

Endemicity of leptospirosis in domestic and wild animal species from Reunion Island (Indian Ocean)

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SUMMARY

Leptospirosis is the major infectious disease on Reunion Island but little is known about the animal reservoir. We conducted a wide-ranging survey that included samples from 574 animals belonging to 12 species. The seroprevalence and prevalence of renal carriage varied greatly depending on the species, with the highest seroprevalence (79·5%) found in Norway rats, and the lowest (13·2%) in tenrecs. The renal carriage rate ranged from 84·6% in mice to 0% in tenrecs. Our results suggest that rodents are the most important reservoirs of leptospirosis on Reunion Island. The epidemiological role that animals play in human infection is discussed. For the first time, we quantified the renal concentration of leptospirae in ten naturally infected mammals. The history of Reunion Island colonization probably explains why the circulating *Leptospira* serogroups were similar to those found in Europe. Our study provides evidence that will help implement preventive measures against this zoonosis.

Key words: Epidemiology, leptospirosis, quantitative PCR, Reunion Island, zoonosis.

INTRODUCTION

Leptospirosis has been reported in more than 150 mammalian species [1]. The disease is maintained by the persistent colonization of the proximal renal tubules of carrier animals. A reservoir animal can remain asymptomatic and shed infectious *Leptospira* in

its urine for a transient period of time or for its entire lifetime [2, 3]. Infection most frequently results from direct contact with infectious urine, although genital transmission [4–7] and contamination by consumption of infected prey [8] have also been described. Direct infection of newborns through breastfeeding has also been reported [9]. The incidence of different *Leptospira* strains in human populations strongly depends on the reservoir hosts present locally and the strains they carry as well as the amount of contact between humans and fauna, ecological conditions, and cultural and agronomical practices [10].

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Reunion Island is a French tropical overseas region located in the Indian Ocean. With an incidence rate of 15–13 cases/100 000 inhabitants in 2010 (33 times higher than the rate observed in metropolitan France) [11], leptospirosis is an understudied major public health problem on this developed island. The only native mammals on the island are bats; however, paralleling human colonization, settlers have progressively introduced a number of farm animals from Europe (cattle, *Bos primigenius*; goat, *Capra aegagrus*; pig, *Sus scrofa*; sheep, *Ovis aeries*). Horses (*Equus ferus*); game species, such as Rusa deer (*Rusa timorensis*) or the tenrec (*Tenrec ecaudatus*); and domestic species, such as dogs (*Canis lupus*) and cats (*Felis sylvestris*) have also been intentionally introduced to the island. The ship rat (*Rattus rattus*), the Norway rat (*Rattus norvegicus*), the mouse (*Mus musculus*), and the shrew (*Suncus murinus*) have been accidentally introduced by ships and boats.

The last available data for animal leptospirosis on Reunion Island date back to the 1980s [12–14], and these data consist of seroepidemiological surveys showing that 40% of stray dogs [12, 14], up to 32% of cattle [12, 13], 5% of pigs [12], and up to 71% of horses [12, 13] were seropositive for leptospirosis. Surprisingly, the rodent reservoir had never been investigated.

The local preventive measures that have been employed against leptospirosis have only focused on health education programmes and rodent control but other potential reservoirs remain neglected. Obtaining information on the prevalence of leptospiral infection among the various animal populations and identification of the animal species that are the predominant carriers is important to inform prevention and control programmes. Thus one of the aims of this work was to conduct a field-to-laboratory survey in order to update data on animal leptospirosis on Reunion Island. To achieve this goal, we first conducted a transversal survey to estimate the seroprevalence of leptospirosis and evaluate the circulating serogroups in nine animal species. Next, we used quantitative polymerase chain reaction (qPCR) to study the prevalence of renal infection at the time of sampling in 12 animal species. To our knowledge, this is the first report of quantitative results of leptospire in the kidney tissues of naturally infected animals. Data detailing the leptospiral status of the animal species living close to humans represent the first step in planning effective control measures to protect humans, and therefore, these data must be regularly updated to guide the

development of preventive measures to counter potential epidemiological changes.

