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# *Phlebotomus sergenti* a common vector of *Leishmania tropica* and Toscana virus in Morocco

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## ABSTRACT

*Background & objectives:* An entomological study using CDC miniature light-traps was performed in El Hanchane locality, where cutaneous leishmaniasis (CL) was emerging during the summer of 2011. The aim of this study is to identify the vectors of *Leishmania* and of phleboviruses.

*Methods:* In the field, a total of 643 sandfly specimens were collected, identified by morphological keys and categorized by sex and species. A total of nine distinct species were morphologically identified where seven belonged to the *Phlebotomus* genus and two species to the *Sergentomyia* genus. *Phlebotomus sergenti* was the most abundant species (76%). Phleboviruses were detected by nested RT-PCR using 30 pooled sandflies while *P. sergenti* females were tested individually for infections of *Leishmania* species.

*Results:* By using ITS1-PCR-RFLP approach, *Leishmania tropica* DNA was detected in 10 females, caught in this emerging focus, and provide additional evidence in favour of the role of *P. sergenti* as vector of *L. tropica* in Morocco. Real-time PCR screening for phlebovirus RNA, using an assay targeting the polymerase gene, showed positive result in one pool of male *P. sergenti*.

*Interpretation & conclusion:* In this study, *P. sergenti* were infected by *L. tropica* and Toscana virus. To our knowledge, actually this is the first time that Toscana virus has been detected in *P. sergenti*.

**Key words** *Leishmania tropica;* Morocco; *Phlebotomus sergenti;* sandfly; Toscana virus

## INTRODUCTION

Phlebotomine sandflies (Diptera: Psychodidae) are small-sized blood-sucking insects feeding on a wide range of hosts, and potentially transmit pathogens to man and other animals. These are vectors of bacteria (e.g. *Bartonella bacilliformis*), viruses (e.g. *Phlebovirus* and *Vesiculovirus*), and protozoa (e.g. *Leishmania* spp)[1–4]. Among > 800 phlebotomine sandfly species estimated to exist, only 98 species of *Phlebotomus* and *Lutzomyia* genera are proven or suspected vectors of human leishmaniasis[5]. This parasitic disease is caused by the unicellular *Leishmania* parasites which affect 12 million of people worldwide[6].

*Leishmania tropica* a causative agent of cutaneous leishmaniasis (CL) is transmitted from person-to-person through *Phlebotomus (Paraphlebotomus) sergenti* in urban settings[5]. It was originally described from Algeria, although the species has a broad range of distribution which covers vast areas of southern mediterranean (Morocco, Algeria and Tunisia), northern mediterranean, Saudi Arabia, Afghanistan, Pakistan and northern parts of India. Such a broad distribution suggests a notable intraspecific variability and since the distribution of the vector is broader than the distribution of the transmitted parasite[7], it may play an important role in the epidemiology of the disease. *Phlebotomus sergenti* has been confirmed as the vector of CL caused by *L. tropica* in Morocco[8], Algeria[9] and Tunisia[10].

Sandflies are also vectors of many viruses belonging to three different genera: (i) the *Phlebovirus* (family *Bunyaviridae*) including sandfly fever *Sicilian virus* (SFSV)[1], sandfly fever *Naples virus* (SFNV)[1], and *Toscana virus* (TOSV)[1]; (ii) the *Vesiculovirus*[11–12]; and (iii) the *Orbivirus*[13]. SFNV and SFSV lead to short-term febrile illness, whereas TOSV virus can cause central nervous system (CNS) infections such as meningitis, encephalitis, meningoencephalitis and peripheral neurologic symptoms. TOSV has been isolated from the *P. perniciosus*[14], *P. perfiliewi*[15], and TOSV RNA was detected in *Sergentomyia minuta*[16].

Several species of *Leishmania* and phleboviruses have a common *Phlebotomus* spp vector. For example, *P. ariasi* is a vector of *L. infantum*[17] and SFSV[18], *P. longicuspis* is

a potential vector of *L. infantum*[19] which have been found to be infected by SFNV and SFSV[20] and *P. perniciosus* can transmit both *L. infantum*[21] and TOSV[5]. The first robust evidence for the existence of an epidemiological relationship between *Leishmania* and phleboviruses, which has been assumed for a long time, was described by Bichaud *et al* [22]. The main objectives of this study were to inventory the Phlebotomine sandfly species in an emerging focus of CL due to *L. tropica*, in the El Hanchane district in the center of Morocco, and to detect *Leishmania* DNA and phlebovirus RNA in sandflies using the internal transcribed spacer 1 (ITS1) gene system and a generic nested RT-PCR, respectively.

