Genetic Polymorphisms and Drug Susceptibility in Four Isolates of *Leishmania tropica* Obtained from Canadian Soldiers Returning from Afghanistan

Marie Plourde, Adriano Coelho, Yoav Keynan, Oscar E. Larios, Momar Ndao, Annie Ruest, Gaétan Roy, Ethan Rubinstein, Marc Ouellette

Centre de Recherche en Infectiologie du Centre de Recherche du CHUQ and Département de Microbiologie, Immunologie et Infectiologie, Faculté de Médecine, Université Laval, Québec, Québec, Canada, Laboratory of Viral Immunology, Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada, Department of Medicine and Laboratory Medicine, Divisions of Infectious Diseases and Microbiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, National Reference Center for Parasitology, McGill University, Montreal General Hospital/Research Institute, Montréal, Québec, Canada, Pavillon Hôtel-Dieu du CHUQ, Québec, Canada

Abstract

**Background:** Cutaneous leishmaniasis (CL) is a vector-borne parasitic disease characterized by the presence of one or more lesions on the skin that usually heal spontaneously after a few months. Most cases of CL worldwide occur in Southwest Asia, Africa and South America, and a number of cases have been reported among troops deployed to Afghanistan. No vaccines are available against this disease, and its treatment relies on chemotherapy. The aim of this study was to characterize parasites isolated from Canadian soldiers at the molecular level and to determine their susceptibility profile against a panel of antileishmanials to identify appropriate therapies.

**Methodology/Principal Findings:** Parasites were isolated from skin lesions and characterized as *Leishmania tropica* based on their pulsed field gel electrophoresis profiles and pteridine reductase 1 (PTR1) sequences. Unusually high allelic polymorphisms were observed at several genetic loci for the *L. tropica* isolates that were characterized. The drug susceptibility profile of intracellular amastigote parasites was determined using an established macrophage assay. All isolates were sensitive to miltefosine, amphotericin B, sodium stibogluconate (Pentostam) and paromomycin, but were not susceptible to fluconazole. Variable levels of susceptibility were observed for the antimalarial agent atovaquone/proguanil (Malarone). Three Canadian soldiers from this study were successfully treated with miltefosine.

**Conclusions/Significance:** This study shows high heterogeneity between the two *L. tropica* allelic versions of a gene but despite this, *L. tropica* isolated from Afghanistan are susceptible to several of the antileishmanial drugs available.

Introduction

Cutaneous leishmaniasis (CL) is a vector-borne parasitic disease characterized by one or more sores or nodules on the skin that often heal spontaneously after a few months, resulting in scar formation. This disease has been frequently diagnosed in military personnel who were returning from duty in Southwest Asia [1,2], with several outbreaks observed in troops deployed to Iraq [3] and Afghanistan [2,4]. Currently, Kabul is believed to be the largest focus of CL worldwide, having an estimated incidence of 67,500 new cases per annum [5]. Whereas CL in Iraq has been mostly caused by *Leishmania major*, CL in Afghanistan can either be due to *Leishmania tropica* or *Leishmania major* [6], and differences in clinical features have been observed between the two species. Notably, *L. tropica* tends to cause more chronic infections and may rarely progress to a systemic form of the disease termed viscerotropic leishmaniasis, a situation requiring special attention [7].

There is a lack of consensus about the best therapeutic options for the treatment of CL, mainly due to the lack of properly controlled clinical trials [8]. Because of the self-healing nature of the illness, the treatment of CL depends on several factors such as the site and number of lesions, the aetiology of the disease, and personal preferences. One of the main therapeutic options that has been used for the treatment of CL for many years relied on the local or systemic administration of pentavalent antimony [9]. Because *Leishmania* species are susceptible to heat, the local application of radio frequency to generate heat at the site of the lesions was also shown to yield cure rates equivalent to systemic pentavalent antimony [10,11]. Nonetheless, the availability of effective oral treatments would constitute attractive therapeutic options against CL, and there is evidence of benefit for the use of oral triazoles like itraconazole and fluconazole against *L. tropica* and *L. major*, respectively [8]. Miltefosine, another orally
administered drug, was shown to be an effective treatment against visceral leishmaniasis in India [12] and cutaneous leishmaniasis in South America [13], but there is only limited data about its efficacy against CL in Southwest Asia [14–16].