METHODS

Field methods

Animal captures were conducted between 12 February 2009 and 20 August 2009 on Reunion Island. Black rats (*R. rattus*), Norway rats (*R. norvegicus*), mice (*M. musculus*), and shrews (*S. murinus*) were trapped in the field using baited live-traps laid out overnight (Manufrance traps for rats and INRA traps for mice and shrews; BTTm, France). Tenrecs (*T. ecaudatus*) were hand-captured alive by local hunters during the official hunting season using traditional hunting methods (prefectural decree no. 1268, 30 May 2008). Small mammals (rats, shrews, mice, tenrecs) were euthanized by injection of pentobarbital, following the recommended procedure [15] and blood was collected by cardiac puncture. Stray dogs and cats were captured by employees of the local animal rescue using a special lariat. These dogs and cats were euthanized by the veterinary services using an intravenous pentobarbital overdose (Doléthal[®], Vétquinol, France), and blood was sampled from the cephalic vein. Cattle, goats, deer (*R. timorensis*), and pigs being prepared for human consumption were subjected to blood sampling from the carotid artery immediately after bleeding and kidney samples were collected at the evisceration and inspection area of the slaughterhouse. For all animals, blood sampling was conducted immediately after death, and the kidneys were removed aseptically after the renal capsule was removed.

Free-tailed bats (*Mormopterus francoismoutoui*) were trapped at nightfall using a black nylon Japanese mist net (Bonardi, 110D, mesh of 16 mm, 12 m long, 2·4 m high, five pockets), and released immediately after urinary sampling. Blood sampling was not conducted on the bats because the quantity of blood required from each individual bat for the microscopic agglutination test (MAT) would be lethal. Bat urine was collected directly after capture at the urethral opening using a sterile rayon swab.

Just after sampling, the blood was centrifuged and the collected serum was stored at –80 °C until analysed. The urine samples were neutralized with an approximately equivalent volume of phosphate-buffered saline (PBS) [16] and frozen at –80 °C. Rayon swabs with urine samples were placed into

Table 1. *Strains used as capture antigens in the microscopic agglutination test*

Species	Serogroups	Serovars	Reference strains
<i>L. interrogans</i>	Australis	Australis	Ballico
<i>L. interrogans</i>	Autumnalis	Autumnalis	Akiyami A
<i>L. interrogans</i>	Bataviae	Bataviae	Van tieren
<i>L. interrogans</i>	Canicola	Canicola	Hond Utrech IV
<i>L. borgpetersenii</i>	Ballum	Castellonis	Castellon 3
<i>L. kirschneri</i>	Cynopteri	Cynopteri	3522 C
<i>L. kirschneri</i>	Grippotyphosa	Grippotyphosa	Moskva V
<i>L. borgpetersenii</i>	Sejroe	Hardjo (hardjobovis)	Sponselee
<i>L. interrogans</i>	Hebdomadis	Hebdomadis	Hebdomadis
<i>L. interrogans</i>	Icterohaemorrhagiae	Copenhageni	Wijnberg
<i>L. noguchii</i>	Panama	Panama	CZ 214 K
<i>L. interrogans</i>	Pomona	Pomona	Pomona
<i>L. interrogans</i>	Pyrogenes	Pyrogenes	Salinem
<i>L. borgpetersenii</i>	Sejroe	Sejroe	M 84
<i>L. borgpetersenii</i>	Tarassovi	Tarassovi	Perepelicin
<i>L. interrogans</i>	Icterohaemorrhagiae	Icterohaemorrhagiae	Verdun
<i>L. kirschneri</i>	Mini	?	Mayotte 2008/01925*

* Strains provided by the Pasteur Institute, Paris were isolated from a patient in Mayotte.

transport media and vortexed before being frozen at -80°C .

A total of 574 animals belonging to 12 species were sampled. Mice, shrews and bats were not sampled for blood. Ten individual urine samples were collected in a small colony of free-tailed bats located in a house. All but one of the pigs slaughtered during our survey were 5- to 6-year-old reproductive sows. None of the animals that we sampled were vaccinated against leptospirosis. We analysed 462 sera samples by MAT, and 546 kidney and 10 bat urine samples were screened for the presence of leptospiral DNA by qPCR.

MAT

Sera were tested for the presence of anti-leptospiral antibodies using the MAT following standard procedures [17]. Seventeen reference strains belonging to 15 serogroups were used as antigens in the MAT (Table 1). We considered 1:100 as the cut-off value for positivity. The serogroup with the highest titre was considered to be the presumptive single infecting serogroup and serum with this result was classified as being infected with the corresponding single *Leptospira* serogroup. In contrast, if two or more serogroups induced equally high titres, we labeled these animal samples as having multiple successive infections or co-infections.

Positive control and standard DNA construction

We cloned the 242-bp fragment of the *lipL32* gene of *L. interrogans* serovar Copenhageni strain Wijnberg into the pGEM[®]-T Easy Vector (Promega, USA) following the manufacturer's recommendations and using the primers LipL32-45F/LipL32-286R (Table 2) [18]. A twofold dilution series of the plasmid DNA was prepared to 10^{-12} to test the analytical sensitivity (determined by the number of plasmid copies that was detected ten times out of ten repetitions) of the real-time qPCR and to construct a standard curve for DNA quantification of positive samples. DNA content was quantified using the QuantIt[™] PicoGreen[®] kit (Invitrogen, France) using the LightCycler 2.0 System (Roche Diagnostics, France) according to the manufacturer's recommendations.

