Increased metacyclogenesis of antimony-resistant *Leishmania donovani* clinical lines

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SUMMARY

Mathematical models predict that the future of epidemics of drug-resistant pathogens depends in part on the competitive fitness of drug-resistant strains. Considering metacyclogenesis (differentiation process essential for infectivity) as a major contributor to the fitness of *Leishmania donovani*, we tested its relationship with pentavalent antimony (Sb^V) resistance in clinical lines. Different methods for the assessment of metacyclogenesis were cross-validated: gene expression profiling (META1 and SHERP), morphometry (microscopy and FACS), *in vitro* infectivity to macrophages and resistance to complement lysis. This was done on a model constituted by 2 pairs of reference strains cloned from a Sb^V-resistant and -sensitive isolate. We selected the most adequate parameter and extended the analysis of metacyclogenesis diversity to a sample of 20 clinical lines with different *in vitro* susceptibility to the drug. The capacity of metacyclogenesis, as measured by the complement lysis test, was shown to be significantly higher in Sb^V-resistant clinical lines of *L. donovani* than in Sb^V-sensitive lines. Together with other lines of evidence, it is concluded that *L. donovani* constitutes a unique example and model of drug-resistant pathogens with traits of increased fitness. These findings raise a fundamental question about the potential risks of selecting more virulent pathogens through massive chemotherapeutic interventions.

Key words: *Leishmania donovani*, visceral leishmaniasis, Nepal, India, antimony resistance, metacyclogenesis, SHERP, META1.

INTRODUCTION

Fifty per cent of the 500 000 annual new cases of visceral leishmaniasis (VL) occur in India, Bangladesh and Nepal (Desjeux, 2004). Since there is no anti-leishmanial vaccine in clinical use, control of VL relies almost exclusively on chemotherapy (Chappuis *et al*. 2007). A few drugs are currently available including pentavalent antimonies (Sb^V) that have been used for more than half a century as a first-line drug (Frézard *et al*. 2009). This drug exhibits several limitations, including toxicity and emergence of drug resistance. This phenomenon is especially well described in the Indian subcontinent, with up to 60% patients in India (Bihar state) and 20% in Nepal (Eastern terai) not responding to Sb^V treatment (Sundar, 2001; Rijal *et al*. 2003). Consequently, this drug was replaced by the new oral drug, Miltefosine, in the frame of the regional VL elimination programme (Thakur *et al*. 2009). Understanding the

dynamics of drug resistance in a clinical context requires studies on the mechanisms leading to its emergence as well as a comprehension of factors favouring the spread of drug-resistant parasites. Parasite fitness defined as the complex integrated skills that allow the organism to replicate and be successfully transmitted (Natera *et al*. 2007) is therefore of uppermost importance.

Metacyclogenesis is the process by which trypanosomatids differentiate metabolically from non-infective procyclic promastigotes (flagellated life stage) to infective metacyclic promastigotes (Sacks, 1989). It is one of the most important adaptive processes during the whole life cycle of the parasite and a major contributor to its fitness. This is well illustrated by transcriptomic studies showing that most significant events of gene expression regulation occur during the metacyclogenesis, with a dramatic down-regulation during the transition from metacyclics to intracellular amastigotes, hereby supporting the hypothesis of pre-adaptation of the parasite to the survival in the macrophage (Alcolea *et al*. 2010). In *Leishmania* species, metacyclogenesis occurs naturally in the sand fly vector, but the process can

Table 1. List of the strains used in this study and their *in vitro* susceptibility to Sb^V

(* AI, Activity Index defined previously as ED$_{50}$ tested strain/ED$_{50}$ reference strain (Rijal *et al*. 2007; Yardley *et al*. 2006).)

International code	Origin	Sb^V susceptibility
Sb^V-sensitive strains		
MHOM/NP/03/BPK282/0 clone 4	Sunsari, Inaruwa	1
MHOM/NP/03/BPK282/0 clone 9	Sunsari, Inaruwa	1
MHOM/NP/02/BPK178/0 clone 3	Sunsari, Inaruwa	2
MHOM/NP/03/BPK294/0 clone 1	Siraha, Bishnupur	1
MHOM/NP/03/BPK206/0 clone 10	Sunsari, Ithahari	1
MHOM/NP/02/BPK080/0 clone 1	Sunsari, Ithahari	1
MHOM/NP/03/BPK288/0 clone 7	Saptari, Sitapur	1
MHOM/NP/02/BPK035/0 clone 1	Saptari, Bhagani	0
MHOM/NP/02/BPK026/0 clone 5	Bhojpur, Bastim	1
MHOM/NP/02/BPK031/0 clone 12	Morang, Bhoctani	0
Sb^V-resistant strains		
MHOM/NP/03/BPK275/0 clone 12	Morang, Schanischare	>6
MHOM/NP/03/BPK275/0 clone 18	Morang, Schanischare	>6
MHOM/NP/02/BPK173/0 clone 3	Sarlahi, Pirari	>6
MHOM/NP/03/BPK173/1 clone 9	Sarlahi, Pirari	>6
MHOM/NP/02/BPK085/0 clone 8	Saptari, Kamalpur	>6
MHOM/NP/02/BPK087/0 clone 11	Morang, Bhodaha	3
MHOM/NP/03/BPK190/0 clone 3	Morang, Govindpur	>6
MHOM/IN/09/BHU568 clone 1	Bihar, Muzzafarpur	9
MHOM/IN/09/BHU573 clone 3	Bihar, Sitamadhi	6
MHOM/NP/02/BPK164/1 clone 11	Dhanusa, Dharapani	>3

be mimicked by *in vitro* parasite growth from a logarithmic to a stationary phase and is induced by acidification of the medium (Saraiva *et al*. 2005). Several methods for monitoring metacyclogenesis were reported independently in the literature, mostly for single strain-analysis: morphometry, functional assays and gene expression analyses (Uliana *et al*. 1999; Knuepfer *et al*. 2001; Saraiva *et al*. 2005; Gamboa *et al*. 2008). Previous studies on a possible link between drug resistance, metacyclogenesis and infectivity showed contrasting results (Sereno and Lemesre, 1997; Silva *et al*. 2004; Samant *et al*. 2007), possibly as a consequence of the species, the drug or the experimental set-up.

