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Short communication

First isolation of *Leptospira interrogans* from the arboreal squirrel *Callosciurus erythraeus* introduced in Argentina

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We isolated *Leptospira* strains from renal tissue samples of Asiatic red-bellied squirrels *Callosciurus erythraeus*, captured in Argentina. In December 2008, we captured 34 squirrels in the province of Santa Fe during a short control campaign, which were screened for *Leptospira* spp. by direct immunofluorescence assays and isolation attempts in renal tissue samples. A prevalence of 38% of *Leptospira* spp. was obtained by direct immunofluorescence. The isolates, genotyped by the MLVA, were identified as *L. interrogans* Canicola (N = 2) and *L. interrogans* Icterohaemorraghiae (N = 1), showing an identical genetic profile to the reference strains Hond Utrech IV (serovar Canicola, serogroup Canicola) and M20 (serovar Copenhageni, serogroup Icterohaemorrhagiae), respectively. Our study shows for the first time that the red-bellied squirrel is a renal carrier of *Leptospira interrogans* and might act as a reservoir of leptospirosis both in native range and areas of introduction within several European countries, Japan and Argentina. The close association of this introduced squirrel species with humans and domestic animals may increase the probability of disease transmission, particularly in favourable environments.

Key words: Asiatic red-bellied squirrel, Callosciurus erythraeus, Leptospira interrogans, leptospirosis, renal carrier, Rolling Pampas, squirrels

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Approximately 60% of human pathogens are zoonotic (Woolhouse 2002), and wildlife is the source of approximately 75% of the diseases that have emerged over the past two decades (Taylor et al. 2001). The increase in wildlife translocation plays an important role in the transmission of zoonotic diseases and in the maintenance of worldwide pathogens, thus, increasing the risk of zoonotic disease outbreaks (Daszak et al. 2000, Bengis et al. 2004, Kruse et al. 2004, Reed et al. 2004) particularly from synanthropic species (McFarlane et al. 2012). Reemerging infectious diseases can be broadly defined as infectious diseases whose geographical range, host range or prevalence has increased in recent years (Bengis et al. 2004). Leptospirosis is a worldwide zoonotic disease caused by the infection of a pathogenic spiroquete of the genus *Leptospira* (Levett 2001, Bharti et al. 2003, Vijayachari et al. 2008) and is considered a reemergent disease given its increase in prevalence and the occurrence of new pathogenic strains (Bengis et al. 2004, Mahajan & Daljeet 2008, Pappas et al. 2008, Vijayachari et al. 2008). A wide variety of animals such as mammals, reptiles and amphibians may serve as reservoir of the pathogenic *Leptospira interrogans* (Seijo et al. 2002, Rossetti et al. 2003, Brihuega & Tealdo 2011) and may be a source of infection to humans and other domestic and wild animals (Michel et al. 2001, 2002, Bharti et al. 2003, Masuzawa et al. 2006). The existence of periodic epizootics may cause significant die-offs in mammals, as reported for example in marine mammals (Cameron et al. 2008). Humans are exposed to leptospirosis infection by commensal and peridomestic wild mammals such as rats and mice *Mus domesticus* that are worldwide carriers of *Leptospira interrogans* (Vijayachari et al. 2008). However, some squirrel species, such as the white neck squirrel *Sciurus stramineus* and the fox squirrel *Sciurus niger*, may also act as carriers of *Leptospira interrogans* (Diesch et al. 1967, Montes Aliaga et al. 2011) and others, such as the southern flying squirrel *Glaucomys volans* and the fox squirrel may transmit leptospirosis to humans (Diesch et al. 1967, Masuzawa et al. 2006).