Internal control (IC) construction

To control for the DNA extraction step and to detect the presence of PCR inhibitors in the biological samples, an IC was chosen based on the following criteria: the IC should not be present in the DNA of the different animal species studied, the IC DNA sequence should not be present in any pathogen that could infect the animals, and the IC DNA sequence should not share similarity with a *Leptospira* DNA sequence. Thus, the IC was constructed using the 63-bp sequence of the DNA polymerase gene of

Table 2. Primers and probes used for qPCR assays

Target	Primer or hydrolysis probe*	Sequence (5' to 3')	Ref.
<i>Leptospira lipL32</i> gene	LipL32-45F	AAGCATTACCGCTTGTGGTG	[18]
	LipL32-286R	GAAGCCAGGACAAGCGCCG	
	LipL32-189P	AAAGCCAGGACAAGCGCCG	
VZV DNA polymerase gene†	VZV UP	CGGCATGGCCCCGTCTAT	[19]
	VZV DP	TCGCGTGCTGCGGC	
	VZV P	ATTAGCAATGGAAACACACGACGCC	

qPCR, Quantitative polymerase chain reaction.

* All probes were labelled with FAM at the 3' end and with TAMRA at the 5' end.

† Gene bank accession no. AB059828-31 and X04370.

varicella-zoster virus (VZV) isolated from a human VZV-positive clinical sample and cloned into the pGEM[®]-T Easy Vector (Promega) using the primers VZV UP/VZV DP (Table 2) [19]. To identify the optimal concentration for use in the qPCR, a tenfold serial dilution of the IC, ranging from undiluted to a 10⁻¹²-fold dilution, were tested by qPCR. The optimal IC concentration was established on the criterion that a reliable IC amplicon was always detected in the samples.

Total DNA extraction

DNA was extracted from 20–25 mg of cortex kidney tissue using the Dneasy[®] Blood and Tissue kit (Qiagen, France) according to the manufacturer's instructions. When adding the proteinase K, we also added 10 µl of the IC plasmid at the optimal IC concentration. Thus, the IC was co-purified with the sample DNA and was detected as a positive control for the extraction process.

Gene amplification by qPCR

The sensitivity of our qPCR assay was 7400 copies/ml. Because one leptospire contains an average of five genome equivalents [20], our analytical sensitivity was calculated to be 1.2 bacteria/µl (2.5–5 bacteria/reaction). All real-time PCR reactions were performed using the LightCycler[®] 480 (Roche Diagnostics). The detection of *Leptospira* DNA was performed using a Taqman probe targeting the *lipL32* gene as previously described by Stoddard *et al.* [18] (Table 2). The absence of PCR inhibition in each sample was assessed in a separate qPCR assay targeting the IC using primers VZV UP/VZV DP and detected with the hydrolysis probe VZV P (Table 2) [19]. For each reaction, the *lipL32*-containing plasmid

was used as a positive control and two negative controls (sterilized water) were included to detect the presence of contaminating DNA. Analyses of samples were performed in triplicate, and to avoid intra-PCR contamination, we repeated the qPCR within different runs for each sample. For each sample, interpretation of the qPCR results could be done when the amplification of the IC was positive, the two negative controls within the run were negative, and the cycle threshold (Ct) value of the positive control was between 24 and 27 cycles. A negative result was assigned in the cases where no amplification of the *lipL32* gene occurred (i.e. the Ct value was greater than 40 cycles). A positive result was assigned to a sample when the three qPCRs targeting the *lipL32* gene were positive.

Statistical methods

All variables were reported as percentage (or mean) ± standard deviation (s.d.). We used a χ^2 test to compare seroprevalences and prevalences of renal carriage between species; *P* values were adjusted using Holm's adjustment method and were considered significant when <0.05. Comparisons of the mean renal concentrations of leptospires between species were not biologically relevant due to wide inter-individual variations within each species. Data analysis was performed using R statistical software [21].

