Species identification and phylogenetic analysis of *Leishmania* isolated from patients, vectors and hares in the Xinjiang Autonomous Region, The People’s Republic of China

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Abstract

**Background**

Visceral leishmaniasis (VL) has been declared as one of the six major tropical diseases by the World Health Organization. This disease has been successfully controlled in China, except for some areas in the western region, such as the Xinjiang Autonomous Region, where both anthroponotic VL (AVL) and desert type zoonotic VL (DT-ZVL) remain endemic with sporadic epidemics.

**Methodology/Principal findings**

Here, an eleven-year survey (2004–2014) of *Leishmania* species, encompassing both VL types isolated from patients, sand-fly vectors and Tarim hares (*Lepus yarkandensis*) from the Xinjiang Autonomous Region was conducted, with a special emphasis on the hares as a potential reservoir animal for DT-ZVL. Key diagnostic genes, ITS1, *hsp70* and *nagt* (encoding *N*-acetylglucosamine-1-phosphate transferase) were used for phylogenetic analyses, placing all Xinjiang isolates into one clade of the *L. donovani* complex. Unexpectedly, AVL isolates were found to be closely related to *L. infantum*, while DT-ZVL isolates were closer to *L. donovani*. Unrooted parsimony networks of haplotypes for these isolates also revealed their relationship.

**Conclusions/Significance**

The above analyses of the DT-ZVL isolates suggested their geographic isolation and independent evolution. The sequence identity of isolates from patients, vectors and the Tarim...
hares in a single DT-ZVL site provides strong evidence in support of this species as an animal reservoir.

**Author summary**

Black faver, also known as visceral leishmaniasis (VL), is caused by pathogens of *Leishmania* species, spread by the bites of infected sand flies. This disease has been successfully controlled in China, except for some areas in the western region, such as Xinjiang. However, the knowledge on *Leishmania* in these areas remains a few important gaps. Particularly, what is the animal reservoir for desert type zoonotic VL (DT-ZVL), as sand flies get infected in areas free of patients or infected dogs? To address this question, an eleven-year survey (2004–2014) in Xinjiang for *Leishmania* species was carried out. We found that VLs in Xinjiang are contributed to *Leishmania donovani* complex, and Tarim hares is likely the reservoir animal for DT-ZVL.

**Introduction**

Visceral leishmaniasis (VL), caused by species of the *Leishmania donovani* complex, is a potentially fatal disease if not treated [1]. In China, before the implementation of the national infectious diseases control programs in 1951, it was one of the major parasitic diseases. A total of approximately half a million VL cases were then reported in 16 provinces north of the Yangtze River [2]. The control programs have successfully eliminated VL in the northeastern plain, but not in the west and northwest regions, where this disease has persisted as three different types: mountain type zoonotic VL (MT-ZVL), anthroponotic VL (AVL) and desert type zoonotic VL (DT-ZVL) [2]. The total number of cases reported from these areas between 2002 and 2011 were 3,169 VL cases, ranging from 140 to 509 cases per year. This study considers the causative agents of AVL and DT-ZVL from the Xinjiang Autonomous Region in the northwest of China.

One old endemic site in Xinjiang is the Kashgar alluvial plain and the Aksu oasis, where the AVL is an endemic disease, whose pathogen is transmitted by the peridomestic vector *Phlebotomus longiductus*. Most patients are over 6 years old (70%) [3]. This type of VL has been considered as anthroponotic on account of its familial clustering and the uncertainty about the existence of potential animal reservoirs [4]. As such, the causative agent has long been referred to as *L. donovani*. More recently, isolates collected from this endemic region, have been identified as *L. infantum* based on the nagt and other single-copy gene analyses (isolates HOM/CN/91/911, HOM/CN/92/921, HOM/CN/97/9701) [5] and the multilocus sequence typing (MLST) of five enzyme-coding genes (e.g. isolate MHOM/CN/80/801) [6].

DT-ZVL is endemic in the northwestern China, mainly in the Bachu and Jiashi counties of Xinjiang, but also in the western part of Inner Mongolia and northern Gansu [3], with wild sand fly species *Phlebotomus wui* and *P. alexandri* as vectors specific for the sandy desert and pebble desert subtypes, respectively. Most patients are infants of 2 years-old or younger (94%). DT-ZVL is considered zoonotic and its pathogen is transmitted by wild sand fly species, *Phlebotomus wui* and *P. alexandri*, which live in rodent burrows as their natural habitats. This is further supported by the lack of familial clustering. Extensive studies in desert rodents, e.g. the great gerbil, have failed to establish their role as a reservoir animal for DT-ZVL, but have implicated the Tarim hare (*Lepus yarkandensis*) as a potential reservoir for DT-ZVL [7]. These
wild hares are unique to the Tarim basin in the Taklamakan desert. They were found to be seropositive for *Leishmania* antigens (rk39) at a high prevalence (25%), microscopically positive for amastigotes in infected tissues and to suffer from the typical VL symptom of splenomegaly, hepatomegaly and sometimes ulcerative lesions of the ears. Moreover, promastigotes were isolated from the spleens of infected hares and found to be virulent for the laboratory-reared susceptible steppe rodent, *Lagurus lagurus*. The DNA (*nagt* locus) sequence identity of hare-, vector- and patient-derived isolates provided the preliminary evidence that the hare could be a potential reservoir of DT-ZVL [7]. The causative agent was considered to be *L. infantum*, since all the *nagt* sequences were identical to those of the reference *L. infantum* isolates from other endemic areas in China and elsewhere [5]. This was further confirmed by the ITS1 locus analysis [8]. In contrast, investigation of similar isolates with the same designated code names (MHOM/CN/00/Wangjie1 as a reference strain) classified them as *L. donovani* based on the ITS1 sequence [9] and MLST analyses [6].

