Comparison of gene expression patterns among *Leishmania braziliensis* clinical isolates showing a different *in vitro* susceptibility to pentavalent antimony

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SUMMARY

Introduction. Evaluation of *Leishmania* drug susceptibility depends on *in vitro* Sb^V susceptibility assays, which are labour-intensive and may give a biased view of the true parasite resistance. Molecular markers are urgently needed to improve and simplify the monitoring of Sb^V-resistance. We analysed here the gene expression profile of 21 *L. braziliensis* clinical isolates *in vitro* defined as Sb^V-resistant and -sensitive, in order to identify potential resistance markers. **Methods**. The differential expression of 13 genes involved in Sb^V metabolism, oxidative stress or housekeeping functions was analysed during *in vitro* promastigote growth. **Results**. Expression profiles were up-regulated for 5 genes only, each time affecting a different set of isolates (mosaic picture of gene expression). Two genes, *ODC* (ornithine decarboxylase) and *TRYR* (trypanothione reductase), showed a significantly higher expression rate in the group of Sb^V-resistant compared to the group of Sb^V-sensitive parasites ($P<0{\cdot}01$). However, analysis of individual isolates showed both markers to explain only partially the drug resistance. **Discussion**. Our results might be explained by (i) the occurrence of a pleiotropic molecular mechanism leading to the *in vitro* Sb^V resistance and/or (ii) the existence of different epi-phenotypes not revealed by the *in vitro* Sb^V susceptibility assays, but interfering with the gene expression patterns.

Key words: *Leishmania (Viannia) braziliensis*, antimony resistance, gene expression profiling, promastigotes, clinical isolates.

INTRODUCTION

Protozoan parasites of the genus *Leishmania* cause a broad spectrum of diseases, collectively known as leishmaniasis, that occur predominantly in tropical and subtropical regions. It is estimated that worldwide there is an annual incidence of 1·5–2 million new cases, with up to 350 million people at risk of infection (Murray *et al*. 2005). Chemotherapy is the main control strategy for leishmaniasis and pentavalent antimonials (Sb^V) remain the mainstay, but their efficacy is threatened by the emergence of drug-resistant *Leishmania* parasites, as described in several endemic regions (Lira *et al*. 1999; Hadighi *et al*. 2006; Rojas *et al*. 2006; Yardley *et al*. 2006).

The *in vitro* intracellular amastigote-macrophage model is currently considered the gold standard for susceptibility testing of *Leishmania* clinical isolates (Vermeersch *et al*. 2009). But the screening entails a complex, labour-intensive and time-consuming protocol, which involves *in vitro* infection of primary macrophages with an infective stage of *Leishmania* parasites (metacyclic promastigotes, axenic amastigotes, or *ex vivo* amastigotes), 3–7 days Sb^V exposure and the final step of microscopical evaluation of the different infections (Neal and Croft, 1984; Vermeersch *et al*. 2009). In view of this scenario, research is needed to identify molecular markers that would by-pass these limitations and would improve the epidemiological surveillance of Sb^V-resistance in the field. These markers are not yet available, due to the limited knowledge of the molecular and biochemical mechanisms underlying resistance to Sb^V, especially in clinical isolates.

Even if intracellular amastigotes may be considered as the clinically relevant stage, the current protocols to study their gene expression require extreme care to ensure reproducibility of the results

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Table 1. Geographical origin, *in vitro* Sb^V and Sb^{III} susceptibility data of Peruvian *Leishmania braziliensis* isolates included in the study and corresponding clinical data: disease (CL, cutaneous leishmaniasis, ML, mucosal leishmaniasis) and treatment outcome (TF, treatment failure; C, cure)

International Code *L. braziliensis*	Geographical origin (state, province)	A.I. $Sb^{V¥}$	A.I. $Sb^{III¥}$	Disease	Treatment ouctome
Sb^V-sensitive isolates					
MHOM/PE/01/PER005/0	Loreto, Ucayali	**1**	ND	CL	TF
MHOM/PE/03/PER130/0	Cusco, Echarate	**1**	0	CL	TF
MHOM/PE/03/PER163/0	Huánuco, Leoncio Prado	**2**	0	CL	C
MHOM/PE/03/PER186/0	Junín, Satipo	**2**	1	CL	C
Sb^V-resistant isolates					
MHOM/PE/01/PER002/0	Madre de Dios, Tambopata	**6**	2	CL	TF
MHOM/PE/01/PER006/1	Junín, Satipo	**6+**	ND	CL	TF
MHOM/PE/01/PER012/1	Cusco, Calca	**6+**	ND	CL	TF
MHOM/PE/01/PER014/0	Junín, Satipo	**6+**	ND	CL	TF
MHOM/PE/02/PER015/0	Ucayali, Coronel Portillo	**6+**	2	CL	TF
MHOM/PE/02/PER016/0	Huánuco, Puerto Inca	**6+**	ND	CL	C
MHOM/PE/02/PER067/0	Cusco, La Convención	**6+**	ND	CL	TF
MHOM/PE/02/PER086/0	Pasco, Oxapampa	**6+**	0	CL	TF
MHOM/PE/02/PER094/0	Huánuco, Puerto Inca	**6**	2	CL	C
MHOM/PE/02/PER104/0	Madre de Dios, Tambopata	**6+**	6+	CL	TF
MHOM/PE/02/PER122/0	Madre de Dios, Tambopata	**6+**	ND	CL	C
MHOM/PE/03/PER157/0	Madre de Dios, Tambopata	**6+**	2	CL	C
MHOM/PE/03/PER182/0	Ayacucho, La Mar	**6**	5	CL	C
MHOM/PE/03/PER201/0	Loreto, Requena	**6**	1	ML	C
MHOM/PE/03/PER215/0	Ucayali, Coronel Portillo	**6**	2	ML	C
MHOM/PE/03/PER231/0	Junín, Satipo	**5**	2	ML	C
MHOM/PE/03/PER260/0	Madre de Dios, Tahuamanu	**6**	2	ML	C

¥ The *in vitro* Sb^V or Sb^{III} susceptibility of a tested isolate was expressed as an 'activity index' (A.I.), i.e. as the ratio of the ED_{50} (50% effective dose) of that tested isolate to the ED_{50} of the WHO reference *L. braziliensis* strain MHOM/BR/75/M2903. Isolates with an A.I. of 0–2 were considered sensitive to Sb^V or Sb^{III} (0, more sensitive than the reference strain M2903), while isolates with an A.I. of 3 or higher were considered resistant. Data shown were reported by Yardley *et al*. 2006.

