

RESEARCH

Open Access



# Laboratory confirmed miltefosine resistant cases of visceral leishmaniasis from India

Saumya Srivastava, Jyotsna Mishra, Anil Kumar Gupta, Amit Singh, Prem Shankar and Sarman Singh\*

## Abstract

**Background:** Miltefosine unresponsive and relapse cases of visceral leishmaniasis (VL) are increasingly being reported. However, there has been no laboratory confirmed reports of miltefosine resistance in VL. Here, we report two laboratory confirmed cases of VL from India.

**Methods:** Two patients with VL were referred to us with suspected VL. The first patient was a native of the VL endemic state of Bihar, but residing in Delhi, a VL non-endemic area. He was treated with broad-spectrum antibiotics and antipyretics but was unresponsive to treatment. The second patient was from Jharkhand state in eastern India (adjoining Bihar), another endemic state for VL. He was refractory to anti-leishmanial treatment, which included administration of miltefosine. Following investigation, both patients were serologically positive for VL, and blood buffy coat from both patients grew *Leishmania donovani*. The isolates derived from both cases were characterized for their drug susceptibility, genetically characterised, and SNPs typed for *LdMT* and *LdROS* gene expression. Both patients were successfully treated with amphotericin B.

**Results:** The in vitro drug susceptibility assays carried out on both isolates showed good  $IC_{50}$  values to amphotericin B ( $0.1 \pm 0.0004$   $\mu\text{g/ml}$  and  $0.07 \pm 0.0019$   $\mu\text{g/ml}$ ). One isolate was refractory to  $Sb^{III}$  with an  $IC_{50}$  of  $> 200$   $\mu\text{M}$  while the second isolate was sensitive to  $Sb^{III}$  with an  $IC_{50}$  of  $36.70 \pm 3.2$   $\mu\text{M}$ . However, in both the isolates,  $IC_{50}$  against miltefosine was more than 10-fold higher ( $> 100$   $\mu\text{M}$ ) than the standard strain DD8 ( $6.8 \pm 0.1181$   $\mu\text{M}$ ). Furthermore, genetic analyses demonstrated single nucleotide polymorphisms (SNPs) ( $_{354}\text{Tyr} \leftrightarrow \text{Phe}$  and  $_{1078}\text{Phe} \leftrightarrow \text{Tyr}$ ) in the *LdMT* gene of the parasites.

**Conclusions:** Here, we document two laboratory confirmed cases of miltefosine resistant VL from India. Our finding highlights the urgent need to establish control measures to prevent the spread of these strains. We also propose that *LdMT* gene mutation analysis could be used as a molecular marker of miltefosine resistance in *L. donovani*.

**Keywords:** Visceral leishmaniasis, Miltefosine, Drug resistance, Bihar, Jharkhand

## Background

Human visceral leishmaniasis (VL), commonly known as kala-azar, is a serious medical and public health issue in India, the Mediterranean region, and in parts of southern Europe, Africa, and South America where it is recognised as a neglected tropical disease [1, 2]. In India, the causative protozoan parasite is *Leishmania donovani* [3]. The parasite is transmitted *via* the bite of a female sand fly from the genera *Lutzomyia* and *Phlebotomus* [1, 3]. The clinical manifestation of leishmaniasis ranges from

asymptomatic to fulminant that can progress to a highly fatal form, if untreated. VL is typically characterized by fever, weakness, progressive weight loss, pancytopenia, and massive hepato-splenomegaly [3].

The first line treatment of VL has been the pentavalent antimonials [sodium antimony gluconate (SAG)] [4]. However, in the last four decades, resistance to SAG has increased to alarming levels, particularly in some parts of India, forcing the national government to stop using of SAG [5]. Currently, the therapeutic options available for VL include amphotericin B (deoxycholate, liposomal or other formulations) and miltefosine [4]. Amphotericin B is highly effective in treating antimony-resistant cases

\* Correspondence: sarman\_singh@yahoo.com; sarman.singh@gmail.com  
Division of Clinical Microbiology and Molecular Medicine, Department of Laboratory Medicine, All India Institute of Medical Sciences, New Delhi, India

[4] but its nephrotoxic side effects and the high cost of liposomal formulations restricts its wider use [6, 7]. Miltefosine, the first oral drug, was licensed for the treatment of VL in 2002 with reported cure rate of 98% [3, 5]. Currently, miltefosine is reported to be safe and well tolerated by adults and children [8] and plays a key role in India's kala-azar elimination program, which aims to reduce the incidence of VL to less than 1 case per 10,000 in the next five years [9].

Recently, there have been an increasing number of cases of unresponsiveness to miltefosine and/or reduced efficacy in the treatment of VL in India and Nepal [9, 10]. Miltefosine is an alkyl-phospholipid, an analogue of phosphocholine. Its chemical similarity to the natural phospholipids of cellular membrane suggests that miltefosine probably inhibits transmembrane signals and the synthesis of the cellular membrane. The exact mode of its action and the developing resistance is not well known. The uptake of miltefosine in *Leishmania* spp. parasites involves the *Leishmania* membrane protein, *Leishmania donovani* miltefosine transporter (*LdMT*), a member of the P4 subfamily of P-type ATPases, and *LdRos*, a potential non-catalytic  $\beta$  subunit of *LdMT*. Both proteins are primarily localized in the *Leishmania* plasma membrane and are required for the rapid intracellular uptake of alkyl-phosphocholine drugs. *LdMT* and *LdRos* form a stable protein complex, which facilitates the translocation of phospholipids in the parasite from the exoplasmic sites to the cytoplasmic sites of the plasma membrane [11–14]. It is reported that even a single point mutation within the two alleles of the *LdMT* gene are responsible for the resistant phenotype through inactivation of the transporter protein [15, 16]. Consequently, mutational changes in *LdMT* (specifically,  $_{856}\text{Leu}\leftrightarrow\text{Pro}$ ,  $_{420}\text{Thr}\leftrightarrow\text{Asn}$ , and  $_{832}\text{Leu}\leftrightarrow\text{Phe}$ ) have shown an increased rate of resistance in both in vitro and in vivo conditions [11, 12]. These mutations result in decreased uptake, increased efflux, faster metabolism [15, 17], and changes in the lipid composition of the parasite membranes [18]. Other mutations identified in the miltefosine resistant *L. donovani* include W210 (in *LdMT*) and M1 (in *LdRos*) [19] and single nucleotide polymorphisms (SNPs),  $_{527}\text{T}\leftrightarrow\text{A}$ , which results in the substitution of  $_{176}\text{Val}\leftrightarrow\text{Asp}$  in *LdMT* gene [20].

Although miltefosine resistant strains of *L. donovani* have not yet been cultured or isolated from the Indian subcontinent [10, 21], parasites with varying degree of miltefosine susceptibility have been reported [19]. In the absence of miltefosine resistant clinical isolates, the only option to study the mechanisms of miltefosine resistance has been to create in vitro resistant strains [14, 20, 22].

