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Research article

ILLEGAL WASTE SITES AS A POTENTIAL MICRO FOCI OF MEDITERRANEAN LEISHMANIASIS: FIRST RECORDS OF PHLEBOTOMINE SAND FLIES (DIPTERA: *PSYCHODIDAE*) FROM SLOVENIA

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Apart from being against the law, illegal waste dumping also poses a threat to human health and to the environment. Solid and decomposing waste is an ideal breeding ground for a number of rodents, insects, and other vermin that pose a health risk through the spread of infectious diseases. The main objective of this study was to survey disease vectors and rodents for the presence of *Leishmania* sp. from waste sites along the Istrian Peninsula in Slovenia and Croatia.

During the survey five sandfly (*Phlebotomus neglectus, P. perniciosus*, *P. papatasi, P. mascitii, Sergentomyia minuta*) and five rodent species were collected (*Rattus rattus*, *Mus musculus*, *Apodemus agrarius*, *A. flavicollis* and *A. sylvaticus*).

Sandflies and rodents were screened using a molecular probe to amplify an approximately 120 bp fragment of the kinetoplast DNA (kDNA) minicircle for the detection of *Leishmania* sp. parasites. *Leishmania infantum* DNA was detected in the spleen of one juvenile black rat (*R. rattus*). Despite few published records on *Leshmania* sp. infection in black rats, the addition of our record highlights the importance of further investigation into the frequency and distribution of such occurrences so that we may better classify the role of rodents as potential reservoirs of leishmaniasis in the Mediterranean basin.

Key words: Leishmania, Phlebotomine sandflies, Rodents

INTRODUCTION

The disposal refuse at illegal waste sites, also known as open dumping or midnight dumping, refers to the improper and/or unauthorized disposal of waste, it is a major problem for many communities throughout Europe. Illegal waste sites are found in isolated locations in both rural and urban areas (along roadsides, in wooded areas and even in national parks and other protected areas). For communities in close proximity

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they have both an environmental and a social impact while also posing potential threats to human health.

Both Slovenia and Croatia have an increasing problem with illegal dumping, it is estimated that there are currently 60,000 illegal waste sites just within Slovenia [1]. Solid and in particular decomposing organic waste discarded at these sites attract rodents, insects, and other vermin. The introduction of the tiger mosquito *Aedes albopictus* is known to have occurred mainly *via* the used tire trade and was enhanced by this species ability to quickly adapt to and colonize new territories [2]. The dumping of old tires and household appliances that readily pool water provide ideal habitats for mosquito species in and around human settlements [3].

Rodents are notorious reservoirs for a number of pathogens and can act as both intermediate infected hosts or hosts for arthropod vectors including ticks, fleas and Phlebotomine sandflies [4]. Rodent-borne zoonoses transmitted from rodent hosts to humans are the cause of significant human morbidity and mortality globally, with several thousand cases diagnosed annually in Europe [5].

There are more than 800 described sandfly species, of which about 100 are suspected or confirmed agents of disease transmission in humans, including 42 *Phlebotomus* species in the Old World [6]. These Diptera are the exclusive vectors of *Leishmania* sp., the protozoan agent of visceral (VL) and cutaneous leishmaniasis (CL) in both human and mammalian hosts [4]. *Leishmania* sp. parasites are transmitted from an animal reservoir (rodents, domestic dogs or wild canids) by the bite of the female phlebotomine sandfly [7]. It can be also anthroponotic where the parasite is transmitted from a human host to another mammal species by a sandfly [8]. Phlebotomines are also vectors of other human pathogens such as bacteria *Bartonella* and phleboviruses [9].

Nevertheless, the greatest impact of sandflies on human health in South East Europe comes from the rural transmission of *Leishmania infantum* by several *Phlebotomus* (*Larroussius*) species. Four *Leishmania* species are present in the Mediterranean basin, *L. infantum* is the most common and is a causative agent of cutaneous (CL) and visceral leishmaniasis (VL), which is fatal if untreated [10].

Domestic dogs are considered to be the major reservoir of Leishmania parasites in the Mediterranean, although some other canids, particularly the red fox (*Vulpes vulpes*), may be involved in the zoonotic cycle [11]. Other mammal species, particularly rodents, have also been recorded with *L. infantum* infection, including *A. sylvaticus*, *R. rattus*, *R. norvegicus*, *Meles meles* (European badger), *Martes martes* (European pine marten), *Mustela nivalis* (Least weasel) and *Genetta genetta* (Common genet). Their role as reservoirs of leishmaniasis is not yet clearly understood [8,11]. It is noteworthy that both European rat species are found to be infected by Leishmania parasites. The ability to transmit infection has been confirmed by xenodiagnosis in black rats suggesting that this species is a likely host [12].