The heterogeneity of Chinese *Leishmania* isolates was first observed by isoenzyme analysis and by DNA hybridization (kinetoplast and nuclear DNA) [10–12]. More recently, the diversity of the Chinese *Leishmania* isolates has been addressed by several other studies using various molecular markers [6,9,13–16]. By analysis of the molecular fingerprints, it was found that the isolates from the three epidemiological disease types were distinguishable (reviewed by [17]). However, there were limitations in these studies. Firstly, only a limited number of isolates were available for each given disease type. Secondly, the parasite samples did not represent the complete transmission cycle. Thirdly, only one isolate (IPHL/CN/77/XJ771) from the sand fly vector found in the DT-ZVL region has been studied thus far. Therefore, the species and their relationship with the epidemiological cycles in the regions of Xinjiang remained unclear. This was especially the case for DT-ZVL which until now had an unknown animal reservoir.

To resolve this dilemma, 20 *Leishmania* isolates were collected from endemic foci of both AVL and DT-ZVL in Xinjiang and analyzed using three different phylogenetic markers (*nagt*, ITS1 and *hsp70*). Our analyses of representative isolates cultured from captured hares, patients and the vector *P. wui* strongly supports the conclusion that the Tarim hare serves as a unique reservoir of DT-ZVL.

**Materials and methods**

**Ethics statement**

Samples collected from patients were approved by The Ethical Committee of Xinjiang Uighur Autonomous Region Center for Disease Control and Prevention under license of #30460120. Formal verbal consent was obtained from each patient or parent of child patient.

**Parasite collection and cultivation**

Samples investigated in this study were collected in Bachu, Jiashi, Minfeng and Shufu counties of Xinjiang province, northwestern China, during an 11 year survey of the VL in the endemic regions of the Tarim Basin (Fig 1 and Table 1). In order to isolate and maintain parasites, bone marrow aspirates from patients, dissected gut of infected sand-flies and homogenates of the lesion or spleen tissue from infected Tarim hares were inoculated into the *Lagurus lagurus* or the grey hamsters. Parasites were recovered from spleen homogenates of infected animals and cultured in modified medium LLM at 27°C [7]. Parasites or spleen samples were stored at 70% ethanol before DNA extraction. Protocols for the use of animals were approved by The Center for Laboratory Animal Research of Xinjiang and Institutional Review Board for Animal Care at Sun Yat-Sen University.
DNA extraction, PCR amplification and sequencing of the ITS1, hsp70 and nagt region

Sample DNA was extracted as described elsewhere [18]. The ITS1, hsp70 and nagt fragments were amplified by using the following primer sets, ITS1: L5.8S (5’ TGA TAC CAC TTA TCG
CAC TT 3’) and LITSR: (5’ CTG GAT CAT TTT CCG ATG 3’) [19]; hsp70: F25 (5’ GGA CGC CGG CAC GAT TKC T 3’) and R1310 (5’ CCT GGT TGT TGT TCA GCC ACTC 3’) [20]; nagt: L1(5’ TCA TGA CTC TTG GCC TGG TAG 3’) and L4 (5’ CTC TAG CGC ACT TCA TCG TAG 3’) using standard conditions as published [5]. PCR products were sequenced by Invitrogen (Life technology) and all sequences were deposited in GenBank under the accession numbers provided in Table 1.

Sequence alignment and phylogenetic analysis

The sequences were checked manually and aligned with a set of Leishmania strains retrieved from GenBank (Table 2) using MEGA, version 5.0 [21] The unaligned 5’ and 3’ ends were removed before phylogenetic analysis. An imported Iranian L. major strain (MHOM/CN/2015/CPOLM-1) collected from a patient found in Guangzhou was used as an outgroup in the ITS1 analysis [22]. The phylogenetic relationships among the isolates were inferred from the phylogenetic tree reconstruction by the Neighbor Joining (NJ) using MEGA 5.0 by default setting and the reliability of the internal branches was tested by 1,000 bootstrap replications.

Haplotype networks

Unrooted parsimony networks of haplotypes for L. donovani complex were constructed using TCS v.1.21 [23], with gaps treated as a fifth state.

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Table 1. *Leishmania* isolates from Xinjiang.

