Cutaneous leishmaniasis in the central provinces of Hama and Edlib in Syria: Vector identification and parasite typing

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Abstract

Background: Cutaneous leishmaniasis is a disease transmitted by sand fly bites. This disease is highly prevalent in Syria where Leishmania major and Leishmania tropica are the known aetiological agents. In 2011, more than 58,000 cases were reported in the country by the Ministry of Health. The central region of the country harbors 20 % of the reported cases. However, the epidemiology of the disease in this area is not well understood. An epidemiological survey was conducted in 2010 to identity the circulating parasite and the sand fly vector in the central provinces of Edlib and Hama.

Methods: Sand fly specimens were collected using CDC light traps and identified morphologically. Total DNA was extracted from the abdomens of female specimens and from Giemsa-stained skin lesion smears of 80 patients. Leishmania parasites were first identified by sequencing the ITS1 gene amplicons. Then polymorphism analysis was performed using the RFLP technique.

Results: A total of 2142 sand flies were collected. They belonged to eight species, among which Phlebotomus sergenti and Phlebotomus papatasi were the most predominant. L. tropica ITS1 gene was amplified from two pools of P. sergenti specimens and from skin smears of cutaneous leishmaniasis patients. This suggests that P. sergenti is the potential vector species in the study area. The digestion profiles of the obtained amplicons by TaqI restriction enzyme were identical for all analysed L. tropica parasites. Moreover, L. infantum ITS1 gene was amplified from two pools of Phlebotomus tobbi in the relatively humid zone of Edlib.

Conclusions: L. tropica is confirmed to be the aetiological agent of cutaneous leishmaniasis cases in the central provinces. RFLP technique failed to show any genetic heterogeneity in the ITS1 gene among the tested parasites. The molecular detection of this parasite in human skin smears and in P. sergenti supports the vector status of this species in the study area. The detection of L. infantum in P. tobbi specimens indicates a potential circulation of this parasite in the humid zone of Edlib. Further epidemiological studies are needed to evaluate the burden of this visceral parasite in the study region.

Keywords: Cutaneous leishmaniasis, Phlebotomus sergenti, Phlebotomus tobbi, Leishmania tropica, Leishmania infantum, ITS1, RFLP, Edlib, Hama, Syria
Background

Cutaneous leishmaniasis (CL) is a disease caused by *Leishmania* parasites and transmitted to humans by sand fly bites in more than 70 countries around the world, in climates ranging from tropical to desert. Syria is considered one of the most endemic countries for CL [1]. Syrian health authority records show that the vast majority (90 %) of reported CL cases in the country are caused by *Leishmania tropica*, whereas the remaining cases are caused by the zoonotic parasite *L. major* [2]. Visceral leishmaniasis due to *Leishmania infantum* is also spread in Syria. The incidence for the period 2004–2008 was 14 cases/year, mainly reported from Edlib province. Dogs are suspected to be the main reservoir [2]. Published data from the Syrian Ministry of Health show that, despite the implementation of several control programmes, the incidence of CL has increased during the last fifteen years from 12,027 cases in 1997 to 58,156 cases in 2011 [3]. Since 2011, the ongoing Syrian war and the resulting massive population displacement have led to a significant increase of the incidence of CL in the country and in neighbouring Turkey, Jordan and Lebanon [4, 5]. In 2013, more than 1000 cases of CL (mainly caused by *L. tropica*) were recorded in Lebanon among Syrian refugees [4].

The northern province of Aleppo accounts for 56 % of CL, followed by the central provinces of Edlib and Hama which together harbor 20 % of all declared cases. Several studies on leishmaniasis have been conducted in the Aleppo area [6–10]. The causative agent was typed as *L. tropica* ZMON-76 [11]. However, the epidemiology of the disease in the central provinces remains poorly studied. Records from Hama province suggest that *L. tropica* is the main circulating parasite [12]. During the summer of 2010, we conducted an entomological survey in the provinces of Hama and Edlib in order to identify the potential vectors of CL and establish an inventory of existing sand fly species. In addition, we analysed Giemsa-stained smears of skin lesions belonging to CL patients residing in these provinces to determine the species identity and polymorphism of the circulating *Leishmania* parasites. Classically, polymerase chain reaction (PCR) based methods performed on various targets such as microsatellite, kinetoplast DNA, or ribosomal RNA have been used for identification of *Leishmania* isolates in various foci [13–15]. The internal transcribed spacers 1 (ITS1) gene, a ribosomal RNA gene, is considered a primary target for this purpose as shown in many studies conducted in endemic areas in the Old and New World [16–22]. In this study, sequence and restriction fragment length polymorphism (RFLP) analyses were performed on the ITS1 amplicons in order to identify the circulating species and to detect potential genetic polymorphism.