Sampling authorization and ethics statement

Rats, mice and shrews are introduced invasive mammals on Reunion Island, thus no particular authorization was required for their capture and study. Euthanasia/slaughtering of animals was conducted ethically by following the recommended procedures of the Parliament and the Council of the European Union [15]. This research adhered to the French legal

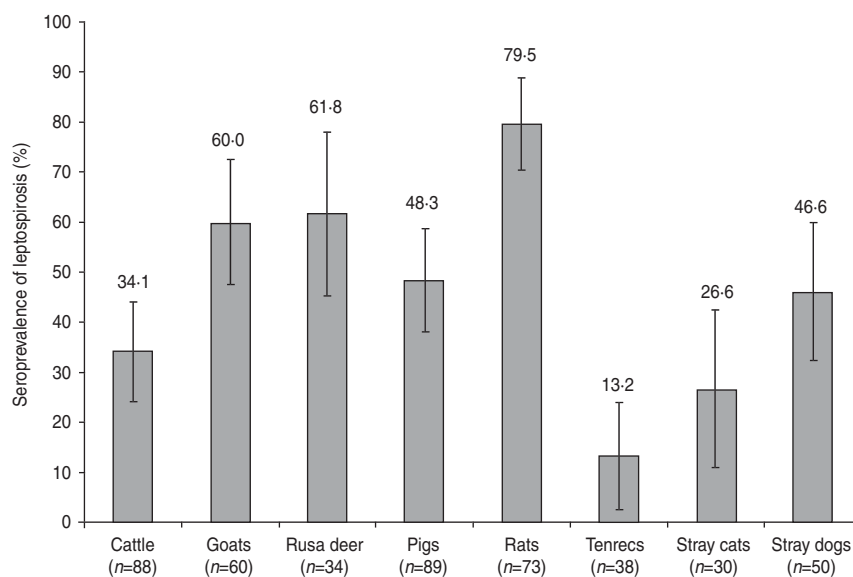


Fig. 1. Seroprevalence of leptospirosis (and standard deviation). Seroprevalence was assessed by MAT on eight animal species from Reunion Island.

requirements articles R.214-93 and R.214-99 to R.214-102 of the French Rural Code and Order of 19 April 1988, giving the authorization to experiment on living animals. For stray carnivores, French law no. 99-5 (6 January 1999) concerning ‘dangerous and stray animals and protection of animals’ was followed. The treatment of livestock followed European Regulation no. 1099/2009 (24 September 2009) and the French Order (12 December 1997) defining ‘the ethical procedures concerning welfare, protection, immobilization, stunning, and euthanasia of livestock animals at the slaughterhouse’. Our study received the approval of the Health Veterinary Inspector, the Director of the Veterinary Services of Reunion Island, the Director of the animal rescue, and the Director of the slaughterhouse of Saint-Pierre. Capture and sampling of the protected bat *M. francoismoutoui* required an official authorization that was issued on 10 March 2009 from the Direction Régionale de l’Environnement (DIREN) of Reunion Island. Sampling of the game species, *T. ecaudatus*, required an official authorization from the Direction de l’Agriculture et de la Forêt (DAF) and from the Hunting Federation of Reunion Island, that we obtained on 13 January 2009 (no. BD/BF/012).

RESULTS

The results of trapping showed that the three species of rodents are sympatric, but the black rat was the most common rodent caught. Black rats accounted

for 85.1% of all rodents sampled, while mice accounted for 9.9%, and the Norway rat for 4.9%.

All results are summarized in Supplementary Table S1.

Serological results

We found that the seroprevalence varied considerably depending on the species (Fig. 1). The seroprevalence (% \pm s.d.) was highest in rats (Norway and black) with $79.5 \pm 9.3\%$ (58/73) of seropositive animals; followed by Rusa deer ($61.7 \pm 16.3\%$, 21/34); goats ($60.0 \pm 12.4\%$, 36/60); pigs ($47.2 \pm 10.4\%$, 43/89); stray dogs ($46.0 \pm 13.8\%$, 23/50); cattle ($34.0 \pm 9.9\%$, 30/88); stray cats ($26.6 \pm 15.8\%$, 8/30); and tenrecs ($13.2 \pm 10.7\%$, 5/38).

The serological results are summarized by species in Tables 3 and 4. Serogroup Icterohaemorrhagiae accounted for 39.7% of all seropositive reactions in Norway and black rats (titres 100–3200), and serogroup Canicola was the second highest serogroup in rats, representing 15.5% of the seropositive reactions (titres 100–400). In tenrecs, the main circulating serogroup was Icterohaemorrhagiae (titres 200–800) but serogroups Canicola and Bataviae were also reported. Stray cats were more frequently seropositive for several serogroups (87.5%) and all antibody titres were low (≤ 400). Canicola was the most prevalent serogroup in stray dogs representing 43.5% of the seropositive animals (titres 100–800), while Icterohaemorrhagiae accounted for 21.7% (titres

Table 3. Results of microscopic agglutination test conducted on animal sera from Reunion Island

