ARE RODENTS A POTENTIAL RESERVOIR FOR *LEISHMANIA INFANTUM* IN ITALY?

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Abstract - Surveys carried out in Southern Italy (Calabria and Sicily) aiming to analyse zoonoses, which occur in wild rodent populations, revealed the presence of *Leishmania* sp. antibodies in murine sera. Polymerase chain reaction (PCR) analysis has revealed *L. infantum*. Capture-Marking-Recapture (CMR) and the Removal Method using "Havahart" live traps, have been performed to estimate wild rodent population in an area of about one hectare. Prevalence of infections has been calculated considering the real population density of the studied species. Research has revealed that the *Rattus rattus* population living in Fiumefreddo Bruzio (Cosenza) (estimated population density = 22.2 rats per ha; CMR Method) had 57.5% of serological and 45% of PCR positiveness; while a *R. norvegicum* population living in a green area (Villa Niscemi) in the city of Palermo (estimated population density = 12.75 rats per ha; Removal Method) had 33.3% of serological positiveness. Results allow us to suppose the presence of new reservoirs in the examined areas. More research is needed in order to confirm this hypothesis.

Riassunto - Durante alcune indagini effettuate in Italia Meridionale (Calabria e Sicilia) sulla presenza di infezioni a carattere zoonosico in popolazioni di roditori, i sieri di alcune specie murine sono stati sottoposti ad analisi per la ricerca di anticorpi nei confronti di *Leishmania* sp.. Sono state effettuate catture con trappole "live" tipo "Havahart" utilizzando il metodo Cattura-marcaggio-ricattura o la Rimozione totale per la stima della densità di popolazione in una superficie di circa un ettaro. Le indagini hanno rivelato per la popolazione di *Rattus rattus* di Fiumefreddo Bruzio (Cosenza) (densità stimata = 22.2 ratti per ha; metodo CMR) una sieropositività del 57.5% e del 45% di positivi alla PCR; invece per la popolazione di *R. norvegicum* di Villa Niscemi, a Palermo (densità stimata = 12.75 ratti per ha; Rimozione Totale) è stata riscontrata una sieropositività del 33.3%. Ulteriori analisi effettuate con metodica PCR (Polymerase chain reaction) hanno permesso di identificare *L. infantum* per la popolazione di *R. rattus* di Fiumefreddo Bruzio. I risultati ottenuti permettono di ipotizzare la presenza di nuovi reservoirs nelle aree esaminate, tuttavia ulteriori indagini dovrebbero essere effettuate per confermare tale ipotesi.

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1. Introduction

The study of the two forms of visceral and cutaneous leishmaniasis, which can be found in the Mediterranean area, identified in dogs the most important reservoir for visceral leishmaniasis from *Leishmania infantum* (Acha & Szyfres, 1989). Some authors have, however, suggested the possibility that other animals, such as Red fox *Vulpes vulpes* and rodents, might be involved in the epidemiological cycle of the protozoan (Ashford & Bettini, 1987). Moreover, it is a well know fact that rodents play a reservoir role in other world areas for other *taxa* of *Leishmania* (Cox, 1993). In particular, the Black Rat (*Rattus rattus*) is claimed to be a possible reservoir of *L. infantum* in Italy, Spain and Saudi Arabia (Gradoni *et al.*, 1983; Agrimi & Mantovani, 1995). Petrovic *et al.* (1978) reports the presence of infections from *L. donovani infantum* in *R. rattus* and *R. norvegicus* in some areas of former Yugoslavia. Although some investigations in Italy have allowed to study in depth the relationships between some murine species and *L. infantum*, their role concerning visceral leishmaniasis is nevertheless unclear (Agrimi & Mantovani, 1995).

Here we present some results of a Research Project, entrusted to the "Istituto Zooprofilattico Sperimentale della Sicilia" by the Ministero della Sanità, concerning the role played by same species of micromammals in maintaining and spreading some important zoonoses.

2. Methods

Surveys were carried out in Southern Italy: a rural area in Fiumefreddo Bruzio, near Cosenza (Calabria) and a garden, Villa Niscemi, in the urban area of Palermo (Sicily), where wild rodent populations have been analysed.

Captures have been carried out using Longworth or Havahart traps in September (Fiumefreddo Bruzio) and December (Palermo) 1997. Capture-Marking-Recapture (Meunier & Solari, 1979) or Removal methods (Seber & Le Cren, 1967) have been used in order to obtain density values.