(Decuypere *et al*. 2008) and are not adapted to the study of a large number of isolates. In contrast, promastigotes can be easily cultivated and harvested with minimal risk of affecting their biological state under well-controlled *in vitro* conditions (Decuypere *et al*. 2008). Furthermore, molecular changes during promastigote differentiation serve as pre-adaptations for parasite transmission and initial infection stages in the vertebrate host (Sacks, 1989; Saxena *et al*. 2003). Hence, a time-course analysis of gene expression changes throughout the promastigote growth cycle may show differences in molecular adaptations of parasites to intracellular life. Our previous study on *L. donovani* clinical isolates indicated that promastigote expression-curves could indeed reveal molecular features distinguishing isolates with different *in vitro* Sb^V susceptibility phenotypes (Decuypere *et al*. 2008). Moreover, considering that the *in vitro* growth rates could vary among different isolates, expression-curves are advisable to guarantee a reliable molecular comparison of identical developmental stages among various isolates under study.

In the present work, we aimed to compare the gene expression patterns of 21 *L. braziliensis* clinical isolates showing 2 different phenotypes of *in vitro* Sb^V susceptibility (respectively sensitive and resistant). Promastigotes were used, as this life-stage can be easily cultured for high-throughput applications. We used real-time quantitative PCR (qPCR) to assess the RNA expression profiles of 13 genes encoding proteins with roles in Sb^V metabolism, oxidative stress or housekeeping functions and compared molecular data to the 2 *in vitro* defined Sb^V phenotypes.

MATERIALS AND METHODS

Parasites and in vitro *culture*

In total, 21 *L. braziliensis* isolates were obtained from confirmed cutaneous or mucosal leishmaniasis patients recruited at the Institute of Tropical Medicine Alexander von Humboldt in Lima, Peru, within the framework of LeishNatDrug-R, a multicentre study on Sb^V treatment failure in leishmaniasis. Isolates were essentially obtained before treatment of patients, typed by PCR-RFLP analysis of *hsp70* and *cpb* genes (Garcia *et al*. 2005) and tested as intracellular amastigotes for their *in vitro* susceptibility to Sb^V (Yardley *et al*. 2006) (see Table 1 for

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summary of isolate features). Promastigote forms were grown at 24 °C in a biphasic medium consisting of rabbit blood agar overlaid with medium 199 (M199; Sigma) containing 20% heat-inactivated fetal bovine serum (FBS; Lonza Bioscience), 25 mM Hepes (pH 7·4), 100 units/ml penicillin and 100 μg/ml streptomycin (Lonza). Growth curves were initiated by inoculating 3×10^6 parasites/ml in 5 ml of M199-20% FBS. Two independently grown cultures and corresponding harvests at 24 h (early-log phase), 72 h (late-log phase), 120 h (early-stationary phase) and 168 h (late-stationary phase) time-points were performed in parallel for each isolate (biological replicates). RNA sampling protocols used here were described previously (Decuypere *et al*. 2005). All studied isolates showed similar growth behaviour during *in vitro* culture, thereby allowing synchronization of the promastigote growth cycle among isolates. The *in vitro* passage numbers (post-isolation from patients) were: (i) in the group of Sb^V-sensitive isolates: mean ± standard deviation (S.D.): 20·5 ± 5·9; (ii) in the group of Sb^V-resistant isolates: mean ± S.D.: 19·9 ± 4·5. There was no statistically significant difference in the mean passage number between isolate groups ($P = 0·9$, *t*-test). Previous work of our group showed that the *in vitro* Sb^V-resistant phenotype was stable far beyond these passage numbers (Laurent *et al*. 2007, and unpublished results).

RNA isolation and real-time quantitative PCR

Total RNA was isolated, analysed and reverse transcribed as described before (Decuypere *et al*. 2005). The resulting cDNA was diluted 10-fold, and 2 μl was used as template in 25 μl of SYBR Green-based quantitative PCR (qPCR) reactions on the iCycler (Bio-Rad), as previously described (Decuypere *et al*. 2005), with only the modification that amplification was done for 34 cycles. We analysed 13 genes. First, a set of 8 genes putatively involved in Sb^V metabolism and/or implicated in laboratory-induced resistance (Ashutosh *et al*. 2007). This set includes genes with predicted function in transport (*LbAQP1, MRPA*), thiol biosynthesis/redox metabolism (*GSH1, GSH2, ODC, TRYR*) and cellular reduction (*ACR2, TDR1*). Secondly, 2 genes resulting from a differential screen of *L. braziliensis* Sb^V-resistant and -sensitive isolates and putatively involved in RNA poly(A)-tail metabolism (*PABP, PAP14*) (Decuypere, 2007). Thirdly, 2 genes coding for housekeeping functions: *Actin* (cytoskeleton function) and *S8* (ribosomal function). Fourthly, the *META1* gene, up-regulated in the infective metacyclic stage of distinct *Leishmania* species (Uliana *et al*. 1999; Gamboa *et al*. 2007), was analysed using primers and conditions reported elsewhere (Gamboa *et al*. 2007).

Full-length gene sequences in *L. major* and *L. infantum* genome databases were used as queries for BLAST searches in the *L. braziliensis* genome database (GeneDB, http://www.genedb.org/). Primers (Table 2) were designed with Primer3 (Rozen and Skaletsky, 2000) and tested for specificity using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

Data analysis and statistics

Analysis was performed on duplicate biological samples that were each assayed in triplicate. The arithmetic average threshold cycle (Ct) was used for data analysis. For each primer set, reaction efficiency estimates were derived from standard curves generated by serial dilutions of a cDNA pool of a promastigote sample. Efficiencies ranged between 1·84 and 2·07 and correlation coefficients were $\geqslant 0·997$ (iCycler 3.1 software, Bio-Rad).

The Ct values of each qPCR run were imported as Excel files into qBase*Plus* (Biogazelle NV, Zulte, Belgium), a software for real-time PCR data analysis based on the geNorm method (Vandesompele *et al*. 2002) and qBase technology (Hellemans *et al*. 2007). Four genes (*ACR2, GSH2, PAP14* and *TDR1*) showed the most stable expression in our sample panel (geNorm stability mean M-value and mean coefficient of variation lower than 0·35 and 15%, respectively) and data were normalized to their geometric mean. The analysis of 13 genes in 168 samples (21 parasite isolates × 4 time-points in duplicate harvests) could be performed as one integrated experiment by performing inter-plate calibrations (based on 3 cDNA samples included in each qPCR run for that purpose). Overall, 1084 out of 1092 data points did not differ by more than 20% in CV (coefficient of variation) of normalized relative quantities between biological replicates, demonstrating the reproducibility of our methodology (Table 2).