Disposing of organic waste in an improper manner at illegal waste sites around and inside human settlements provides food for sandfly larval instars and shelters for

adults. In conjunction with the attraction of rodent species to these sites, life cycle of *Leishmania* sp. parasites could be promoted with consequent impacts upon human health.

In this study, we investigated the potential risk of illegal waste sites for the spread of leishmaniasis by using a DNA probe to detect the presence of these parasites in sandfly and rodent hosts.

MATERIAL AND METHODS

The study was conducted on the Istrian peninsula, which is the largest region on the Adriatic Sea located in its Northeastern part and includes portions of Croatia, Slovenia and Italy. The climate of the region is Mediterranean and Sub-Mediterranean with dry and warm summers and mild winters. The average annual air temperature along the northern coast is around 14°C and 16°C in the southern area and islands. It snows very rarely. The entomological and rodent surveys were carried out at selected and marked illegal waste sites along the study area between April 2011 and May 2013 (Figure 1).

Figure 1. Map of the Istrian peninsula showing locations of investigated illegal waste sites (●positive sandflies sites; ●negative sandflies sites)

Sandflies collection and identification

Phlebotomine sandflies were collected, both outside and within human settlements, by standard white-light Center for Disease Control and Prevention (CDC) and BG-Sentinel traps with CO_2. Traps were running during, at least three successive nights at various peridomestic, domestic and other locations, near waste sites. The traps were inspected each morning and individual sandflies were separated from other insects and kept either dry or in 70% ethanol. Indoors, inside houses collection was carried out by mouth or electrically powered aspirators.

Species identification of the Phlebotomine sandflies was based on morphology with the head and terminal part of the abdomen separated and mounted on microscopic slides for inspection [13-16].

Rodents collection and species identification

All rodents were collected using baited Sherman traps, in order to avoid possible death, injuries and suffering from heat all sampling was conducted from 7:00 pm until early morning hours when they were collected. Species identification was based on morphological characteristics including the color of the fur, ear/tail hair composition and morphometric analysis of the skull and sole as described in the most relevant taxonomic keys [17,18].

Molecular detection of Leishmania parasites

Genomic DNA was extracted from sandflies using QIAmp DNA Mini Kits (Qiagen, Germany) from the thorax, wings and legs while in rodents spleen tissue was used. All extractions were carried out using the *manufacturer's protocols*.

Each PCR reaction had a final volume of 25µl, and was performed using 3µl of extracted DNA as template, 100pmol of each of the two primer and KAPA HiFi HotStart ReadyMix 2X (Kapa Biosystems, Inc., United States) containing KAPA dNTPs, reaction buffer and Mg^{2+} at a 1X final conc. of 2.5 mM and water. The two primer sequences used in the PCR were 13A 5'-GTGGGGGAGGGGCGTTCT-3' and 13B 5'-ATTTTCCACCAACCCCCAGTT-3' which amplified a sequence of 120 bp of the kDNA minicircles. This conserved sequence is present in all minicircle classes of all Leishmania species and due to its small size the PCR using primers 13A/13B was highly sensitive and suitable for screening. Samples were subjected to the following thermocycling profile: 1 cycle of 3 minutes at 94°C, 30 cycles of 94°C for 30 seconds, 20 seconds at 52°C and 15 seconds at 68°C, with a final extension of 5 minutes at 72°C. Negative (without DNA) and positive (with DNA) controls were included in all assays. PCR products were analyzed by gel electrophoresis in 2% agarose gel containing Midori Green DNA Stain (Nipon Genetics Europe GmbH) [19].

Ethical issue

Ethical permission to kill small mammalian was issued by the *Ministry of Agriculture* and the Environment of the *Republic of Slovenia (document numbers 34401-36/2012/8 and 34401-36/2012/9)* in accordance with the *European Union* regulations regarding animal research, primarily Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

RESULTS

A total of 119 specimens of Phlebotomine sandflies were collected. It is important to stress that illegal waste sites, which are usually located in the middle of the forest or near regional roads, are not the favorite habitats of sand flies which is why the number of collected specimens is relatively small. Five species were identified with the majority belonging to a species with no known epidemiological importance, *Sergentomyia minuta* (48.7%). The remaining four species were identified as *Phlebotomus perniciosus* (30.3%), *P. papatasi* (13.4%), *P. neglectus* (5%) and *P. mascitii* (2.6%). All sandfly species were collected within or close to waste sites, except *P. papatasi* which was collected inside a farm house in the Slovenian village Velike Žablje (Tab. 1).