## MATERIAL & METHODS

*Study sites and collection of sandflies*

Sandfly sampling was carried out from July to September 2011 (during the period of sandfly peak prevalence), in the district of El Hanchane (31° 31' 11" N and 9° 26' 02" W) which is 30 km near the town of Essaouira in the centre of Morocco. Population size was 5349 and this semi-rural locality was selected based on the emergence of CL cases. The sandflies were collected using CDC light-traps placed in or near human dwellings or horse stables. The traps were set in the late afternoon and sandflies were collected from the traps the following morning. All the sandflies were identified morphologically by dissecting genital organs according to morphological keys modified by Boussaa[23].

For detection of phlebovirus RNA, sandflies were pooled with a maximum of 30 individuals per pool, based on trapping origin, species and sex, and were placed in 1.5 ml tubes and stored at –80°C. *Phlebotomus sergenti* females were tested individually for infection by *Leishmania* species, a part of nucleic acid (NA) extracted was pooled for the detection of phlebovirus RNA.

*Extraction of NA from sandflies*

RNA extraction for 33 pools of sandflies was performed using the "Mini kit MACHEREY-NAGEL Nucleospin RNA II" (Dûren, Germany) according to the manufacturer's instructions. In 273 females of *P. sergenti* total nucleic acid was extracted via Biorobot EZ1, by Qiagen kit according to the instructions of the manufacturer.

*Detection of Phlebovirus and Leishmania infection*

*Phlebovirus:* The RNA was transcribed into cDNA with the "Transcriptor First Strand cDNA Synthesis Kit, Roche" (Mannheim, Germany), PCRs were performed as previously reported[24]. To avoid the risks of contamination, positive controls were not included in the experiment. The PCR product was cloned and sequenced in both directions.

*Leishmania typing:* The ribosomal internal transcribed spacer 1 (ITS1) was subjected to PCR, using the LITSR and L.5.8S primers[25]. Amplification reaction was performed in a volume of 50 µl. The reaction was performed in a thermocycler (S1000™ Thermal Cycler, Bio-Rad) with the following steps: initial denaturation at 94°C for 2 min followed by 32 cycles each, consisting of denaturation at 94°C for 20 s, annealing at 53°C for 30 s and extension at 72°C for 1 min and in the end post-extension phase at 72°C for 6 min. PCR products were analyzed using a 1.5% agarose gel together with a 100 bp ladder marker, and were visualized by UV-light transillumination.

For *Leishmania* species discrimination, ITS1-PCR products were assigned to RFLP analysis. The PCR products were digested with the restriction endonuclease *Hae*III (fermentase-lithvani) for 2 h at 37°C. The produced fragments were separated by electrophoresis on 2% agarose gel and compared with those of WHO reference strains of *L. major* (MHOM/SU/73/5ASKH), *L. tropica* (MHOM/SU/74/K27) and *L. infantum* (MHOM/TN/80/IPT1). Contamination was monitored by negative controls during processes of NA extraction and the PCR amplifications. Genomic NA was extracted in a room where the amplified DNAs were never processed and the PCRs were done in a separate room.

## RESULTS

*Sandflies collected*

All the sandfly samples trapped during this study were identified individually to the species level. A total of 643 were collected (374 females and 269 males). *Phelobotomus sergenti* was the most abundant species (76.67%), followed by *P. longicuspis* (11.51%), *S. minuta* (3.89%), *P. alexandri* (3.42%), *S. antennata* (2.18%), and *P. perniciosus* (1.24%), respectively. The remaining species were *P. langeroni* (0.62%), *P. kazeruni* (0.31%), and *P. bergeroti* (0.16%) (Table 1).