The fold change of gene expression between log- and stationary-phase promastigotes (time-points 24 h and 72 h *vs* 120 h and 168 h of the growth curves, respectively) was determined for each parasite isolate, further called FC-PRO. The linear component of the variability of the expression level of each gene during *in vitro* growth was modelled in a multiple linear regression. Predictors tested were the examined time-points during *in vitro* promastigote growth and the Sb^V susceptibility of the studied isolates. The significance of the regression coefficient corresponding to the Sb^V-susceptibility was used to test difference in intercepts (change in baseline or initial gene expression level) between the Sb^V-sensitive (further called Sb^V-S) and Sb^V-resistant (further called Sb^V-R) isolates. The interaction between the examined time and the Sb^V susceptibility was

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Table 2. Genes selected for expression profiling of *Leishmania braziliensis*: primer sequences, qPCR conditions and reproducibility of assays

Gene	Product	Function/Relevance	GeneDB ID	Forward/Reverse Primer[a]	Primer final concentration (nM)	% CV[b]
LbAQP1[c]	aquaglyceroporin	membrane channel for transport of water and small solutes; possible role in volume regulation and osmotaxis	LbrM31_V2.0020	5′ CTTTGCGGTGTGGAGTGAGATA 3′ 5′ CCAGAGTTGATACCTGTCGTGATAC 3′	600	7·6
MRPA	ABC-thiol transporter	intracellular ABC transporter, presumably involved in sequestration metal-thiol conjugates	LbrM23_V2.0280	5′ AAGTGGGCAGCGACTCAAA 3′ 5′ CCAGTTCAGCGTCTCCGTT 3′	400	4·6
ACR2[¥]	hypothetical protein, conserved	putative arsenate reductase 2; protein tyrosine phosphatase activity and metal reductase activity in presence of glutathione	LbrM32_V2.2980	5′ CAAACCTGATTCGTTCGCCA 3′ 5′ CAATGAAGCCGCAGTCTCG 3′	500	3·3
TDR1	thiol-dependent reductase 1	homologue of glutathione S-transferase	LbrM31_V2.0550	5′ GCTTCTTCCTGGACAACGC 3′ 5′ CTCAAACTCAGCCTTCGCATC 3′	500	3·9
GSH1[c]	gamma-glutamylcysteine synthetase, putative (γ-GCS)	key enzyme in glutathione biosynthesis (rate limiting step)	LbrM18_V2.1700	5′ CTACGACTCTATCTCCATCTTCATCA 3′ 5′ CACACCAGCCTTCTCCAGC 3′	600	5·7
GSH2	glutathione synthetase, putative (GS)	second step in glutathione biosynthesis (downstream of γ-GCS)	LbrM14_V2.0880	5′ ACGAAGAGCGACGACCCA 3′ 5′ GTGACGCCGTAGATGCCA 3′	400	3·0
ODC	ornithine decarboxylase, putative	key enzyme in spermidine biosynthesis	LbrM12_V2.0300	5′ CAGTCTGTCGTGCCGATGGA 3′ 5′ CGTTCAGCAGGAGAAGCGTC 3′	500	4·9
TRYR	trypanothione reductase	central oxidoreductase for maintenance cellular redox potential	LbrM05_V2.0350	5′ GAAAAGGATGGCGAGGTGC 3′ 5′ AGATGCCTACGCTCTGAATGAT 3′	500	6·2
Actin[d]	actin	cytoskeletal structural protein	LbrM04_V2.1250	5′ GGCGAACGAGGAGTCATTTG 3′ 5′ ATCAGCGACGGCTTGAACAG 3′	500	5·1
S8[c,d]	40S ribosomal protein S8, putative	structural constituent of large subunit ribosome	LbrM24_V2.2160	5′ CGACTTGGATGCGGGGA 3′ 5′ GGCGAAGCCTTGTTCACG 3′	600	6·0
PAP14[c,d]	poly(A) polymerase, putative	polyadenylation precursor mRNA	LbrM14_V2.1350	5′ CCTGCTACAATGTTACCCTCACC 3′ 5′ GAACTTCGCCTCCGCCTC 3′	300	3·4
PABP[d]	RNA-binding protein, putative	putative poly(A)-binding protein; co-factor for polyadenylation	LbrM30_V2.2560	5′ CGCCAGGGAAACCCAAAG 3′ 5′ CCGTGTAGAGACTGCCCATCC 3′	400	3·1
META1[c]	hypothetical protein, conserved	putative infective insect stage-specific protein META1	LbrM17_V2.0980	5′ GGGCAGCGATGACTTGAT 3′ 5′ CACCAACTTGCCATCCTC 3′	400	7·4

GeneDB ID, gene Accession number on GeneDB (http://www.genedb.org/); Product, predicted gene function based on orthologues in the different TriTrypDB organisms [annotation from GeneDB and TriTrypDB (http://tritrypdb.org/tritrypdb/) databases].

[a] *Leishmania*-specific primer sets.

[b] Mean coefficient of variation [CV = (standard deviation/mean)*100] of normalized relative quantities between biological replicates (calculated across all measurements).

[c] Primers and qPCR conditions as reported previously (Gamboa *et al*. 2007).

[d] Primers developed by Decuypere (2007).

[¥] Potential orthologous gene and protein sequences in *L. major* (LmjF32.2740 at GeneDB) are annotated as *ACR2* and Sb(V)-As(V) reductase (LmACR2) at GenBank (Accession numbers: AY567836.1, AAS73185.1, respectively).