	Black rats	Norway rats	Tenrecs	Cats	Dogs	Cattle	Goats	Rusa deer	Pigs	Total
Icterohaemorrhagiae	22	1	3	0	5	0	0	2	2	35
Canicola	7	2	1	0	10	1	0	0	0	21
Sejroe	2	1	0	0	1	17	0	0	0	21
Panama	0	1	0	1	3	2	25	8	16	56
Pyrogenes	0	0	0	0	0	2	6	5	11	24
Tarassovi	0	0	0	0	2	1	0	0	0	3
Mini	1	0	0	0	0	0	0	1	2	4
Ballum	0	0	0	0	2	1	0	0	1	4
Cynopteri	1	0	0	0	0	0	0	0	2	3
Autumnalis	0	0	0	0	0	0	1	0	0	1
Bataviae	0	0	1	0	0	0	0	0	0	1
Co-agglutinations*	19	1	0	7	0	6	4	5	9	51
Negative	14	1	33	22	27	58	24	13	46	238
Total	66	7	38	30	50	88	60	34	89	462

* A serum showing agglutinations for more than one serogroup with no serogroup presenting a highest titre.

Table 4. Titres observed by microscopic agglutination test by species and serogroup. Minimum and maximum titres are presented. If one or two animals were seropositive to a serogroup, the titres are presented. Seropositive reactions to more than one serogroup are not included

	Black rats	Norway rats	Tenrecs	Cats	Dogs	Cattle	Goats	Rusa deer	Pigs
Icterohaemorrhagiae	100–3,200	400	200–800	–	100–1600	–	–	400	200, 400
Canicola	100–400	200, 400	100	–	100–800	200	–	–	–
Sejroe	100, 400	200	–	–	400	100–3,200	–	–	–
Panama	–	100	–	100	100	100	100–400	100–200	100–400
Pyrogenes	–	–	–	–	–	100	100–1600	100	100–400
Tarassovi	–	–	–	–	200, 400	400	–	–	–
Mini	100	–	–	–	–	–	–	800	100, 400
Ballum	–	–	–	–	100	100	–	–	100
Cynopteri	3,200	–	–	–	–	–	–	–	100, 100
Autumnalis	–	–	–	–	–	–	200	–	–
Bataviae	–	–	100	–	–	–	–	–	–

100–1600). In cattle, 56.6% of positive animals had antibodies to serogroup Sejroe, and titres reached 3200. Panama was the main serogroup found in goats, Rusa deer, and pigs accounting for 69.4%, 38.1%, and 37.2% of the seropositive reactions for these animals, respectively. Pyrogenes was the second highest serogroup found in these species, representing 16.6%, 23.8%, and 25.6% of the seropositive results for goats, Rusa deer, and pigs, respectively. Antibody titres to *Leptospira* were low in goats, Rusa deer, and pigs (≤ 400) except for two goats that had titres of 800 and 1600 to serogroup Pyrogenes.

The seroprevalence was significantly higher in rats than in tenrecs ($P=3.1 \times 10^{-6}$), cats ($P=0.008$), and cattle ($P=0.0003$). Seroprevalence was also significantly higher in goats ($P=0.041$) than in cattle,

whereas seroprevalence in tenrecs was significantly lower than seroprevalence in goats ($P=0.0003$), deer ($P=0.018$), and pigs ($P=0.011$) (pairwise P values are shown in Supplementary Table S2).

Renal carriage

Of the 541 kidney samples, 78 contained qPCR inhibitors; thus, only 463 kidneys samples could be analysed for the presence of *Leptospira*. Renal carriage (% \pm s.d.) of *Leptospira* was most prevalent in mice with $84.6 \pm 19.6\%$ (11/13) positive animals, followed by rats ($65.9 \pm 10.3\%$, 54/82), shrews ($31.2 \pm 13.1\%$, 15/48), stray dogs ($29.2 \pm 19.3\%$, 7/24), stray cats ($28.6 \pm 18.2\%$, 6/21), goats ($26.5 \pm 12.4\%$, 13/49), Rusa deer ($18.8 \pm 13.5\%$, 6/32), cattle