In the present paper, we aimed to analyse the possible link between Sb^V-susceptibility and metacyclogenesis in *L. donovani* clinical lines. We first adapted the different methods reported in the literature to *L. donovani* and cross-validated them on a model constituted by 2 pairs of reference strains cloned from an Sb^V-resistant and -sensitive isolate. Secondly, we selected the most adequate parameter and extended the analysis of metacyclogenesis diversity to a sample of 20 clinical lines with different *in vitro* susceptibilities to the drug.

MATERIALS AND METHODS

Parasites

In total, 20 *L. donovani* cloned strains were used for this study (see Table 1). They were derived from 18 clinical isolates (2 of them providing 2 strains each). Sixteen strains were obtained from Nepalese patients with confirmed visceral leishmaniasis. Those were recruited between 2002 and 2004 at the B.P. Koirala Institute of Health Sciences, Dharan, Nepal, in the frame of a research project on antimony resistance (Rijal *et al*. 2007). The other 2 strains were obtained from North Indian patients in May 2009 in the frame of the Kaladrug project. Written informed consent was obtained from the patients and in the case of children, from the parents or guardians. Ethical clearance was obtained from the institutional review boards of the Nepal Health Research Council, Kathmandu, Nepal, Banaras Hindu University, Varanasi, India and the Institute of Tropical Medicine, Antwerp, Belgium. All used clones were tested as intracellular amastigotes for their *in vitro* antimonial susceptibility as described elsewhere (Rijal *et al*. 2007). The calculation of an activity index (AI) allowed us to classify the clones in Sb^V-sensitive and Sb^V-resistant groups. Thus, clones with an AI of 0–2 were considered as sensitive, while those showing an AI of 3 or higher were considered to be resistant. Promastigotes were grown in haemoflagellate-modified minimal essential medium (HOMEM) (Invitrogen) supplemented with 20% (v/v) heat-inactivated foetal calf serum (PAA Laboratories GmbH), pH 7·5, at 26 °C over 7–8 days. The cultures were initiated by inoculating day 3–4 stationary phase parasites in 5 ml of culture medium at a final concentration of 5×10^5 parasites/ml. The parasite

Table 2. List of genes targeted in real-time qPCR analyses

Gene	Product	Accession number	Forward/reverse primer	Final concentration
SHERP	Small Hydrophilic Endoplasmic Reticulum-associated Protein	LinJ23.1190	5′ CAACAAGATCCAGGAGCTGAA 3′ 5′ GCCGCTTATCTTGTCCTTGA 3′	300 nM
META1	Metacyclic gene	LinJ.17.0990	5′ TTGAAGTGGCAACGAGGAGAC 3′ 5′ GGGGGAAAAGAGCAAATAGGG 3′	300 nM
ACR2	Arsenate reductase 2	LinJ32.3240	5′ GCCCAGTCGCTCATACGG 3′ 5′ AGAACGCCTCCCACCCAC 3′	400 nM
CBS	Cystathionine β-synthase	LinJ17.0280	5′ CGCCGATGTCAACTGGATG 3′ 5′ GCTCCTTCTTCAGCGTGTCG 3′	300 nM
PRP1	Pentamidine resistance protein 1	LinJ31.1810	5′ TGATTCCCTTTTTGGGCATTA 3′ 5′ CGTAGAACTTGAGCAGGAGCAC 3′	400 nM

density was determined every 24 h using disposable count chambers Uriglass (Menarini diagnostics) to follow up the growth and differentiation profile. Parasites were harvested at different time-points of the growth curves (specific time-points per experiment are specified in the text). During the modelling phase (evaluation of different parameters for assessment of metacyclogenesis), biological duplicates were used.

RNA isolation and real-time quantitative PCR

Parasite cultures at logarithmic or stationary phase of Sb^V-resistant or Sb^V-sensitive strains, were washed twice in phosphate-buffered saline (PBS) and immediately disrupted in RNAqueous Lysis/ Binding solution (Ambion) and frozen at −80 °C until RNA extraction. Total RNA was extracted, treated and analysed as described elsewhere (Decuypere *et al.* 2008). Reverse transcription of total RNA and expression profiling of the 5 genes listed in Table 2 were performed as described elsewhere (Decuypere *et al.* 2008). Normalization of gene expression was done by determining the 3 most stable expressed genes from the set of tested genes, as described elsewhere (Vandesompele *et al.* 2002). The following controls were included in each run for each gene: (i) negative controls of cDNA synthesis (i.e. without reverse transcriptase), and (ii) no-template controls. All reactions were done in triplicate, with their arithmetic average threshold cycle (CT) used for data analysis. The geNorm VBA applet for MS Excel was used to determine the 3 most stable expressed genes from the set of 5 tested genes in a given sample panel. The qbase^{plus} (Biogazelle) software was used for the determination of the relative (normalized) expression levels of target gene.