In this report, we describe the molecular characterization and in vitro drug susceptibility profiles of Leishmania parasites isolated from four Canadian soldiers suffering from CL after returning from Afghanistan. Primary treatment based on oral fluconazole failed to improve the appearance of lesions in three of them. We show that L. tropica was responsible for the lesions in every patient and that the parasites are highly heterogeneous but nonetheless remained sensitive to most known antileishmanial drugs (antimonials, miltefosine, amphotericin B, paromomycin) but were insensitive to fluconazole. This study suggests a number of therapeutic regimens for treating cutaneous leishmaniasis caused by L. tropica among patients and soldiers returning from Afghanistan. Canadian soldiers from this study were successfully treated with miltefosine.

Methods

Ethics Statement

The skin biopsies were taken after appropriate informed consent was obtained, and as part of the routine patient care, Leishmania parasite isolates were submitted for susceptibility testing in order to assist in the clinical management of individuals with suboptimal response to fluconazole. No additional samples or procedures were done.

Parasites and culture

Fresh tissue samples obtained through biopsy of the skin lesions were collected from three Canadian soldiers who returned from duty in southern Afghanistan with suspected CL lesions at the Department of Medical Microbiology and Infectious Diseases of the University of Manitoba in Winnipeg. Samples were submitted to culture, pathological examination, and PCR analyses. The histology revealed the presence of granulomatous inflammation. The isolates identified as 017102, 431462, and 072218 underwent routine clinical laboratory studies at the National Reference Center for Parasitology in Montreal, QC. An additional skin lesion sample (identified as 19897) was collected from a Canadian soldier also returning from Afghanistan and suspected of suffering from CL at the CHUQ in Quebec, QC. Parasites were isolated from the biopsy in SDM-79 medium supplemented with 20% heat-inactivated fetal calf serum, 5 μg/ml hemin and 10 μM biotin at pH 7.0 and 25°C. The molecular characterization of parasites was done at the Centre de Recherche en Infectiologie du Centre de Recherche du CHUL, Quebec, QC. The L. tropica strains 175 and 482, isolated from Iranian patients [17], and L. tropica MHOM/SU/74/K27, obtained from the ATCC, were used as reference isolates.

Phylogenetic analysis

Multiple sequence alignments were performed on the amino acid sequence of the PTR1 coding region using ClustalW [19] with the default settings. The resulting multiple alignments were subjected to phylogenetic analysis using the neighbor-joining method [20] with the Poisson correction distance method as implemented in the MEGA3.1 software [21]. The reliabilities of each branch point were assessed by the analysis of 1000 bootstrap replicates.

Viability test

The 50% inhibitory concentrations (IC50) of drugs on macrophages were established by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, THP-1 cells were differentiated in 96-well flat-bottom microtiter plates in a volume of 100 μl of complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 20 ng/ml phorbol myristate acetate. Plates were incubated at 37°C in the presence of 5% CO2 for 3 days. Drugs were added at 1/10 of the final concentration in a volume of 10 μl in duplicate. After 96 h of incubation, 10 μl of MTT (10 mg/ml) was added to each well and plates were further incubated for 4 h. The enzymatic reaction was stopped by the addition of 100 μl of 50% ethanol-10% sodium dodecyl sulfate. The plates were
incubated for an additional 30 minutes under agitation at room temperature before reading the optical density at 570 nm with a 96-well scanner. The viability assays were performed in duplicates. As a control, the cytotoxicity of reagents used to solubilize the drugs was determined and no substantial toxicity was found.

Drug susceptibility assays

*L. tropica* promastigote parasites were transfected with the firefly luciferase-containing vector pSP1.2 LUC αHYGα as previously described [22]. THP-1 cells were differentiated by incubation at 37°C in the presence of 5% CO2 for 3 days in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 20 ng/ml phorbol myristate acetate. The cells were washed with pre-warmed medium and subsequently infected with *L. tropica* promastigotes at a parasite/macrophage ratio of 15:1 for 3 h. Non-internalized parasites were removed by several washes. Luciferase activity was measured after 4 days of exposure to fluconazole, Pentostam, amphotericin B, miltefosine, paromomycin or Malarone as described elsewhere [23].

