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**Survey on the presence of *Leishmania* sp. in peridomestic rodents from the Emilia-Romagna Region (North-Eastern Italy)**

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## Abstract

Leishmaniasis is a neglected vector-borne parasitic disease caused in Italy only by the species *Leishmania infantum* of the *Leishmania donovani* complex, which is the causative agent of the zoonotic visceral leishmaniasis (VL), and the sporadic cutaneous leishmaniasis (CL) in humans, and of the canine leishmaniasis (CanL). The disease is considered endemic in southern, central, and insular Italian regions and recognizes phlebotomine sand flies as vector and dogs as main reservoir. However, a specific north-eastern region, namely Emilia-Romagna, always showed a peculiar epidemiological situation when compared to the other northern Italian regions and recent studies are indeed questioning the role of dog as main reservoir of *L. infantum*. Due to their synanthropic relationship with humans, rodents have been tested for *Leishmania* spp. in several European countries. The aim of this study was to assess the presence of *Leishmania* spp. in peridomestic rodents in the Emilia-Romagna

Region. The study was carried out on 136 peridomestic rodents collected by professional rodent control services: 47 brown rats (*Rattus norvegicus*), 39 black rats (*Rattus rattus*) and 50 mice (*Mus musculus*). Specimens of earlobe skin, spleen, liver and prescapular lymph nodes were tested with a real-time PCR. Fifteen (11 %) rodents, tested positive for *L. infantum*. Positivity was obtained from different target organs; notably 33% of the rodents tested positive in earlobe skin samples. These findings revealed the presence of *Leishmania* spp. in peridomestic rodents of the Emilia-Romagna Region, also in two species never tested before in Italy, namely *R. norvegicus* and *M. musculus*.

**Keywords**: Leishmaniasis, Italy, *Mus musculus, Rattus norvegicus*, *Rattus rattus*

## Background

Leishmaniasis is a neglected vector-borne parasitic disease endemic in southwestern Europe. With reference to Italy, *Leishmania infantum* of the *Leishmania donovani* complex is the only species responsible for visceral leishmaniasis (VL), for sporadic cutaneous leishmaniasis (CL) in humans and for canine leishmaniasis (CanL) (Gramiccia and Gradoni 2005; Rugna et al. 2018). The parasite is transmitted by phlebotomine sand flies, and in Italy dogs have long been recognized as the major reservoir in southern, central and insular regions, where the disease is considered endemic. Among the northern Italian regions, Emilia-Romagna has always had a different epidemiological scenario: CL has been widely reported since 1934, and between 1950-1958 up to 2,670 cases were diagnosed in the province of Forlì (Pampiglione 1975). In contrast, until the early 1970's, in this region VL appeared sporadically, with only 4 autochthonous cases observed, one in the province of Bologna and 3 in the province of Forlì. In the same period and within the same area, no ascertained autochthonous cases of CanL were reported (Pampiglione 1975). In 1971-1972, in two municipalities of Bologna province located in a foothill area a dramatic outbreak of VL was reported, involving 60 patients with a lethality of 21.7% (Pampiglione 1975). Since then, the geographic distribution of human and canine leishmaniasis has notably increased and the disease spread even in other regions of northern Italy, where many autochthonous cases of VL, CL and CanL have been reported (Gaspari et al. 2017; Mendoza-Roldan et al. 2020). This epidemiological change may be due to environmental issues, occurrence of competent insect vectors and movement of infected dogs from endemic areas (Santi et al. 2014). However, such changes might not be sufficient to explain the recurrent VL and CL foci recorded in Emilia-Romagna Region (Gaspari et al. 2017), especially considering that molecular studies carried out on strains isolated from autochthonous cases of VL are questioning the role of dogs as reservoirs of *L. infantum* in this region, as earlier suggested (Pampiglione 1975; Rugna et al. 2018).

The role of wildlife has long been recognized as crucial in the transmission and maintenance of zoonotic agents and several sylvatic species are known to be susceptible to leishmaniasis. Considering their synanthropic relationship with humans and their abundance the role of rodents as possible leishmaniasis reservoirs has been questioned in different European countries (Alcover at al. 2021). Several studies established the presence of *L. infantum* in these hosts in Greece (Papadogiannakis et al. 2009; Tsakmakidis et al. 2017), Portugal (Helhazar et al. 2013) and Spain (Navea-Pérez et al. 2015; Galán-Puchades et al. 2019; Martín-Sánchez et al. 2020).