Morphometric analysis

Promastigotes from logarithmic and stationary growth phases were washed in PBS, air-dried on a glass slide, fixed with methanol, stained with 10% (v/v) Giemsa, and analysed by optical microscopy. Cell body length (L), width (W) and flagella length (F) were measured for a minimum of 100 promastigotes in each slide examined using Cell D Imaging Software (Olympus Microscopy). The values of L×W and F/L were then calculated for each promastigote. Parasite populations corresponding to sensitive and resistant clones were clustered in 2 subpopulations (logarithmic and metacyclic-like parasites). A cluster analysis was implemented to determine cut-off values for the 2 parameters (L×W and F/L) and for the resistant and sensitive parasites, clustering the total sample in 2 subpopulations (done twice, once for L×W and once for F/L). Based on an adapted version of the methodology described elsewhere (Cardoen *et al.* 2009; Havelaar *et al.* 2010), groups of different importance were identified by Classification and Regression Tree analysis (CART Version 6.0, Salford Systems, San Diego, California, USA). The aim was to obtain 2 subgroups with minimal within group variance (grouping samples with similar L×W and F/L values). Starting with all the samples the method will obtain a binary split into 2 groups (nodes) that are most homogeneous with respect to the L×W and F/L values. The technique is explained in more detail elsewhere (Speybroeck *et al.* 2004). A percentage of metacyclic-like parasites was then calculated for parasite populations using the command prop.test implemented in the R software. Statistic analysis was done using a 2-sample test for equality of proportions without continuity correction.

Flow cytometry

Logarithmic and stationary growth-phase parasites were washed and re-suspended in phosphate-buffered saline (PBS) in the presence of 1 μg/ml propidium iodide (Sigma, St Louis, MO, USA) to assess viability. Live parasites were then analysed by

flow cytometry using forward scatter (FSC) side scatter (SSC) and fluorescence detection. Dot plots of FSC *vs* SSC represent the acquisition of 20 000 events. Data were collected in a BD FACSCalibur and list mode files were analysed by FlowJo 7.6.1 software. (Tree Star, Inc. Oregon, USA)

Complement-mediated lysis

Promastigotes in the exponential phase or stationary phase of growth *in vitro* were tested for their resistance to complement-mediated lysis as described elsewhere (Gamboa *et al.* 2008) with modifications. Briefly, promastigotes were tested at a concentration of 5×10^6 parasites/ml in doubling dilutions of human serum from 1:1 to 1:128 at 26 °C for 1 h. The results were expressed as the percentage of living parasites compared to controls that were not exposed to serum. GraphPad Prism 5 software was used to determine EC_{50} (50% effective concentration) corresponding to each sample tested using a sigmodial dose-response model with variable slope. Each experiment was performed twice. For the second part of the work (validation of the results), a high-throughput complement-mediated lysis test was developed using resazurin to assess the viability of the parasites (Mikus and Steverding, 2000). Briefly, promastigotes were diluted in homem 20% FCS to 1×10^6 parasites /ml and then subjected to the different concentrations of human serum in triplicate in a final volume of 200 µl. After an incubation of 1 h at 26 °C, resazurin was added to each well at a final concentration of 0·125 mg/l and plates were further incubated for 24 h at 26 °C. The fluorescence at 560 and 590 nm was measured simultaneously using the Victor X3 Multilabel Reader (PerkinElmer). The fluorescence in the absence of serum was set as the 100% control. The percentage survival was exported to GraphPad Prism 5 to calculate the 50% effective concentration (EC_{50}) using a sigmodial dose-response model with variable slope, to visualize results and to statistically analyse the data with a *t*-test.

In vitro *infectivity*

The ability of promastigotes to invade and multiply within macrophages *in vitro* was assessed. Murine peritoneal macrophages were harvested from BALB/c mice, plated out at 2×10^5 cells per 200 µl per well in RPMI medium supplemented with 10% FCS and 100 U/ml penicillin and streptomycin (= full RPMI) in 16 well Lab-Tek chamber slides (Nunc) incubated at 37 °C and 5% CO_2 for 24 h. Then, macrophages were infected with stationary phase cultures with a ratio of 10 parasites per 1 macrophage. After 24 h of incubation, free parasites were removed by repeated washings in a serum-free medium and incubated for an additional 3 days in the same conditions. For each strain tested at least 400 macrophages were counted to determine the percentage of infected macrophages and an Infection Index (= % of infected macrophages × average number of amastigotes/ macrophage).

RESULTS

Modelling

At this stage, a series of parameters for the assessment of metacyclogenesis was evaluated on 4 cloned strains: 2 derived from clinical isolate BPK275/0 (Sb^V- resistant) and 2 others derived from BPK282/0 (Sb^V-sensitive). Growth curves were shown to be very similar among the 2 strains derived from each clinical isolate, but differences were observed between the 2 pairs of strains, the 2 BPK282/0 strains growing more quickly and reaching earlier the stationary phase (Fig. 1). Therefore, collection of the samples for the next analyses was adapted to each growth curve: (i) logarithmic phase (day 3 for the 4 strains, further called L-samples) and (ii) stationary phase (days 6 and 8, for BPK282/0 and BPK275/0 strains, respectively, further called S-samples).

First, molecular assays were applied to the different samples. The expression level of META1 and SHERP, 2 genes reported to be over-expressed in metacyclics (Uliana *et al.* 1999; Knuepfer *et al.* 2001), was monitored all along the growth curve (Fig. 1A and B). In the 4 lines, the 2 genes showed an increased expression during the progression from logarithmic to stationary phase and this reached a maximum between days 6 and 8. However, over-expression was much higher in the 2 Sb^V-sensitive lines (twice for META1 and 2·7 times for SHERP).