Methods

Description of the study area

The provinces of Hama (8883 km²) and Edlib (5933 Km²) are located in the central region of Syria (Fig. 1), which is characterized by an arid climate with an annual average rate of precipitation lower than 200 mm. The western side of this region is influenced by the Mediterranean climate and, therefore, is obviously more humid. Agriculture is the main economic resource for the two million residents of this area.

Sample collection

Sand flies were captured during July and August 2010, in 11 geographical locations in the province of Hama and 17 locations in the province of Edlib (Fig. 1), from where human CL cases were reported in 2010 by the Syrian Ministry of Health. CDC miniature light traps (John Hock Co., Gainesville, FL, USA) were placed in indoor (bedrooms, animal shelters) and outdoor habitats (private gardens). Collected specimens were preserved in 90 % ethanol. In addition, Giemsa-stained skin smear slides of patients with confirmed CL were provided to us by the Ministry of Health authorities. These smears were performed on skin lesions of patients seeking diagnosis and treatment at local primary health care centers in Hama and Edlib provinces. All tested smears were confirmed positive by observing *Leishmania* amastigotes microscopically.

Sand fly identification

Male specimens were cleared in Marc-André solution [23] and then mounted on slides in chloral gum medium [24]. Female specimens were dissected; their head and genitalia were cleared and then mounted, whereas their abdomen and thorax were preserved in ethanol for further molecular analysis. Female and male specimens were morphologically identified by observing head and genital structures under the microscope using local and regional identification keys [25, 26].

DNA extraction

Abdomens of female sand flies were pooled (up to 10 per pool) according to species and capture locality. These samples were then ground in microtubes (1.5 mL). Slides of skin lesion smears were individually scraped using sterile blade scalpels. The scraping product was collected in microtubes. Total DNA was extracted from both types of materials using the extraction kit QIAamp DNA Mini Kit (Qiagen, Valencia, CA).

PCR amplification and sequencing of ITS1 gene

DNA extracts were screened for *Leishmania* DNA by conventional PCR technique. A 339-bp fragment of the internal transcribed spacer region (ITS1) of the small
subunit ribosomal DNA was amplified using specific primers: L5.8S: 5′-TGATACCACTTATCGCACTT-3′ and LITSR: 5′-CTGGATCATTTTCCGATG-3′ [17]. The PCR mix comprised 2.5 U Taq polymerase (Promega, Madison, WI), 0.6 pM of each primer, 1.5 mM MgCl₂, 0.1 mM dNTPs, and 5 μL DNA template in a final volume of 50 μL. Cycling conditions were as follow: 95 °C, 120 s and (94 °C, 30 s; 53 °C, 60 s; 72 °C, 60 s) repeated 37 times.

Amplicons were visualized after electrophoresis in 1.5 % agarose gel in TBE (1X) buffer. DNA extracts from *L. infantum* parasite of the reference strain MHOM/FR/78/LEM 75 and male sand fly specimen were used respectively as positive and negative controls. Amplicons were purified using the Qiagen PCR Clean up Kit (Qiagen, Valencia, CA). Fifty fmol of purified DNA were sequenced in the forward direction with an ABI Prism 3130 Genetic Analyzer, Big Dye Terminator technology (Applied Biosystems, Foster City, CA, USA) using 1.6 pmol of each primer. Sequences were manually edited and used to query GenBank for species identity.

**RFLP analysis of amplified ITS1**
In order to detect potential genetic polymorphism among the identified *Leishmania* parasites, 10 μL of non-purified ITS1 amplicons were digested with Taq I restriction enzyme as indicated by the manufacturer (New England Biolabs, Ipswich, MA, USA). The digested products were run on 1.5 % electrophoresis gel agar at 100 V in TBE buffer and visualized under ultraviolet light. Bands were stained by adding GelRed dye (Phenix research, USA) to the gel preparation.

**Results**

**Identification of collected sand fly specimens**
The total number of sand flies captured was 2142, including 603 in Hama province and 1539 in Edlib province. Their identification revealed eight species, of which seven belonged to the *Phlebotomus* genus and one to the *Sergentomyia* genus (Tables 1 and 2).