Results

Molecular characterization of *Leishmania* isolates

Parasites recovered from biopsy samples of four Canadian military personnel who returned from deployment in Kandahar, Afghanistan, with clinical manifestations of CL were characterized by pulsed field gel electrophoresis (PFGE). PFGE conditions optimized for the analysis of larger chromosomes did not show any major differences in the chromosome numbers and sizes between our isolates (Fig. 1A) and revealed that they were genetically closely related to the ATCC *L. tropica* strain MHOM/SU/74/K27 and to a *L. tropica* isolate recovered from a patient suffering from CL in Iran (*L. tropica* 175) [17,24]. The analysis of smaller chromosomes revealed considerable karyotype differences, however (Fig. 1B).

![Figure 1. Karyotypes of Afghan and Iranian *Leishmania tropica* isolates as characterized by PFGE.](https://www.plosntds.org/doi/10.1371/journal.pntd.0001463.g001)

**Figure 1.** Karyotypes of Afghan and Iranian *Leishmania tropica* isolates as characterized by PFGE. Cells were embedded and lysed in agarose and their chromosomes were electrophoresed and stained with ethidium bromide. **A.** 600–1300 kb electrophoresis. **B.** 100–500 kb electrophoresis. The field isolates 017102, 431462, 072218 and 18693 from Afghanistan have closely related karyotype to the *L. tropica* reference strains 175 and MHOM/SU/74/K27. M, yeast chromosomes molecular weight marker (BioRad).

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![Figure 2. Phylogenetic analysis of the PTR1 sequences.](https://www.plosntds.org/doi/10.1371/journal.pntd.0001463.g002)

**Figure 2.** Phylogenetic analysis of the PTR1 sequences. Amino acid sequences were aligned using the ClustalW algorithm. The resulting multiple alignment was subjected to phylogenetic analysis by using the neighbor-joining method with Poisson correction as implemented in the MEGA3.1 software. The field isolates 017102, 431462, 072218, and 18693 from Afghanistan are clustering with the *L. tropica* reference strains 175 and MHOM/SU/74/K27. The reliabilities of each branch point were assessed by the analysis of 1000 bootstrap replicates.

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| Genes | Nucleotides | Amino acids |
|-------|-------------|-------------|
| PTR1  | G 243 A     | Ala 81 Ala  |
|       | G 561 T     | Pro 187 Pro |
|       | T 624 A     | Ala 208 Ala |
|       | A 642 G     | Pro 214 Pro |
|       | T 690 C     | Ala 230 Ala |
|       | A 698 G     | His 233 Arg |
|       | C 752 G     | Pro 251 Arg |
|       | G 828 T     | Ala 276 Ala |
|       | A 1469 C    | Gln 490 Ala |
|       | C 189 T     | Ser 63 Ser  |
|       | C 1251 T    | Gly 417 Gly |
|       | T 1335 A    | Asp 445 Glu |
| G109  | G 109 T     | Ala 37 Ser  |
|       | C 135 G     | Ala 45 Ala  |
| G230  | G 230 A     | Ser 77 Asn  |
|       | C 383 T     | Ala 128 Val |
|       | C 699 T     | Asn 233 Asn |
|       | C 888 T     | Val 296 Val |
|       | T 1290 C    | Ile 430 Ile |
| PGD   | G 54 C      | Ala 18 Ala  |
|       | A 114 G     | Thr 38 Thr  |
|       | G 249 T     | Thr 83 Thr  |
|       | A 447 G     | Pro 149 Pro |
|       | A 489 G     | Ala 163 Ala |
|       | G 623 T     | Arg 208 Leu |
|       | G 666 T     | Gln 222 Asp |
|       | A 679 G     | Asn 227 Asp |
|       | G 876 C     | Met 292 Ile |
|       | A 884 G     | Tyr 295 Cys |
|       | G 903 C     | Ala 301 Ala |
|       | G 1056 C    | Leu 352 Leu |
|       | A 1228 G    | Asn 410 Lys |
|       | C 1293 T    | Ala 431 Ala |
|       | G 1383 C    | Gly 461 Gly |
|       | A 55 C      | Lys 19 Gln |
|       | A 978 C     | Ala 326 Ala |
|       | C 1141 T    | Pro 381 Ser |
| G6PDH | A 178 G     | Asn 60 Asp |
|       | A 198 A     | Glu 66 Glu |
|       | C 294 T     | Gly 98 Gly |
|       | A 463 G     | Asn 155 Asp |
|       | T 669 C     | Gly 223 Gly |
|       | A 1038 G    | Pro 346 Pro |
|       | A 1039 G    | Ile 347 Val |
|       | G 1083 C    | Ala 361 Ala |
|       | C 1191 T    | Gly 397 Gly |

The isolates were further characterized on the basis of the pteridine reductase 1 (PTR1) sequence [17]. PCR fragments of the coding region of PTR1 were amplified from genomic DNA extracted from the clinical isolates and sequenced. The sequences generated were compared to those of eight Leishmania reference strains and were shown to be closely related to L. tropica sequences (data not shown). A neighbor-joining phylogenetic analysis generated from the translated PTR1 sequences further confirmed that the four CL strains derived from Canadian soldiers were L. tropica parasites (Fig. 2).