The red-bellied squirrel *Callosciurus erythraeus* is a sciurid rodent native to southeastern Asia (Corbet & Hill 1992) which was intentionally introduced into Argentina (Aprile & Chicco 1999), Belgium (Dozières et al. 2010), France (Gerriet 2009), Italy (LIFE09 NAT/IT/095 EC-Square Project 2012), Japan (Miyamoto et al. 2004) and The Netherlands (Dijkstra et al. 2009). In 1970, 10 red-bellied squirrels were introduced for ornamental purposes into the Buenos Aires province, Argentina (Aprile & Chicco 1999), where an expanding, large wild population is now established, and further intentional releases have created new invasion foci within the country (Benitez et al. 2013). This introduced rodent can now be found in three Argentinean provinces, two of which have environmental characteristics that favour leptospirosis infection (Martin et al. 2002, Seijo et al. 2002, Musacchio et al. 2010); it inhabits rural and urban areas in close association with humans and domestic animals, and is also illegally traded as pet (Guichón et al. 2005, Benitez et al. 2013). Red-bellied squirrels serve as an occasional host of local parasites acquired mainly from sympatric wild mammals (Gozzi et al. 2013), cause economic damages in cables and irrigation systems, and in fruit and timber plantations (Guichón et al. 2005), and were therefore declared a harmful species in various districts where numerous local producers and residents demand control actions (CDL 2011, DFF 2012).

To our knowledge, no studies have evaluated the role that red-bellied squirrels may play in the maintenance and transmission of leptospirosis either in their native or introduced ranges. The aim of our study was to evaluate if red-bellied squirrels are renal carriers of *Leptospira interrogans* in Argentina and may constitute a health risk.

Material and methods

Our study site is located in Cañada de Gómez (32°48'S, 61°23'W), southern Santa Fe province, Argentina, belonging to the Rolling Pampa region, which is the main agricultural area of the country. Mean annual temperature is 17.5°C and annual rainfall is 1,019 mm, with a relatively high mean annual humidity of 74% (EEAO INTA 2008). In Cañada de Gómez, red-bellied squirrels were released in ca 1999 within a private ranch mainly used for agriculture, tourism and equine activities. Bark stripping by these squirrels was the major damage recorded 10 years after liberation of the species when the ranch owner conducted a short control campaign for five consecutive days in a 7-ha grid to reduce damage caused by squirrels. Within this context, 34 squirrels were live-trapped in December 2008 using baited cage traps (30 x 13 x 13 cm) on tree branches, and were euthanised following animal handling procedures approved by international guidelines (American Veterinary Medical Association 2007, Animal Care and Use Committee 1998). Captured squirrels looked healthy and had no evident lesions. Kidneys were obtained aseptically by necropsy in the field, frozen and then analysed in the Leptospirosis Laboratory, OIE Reference, INTA. Logistical limitations prevented obtaining other kind of samples (e.g. urine, blood and serum) and the immediate inoculation of the kidneys into the culture medium when extracted in the field. Direct immunofluorescence (DI) assays were used to test for the presence of leptospires in kidney tissue smears ($N = 68$), using a conjugate polyvalent USDA (USA). For isolation attempts, aliquots of squirrel renal tissue were plated in modified Ellinghausen McCullough-Johnson-Harris (EMJH) liquid medium and the Fletcher semi-solid medium, under a biosafety cabin of the Leptospirosis Laboratory. Isolation is the best confirmatory method to evaluate whether an animal is a renal carrier of leptospires, while DI was used as a complementary test. Cultures were incubated at 30°C and weekly assessed under dark field microscopy. Isolations were made within 20-60 days postculture. Molecular characterisation of the strains isolated was carried out by Multiple-Locus Variable-number tandem repeat Analysis (MLVA), using the loci VNTR4, VNTR7, VNTR9, VNTR10, VNTR19, VNTR23 and VNTR31, as described by Majed et al. (2005).

Results

A total of 13 individuals out of 34 captured squirrels were positive for *Leptospira* with direct immunofluorescence assays, resulting in a prevalence of 38% (95% CI = 22-54; Table 1). *Leptospira* strains were isolated in Fletcher medium, but no isolations were obtained with the modified (EMJH) liquid medium. The three isolates (corresponding to three different individuals) were genotyped by the MLVA and identified as *L. interrogans* Canicola (N = 2) and *L. interrogans* Icterohaemorraghiae (N = 1), showing an identical genetic profile to the reference strains Hond Utrech IV (serovar Canicola, serogroup Canicola) and M20 (serovar Copenhageni, serogroup Icterohaemorrhagiae), respectively.