In Italy, a study performed in Sicily detected *L. infantum* by PCR in 45% of black rats, even if in this region the role of the dog as reservoir has been well established (Di Bella et al. 2003). However, a study performed in Montecristo Island (Tuscany), revealed the presence of *L. infantum* in the 15.5% of rodents examined, even in the absence of domestic carnivores (Zanet et al. 2014).

The aim of this survey was to assess the presence of *Leishmania* spp. in peridomestic rodents collected in the Emilia-Romagna Region, Italy.

## Materials and Methods

From June 2019 to June 2021, 136 peridomestic rodent carcasses were sampled during pest control programs from the provinces of Ferrara, Forlì-Cesena and Ravenna (Emilia Romagna) (figure 1): 47 brown rats, *Rattus norvegicus* (20 females and 27 males), 39 black rats, *R. rattus* (21 females and 18 males), 50 mice, *Mus musculus* (22 females and 28 males) were collected from the territory by professional rodent control services and stored at -20 °C before processing.

The entire carcass was examined; species, sex and age classes were identified by morphological and metrical evaluation (CDC). Necropsies and samples collection were performed with sterile surgical instruments and when possible, according to the state of the carcasses, 25 mg of tissue were collected from earlobe skin, prescapular lymph node and liver, and 10 mg from the spleen (Helhazar et al. 2013). Due to the corruption of the remains, lymph nodes were not collected from 16 subjects. Samples were placed in sterile 1.5 ml tubes and stored at -20 °C.

DNA extraction was performed with PureLink® Genomic DNA Mini Kit (Invitrogen, ThermoFisher Scientific) following the manufacturer's instructions. For DNA amplification a real-time PCR protocol was performed targeting a 71-bp region of the minicircle kinetoplast DNA using primer pair Leish71Up (5′-CCAAACTTTTCTGGTCCTYCGGGTAG-3′) and Leish71Do (5′-TGAACGGGATTTCTGCACCCATTTTTC -3′) (Tsakmakidis et al. 2017). Reactions were carried out in a total volume of 20 μL with 10 μL of PowerUP™ SYBR™ Green master mix (2X), 0.3 μM of each primer and 2 μL of DNA. The amplification was performed in

StepOnePlus Real-Time PCR System (Applied Biosystems) and the thermal cycling profile was as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 sec., 60 °C for 30 sec. At the end of the amplification, a melting curve analysis was performed from 60 °C to 95 °C, with a slope of 0.3 °C to monitor primer dimers of non-specific product formation. Each sample was amplified in triplicate, the average temperature of melting (Tm) observed was 79.39 ± 0.15 °C and the average standard deviation observed in cts was 0.65. The standard curve was created with serial dilution of *L. infantum* DNA ranging from 10,000 to 0.1 parasites per reaction. Each reaction was carried out by three replicates per dilution, in three independent experiments. The ct value cut-off was settled at mean ct value of 39.3 which corresponds to 1 parasite per mL of the original parasite suspension.

As a positive control the reference strain *L. infantum* MHOM/TN/80/IPT1, kindly provided by the Unit of Clinical Microbiology, Regional Reference Centre for Microbiological Emergencies (CRREM), St. Orsola-Malpighi University Hospital, Bologna, Italy, was used. Confidence intervals were calculated by R Studio (RStudio Team 2020).

## Results and Discussion

Out of 136 subjects examined, 15 (11 %; 95% CI=5.7-16.3) were positive for *Leishmania* spp. In particular, 10.6% (95% CI=1.8-19.4) of brown rats, 12.8% (95% CI=2.5-23.7) of black rats and 10% (95% CI=1.7-18.3) of mice (Table 1). Of the five positive mice, three tested positive in two target organs - spleen and earlobe skin or spleen and liver or spleen and lymph nodes - the remaining two subjects tested positive only in lymph nodes or liver, respectively. The geographical distribution of the positive subjects appears homogeneous between the sampled sites (figure 1).

The present survey assessed the presence of *Leishmania* spp. in synanthropic rodents of the Emilia-Romagna Region. The conditions settled by the WHO (2010) for a species to be recognized as reservoir is the prevalence of infection > 20% and the availability of the parasite in blood and skin in sufficient amount to be ingested by a sand fly. In the Mediterranean area such conditions were globally assessed only for *M. musculus*, while *R. norvegicus* and *R. rattus* showed lower prevalence of infections (16.4% and 9.9%, respectively) (Alcover et al. 2021).

