Using a top predator as a sentinel for environmental contamination with pathogenic bacteria: the Iberian wolf and leptospires

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*The Iberian wolf (*Canis lupus*) is the top predator in the Iberian environments in which it lives, feeding on a wide range of species, thus encountering a wide range of disease agents. Therefore, the wolf can serve as sentinel of environmental contamination with pathogens. We investigated the exposure of free-living wolves to 14 serovars of* Leptospira interrogans sensu lato*. Kidney samples from 49 wolves collected from 2010-2013 in northwestern Spain were analysed by culture, direct immunofluorescence and polymerase chain reaction. Tissue fluids were analysed for antibodies by a microscopic agglutination test. Ten wolves (observed prevalence: 20%, 95% confidence interval = 11-33%) showed evidence of contact with leptospires, eight through direct detection and nine through serology (7 wolves were positive according to both techniques). Titres below the cut-off level were also detected in seven cases. Serovars confirmed were Canicola (n = 4), Icterohaemorrhagiae (n = 3) and Sejroë, Ballum and Grippotyphosa (n = 1 each), indicating that wolves were infected with serovars for which dogs, rodents and ungulates, are the natural hosts and supporting the utility of the wolf and other large predators as environmental sentinels for pathogens.*

Key words: Carnivora - *Leptospira borgpetersenii* - spirochete

Leptospirosis is a disease of public health and veterinary importance caused by pathogenic spirochetes of the genus *Leptospira*. The disease is distributed worldwide and is known to affect humans, domestic animals and wildlife. Leptospires exploit some mammal species as reservoir hosts by establishing chronic infections in the renal tubules of the kidneys ("carrier phase") that can persist for months or longer. From this niche, bacteria are shed in urine, contaminating the environment. Susceptible hosts thus acquire infection indirectly from infected animals by coming into contact with environmental bacteria (Birtles 2012), though direct contact can also take place (Levett 2001). The Genus *Leptospira* includes more than 17 species and is subdivided into a large number of antigenically distinct serovars, many of which have been associated with particular mammalian reservoir hosts and therefore have ecological relevance (Birtles 2012).

A sentinel species is one used to collect information about disease. For example, scavenging or carnivorous species screen a large number of species at a lower trophic level. Measuring evidence of disease in the carnivore/scavenger provides an index to the relative frequency of occurrence of disease (Wobeser 2007). Most members of the order Carnivora are wide-ranging, generalist species that can be useful as sentinels to determine the presence and relative importance of different serovars of *Leptospira* in the environment (Millán et al. 2009). The wolf (*Canis lupus*) is one of the three large predators inhabiting the Iberian Peninsula, showing a remarkable range in the region (ca. 140,000 km^2, 25% of Iberia) (Blanco & Cortés 2012) and a high level of tolerance to human activities (Llaneza et al. 2012). Iberian wolves feed on a wide range of preys, from small mammals to large ungulates, both wild and domestic, poultry and carrion (Cuesta et al. 1991). Taking into account the ecological role of wolves in ecosystems, its generalist nature and its range, Iberian wolves may serve as good sentinels for environmental monitoring of infectious agents or pollutants in Iberia. The aim of this study was to explore the potential role of wolves as sentinels of environmental contamination with pathogenic leptospires.

MATERIALS AND METHODS

We collected samples of 49 dead wolves (causes of death: legal hunting, road kill and illegal killing) from 2010-2013 in two regions of northwestern Spain: Asturias (43º28'N 5º27'O) and Galicia (43º17'N 7º41'O). Carcasses were collected as part of a long-term collection protocol of wolf samples approved by the Regional

