Real-time PCR detection of *Leptospira* sp. in rodents from Toulon harbour (France)

A. LEVIEUGE1*, M. H. ABOUBAKER2, O. TERRIER1, M. DRANCOURT2, B. DAVOUST3

1Secteur vétérinaire de Toulon, BP 20549, 83 041 Toulon Cedex 9, FRANCE.
2Unité de recherche sur les maladies infectieuses et tropicales émergentes, UMR CNRS 6236, IRD 198, IFR 48, Faculté de médecine, 13 000 Marseille, FRANCE.
3Direction régionale du service de santé des armées de Toulon, BP 20549, 83 041 Toulon cedex 9, FRANCE.

*Corresponding author: a_levieuge@hotmail.com

**SUMMARY**

Fifty-two rodents were trapped in the port of Toulon (south-east of France) to evaluate the prevalence of *Leptospira* infection in animals living in an inner city, commensally with men. Kidney samples were taken on each animal and real time Polymerase Chain Reaction amplification was performed. Almost 20% of specimens were found positive for *Leptospira* sp. DNA and more than 17% for *Leptospira interrogans*, pathogen specie for humans. This study confirms the high rate of natural leptospirosis infection of rodents in moderate climate, corresponding to a zoonotic risk, and it suggests the necessity to limit the rodent population.

**Keywords:** *Leptospira* sp., PCR, Rodents, Kidneys.

**Introduction**

Leptospirosis is a public health problem, classified as a priority zoonotic disease. Each year, the French national reference centre counts about 300 now human cases [4]. Our neighbour country Italy counts about one hundred cases with a specific mortality rate of 22.6% [10]. This infectious disease is thought to be misdiagnosed for two reasons. Firstly, its clinical presentation is difficult to distinguish from the other febrile illnesses. Moreover, pathogen bacteria are highly widespread in the environment; they infect both domestic and wild animals and excreted bacteria are able to survive in the environment until contacts with a new host [12]. The agent of human leptospirosis belongs to the class of Spirochetes genus *Leptospira* which contains saprophytic species (*Leptospira biflexa* s.l.) and pathogen species (*Leptospira interrogans* s.l.). This pathogen species is further divided into more than 200 serovars. The aim of this study was to evaluate the infection prevalence in urban rodents commensally living with humans in the vicinity of one port and to evaluate the zoonotic risk.

**Materials and Methods**

**RODENTS**

The field work was carried out during two weeks in October 2006 in the harbour of Toulon (43°07N-5°55E), in the Mediterranean south-east of France. Every morning 36 traps containing apple and dry bread with fish oil were placed near to the water. Every morning, the traps were checked and the caught rodents taken. During this season, most individuals were adults. In total we collected 52 rats. All of them were identified as *Rattus norvegicus*, including 21 (40%) females and 31 (60%) males. The average weights were 196 g and 281 g for males and females respectively. All captured rats were apparently healthy. Living rats were anaesthetized to collect blood then euthanized by intraperitoneal injection of pentobarbital solution accordingly the animal experimentation ethic standards. A post-mortem examination was performed and kidney samples were collected for further analysis (one kidney per rat).

**LEPTOSPIRA DETECTION**

*Leptospira biflexa* Patoc, *Leptospira interrogans* ATCC 23470, *L. interrogans* Icterohaemorrhagiae and *Leptospira fainei* suspensions were calibrated to 2.5x10^8 organisms / mL by microscopic counting after acridine orange staining [2]. For each suspension, 100 µL were mixed with 260 µL lysis buffer and proteinase K (40 µL, activity > 600 U/L), incubated at 72°C for 10 minutes then at 95°C for 10 additional minutes. Thereafter, the lysate (250 µL) was placed in the Mag NA Pure apparatus for DNA extraction using Mag NA Pure LC DNA isolation Kit III (Roche Diagnosis, Meylan, France). In the same way, the kidney tissues from rodents...
were incubated in solution containing lysis buffer (Roche Diagnosis) (260 µL) and protease K (40 µL, activity > 600 U/L) at 56°C overnight then 250µL of lysate were placed in the Mag NA Pure for the DNA extraction using Mag NA Pure LC DNA isolation Kit III.