Recently, a case of VL was reported from Nepal with clinical relapse after successful treatment with miltefosine; however, the parasite could not be isolated from the patient [23]. Two cases of miltefosine unresponsive

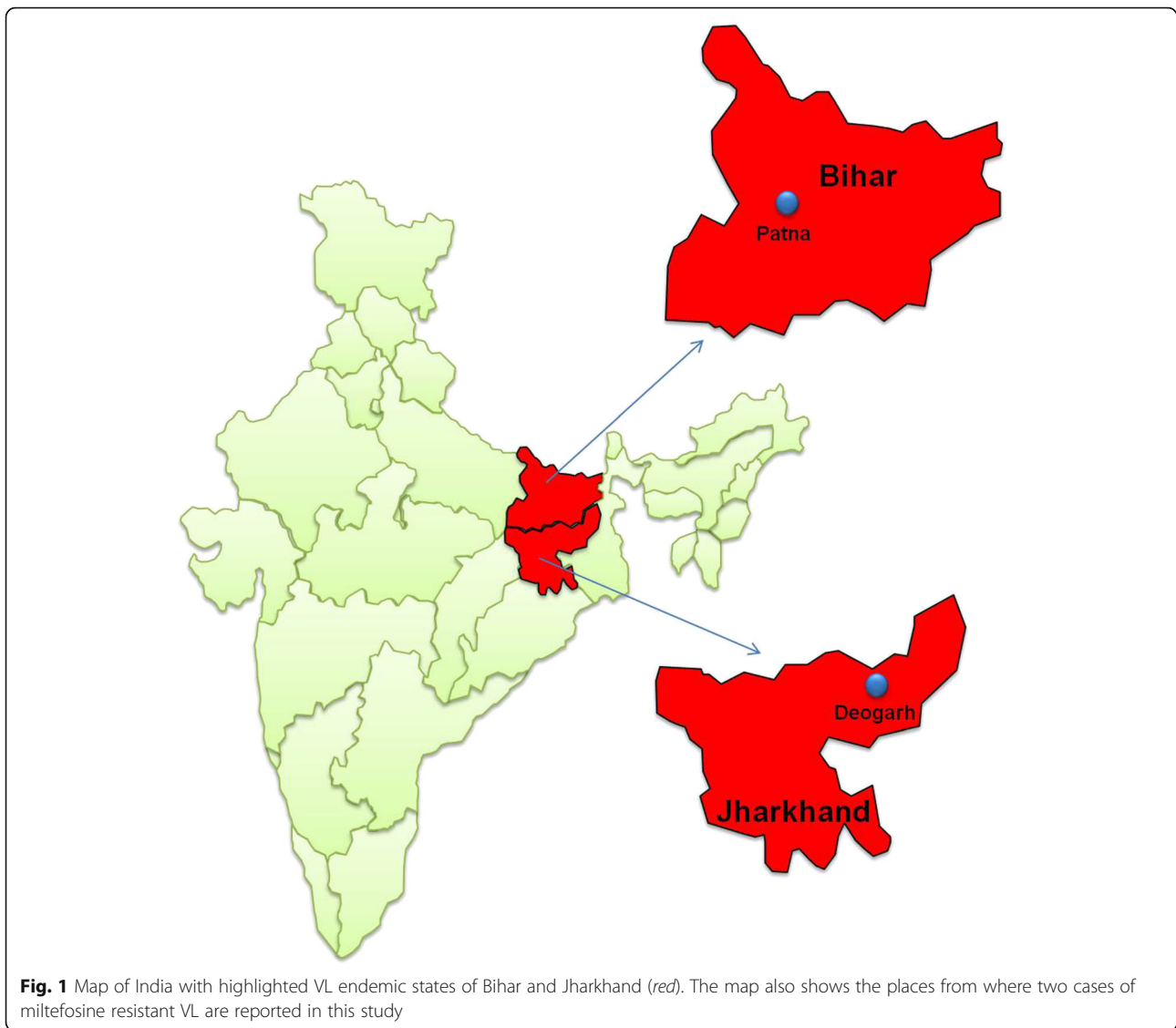
cutaneous leishmaniasis are also on record; one from Ecuador and another from Venezuela, but again without isolation of the parasite [24, 25]. Here we are reporting two cases of laboratory confirmed miltefosine resistant VL (*L. donovani*) from the VL endemic area of India with phenotypic and genotypic characterization of the isolates.

## Methods

### Patient history

Two patients with suspected drug-resistant VL were referred to our laboratory for confirmatory diagnosis in the first 6 months of 2011. Both patients were from VL endemic areas. The first patient (LD843) was a 12-year-old boy native to the Patna district of Bihar (Fig. 1), but who had been residing in Delhi (a VL non-endemic area) for more than 5 years, although he frequently returned to his native village in Bihar. Referred in March 2011 from the Kalawati Saran Children Hospital, New Delhi, the patient had started having a high-grade fever for the past 20 days, which was not responding to antipyretics and broad-spectrum antibiotics (given locally). On clinical and laboratory examination, he was found to have moderate hepato-splenomegaly and was severely anaemic with haemoglobin levels of 5.2 g/dl [normal range (NR) 12–14 g/dl]. The patient had previously visited his native village in Bihar in November 2010.

The second patient (LD860), a 37-year-old man from the Deogarh District of Jharkhand (Fig. 1), was referred in April 2011. This individual presented at a local hospital in Jharkhand in November 2009, with complaints of loss of appetite and body weight. LD860 had a history of persistent fever for more than two weeks that was refractory to antipyretics and antibiotics. On clinical examination, the patient was anaemic, febrile, had moderate hepato-splenomegaly, with a palpable liver and spleen measuring 142 and 152 mm, respectively. He had a white blood cell count of  $10,600\text{ mm}^3$  (NR 4,000–10,000  $\text{mm}^3$ ), haemoglobin of 8.6 g/dl (NR 12–14 g/dl), with 50% neutrophils (NR 40–70%) and 44% lymphocytes (NR 20–35%). Following a positive serum aldehyde test for VL, in January 2010 SAG was administered at 20 mg/kg/day for a period of 21 days. Within 5 days his general condition improved and the patient became afebrile. However, after 6 months (August 2010) his clinical symptoms returned. At this point, he was severely anaemic with haemoglobin levels of 5.2 g/dl, leukocyte count of  $8,800\text{ mm}^3$ , with 63% neutrophils and 34% lymphocytes. Bone marrow aspirates showed an abundant number of Leishman-Donovan (LD) bodies. This time the patient was administered amphotericin B at 1.0 mg/kg/day for 15 days. However, treatment was interrupted due to poor tolerance. The clinical and laboratory signs of VL persisted and his repeat bone marrow remained positive for LD bodies. The patient's liver enzymes were significantly increased with an SGPT value of 92 IU/l (NR 0–41 IU/l) and



serum bilirubin of 2.6 mg/dl (NR 0.2–1.0 mg/dl). However, after cessation of amphotericin B treatment, his haemoglobin increased to normal limits i.e. 11.7 g/dl. In September 2010, miltefosine was administered to the patient at a dose of 50 mg/twice a day for 28 days. Although the patient became afebrile within a week and the laboratory findings were satisfactory, his liver enzymes and bilirubin levels remained high, for which he was given nutritional supplements and managed symptomatically. Again, in February 2011, the patient complained of fever, jaundice, and heaviness in the right subcostal region. Laboratory investigations revealed increased liver enzymes [SGOT] and reversed albumin-globulin ratio. Ultrasonography of the abdomen showed mild hepatomegaly and moderate splenomegaly. Finally, on 8th April 2011, the local physician referred the patient to the All India Institute of Medical Sciences, New Delhi, for further investigation and management.