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Governments of Galicia and Asturias. This sample size represents about 2.5% of the wolves inhabiting the Iberian Peninsula (estimated in 2,000 individuals) (Blanco & Cortés 2012). Kidney samples were obtained and kept frozen until analyses. Once in the laboratory, samples were subjected to three types of analyses. Direct immunofluorescence (IF) was carried out using Rabbit IgG against a pool of the studied serovars of *Leptospira* (made in-house) and goat anti-rabbit IgG-fluorescein isothiocyanate conjugate (Nordic Immunological Laboratories, Netherlands) following the procedure of León-Vizcaíno et al. (1987). The serovars investigated were Australis, Autumnalis, Ballum, Bataviae, Bratislava, Canicola, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Pomona, Saxkoebing, Sejroë and Tarassovi. The strains used can be found in Millán et al. (2009). Culture was carried out using a standard Ellinghausen-McCullough-Johnson-Harris (EMJH) (Difco Laboratories, Madrid, Spain) liquid medium following the procedures of Baranton and Postic (1989). Kidney samples were homogenised in a 1:10 dilution with EMJH medium (Difco). Homogenate samples were subjected to DNA extraction, performed as described by Rojas et al. (2010) using a commercial kit (QIAamp DNA Mini Kit; Qiagen Iberia, Spain). Extracted DNA was quantified using spectrophotometry at 260 nm. Polymerase chain reaction (PCR) was performed as described by Moreno and Agudelo-Flórez (2010) using primers LipL32/270F (5'-CGCTGAAATGGGAGTTCGTATGATT-3') and LipL32/662R (5'-CCAACAGATGCAACGAAAGATCCTTT-3) as reported by Levett et al. (2005). No-template controls were included in PCRs. PCR was performed in a Biometra T Personal (Biometra, Germany) thermocycler and PCR products were visualised through electrophoresis on a 1.5% agarose gel stained with ethidium bromide and viewed under ultraviolet light. Positive DNA extractions were amplified by PCR in order to differentiate *Leptospira* serovars detecting the oligonucleotide i-Repl as a target of the amplification (Barocchi et al. 2001, Rojas et al. 2010). PCR was performed as described by Rojas et al. (2010) using the primers Rep1 (5'- AGCGGGTATGACTCCGC-3') and iRep1 (5'-GCGGACTCATACCCGCT-3') (Barocchi et al. 2001). To detect antibodies against such serovars, kidney tissue exudates were analysed by the indirect microscopic agglutination test following procedures outlined in Faine (1982), which is the standard method to detect antibodies against *Leptospira* serovars. We considered 1:100 the cut-off point for positive sera. Differences in prevalence between seasons, regions and age (pups: younger than 1 year; subadult: between 1-2 years; adult: older than 2 years) and sex groups were tested with the χ^2 test or Fisher's exact test using PASW Statistics 17.0.

RESULTS

Ten wolves (observed prevalence: 20.1%, 95% confidence interval = 11-33%) showed evidence of contact with leptospires (Table). Eight wolves were actively infected as revealed by direct detection (16.3%); of these, three were positive by PCR and IF and five only by PCR. All wolves were negative for culture. Nine wolves had antibodies

TABLE

Serovars detected in 10 out of 49 wolves analysed for pathogenic leptospires

			Direct detection in kidney		Serology in kidney exudates	
Reference	Region	Age/sex	Serovar	Positive by[b]	Serovar (titration)	Other titres below the cut-off level[a]
CL-012	Galicia	Subadult/female	-	-	Canicola (1:400)	Ballum (1:10)
CL-016	Galicia	Adult/male	Canicola	PCR	Canicola (1:100)	-
CL-017	Galicia	Pup/female	Canicola	PCR	Canicola (1:400)	-
CL-022	Asturias	Pup/male	-	-	Ballum (1:800)	Canicola (1:20)
CL-024	Galicia	Adult/female	Canicola	PCR, IF	Canicola (1:100)	-
CL-026	Asturias	Subadult/female	Icterohaemorrhagiae	PCR	Icterohaemorrhagiae (1:100)	-
CL-034	Asturias	Subadult/male	Icterohaemorrhagiae	PCR, IF	-	Icterohaemorrhagiae (1:20)
CL-035	Asturias	Subadult/male	Icterohaemorrhagiae	PCR	Icterohaemorrhagiae (1:200)	Australis (1:50)
CL-063	Asturias	Subadult/male	*Leptospira interrogans* (serovar ND)	PCR	Grippotyphosa (1:400)	-
CL-067	Asturias	Adult/female	Sejroë	PCR, IF	Sejroë (1:100)	-

a: three further wolves showed titres below the cut-off level: Sejroë (1:50), Sejroë (1:10) and Ballum (1:10); *b*: all samples were negative by culture; IF: direct immunofluorescence; ND: not determined; PCR: polymerase chain reaction.

(18.3%). Seven wolves were positive for both antibodies and leptospires. Overall, serovars detected were Canicola (n = 4, 40% of the positive cases), Icterohaemorrhagiae (n = 3, 30%) and Sejroë, Ballum and Grippotyphosa (n = 1, 10% each). Four of the seropositive wolves and three further wolves showed titres against other serovars, including serovar Australis, but below the cut-off level (Table). No differences were found in prevalence depending on the diverse factors studied (see Materials and Methods). Interestingly, a wolf pup had high antibody titres against serovar Ballum, but was apparently uninfected.