The prevalence values observed in the current study are below the average found in Portugal or Spain (Barcelona) where the 33.3% of examined rodents (*M. musculus* and *R. norvegicus*, Helhazar et al. 2013; *R. norvegicus,* Galán-Puchades et al. 2019) resulted positive, or the one reported in Granada (Spain) in mice (88.9%) (Martín-Sánchez et al. 2020) or in different rodent species (*R. rattus*, *M. musculus* and *Apodemus sylvaticus*) (27%) by Navea-Pérez et al. (2015), whilst it is higher than the prevalence observed in brown rats (5.9%) in Greece (Papadogiannakis et al. 2009). Further studies performed in Greece by Tsakmakidis et al. (2017) on spleen of *R. norvegicus*, *R. rattus* and *M. musculus* revealed a prevalence of 19.58% comparable to the one herein reported. The majority of the studies evaluated the presence of the parasite in more than one target organ including skin, liver, spleen and blood (Helhazar et al. 2013; Martín-Sánchez et al. 2020; Navea-Pérez et al. 2015; Tsakmakidis et al. 2017) while few studies examined only the spleen as target organ (Galán-Puchades et al. 2019; Papadogiannakis et al. 2009). Testing more than one target tissue allow to increase the possibility to detect *Leishmania* spp. as observed also in our study. Three *M. musculus* here examined showed the presence of the parasite DNA in two different target organs (spleen

+ lymph nodes and spleen + liver). Although the spleen is traditionally recognized as *Leishmania* spp. target organ for PCR in different animal species (Papadogiannakis et al. 2009), our results showed the presence of *Leishmania* spp. in the earlobe skin samples from 33 % of the positive rodents pointing out that this tissue should be also considered. In fact, wild animals are frequently collected in decomposition state and the putrefaction of the target tissues, like visceral organs, may affect the integrity of the kinetoplast DNA (Mũnoz-Madrid et al. 2013).

In Italy, the role of black rats in the transmission of *L. infantum* has long been investigated, starting from surveys performed in Tuscany in the 1980's (Pozio et al. 1985). Further studies showed that *Phlebotomus perniciosus* and *P. perfiliewi* are attracted to *R. rattus* and that these sand fly species become infected when they feed on black rats experimentally infected with *L. infantum* (Pozio et al. 1985). More recent study carried out in Calabria (Italy) by Di Bella et al. (2003) showed 45% positivity in the spleen of 22 *R. rattus* although in this region the role of dogs as reservoirs has long been established. Zanet et al. (2014), reported 15.5% prevalence in black rats examined in the Montecristo Island (Tuscany, Italy) where *L. infantum* was recorded even in absence of domestic carnivore hosts. This value is similar to the one (12.8%) obtained in the same host in our study, that moreover provided also data on *R. norvegicus* and *M. musculus* (10.6% and 10% respectively) species not previously tested for *L. infantum* in Italy.

Leishmaniasis in Emilia-Romagna has a peculiar epidemiological scenario compared to the other Northern Italian regions. Recently Rugna et al. (2018), by Multilocus Microsatellite Typing (MLMT) detected differences between *Leishmania* strains from men and sand flies to the ones from dogs. The MLMT profiles showed all canine samples belonged to one group genetically related to Mediterranean MON-1 strain and similar to the VL samples from other Italian regions, while all but one VL Emilia-Romagna case, and the isolates from sand fly fell into a different group. Therefore, in this region the co-circulation of two distinct groups of *L. infantum* seems to occur, and the VL in humans could have different cycles involving *P. perfiliewi* as a vector (Rugna et al. 2018; Calzolari et al. 2019) and might include other vertebrates, besides dogs, as reservoirs.

In two of the three provinces studied, Ravenna and Forlì-Cesena, foci of VL, usually located in hilly areas, were historically described. The rodent samples analyzed were collected in an area not higher than 50 m above sea level, where the density of phlebotomines is scant and, according to leishmaniasis regional control plan, in 2020 only CL cases have been reported (Santi et al. 2021). Further research should focus on studying which strains circulate in this area.