| Name          | WHO code | Source/Host (Age) | Time of isolation | Location* | Type | Haplotype | Genbank accession No. (ITS1, hsp70, nagt) |
|---------------|----------|-------------------|------------------|-----------|------|-----------|-----------------------------------------|
| 934 MHOM/CN/2004/KBC-1 | Patient (11 months) | Dec. 2004 | Bachu DT-ZVL | I2 H2 | KU975140.1, KX150477.1, KX150463.1 |
| 2703 IMJW/CN/2005/KBC-2 | P. wui | Jun. 2005 | Bachu DT-ZVL | I2 - | KU975141.1, NA, NA |
| 2681 MLEP/CN/2005/KBC-1 | Tarim hare | Dec. 2005 | Bachu DT-ZVL | I2 - | KU975142.1, NA, NA |
| 418 MLEP/CN/2004/KBC-2 | Tarim hare | Dec. 2004 | Bachu DT-ZVL | I2 H2 | KU975143.1, KX150478.1, KX150464.1 |
| 432 MLEP/CN/2004/KBC-3 | Tarim hare | Dec. 2004 | Bachu DT-ZVL | I2 H2 | KU975144.1, KX150479.1, NA |
| 3153 MHOM/CN/2009/KJS-1 | Patient (1 year) | Jan. 2009 | Jashi DT-ZVL | I2 - | KU975145.1, KX150480.1, KX150465.1 |
| 3081 MHOM/CN/2009/KJS-3 | Patient (11 months) | Jan. 2009 | Jashi DT-ZVL | I2 H2 | KU975146.1, KX150481.1, NA |
| 3208 MHOM/CN/2009/KJS-4 | Patient (7 months) | Feb. 2009 | Jashi DT-ZVL | I2 - | KU975147.1, NA, NA |
| 3227 MHOM/CN/2009/KJS-5 | Patient (7 months) | Nov. 2009 | Jashi DT-ZVL | I2 H2 | KU975148.1, KX150482.1, KX150466.1 |
| 3228 MHOM/CN/2009/KJS-6 | Patient (6 months) | Nov. 2009 | Jashi DT-ZVL | I2 H2 | KU975149.1, KX150483.1, KX150467.1 |
| 2693 IMJW/CN/2008/KJS-1 | P. wui | Jul. 2008 | Jashi DT-ZVL | I2 H2 | KU975150.1, KX150484.1, KX150468.1 |
| 3044 MLEP/CN/2007/KJS-1 | Tarim hare | Dec. 2007 | Jashi DT-ZVL | I2 H2 | KU975151.1, KX150485.1, KX150469.1 |
| 3410 IMJW/CN/2014/HMF-1 | P. wui | Sep. 2014 | Minfeng DT-ZVL | I2 H2 | KU975152.1, KX150486.1, KX150470.1 |
| 3416 IMJW/CN/2014/HMF-2 | P. wui | Sep. 2014 | Minfeng DT-ZVL | I2 H2 | KU975153.1, KX150487.1, KX150471.1 |
| 3009 MHOM/CN/2009/KSF-1 | Patient (2 years) | Jan. 2009 | Shufu AVL | I1 H1 | KU975154.1, KX150488.1, NA |
| 2616 MHOM/CN/2009/KSF-2 | Patient (24 years) | Feb. 2009 | Shufu AVL | I1 H1 | KU975155.1, KX150489.1, KX150472.1 |
| 3149 MHOM/CN/2009/KSF-3 | Patient (10 years) | Mar. 2009 | Shufu AVL | I1 H1 | KU975156.1, KX150490.1, KX150473.1 |
| 2618 MHOM/CN/2009/KSF-4 | Patient (53 years) | Mar. 2009 | Shufu AVL | I1 H1 | KU975157.1, KX150491.1, KX150474.1 |
| 3219 MHOM/CN/2009/KSF-6 | Patient (4 years) | May. 2009 | Shufu AVL | I1 H1 | KU975158.1, KX150492.1, KX150475.1 |
| 3344 MHOM/CN/2009/KSF-7 | Patient (3 years) | Feb. 2009 | Shufu AVL | I1 - | KU975159.1, NA, NA |

*Bachu, Jashi, Minfeng and Shufu are counties in Xinjiang Autonomous Region, the People’s Republic of China.

-, not available; NA, no amplicon.

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Table 2. List of reference strains used in this study.