Discussion

Studies assessing the potential role of squirrels as maintenance hosts of *L. interrogans* all over the world are scarce and no reports of dead squirrels or individuals showing clinical symptoms of leptospirosis have been found. Squirrels of the genus *Sciurus* are known to be seropositive or carriers of eight serogroups of *Leptospira* sp. (see Table 1). *Leptospira kirschneri* was reported for the southern flying squirrel *Glaucomys volans*, while five other serogroups, which are different from the two serogroups identified in our study, were detected in *Callosciurus* squirrels (see Table 1). The prevalence of *Leptospira* spp. obtained in the red-bellied squirrel with direct immunofluorescence assays was amongst the highest reported values (see Table 1), even if fieldwork was conducted during a particularly drought period and other studies used laboratory techniques more sensible, such as a Microscopic Agglutination Test (MAT). The serogroups Icterohaemorrhagiae and Canicola found in our study have only been previously reported for the grey squirrel *Sciurus*

Table 1. Squirrel species as reservoirs of *Leptospira*, indicating collection site, laboratory tests (MAT: microagglutination test, DI: direct inmunofluorescence or Isolation), *Leptospira* species or serogroup, number of positive squirrels of the total number of squirrels analysed and references. The abbreviations are: Ictero = Icterohaemorragiae; Can = Canicola; Geor = Georgia; Aus = Australis; Pom = Pomona; Hard = Hardjo; Grippo = Grippotyphosa. [a] Corresponds to *Callosciurus erythraeus taiwanensis* (Corbet & Hill 1992) Ascribes to: [b] *L. interrogans* (Bharti et al. 2003); [c] *L. santarosai* (Bharti et al. 2003); [d] *L. kirschneri* (Bharti et al. 2003); [e] *L. borgpetersenii* (Bharti et al. 2003); [f] Lepto 175 *(Sarawak)* is a poorly known strain endemic to Malaysia, but its pathogenic potential is still unknown (Thayaparan et al. 2013).

Species	Site	Test/Isolation	*Leptospira*/Serogroup	Positive squirrels	Reference
Sciurus stramineus	Peru	MAT	Ictero[b], Can[b], Aus[b], Geor[c]	29/35	Montes Aliaga et al. 2011
Sciurus carolinensis	USA	MAT	Ictero[b], Can[b], Pom[b], Hard[b],Grippo[d]	1/20	Richardson & Gauthier 2003
	USA	Isolation	Ballum[e]	1/26	Shotts 1975
Sciurus niger	USA	Isolation	Grippo[d]	1/27	Shotts 1975
	USA	Isolation	*Leptospira gryppotyphosa*[d]	1/9	Diesch et al. 1967
Glaucomys volans	Japan	Isolation	*Leptospira kirschneri*	5/10	Masuzawa et al. 2006
Callosciurus caniceps	Malaysia	MAT	Djasiman[b]	1/71	Gordon Smith et al. 1961
Callosciurus nigrovitattus	Malaysia	MAT	Hebdomadis[b]	1/36	Gordon Smith et al. 1961
Callosciurus notatus	Malaysia	MAT	Pom[b], Lepto 175[f]	4/4	Thayaparan et al. 2013
Callosciurus flavimanus taiwanensis[a]	Taiwan	Isolation	*Leptospira javanica*[e]	1/2	Tsai & Kundin 1970
Callosciurus erythraeus	Argentina	Isolation	Ictero[b], Can[b]	3/34	Our study
	Argentina	DI	Leptospira spp.	13/34	Our study

Table 2. Wild and domestic mammals reported to be seropositive or carriers of *L. interrogans* Canicola and Icterohaemorragiae serogroups in Argentina, indicating laboratory tests, serogroup and references. The abbreviations are: Can = Canicola; Ictero = Icterohaemorragiae.