Both patients underwent thorough laboratory investigations at the All India Institute of Medical Sciences, New Delhi. Sampling was conducted by the expert phlebotomist in the institute’s central collection facility and sent to us for routine investigations, including for leishmaniasis. The patient’s serum samples were tested by anti-rK39 dipstick test (kala-azar Detect™ Rapid Test, In Bios International, Inc., Seattle, WA, USA) and anti-rKE16 spot test (Signal®-KA, and Crystal® KA, Arkray Healthcare Private Limited [formerly Span Diagnostics Limited], Surat, Gujarat, India). The latter tests are based on a novel recombinant antigen [26] prepared from the Indian strain (KE16) of *L. donovani* (MHOM/IN/1998/KE16). Both are commercially available rapid tests and approved by the Government of India for confirmatory diagnosis of leishmaniasis. The Signal®-KA flow through spot test and Crystal® KA (rKE16) lateral flow dipstick

test and kala-azar Detect™ Rapid Test (rK39) [27] were strongly positive. The aetiological agent of disease was further confirmed by microscopic demonstration of LD bodies in smears prepared from blood buffy coat. A samples of blood buffy coat from both the patients were used for isolation of the parasite on Novy-MacNeal-Nicolle (NNN) medium.

#### Parasite culture

To prepare NNN medium, 1.4 g of agar and 0.6 g of NaCl were suspended in 90 ml of distilled water, which was then autoclaved at 121 °C for 20 min. The solution was cooled to 50 °C before 10 ml of defibrinated rabbit blood was added. Two and a half ml of the mixture was then aliquoted into 7 ml Bijou tubes and allowed to cool and solidify at room temperature and finally stored at 4 °C. One aliquot was used for quality control. Just before inoculating the sample, 2 ml of M199 medium, supplemented with 20% v/v heat inactivated foetal bovine serum (FBS) and antibiotic solution containing 50 µg/ml gentamicin (Sigma-Aldrich, St. Louis, Missouri, USA) and 100 µg/ml of streptomycin sulphate salt (Sigma-Aldrich, St. Louis, MO, USA), was added. After that, buffy coat samples (washed two times with 1× PBS) were inoculated in NNN media and transferred to biochemical oxygen demand incubator (BOD) to incubate at 25 °C. Fungal contamination was checked after 4 days, and repeated every 4 days therefore to monitor the growth of *Leishmania* spp. until day 30th of inoculation. The culture was then passaged every seven days into fresh NNN medium. Parasites were then progressively adapted to medium M-199 supplemented with 10% FBS, gentamycin and streptomycin (complete medium) for mass culture. Each isolate was typed to the level of species using sequence data from the internal transcribed spacer (ITS) of DNA, following PCR (see below). The isolates were then characterized for their drug susceptibility. The parasites were tested for their in vitro susceptibility to standard anti-leishmanial drugs within ten passages from isolation. Both the isolates were subjected to nucleotide sequencing and SNPs analysis of *LdMT* and *LdRos* gene using Torrent variant caller software v5.0.1, as detailed below.

#### Genetic characterization of species

Parasite DNA from both clinical samples were subject to PCR amplification of *Leishmania*-specific ITS regions. In brief, total genomic DNA was isolated from blood buffy coat using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), as per manufacturer's instructions. The complete ribosomal ITS region (~1.1 kb) was then amplified using *Leishmania*-specific primers (forward, 5'-CTG GAT CAT TTT CCG ATG-3'; and reverse, 5'-ACA CTC AGG TCT GTA AAC-3') [28]. The amplification was performed in the thermal cycler (Eppendorf,

Hamburg, Germany) and initially heated to 95 °C for 2 min followed by 34 cycles of 95 °C for 20 s, 53 °C for 30 s and 72 °C for 1 min. The final extension was carried out at 72 °C for 1 min. The PCR amplicon was excised for purification using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) from a 1.8% agarose gel prepared in 1× TAE buffer, The purified PCR amplicon was then sequenced using the ABI Prism Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA).

#### In vitro drug susceptibility test (MTT Assay)

The promastigote viability of the isolates (LD843 & LD860) compared to standard strain DD8, (routinely maintained and passaged in vivo in Syrian hamster to maintain virulence and infectivity) [29] was determined against 3 standard drugs using the MTT assay [30]. Cells ( $1 \times 10^4$  cells/100 µl/well) were incubated in flat bottomed 96-well plate (Nunc, Roskilde, Denmark) using different concentrations of Sb<sup>III</sup>, an active component of SAG [4], amphotericin B and miltefosine for 48 h at 26 °C. The drugs were obtained from Sigma-Aldrich (Sb<sup>III</sup>), Lifecare Innovations Gurgaon, Haryana, India (Amphotericin B), and Zentaris Frankfurt, Germany (Miltefosine). In brief, stock solutions of all three drugs were dissolved in sterile distilled water. Each was further serially diluted up to seven-fold with freshly prepared complete medium and incubated as described above for 48 h at 26 °C in a BOD incubator. After 48 h of incubation, 25 µl of sterile MTT (5 mg/ml dissolved in 1× PBS) was added to each well and plates were incubated for 2 h at 37 °C. After incubation, 150 µl of pure dimethyl sulfoxide (DMSO) was added in each well to stop the reaction. The absorbance was measured in a microplate reader at 570 nm wavelengths. The inhibitory effect of the specific drug was expressed as 50% inhibitory concentration (IC<sub>50</sub>), i.e. the concentration of a drug which is required for 50% inhibition of cell viability compared to the control cells in culture medium without drugs. The IC<sub>50</sub> was calculated from the graph showing different concentrations of the standard drugs plotted against percentage cell growth. Each test was performed in duplicate with three independent experiments.

#### Anti-amastigote assay

The J-774A.1 mouse macrophage cell line was used to evaluate the activity of miltefosine against intracellular amastigotes transformed from clinical isolates. This was then compared against the WHO standard strain DD8, which is a pan-susceptible strain, isolated in 1968. Amastigote studies against SAG and amphotericin B were not conducted because the promastigote stage of clinical isolates was sensitive to these drugs. Cells were



seeded in 16-well tissue culture chamber slides at a density of  $4 \times 10^4$  cells/ml in RPMI 1640 medium with 10% FBS. The slides were incubated at 37 °C with 5% CO<sub>2</sub>. After 24 h the medium was replaced with medium containing meta-cyclic promastigotes at a macrophage/parasite ratio of 1:10. This ratio allows promastigotes to be engulfed by macrophages and transformed into amastigotes within 24 h. After 24 h of incubation at 37 °C with 5% CO<sub>2</sub>, one slide was fixed in 100% methanol and stained using Giemsa to determine the initial level of infection. Once a satisfactory level of infection was achieved, slides were exposed to miltefosine at doses of 100, 50, 25, 12.5, 6.25 and 3.12 μM (in duplicate) and incubated for 48 h at 37 °C and 5% CO<sub>2</sub>. Following incubation, slides were stained with Giemsa and microscopically examined. Parasite burden was calculated as [the percentage of infected macrophages × (mean number of amastigotes/macrophage)] and compared to the parasite burden in untreated infected control cells. Most of the macrophages were destroyed at higher concentrations of miltefosine (> 25 μM) and could not be evaluated. The results were calculated as the percent inhibition of the total parasite burden, and the IC<sub>50</sub> was calculated from the graph showing different concentrations of miltefosine plotted against percentage inhibition. These tests were performed in duplicate with three independent repeats.