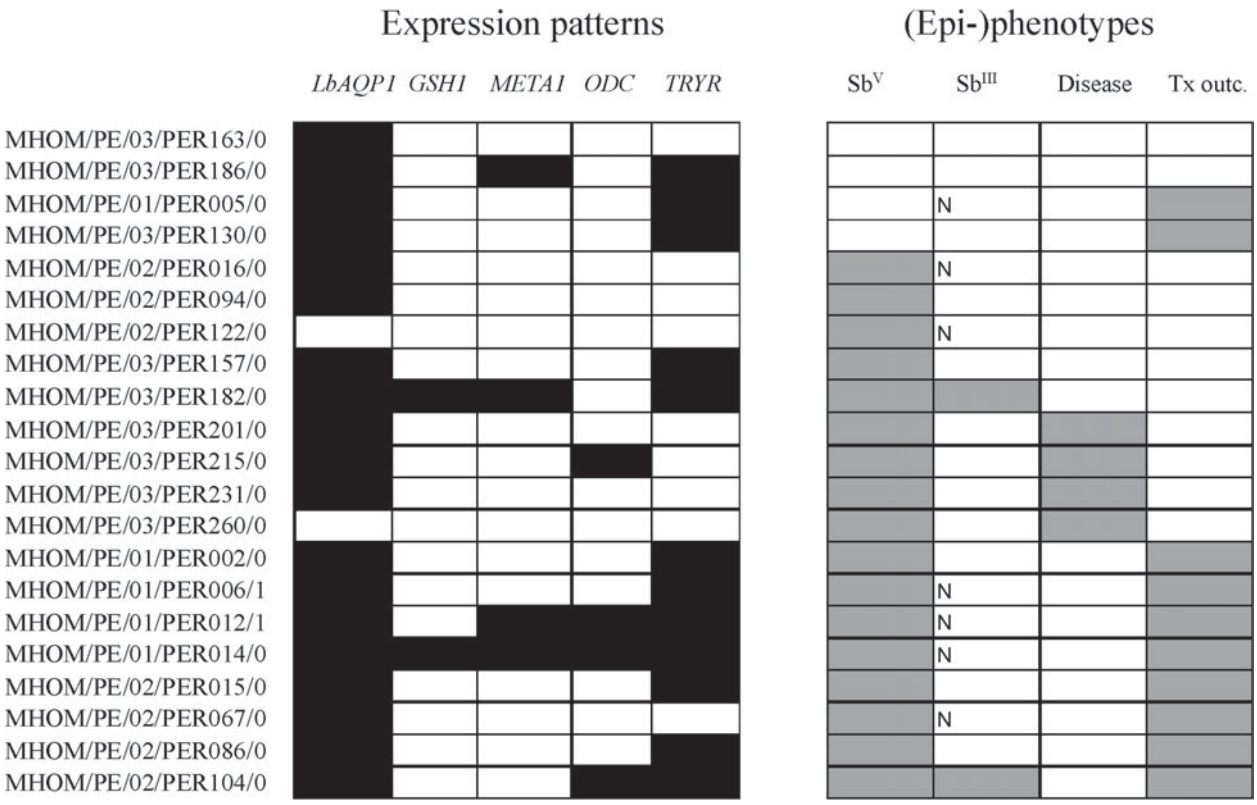

Fig. 1. Gene expression and phenotype mosaic. Schematic representation of the significantly modulated gene expression (highlighted in black if FC-PRO $\geqslant 2$, see Materials and Methods section) in the 21 isolates here studied. Schematic representation of the phenotype diversity: (i) *in vitro* Sb^V susceptibility (grey if resistant), (ii) *in vitro* Sb^{III} susceptibility (grey if resistant; N, not available), (iii) clinical form (grey if ML, otherwise CL) and (iv) treatment outcome (grey if failure, otherwise cure).

included, and the significance of the regression coefficient corresponding to the interaction term was used to test difference in slopes (change in gene expression level along time) between the Sb^V-S and Sb^V-R isolates. To increase the strength of the evidence, a significance level of 0·01 was considered. To achieve biological significance we only considered cases where the slope ratio or the intercept ratio (between the Sb^V-S and Sb^V-R groups) was $\geqslant 2$, as recommended elsewhere (McCarthy and Smyth, 2009).

A multiple logistic regression to model the Sb^V susceptibility using the *ODC* and *TRYR* slopes (gene expression rate) and intercepts (baseline gene expression level) as predictors for all the isolates was performed. A linear score to estimate the probability of Sb^V-resistance was developed based on the best regression model. An isolate was predicted to be Sb^V-resistant if the probability was greater than the best cutoff (Pr-cutoff). A Receiver Operating Characteristic curve (ROC) was calculated and the Pr-cutoff was estimated in order to maximize the Youden's J-index (Sensitivity + Specificity − 1) (Youden, 1950). The 95% Confidence Intervals (CI) for the estimated sensitivity and specificity were calculated for proportions following a binomial distribution. All the analysis was performed using the statistical software Stata 10 (StataCorp).

RESULTS

Gene expression profiling throughout the promastigote growth cycle of L. braziliensis *isolates*

In order to assess for each gene the extent of expression regulation during *in vitro* promastigote growth/differentiation of *L. braziliensis*, we examined the fold change in mRNA abundance from log- to stationary phase (FC-PRO) in all isolates. The expression level of 8 genes did not change significantly during the promastigote growth cycle (FC-PRO <2), a pattern common to all 21 isolates. Genes in this category include: *ACR2, Actin, GSH2, MRPA, PABP, PAP14, S8* and *TDR1*. Five genes showed a significant up-regulation of expression (FC-PRO $\geqslant 2$), each of them in a different set of isolates, hereby providing a 'mosaic' picture of gene expression in the present sample (Fig. 1). Three of these genes showed significant up-regulation in very few isolates: *GSH1* (2 isolates, both Sb^V-R), *META1* (4 isolates – one Sb^V-S and 3 Sb^V-R) and *ODC* (4 isolates, all Sb^V-R). The last two genes, *LbAQP1*

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and *TRYR*, showed a FC-PRO $\geqslant 2$ in more than 50% of the isolates (19/21 isolates for *LbAQP1*; 12/21 isolates for *TRYR*). The isolates showing growth-dependent changes in *LbAQP1* corresponded to the 4 Sb^V-S isolates and 15/17 Sb^V-R isolates. As for *TRYR*, 3/4 Sb^V-S isolates and 9/17 Sb^V-R isolates showed modulated expression during promastigote growth. Interestingly, when other phenotypes associated with the parasites were taken into consideration, it appeared that 9/10 isolates from patients with treatment failure showed a significant up-regulation of *TRYR* expression during growth, *vs* 3/11 from cured patients (Fig. 1).

Comparison of gene expression profiles during promastigote growth between L. braziliensis *isolates, in relation to the* in vitro *Sb^V susceptibility*

Next, we compared the gene expression patterns between Sb^V-S and Sb^V-R *L. braziliensis* isolates. We did a multiple linear regression in order to identify genes showing differential expression between these 2 groups of isolates. We did not find significant differences in the baseline expression level (intercept); only the slopes were informative, hereby further validating the importance of analysing gene expression curves. A significant difference was observed between Sb^V-S and Sb^V-R isolates in slopes for 2 genes, *ODC* and *TRYR* (Table 3). The change in expression level along time (slope) of both genes in the group of Sb^V-R isolates was significantly higher than that in the group of Sb^V-S isolates ($P<0{\cdot}01$) (Table 3, Fig. 2). For the rest of the examined genes, the slopes of the expression curves were similar between the 2 groups of Sb^V-S and Sb^V-R isolates (Table 3).

Focusing on *ODC* and *TRYR* genes, we also assessed the slopes of the expression curves of individual isolates (Fig. 3A,B) and calculated the sensitivity and specificity of each marker by comparison to the *in vitro* phenotype of Sb^V-susceptibility. The best logistic model to predict Sb^V-resistance only included the expression rate (slope) of *ODC* gene ($P=0{\cdot}06$). Based on the Pr-cutoff that maximized the Youden's J index, the sensitivity and specificity of the *ODC* expression rate to detect Sb^V-resistance was 100% (95% CI: [100%, 100%]) and 50% (95% CI: [28%, 71%]) respectively. Reciprocally, specificity of 100% was associated with a sensitivity of 24%. The sets of sensitivity/specificity and their relationship are shown in Fig. 4a. The assay efficacy, measured by Youden's J index, was 0·5, and the area under the ROC curve was 0·72. With regard to data of *TRYR*, the logistic model to predict Sb^V-resistance did not achieve statistical significance ($P=0{\cdot}61$). Based on the Pr-cutoff that maximized the Youden's J index, the sensitivity and specificity of the *TRYR* expression rate to detect Sb^V-resistance was 76% (95% CI: [51%, 92%]) and 50% (95% CI: [44%, 65%]) respectively (Fig. 4c). The assay efficacy, measured by Youden's J index, was 0·26, and the area under the ROC curve was 0·53.