DISCUSSION

The prevalence detected in the present survey is higher than reported for wolf populations elsewhere. Very low seroprevalences were found in Alaska (Zarnke et al. 2004) and Scandinavia (Akerstedt et al. 2010). In Minnesota, about 10% of wolves were seropositive (Khan et al. 1991). Warmer winters in northwestern Spain, as compared to these northern locations and relatively wet summers, probably enhance the survival of spirochetes in the environment. However, the difference observed with previous studies might only be a result of the convenience sampling used in this study compared with samples obtained from live-trapped animals in all other studies quoted (Khan et al. 1991, Zarnke et al. 2004, Akerstedt et al. 2010).

Our study reveals, for the first time, active *Leptospira* infection in wolves. It is noteworthy that seroprevalence was only slightly higher than prevalence of active infections, whereas previous studies in wild carnivores using both techniques showed that this difference is usually marked (Millán et al. 2009, Moinet et al. 2010). The lack of such differences in the present study may result from a poor performance of the serological test due to the use of tissue exudates instead of serum. Alternatively, discrepancies may also indicate a short life of circulating antibodies in wolves or a low degree of seroreactivity. In fact, antibody titres were low (1:100) in four wolves that were concurrently infected. In addition, titres below the cut-off level were detected in other cases, suggesting the existence of chronic infections in wolves that could be acting as renal carriers (Table).

Our results confirm wolf exposure to leptospires of different origins. Dogs are considered the natural host for serovar Canicola (André-Fontaine 2006), though other species can be found infected by this serovar (García et al. 2013). Given the genetic proximity between wolves and dogs, it is not surprising that this was the most prevalent serovar detected in the present survey. However, this was not the case in the study by Khan et al. (1991) in Minnesota, where this serovar was only the fourth most frequently detected. These differences may reflect the fact that Iberian wolves live in a more anthropised environment (Llaneza et al. 2012) and that wolves occurring in human-dominated landscapes may be frequently exposed to canine pathogens.

The main reservoirs for the serovars Icterohaemorragiae, Sejroë and Ballum are rodents (Turk et al. 2003, André-Fontaine 2006). No information is available about leptospiral infections in rodents in the study areas. In a nearby region in western Spain, the most common serovar found was Ballum (García et al. 2013). The abovementioned serovars were almost absent in wild ungulates in Asturias, as recently published (Espí et al. 2010). Therefore, wolves may come into contact with these serovars after consumption of rodents or perhaps lagomorphs, as suspected in other studies analysing wild carnivores in Southern Europe (Millán et al. 2009, Moinet et al. 2010). However, the proportion of these food items is very low or absent in the diets of wolves from the study areas (Llaneza et al. 1996, López-Bao et al. 2013). An alternative hypothesis may be that the wolves became infected after drinking from rodent-contaminated water sources or from consuming other preys or carrion of ungulates that can occasionally be infected by these serovars (Slavica et al. 2008, Espí et al. 2010).

Serovar Grippotyphosa, also detected in the present survey, was the most seroprevalent in wolves from Minnesota, representing one in four positive cases (Khan et al. 1991). Antibodies against this serovar were among the most frequently detected in the survey of wild ungulates in Asturias by Espí et al. (2010), especially in fallow deer (*Dama dama*) and wild boar (*Sus scrofa*). Antibodies against serovar Grippotyphosa were also relatively prevalent in cattle farms in Galicia and Asturias (Espí et al. 2000, Guitián et al. 2001). Thus, wolves probably come into contact with this serovar after consumption of wild or domestic ungulates or their products.

The disease significance of infection with leptospires for wolves is unknown. Lesions (chiefly chronic interstitial nephritis) associated with leptospiral infection have been recorded in other species of wild carnivores (Millán et al. 2009). In dogs, leptospires can cause hepatic and renal failure and, occasionally, death (Greene et al. 2012). It is generally assumed that serovars Icterohaemorrhagiae and Canicola are responsible for the acute forms of canine leptospirosis. However, typical acute leptospirosis can also be induced by strains belonging to other serogroups, including Grippotyphosa (André-Fontaine 2006).

In conclusion, our survey revealed infection and exposure in wolves with serovars maintained by different reservoirs such as dogs, rodents and ungulates, confirming the usefulness of the wolf and other large predators as an environmental sentinel for leptospires and probably other environmental pathogens.

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