*Toscana virus infections identified in sandflies*

Out of 33 pools, only one pool of male *P. sergenti* contained phlebovirus RNA. This 201-nucleotide sequence (excluding primers) is identical at the nucleotide level with the sequence of Toscana virus detected in Morocco (TOSV_Morocco_SLP1_JN832571) handled

Table 1. Species and gender of sandflies processed for phleboviruses and *Leishmania*

| Species | Sandflies | | Pools | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| *P. sergenti* | 220 | 273 | 9 | 10 |
| *P. longicuspis* | 22 | 52 | 1 | 2 |
| *P. alexandri* | 0 | 22 | 0 | 1 |
| *P. perniciosus* | 4 | 4 | 1 | 1 |
| *P. kazeruni* | 0 | 2 | 0 | 1 |
| *P. langeroni* | 4 | 0 | 1 | 1 |
| *P. bergeroti* | 0 | 1 | 0 | 1 |
| *S. minuta* | 6 | 19 | 1 | 1 |
| *S. antennata* | 13 | 1 | 1 | 1 |
| Sub total | 269 | 374 | 14 | 19 |
| Total | 643 | | 33 | |

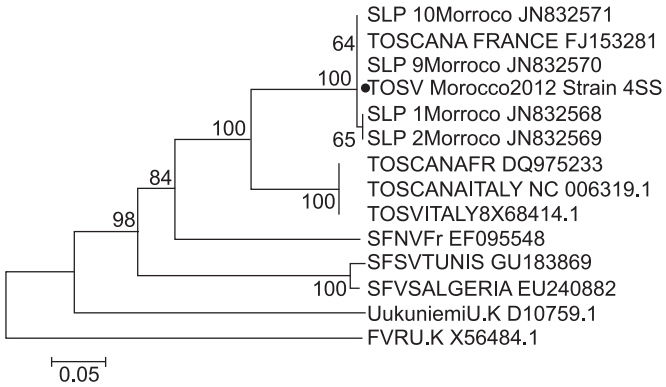


*Fig. 1:* Phylogenetic analysis of TOSV Morocco based on nucleotide in L-polymerase gene. Groupings were determined by the neighbour-joining method with the MEGA 5 programme. Bootstrap values are indicated and corresponded to 500 replications. Black bullet point indicate virus name that corresponds to sequences determined in this study. Scale bars indicate pair-wise nucleotide distances (0.05 = 5%). The sequence from the TOSV strain detected in the present study was named TOSV Morocco 2012 Strain 4SS.

2 yr before. The phylogenetic analysis indicated that this sequence was most closely (100% bootstrap support) related to the Toscana virus France strain (GenBank Accession No. FJ153281).

Groupings were determined by a neighbour-joining method with MEGA v.5.05, and the robustness of the groups were tested using 500 bootstrap pseudo-replicates (Fig. 1).

*Leishmania infections identified in sandflies*

A total of 10 females of *P. sergenti* were found positive for *L. tropica* (3.66%) showing a band of approximately 320 bp. The RFLP profiles for 10 infected *P. sergenti* consisted of two bands (185 and 57/53 bp) were identical to that of the *L. tropica* (Fig. 2). In another context, this result is confirmed by sequencing of ITS-5, 8rRNA gene which allowed the identification of six different sequences of *L. tropica* (Data not shown).

## DISCUSSION

Infections by TOSV and *Leishmania* represent an important public health problem in the countries where these pathogens circulate. TOSV has a tropism for the central nervous system and is a major cause of meningitis and encephalitis in parts of the mediterranean basin. In humans, infection with *Leishmania* spp ranges from asymptomatic form to severe visceral involvement.

*Phlebotomus sergenti*, the most abundant species in this survey is related to the semi-arid bioclimatic belt where the vegetation is characterized by Thuja (*Tetraclinis articulata)*, Junipers (*Juniperus* spp) and to a lesser degree, to the arid belt, by Argan (*Argania spinosa)* and common Jujube (*Ziziphus lotus*)[26]. Indeed, this type of vegetation is characteristic of El Hanchane area especially