Table 1. Distribution of collected sandfly species

Species	***P. neglectus***		***P. perniciosus***		***P. mascitii***		***P. papatasi***		***S. minuta***	
Locality	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**	**male**	**female**
Croatia										
Pula		1								
Veliki Brijun	1		18	4					14	43
Umag	1	1								
Slovenia										
Malija	2									
Rakitovec									1	
Velike Žablje			9	5		3	4	12		
Total	6 (5%)		36 (30.3%)		3 (2.6%)		16 (13.4%)		58 (48.7%)	
					119					

In total 173 small rodents were collected, 6 *R. rattus*, 76 *M. musculus*, 47 *A. agrarius*, 26 *A. flavicollis* and 18 *A. sylvaticus*.

All of the female sandflies tested for the presence of Leishmania parasites were negative. Parasitic DNA was detected in the spleen tissue of one juvenile *R. rattus* caught in an illegal waste site near Umag (Croatia) (Fig. 2).

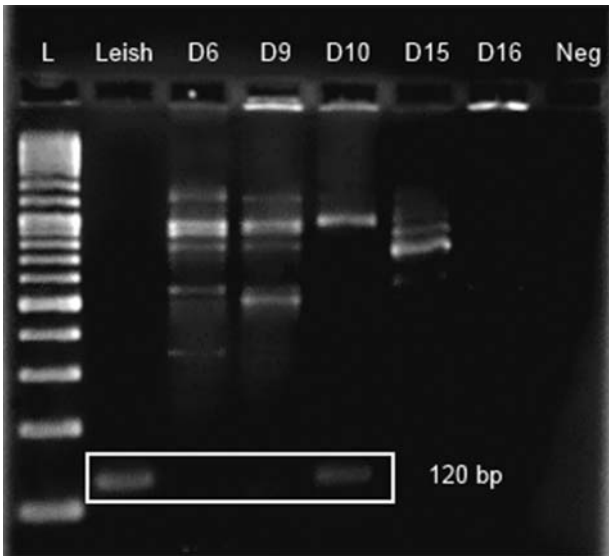

Figure 2. A positive PCR result for the amplified 120bp Leishmania kDNA minicircles fragment; L) ladder, Leish) positive control, D10) positive sample, Neg) negative control.

Independent verification was carried out on the spleen tissue that tested positive by the Parasitology French Reference Centre on Leishmaniasis, University of Montpellier in France. This organization performed additional molecular analyses that identified the infective agent as *L. infantum*.

DISCUSSION

Illegal waste sites have gone from being viewed primarily as an aesthetic problem to a broader environmental issue with wide ranging public health concerns. Although illegally dumped waste is mostly construction and demolition waste, abandoned automobile parts and various appliances, it may also include household trash that contains food scraps and other organic material. Locations where the latter have been dumped make them an ideal for various kinds of vermin.

Phlebotomine sandflies display various resting and breeding habitat preferences in the Mediterranean area. Generally, these are traditional stone walls (or so-called "*barbacanes*"), rock crevices, tree trunks, caves, wells and rocks [20,21]. However, it has been demonstrated that animal burrows, particularly those of rodents, are a preferred habitat of Old World sandflies [22-24]. In Mediterranean countries several rodent species are involved in the life cycle of the most prevalent *Leishmania* sp. parasites (*L. infantum* and *L. tropica*), the most common of which are *Psammomys obesus*, *Meriones sp.* and *Rhombomys opimus*. Additional species of emerging concern include *Microtus guentheri* and *M. tristrami* [25,26].

Although the European rat species *R. rattus* and *R. norvegicus* have occasionally been found with Leishmania parasite infections, the role of these two species is yet to be clarified. Detection of amastigotes in spleen smears of *R. norvegicus* has been reported in Ismailia, Egypt. Near Amman (Jordan) the same rat species and one *Meriones* sp. have also been found with *Leishmania* infection but no isolations were made [25]. Furthermore, *L. infantum* was detected in the spleen tissue of one *R. norvegicus* trapped in Greece using genetic techniques [27].

Rattus rattus is also considered a possible reservoir of *L. infantum* in Italy and Spain [28]. Five black rats, collected in central Serbia, were reported with *Leishmania* parasite infection but the parasites were not identified [29]. In the province of Granada (Spain) *L. infantum* was isolated from *R. rattus* [25]. A survey carried out in Southern Italy [30] revealed unusually high infection rates in both *R. rattus* (57.5% serologically positive and 45% PCR positive, using the same DNA marker as in this study) and *R. norvegicus* (33.3% serologically positive). Using the same DNA marker as in latter Italian study we found that the prevalence of Leishmania infection in *R. rattus* was 16.6% (1/6). Other collected rodent species were not positive.