#### Whole genome sequencing, assembly and SNPs analysis of LD843

For genome analysis, total genomic DNA (gDNA) was extracted from LD843 strain using the Qiagen Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). The integrity and purity of gDNA was analysed on a 0.8% agarose gel prepared in 1× TAE. The whole-genome sequencing of LD843 strain was carried out using 2 × 400 bp paired-end reads using an Ion Torrent Personal Genome Machine (PGM, ThermoFisher, MA, USA) and the sequencing chip 316v2 (ThermoFisher, MA, USA). Low quality and adapter sequences were removed from the reads based on the phred quality score (Q=30) of individual bases. Reads were assembled into longer scaffolds using SPAdes assembly v3.1 [31]. Miscalled single bases and insertions/deletions (indels) in the assembled contigs were corrected with Iterative Correction of Reference Nucleotides (iCORN) software [32]. The sequence quality and read-depth coverage analysis were completed using Torrent coverage analysis v4.2 software [33]. The Integrative Genomics Viewer (IGV) [34] was used to visualize the individual reads for the analysis of read and mapping quality of contigs.

Non-mapping and replica reads were removed from the assembled reads based on lower mapping quality scores BQ (< 30). Sequencing analysis for LD860 is presently in process.

#### SNPs analysis of the gene(s) involved in miltefosine resistance

SNPs analysis was carried out following alignment of the reference sequences for *LdMT* and *LdRos* gene sequences with the de novo aligned sequence of LD843 using Torrent variant caller software v5.0.1 [35]. The regions which had a read depth of less than 100-fold coverage and a maximum of three polymorphisms in any given seven base regions were selected as candidate variable sites, while INDELs were identified with the Variant caller v5.1 software. The accepted read coverage of the variant bases were > 3 for forward and reverse strand and > 6 for SNP quality and minimum one distant read from contig gap. CIGAR scores displayed the mapping quality and SNPs for all reads at a specific site. For heterozygous SNPs, the variant BQ or CIGAR score was checked to make certain it was not considerably worse than the reference genotype BQ. To reduce sequencing error, a series of SNPs quality validation steps were adopted, including poor-quality SNPs detection. The position of individual SNP was visualized using the IGV software for the analysis of read depth, filtering quality, and haplotype score [34].

#### Statistical analysis

The data are shown as the mean and standard deviation (± SD) of three independent experiments. The percent cell viability and IC<sub>50</sub> values were calculated using Microsoft Excel 2007 (Microsoft Corp, WA, USA).

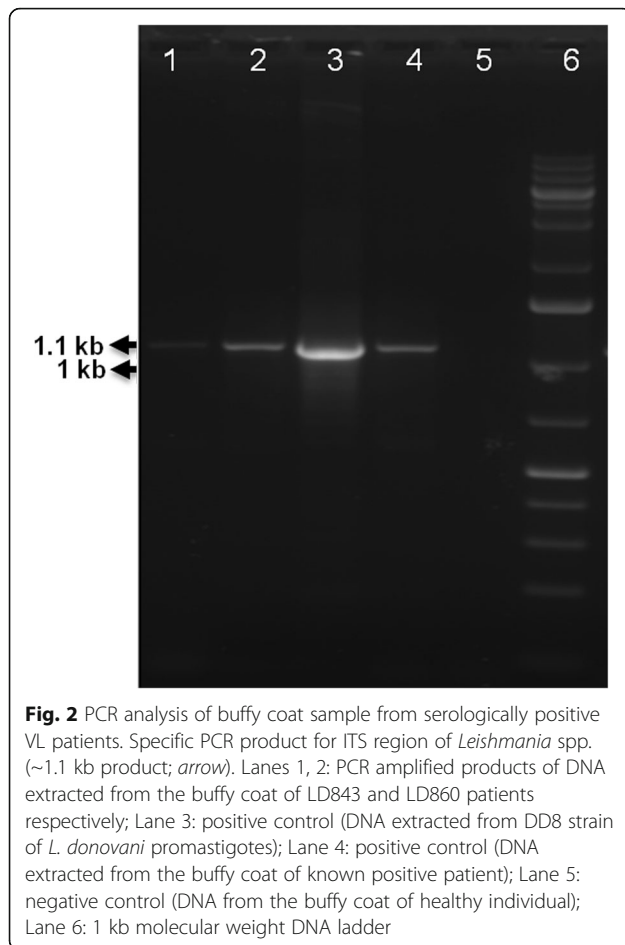
## Results

#### Management of patients

Both patients received intravenous amphotericin B deoxycholate (1.0 mg/kg/day) for 15 days under strict medical supervision. LD843 was treated at the Kalawati Saran Children Hospital, New Delhi while LD860 was treated at the All India Institute of Medical Sciences, New Delhi. The treatment was successful with complete remission of the clinical symptoms within 2 weeks. Both patients were discharged from hospital. Their laboratory parameters also returned to normal within one month. The patients underwent routine medical checkups for 1 year following treatment. During this period neither of the patient reported relapse of symptoms.

#### Characterization of the *Leishmania* spp.

Parasite DNA from both clinical samples was subjected to PCR amplification (~1.1 kb) of the *Leishmania* specific ITS region (Fig. 2). Sequencing of these amplicons and subsequent comparison to publicly available sequence data indicated that sequences determined here showed 99% similarity with the *L. donovani* isolates (NICD/IN/50; accession number EU753224.2). Sequencing analysis,



further confirmed that the cases were of *L. donovani* infection. The sequences obtained from the ITS region of DNA isolated from strain LD843 and LD860 have been submitted to GenBank under accession nos. JQ029056.1 and JQ780821, respectively.

#### Drug susceptibility test

The  $IC_{50}$  for all three drugs ( $Sb^{III}$ , amphotericin B and miltefosine) were determined by plotting drugs concentration vs percentage cell growth. The DD8 strain was found to be sensitive to all three drugs;  $IC_{50}$  values of  $32.78 \pm 1.74 \mu M$ ,  $0.1 \pm 0.0028 \mu g/ml$  and  $6.8 \pm 0.1181 \mu M$ , respectively, were determined (Fig. 3). Both the clinical isolates, LD843 and LD860, showed good response to amphotericin B with  $IC_{50}$  values of  $0.1 \pm 0.0004 \mu g/ml$  and  $0.07 \pm 0.0019 \mu g/ml$ , respectively. However, both isolates showed > 10-fold increase in  $IC_{50}$  (> 100  $\mu M$ ) to miltefosine, compared to the DD8 strain ( $6.8 \pm 0.1181 \mu M$ ). In addition, LD860 was found to be resistant to  $Sb^{III}$  with an  $IC_{50}$  of > 200  $\mu M$ , while LD843 was sensitive to  $Sb^{III}$  with an  $IC_{50}$  of  $36.70 \pm 3.2 \mu M$  (Fig. 3).