The presence of *Leishmania* sp. antibodies in the murine sera has been investigated. Serological analysis has focused on populations of *Rattus norvegicus* (Villa Niscemi, Palermo), and of *Rattus rattus* (Fiumefreddo Bruzio, Cosenza). Occasional captures of *Mus musculus* were carried out in Villa Niscemi and Fiumefreddo Bruzio areas, and of *Rattus rattus* in Villa Niscemi too, but no data about density has been collected. Sample size of the studied population guaranteed to find, at least, one positive case, considering a prevalence of 2%, with 99% confidence limits (Canon & Roe, 1982). For this reason we have almost analysed the same number of sera of the captured animals of which population density was estimated.
Leishmania antibodies have been searched thanks to an indirect immunofluorescence technique on 10-pit slides prepared by Laboratorio Antigeni of the Istituto Zooprofilattico Sperimentale della Sicilia, starting from *Lieshmania infantum* grown on Tobie's ground. Sera have been tested at 1/40 dilution. Anti Rat- IgG and anti Mouse IgG conjugated with fluorescein isothyocyanate have been used at the dilution indicated for the acquired lot. Serological analyses were performed on a total of 24 specimens of *R. rattus*, and 4 M. *musculus* collected at Fiumefreddo Bruzio, as well as on 9 *R. norvegicus*, 4 *R. rattus* and 13 *Mus musculus* caught at Villa Niscemi. Furthermore we performed a PCR analysis (Osman *et al.*, 1997) on spleen samples from *R. rattus* caught at Fiumefreddo Burzio, searching for DNA fragments belonging to *L. Infantum*.
The applied methodology was subdivided in the following steps:

DNA extraction from spleen samples
Spleen samples from each animal were dissected and 25 mg of organ were submitted to the extraction technique according to the procedure described by Kit Quiagen, specific for tissues.

PCR
Amplification reaction was performed using a mix of reaction sufficiently clasping in order to carry out conditions of maximum specificity. Primers used correspond to the 2 specific DNA fragments of kinetoplast named 13A and 13B, and described by Sambrook *et al.* (1989), Rodgers *et al.* (1990). Amplification product is formed by a fragment of approximately 120 bp. The extracted DNA has been submitted to 30 amplification cycles and amplification products were analysed by means of electrophoresis on 1% agarose gel for comparison with a molecular weight marker (DNA of phagos l digested with Hind III) (Sambrook *et al.*, 1989).

Preparation of the probe
Probe used for hybridation tests was obtained by sequence analysis of the amplified fragment and corresponds to an internal of 70 bp which was subsequently marked with Digossigenina, according to the random primer technique (DIG DNA labelling kit Boheringer).

Southern blot
After electrophoretic analysis, gel containing amplified DNA was employed for transfer on nylon filter by means of Southern blot (Sambroook et al., 1989; Gramiccia et al., 1992). Finally, DNA was fixed on the filter for 3 minutes of UV exposure and hybridised with 100 ng of the probe marked with Digossigenina at 55°C for 12 hours. The hybridation sign was then revealed thanks to DIG Luminescent Detection Kit (Boheringer) on autoradiographic plate.

2.1 Field methods
Fiumefreddo Bruzio (Cosenza)
The Calabrian study area is situated in a typical rural settling in Marina di Fiumefreddo Bruzio (Cosenza), a locality of the mid-Calabrian Tyrrhenian coast, in the province of Cosenza. The area, though placed at an altitude of 5 m.a.s.l., , from the bioclimatic point of view, is included in the sannitic vegetation belt on the broad leaved bioma.
The trap grid was placed in October 1997 within a rural settling formed by houses inhabited only during summertime, a small poultry pen, an orange tree grove and a small vine-yard surrounded by an embankment covered by dense scrubs of *Robus* sp., *Robinia pseudoacacia, Mirtus communis* and *Ailanthus altissima*. It is the typical rural Mediterranean environment, where a mosaic of vegetation, crops and farming of domestic animals are present, favouring in particular the settling of murid colonies (mainly of R. *rattus* and M. *Musculus* and less commonly the *R. norvegicus*).
We have used *Havahart* traps placed in 5 rows of ten traps each, at a distance of 15 meters from each other, in order to include the different typologies of the environment. Capture area considered had a surface of 1.125 hectares and its value has been calculated adding to each side of the grid a belt whose width is equal to the half of the distance between traps (7.5

meters). As bait in traps we used a mixture of bread, anchovy paste, cereal flakes and apple; we also placed some cotton wool as nesting material. In order to estimate the population density we applied the Chapman Method (Meunier & Solari, 1979) with four days of marking and two days of recapture on individuals of *R. rattus*. Marking was performed by hair clipping, after having immobilised the animal inside a polythene bag.