| Species            | WHO code       | Origin         | Gene | Haplotype number | Accession  |
|--------------------|----------------|----------------|------|------------------|------------|
| *L. donovani*      | MHOM/CN/00/Wangjie1 | China          | ITS1 | I2               | AJ000294  |
| *L. donovani*      | IPHL/CN/77/XJ771   | Bachu, China   | ITS1 | I2               | HM130608  |
| *L. donovani*      | MCAN/CN/60/GS1     | Gansu, China   | ITS1 | I5               | HQ830354  |
| *L. donovani*      | MHOM/ET/67/HU3     | Ethiopia       | ITS1 | I3               | AJ634373  |
| *L. donovani*      | MHOM/IN/80/DD8     | India          | ITS1 | I4               | AJ000292  |
| *L. donovani*      | MHOM/SD/93/9S      | Sudan          | ITS1 | I3               | AJ634372  |
| *L. donovani*      | MHOM/IQ/1981/SUKKAR2 | Iraq           | ITS1 | I6               | AM901452  |
| *L. donovani*      | MHOM/KE/83/NLB189  | Kenya          | ITS1 | I4               | AJ634374  |
| *L. infantum*      | MHOM/CN/08/Jiashi-1 | Jiashi, China  | ITS1 | I2               | GQ367486  |
| *L. infantum*      | MHOM/CN/54/Peking  | Beijing, China | ITS1 | I1               | AJ634345  |
| *L. infantum*      | MHOM/CN/78/D2      | Xinjiang, China| ITS1 | I1               | AJ000303  |
| *L. infantum*      | MHOM/TF/87/LEM75   | Tunisia        | ITS1 | I1               | AJ634339  |
| *L. infantum*      | MCAN/ES/86/LEM935  | Spain          | ITS1 | I1               | AJ634355  |
| *L. infantum*      | MHOM/IT/94/ISS1036 | Italy          | ITS1 | I1               | AJ634353  |
| *L. infantum*      | MHOM/IJ/2012/Savodjbolagh11 | Iran    | ITS1 | I1               | KC347299  |
| *L. infantum*      | MHOM/UZ/2007/KU    | Uzbekistan     | ITS1 | I1               | FM164420  |
| *L. major*         | MHOM/CN/2015/CPOLM-1 | China, imported | ITS1 | -                | KU975160  |
| *L. donovani*      | MHOM/CN/00/Wangjie1 | China          | hsp70| H2               | HF586394  |
| *L. donovani*      | MHOM/CN/90/9044    | Shandong, China| hsp70| H1               | JX021428  |
| *L. donovani*      | MHOM/CN/86/SC6     | Sichuan, China | hsp70| H1               | JX021429  |
| *L. donovani*      | IWUI/CN/77/771     | Bachu, China   | hsp70| H2               | JX021425  |
| *L. donovani*      | MHOM/NP/2003/BPK282 | Nepal         | hsp70| H3               | XM_003862348 |
| *L. donovani*      | MHOM/ET/67/HU3     | Ethiopia       | hsp70| H4               | X52314   |
| *L. donovani*      | MHOM/IN/00/DEV1    | India          | hsp70| H3               | FN395028  |
| *L. donovani*      | MHOM/MA/95/CRE72   | Morocco        | hsp70| H3               | HF586352  |
| *L. donovani*      | MHOM/SD/87/UGX-MARROW | Sudan       | hsp70| H3               | HF586386  |
| *L. infantum*      | MHOM/EG/87/RTC2    | Egypt          | hsp70| H1               | HF586350  |
| *L. infantum*      | MCAN/IL/97/LRC-L720 | Israel       | hsp70| H1               | HF586393  |
| *L. infantum*      | MHOM/MA/67/ITMAP263 | Morocco      | hsp70| H1               | FN395033  |
| *L. infantum*      | MHOM/PT/00/IMT260  | Portugal       | hsp70| H1               | FN395032  |
| *L. infantum*      | MHOM/MT/78/Buck    | Malta          | hsp70| H1               | FN395031  |
| *L. infantum*      | MHOM/MA/67/ITMAP263 | Morocco      | hsp70| H1               | FN395033  |
| *L. infantum*      | MHOM/BR/07/ARL     | Brazil         | hsp70| H1               | FN395037  |
| *L. infantum*      | MCAN/ES/98/LLM-877 | Spain          | hsp70| H1               | XM_001470287 |
| *L. donovani*      | MHOM/CN/80/801     | Kashi, China   | hsp70| H1               | JX970993  |
| *L. donovani*      | MHOM/CN/86/SC9     | Sichuan, China | hsp70| H1               | JX021430  |
| *L. donovani*      | MCAN/CN/97/WDD23   | Gansu, China   | hsp70| H1               | JX970994  |
| *L. donovani*      | MHOM/CN/96/KS6     | Kashgar, China | hsp70| H1               | JX970996  |
| *L. braziliensis*  | MHOM/CO/90/LEM2216 | Colombia       | hsp70| H5               | FN395043  |
| *L. guyanensis*    | MHOM/PE/02/LH2372  | Peru           | hsp70| H6               | FN395051  |
| *L. aethiopica*    | MHOM/ET/72/L100    | Ethiopia       | hsp70| H7               | FN395021  |
| *L. tropica*       | MHOM/IN/79/DD7     | India          | hsp70| H8               | FN395025  |
| *L. major*         | MHOM/IL/67/LRC-L137 | Israel       | hsp70| H9               | FN395023  |
| *L. amazonensis*   | MHOM/BR/73/M2269   | Brazil         | hsp70| H10              | EU599090  |
| *L. mexicana*      | MNYC/BZ/62/M379    | Belize         | hsp70| H11              | EU599091  |
| *L. infantum*      | MHOM/KE/84/NLB_323 | Kenya          | nagt | -                | DQ836148  |

(Continued)
Results

Sample collection

Samples were collected from 2004 to 2014. In total, 20 *Leishmania* isolates were obtained from patients, sand fly vector (*P. wui*) and Tarim hares (Table 1). The collection was carried out during the annual surveys for VL by the Xinjiang Center for Disease Control and Prevention in the AVL-endemic Shufu county in the alluvial plain and in the DT-ZVL-endemic regions in the Bachu, Jiashi and Minfeng counties in the desert area (Fig 1). A total of 12 samples were collected from the patients, of which six were under 1 year old from the DT-ZVL-endemic Bachu or Jiashi counties and six were 2 years old or older from the AVL-endemic Shufu county. The demographic data are consistent with the designation of the VL as the two different types indicated. The remaining eight samples were isolated from the vector *P. wui* and Tarim hares in the DT-ZVL-endemic Bachu, Jiashi and Minfeng counties.

PCR amplification of *nagt*, ITS1 and *hsp70* sequences and phylogenetic analyses

DNA isolated from cultured promastigotes of all 20 samples were subjected to PCR amplification. PCR amplification of the DNA samples with the primer set for *nagt* yielded a single product of the expected size (~1.4 kb) from 13 of the 20 isolates. The reason for the lack of amplification might be caused by several reasons. For instance, *nagt* is a single copy gene which may not have been successfully amplified due to poor sample quality in some cases. These *nagt* sequences were subjected to phylogenetic analysis together with those from representative sequences of other strains/species (Tables 1 and 2). The results clearly showed the segregation of all samples analyzed here into two groups according to the two VL types in the same clade with the reference sequences of the *L. donovani/L. infantum* complex. They were all distant from the other species complexes of the subgenus *Leishmania* (e. g. *L. major, L. tropica, L. mexicana*) and the subgenus *Viannia* (*L. braziliensis*) (Fig 2).