**Table 1. Cont.**

| Genes | Nucleotides | Amino acids |
|-------|-------------|-------------|
|       | A 1542 G    | Pro 514 Pro |

*a*Nucleotide polymorphisms and corresponding amino acid changes are shown.  
bThe polymorphisms in bold cause an amino acid substitution.  
cThe polymorphisms indicated were common to every L. tropica isolates analyzed, except for PGD, G6PDH, and SAD, which were not polymorphic in L. tropica MHOM/SU/74/K27.  
dFor nucleotide numbering, +1 corresponds to the A of the ATG translation initiation codon.

### Leishmania tropica genetic heterozygosity

The PTR1 nucleotide sequences of the four *L. tropica* isolates from Canadian soldiers revealed the presence of single nucleotide polymorphisms (SNPs) at five different positions (Table 1). The changes in nucleotide were conservative (Table 1), and the same polymorphisms were also observed in two other strains of *L. tropica* (strains 482 and MHOM/SU/74/K27) that we have analyzed (Table 2). The heterozygous gene sequences were detected as split peaks in the chromatogram generated by the sequencing of the polymerase chain reaction (PCR) products (strains 482 and MHOM/SU/74/K27). The heterozygous gene sequences were detected as split peaks in the chromatogram generated by the sequencing of the polymerase chain reaction (PCR) products (strains 482 and MHOM/SU/74/K27).

To assess whether these polymorphisms corresponded to population heterogeneity or to parasite heterozygocity, the *PTR1* sequence of cloned parasites from three distinct *L. tropica* strains (MHOM/SU/74/K27) was determined (3 clones for each strain). Again, the same *PTR1* polymorphisms were detected in every clone tested. Each allele was detected at a frequency of 50%, which suggests that parasites were harbouring two distinct alleles. The same two alleles were detected in every *L. tropica* strain studied (Table 1), which is reflected by the homogenous clustering of the *L. tropica* isolates in the neighbour-joining phylogenetic analysis (Fig. 2).

To assess the extent of the genetic polymorphism in our panel of *L. tropica* isolates, nine additional genes located on distinct chromosomes, i.e. glucose-6-phosphate isomerase (*GPI*), nucleoside hydrolase 1 (*NH1*), dihydrofolate reductase-thymidylate synthase (*DHFRTS*), stearic acid desaturase (*SAD*), mannose phosphate isomerase (*MPI*), aspartate aminotransferase (*ASAT*), 6-phosphogluconate dehydrogenase (*PGD*), glucose-6-phosphate dehydrogenase (*G6PDH*), and cytochrome B (*CYTB*), were also sequenced in clones of strains 482, 072218 and MHOM/SU/74/K27. Heterozygous sites were observed at every locus (Table 1) except for *MPI* and *CYTB* (Table 2). In addition, the same alleles were detected in every *L. tropica* strain studied, except for the *ASAT* gene, which had three additional polymorphic sites common.
Table 2. Single nucleotide polymorphisms within *Leishmania* species for 10 genes.

| Gene     | GPI | PTR1 | NH1 | PGD | ASAT | G6PDH | DHFR-TS | SAD | MPI | CYTB |
|----------|-----|------|-----|-----|------|--------|---------|-----|-----|------|
| Chromosome | 12  | 23   | 29  | 35  | 24   | 34     | 6       | 14  | 32  | *    |
| *L. major* Friedlin | 0   | 0    | 0   | 0   | 0    | 0      | 0       | 0   | 0   | 0    |
| *L. infantum* JPCM5 | 0   | 0    | 0   | 0   | 0    | 0      | 0       | 0   | 0   | 0    |
| *L. infantum* LEM 3843 | 0   | 0    | 0   | ND  | ND   | ND     | ND      | ND  | ND  | ND   |
| *L. donovani infantum* | 0   | 0    | 0   | ND  | ND   | ND     | ND      | ND  | ND  | ND   |
| *L. tropica* MHOM/SU/74/K27 | 7   | 5    | 4   | 0   | 3    | 0      | 4       | 0   | 0   | 0    |
| *L. tropica* IRAN 482 | 7   | 5    | 4   | 15  | 6    | 10     | 4       | 3   | 0   | 0    |
| *L. tropica* 072218 | 7   | 5    | 4   | 15  | 6    | 10     | 4       | 3   | 0   | 0    |

ND – not done.