Table 3. Comparison of slope and intercept parameters of modelled expression levels of examined genes between Sb^V-sensitive and Sb^V-resistant *Leishmania braziliensis* isolates

Gene	Sb^V-S isolates ($n=4$) Slope/ Intercept§	Sb^V-R isolates ($n=17$) Slope/ Intercept§	Slope/ Intercept difference Sb^V-S vs. Sb^V-R isolates *P*-value
ACR2	*0·0011*	*−0·0004*	0·0410
	0·9284	0·9857	0·2400
Actin	*−0·0014*	*−0·0033*	0·3990
	1·0556	1·3199	0·0690
LbAQP1	*0·0122*	*0·0087*	0·2910
	0·0921	0·2699	0·4230
GSH1	*0·0047*	*0·0050*	0·4230
	0·6191	0·5966	0·8220
GSH2	*0·0004*	*−0·0004*	0·3450
	0·9506	1·0091	0·2670
META1	*0·0083*	*0·0010*	0·0430
	0·4905	0·7126	0·3450
MRPA	*0·0015*	*0·0011*	0·7080
	0·8588	0·9118	0·4920
ODC	***0·0029***	***0·0092***	**0·0010**
	0·6869	0·4715	0·0850
PABP	*0·0006*	*0·0006*	0·9960
	1·0421	0·9760	0·5400
PAP14	*0·0002*	*0·0011*	0·1720
	1·0495	0·9214	0·0050
S8	*−0·0009*	*0·0004*	0·4050
	1·0409	1·0644	0·8180
TDR1	*−0·0015*	*−0·0006*	0·4420
	1·0946	1·1209	0·7120
TRYR	***0·0038***	***0·0121***	**0·0000**
	0·5116	0·3703	0·2410

Sb^V-S, *in vitro* sensitive to pentavalent antimony; **Sb^V-R**, *in vitro* resistant to pentavalent antimony; *n*, number of isolates in each group.

§ Shown are the slopes (first line in italics) and the intercepts (second line) derived from multiple linear regression analysis of the variability of the expression level of each gene during promastigote growth/differentiation. Genes for which (i) statistically significant comparisons ($P<0{\cdot}01$) between the groups of Sb^V-S and Sb^V-R isolates and (ii) ratio between slopes or intercepts was $\geqslant 2$ (see Materials and Methods section) are highlighted in bold text.

Comparison of gene expression profiles during promastigote growth between L. braziliensis *isolates, in relation to the treatment outcome*

Finally, we analysed among individual isolates the sensitivity and specificity of *ODC* and *TRYR* slope values in comparison to the antimonial treatment outcome. With regard to data of *ODC* and *TRYR*,

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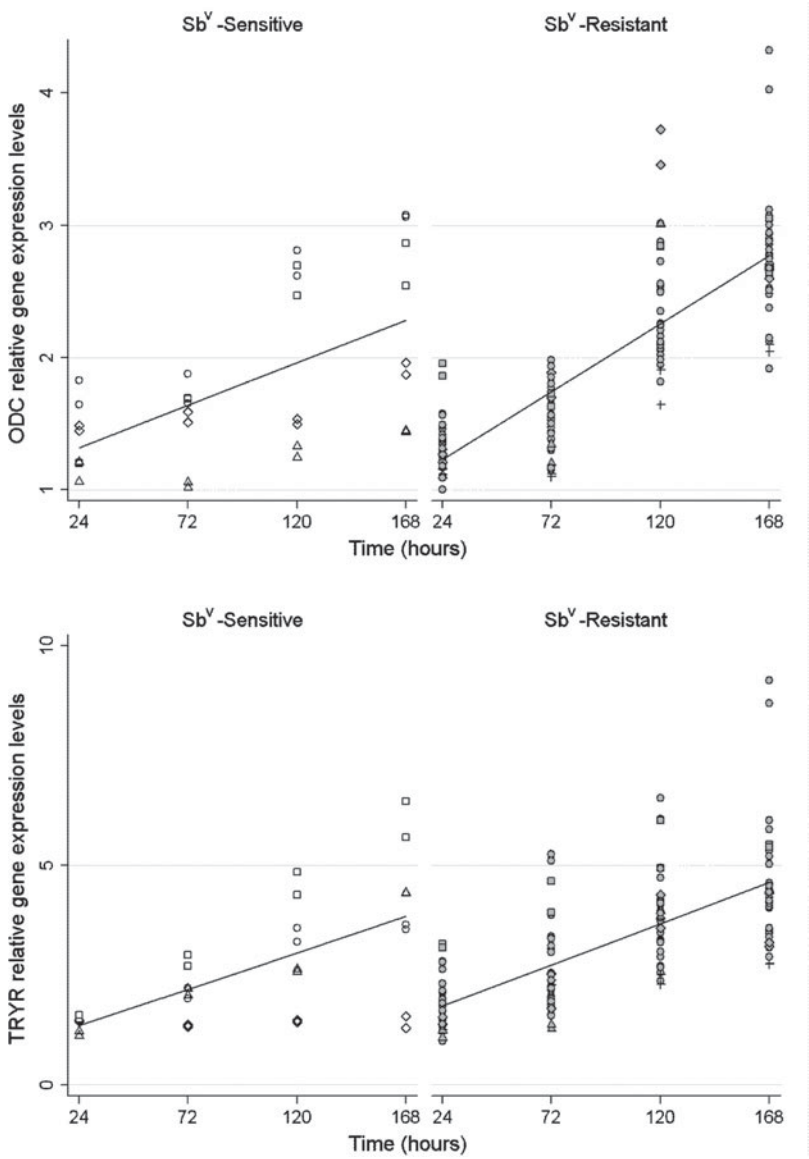

Fig. 2. Differential expression rate of genes *ODC* and *TRYR* between Sb^V-sensitive ($n=4$) and Sb^V-resistant ($n=17$) *Leishmania braziliensis* isolates. The relative expression levels of each gene [re-scaled relative to the sample with the lowest expression] and the estimated linear regression lines are shown for each group of isolates. The 2 independent biological replicates analysed at each time-point for a given isolate are labelled with the same symbol. Time-points during *in vitro* promastigote growth/differentiation: 24 h, parasites harvested at early-log phase; 72 h, parasites harvested at late-log phase; 120 h, parasites harvested at early-stationary phase; 168 h, parasites harvested at late-stationary phase.

the logistic model to predict treatment outcome did not achieve statistical significance ($P=0{\cdot}291$ for *ODC*, and $P=0{\cdot}229$ for *TRYR*). Based on the Pr-cutoff that maximized the Youden's J index, the sensitivity and specificity of the *ODC* expression rate to detect antimonial treatment failure was 100% (95% CI: [100%, 100%]) and 36% (95% CI: [15%, 57%]) respectively (Fig. 4b). The assay efficacy, measured by Youden's J index, was 0·36, and the area under the ROC curve was 0·6364. With regard to data of *TRYR*, the sensitivity and specificity of the *TRYR* expression rate to detect antimonial treatment failure was 50% (95% CI: [28%, 71%]) and 90% (95% CI: [77%, 100%]) respectively (Fig. 4d). The assay efficacy, measured by Youden's J index, was 0·41, and the area under the ROC curve was 0·70.