Secondly, morphometry of the parasites was assessed by 2 methods and we focused on samples from stationary phase (S, samples: days 6 and 8 respectively for BPK282/0 and BPK275/0 strains, respectively). On the one hand, optical microscopy was used. Visual inspection of the plates revealed the reported dimorphism of promastigotes: parasites with large cell bodies and a flagellum of similar length (typical of procyclics) and short slender cells with a flagellum measuring at least twice the length of the cell body (typical of metacyclics). For an objective comparison and quantification, a morphometric analysis of L- and S-samples was performed for the 4 strains. A bi-modal distribution was observed for both parameters L*W and F/L and, a cut-off was defined using the classification and regression tree technique. Noteworthy, the cut-off was different for the 2 pairs of strains, likely reflecting intrinsic larger size of BPK282/0 strains. According to the respective cut-offs, the proportion of metacyclic-like promastigotes was estimated in all samples. For each strain, the percentage of metacylic-like promastigotes was found to be significantly higher in S-samples than in L-samples ($P < 0·05$). A significantly higher
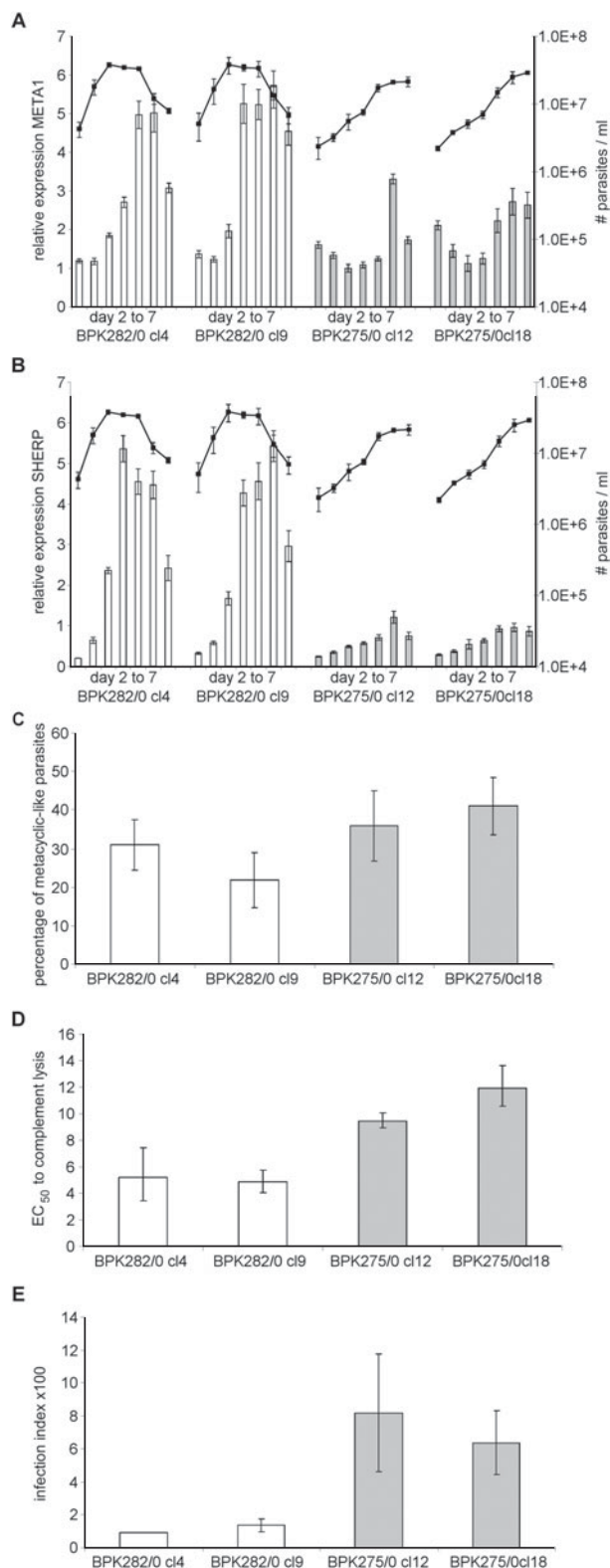
Fig. 1. Cross-validation of methods for assessment of metacyclogenesis in 2 Sb^V-sensitive (white) and 2 Sb^V-resistant (grey) strains of *Leishmania donovani*. Gene expression analysis (META1 and SHERP, panels A and B respectively) was monitored each day during the growth of the parasites (see growth curves above the histograms of relative expression). (C–E) Results obtained with samples from stationary phase: (C) morphometry by optical microscopy, (D) resistance to complement lysis (EC$_{50}$, Percentage serum causing 50% cell lysis),

percentage of metacylic-like promastigotes was observed in the 2 resistant strains (39%) when we compared them to the 2 sensitive strains (27·7%), $P = 0·004$. On the other hand, the same samples were also analysed by flow cytometry (Fig. 2). The populations of parasites were gated in 2 subpopulations displaying clearly different FSC intensities corresponding to different cell sizes as described elsewhere (Saraiva *et al*. 2005). As for the microscopy, the cut-off between the 2 populations was not the same among strains and also indicated an intrinsically larger size of BPK282/0 strains. When comparing sensitive and resistant strains, the percentage of FSC^{low} parasites (metacyclic-like) showed an increase from L-samples to S-samples, however this percentage is higher in S-samples of resistant strains (40·0 and 42·0%) than in sensitive strains (20·6 and 26·9%).

Thirdly, parasites were submitted to 2 types of functional assays. On the one hand, they were subjected to complement-mediated lysis, a phenomenon to which metacyclics are known to resist more than procyclics (Gamboa *et al*. 2008). At this stage, the percentage of viable promastigotes was estimated using movement of the flagellum as a sign of viability (Zakai *et al*. 1998). S-samples of the 2 resistant strains showed a higher resistance to complement-mediated lysis than those of the 2 sensitive strains (EC$_{50}$ = 10·66% and 5·02%, respectively) (Fig. 1D). This indicated a higher proportion of metacyclics in resistant strains than in sensitive strains in stationary-phase cultures. On the other hand, stationary-phase promastigotes of the 4 strains were used to infect peritoneal macrophages *in vitro*: 72 h after infection, the infection index corresponding to each strain was determined (Fig. 1E). The 2 BPK275/0 strains appeared to be at least 4 times more infective than the 2 BPK282/0 strains.