*For each gene, the number of heterozygous sites is indicated.

The CYTB gene is located on kinetoplast DNA.

Abbreviations: PTR1 – pteridine reductase 1; NH1 – nucleoside hydrolase 1; DHFR-TS – dihydrofolate reductase-thymidylate synthase; SAD – stearic acid desaturase; GPI – glucose-6-phosphate isomerase; PGD – 6-phosphogluconate dehydrogenase; ASAT – aspartate aminotransferase; G6PDH – glucose-6-phosphate dehydrogenase; MPI – mannose phosphate isomerase; CYTB – Cytochrome B.

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Table 3. Susceptibility of *L. tropica* clinical isolates to antileishmanial drugs.

| Strains | AmphotericinB (µg/ml) | Paromomycin (µg/ml) | M afloconazole (µg/ml) | Pentostam (µg/ml) | Malarone (µg/ml) | Fluconazole (µg/ml) |
|---------|-----------------------|---------------------|------------------------|------------------|-----------------|--------------------|
| 175     | 0.51±0.08             | 19.0±8.1            | 0.19±0.07              | 300±32           | >8              | >275               |
| 017102  | 0.59±0.29             | 7.4±1.7             | 0.11±0.004             | 188±39           | 6.6±0.1         | >275               |
| 431462  | 0.32±0.18             | 3.2±0.9             | 0.09±0.004             | 142±4            | 3.6±0.6         | >275               |
| 072218  | 0.51±0.07             | 9.8±1.8             | 0.15±0.07              | 142±12           | 4.4±1.1         | >275               |
| 18693   | 0.72±0.33             | 5.8±4.8             | 0.65±0.33              | ND               | >8              | >275               |

*Determinas of amastigotes in THP-1 cells.

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cutaneous lesions cicatrized while being on Malarone prophylaxis, so we tested whether Malarone had any activity against L. tropica isolates using the intracellular amastigote assay. The toxicity of Malarone to the THP-1 cells was first established by MTT viability assay, and this cell line was found to display a Malarone IC50 of 32 μg/ml. No THP-1 cytotoxicity was observed for Malarone concentrations up to 10 μg/ml. Using drug concentrations below 10 μg/ml, we found that three L. tropica strains were sensitive to Malarone as intracellular amastigotes (Table 3), including the strain that was isolated from the patient whose CL regressed during Malarone prophylaxis.

Discussion

We describe here the drug susceptibility and molecular characterization of L. tropica isolates derived from Canadian soldiers returning from Afghanistan. The isolates were identified as L. tropica by phylogenetic studies based on the PTR1 sequence, an approach proven to be useful for the molecular identification of Leishmania species [17]. Moreover, the PFGE karyotypes of the recovered Leishmania parasites were similar to those of L. tropica reference strains. This is consistent with epidemiological data that showed the majority of CL cases in Afghanistan being due to this species [5,25]. Interestingly, the sequence of PTR1 revealed several SNPs in distinct L. tropica isolates. This phenomenon appeared to be widespread across the L. tropica genome, as it was also observed at other genetic loci on different chromosomes. Most of the loci analyzed code for proteins that are part of the panel of enzymes used for the characterization of Leishmania species by multilocus enzyme electrophoresis [26]. Among these, six (GPI, NHI, ASAT, G6PDH, PGD, and MPI) were further shown to be useful markers for the molecular characterization of Leishmania strains and species [27–29]. CITB was chosen as a mitochondrial gene representative, since it has also been reported to be phylogenetically informative [30,31]. The SAD and DHFR-Ts loci were randomly chosen. DNA sequencing of cloned parasites revealed a number of heterozygous sites at these loci, some of which led to non-conservative changes. Although the prevailing mode of reproduction of Leishmania appears to be clonal [32], heterozygosity at several sites within genes or at distinct loci is suggestive of genetic exchange between strains [27], and this phenomenon has previously been observed in other Leishmania species [27,28,32–35]. Most of these studies used microsatellite markers with high mutation rates as indicators of heterozygocity, however, and this is the first report about extensive heterozygocity within coding regions in L. tropica. Nonetheless, the heterozygosity of the L. tropica isolates appears to be fixed, the same alleles being found among strains for most of the loci studied except for the reference L. tropica MHOM/SU/74/K27. This is suggestive of clonal propagation within foci of endemicity and is consistent with the anthropoponic mode of transmission of L. tropica in urban and peri-urban environments of Afghanistan [5]. L. tropica parasites were known to display genetic heterogeneity at the population level [34–37] and to be responsible for a spectrum of clinical manifestations including cutaneous, chronic, or visceral leishmaniasis [7]. Unfortunately, the small number of isolates available for analysis prevented correlating heterozygocity with clinical data or drug susceptibility. However, this seems to be unique to L. tropica since other species did not show this level of allelic polymorphism (Table 2).