#### Anti-amastigote assay findings

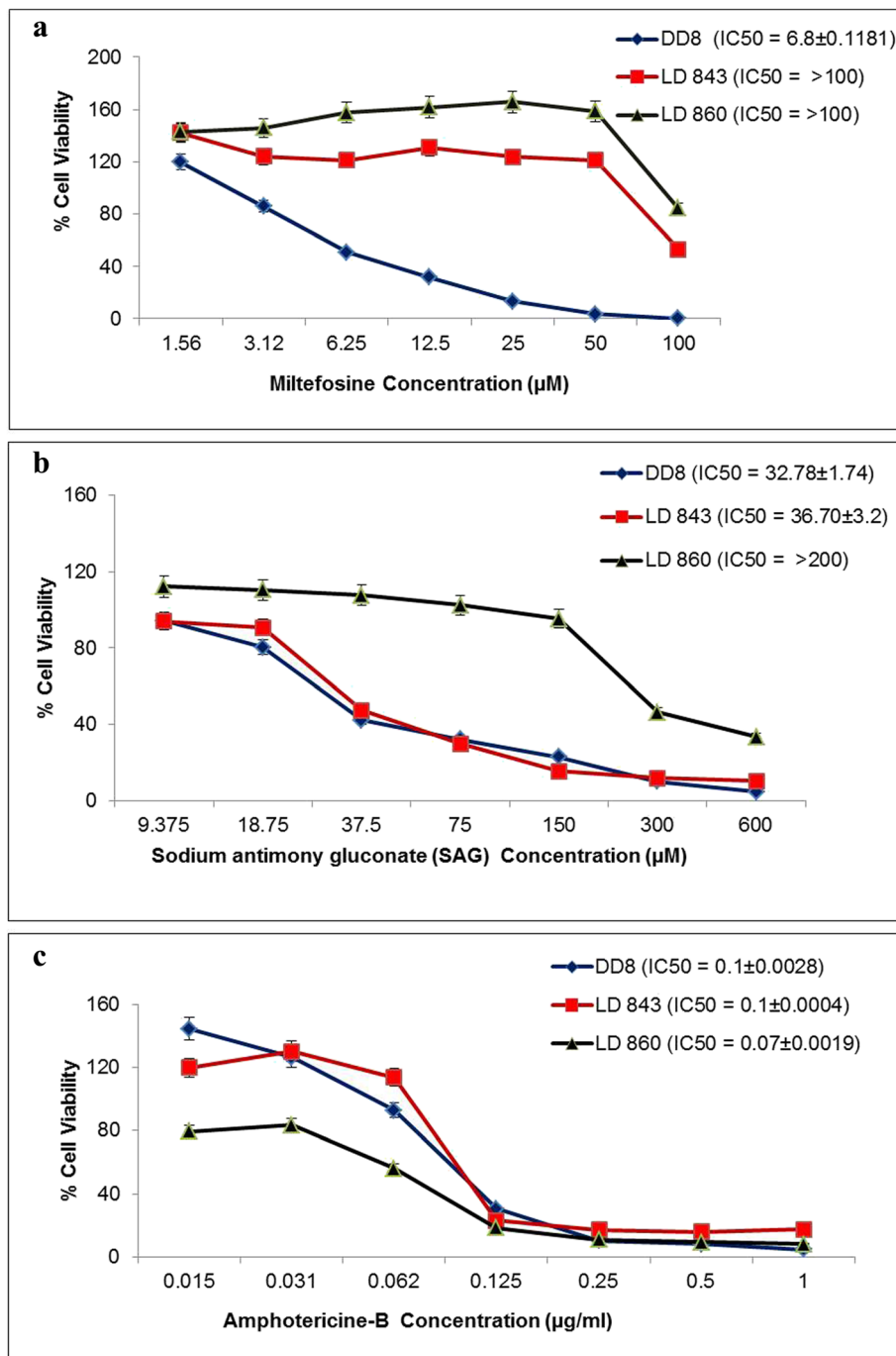
All amastigote assays were carried out in the mouse macrophage cell line J-774A.1 (Fig. 4a). As the promastigote assays showed no resistance to  $Sb^{III}$  and amphotericin B, the amastigote assays were only performed using miltefosine. Like promastigotes, amastigotes also showed that miltefosine had no inhibitory effect on these clinical isolates. After 48 h, the percentage of parasites internalized by macrophages was higher in both isolates compared to those of the DD8 strain (Fig. 4b). After exposure of cells to miltefosine, the clearance of the internalized amastigotes was almost complete DD8 (Fig. 4c). Interestingly, the percentage of parasites internalized by macrophages was slightly higher in both the clinical isolates (Fig. 4d), but clearance of amastigotes in the miltefosine treated cells was negligible (Fig. 4e). Both the clinical isolates showed resistance to miltefosine and had an  $IC_{50}$  value of > 25  $\mu M$  when compared to DD8, which had an  $IC_{50}$  value of  $13.56 \pm 4.17 \mu M$  (Fig. 5). All the experiments were repeated in triplicate. There were no significant differences between both the in vitro assays ( $P = 0.0485$ ).

#### Whole genome sequencing and assembly of LD843

The whole genome sequencing performed using an Ion PGM system produced a total genome length of 31,356,640 bp, or 31.35 Mb, at an average coverage of 23 $\times$  depth (covering 99.76% of the genome), and a GC content of 56.68%. The *de novo* assembled genome of the LD843 strain of *Leishmania* consisted of 3,670 scaffolds, with the largest of these 137,235 bp, and an N50 of 21,796 and an N75 of 10,357 bp.

#### SNPs analysis of gene involved in miltefosine resistance

A total of 31,356,640 nucleotide from the *de novo* assembled *Leishmania* genome (830.6 $\times$  mean coverage depth of reference) were aligned to the reference sequences of *LdMT* (accession no. XM 003859320.1) and *LdRos* (accession no. FR799619.2) genes (Additional file 1). Twenty-one variants (indels) were detected in *LdMT*, while 13 were detected in *LdRos*. In-depth analysis of all variants conferred that five heterozygous SNPs were detected in *LdMT* whereas no significant SNPs were detected in *LdRos*. Three of the SNPs in *LdMT* were synonymous and two were non-synonymous. As synonymous SNPs do not affect the structural and functional activity of the protein, we only focused on the two non-synonymous SNPs ( $_{1061}A \leftrightarrow T$  and  $_{3233}T \leftrightarrow A$ ), which resulted in the substitutions  $_{354}Tyr \leftrightarrow Phe$  and  $_{1078}Phe \leftrightarrow Tyr$ , respectively (Table 1, Fig. 6). The nucleotide substitutions are significant and perturb the protein's structural stability. The nucleotide sequence of *LdMT* has been submitted to GenBank (KX827627).