Species	Test/Isolation	Serogroup	Reference
Armadillo *Chaetophractus villosus*	Isolation	Can, Ictero	Myers et al. 1977, Seijo et al. 2002
Opossum *Didelphis albiventris*	Isolation	Can	Brihuega et al. 2007, Pavan et al. 2010, Perez Carusi et al. 2009
	Serology	Ictero	Perez Carusi et al. 2009
Fox *Dusicyon culpaeus*	Serology	Can, Ictero	Martino et al. 2004
Coypu *Myocastor coypus*	Isolation	Ictero	Seijo et al. 2002
Cavy *Cavia aperea pamparum*	Isolation	Ictero	Brihuega & Tealdo 2011
Sigmodontine rodent *Akodon azarae*	Isolation	Ictero	Seijo et al., 2002
Black rat *Rattus rattus*	Isolation/Serology	Ictero, Can	Arango et al. 2001, Seijo et al. 2002, Vanasco et al. 2003
Brown rat *Rattus norvegicus*	Isolation	Ictero	Arango et al. 2001
Dog *Canis familiaris*	Serology/ Isolation	Can	Vanasco et al. 2000, Seijo et al. 2002
Cow *Bos* spp.	Isolation	Can	Pavan et al. 2010
Pig *Sus scrofa domesticus*	Isolation	Ictero	Pavan et al. 2010

carolinensis and the white neck squirrel *S. stramineus* (see Table 1). Both serogroups have a universal distribution (Levett 2001, Bharti et al. 2003) being frequently found in domestic and wild mammals such as rats, mice, marsupials, deer, cattle and dogs (Bharti et al. 2003, Brihuega et al. 2007). The susceptibility of humans and cattle to these serogroups reinforces their sanitary and economic importance (Szyfres 1976, Vanasco et al. 2000, Acha & Szyfres 2001).

Several wild and domestic mammals in Argentina are found to be seropositive or carriers of *L. interrogans* Canicola and Icterohaemorrhagiae serogroups (Table 2). The red-bellied squirrel coinhabit with many of these wild (e.g. rats *Rattus* spp., sigmodontine rodents *Akodon azarae* and *Oligoryzomys* spp. and opossums *Didelphis albiventris*) and domestic mammals given their rural-urban habits, which could explain the isolation of these two serogroups from the squirrels captured in the Santa Fe province. This region of Argentina is considered to have high risk of leptospirosis infection (Vanasco et al. 2000, Seijo et al. 2002, Vanasco et al. 2008, Musacchio et al. 2010), where reported cases of human leptospirosis have been associated to urban and suburban areas exposed to floods and the high number of residents close to backwaters and landfills, together with abundant pigs, dogs and rodents nearby (Vanasco et al. 2000, 2008). The two serogroups identified in the red-bellied squirrel in our study are within the four serogroups potentially associated with cases of human leptospirosis in this region (Vanasco et al. 2000).

Our study shows that the red-bellied squirrel introduced into Argentina is a renal carrier of *L. interrogans*. Our study is the first to evaluate the role of the red-bellied squirrel in leptospira infection worldwide (see Table 1) and suggests that this species may act as a reservoir of *L. interrogans* playing a role in the epidemiology of leptospirosis. Further studies are needed to be able to make conclusions about the status of the red-bellied squirrel as a reservoir of leptospirosis both in native and introduced ranges. Squirrels have been reported to transmit leptospirosis to humans, as occurred with the fox squirrel in the United States (Diesch et al. 1967) and the introduced southern flying squirrel in Japan (Masuzawa et al. 2006). The introduction of wild fauna may result in an increased prevalence of *L. interrogans* in the environment, increasing the risk of leptospirosis transmission. It must be noted that the introduced red-bellied squirrel lives in close association with humans and domestic animals as they inhabit urbanised and agricultural areas not only in Argentina, but also in Japan and European countries. Moreover, these squirrels are illegally translocated and traded as a pet, increasing the risk of disease transmission particularly in favourable environments. The outcome of our study will inform new regulations for preventing the capture, possession, trade and translocation of the red-bellied squirrel to minimise the associated sanitary risks.

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