Although CL is generally self-limiting, the complexity of the clinical spectrum associated with L. tropica infections emphasizes the need for treatment. Evidence suggested that the disruption of ergosterol biosynthesis by oral azoles is an effective treatment against CL [38,39]. However, a species-specific effect was found to be important to the clinical outcome conferred byazole molecules, with itraconazole and fluconazole being more active against L. tropica and L. major, respectively [8]. Here, the failure of oral fluconazole to improve the appearance of cutaneous lesions was indeed explained by the intrinsic resistance of our L. tropica isolates, the amastigote parasites being insensitive to the highest fluconazole concentration achievable in vitro using an established intracellular assay (Table 3). All isolates were sensitive to the other drugs tested, however, with the exception of Malarone, for which variable levels of susceptibility were observed. While anecdotal, CL regressed in one soldier during Malarone prophylaxis. Although we cannot exclude spontaneous healing, it might be worthwhile to evaluate the usefulness of this drug against CL in properly controlled experiments.

Miltefosine is an orally administered antileishmanial approved for the treatment of visceral leishmaniasis in India [12], with demonstrated efficacy against CL in some regions of South America [13]. In contrast, mostly sporadic data have been reported regarding the efficacy of miltefosine against CL in Southwest Asia [14–16]. Based on the results of our drug susceptibility screening, soldiers were treated with miltefosine and healing of their CL lesions was observed [40].
treated with Malarone received miltefosine but elected to discontinue therapy due to abdominal pain and in the face of a contracting lesion. The other soldiers tolerated medication well and lesions resolved at follow up.

Supporting Information

Table S1 Primers used in this study.

| Primer | Sequence |
|--------|----------|
| F      | 5'-...   |
| R      | 5'-...   |