Diversity of metacyclogenesis among clinical strains of L. donovani

In the second part of the work we extended the analysis of metacyclogenesis diversity to a broader sample of strains. Among the 5 aforementioned parameters evaluated, we selected one, the test of resistance to complement lysis, being a functional test and easier to implement as a high throughput application. In total, 20 lines were analysed, derived from 18 different clinical isolates, 9 Sb^V-sensitive and 9 Sb^V-resistant; all measures were done at day 7 of the growth curve (Fig. 3). Accordingly,

(E) *in vitro* infectivity to murine peritoneal macrophages (infection index = % of infected macrophages multiplied by the average number of amastigotes/macrophage). Biological duplicates were used in all experiments.
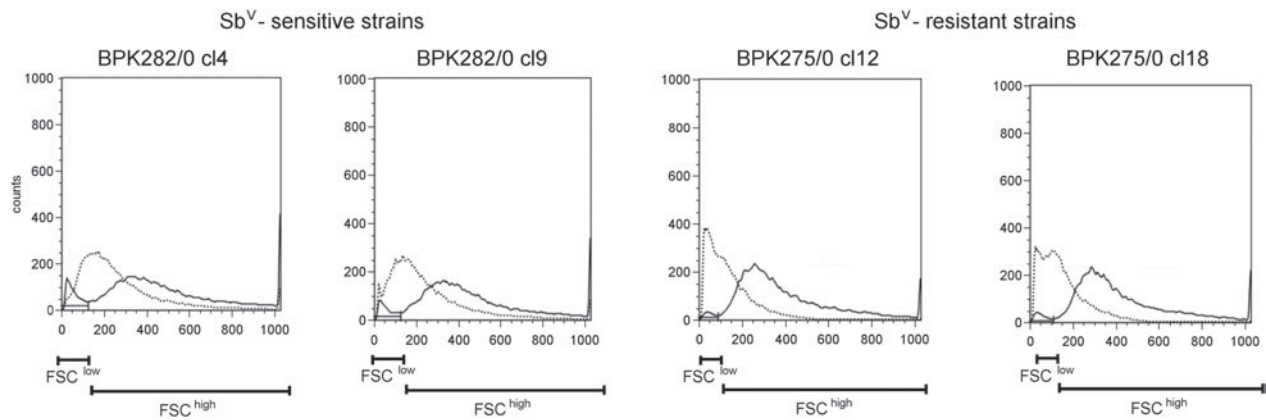
Fig. 2. Flow cytometric analysis of 2 Sb^V-sensitive strains and 2 Sb^V-resistant strains in samples collected in logarithmic (full line) and stationary (dotted line) phases. FSC, forward-angle light scatter. The FSC cut-off between short (FSC^{low}) and large (FSC^{high}) promastigotes was defined at the minimum of the curve of logarithmic samples.
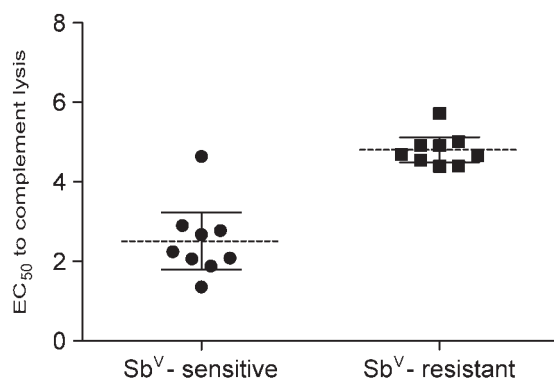

Fig. 3. Resistance to complement-mediated lysis in 9 Sb^V-sensitive (dots) and 9 Sb^V-resistant (squares) strains in stationary phase (all harvested at day 7). The Alamar blue was used as parasite viability indicator: EC$_{50}$, percentage serum causing 50% cell lysis. Each point represents a tested parasite strain. Dashed line represents the mean value observed in the 2 populations.

Sb^V-resistant parasites showed a significantly higher ($P = 0.0003$) resistance to complement lysis (mean EC$_{50}$ = 4.80% ± 0.41) in stationary phase cultures when compared to Sb^V-sensitive strains (mean EC$_{50}$ = 2.51% ± 0.93). This showed, thus, the occurrence of a higher metacyclogenesis rate among the Sb^V-resistant lines of *L. donovani* here studied.

DISCUSSION

In the present study we first cross-validated on a limited number of strains a series of parameters for the assessment of metacyclogenesis in *L. donovani*. It was found that all morphological and functional parameters agreed and showed a higher proportion of metacyclics in the 2 Sb^V-resistant lines. This contrasted with molecular parameters. While META1 and SHERP genes showed a higher expression during metacyclogenesis of each individual strain, inter-strain comparison showed lower levels of expression in stationary forms of SbV-resistant parasites. Our data thus do not validate META1 and SHERP as adequate markers for inter-strain comparison of metacyclogenesis. This could indicate the occurrence of different molecular adaptations in resistant metacyclics, but needs to be interpreted with caution, as post-transcriptional regulation is common in trypanosomatids (Martínez-Calvillo *et al.* 2010). In a second stage, extending our sample size and focusing on 1 parameter (functional, i.e. complement lysis), we found that the metacyclogenesis rate was about twice as high in clinical lines of *L. donovani* resistant to Sb^V than in Sb^V-sensitive lines.

