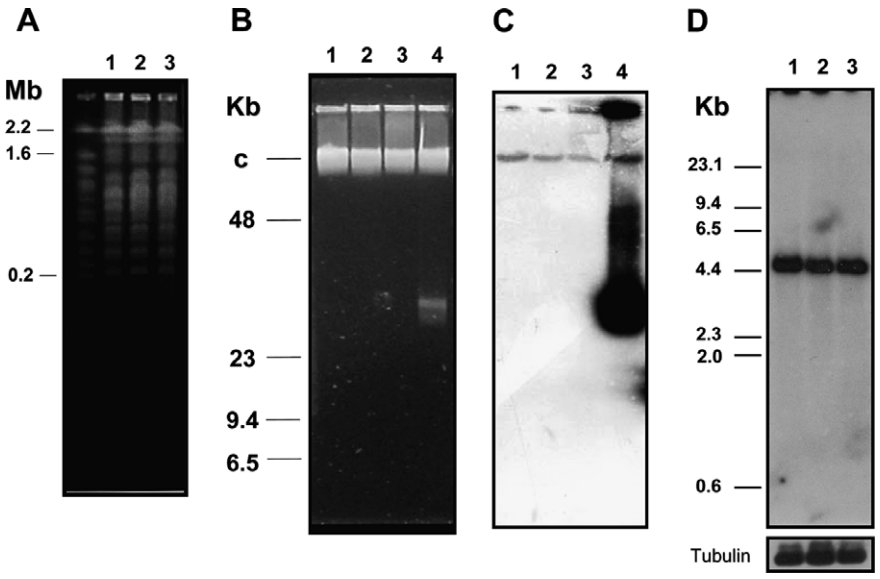

Fig. 2. Analysis of DNA amplification and extrachromosomal elements in wild-type and PEN^r 0.5 and PEN^r 5 mutants of *L. amazonensis*. (A) Molecular karyotypes of wild-type and mutants lines. Chromosomal bands were separated by CHEF electrophoresis and stained with ethidium bromide. (B and C) Identification of circular amplicons in mutant lines by FIGE. Total DNA was separated by FIGE, stained with ethidium bromide (B), transferred onto nylon membranes and hybridized with a *PRP1* gene probe (an 1.2-kb *Pvu*II fragment encoding the 2nd transmembrane domain of PRP1 from *L. major*) (C). (D) Southern blot analysis of *PRP1* gene amplification. DNA of parasites was digested with *Pst*I and hybridized with the same probe described above. This filter was rehybridized with the tubulin probe. Lanes: 1: *L. amazonensis* (wild-type); 2: PEN^r 0.5; 3: PEN^r 5; 4: *L. amazonensis* transfected with pSNBR/8kb *Sma*I-A. c—compression zone. Molecular size markers are indicated on the left.

et al., 2006). Transcripts of ABC transporters by Northern blot analysis in wild-type *Leishmania* parasites is difficult to be detected due to the low levels of gene expression (Coelho et al., 2006; Henderson et al., 1992; Papadopoulou et al., 1994). In order to evaluate *PRP1* gene expression, we carried out a real time RT-PCR analysis of PEN^r 5, PEN^r 0.5, and wild-type promastigotes and the levels of *PRP1* transcripts detected for the three lines were the same (data not shown). No difference in *PRP1* transcripts levels was observed between sensitive and resistant parasites to pentavalent antimonials (Decuypere et al., 2005). These data show that expression of *PRP1* gene is not modulated during life cycle of the parasites or between parasites sensitive and resistant to drugs.

In pentamidine-resistant parasites, the drug remains free in the cytosol and is not accumulated in the mitochondrion, the main target of the drug (Basselin et al., 2002; Mukherjee et al., 2006). Similarly, a significant reduction in accumulation of DAPI, a fluorescent analogue of pentamidine, was observed in PEN^r 5 parasites when compared to wild-type parasites (Fig. 3), the same phenotype observed in *L. mexicana* and *L. donovani* promastigotes resistant to pentamidine (Basselin et al., 2002; Mukherjee et al., 2006). Pentamidine accumulated in the cytosol would be removed throughout an ABC transporter but agents like verapamil, trifluoperazine, and prochlorperazine may lead pentamidine to accumulate in the cytosol to levels which drive accumulation in the mitochondrion (Basselin et al., 2002). In *Leishmania*, verapamil was already reported to be a classical inhibitor of ABC transporters associated to efflux activity (Essodaigui et al., 1999). Herein, we observed the same phenotype, reversal of pentamidine resistance in both resistant parasites. A new and so far not described ABC transporter could be responsible to remove pentamidine from the cytosol, and PRP1 may not be involved except by gene mutation(s), as discussed above.

In *L. major* genome, 42 members of the ABC transporter family were described, and ABCC subfamily contains eight members of which PRP1 is one of the members (Leprohon et al., 2006). It should be considered that other studies, as DNA microarrays, are necessary for identification of genes associated with drug resistance and it is likely that some of these ABC transporters could be involved in the pentamidine efflux in resistant mutants of *Leishmania*. Interestingly, this efflux activity was not observed in mutants of *L. donovani* resistant to pentamidine when treated with the ABC transport inhibitors prochlorperazine and trifluoperazine (Mukherjee et al., 2006). Differences at the molecular level between *Leishmania* species may affect the activity of these ABC transporters in the drug efflux.

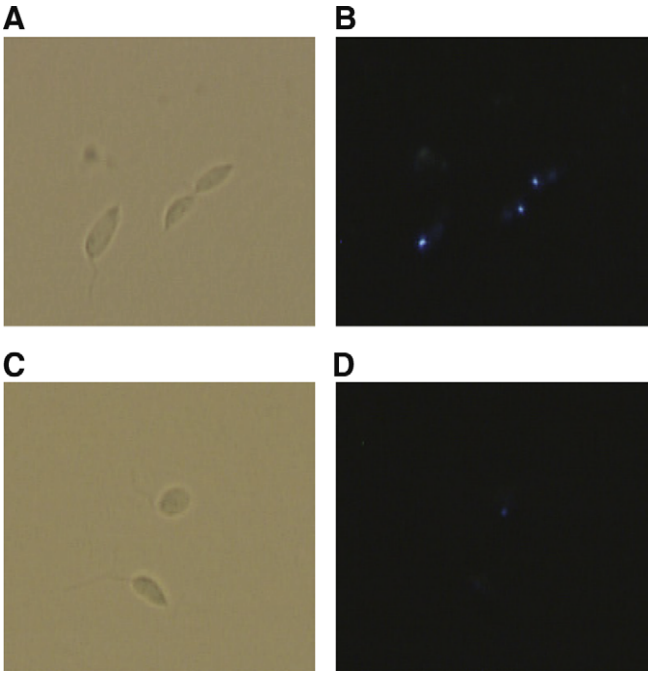

Fig. 3. Fluorescent images of wild-type and PEN^r 5 promastigote parasites stained with DAPI, a fluorescent analogue of pentamidine. Bright blue fluorescence mainly associated with kinetoplast DNA is observed in wild-type parasites (A and B) but not in PEN^r 5 parasites (C and D). (A and C), phase contrast; (B and D), DAPI fluorescence.

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