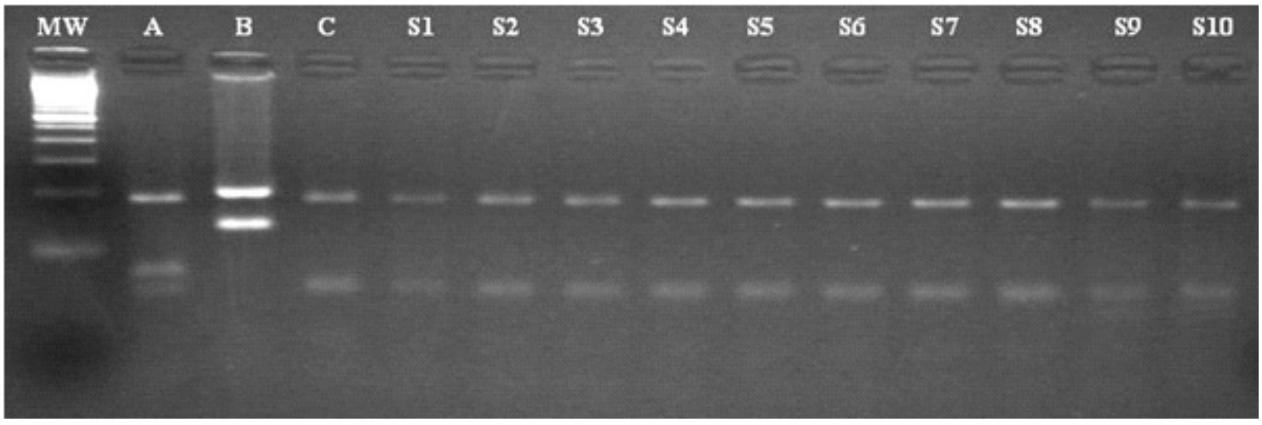


*Fig. 2:* PCR-RFLP analysis of ITS1 region of *Leishmania* species using *HaeIII* restriction enzyme. MW: 100 bp molecular weight; Reference strains: A—*L. infantum* (MHOM/TN/80/IPT1); B—*L. major* (MHOM/SU/73/5ASKH); and C—*L. tropica* (MHOM/SU/74/K27); and S1—S10: *P. sergenti.*

the *Tetraclinis articulata* and *Argania spinosa*.

In Morocco, *L. tropica* had been isolated from *P. sergenti* for the first time over three decades ago[27]. Since then, *P. sergenti* was suggested as a vector in several CL foci based only on epidemiological and entomological findings, including the abundance and endophily of this species. This is the first report of detection of *L. tropica* DNA within *P. sergenti* naturally infected in an emerging CL focus in the country.

*Leishmania* infection of sandfly has classically been examined by dissecting fresh individual sandflies under a microscope. This method needs dissecting expertise and a large number of specimens, since the *Leishmania* infection rate in sandflies is usually very low (0.01–1%) even in the highly endemic areas[28]. In recent years, molecular techniques based on PCR[29–30] are used to identify *Leishmania* infection and could be a useful tool in epidemiological studies and strategic planning for the control of human leishmaniasis[5, 31–32]. The infection rate is an essential parameter for estimating the vectorial capacity of the sandfly. In the present study, the rate of *P. sergenti* females naturally infected by *L. tropica* was ~3.66%. This value is lower than the rates recently described in Al-madinah Al-munawarah province where 31% of *P. sergenti* females were found positive against kDNA of *L. tropica*[33].

One pool of *P. sergenti* males was infected by TOSV; to our knowledge, this is the first time that TOSV has been detected in phlebotomine sandflies other than *P. perniciosus, P. perfiliewi* and *S. minuta*. Since, male sandflies are non-haematophagous, virus detection has been interpreted as evidence of transovarial transmission of this phlebovirus under natural conditions. In Morocco, TOSV has been previously detected in male *P. perniciosus* in Sefrou province[34].

Since, TOSV was previously detected from sandflies in our laboratory, the question of contamination should be discussed. First, we do not grow the virus in cell culture and never use positive controls for RT-PCR screening. Second, previous detection of TOSV RNA and RT-PCR was done more than two years earliar to this finding: meanwhile, a total of 22 RT-PCR experiments were conducted without finding any positive results. Third, sequencing of this RT-PCR product was performed not in the laboratory, but in Marseille where the material was shipped. For these reasons, it was believed that the finding of TOSV RNA in these sandflies is valid.

We demonstrated that *L. tropica* and TOSV could infect the common arthropod vector (*P. sergenti*). It has been also reported that both TOSV and *L. infantum* are transmitted by *P. perniciosus*. Indeed, Bichaud *et al*[22] confirmed the existence of an epidemiological relationship between *L. infantum* and TOSV infections and serological analyses were based on the IgG detection for both *Leishmania* and TOSV. Another example of this association between Karimabad virus infection and CL was reported in Iran[35].

## CONCLUSION

In conclusion, our data showed that *P. sergenti* is a potential vector of *L. tropica* and TOSV. Therefore, more epidemiological investigations are proposed to elucidate the role of *P. sergenti* in the ecology of TOSV and further serological studies are required to estimate the population risk-factors of infections by TOSV and *L. tropica* in this focus, because persons exposed to *Leishmania* parasite infections are at greater risk of being infected with TOSV.

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