Mathematical models predict that the future of epidemics of drug-resistant pathogens depends in part on the competitive fitness of drug-resistant strains (Borrell and Gagneux, 2009). Several studies on prokaryotes and eukaryotes have demonstrated that drug resistance generally confers a reduction in fitness expressed as reduced growth, virulence or transmission (Hastings and Donnelly, 2005; Andersson and Hughes, 2010). In some cases, compensatory mutations may occur which restore the fitness of mutants to that of sensitive forms (Walliker *et al.* 2005). Considering metacyclogenesis as a major adaptive process in the life cycle of the parasite, our results suggest a higher fitness of Sb^V-resistant *L. donovani* parasites. Other published data converge to support this concept. Samant *et al.* (2007) showed that Sb^V-resistant clinical isolates of *L. donovani* expressed more proteophosphoglycans, which are considered as an important virulence factor in both vector and mammalian hosts. Indeed, a recent report highlighted the inhibition of Sb^V-induced dendritic cell activation by Sb^V-resistant *L. donovani* isolates (Haldar *et al.* 2010). Another study showed that resistant parasites were more virulent in experimental *in vivo* models (Vanaerschot *et al.* 2010), in agreement with the clinical observation that patients with treatment failure presented a higher parasite

load before treatment compared to responder patients (Thakur *et al*. 2003). The exceptional nature of the increased fitness of Sb^V-resistant *L. donovani* could be explained by a unique synergy between the survival strategy of the parasite in the macrophage and the mode of action of antimonials. On the one hand, *L. donovani* is an expert in manipulating macrophages, interfering with cell signalling and inhibiting a series of effectors like reactive oxygen and nitrogen species (ROS/RNS) (Mookerjee *et al*. 2006). On the other hand, Sb^V enables host cells to re-activate their ROS/RNS attack on the intracellular amastigotes (Mookerjee *et al*. 2006). Moreover, several authors reported a cross-resistance of *Leishmania* to antimonials and nitric oxide (Carter *et al*. 2005; Holzmuller *et al*. 2005). Metacyclics are intrinsically insensitive to SbV (da Luz *et al*. 2009), and amastigotes are the only stage naturally submitted to the pressure of the drug or other effectors. The link between the selective pressures exerted on the amastigote stage and the phenotype here measured on promastigotes must be analysed further in the light of the global molecular adaptations of the resistant parasites (t'Kindt *et al*. 2010) and the pre-adaptations that metacyclics develop for their survival in the macrophage (Alcolea *et al*. 2010).

The results obtained on *L. donovani* contrast with findings made on *L. mexicana*. Sereno and Lemesre (1997) induced pentamidine resistance in *L. mexicana* and found no difference in the infectivity for resident mouse macrophages between the wild-type and pentamidine-resistant clones. Another study showed that metacyclogenesis and infectivity were reduced in a *L. mexicana* strain made resistant to Gliben-clamide®, an ATP-binding cassette-transporter blocker (Silva *et al*. 2004). Further work would be required to understand if the differences with our findings are related to the species considered, the nature of the drug or the experimental set-up. Indeed, in the *L. mexicana* studies, drug resistance was induced while we analysed drug-resistant clinical isolates and we previously showed that mechanisms for natural resistance could differ from those revealed during experimental induction (Decuypere *et al*. 2005). Further work is also needed to further dissect the metacyclogenesis in *L. donovani* and explore the cooperation between apoptotic and viable metacyclics in SbV-resistant strains, as this was reported to enhance pathogenesis in other *Leishmania* species (Wanderley *et al*. 2009).

Could our results imply that decades of antimonial drug pressure (already used since 1924 with progressively higher drug regimens) on *L. donovani* contributed to select fitter and more virulent pathogens? One might speculate arguing on (i) the success of this species in 're-colonizing' the Indian subcontinent after a major evolutionary bottleneck in the 60's (associated with DDT spraying) (Alam *et al*. 2009) and (ii) the high pathogenicity of that species.

Reciprocally, it could also be possible that Sb^V-resistance emerged among the fittest strains, i.e. the ones that were already best pre-adapted to the macrophage effectors. This alternative hypothesis is supported by the observation of abundant cases of primary Sb^V-resistance in *L. braziliensis* (Yardley *et al*. 2006), a species endemic in Latin America, for which the main reservoir is constituted by wild animals, on which there is obviously no drug pressure. Surveillance of *L. donovani* populations in the Indian subcontinent is of uppermost importance to follow the fate of the Sb^V-resistant parasites in the context of the new selective pressures brought by new drugs like miltefosine (now used in first line in the region). Our unpublished data do not indicate yet the occurrence of cross-resistance between both drugs, but 'fitter' parasites might be the first in which new molecular adaptations might emerge. A close monitoring of this process is foreseen in the context of the current VL elimination programme (see www.leishrisk.net/kaladrug).

REFERENCES

Alam, M. Z., Kuhls, K., Schweynoch, C., Sundar, S., Rijal, S., Shamsuzzaman, A. K., Raju, B. V., Salotra, P., Dujardin, J. C. and Schönian, G. (2009). Multilocus microsatellite typing (MLMT) reveals genetic homogeneity of *Leishmania donovani* strains in the Indian subcontinent. *Infection Genetics and Evolution* **9**, 24–31. doi: 10.1016/j.meegid.2008.09.005

Alcolea, P. J., Alonso, A., Gómez, M. J., Moreno, I., Domínguez, M., Parro, V. and Larraga, V. (2010). Transcriptomics throughout the life cycle of *Leishmania infantum*: high down-regulation rate in the amastigote stage. *International Journal for Parasitology* **40**, 1497–1516. doi: 10.1016/j.ijpara.2010.05.013

Andersson, D. I. and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Review Microbiology* **8**, 260–271. doi: 10.1038/nrmicro2319

Borrell, S. and Gagneux, S. (2009). Infectiousness, reproductive fitness and evolution of drug-resistant Mycobacterium tuberculosis. *The International Journal of Tuberculosis and Lung Disease* **13**, 1456–1466.