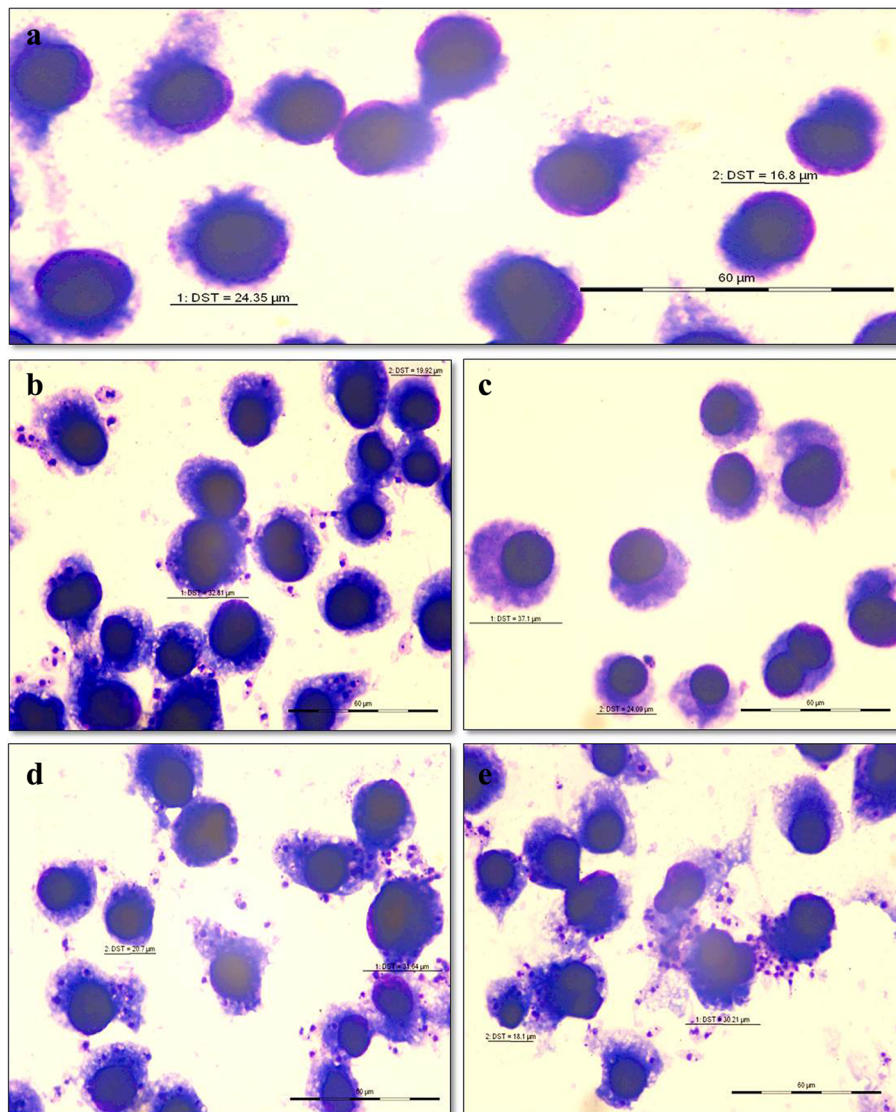


**Fig. 3** Representative plots of susceptibility profile (IC<sub>50</sub>) of parasite isolates from VL cases and standard strain DD8 to (a) Miltefosine, b SAG (Sb<sup>III</sup>) and (c) Amphotericin B. Each individual graph represents the mean from three separate assays

**Discussion**

Visceral leishmaniasis is highly fatal if not treated [36]. In the absence of effective vaccines or vector control measures, the only option to control the disease is chemotherapy. For the last few decades, antimonials have been the predominant drugs of choice [4]. However, clinical

resistance to these drugs emerged in the late 1980s and has become a principle obstacle in VL treatment and control [4, 36, 37]. Moreover, severe cardiac toxicity has further limited its use [38]. Alternative treatment options include amphotericin B and miltefosine. Both have similar efficacy, but miltefosine is an oral drug while amphotericin B



**Fig. 4** The *in vitro* miltefosine resistance demonstrated in clinical isolates using J774-A1 cell line. **a** Uninfected J774-A1 macrophages. **b** Macrophages cells infected with DD8 strain but untreated with miltefosine showing amastigotes within macrophages. **c** Miltefosine-treated DD8 infected macrophages showing clearance of amastigotes. **d** Macrophage cells infected with the clinical isolate but untreated with miltefosine and showing amastigotes within macrophages. **e** Macrophages infected with clinical isolate and treated with miltefosine showing no effect on the clearance of amastigotes. Giemsa stained macrophages cells were photographed at 1000x magnification using a light microscope

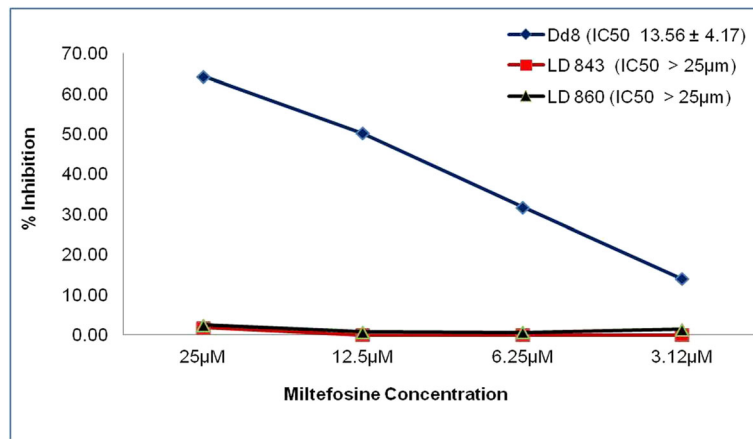
requires intravenous infusion and hospitalization. The liposomal forms of amphotericin B are safer but prohibitively costly [6, 39].

Although miltefosine was the preferred treatment option for VL, with a cure rate as high as 98% [4], the long course of disease treatment, and the longer half-life (around 150 h), has rendered this drug highly vulnerable to the development of resistance [40]. This hypothesis is further supported by preliminary data of the post phase 4 trials of miltefosine showing the cure rate dropping to about 82–87% only [41]. Its

vulnerability is further proved with the fact that miltefosine-resistant mutants of *Leishmania* can be generated very easily *in vitro* [42].

Although the exact method by which miltefosine resistance develops remains unknown, it is well documented that miltefosine uptake by *Leishmania* parasites involves multiple integral membrane proteins including *LdMT*, a member of the P4 subfamily of P-type ATPases, and *LdRos*, a potential noncatalytic  $\beta$  subunit of *LdMT* [11–14]. These trans-membrane proteins are required for the rapid intracellular uptake





**Fig. 5** Representative plot of anti-amastigote assay of parasites isolated from kala-azar cases and standard strain DD8. The graph represents the % inhibition and mean IC<sub>50</sub> of the results from three separate assays

of alkylphosphocholine drugs such as miltefosine. It is conceivable that resistance may develop following the occurrence of non-synonymous point mutations in the genes coding for the respective proteins. Comparison of our genome sequence from strain LD843 with data publicly available for *LdMT* (3,294 bp) and *LdRos* (1,142 bp) revealed two novel SNPs in *LdMT*; <sub>354</sub>Tyr↔Phe and <sub>1078</sub>Phe↔Tyr. Tyrosine and phenylalanine play a key role in cell proliferation and differentiation via phosphorylation [43]. These non-synonymous SNPs in *LdMT* may cause conformational changes in this structural protein that may alter the plasma membrane and/or induced changes to miltefosine uptake by the parasite leading to the development of miltefosine resistance.

The present report highlights the existence of field strains of VL in India that are resistant to miltefosine, and provides laboratory confirmation of this finding. The high IC<sub>50</sub> of the clinical isolates indicates in vivo selection of the miltefosine resistant clones. However, the paediatric case is of rather more concern as it indicates the possibility of transmission of the miltefosine resistant strain in the neighbouring area within a span of a few months only. The distance between Patna (Bihar) and Deogarh (Jharkhand) is approximately 300 km.

Ultimately, this report sends a strong warning to VL control programs and WHO that miltefosine-resistant strains have emerged in India and that improved methods of treatment and control are required in order to prevent the spread of resistant strains to other areas within and outside India.