PCR amplification of the same DNAs for the ITS1 locus yielded ~320 bp products from all 20 samples. Sequence analyses of these products separated the 20 samples again into Type 1 and Type 2, in accordance with the two VL types (Table 1).

Alignment of Types 1 and 2 ITS1 sequences revealed C/T-substitution at position 78 and A-deletion at location 119 in the latter (Fig 3). Type 1 includes all isolates from the 6 patients

| Species | WHO code | Origin     | Gene | Haplotype number | Accession |
|---------|----------|------------|------|------------------|-----------|
| L. infantum | unknown | Turkey     | *nagt* | -                | AF205934  |
| L. infantum | MHOM/CN/50/Bman | China | *nagt* | -                | DQ836147  |
| L. infantum | MHOM/IR/11/Kazeroun | Iran | *nagt* | -                | KF701211  |
| L. infantum | MHOM/IR/11/Lamerd1 | Iran | *nagt* | -                | KF701212  |
| L. donovani | HOM/IN/97/JD | India  | *nagt* | -                | DQ836150  |
| L. major | MHOM/IR/11/Farashband | Iran | *nagt* | -                | KF701209  |
| L. tropica | MHOM/IR/11/Ghir-Karzin3 | Iran | *nagt* | -                | KF701206  |
| L. mexicana | HOM/CO/94/1182 | Colombia | *nagt* | -                | DQ836161  |
| L. braziliensis | HOM/BR/75/M2903 | Brazil | *nagt* | -                | DQ836162  |
| L. turanica | MRHO/IR/11/Gol-6 | Iran  | *nagt* | -                | JX103553  |
| L. gerbilli | MRHO/IR/10/Gol-9 | Iran  | *nagt* | -                | JX103531  |

*, designation of the strain does not represent the genotype; -, not available.

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in the AVL-endemic Shufu county, while Type 2 includes the remaining 14 isolates, from patients, the vector *P. wui* and Tarim hares in the DT-ZVL-endemic Bachu, Jiashi and Minfeng counties.

The types 1 and 2 ITS1 sequences were subjected to phylogenetic analyses together with those of the reference strains in the *L. donovani/infantum* complex from other geographical origins (Table 2). *L. major* sequence was used as the outgroup (Fig 4). All 20 sequences in question were found to group with members of the *L. donovani/infantum* complex in a primary clade, which was then separated into three subclades. One subclade contains all six samples from the AVL foci (Shufu county) that were identical in sequence with those of the *L. infantum* strains that are widely distributed in Mediterranean regions (France, Spain, and Italy), Middle East (Iran), Central Asia (Uzbekistan), and China (Beijing, Xinjiang). Another subclade contains the 14 samples from the DT-ZVL foci that were identical in sequence to those of some Chinese isolates, which were previously typed either as *L. infantum* or

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**Fig 2. Phylogenetic tree based on the alignment of amplified section of the nagt sequences.** The phylogenetic tree was constructed using the Neighbor-Joining (NJ), Maximum likelihood (ML) and Minimum Evolution (ME) methods, bootstrap values were provided next to nodes (NJ/ML/ME), a value lower than 50 was indicated as (- -). Information on the origins and hosts of isolates are provided. The terrain where isolates were collected in this study is indicated with triangle (▲, DT-ZVL foci) or square (■, AVL foci). don, *L. donovani*; inf, *L. infantum*; maj, *L. major*; aet, *L. aethiopica*; tro, *L. tropica*; tur, *L. turanica*; ger, *L. gerbilli*; mex, *L. mexicana*; bra, *L. braziliensis*; *, the species is suggested to be *L. donovani*.

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The third subclade, more distant from the first two, contains samples of mixed origins, ranging from Africa to the Middle East to Asia (China and India). We successfully PCR-amplified the \textit{hsp70} (~1,286 bp) from 16 of the 20 samples. Alignment of their sequences also revealed differences according to their origin from AVL and DT-ZVL (see Table 1 for sequence types). Isolates from the AVL foci had the identical \textit{hsp70} sequence haplotype H1, while isolates from the DT-ZVL foci were all H2. A set of \textit{hsp70} sequences from reference strains, representing both Old and New world species, was retrieved from GenBank (Table 2). Phylogenetic analyses of the \textit{hsp70} sequences also support the conclusion that all 20 isolates belong to the \textit{L. donovani/infantum} complex (Fig 5). Consistent with the ITS1 analysis, isolates from the AVL foci clustered with \textit{L. infantum} strains. Isolates from the DT-ZVL foci were separated from the isolates of the AVL foci. Collectively, \textit{Leishmania} isolates from AVL and DT-ZVL in Xinjiang clearly separate into two genetically distinct groups, as determined by sequence analysis of the three different genetic markers. The potential of the Tarim hare as a reservoir for DT-ZVL is strongly suggested by the sequence identity of its isolates with those from patients and vectors for all three phylogenetic markers examined.