DISCUSSION

The present paper is, to our knowledge, the first to report results of the molecular characterization of clinical isolates of *L. braziliensis* in the context of *in vitro* Sb^V susceptibility. Our targeted approach focused on the analysis of expression profiles of genes putatively involved in Sb^V metabolism and/or implicated in laboratory-induced resistance (Ashutosh *et al.* 2007) in the present collection of *L. braziliensis* isolates, naturally Sb^V-resistant or

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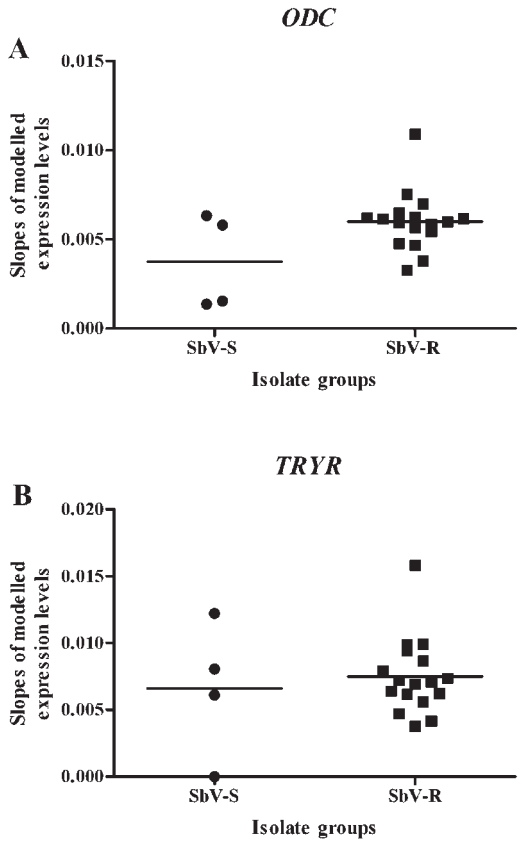

Fig. 3. Distribution of slopes corresponding to the gene expression profile of individual isolates resistant or sensitive to Sb^V, for *ODC* (A) and *TRYR* (B). The horizontal line denotes the mean of slope values in each group of isolates.

-sensitive. It allowed assessment of the extent and types of variability in gene expression patterns that occur under a natural context.

Herein, we first analysed the gene expression patterns throughout the promastigote growth cycle of *L. braziliensis* isolates, independently of their *in vitro* Sb^V susceptibility. This revealed for 8 genes a striking picture of non-modulated transcript patterns during the growth cycle. In the 5 remaining genes (*GSH1, META1* and *ODC, LbAQP1* and *TRYR*), there was a 'mosaic' of up-regulated expression among the studied isolates. In the context of stage-related gene expression, the *META1* gene deserves specific attention. Indeed, it has already been shown to be up-regulated in the infective metacyclic stage of distinct *Leishmania* species (Uliana *et al*. 1999; Gamboa *et al*. 2007), but in the present study, less than 25% of isolates exhibited a significantly higher expression in late-stationary phase (FC-PRO $\geqslant$2). Even if all our isolates were infective (as demonstrated by the *in vitro* susceptibility assays undertaken in macrophages, Yardley *et al*. 2006; Rijal *et al*. 2007), further work is needed to determine (i) if this low proportion of *META1* expression up-regulation reflects variations in the level of metacyclogenesis among our isolates as reported elsewhere (Da Silva and Sacks, 1987) or (ii) if the validity of *META1* as a metacyclogenesis marker should be questioned.

In a second stage, we analysed the gene expression patterns in the context of the *in vitro* Sb^V resistance as defined by the reference biological assays. Two genes, *ODC* and *TRYR*, showed a significantly different change in expression level (slope of the expression curve) between the 2 groups of *in vitro* Sb^V-S and Sb^V-R isolates. However, analysis of individual slopes revealed an overlap in the distribution of slopes, with a direct impact on the sensitivity and specificity of the respective markers. High sensitivity was generally accompanied by low specificity and vice-versa. Taking an arbitrary cut-off with the highest specificity, *ODC* slopes provided the best performances among the genes studied, but this corresponded to a sensitivity of 24% only. In other words, when using this marker under stringent conditions, it could only identify a fourth of the *in vitro* Sb^V resistant isolates of the present sample.

The low sensitivities of *ODC* and *TRYR* markers by comparison to the *in vitro*-defined Sb^V susceptibility could be explained in different ways. First, different mechanisms of resistance might have been selected by different *L. braziliensis* subpopulations. Thus, in a subpopulation of *L. braziliensis* isolates, alteration of *ODC* gene expression might be linked with resistance, a phenomenon reported in metal-resistant laboratory mutants (Haimeur *et al*. 1999). In another *L. braziliensis* subpopulation, other gene (s) would be involved, like *TRYR*, also reported in *L. donovani* to be related with resistance (Mandal *et al*. 2007; Mittal *et al*. 2007). In other subpopulations, other genes not detected yet could be involved. The hypothesis of a pleiotropic response of the parasite to drug pressure is supported by previous observations in *L. donovani* (Decuypere, 2007; Laurent *et al*. 2007). Analysis of additional genes possibly involved in antimony resistance might provide a clue to this question, but with the avenue of new high-throughput sequencing technologies, it would be more powerful in a next exploration stage to address the whole genome or transcriptome than to pursue with targeted, and hereby possibly biased, studies (Dujardin, 2009). Later, the most robust (set of) markers could be assembled in a simple monitoring tool.