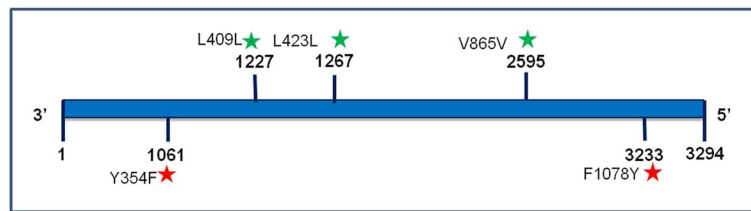
**Conclusions**

Miltefosine was considered a highly effective oral drug for the treatment of VL in India, but these two cases of laboratory confirmed resistance in *L. donovani* against this drug sends an urgent signal for the need to search for new drugs or drug combinations. Although miltefosine induces an early clinical response in patients, increasing numbers of relapses indicate an urgent need for studying new drug development and use of combination therapies. As cell-mediated immunity against *Leishmania* parasites is compromised during the acute phase of VL, administration of miltefosine with agents that are known to have immunomodulatory, effects can be a good choice. We also propose that <sub>354</sub>Tyr↔Phe and <sub>1078</sub>Phe↔Tyr mutations in the *LdMT* gene can be used as molecular markers of miltefosine resistance in *L. donovani*.

**Table 1** Gene mutations identified in miltefosine resistant strain LD843

S. No.	Chromosome no.	SNP type	Position in gene	Reference base	Reference cds	Base in LD843	LD843 cds	Amino acid change
1	13	Non-syn	1061	A	TAC	T	TTC	Y354F
2	13	Syn	1227	T	CTA	C	CTC	–
3	13	Syn	1267	T	TTG	C	CTG	–
4	13	Syn	2595	T	GTT	C	GTC	–
5	13	Non-syn	3233	T	TTC	A	TAC	F1078Y

Abbreviations: S serial; SNP single nucleotide polymorphism; cds codons; Syn synonymous; Non-syn non-synonymous



**Fig. 6** SNPs analysis of *LdMT* gene after genome sequencing of clinical isolates are indicated above the gene by star: red colour indicates non-synonymous mutations and green colour indicates synonymous mutations

### Additional file

**Additional file 1:** Sequence alignment and SNPs analysis of *LdMT* gene in LD843 strain (*LdMT\_M*) with wild type sequence (*LdMT\_W*). (PDF 268 kb)

### Abbreviations

DW: Distilled water; FBS: Foetal bovine serum; gDNA: Genomic DNA; IC<sub>50</sub>: 50% inhibitory concentration; ITS: Internal transcribed spacer; *LdMT*: *Leishmania donovani* miltefosine transporter; NR: Normal range; Phe: Phenylalanine; SAG: Sodium antimony gluconate; SNP: Single nucleotide polymorphism; Tyr: Tyrosine; VL: Visceral leishmaniasis

### Acknowledgments

The authors wish to thank both the patients for their patience and cooperation and for giving us the opportunity to publish their cases. Furthermore, we thank the team of doctors (Kalawati Saran Children’s Hospital, New Delhi) for referring this case. We thank Zentaris (formerly ASTA Medica) for providing the miltefosine. Authors wish to acknowledge the critical review of the manuscript by Dr. Lesley Drake, Imperial College London, School of Public Health, Faculty of Medicine, Norfolk Place, London, UK. We also acknowledge Ms. Shruti Kahol for her technical help.

### Funding

This study was partially supported by the European Commission (HEALTH-F3-2013-602773) under category FP7-Health-2013-Innovation, KINDReD (Kinetoplastid Drug Development: Strengthening the preclinical pipeline) to SSingh. Research fellowship from Indian Council of Medical Research, New Delhi and All India Institute of Medical Sciences, New Delhi to SS and PS, respectively, are highly acknowledged.

### Availability of data and material

The nucleotide sequence of *LdMT* has been submitted to GenBank with accession no. KX827627.

### Authors’ contributions

SS performed the study and drafted the manuscript. JM and PS participated in performing the analyses and gave suggestion. PS critically revised the manuscript content. AKG and AS helped in sequencing analysis. SSingh arranged funds for the work, provided active supervision and inputs in the study design and finalized the manuscript structure and contents. All authors read and approved the final manuscript.

### Competing interests

The authors declare that they have no competing interests.

### Consent for publication

Not applicable.

### Ethics approval and consent to participate

The Institutional Ethics Committee of the All India Institute of Medical Sciences, New Delhi approved this study (vide reference number IEC/NP-93/2011). Written consent of both the patients was obtained for both participation and publication.

Received: 24 June 2016 Accepted: 4 January 2017

Published online: 31 January 2017

### References

- Guerin PJ, Olliaro P, Sundar S, Boelaert M, Croft SL, Desjeux P, et al. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect Dis.* 2002;2:494–501.
- Berman J. Visceral leishmaniasis in the New World and Africa. *Indian J Med Res.* 2006;123:289–94.
- Singh S, Sivakumar R. Challenges and new discoveries in the treatment of leishmaniasis. *J Infect Chemother.* 2004;10:307–15.
- Mishra J, Madhubala R, Singh S. Visceral and post-Kala-Azar dermal leishmaniasis isolates show significant difference in their in vitro drug susceptibility pattern. *Parasitol Res.* 2013;112:1001–9.
- Sundar S, More DK, Singh MK, Singh VP, Sharma S, Makharia A, et al. Failure of pentavalent antimony in visceral leishmaniasis in India: report from the center of the Indian epidemic. *Clin Infect Dis.* 2000;31:1104–7.
- Lachaud L, Bourgeois N, Plourde M, Leprohon P, Bastien P, Ouellette M. Parasite susceptibility to amphotericin B in failures of treatment for visceral leishmaniasis in patients coinfecting with HIV Type 1 and *Leishmania infantum*. *Clin Infect Dis.* 2009;48:16–22.
- Ellis D. Amphotericin B: spectrum and resistance. *J Antimicrob Chemother.* 2002;49 Suppl 1:7–10.
- Sundar S, Singh A, Rai M, Prajapati VK, Singh AK, Ostyn B, et al. Efficacy of miltefosine in the treatment of visceral leishmaniasis in India after a decade of use. *Clin Infect Dis.* 2012;55:543–50.
- WHO. Intercountry consultation on elimination of Kala-Azar in the South-East Asia region. Technical Report Regional Office for South-East Asia; 2012.
- Rijal S, Ostyn B, Uranw S, Rai K, Bhattarai NR, Dorlo TPC, et al. Increasing failure of miltefosine in the treatment of Kala-azar in Nepal and the potential role of parasite drug resistance, reinfection, or noncompliance. *Clin Infect Dis.* 2013;56:1530–8.
- Perez-Victoria FJ, Gamarró F, Ouellette M, Castanys S. Functional cloning of the miltefosine transporter. A novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem.* 2003;278:49965–71.
- Cojean S, Houze S, Haouchine D, Huteau F, Lariven S, Hubert V, et al. *Leishmania* resistance to miltefosine associated with genetic marker. *Emerg Infect Dis.* 2012;18:704–6.
- Hanson PK, Malone L, Birchmore JL, Nichols JW. Lem3p is essential for the uptake and potency of alkylphosphocholine drugs edelfosine and miltefosine. *J Biol Chem.* 2003;278:36041–50.
- Perez-Victoria FJ, Sanchez-Canete MP, Castanys S, Gamarró F. Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites. *J Biol Chem.* 2006;281:23766–75.
- Seifert K, Perez-Victoria FJ, Stettler M, Sanchez-Canete MP, Castanys S, Gamarró F, et al. Inactivation of the miltefosine transporter, LdMT, causes miltefosine resistance that is conferred to the amastigote stage of *Leishmania donovani* and persists in vivo. *Int J Antimicrob Agents.* 2007;30:229–35.
- Seifert K, Matu S, Javier Perez-Victoria F, Castanys S, Gamarró F, Croft SL. Characterisation of *Leishmania donovani* promastigotes resistant to hexadecylphosphocholine (miltefosine). *Int J Antimicrob Agents.* 2003;22:380–7.
- Perez-Victoria FJ, Sanchez-Canete MP, Seifert K, Croft SL, Sundar S, Castanys S, et al. Mechanisms of experimental resistance of *Leishmania* to miltefosine: Implications for clinical use. *Drug Resist Updat.* 2006;9:26–39.