The dominant species among collected specimens was *Phlebotomus sergenti* in both provinces, followed by *Phlebotomus papatasi*. These species are known vectors of anthroponotic and zoonotic forms of CL respectively [27–32]. *Larroussius* subgenus is dominantly represented by *Phlebotomus gallilaeus*. Specimens of this species were mainly collected from the locality of Khamrieh in the humid area west of Edlib, near the Turkish border. *P. tobbi* is another *Larroussius* member found in the study area. This species is spread mainly in countries of the mid-northern to eastern sides of the Mediterranean basin. Moreover, *P. tobbi* is the vector of *L. infantum* in Cyprus [33] and a suspected vector of this parasite in several countries, including Turkey and Iran [34, 35].
Parasite identification

The presence of ITS1 gene of *Leishmania* parasite was investigated by PCR on 1172 specimens of female sand flies, divided into 216 pools, as well as on skin smears of 80 patients of CL (40 from each province). Amplification results identified two positive pools of *P. sergenti*, one collected in the locality of Deir Charki (Edlib), the other in Smakh (Hama) (Fig. 2). In total, 489 female specimens of this species were tested in the study zone, including 254 from Edlib province (of which 14 were from Deir Charki) and 235 from Hama province (16 from Smakh). We also identified two *Leishmania* positive pools of *P. tobbi*, which were collected in the localities of El Jebb and El Safsafeh in Edlib. In this province, a total of 85 female specimens of *P. tobbi* were collected, of which only 6 were captured in El Jebb and 19 in El Safsafeh. On the other hand, ITS1 gene amplicons were obtained from 70 skin smears (34 from Edlib and 36 from Hama) out of the 80 tested samples.

All the obtained amplicons were sequenced. Their analysis revealed that the gene segments amplified from the two pools of *P. sergenti* and those from all analysed skin smears from both Syrian provinces are identical and belong to *L. tropica*. These sequences perfectly matched with ITS1 sequences of *L. tropica* isolate MHOM/AF/88/KK27 from Afghanistan and MHOM/IR/2012/Savadjbolagh isolate from Iran [GenBank: GQ913688.1, GenBank: KC574386.1]. Moreover, analysis of ITS1 gene sequences amplified from the two pools of *P. tobbi* from Edlib showed that the parasite belongs to *L. infantum* and is identical to the Iranian strain ITOB/IR/2009/Bilesavar [GenBank: HQ535858.1], which was also isolated from *P. tobbi*.

RFLP results

After species identification, all PCR products of *L. tropica* from the skin smears of CL patients and from *P. sergenti* pools were digested by Taq1 restriction enzyme in order to detect potential genetic polymorphism. Digestion products showed that all isolates displayed two DNA bands of approximately 140 and 190 bp (Fig. 3).

Discussion

The objectives of the current study were to identify the sand fly vectors and *Leishmania* species responsible for the CL cases in the central provinces of Hama and Edlib. Sequences of the ITS1 gene amplified from skin lesions of 70 patients showed that *L. tropica* is the causative agent of CL in both provinces. This species is also the main agent of CL in the highly endemic Aleppo province, northern Syria [9, 36] and in several foci in neighbouring countries [37, 38]. PCR-RFLP analysis of the ITS1 gene is commonly used to detect intraspecific genetic diversity among *L. tropica* species [21, 22]. This method is usually

### Table 1 Species identity of sand fly specimens collected from 11 localities in the province of Hama

| Collection locality | Geographical coordinates | P. (Ph) papatasi | P. (Para) sergenti | P. (Para) jacusieli | P. (L) tobbi | P. (L) gallilaeus | P. (L) syriacus | S. (S) dentata | Total |
|---------------------|--------------------------|-----------------|-------------------|-------------------|-------------|-----------------|---------------|-------------|-------|
| Aouja               | 35° 9’13.26”N 36° 52’52.99”E | 15              | 5                 | 0                 | 0           | 0               | 0             | 20          | 40    |
| Asileh              | 35° 11’57.51”N 36° 29’16.17”E | 4               | 10                | 0                 | 0           | 1               | 0             | 5           | 20    |
| Deir El Salib       | 35° 5’9.76”N 36° 26’49.24”E | 8               | 41                | 0                 | 5           | 0               | 3             | 2           | 59    |
| EL Rasafah          | 35° 1’38.98”N 36° 18’8.62”E | 6               | 24                | 4                 | 4           | 0               | 4             | 9           | 51    |
| Hamadi El Chihan    | 35° 7’54.30”N 36° 51’55.38”E | 3               | 1                 | 0                 | 0           | 0               | 0             | 0           | 4     |
| Kaskas              | 35° 4’7.30”N 36° 29’28.72”E | 28              | 231               | 0                 | 2           | 0               | 3             | 2           | 266   |
| Kasoun El Jabal     | 35° 9’17.65”N 36° 50’50.02”E | 7               | 4                 | 0                 | 0           | 0               | 0             | 2           | 13    |
| Kfarakid            | 35° 3’44.01”N 36° 26’34.99”E | 11              | 31                | 1                 | 9           | 0               | 2             | 1           | 55    |
| Maarrine            | 35° 9’40.38”N 36° 27’24.21”E | 7               | 11                | 0                 | 0           | 1               | 0             | 3           | 22    |
| Mahrouseh           | 35° 9’9.60”N 36° 24’34.05”E | 8               | 31                | 0                 | 4           | 0               | 0             | 3           | 46    |
| Smakh               | 35° 5’39.27”N 36° 53’56.72”E | 9               | 16                | 0                 | 0           | 0               | 0             | 2           | 27    |
| Total               |                          | 106             | 405               | 5                 | 24          | 2               | 12            | 49          | 603   |