Also notable is the presence of a positive brown rat in the province of Ferrara, where autochthonous cases of leishmaniasis in both dogs and humans have never been recorded: the specimen was collected in a locality on the border between the provinces of Ferrara and Ravenna where the phlebotomine population is recorded as being moderate (Santi et al. 2021). This finding, considering the consistent increase in geographical distribution of the disease and its vector, will require further investigation.

*L. infantum* is a vector-borne parasite and in its epidemiology many mammal species are involved, hence identifying which one may act as a reservoir in the Emilia-Romagna Region is an ambitious task due to the presence of different environments i.e. hilly or flatlands and different distribution of sylvatic and peridomestic animals, which may possibly be involved in the parasite cycle. Even if the presence of the parasite in mammalian hosts is crucial, in order to fully understand his meaning as main reservoir or epiphenomena it should be associated with studies on the blood preferences of the phlebotomine vector.

The total prevalence observed in the present study (11%), despite being lower to the one required from WHO (2010) to establish a role of reservoir is comparable to the Mediterranean's one. As reported in previous studies, this value is far from being trivial: considering their close relationship with humans, their ability to colonize new environments and their impact on human health, rodents should not be neglected for their potential role in the transmission of Leishmaniasis, especially in urban areas (Alcover et al. 2021).

Although these preliminary findings are not sufficient to prove the role of peridomestic rodents as reservoirs of *L. infantum*, they nevertheless indicate the opportunity to further investigate their possible role in the epidemiology of different strains of *L. infantum* circulating in the Emilia-Romagna Region.

**List of abbreviations**

VL = Visceral Leishmaniasis

CanL = Canine Leishmaniasis

CL = Cutaneous Leishmaniasis

**Declarations:**

*-Ethics approval and consent to participate*

No ethical approval is officially required since the rodents examined had been subjected to pest control are considered pest species.

*- Consent for publication*

Not applicable

*- Availability of data and materials*

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

*- Competing interests*

The authors declare that they have no competing interests

*- Funding*

This study received no external funding

*- Authors' contributions*

MF and RG conceived the study. AM performed field work. AM and MC performed laboratory work and analyzed data. AM and MC wrote the first draft of the manuscript. MF and RG reviewed the manuscript. All authors read and approved the final manuscript

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Table 1: Real time PCR positive samples

| ID | Specimen | Locality | Real-Time PCR | | | |
|---|---|---|---|---|---|---|
| | | | Earlobe Skin | Spleen | Liver | Lymph Node |
| 57 | *Mus musculus* | Bizzuno (RA) | ct= 32.7 (87) | ct= 29.68 (676) | Negative | NA |
| 59 | *Mus musculus* | Bizzuno (RA) | Negative | ct= 30.77 (316) | ct= 31.97 (143) | NA |
| 67 | *Mus musculus* | Bizzuno (RA) | Negative | Negative | Negative | ct= 33.61 (47) |
| 98 | *Mus musculus* | S. Alberto (RA) | Negative | ct= 36.71 (5.8) | Negative | ct= 37.07 (4.5) |
| 111 | *Mus musculus* | Bizzuno (RA)) | Negative | Negative | ct= 35.9 (10) | Negative |
| 4 | *Rattus norvegicus* | Ravenna (RA) | ct= 34.25 (30.9) | Negative | Negative | Negative |
| 86 | *Rattus norvegicus* | Godo (RA) | Negative | Negative | ct= 36.47 (6.8) | Negative |
| 141 | *Rattus norvegicus* | Ravenna (RA) | ct= 37.75 (2.9) | Negative | Negative | Negative |
| 175 | *Rattus norvegicus* | Forlì (FC) | Negative | Negative | ct= 36.27 (7.8) | Negative |
| 178 | *Rattus norvegicus* | Argenta (FE) | Negative | Negative | ct= 36.67 (5.8) | Negative |
| 37 | *Rattus rattus* | Forlì (FC) | Negative | ct= 36.47 (6.8) | Negative | Negative |
| 60 | *Rattus rattus* | San Pietro in Campiano (RA) | ct= 36.86 (6.2) | Negative | Negative | Negative |
| 95 | *Rattus rattus* | Montaletto di Cervia (RA) | Negative | ct= 37.44 (6.2) | Negative | Negative |
| 179 | *Rattus rattus* | Montaletto di Cervia (RA) | Negative | Negative | Negative | ct= 36.63 |
| 206 | *Rattus rattus* | Longastrino (RA) | ct= 37.89 (2.6) | Negative | Negative | Negative |

Legend: Ct values are reported as mean ct of observed in different target organs with the estimated quantity of parasites/ml (mean standard deviation observed ±0.65). Localities are as well reported with reference to the province: Ferrara (FE), Forlì-Cesena (FC) and Ravenna (RA).