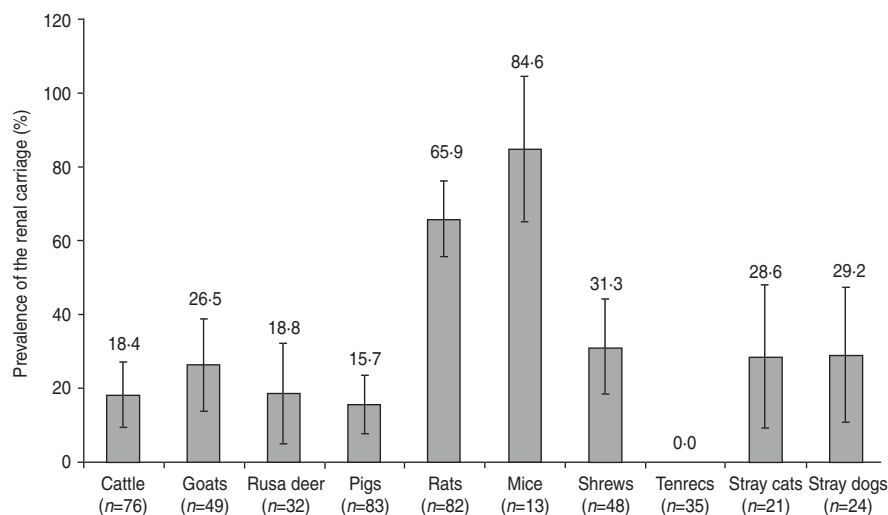


Fig. 2. Prevalence of renal carriage (and standard deviation). Presence of leptospires in kidney tissue was assessed by qPCR targeting the *lipL32* gene on ten animal species from Reunion Island.

($18.2 \pm 8.7\%$, 14/77), and pigs ($15.6 \pm 7.8\%$, 13/83). No leptospiral DNA was detected in tenrec kidneys ($0.0 \pm 5.0\%$, 0/18) (Fig. 2). The carriage rate between rats and mice was not significantly different, but both of these rodents showed significantly higher prevalence of renal carriage than the other species (pairwise *P* values are shown in Supplementary Table S3). There was no other significant difference in the prevalence of renal carriage between species.

The mean concentrations of leptospires in the kidneys for each species are presented in Table 5.

Urinary shedding in bats

Eight out of ten individual samples of bat urine contained qPCR inhibitors but both of the amplifiable urine samples were positive for the presence of *Leptospira* DNA.

DISCUSSION

Although it is often neglected, descriptive epidemiology is the first step required for in-depth studies of leptospirosis (i.e. molecular epidemiology and phylogenetic classification). The aim of this study was to update the data on animal leptospirosis on Reunion Island. The epidemiological investigation of 12 out of the 16 mammalian species living on Reunion Island contributes to global knowledge of the disease at the international level (hare, horse, sheep, and the protected insectivorous bat *Taphozous mauritanus* were not investigated due to difficulties in the sampling

protocol and/or in gaining the appropriate authorizations).

Our results indicate that *Leptospira* infection is unexpectedly common in all of the investigated mammals on Reunion Island (Figs 1 and 2, Supplementary Table S1). In this study, PCR-based detection of leptospiral DNA in kidney or urine samples was used as the definitive proof of carrier or infectious status [22]. Because leptospires only transiently colonize the kidneys in non-reservoir species [23], the detection of leptospires in the various kidney samples was expected to be minimal if the species was not a reservoir ($\sim 1\%$). Interestingly, our results indicate that all of the mammals on Reunion Island, with the exception of tenrecs, are potential renal carriers of *Leptospira* (Fig. 2). The absence of leptospiral DNA in the kidney samples of tenrecs and the low seroprevalence (compared to other species) may indicate that tenrecs are either less exposed than other species or that they may be susceptible to infection but are most likely not chronic reservoirs of *Leptospira*. In contrast, our results showed that 31.2% of shrews are carriers of pathogenic *Leptospira*, which is lower than the prevalence rate recently reported in Madagascar (43.5%, $n=26$) [24]. Nevertheless, our study corroborates other findings and provides further support that this insectivore may act as a maintenance host for *Leptospira* [24, 25].

Furthermore, we demonstrated that stray carnivores are frequently renal carriers of *Leptospira*. Although dogs are known to be a potential zoonotic reservoir host of leptospirosis [26], the possibility of persistent renal infection in cats by *Leptospira* is

Table 5. Mean leptospiral load in kidney tissue by species. The concentration of leptospires in kidney tissue was assessed by qPCR targeting lipL32 gene in each carrier animal

Species	Mean bacterial load \pm s.d. (genome copies/mg kidney tissue)
Black rat	$5.7 \times 10^6 \pm 3.3 \times 10^7$
Norway rat	$7.8 \times 10^2 \pm 1.2 \times 10^3$
Mouse	$6.4 \times 10^6 \pm 8.5 \times 10^3$
Shrew	$2.1 \times 10^3 \pm 3.2 \times 10^3$
Stray dog	$1.9 \times 10^3 \pm 3.0 \times 10^3$
Stray cat	$4.3 \times 10^2 \pm 4.7 \times 10^2$
Cattle	$8.8 \times 10^2 \pm 1.2 \times 10^3$
Goat	$9.2 \times 10^2 \pm 7.8 \times 10^2$
Rusa deer	$2.9 \times 10^3 \pm 3.7 \times 10^3$
Swine	$8.6 \times 10^2 \pm 1.7 \times 10^3$

qPCR, Quantitative polymerase chain reaction; s.d., standard deviation.