Villa Niscemi (Palermo)
Sampling was carried out in a suburban environment of the town of Palermo; the area, from a potential vegetational point of view, can be considered as the climax of *Olea europea* var. oleaster and *Ceratonia siliqua* or O. *europea* var. oleaster and *Pistacia lentiscus* (Tomaselli, 1973). The trapping grid was placed in a portion of a urban garden, Villa Niscemi, not open to the public, but used as a maintenance site and a deposit of various materials. Villa Niscemi is situated close to "Monte Pellegrino e Parco della Favorita" Natural Reserve and is adjacent to a densely inhabited residential zone, in a working-class neighbourhood. The place is typical of a suburban environment, with populated buildings, natural green areas (though degraded and rearranged) and cultivated areas (citrus groves, orchards, gardens with introduced botanical species). Inside the villa's premises, the remains of the ancient irrigation system dating from the Arab occupation of Sicily can still be seen. It is typical of Sicilian citrus orchards and is represented by the so-called *saje* (small irrigation drains) and small collecting wells, now disused where water is stagnant. Not far away from the trapping grid is present a small man-made pond. The capture grid was obtained placing 49 traps (Havahart model) in 14 rows within a triangular shaped area, with 2 sides delimited by the villa's boundaries, with a distance of 10 meters between traps and a total sampling surface of 0.490 hectares. Within the area, vegetation is mainly herbaceous and characterised by nitrogen-loving plants, such as *Acanthus, Oxalis, Urtica, Malva*, whose presence is due to the accumulation of organic scrap material (sawdust and manure). Among shrubs and trees, *Phyllirea, Viburnum, Cupressus, Eriobothrya*, oaks, evergreen oak and citrus species are prominent.
In order to estimate the population density we have applied the method of total removal (Seber & Le Cren, 1967). Trapping was performed in December 1997, after one day of prebaiting, during a total period of 6 days of captures. Removal method was preferred in this study area, as the CMR method, which was employed at Fiumefreddo Bruzio, shows same disadvantages concerning the size estimate of population of *R. norvegicus*. Disadvantages are mainly linked to the difficulty in recapturing the individuals which had been previously exposed to trapping, and to their social structure which does not allow homogeneous redistribution of the already trapped individuals (Turillazzi, 1996). Furthermore, it is well known the particular neophobia which distinguishes commensal populations of *R. norvegicus* (Cagnin, 1987). As we have also recorded the presence of small murines (*A. sylvaticus* and M. *musculus*) in the same area, we placed also some "Longworth" traps.

3. Results
Among the examined sera we have altogether found positive cases towards *Leishmania* in the populations of *R. rattus* of Fiumefreddo Bruzio and of *R. norvegicus* and *Mus musculus* of Villa Niscemi. The results of our investigations are shown in table 1.
It is interesting to stress the fact that sera deriving from the population of *R. rattus* of Fiumefreddo Bruzio (n = 22) proved positive towards *Leishmania* with a percentage of 57.5%. This value is particularly high when compared to the data available from the literature (Bettini *et al.*, 1980; Pozio *et al.*, 1981). A lower serum prevalence was reported, on the contrary, in *R. norvegicus* at Villa Niscemi (Palermo): 33.3% (n = 9). In this last station we have also recorded a case of serological positivity towards *Leishmania* in a single individual of M. *musculus*.
The results of PCR, concerning analyses performed upon spleen samples from specimens of *R. rattus* trapped at Fiumefreddo Bruzio, have allowed to identify *L. infantum* as responsible of the infection, with a calculated prevalence of 45% (n = 20). It is important to stress (table 2) how some sera, which resulted positive serologically, proved to be negative to PCR, and viceversa some spleen samples which proved positive to PCR did not come from specimens which resulted serologically positive.