References

1. Goldrick BA (2004) Another hazard of war: infectious diseases: leishmaniasis, malaria threaten troops, millions in use drug screening. Antimicrob Agents Chemother 48: 1168–1173.
2. van ThiPP, Leenstra T, de Vries HJ, van der Suijs A, van Gool T, et al. (2010) Cutaneous leishmaniasis (Leishmania major infection) in Dutch troops deployed in northern Afghanistan: epidemiology, clinical aspects, and treatment. Am J Trop Med Hyg 83: 1295–1300.
3. Weina PJ, Neafie RC, Wortmann G, Polhemus M, Aronson NE (2004) Old world leishmaniasis: an emerging infection among deployed US military and civilian workers. Clin Infect Dis 39: 1674–1680.
4. Faulde M, Schilder J, Heyl G, Amirthi H, Hoornaar A (2008) Zoonotic cutaneous leishmaniasis outbreak in Mazar-e Sharif, northern Afghanistan: an epidemiological evaluation. Int J Med Microbiol 298: 543–550.
5. Reithinger R, Molen M, Asflit K, Saidl M, Erasmus P, et al. (2003) Anthroponotic cutaneous leishmaniasis, Kabul, Afghanistan. Emerg Infect Dis 9: 727–729.
6. Mauli A, Grosl M, Gasser JR, RA, Sun W, Oster CN (1993) Visceral infection caused by Leishmania tropica in veterans of Operation Desert Storm. N Engl J Med 329: 1381–1387.
7. Gonzalez U, Pinart M, Reviz L, Alvar J (2008) Interventions for Old World cutaneous leishmaniasis. Cochrane Database Syst Rev. pp CD000567.
8. Wortmann G, Miller RS, Oster G, Jackson J, Aronson N (2002) A randomized, double-blind study of the efficacy of a 10- or 20-day course of sodium stibogluconate for treatment of cutaneous leishmaniasis in United States military personnel. Clin Infect Dis 35: 261–267.
9. Aronson NE, Wortmann GW, Byrne WR, Howard RS, Bernstein WB, et al. A randomized controlled trial of local heat therapy versus intra venous sodium stibogluconate for the treatment of cutaneous Leishmania major infection. PLoS Negl Trop Dis 4: e620.
10. Reithinger R, Asflit K, Kolaczinski J, Molen M, Hani S (2003) Social impact of leishmaniasis, Afghanistan. Emerg Infect Dis 11: 634–636.
11. Sandler S, Jia TK, Thakur CP, Engil J, Sanderman H, et al. (2002) Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med 347: 1739–1746.
12. Soto J, Arana RA, Toledo J, Rizo N, Vega JC, et al. (2004) Miltefosine for new world cutaneous leishmaniasis. Clin Infect Dis 38: 1266–1272.
13. Stojkovic M, Junghanss T, Krause E, Davidson RN (2007) First case of typical Old World cutaneous leishmaniasis treated with miltefosine. Int J Dermatol 46: 392–395.
14. Mohabed M, Fotouhi A, Hooshmand B, Zarei Z, Akhoundi B, et al. (2007) Anthroponotic cutaneous leishmaniasis outbreak in Mazar-e Sharif, northern Afghanistan: an epidemiological evaluation. Int J Med Microbiol 298: 543–550.
15. Aronson NE, Wortmann GW, Byrne WR, Howard RS, Bernstein WB, et al. A randomized controlled trial of local heat therapy versus intra venous sodium stibogluconate for the treatment of cutaneous Leishmania major infection. PLoS Negl Trop Dis 4: e620.
16. van ThiPP, Leenstra T, Kager PA, de Vries HJ, van Vught M, et al. Miltefosine treatment of Leishmania major infection: an observational study involving Dutch military personnel returning from northern Afghanistan. Clin Infect Dis 50: 80–83.
17. Hadlidi R, Mohabed M, Boucher P, Hajarja H, Khamesiour A, et al. (2006) Unresponsiveness to Glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant Leishmania tropica parasites. PLoS Med 3: e162.
18. Sereno D, Roy G, Lemere IL, Papadopoulou B, Ouellette M (2001) DNA transformation of Leishmania infantum axenic amastigotes and their use in drug screening. Antimicrob Agents Chemother 45: 1168–1173.
19. Roy G, Dumas C, Sereno D, Wu Y, Singh AK, et al. (2008) Epidermal and stable expression of the luciferase reporter gene for quantifying Leishmania spp. infections in macrophages and in animal models. Mol Biochem Parasitol 110: 195–206.
20. Hadlidi R, Boucher P, Khamesiour A, Mecarm AR, Roy G, et al. (2007) Glucantime-resistant Leishmania tropica isolated from Iranian patients with cutaneous leishmaniasis are sensitive to alternative antileishmania drugs. Parasitol Res 101: 1319–1322.
21. Ashford RW, Kosterlany RV, Karimzad MA (1992) Cutaneous leishmaniasis in Kabul: observations on a ‘prolonged epidemic’. Ann Trop Med Paratol 86: 271–274.
22. Rougeron V, De Meeus T, Hide M, Waleckx E, Bermudez H, et al. (2009) Phylogenetic analysis of the genus Leishmania by cytochrome b gene sequencing. Exp Parasitol 128: 483–491.
23. Zemanova E, Jiráks M, Mauricio IL, Horák A, Miles MA, et al. (2007) The Leishmania donovani complex: genotypes of five polymorphic metabolic enzymes (ASAT, GPI, NHI, N2H, PGD). Int J Parasitol 37: 757–767.
24. Sereno D, Roy G, Lemere IL, Papadopoulou B, Ouellette M (2001) DNA transformation of Leishmania infantum axenic amastigotes and their use in drug screening. Antimicrob Agents Chemother 45: 1168–1173.
25. Sereno D, Roy G, Lemere IL, Papadopoulou B, Ouellette M (2001) DNA transformation of Leishmania infantum axenic amastigotes and their use in drug screening. Antimicrob Agents Chemother 45: 1168–1173.
26. Sereno D, Roy G, Lemere IL, Papadopoulou B, Ouellette M (2001) DNA transformation of Leishmania infantum axenic amastigotes and their use in drug screening. Antimicrob Agents Chemother 45: 1168–1173.