contentious [27–29]. To our knowledge, leptospires have rarely been found in cat kidneys: *Leptospira* from serogroup Canicola was isolated from a cat on the island of Trinidad (West Indies) [27], while in Spain, Millán *et al.* [30] reported a prevalence of renal carriage in 20% (5/25) of the roaming cats investigated. Additionally, a recent study described three clinical cases of leptospirosis in naturally infected cats [31]. Our study adds to these data and unambiguously indicates that cats could be renal carriers of *Leptospira* and therefore possibly be a source of infection to humans. We hypothesize that stray dogs and cats may be exposed to *Leptospira* via consumption of small infected mammals (rodents and shrews) [8] which are known to be *Leptospira* carriers, or by roaming in contaminated environments [32].

The significant prevalence of renal carriage in livestock (Fig. 2) probably reflects the fact that these animals are chronically infected, which raises a significant concern due to the zoonotic nature of the disease. Widespread circulation of leptospirosis in livestock may also have financial implications as infection may result in a decrease in milk quality and quantity, an increase in abortions, higher culling rates, more stillbirths, and death [33–35].

The carriage rate reported in rats in our study (65.9%) was higher than the prevalence rate generally found on other tropical islands such as Madagascar where 40% of renal carriers were reported in rats ($n=190$) [24], or Hawaii, where 60.2% of renal carriers were found in the *R. norvegicus* population

($n=510$) [36], or even New Caledonia, where 26.7% of rats were carriers of *Leptospira* [37]. Nevertheless, our prevalence rate was close to that reported in rats in Metro Manila in the Philippines (63%, $n=56$) [38] and lower than the prevalence rate described in Norway rats in Brazil (80.3%, $n=142$) [39]. We reported a prevalence of renal carriage of 84.6% in mice, which was in accord with other studies conducted in Hawaii (79.5%, $n=31/39$) [36], and the Azores (85.4%, $n=41$) [40]. The differences observed in carriage prevalence could be due to study design and the detection strategies employed but may also reflect different dynamics in the different ecosystems.

In consideration of the fact that the bat, *M. francismoutoui*, is a protected species, we chose to conduct non-invasive sampling on this mammal. The number of non-amplifiable bat urine samples (8/10) may indicate that the sampling technique used for bats was not optimal and that contamination by PCR inhibitors occurred. Nevertheless, two bats were shown to be urinary shedders of *Leptospira*. The role that bats play in the transmission of *Leptospira* is not fully understood. Most of the studies focused on domestic animals because of their close association with humans; however, because of their abundance, and close contact with both domestic and wild animals and humans, bats may be involved in the maintenance and transmission of leptospires on Reunion Island, as reported elsewhere [22, 41]. Studies in Peru reported that bats can be reservoirs of leptospirosis [41, 42] and the survey conducted by Matthias *et al.* [42] described a prevalence of renal carriage of 3.4% ($n=589$) in insectivorous bats. Furthermore, on Reunion Island, bat guano is used for agricultural purposes and collecting the guano under a roosting site could expose people to *Leptospira* infection.

Seroprevalence was high in all the species investigated (Fig. 1) suggesting that contacts with *Leptospira* are frequent. We report a higher seroprevalence of *Leptospira* infection in rats than that commonly reported [40, 43], except in the Philippines, where 92% of rats showed antibodies to *Leptospira* [38].

In this study, we demonstrated that the seroepidemiological results in dogs were similar to those found in 1979 (seroprevalence 41% in 1979 [12] vs. 46.0% in our study) and that Canicola has remained the main circulating serogroup in stray dogs (69% of those seropositive in 1979 [12] vs. 43.5%) followed by Icterohaemorrhagiae (26% [12] vs. 21.7%).