Our study also investigated two most important *Leishmania* sandfly vectors collected in illegal waste sites *P. neglectus* and *P. perniciosus,* respectively. Both species are linked to rats as possible reservoirs involved in *Leishmania* parasite transmission in Greece and Italy [27,28,31]. The transmission of *L. infantum* to *R. rattus* by *P. perniciosus* has been demonstrated in laboratory experiments, with additional indications that this species of sandfly has an affinity towards dogs and other farm animals [31]. We tested all of the female sandflies for the presence of *Leishmania* parasites but the results were negative.

Although a large number of studies have already stressed the harmfulness of illegal landfills, our study indicates their possible contribution to the spread of leishmaniasis. We have demonstrated presence of sandfly vectors, *Leishmania* pathogens and infected rodents as potential reservoirs, in some of the investigated sites. It was expected for sandfly to be found since they are abundant along the Mediterranean cost, but rodents infected by *Leishmania infantum* is something that should be of our concern. Within current trends of environmental change species migrations are leading to an expansion in the range and density of leishmaniasis vectors and hosts, as a result we may expect the prevalence of this disease to increase.

As a bridge between Eastern and Western populations, the region of Slovenia hosts an unknown number of sandfly species. We have identified five, including *P. neglectus*, *P. perniciosus* and *P. papatasi*, some of the most important vectors of *Leishmania* parasites and these are the first published records of Phlebotomine sandflies fauna in this country (Tab. 1). Due to climate variability, environmental transformations and human activity there may be additional species such as *P. kandelaki*, a proven vector of *Leishmania* parasites in the Middle East, present in this region. In 2003, the westernmost range of *P. kandelakii* was in Montenegro [32], while in 2008 it was collected further to the west,

at the coast of Croatia in Krk Island (Ivović, unpublished data). Although reported as present in the past the status of leishmaniasis as endemic in Slovenia is not confirmed [11].

CONCLUSIONS

Recent faunistic and epidemiological research has revealed an increased prevalence of autochthonous leishmaniasis in the European human populations. However, there are still many gaps in our understanding of the distribution of the vectors and hosts of this disease, importantly we have not yet established a methodology to map likely leishmaniasis reservoirs. While there has been significant progress towards understanding what the infection risks of Leishmania are further research is required to provide a comprehensive risk framework for the Euro-Mediterranean region to ensure the best possible public health outcomes.

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DIVLJE DEPONIJE KAO POTENCIJALNA ŽARIŠTA LAJŠMANIOZE NA PODRUČJU MEDITERANA: PRVI NALAZ FLEBOTOMINA (DIPTERA: PSYCHODIDAE) U SLOVENIJI

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Osim što je protivzakonito, ilegalno odlaganje odpada predstavlja pretnju kako za ljudsko zdravlje tako i za životnu sredinu. Takođe, čvrst a posebno organski otpad u stanju truljenja, predstavlja idealno mesto za razmnožavanje i razvoj brojnih glodara, insekata, i drugih štetočina koje mogu da šire zarazne bolesti. Glavni cilj ove studije bio je utvrđivanje stepena inficiranosti parazitom iz roda *Leishmania* sp., flebotomina kao vektora i glodara kao rezervoara koji su sakupljeni na ilegalnim deponijama u delu Istarskog poluostrva.

Rezultati istrživanja su ukazali na prisustvo pet vrsta flebotomina (*Phlebotomus neglectus, P. perniciosus*, *P. papatasi, P. mascitii* i *Sergentomyia minuta*) i pet vrsta glodara.

Sakupljene flebotomine i glodari su testirani molekularno-biološkom metodom *PCR* (lančana reakcija polimerizacije) kojom je umnožavan region od oko 120 baznih parova kDNK (kinetoplast DNA minicircle) parazitskog porekla. DNK vrste *L. infantum* nije detektovana u analiziranim ženkama flebotomina ali je detektovana u uzorku slezine jednog od juvenilnih crnih pacova (*R. rattus*).

Bez obzira na mali broj publikovanih nalaza koji ukazuju na prisustvo parazita *Leishmania* sp. kod pacova, rezultat ove studije ukazuje na značaj obimnijih istraživanja učestalosti i distribucije infekcije kod ove vrste glodara, na osnovu kojih bi se razjasnila uloga sinantropnih glodara kao rezervoara lajšmanioze u Mediteranu.