Secondly, it is possible that molecular adaptations in *L. braziliensis* promastigote stages could be different than in *L. donovani*, impeding the discrimination of Sb^V-resistant and -sensitive isolates by gene expression studies. Our previous study on Nepalese *L. donovani* clinical isolates indicated that promastigote expression-curves could reveal molecular features distinguishing isolates with different *in vitro* Sb^V susceptibility phenotypes (Decuypere *et al*. 2008). The comparative genomic analysis of *L. major, L. infantum* and *L. braziliensis* (Peacock

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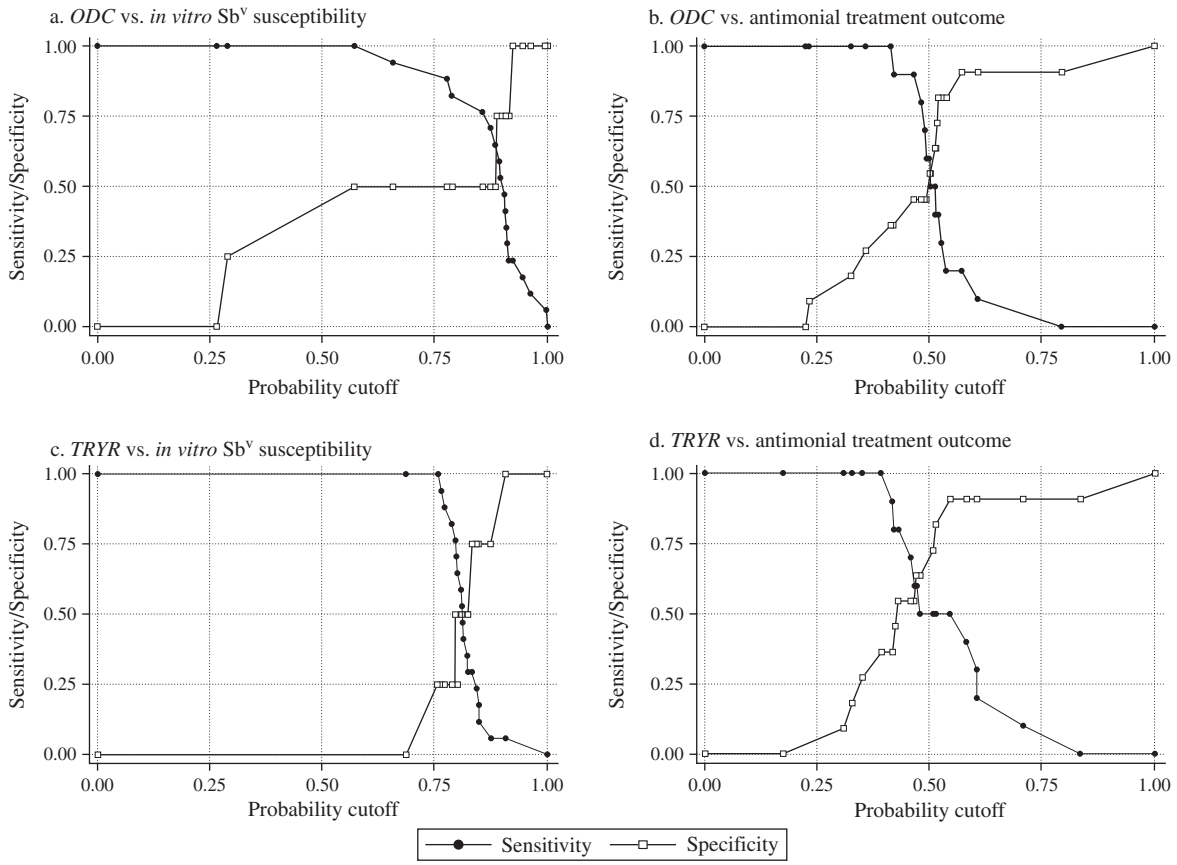

Fig. 4. Sensitivity/Specificity curves calculated from the multiple logistic regression model using the *ODC* or *TRYR* slopes as predictors of *in vitro* Sb^V susceptibility (a, c) or antimonial treatment outcome (b, d) for all the isolates.

et al. 2007; Smith *et al*. 2007) showed the presence of a putative RNAi machinery in the latter species, which might have an effect on gene expression regulation. In line with this, the comparison of the organization of the H locus conserved repeats between *L. braziliensis* and *L. major* resistant mutants did not show amplified episomal molecules in *L. braziliensis* (Dias *et al*. 2007). Furthermore, we cannot exclude that point mutations in important functional sites could be also related to resistance in *L. braziliensis*.

Thirdly, the antimony susceptibility phenotype definition, as currently established by the *in vitro* macrophage-amastigote assays, should also be considered. Indeed, different epi-phenotypes to which the parasites could have developed specific adaptations might remain hidden in the *in vitro* Sb^V susceptibility assays, but interfering with the gene expression patterns. For instance, (i) the variable adaptation to the macrophage effectors in the immunological context of the clinical infection (absent in the *in vitro* susceptibility assays) or (ii) the resistance to the reduced form of the drug, Sb^{III} (Yardley *et al*. 2006; Rijal *et al*. 2007). These epi-phenotypes would explain the incongruences between the parasites' *in vitro* Sb^V resistance and chemotherapy failure reported elsewhere (Yardley *et al*. 2006; Rijal *et al*. 2007) and illustrated in Table 1. More specifically, half of the parasites isolated before treatment from patients who later showed a definitive cure were found *in vitro* to be resistant to Sb^V. Interestingly, the *P*-value of the logistic model for *TRYR* results was much lower in the context of treatment outcome (0·229) than in the context of *in vitro* Sb^V resistance (0·61). This is in line with the observations made when classifying the isolates according to the significant up-regulation (FC-PRO $\geqslant 2$) of *TRYR* expression during growth: (i) 9/10 and 3/11 in isolates from patients with treatment failure and success respectively and (ii) 9/17 and 3/4 in isolates respectively Sb^V-resistant and sensitive. In a parallel study of Brazilian isolates, using the same protocol as here described, we did not find any informative difference in *L. braziliensis* isolates from Sb^V treatment failure, but sample size was much smaller than in the present study (Torres *et al*. 2010). However, analysis of 25 *L. guyanensis* isolates revealed an overexpression of *GSH1* in promastigotes of strains from treatment failure, hereby further validating our methodology (Torres *et al*. 2010). In the present study, *GSH1* was not as discriminative in *L. braziliensis* and this could reflect differences between species or geographical populations (Arevalo *et al*. 2007).

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Further work with larger cohorts would be needed to verify these trends. Special attention could also be paid to the analysis of parasites isolated after treatment failure (in the present study, most of them were isolated before treatment), as drug pressure could also affect or select the expression pattern of several genes.

Variation in the gene expression of *ODC* and *TRYR* might thus contribute to explain only a small part of the variation in phenotype of *in vitro* Sb^V susceptibility in the present sample of *L. braziliensis* isolates. Further work is needed to identify a marker or a set of markers of Sb^V resistance as well as the best parasite stage in which to apply it. This molecular exploration should take into account a phenotype definition that may be more complex than that revealed by the *in vitro* Sb^V biological assays. Certainly, a second step in our exploration should consider the amastigote stages. Indeed, this latter model already implied overexpressed genes in Sb^V-resistant *L. donovani* isolates (Decuypere *et al*. 2005; Mukherjee *et al*. 2007). However, this life stage is extremely sensitive to minimal disturbance of the parasite's environment during harvesting, hence extreme care is needed to 'freeze' gene expression levels instantly (Decuypere *et al*. 2008). In that sense, axenic amastigotes might represent an interesting biological compromise between promastigotes and intracellular amastigotes, but axenization is not straightforward for some species and for clinical isolates.

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REFERENCES

Ashutosh, Sundar, S. and Goyal, N. (2007). Molecular mechanisms of antimony resistance in *Leishmania*. *Journal of Medical Microbiology* **56**, 143–153.