Cardoen, S., Van Huffel, X., Berkvens, D., Quoilin, S., Ducoffre, G., Saegerman, C., Speybroeck, N., Imberechts, H., Herman, L., Ducatelle, R. and Dierick, K. (2009). Evidence-Based semiquantitative methodology for prioritization of foodborne zoonoses. *Foodborne Pathogens and Disease* **6**, 1083–1096. doi: 10.1089/fpd.2009.0291

Carter, K. C., Hutchison, S., Boitelle, A., Murray, H. W., Sundar, S. and Mullen, A. B. (2005). Sodium stibogluconate resistance in *Leishmania donovani* correlates with greater tolerance to macrophage antileishmanial

responses and trivalent antimony therapy. *Parasitology* **131**, 747–757. doi: 10.1017/S0031182005008486

Chappuis, F., Sundar, S., Hailu, A., Ghalib, H., Rijal, S., Peeling, R. W., Alvar, J. and Boelaert, M. (2007). Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology* **5**, 873–882. doi: 10.1038/nrmicro1748

da Luz, R. I., Vermeersch, M., Dujardin, J. C., Cos, P. and Maes, L. (2009). In vitro sensitivity testing of *Leishmania* clinical field isolates: preconditioning of promastigotes enhances infectivity for macrophage host cells. *Antimicrobial Agents Chemotherapy* **53**, 5197–5203. doi: 10.1128/AAC.00866-09

Decuypere, S., Rijal, S., Yardley, V., De Doncker, S., Laurent, T., Khanal, B., Chappuis, F. and Dujardin, J. C. (2005). Gene expression analysis of the mechanism of natural Sb(V) resistance in *Leishmania donovani* isolates from Nepal. *Antimicrobial Agents and Chemotherapy* **49**, 4616–4621. doi: 10.1128/AAC.49.11.4616-4621.2005

Decuypere, S., Vanaerschot, M., Rijal, S., Yardley, V., Maes, L., de Doncker, S., Chappuis, F. and Dujardin, J. C. (2008). Gene expression profiling of *Leishmania (Leishmania) donovani*: overcoming technical variation and exploiting biological variation. *Parasitology* **135**, 183–194. doi: 10.1017/S0031182007003782

Desjeux, P. (2004). Leishmaniasis: current situation and new perspectives. *Comparative Immunology, Microbiology and Infectious Diseases* **27**, 305–318. doi: 10.1016/j.cimid.2004.03.004

Frézard, F., Demicheli, C. and Ribeiro, R. R. (2009). Pentavalent antimonials: new perspectives for old drugs. *Molecules* **14**, 2317–2336. doi: 10.3390/molecules14072317

Gamboa, D., Torres, K., De Doncker, S., Zimic, M., Arevalo, J., Dujardin, J. C. (2008). Evaluation of an *in vitro* and *in vivo* model for experimental infection with *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana*. *Parasitology* **135**, 319–326. doi: 10.1017/S0031182007003848

Haldar, A. K., Yadav, V., Singhal, E., Bisht, K. K., Singh, A., Bhaumik, S., Basu, R., Sen, P. and Roy, S. (2010). *Leishmania donovani* isolates with antimony-resistant but not -sensitive phenotype inhibit sodium antimony gluconate-induced dendritic cell activation. *PLoS Pathogens* **6**, e1000907. doi: 10.1371/journal.ppat.1000907

Hastings, I. M. and Donnelly, M. J. (2005). The impact of antimalarial drug resistance mutations on parasite fitness, and its implications for the evolution of resistance. *Drug Resistance Updates* **8**, 43–50. doi: 10.1016/j.drup.2005.03.003

Havelaar, A. H., van Rosse, F., Bucura, C., Toetenel, M. A., Haagsma, J. A., Kurowicka, D., Heesterbeek, J. H., Speybroeck, N., Langelaar, M. F., van der Giessen, J. W., Cooke, R. M. and Braks, M. A. (2010). Prioritizing emerging zoonoses in the Netherlands. *PLoS ONE* **5**, e13965. doi: 10.1371/journal.pone.0013965

Holzmuller, P., Sereno, D. and Lemesre, J. L. (2005). Lower nitric oxide susceptibility of trivalent antimony-resistant amastigotes of *Leishmania infantum*. *Antimicrobial Agents and Chemotherapy* **49**, 4406–4409. doi: 10.1128/AAC.49.10.4406-4409.2005

Knuepfer, E., Stierhof, Y. D., McKean, P. G. and Smith, D. F. (2001). Characterization of a differentially expressed protein that shows an unusual localization to intracellular membranes in *Leishmania major*. *The Biochemical Journal* **356**, 335–344.

Martínez-Calvillo, S., Vizuet-de-Rueda, J. C., Florencio-Martínez, L. E., Manning-Cela, R. G. and Figueroa-Angulo, E. E. (2010). Gene expression in trypanosomatid parasites. *Journal of Biomedicine and Biotechnology* 525241. Epub. doi: 10.1155/2010/525241

Mikus, J. and Steverding, D. (2000). A simple colorimetric method to screen drug cytotoxicity against *Leishmania* using the dye Alamar Blue. *Parasitology International* **48**, 265–269.