**Discussion**

In the past, most of the work on leishmaniasis in China has been focused on prevention and control programs. Meanwhile the biological characteristics of the persisting \textit{Leishmania} spp. remains unclear, especially in some of the northwestern regions where VL is still endemic. In this study, two major findings were made from analyses of more recent isolates. Firstly, we demonstrated a clear separation of the AVL and DT-ZVL isolates into 2 different groups in the same \textit{L. donovani/infantum} clade. Secondly, the sequence identity of patient-, vector- and Tarim hare-derived isolates strongly suggests they have a zoonotic transmission cycle and that the Tarim hare acts as a potential reservoir of DT-ZVL. The role of Tarim hares as a reservoir is further supported by the fact that other lagomorphs have been reported in this role for...
zoonotic VL in Spain [24–26]. This also raises the question as to whether lagomorphs should be studied, at the global level, as a potential reservoir for *Leishmania* spp. The pathogen is thought to be transmitted among the natural hosts by sand flies. Humans are occasionally infected when they enter the region where sylvatic infected sand flies exist.
Fig 5. Haplotype network and phylogenetic tree based on the hsp70 sequences of the *L. donovani* complex. A haplotype network constructed using Median Joining and post processing with an MP calculation. The haplotypes of H1 to H11, as given in Fig 2, Tables 1 and 2, are represented by yellow circles. Small red solid circles represent median joining points, and mutational sites between two circles are shown next to the connecting line. The subgenera *Leishmania* and *Viannia* are separated by a thick black line. The phylogenetic tree was
Our study has provided preliminary evidence for the genetic difference in *Leishmania* isolates from the AVL and DT-ZVL regions in Xinjiang. In our study, we used genetic markers which were either single copy (*nagt*) or multicopy (ITS1 and *hsp70*). The latter proved more successful for amplification due to their multicopy nature when used for these challenging samples. However, discriminatory markers or typing methods with a higher resolution such as multilocus microsatellite typing (MLMT) or multilocus sequence typing (MLST) are encouraged to be considered for further analyses [6,27]. Ideally, whole genome sequencing would provide the ultimate solution to identify all possible genetic factors correlated with adaptation to different disease conditions [28–30]. Another possible limitation in this study was that only cultivable parasites were used for the analyses. These might represent a bias in the parasite population studied. Thus, phylogenomic analysis of lesion-derived amastigotes from mammalian hosts or promastigotes from sand-fly gut material will be a necessity for further study.

When we compared published *nagt*, ITS1 and *hsp70* sequences (MHOM/IN/1983/AG83; MHOM/IN/00/DEV1; MHOM/IN/80/DD8) from Indian *L. donovani* clinical samples, they were 100% identical to the Indian representative sequences we used in this study—we did not, therefore, include these in our phylogenetic study. To confirm the role of the Tarim hare as an animal reservoir, further detailed studies are required which need to involve collection of a larger sample set of infected animals (and human hosts) and analyse lesion-derived amastigotes with the multilocus markers or genomics analyses described above. However, due to the comprehensive control of leishmaniasis in China, both patient and animal infections are rare, and therefore some of the questions posed above may not be able to be answered.

There is little doubt that *Leishmania* spp. causing persistent VL in Xinjiang belongs to the *L. donovani* complex, but we found an atypical association between *L. infantum/donovani* and the epidemiology of the ZVL/AVL types. For the isolates from Shufu county, a well-known foci of AVL [3], sequence analysis indicated that they are, most likely, *L. infantum*. This is unusual, considering that the disease type is AVL. First, these isolates were mainly found in patients over 2 y.o. (Table 1), which is considered the main characteristic of *L. donovani* infection [31]. In addition, no animal reservoir has been found for the Xinjiang *L. infantum* species. A possibility that a hidden ZVL co-exists with AVL in the Shufu county was discounted, since all 6 *Leishmania* isolates in this region display the same ITS1 and *hsp70* haplotype. The existence of *L. infantum* in AVL has also been reported in other studies. A previously investigated isolate from Kashi City (an AVL focus), MHOM/CN/80/801, with an identical *hsp70* sequence to the *L. infantum* clade, was suggested to be *L. infantum* by MLST in another study [6]. In addition, several adult cases of *L. infantum* infection were reported in Spain where *L. infantum* was not considered to be a local species [32]. Thus, we conclude that the Xinjiang AVL isolates from the oases of the Kashgar county are *L. infantum* with atypical clinical manifestations and no identified animal reservoir.

On the other hand, all 14 isolates from the DT-ZVL foci, i.e. Bachu, Jiashi and Minfeng counties, are genetically close to *L. donovani*, even though they are responsible for zoonotic disease. The ITS1 phylogenetic tree showed a similar tree topology to a previous study [8], in
which all the isolates formed a sub-clade within the *L. infantum* cluster. However, further analysis revealed that their ITS1 sequences were identical to the MLEE-typed strain *L. donovani* MHOM/CN/00/Wangjie1, and also the strain IPHL/CN/77/XJ771 from Bachu county, which were deemed previously to have identity with *L. donovani* [9,13]. Additionally, our hsp70 data also suggested that these isolates are Xinjiang-specific *L. donovani* strains that are genetically close to the common *L. donovani* strains (H3) from India, Sudan, Nepal and Morocco in the haplotype network (Fig 5).