Letters between brackets indicate the species subgenus: (Ph) Phlebotomus, (Para) Paraphlebotomus, (L) Larroussius, (S) Sergentomyia
Table 2  Species identity of sand fly specimens collected from 17 localities in the province of Edlib

| Collection locality | Geographic coordinates | P. (Ph) papatasi | P. (Para) sergenti | P. (Para) jacusieli | P. (Para) alexandri | P. (L) tobbi | P. (L) galiilaeus | P. (L) syriacus | S. (S) dentata | Total |
|---------------------|------------------------|------------------|-------------------|-------------------|-------------------|-------------|-----------------|----------------|--------------|-------|
| Al Allani           | 36° 9'39.87"N 36° 23'7.62"E | 0                | 0                 | 0                 | 6                 | 0           | 0              | 0              | 0            | 6     |
| Al Bakdash          | 35° 44'38.41"N 36° 21'53.93"E | 12               | 13                | 0                 | 0                 | 7           | 1              | 1              | 17           | 51    |
| Biskla              | 35° 360.23"N 36° 34'47.02"E | 12               | 19                | 0                 | 0                 | 2           | 0              | 1              | 0            | 34    |
| Deir Charki         | 35° 36'18.17"N 36° 42'53.65"E | 3                | 18                | 0                 | 0                 | 0           | 0              | 0              | 0            | 21    |
| Deir Gharbi         | 35° 35'34.94"N 36° 41'12.83"E | 28               | 129               | 0                 | 0                 | 0           | 0              | 0              | 7            | 164   |
| Delbieh             | 36° 7'38.69"N 36° 24'8.31"E | 7                | 0                 | 0                 | 0                 | 4           | 0              | 0              | 0            | 11    |
| El Jakarah          | 36° 8'55.49"N 36° 23'20.67"E | 7                | 0                 | 0                 | 0                 | 2           | 1              | 0              | 1            | 11    |
| El Jebb             | 36° 10'7.98"N 36° 24'16.58"E | 16               | 98                | 0                 | 0                 | 9           | 4              | 5              | 0            | 132   |
| El Kah              | 35° 32'25.94"N 36° 43'44.11"E | 17               | 44                | 0                 | 0                 | 1           | 0              | 0              | 23           | 85    |
| El khamrieh         | 36° 630.90"N 36° 23'21.13"E | 29               | 2                 | 0                 | 1                 | 31          | 270             | 12             | 0            | 345   |
| El Safafeh          | 36° 89.89"N 36° 24'0.53"E | 3                | 0                 | 0                 | 1                 | 23          | 11             | 4              | 1            | 43    |
| Freikheh            | 35° 45'40.77"N 36° 21'48.07"E | 2                | 2                 | 0                 | 0                 | 0           | 0              | 0              | 0            | 4     |
| Hass                | 35° 37'59.47"N 36° 38'1.72"E | 42               | 13                | 1                 | 0                 | 1           | 0              | 2              | 2            | 61    |
| Kafroumeh           | 35° 38'15.85"N 36° 39'1.24"E | 138              | 248               | 0                 | 0                 | 2           | 0              | 4              | 23           | 415   |
| Sallet El Zouhour   | 35° 44'53.77"N 36° 21'48.74"E | 5                | 4                 | 0                 | 0                 | 3           | 0              | 0              | 0            | 14    |
| Tekanah             | 35° 3331.92"N 41'3.85"E | 31               | 66                | 0                 | 0                 | 1           | 0              | 0              | 0            | 98    |
| Wadi el Dahab       | 36° 86.74"N 36° 27'10.03"E | 1                | 0                 | 0                 | 0                 | 10          | 23             | 0              | 10           | 44    |
| Total               |                       | 353              | 656               | 1                 | 2                 | 102         | 310            | 29             | 86           | 1539  |