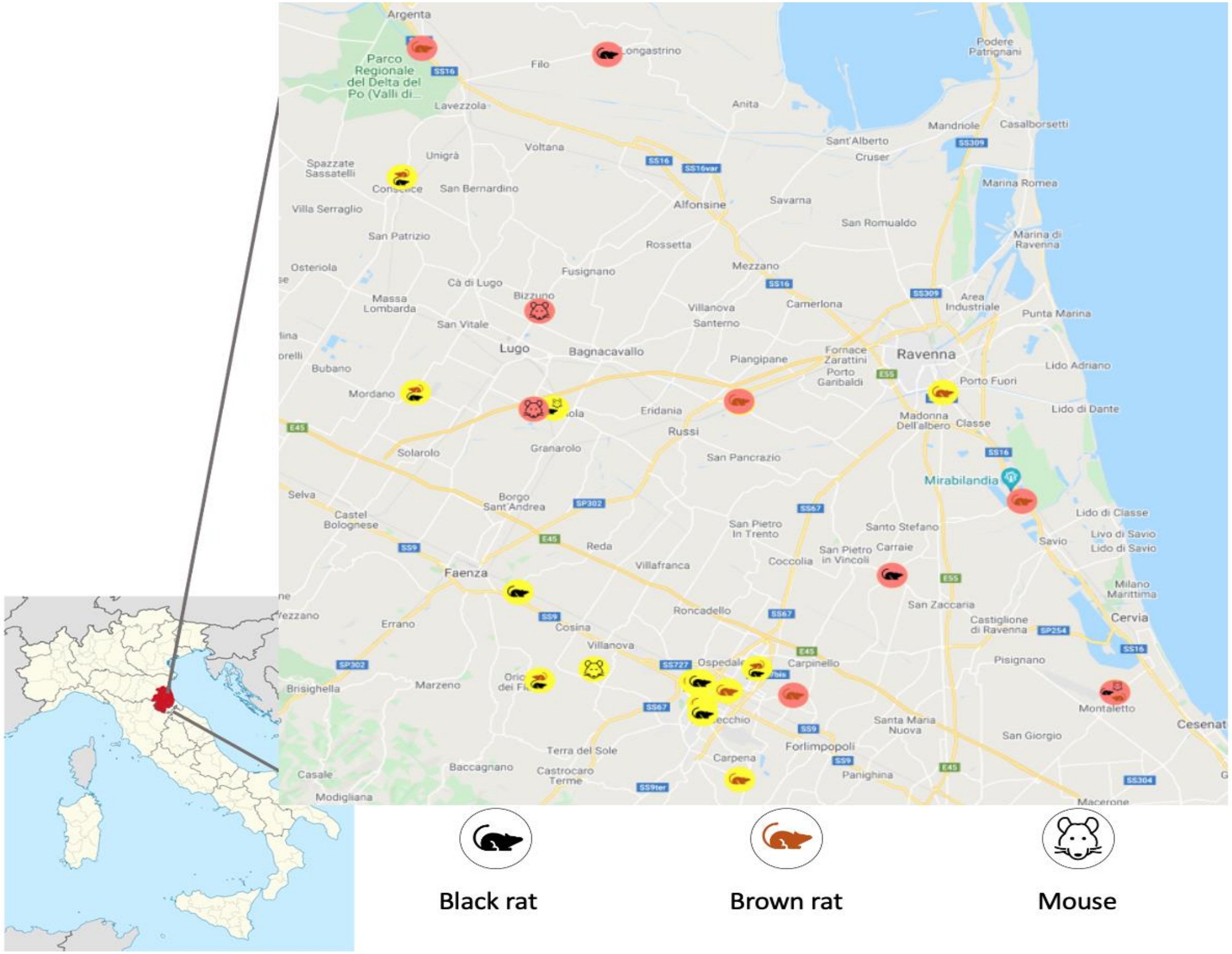


Figure 1. Map of the sampling area in the Emilia-Romagna Region. Dots are representative for sampling sites; red dots: at least one specimen positive, yellow dots: all the specimen negative.