4. Discussion
Methods used for the study of the infection from *L. infantum* in murine populations have allowed to obtain prevalence data which represents an important preliminary result for future

Table 1 - Results of the present investigations

Trapping locality	Fiumefreddo Bruzio(Cosenza) Rural area,5 m a.s.l. situated on the Calabria coast; Grid n 50 Havahart traps, at a distance of 15 m from each other, 5x10 lines;	Villa Niscemi (Palermo) Town garden 5 m a.s.l.situated in the city of Palermo; Grid: n. 49 Havart traps at a distance of 10 m from each other, 14 lines;
Population density of the species considered	Species: *Rattus rattus*, valued density =22.2 rats per ha (CMR method);	Species: *Rattus norvegicus*, valued density = 12.75 rats per ha (removal method);
Serelogical results	57.5% positive *R.rattus* (n=22)	Leshmania: 33.3% positive for *R.norvegicus* (n=9), 1 positive for *Mus musculus* (N=13);
Results of PCR	45% positive for *R.rattus* (n=20);	

Table 2 - Comparison between results of PCR and serological anayses performed on biological samples of *R.rattus* trapped at Fiumefreddo Bruzio (n.d. = not determinated)

	PCR	SEROLOGICAL		PCR	SEROLOGICAL
1	n.d	Positive	13	Negative	Positive
2	n.d	Positive	14	Negative	Positive
3	Positive	Negative	15	Negative	Positive
4	n.d.	Negative	16	Positive	Positive
5	n.d.	Negative	17	Positive	Negative
6	Negative	Positive	18	Positive	Negative
7	Negative	Positive	19	Positive	Negative
8	Negative	Positive	20	Negative	Negative
9	Positive	Positive	21	Negative	Negative
10	Negative	Positive	22	Positive	Positive
11	Negative	Positive	23	Negative	n.d
12	Positive	Negative	24	Positive	n.d.

studies, with a more precise target.
Several surveys conducted in Tuscany (Bettini *et al.*, 1980; Pozio *et al.*, 1981) had already highlighted the infection from *L.infantum* in specimens of *R. rattus*, and have given an input to a series of investigations (Gradoni *et al.*, 1983; Pozio *et al.*, 1985) aiming at an in-depth study on the relationships between *R. rattus, L. infantum* and the possible vectors involved in the transmission (*Phlebotomus perniciosus* and *P. perfiliewi*). The results of these investigations permitted to consider the Black Rat as a possible reservoir in the epidemic cycle of the protozoan (Gradoni *et al.*, 1983; Ashford & Bettini, 1987). In particular, Ashford & Bettini (1987) wrote: *"having satisfied all conditions necessary for the incrimination of species as reservoir of Leishmania, according to the suggestions of Killick-Kendrick & Ward (1981), the black rat's role as a natural reservoir host of L. donovani infantum in Tuscany deserves serious considerations"*.
Some recent considerations, however, have scaled down the epidemiological role of *R. rattus*, attributing to this rodent a possible role of epiphenomenon in the epidemiological cycle of *L. infantum* (Lainson, 1982; Gradoni com. pers., in Agrimi & Mantovani, 1995).
Data on the prevalence of the infections from

L. infantum recorded in *R. rattus* at Fiumefreddo Bruzio, differs considerably from the results reported from the Tuscan populations (3 isolations from a total of 143 specimens trapped between November 1977 - November 1978, at Baccinello (Grosseto) - Bettini *et al.*, 1980; one isolation among 94 individuals trapped in some rural areas of Monte Argentario (Grosseto) - Pozio *et al.*, 1981), stressing the importance held by the Black Rat as a reservoir of the protozoan in the researched Calabrian locality.

As regards the infection from *L. infantum* in *R. norvegicus*, it has been already reported for former Yugoslavia (Petrovic *et al.*, 1975), but this represent the first case of positivity confirmed for Italy. Further in depth studies in order to complement these results with PCR analyses are currently in progress. Studies on the experimental infection of *R. norvegicus* with *L. infantum*, using vectors that could be found in the area of Villa Niscemi, could explain the role played by this rodent in the protozoan's cycle.

The few captures of M. *musculus* at Fiumefreddo Bruzio and of *R. rattus* at Villa Niscemi do not allow us to exclude the presence of the infection in these populations; it is however interesting to remark the serological positivity in a single M. *musculus* trapped at Villa Niscemi.

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