*L. donovani* is considered to be mainly anthropootic while *L. infantum* is zoonotic with dogs serving as a primary reservoir. There have been no reports of AVL caused by *L. infantum*, while ZVL caused by *L. donovani* has been documented before. Thus, all the Xinjiang *Leishmania* isolates we have studied display atypical epidemiological features. This suggests that more attention needs to be paid when classifying these species on clinical grounds, since there might also be underreporting or mis-reporting occurrences elsewhere.

In conclusion, species of the *L. donovani* complex are responsible for AVL and DT-ZVL in Xinjiang Autonomous Region of China. We consider that the two types of VL are caused by two different groups of parasite. Epidemiological conditions have a great impact on shaping the endemic area occupied by these parasites. Our results further support that the Tarim hare is most likely the reservoir for *L. donovani* and the source of infection in the desert region. Further control measures targeting these wild animals may be needed for the effective control of this disease. More discriminatory methods, particularly direct whole genome sequencing of parasites from host tissues, will be the preferred approach to clearly dissect the complicated situation in these Chinese *Leishmania* parasites.

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**References**

1. Bruschi F, Gradoni L. The leishmaniases: old neglected tropical diseases. 1st ed. Berlin: Springer; 2018.
2. Lun ZR, Wu MS, Chen YF, Wang JY, Zhou XN, Liao LF, et al. Visceral leishmaniasis in China: an endemic disease under control. Cil Microbiol Rev. 2015; 28: 987–1004. https://doi.org/10.1128/CMR.00080-14 PMID: 26354822

3. Wang JY, Cui G, Chen HT, Zhou XN, Gao CH, Yang YT. Current epidemiological profile and features of visceral leishmaniasis in People’s Republic of China. Parasit Vectors. 2012; 5: 31. https://doi.org/10.1186/1756-3305-5-31 PMID: 22316234

4. Guan LR. Current status of kala-azar and vector control in China. Bull World Health Organ. 1991; 69: 595–601. PMID: 1959161

5. Waki K, Dutta S, Ray D, Kolli BK, Akman L, Kawazu S, et al. Transmembrane molecules for phylogenetic analyses of pathogenic protists: Leishmania-specific informative sites in hydrophilic loops of trans-endoplasmic reticulum N-acetylglucosamine-1-phosphate transferase. Eukaryot Cell. 2007; 6: 198–210. https://doi.org/10.1186/1476-5958-5-59 PMID: 17142569

6. Zhang CY, Lu XJ, Du QX, Jian J, Shu L, Ma Y. Phylogenetic and evolutionary analysis of Chinese Leishmania isolates based on multilocus sequence typing. PLoS One. 2013; 8: e63124. https://doi.org/10.1371/journal.pone.0063124 PMID: 23646184

7. Liao LF, Yan SS, Bate W, Wu M, Xu B, Zhang Y, et al. Leishmania infantum firstly isolated from Yarkend hare (Lepus yarkandensis). Chin J Vector Bio & Control. 2009; 20: 45–47. (in Chinese)

8. Wang JY, Gao CH, Yang YT, Chen HT, Zhu XH, Lv S, et al. An outbreak of the desert sub-type of zoonotic visceral leishmaniasis in Jiashi, Xinjiang Uygur Autonomous Region, People’s Republic of China. Parasitol Int. 2010; 59: 331–337. https://doi.org/10.1016/j.parint.2010.04.002 PMID: 20434585

9. Yang BB, Guo XG, Hu XS, Zhang JG, Liao L, Chen JL, et al. Species discrimination and phylogenetic inference of 17 Chinese Leishmania isolates based on internal transcribed spacer 1 (ITS1) sequences. Parasitol Res. 2010; 107: 1049–1065. https://doi.org/10.1007/s00436-010-1969-9 PMID: 20617444

10. Xu ZB, Le Blancq S, Evans DA, Peters W. The characterization by isoenzyme electrophoresis of Leishmania isolated in the People’s Republic of China. Trans R Soc Trop Med Hyg. 1984; 78: 689–693. https://doi.org/10.1016/0033-9203(84)90243-8 PMID: 23646184

11. Xu ZB, Liu ZT, Long JY, Chai JJ, Chen WK. Further characterization of Chinese Leishmania isolates by isoenzyme electrophoresis. Chin Med J (Engl). 1989; 102: 679–685. PMID: 2517080

12. Lu HG, Zhong L, Guan LR, Qu JQ, Hu XS, Chai JJ, et al. Separation of Chinese Leishmania isolates into five genotypes by kinetoplast and chromosomal DNA heterogeneity. Am J Trop Med Hyg. 1994; 50: 763–770. https://doi.org/10.4269/ajtmh.1994.50.763 PMID: 8024072

13. Cao DP, Guo XG, Chen DL, Chen JP. Species delimitation and phylogenetic relationships of Chinese Leishmania isolates reexamined using kinetoplast cytochrome oxidase II gene sequences. Parasitol Res. 2011; 109: 163–173. https://doi.org/10.1007/s00436-010-2239-6 PMID: 21221640

14. Guan W, Cao DP, Sun K, Xu JN, Zhang JR, Chen DL, et al. Phylogenetic analysis of Chinese Leishmania isolates based on small subunit ribosomal RNA (SSU rRNA) and 7 spliced leader RNA (7SL RNA). Acta Parasitol. 2012; 57: 101–113. https://doi.org/10.2478/actapa.2012.0019 PMID: 22807046