Mookerjee Basu, J., Mookerjee, A., Sen, P., Bhaumik, S., Sen, P., Banerjee, S., Naskar, K., Choudhuri, S. K., Saha, B., Raha, S. and Roy, S. (2006). Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. *Antimicrobial Agents and Chemotherapy* **50**, 1788–1797. doi: 10.1128/AAC.50.5.1788-1797.2006

Natera, S., Machuca, C., Padrón-Nieves, M., Romero, A., Díaz, E. and Ponte-Sucre, A. (2007). *Leishmania* spp.: proficiency of drug-resistant parasites. *International Journal of Antimicrobial Agents* **29**, 637–642. doi: 10.1016/j.ijantimicag.2007.01.004

Rijal, S., Chappuis, F., Singh, R., Bovier, P. A., Acharya, P., Karki, B. M., Das, M. L., Desjeux, P., Loutan, L. and Koirala, S. (2003). Treatment of visceral leishmaniasis in south-eastern Nepal: decreasing efficacy of sodium stibogluconate and need for a policy to limit

further decline. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **97**, 350–354.

Rijal, S., Yardley, V., Chappuis, F., Decuypere, S., Khanal, B., Singh, R., Boelaert, M., De Doncker, S., Croft, S. and Dujardin, J. C. (2007). Antimonial treatment of visceral leishmaniasis: are current in vitro susceptibility assays adequate for prognosis of in vivo therapy outcome? *Microbes and Infection* **9**, 529–535. doi: 10.1016/j.micinf.2007.01.009

Sacks, D. L. (1989). Metacyclogenesis in *Leishmania* promastigotes. *Experimental Parasitology* **69**, 100–103.

Samant, M., Sahasrabuddhe, A. A., Singh, N., Gupta, S. K., Sundar, S. and Dube, A. (2007). Proteophosphoglycan is differentially expressed in sodium stibogluconate-sensitive and resistant Indian clinical isolates of *Leishmania donovani*. *Parasitology* **134**, 1175–1184. doi: 10.1017/S0031182007002569

Saraiva, E. M., Pinto-da-Silva, L. H., Wanderley, J. L., Bonomo, A. C., Barcinski, M. A. and Moreira, M. E. (2005). Flow cytometric assessment of *Leishmania* spp metacyclic differentiation: validation by morphological features and specific markers. *Experimental Parasitology* **110**, 39–47. doi: 10.1016/j.exppara.2005.01.004

Sereno, D. and Lemesre, J. L. (1997). *In vitro* life cycle of pentamidine-resistant amastigotes: stability of the chemoresistant phenotypes is dependent on the level of resistance induced. *Antimicrobial Agents and Chemotherapy* **41**, 1898–1903.

Silva, N., Camacho, N., Figarella, K. and Ponte-Sucre, A. (2004). Cell differentiation and infectivity of *Leishmania mexicana* are inhibited in a strain resistant to an ABC-transporter blocker. *Parasitology* **128**, 629–634.

Speybroeck, N., Berkvens, D., Mfoukou-Ntsakala, A., Aerts, M., Hens, N., Van Huylenbroeck, G. and Thys, E. (2004). Classification trees versus multinomial models in the analysis of urban farming systems in Central Africa. *Agricultural Systems* **80**, 133–149.

Sundar, S. (2001). Drug resistance in Indian visceral leishmaniasis. *Tropical Medicine & International Health* **6**, 849–854.

Thakur, C. P., Meenakshi Thakur, A. K. and Thakur, S. (2009). Newer strategies for the kala-azar elimination programme in India. *The Indian Journal of Medical Research* **129**, 102–104.

Thakur, C. P., Mitra, D. K. and Narayan, S. (2003). Skewing of cytokine profiles towards T helper cell type 2 response in visceral leishmaniasis patients unresponsive to sodium antimony gluconate. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **97**, 409–412.

t'Kindt, R., Scheltema, R. A., Jankevics, A., Brunker, K., Rijal, S., Dujardin, J. C., Breitling, R., Watson, D. G., Coombs, G. H. and Decuypere, S. (2010). Metabolomics to unveil and understand phenotypic diversity between pathogen populations. *PLoS Neglected Tropical Diseases* **4**, e904. doi: 10.1371/journal.pntd.0000904

Uliana, S. R., Goyal, N., Freymüller, E. and Smith, D. F. (1999). *Leishmania*: overexpression and comparative structural analysis of the stage-regulated meta 1 gene. *Experimental Parasitology* **92**, 183–191. doi: 10.1006/expr.1999.4410

Vanaerschot, M., Maes, I., Ouakad, M., Adaui, V., Maes, L., De Doncker, S., Rijal, S., Chappuis, F., Dujardin, J. C. and Decuypere, S. (2010). Linking *in vitro* and *in vivo* survival of clinical *Leishmania donovani* strains. *PLoS One* **5**, e12211. doi: 10.1371/journal.pone.0012211

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, RESEARCH0034.

Walliker, D., Hunt, P. and Babiker, H. (2005). Fitness of drug-resistant malaria parasites. *Acta Tropica* **94**, 251–259. doi: 10.1016/j.actatropica.2005.04.005

Wanderley, J. L., Pinto da Silva, L. H., Deolindo, P., Soong, L., Borges, V. M., Prates, D. B., de Souza, A. P., Barral, A., Balanco, J. M., do Nascimento, M. T., Saraiva, E. M. and Barcinski, M. A. (2009). Cooperation between apoptotic and viable metacyclics enhances the pathogenesis of Leishmaniasis. *PLoS One* **4**, e5733. doi: 10.1371/journal.pone.0005733

Yardley, V., Ortuno, N., Llanos-Cuentas, A., Chappuis, F., Doncker, S. D., Ramirez, L., Croft, S., Arevalo, J., Adaui, V., Bermudez, H., Decuypere, S. and Dujardin, J. C. (2006). American tegumentary leishmaniasis: Is antimonial treatment outcome related to parasite drug susceptibility? *Journal of Infectious Diseases* **194**, 1168–1175. doi: 10.1086/507710

Zakai, H. A., Chance, M. L. and Bates, P. A. (1998). *In vitro* stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana*. *Parasitology* **116**, 305–309.