The detection of Leptospira sp. DNA was carried out by using real-time PCR amplification of 16S rDNA [8]. Two systems were run in parallel. The first system targeting a 58-bp fragment used primer pair [Lepto1 Forward: 5'-GCG-GCG-AAC-AAA-GGA-TGA-A-3' and Lepto1 Reverse: 5'-GG-AAG-TTA-TCC-CAG-AC-T-C-3' with probe 6-FAM-ACG-TGG-GTA-ATC-TTG-MGB] amplifies L. noguchii, L. inadai, L. borgpetersenii, L. weilii, L. genomospecies 5, L. interrogans, L. alexanderi, L. kirschneri, L. fainei and L. genomospecies 1. The second system targeting a 331-bp fragment used primer pair [Lepto2 Forward: 5'-TG-GCG-AAC-AAA-GGA-TGA-A-3' and Lepto2 Reverse: 5'-GG-AAC-ACC-ACA-TTC-GGT-ATT-3' with probe 6-FAM-GAT-GGA-TAA-CCT-ACC-TAG-AAG-MGB] amplifies L. meyeri, L. biflexa, L. weilii, L. genomospecies 3 and L. wolbachii. A third system (rpoB system) based on the detection of specific gene rpoB encoding the β-subunit of RNA polymerase of L. interrogans was used to confirm the strain identification [7, 11]. This system is Lepto Rpob Forward 5'-CAG-AAT-TTG-TAT-CGT-AAA-CAA-ACA-C 3' and Reverse 5'-CGC-CCT-TTC-TTT-CTA-CAA-CG 3' with probe sequence 6-FAM-CGT-ACC-TCT-GAT-GGA-ACC-GTA-AT-3' and Lepto2 Reverse: 5'-GTG-GCG-AAC-AAA-GGA-TGA-A-3'.

Negative controls consisting in PCR mix without DNA remained negative in all PCR experiments. Serial tenfold dilutions of a suspension of L. interrogans Icterohemorrhagiae (2.5x10^8 organisms / mL) yielded positive amplification with the number of cycles (Ct) ranging from 18.57 (equivalent to 2.5x10^8 organisms / mL) to 36.09 (equivalent to 2.5x10^3 organisms / mL) for system 1, and from Ct = 18.16 (equivalent to 2.5x10^8 organisms / mL) to 37.99 (equivalent to 2.5x10^3 organisms / mL) for system 2 using L. biflexa Patoc.

**Results**

Ten kidney tissue specimens (19.2%) were found positive only with the first system and amplified fragments were evidenced with the rpoB system in 9 cases / 10, confirming the presence of L. interrogans.

**Discussion**

In this study, almost 20% of kidney specimens tested for the presence of Leptospira sp. DNA were found positive. By combining 3 different real-time PCR systems with probes targeting the 16S rDNA and rpoB, organisms could be identified as L. interrogans in 9/10 animals and one unidentified Leptospira sp., different from L. interrogans, in one specimen. These results show that an important proportion of rodents were detected positive by the real time PCR method, meaning that Leptospira have established a renal colonization. Moreover, individuals with renal Leptospirosis infection are supposed to have a consecutive urinary excretion of Leptospira in high concentration [3, 12]. Quantifiable detection in kidney and urine would make sense to evaluate the concentration of excreted bacteria but DNA amplification by real time PCR does not allow discriminating viable and dead organisms [3]. More than 17% of rodents (9/52) are undoubtedly infected with L. interrogans, a specie pathogen for humans. To our knowledge, all serovars of this specie may be pathogen for humans, depending more on virulence, inoculation and individual sensibility [6].

Most of the rodent leptospirosis works in France concern serological studies. The results of the present study are in agreement with the observations of other PCR-based studies in metropolitan France: the leptospirosis prevalence in rodents ranged between 27% and 30% [1, 5, 12] but the prevalence found in tropical countries as Latin America, India, or south eastern Asia is always higher [4]. None of the experimental works could establish a link between the prevalence of rodent’s leptospirosis and the human’s one. It just allows identification of several risk factors, more particularly for some occupations. Stagnant water is the principal risk of infection for humans [9]. In the city, rain puddle may be contaminated with rodent’s urine. That’s why some human activities should be considered as acute risk factors. For the global population, activities in contact with soft water possibly contaminated by rodent’s urine are risky: bathing, fishing, canoeing, rafting, and any water sports or leisure [4, 9]. Hunters and rodent pet owners are exposed too. Moreover, some specific occupational risks must be mentioned: sewer workers, knackers and slaughterhouse technicians, miners, rodents control workers, soldiers, septic tank cleaners, gamekeepers, or farmers. Even in an inner city, urban dwellers may be exposed to L. interrogans excreted by rodents because these animals are used to living in sea ports, sewers or in any location with fresh water [12]. Domestic animals could be naturally infected and represent a source for human infection, but nowadays a large part of them are vaccinated in France. However, wild animals have an important role in leptospirosis epidemiology. The control of rodent populations may change epidemiology of leptospirosis in domestic animals and humans. Indeed, rodents are maintenance hosts and they may be highly infected by Leptospira without any demography changing; they carry and excrete Leptospira in a continuous cycle of transmission.

This study confirms the natural high infection rate of urban rodents with pathogen Leptospira which implies a potential hazard for human health. Not only professionals who are in contact with rodents but also for the public even townmen in recreational settings are exposed to pathogen Leptospira [4, 9]. The contact may occur directly with rodents or indirectly near to the water spoiled with urine containing bacteria. Human protection should mainly be based on four items:
control of rat populations should be continued, information of zoonotic risk to the public needs to be maintained or reinforced, drainage of soft water accumulation should be performed, and basic hygiene rules must be applied, particularly for specific occupational risks: concerned people should carry protective clothing.

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References