Arevalo, J., Ramirez, L., Adaui, V., Zimic, M., Tulliano, G., Miranda-Verastegui, C., Lazo, M., Loayza-Muro, R., De Doncker, S., Maurer, A., Chappuis, F., Dujardin, J. C. and Llanos-Cuentas, A. (2007). The influence of *Leishmania (Viannia)* species on the response to antimonial treatment of patients with American Tegumentary Leishmaniasis. *Journal of Infectious Diseases* **195**, 1846–1851.

Da Silva, R. and Sacks, D. L. (1987). Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. *Infection and Immunity* **55**, 2802–2806.

Decuypere, S. (2007). Antimonial treatment failure in anthroponotic visceral leishmaniasis: towards improved tools and strategies for epidemiological surveillance and disease control. Ph.D. thesis. University of Antwerp, Antwerp, Belgium.

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.

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.

Dias, F. C., Ruiz, J. C., Lopes, W. C., Squina, F. M., Renzi, A., Cruz, A. K. and Tosi, L. R. (2007). Organization of H locus conserved repeats in *Leishmania (Viannia) braziliensis* correlates with lack of gene amplification and drug resistance. *Parasitology Research* **101**, 667–676.

Dujardin, J. C. (2009). Structure, dynamics and function of *Leishmania* genome: resolving the puzzle of infection, genetics and evolution? *Infection, Genetics and Evolution* **9**, 290–297.

Gamboa, D., Van Eys, G., Victoir, K., Torres, K., Adaui, V., Arevalo, J. and Dujardin, J. C. (2007). Putative markers of infective life stages in *Leishmania (Viannia) braziliensis*. *Parasitology* **134**, 1689–1698.

Garcia, A. L., Kindt, A., Quispe-Tintaya, K. W., Bermudez, H., Llanos, A., Arevalo, J., Bañuls, A. L., De Doncker, S., Le Ray, D. and Dujardin, J. C. (2005). American tegumentary leishmaniasis: antigen-gene polymorphism, taxonomy and clinical pleomorphism. *Infection, Genetics and Evolution* **5**, 109–116.

Hadighi, R., Mohebali, M., Boucher, P., Hajjaran, H., Khamesipour, A. and Ouellette, M. (2006). Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. *PLoS Medicine* **3**, e162.

Haimeur, A., Guimond, C., Pilote, S., Mukhopadhyay, R., Rosen, B. P., Poulin, R. and Ouellette, M. (1999). Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant *Leishmania*. *Molecular Microbiology* **34**, 726–735.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F. and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8**, R19.

Laurent, T., Rijal, S., Yardley, V., Croft, S., De Doncker, S., Decuypere, S., Khanal, B., Singh, R., Schönian, G., Kuhls, K., Chappuis, F. and Dujardin, J. C. (2007). Epidemiological dynamics of antimonial resistance in *Leishmania donovani*: genotyping reveals a polyclonal population structure among naturally-resistant clinical isolates from Nepal. *Infection, Genetics and Evolution* **7**, 206–212.

Lira, R., Sundar, S., Makharia, A., Kenney, R., Gam, A., Saraiva, E. and Sacks, D. (1999). Evidence that the high incidence of treatment failures in Indian

kala-azar is due to the emergence of antimony-resistant strains of *Leishmania donovani*. *The Journal of Infectious Diseases* **180**, 564–567.

Mandal, G., Wyllie, S., Singh, N., Sundar, S., Fairlamb, A. H. and Chatterjee, M. (2007). Increased levels of thiols protect antimony unresponsive *Leishmania donovani* field isolates against reactive oxygen species generated by trivalent antimony. *Parasitology* **134**, 1679–1687.

McCarthy, D. J. and Smyth, G. K. (2009). Testing significance relative to a fold-change threshold is a TREAT. *Bioinformatics* **25**, 765–771.

Mittal, M. K., Rai, S., Ashutosh, Ravinder, Gupta, S., Sundar, S. and Goyal, N. (2007). Characterization of natural antimony resistance in *Leishmania donovani* isolates. *American Journal of Tropical Medicine and Hygiene* **76**, 681–688.

Mukherjee, A., Padmanabhan, P. K., Singh, S., Roy, G., Girard, I., Chatterjee, M., Ouellette, M. and Madhubala, R. (2007). Role of ABC transporter MRPA, gamma-glutamylcysteine synthetase and ornithine decarboxylase in natural antimony-resistant isolates of Leishmania donovani. *Journal of Antimicrobial Chemotherapy* **59**, 204–211.

Murray, H. W., Berman, J. D., Davies, C. R. and Saravia, N. G. (2005). Advances in leishmaniasis. *Lancet* **366**, 1561–1577.

Neal, R. A. and Croft, S. L. (1984). An *in-vitro* system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. *Journal of Antimicrobial Chemotherapy* **14**, 463–475.

Peacock, C. S., Seeger, K., Harris, D., Murphy, L., Ruiz, J. C., Quail, M. A., Peters, N., Adlem, E., Tivey, A., Aslett, M., Kerhornou, A., Ivens, A. and others. (2007). Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. *Nature Genetics* **39**, 839–847.

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.

Rojas, R., Valderrama, L., Valderrama, M., Varona, M. X., Ouellette, M. and Saravia, N. G. (2006). Resistance to antimony and treatment failure in human *Leishmania (Viannia)* infection. *The Journal of Infectious Diseases* **193**, 1375–1383.

Rozen, S. and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132**, 365–386.

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

Saxena, A., Worthey, E. A., Yan, S., Leland, A., Stuart, K. D. and Myler, P. J. (2003). Evaluation of differential gene expression in *Leishmania major* Friedlin procyclics and metacyclics using DNA microarray analysis. *Molecular and Biochemical Parasitology* **129**, 103–114.

Smith, D. F., Peacock, C. S. and Cruz, A. K. (2007). Comparative genomics: from genotype to disease phenotype in the leishmaniases. *International Journal for Parasitology* **37**, 1173–1186.

Torres, D. C., Adaui, V., Alves, M. R., Romero, G. A. S., Arevalo, J., Cupolillo, E. and Dujardin, J. C. (2010). Gene expression profiling in *Leishmania braziliensis* and *Leishmania guyanensis* clinical isolates with different treatment outcome from Brazil. *Infection Genetics and Evolution* May 15 (Epub ahead of print).

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.

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.I-0034.II.

Vermeersch, M., da Luz, R. I., Toté, K., Timmermans, J. P., Cos, P. and Maes, L. (2009). *In vitro* susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. *Antimicrobial Agents and Chemotherapy* **53**, 3855–3859.

Yardley, V., Ortuño, N., Llanos-Cuentas, A., Chappuis, F., De Doncker, S., 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? *The Journal of Infectious Diseases* **194**, 1168–1175.

Youden, W. J. (1950). Index for rating diagnostic tests. *Cancer* **3**, 32–35.