18. Rakotomanga M, Saint-Pierre-Chazalet M, Loiseau PM. Alteration of fatty acid and sterol metabolism in miltefosine-resistant *Leishmania donovani* promastigotes and consequences for drug-membrane interactions. *Antimicrob Agents Chemother*. 2005;49:2677–86.
19. Bhandari V, Kulshrestha A, Deep DK, Stark O, Prajapati VK, Ramesh V, et al. Drug susceptibility in *Leishmania* isolates following miltefosine treatment in cases of visceral leishmaniasis and post kala-azar dermal leishmaniasis. *PLoS Negl Trop Dis*. 2012;6:1657.
20. Kulshrestha A, Sharma V, Singh R, Salotra P. Comparative transcript expression analysis of miltefosine-sensitive and miltefosine-resistant *Leishmania donovani*. *Parasitol Res*. 2014;113:1171–84.
21. Prajapati VK, Sharma S, Rai M, Ostyn B, Salotra P, Vanaerschot M, et al. In vitro susceptibility of *Leishmania donovani* to miltefosine in Indian visceral leishmaniasis. *Am J Trop Med Hyg*. 2013;89:750–4.
22. Shaw CD, Lonchamp J, Downing T, Imamura H, Freeman TM, Cotton JA, et al. In vitro selection of miltefosine resistance in promastigotes of *Leishmania donovani* from Nepal: genomic and metabolomic characterization. *Mol Microbiol*. 2016;99:1134–48.
23. Pandey BD, Pandey K, Kaneko O, Yanagi T, Hirayama K. Relapse of visceral leishmaniasis after miltefosine treatment in a Nepalese patient. *Am J Trop Med Hyg*. 2009;80:580–2.
24. Calvopina M, Gomez EA, Sindermann H, Cooper PJ, Hashiguchi Y. Relapse of New World diffuse cutaneous leishmaniasis caused by *Leishmania (Leishmania) mexicana* after miltefosine treatment. *Am J Trop Med Hyg*. 2006;75:1074–7.
25. Zepa O, Ulrich M, Blanco B, Polegre M, Avila A, Matos N, et al. Diffuse cutaneous leishmaniasis responds to miltefosine but then relapses. *Br J Dermatol*. 2007;156:1328–35.
26. Sivakumar R, Sharma P, Chang K-P, Singh S. Cloning, expression, and purification of a novel recombinant antigen from *Leishmania donovani*. *Protein Expr Purif*. 2006;46:156–65.
27. Singh S, Gilman-Sachs A, Chang KP, Reed SG. Diagnostic and prognostic value of K39 recombinant antigen in Indian leishmaniasis. *J Parasitol*. 1995;81:1000–3.
28. El Tai NO, El Fari M, Mauricio I, Miles MA, Oskam L, El Safi SH, et al. *Leishmania donovani*: intraspecific polymorphisms of Sudanese isolates revealed by PCR-based analyses and DNA sequencing. *Exp Parasitol*. 2001;97:35–44.
29. Aslan H, Dey R, Meneses C, Castrovinci P, Jeronimo SMB, Oliva G, et al. A new model of progressive visceral leishmaniasis in hamsters by natural transmission via bites of vector sand flies. *J Infect Dis*. 2013;207:1328–38.
30. Dutta A, Bandyopadhyay S, Mandal C, Chatterjee M. Development of a modified MTT assay for screening antimonial resistant field isolates of Indian visceral leishmaniasis. *Parasitol Int*. 2005;54:119–22.
31. Algorithmic Biology Lab. Available at <http://bioinf.spbau.ru/spades>. Accessed 27 Dec 2016.
32. Otto TD, Sanders M, Berriman M, Newbold C. Iterative correction of reference nucleotides (iCORN) using second generation sequencing technology. *Bioinformatics*. 2010;26:1704–7.
33. IonTorrent. New Generation Sequencer Software. Available at <http://129.130.90.13/ion-docs/Coverage-Analysis-Plugin.html>. Accessed 27 Dec 2016.
34. Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. *Nat Biotechnol*. 2011;29:24–6.
35. IonTorrent. Variant Caller. <http://129.130.90.13/ion-docs/Torrent-Variant-Caller-Plugin.html>. Accessed 8 Jan 2016.
36. Murray HW, Berman JD, Davies CR, Saravia NG. Advances in leishmaniasis. *Lancet Lond Engl*. 2005;366:1561–77.
37. Olliaro PL, Guerin PJ, Gerstl S, Haaskjold AA, Rottingen J-A, Sundar S. Treatment options for visceral leishmaniasis: a systematic review of clinical studies done in India, 1980–2004. *Lancet Infect Dis*. 2005;5:763–74.
38. Thakur CP, Sinha GP, Pandey AK, Kumar N, Kumar P, Hassan SM, et al. Do the diminishing efficacy and increasing toxicity of sodium stibogluconate in the treatment of visceral leishmaniasis in Bihar, India, justify its continued use as a first-line drug? An observational study of 80 cases. *Ann Trop Med Parasitol*. 1998;92:561–9.
39. Berman JD. Editorial Response: U.S. Food and Drug Administration approval of AmBisome (liposomal amphotericin B) for treatment of visceral leishmaniasis. *Clin Infect Dis*. 1999;28:49–51.
40. Berman J, Bryceson ADM, Croft S, Engel J, Gutteridge W, Karbwang J, et al. Miltefosine: issues to be addressed in the future. *Trans R Soc Trop Med Hyg*. 2006;100:41–4.
41. Sundar S, Murray HW. Availability of miltefosine for the treatment of kala-azar in India. *Bull World Health Organ*. 2005;83:394–5.
42. Mishra J, Singh S. Miltefosine resistance in *Leishmania donovani* involves suppression of oxidative stress-induced programmed cell death. *Exp Parasitol*. 2013;135:397–406.
43. Nascimento M, Abourjeily N, Ghosh A, Zhang WW, Matlashewski G. Heterologous expression of a mammalian protein tyrosine phosphatase gene in *Leishmania*: effect on differentiation. *Mol Microbiol*. 2003;50:1517–26.

Submit your next manuscript to BioMed Central and we will help you at every step:

- We accept pre-submission inquiries
- Our selector tool helps you to find the most relevant journal
- We provide round the clock customer support
- Convenient online submission
- Thorough peer review
- Inclusion in PubMed and all major indexing services
- Maximum visibility for your research

Submit your manuscript at  
[www.biomedcentral.com/submit](http://www.biomedcentral.com/submit)