Serogroups Sejroe, Mini, and Cynopteri in rats, as well as Sejroe, Panama, Tarassovi, and Ballum in dogs (Tables 3 and 4) showed low seroprevalence and/or low titres, suggesting that contact with these serogroups was rare or was detected in our study as the result of non-specific co-agglutination. Between 1998 and 2009, serogroup Icterohaemorrhagiae and Canicola were reported to represent 59.3% and 17.5%, respectively, of the human cases diagnosed on Reunion Island [11]. Our results suggest that rats and dogs are the main source of human infection and corroborate recent findings reporting that rats may also act as a reservoir for serogroup Canicola [24, 44]. The seroprevalence in cattle was similar to that reported by two studies conducted on Reunion Island in 1979 (29% [13] to 32% [12]), and serogroup Sejroe has remained the major serogroup circulating in this species. Eight out of 17 cattle seropositive to serogroup Sejroe had antibody titres ≥ 800 , probably indicating a recent infection [45]. Between 1998 and 2009, Sejroe was involved in 5.4% of human infections [11], suggesting that cattle are seldom involved in human contamination on Reunion Island. Panama was the most prevalent serogroup circulating in Rusa deer, goats, and pigs, whereas Pyrogenes was the second most common serogroup in these three species (Table 3). Titres were most frequently low (≤ 400) suggesting a chronic or prior infection. High animal density and humid breeding conditions could explain the high seroprevalence rate of leptospirosis in farm animals. The Panama and Pyrogenes serogroups have been involved in 0.2% and 1.4% of human infections, respectively [11]. Two hypotheses could explain the differential frequencies of these serogroups in humans and animals: the frequency of transmission from pigs, goats, and deer to humans is low, or infections of humans by serogroup Pyrogenes and Panama are asymptomatic or mild, as previously observed in Cambodia and Mexico [46, 47] and are therefore rarely diagnosed at laboratory level. The presence of anti-*Leptospira* antibodies has rarely been reported in cats [30, 48], but we report a seroprevalence of 26.6% in this species. Most of the seropositive cats had antibodies to several serogroups, indicating that exposure to multiple strains is frequent in this species and that no specific serogroup seems to be maintained in the cat population.

The main serogroups found in cattle (Sejroe), rats (Icterohaemorrhagiae), and dogs (Canicola) have also been reported to infect the same European species. This finding is not surprising given that all of the farm

and wild species living on Reunion Island originated from Europe [49] and that the black rat introduced to the island is genetically similar to those found in Europe [50]. Considering that leptospires were probably introduced on Reunion Island via the non-native animal hosts that were introduced by humans, it is not surprising to find the same epidemiological patterns of the disease that have been reported in Europe.

MAT is a serogroup rather than a serovar-specific assay and because of the high degree of cross-reactivity that occurs between different serogroups, the serological data should be used only to gain a broad idea of the serogroups present at the population level [51]. Moreover, paradoxical reactions and cross-reactions between serogroups are common [52, 53]. Furthermore, an important limitation of MAT is the number of antigens used, which corresponds to the antigens that are expected to be present in the area being tested and thus minimizes the probability of detecting a response to a serogroup that is not expected [51, 52]. Another confounding factor in areas of high endemicity is the possibility of multiple successive or even co-infections with multiple serovars [51]. Culturing and typing of infectious isolates and/or direct molecular typing of strains from clinical samples [54] could be used for further epidemiological studies.

To our knowledge, the current study is the first to report the quantification of *Leptospira* in the kidneys of non-laboratory mammals. We showed that the concentration of leptospires in the kidney of carrier animals presented a large amount of inter-individual variation (standard deviations are shown in Table 5). A large variation in the concentration of leptospires in the urine of experimentally infected rats [55] and in naturally infected deer [56] has been described. This variation could be related to variable concentrations of leptospires in the kidney tubules. Various factors could impact the renal concentration of leptospires, including the age of the infected animal [57], other factors inherent in the host [57], the amount of time that has elapsed since infection, and the infecting *Leptospira* serogroup [2]. Moreover, common associations between particular serovars and their animal reservoirs have largely been recognized [17]. Isolation and serotyping or direct molecular typing of the strains would be essential for better understanding of these data. Rats do not develop disease from *Leptospira* infection [2], and our results suggest that naturally infected animals belonging to different species have the same capacity to harbour leptospires

in their kidneys. These results constitute the basis of our plan for further studies aimed at evaluating the contribution of each species to *Leptospira* environmental contamination in the field. At the herd level, the detection or quantification of leptospires in the kidneys of slaughtered animals might be correlated with indicators of herd productivity and could help in the development of veterinary control programmes.

On Reunion Island, the tropical climate and presence of flooded areas increase the environmental exposure of humans and animals to *Leptospira* [58]. As a consequence, the risk of transmission should be considered at the ecosystem level. Rodents, dogs and most likely shrews are the major sources of human infection whereas livestock and cats seem to mostly maintain leptospire reservoirs of lower relevance to human health. Thus, preventive measures aimed at reducing the burden of leptospirosis in the human population should first focus on relevant control measures against rodents and stray dogs. In livestock, intra-herd transmission could be reduced by the detection of carriers (in particular reproductive animals used for natural breeding) [4, 6] and relevant treatment, whereas inter-herd transmission could be reduced by the control of introduced animals, environmental control measures [59], and management of the pasture [59, 60]. Knowledge of the animal hosts involved in leptospirosis epidemiology on a small geographical scale contributes to a better understanding of the disease on a global scale.

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SUPPLEMENTARY MATERIAL

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DECLARATION OF INTEREST

None.

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