Letters between brackets indicate the species subgenus: (Ph) Phlebotomus, (Para) Paraphlebotomus, (L) Larroussius, (S) Sergentomyia

Fig. 2 Electrophoresis gel showing PCR products of the ITS1 gene amplified from (a) two sand fly pools of *P. sergenti* (lanes 4 and 6) (b) two sand fly pools of *P. tobbi* (lanes 6 and 8)
zymodeme (MON-76) among 51 tested samples of Psammomys obesus seems to circulate in the study area, as it – L. infantum and P. tobbi are the most as the vector of this L. infantum L. tropica. The rodent reservoir of L. tropica (Procavia capensis) is the in the humid part of Edlib. These isolates with different digestion patterns, reflecting the existence of a genetic polymorphism at the level of the ITS1 gene. Moreover, our results suggest that the sand fly vector responsible for L. tropica transmission in the study area is P. sergenti. Two pools of sand flies belonging to this species were positive for Leishmania ITS1 gene. PCR-RFLP analysis of the amplified gene produced the same digestion pattern as that observed for all L. tropica isolates from skin lesions. Besides, P. sergenti is the dominant sand fly (43 %) in our collections. This species is known to be the vector of L. tropica in several countries [27–30]. L. tropica is an anthropo- nic parasite; nevertheless, zoonotic transmission has been documented in certain foci in the Middle East. The rock hyrax (Procavia capensis) is the main reservoir of L. tropica in certain Israeli and Palestinian foci [40]. In addition, dogs (and other Canidae) are suspected to be involved in the transmission of this Leishmania species in Morocco, Iran and Israel [41–43]. The high endemicity of L. tropica in the central provinces of Syria suggests an anthropo- nic transmission pattern of this parasite. However, the involvement of dogs as reservoirs should be investigated. It is worth noting the absence of L. major, the agent of zoonotic CL, among the tested skin smears, despite the important spread of its specific vector, P. papatasi. The rodent reservoir of this species (Psammomys obesus) does not occur in the central provinces, while it is found mainly in the desert area, east of Damascus [44]. L. infantum seems to circulate in the study area, as it was detected in two pools of female specimens of P. tobbi collected in the humid area of Edlib. L. infantum is the known agent of visceral leishmaniasis in the Mediterranean basin. Few cases of VL are reported annually in Syria. Recent reports showed an incidence of VL of 14 cases per year, based on a 2–4-fold underreporting ratio [1]. The involvement of P. tobbi in the transmission of visceral Leishmania parasites was also recorded during a study conducted in the province of Lattakia that borders the western side of the study zone [45]. Nevertheless, L. infantum was also proven to be a causative agent of CL in the Mediterranean basin [46–50]. In the neighbouring Anatolia (South of Turkey), Svobodova and collaborators (2009) identified L. infantum as the causative agent of cutaneous lesions [33]. These authors incriminated P. tobbi as the vector of this dermatropic form. We expect a similar epidemiological situation in the humid zones west of Syria. Analysis of skin smears of patients originating from these zones is required to confirm the circulation of dermatropic L. infantum.

Conclusions
Using PCR-ITS1 method, L. tropica was identified as the parasitic agent responsible for the high number of CL cases in the central provinces of Syria. RFLP analysis of the ITS1 gene amplicons obtained from skin lesions and from the sand fly vector did not reveal any polymorphism among the circulating parasites. Entomological data showed that P. sergenti and P. papatasi are the most dominant species in our collections. The detection, for the first time in Syria, of L. tropica ITS1 gene in two pools of P. sergenti, supports the vector status of this species. The amplification of L. infantum ITS1 gene from two pools of P. tobbi in the humid part of Edlib suggests an active circulation of this parasite. Further studies in humans, vectors and potential reservoirs are needed to determine the burden of L. infantum in the study area and in other parts of Syria.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
NH was involved in the study design, conducted field work (Sand fly collection), performed molecular study, contributed to the sand fly identification and drafted the manuscript. HS identified the sand flies and performed molecular work. AA organized and participated in field work (samples collection), JV was involved in the optimization of the molecular work protocol. SA initiated the study, provided local contacts, contributed to the study design, samples collection and manuscript reviewing. All authors read and approved the final manuscript.
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