15. Zhang CY, Zhou J, Ding B, Lu XJ, Xiao YL, Hu XS, et al. Phylogenetic analysis of lack gene sequences for 22 Chinese Leishmania isolates. Infect Genet Evol. 2013; 17: 79–86. https://doi.org/10.1016/j.imeegid.2013.03.026 PMID: 23641410

16. Alam MZ, Nakao R, Sakurai T, Kato H, Qu JQ, Chai JJ, et al. Genetic diversity of Leishmania donovani/infantum complex in China through microsatellite analysis. Infect Genet Evol. 2014; 22: 112–119. https://doi.org/10.1016/j.meegid.2014.01.019 PMID: 24480049

17. Ma Y, Bu L, Hua X, 20-year search on molecular markers of Leishmania isolates from different Kala-azar foci in China to confirm whether genetic fingerprints of Kala-azar pathogens correlate with disease types. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi. 2011; 28: 997–1000. (in Chinese) PMID: 22097271

18. Sambrook J, Russell DW. Molecular cloning: a laboratory manual. 3rd eds. Newyork: Cold Spring Harbor Laboratory; 2001.

19. el Tai NO, Osman OF, el Fari M, Presber W, Schonian G. Genetic heterogeneity of ribosomal internal transcribed spacer in clinical samples of Leishmania donovani spotted on filter paper as revealed by single-strand conformation polymorphisms and sequencing. Trans R Soc Trop Med Hyg. 2000; 94: 575–579. https://doi.org/10.1016/s0035-9203(00)90093-2 PMID: 11132393

20. Montalvo AM, Fraga J, Maes I, Dujardin JC, Van der Auwera G. Three new sensitive and specific heat-shock protein 70 PCRs for global Leishmania species identification. Eur J Clin Microbiol Infect Dis. 2012; 31: 1453–1461. https://doi.org/10.1007/s10096-011-1463-z PMID: 22083340

21. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGAS: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011; 28: 2731–2739. https://doi.org/10.1093/molbev/msr121 PMID: 21546353
22. Lai D-H, Wu N, Xie Y-T, Hong XK, Chen YF, Liao L-F, et al. Pathogen identification in an imported case of cutaneous leishmaniasis. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi. 2016; 34, 295–296 & cover 3. (in Chinese) PMID: 30129743

23. Clement M, Posada D, Crandall KA. TCS: a computer program to estimate gene genealogies. Molecular Ecology. 2000; 9: 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x PMID: 11050560

24. Molina R, Jimenez MI, Cruz I, Iriso A, Martin-Martin I, Sevillano O, et al. The hare (Lepus granatensis) as potential sylvatic reservoir of Leishmania infantum in Spain. Vet Parasitol. 2012; 190: 268–271. https://doi.org/10.1016/j.vetpar.2012.05.006 PMID: 22677135

25. Jimenez M, Gonzalez E, Martin-Martin I, Hernandez S, Molina R. Could wild rabbits (Oryctolagus cuniculus) be reservoirs for Leishmania infantum in the focus of Madrid, Spain? Vet Parasitol. 2014; 202: 296–300. https://doi.org/10.1016/j.vetpar.2014.03.027 PMID: 24774435

26. Moreno I, Alvarez J, Garcia N, de la Fuente S, Martinez I, Marino E, et al. Detection of anti-Leishmania infantum antibodies in sylvatic lagomorphs from an epidemic area of Madrid using the indirect immunofluorescence antibody test. Vet Parasitol. 2014; 199: 264–267. https://doi.org/10.1016/j.vetpar.2013.10.010 PMID: 24211046

27. Kuhls K, Keilonat L, Ochsenreither S, Schaar M, Schweynoch C, Presber W, et al. Multilocus microsatellite typing (MLMT) reveals genetically isolated populations between and within the main endemic regions of visceral leishmaniasis. Microbes Infect. 2007; 9: 334–343. https://doi.org/10.1016/j.micinf.2006.12.009 PMID: 17307010

28. d’Avila-Levy CM, Boucinh a C, Kostygov A, Santos HL, Morelli KA, Grybchu k-Ieremenko A, et al. Exploring the environmental diversity of kinetoplastid flagellates in the high-throughput DNA sequencing era. Mem Instituto Oswaldo Cruz. 2015; 110: 956–965. https://doi.org/10.1590/0074-02760150253 PMID: 26602872

29. Votýpka J, d’Avila-Levy CM, Grellier P, Maslov DA, Lukeš J, Yurchenko V. New approaches to systematics of Trypanosomatidae: criteria for taxonomic (re) description. Trends Parasitol. 2015; 31: 460–469. https://doi.org/10.1016/j.pt.2015.06.015 PMID: 26433249

30. Lukeš J, Butenko A, Hashimi H, Maslov DA, Votýpka J, Yurchenko V. Trypanosomatids are much more than just trypanosomes: clues from the expanded family tree. Trends Parasitol. 2018; 34(6), 466–480. https://doi.org/10.1016/j.pt.2018.03.002 PMID: 29605546

31. World Health Organization. Control of the leishmaniases. World Health Organ Tech Rep Ser. 2010; (949): xii-xiii, 1–186, back cover. PMID: 21485694.

32. Horrillo L, San Martin JV, Molina L, Madronal E, Matia B, Castro A, et al. Atypical presentation in adults in the largest community outbreak of leishmaniasis in Europe (Fuenlabrada, Spain). Clin Microbiol Infect. 2015; 21: 269–273. https://doi.org/10.1016/j.cmi.2014.10.017 PMID: 25658537