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Abstract

Wild boar (Sus scrofa) is considered a potential source of several viral and bacterial pathogens that represent a risk to humans and other mammals. Among these, the spirochete of the genus Leptospira causes Leptospirosis, a neglected zoonotic disease. This study investigates the presence of antibodies against pathogenic Leptospira spp. serovars in wild boar in different areas of Lombardy region (northern Italy) and the risk factors associated with its presence in a specific population. Blood and tissue samples from wild boars were collected from 2008 to 2013 during a wildlife survey. A total of 2101 serum samples were analysed using a microscopic agglutination test (MAT) to detect antibodies against Leptospira interrogans sensu lato. Culture isolation and Leptospira DNA detection by PCR were carried out using 189 kidney and 159 urine samples, respectively. Antibodies against 5 serovars were detected in 321 serum samples (15.3%). Bratislava was the most frequently identified serovar (14.6%; 95% C.I. 13.1–16.2%), followed by Copenhagen (1.48%; 95% C.I. 1.0–2.1%), Grippotyphosa and Pomona (0.48%; 95% C.I. 0.23–0.87%), and Canicola (0.05%; 95% C.I. 0–0.3%). Genotyping by multilocus sequence typing and multilocus variable number tandem repeat analysis of a single leptospire isolate confirmed the presence of L. interrogans serovar Bratislava with the same genetic profile as Jez Bratislava. The statistical analyses confirmed the wild boar’s age class as an important risk factor for the seroprevalence of leptospirosis, whereas no effect of wild boar abundance on seroprevalence was observed. In addition, an increasing seroprevalence was observed, in particular that of Australis Bratislava showed a general increasing pattern over the years. Our results confirmed that wild boars are a potential source of pathogenic Leptospira spp., which can infect humans, domestic animals and other wild animal species in low-density regions, such as those on the Alps.

Introduction

Leptospirosis is a re-emerging and widespread public health problem, caused by pathogenic serovars of Leptospira spp. It has a wide distribution and occurs in tropical, subtropical and temperate zones, favoured by a large variety of both wild and domestic mammals that can play the role of natural carriers of leptospires (Faine et al., 1999). Through urine shedding, Leptospira spp. are spread through the environment and can survive for long periods of time, causing contamination of surface water, soil and muddy areas. Among wildlife species, small rodents are considered to be the most common carriers and reservoirs of the infection (Hartskeerl and Terpstra, 1996). The transmission of leptospires to other mammals, including humans, is due to direct contact with the urine of infected animals, but also through indirect contact with leptospires in the environment (Levett, 2001; Cvetnic et al., 2003). The general increase in the incidence of leptospirosis in some geographical areas is related to climatic and ecological changes, as well as changes in agriculture and farming practices that can influence wildlife population dynamics (Vijayachari et al., 2008; Hartskeerl et al., 2011; WHO, 2011).

Wild boar (Sus scrofa) is a known animal host of Leptospira spp., and it is considered a potential source of leptospires that then infect humans and domestic animals. An increase in the population density of wild boars has been documented in many European countries, including Italy (Vicente et al., 2002; Ebani et al., 2003; Vengust et al., 2008; Massei et al., 2015; Pedersen et al., 2015; Vale-Gonçalves et al., 2015; Žmudzki et al., 2016). The high dispersion rate of this species and the consequential increase in potential interactions (both direct and indirect) among wild boars, humans, domestic animals and other wildlife species could increase the dissemination risk of such a disease (Jansen et al., 2006, 2007; Ruiz-Fons, 2015). In fact, this species seems to be a potential transmission source of pathogenic leptospires to other mammal species that share the same geographical areas, thereby, playing an important role in the epidemiological cycle of leptospirosis (Vale-Gonçalves et al., 2015).

Through the long term surveillance of specific alpine hunting areas in Northern Italy, and by applying serological, microbiological and molecular testing, our goals were to describe the temporal dynamics of Leptospira interrogans sensu lato infections in free-ranging wild boar populations and define the host risk factors associated with its presence.

Materials and methods

Sampling

A total of 2101 sera samples were collected during five hunting seasons (from 2008–09 to 2012–13) from hunted free-living wild boar in eight hunting districts in the Province of Brescia (45°38′ N, 10°18′ E), Northern Italy (Fig. 1). The hunting districts are characterized by a footstep mountain habitat where wild boar is completely free-living and is not specifically managed for hunting. The numbers of sera samples collected during each hunting season were 519, 434, 420, 373 and 355,
respectively. The age of the animals was determined based on tooth eruption patterns (Sáez-Royuela et al., 1989): individuals were considered “young” at < 12 months of age, “sub-adult” at 13–24 months of age and “adult” at > 24 months of age. Tested sera were obtained from “young” (n=417), “sub-adult” (n=578) and “adult” (n=958) wild boar (148 not recorded), and the sex composition was 989 males and 1055 females (57 not recorded). The blood samples were taken immediately after hunting, and the serum was obtained by centrifugation and then stored at -20 °C until analysed at the National Reference Center for Animal Leptospirosis (NRCL) located at the Istituto Zootecnico Sperimentale Lombardia ed Emilia Romagna (IZSLER) in Brescia.

During the hunting seasons from 2008–09 to 2012–13, kidneys and urine samples from 245 wild boars were collected after necropsy and immediately processed and analysed.

**Serological test**

Sera were examined using the microscopic agglutination test (MAT) (OIE, 2008) for antibodies against a reference panel of the eight most representative *Leptospira* (*L. interrogans* s.l.) serogroups in Italy: Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, Sejroe and Tarassovi. For each one, the following serovars were used: Bratislava, strain Riccio 2; Ballum, strain Mus 127; Canicola, strain Alarik; Grippotyphosa, strain Moskva V; Copenhageni, strain Wijnberg; Pomona, strain Pomona; Hardjo, strain Hadjoprajitno; Tarassovi, strain Mitis-Johnson. Samples showing titres $\geq 40$ were repeated.

**PCR detection**

DNA was extracted from 0.5–4.0 ml of centrifuged urine samples using the PureLink Genomic DNA kit (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. A Taqman-based PCR assay targeting the lipL32 gene was used to detect pathogenic leptospires with primers described previously (Stoddard et al. 2009). The PCR was performed in a 25-µl final volume, using 5 µl of extracted DNA, 5 µl of 5× Mastermix Quantfast (Quantifast Pathogen + IC Kit, Qiagen, Hilden, Germany), 2.5 µl of 10× Internal Control assay, 700 nM of primers and 200 nM of the probe. The assay was performed on a BIO-RAD CFX96 System with the following thermal conditions: a holding stage of 95 °C for 5 min, and 45 cycles of 95 °C for 15 s and 60 °C for 30 s. Samples with Ct lipL32 $< 35$ were considered positive and those with $35 \leq Ct_{lipL32} \leq 40$ were repeated.

**Isolation and typing**

Isolation was performed using Ellingham-Hausen-McCullough-Johnson-Harris (EMHJ) semisolid selective medium (Ellingham and McCullough, 1965) and 10% homogenate of kidney samples. Incubation of 3 ml of medium with 0.3 ml of the homogenate was done at 30 ± 1 °C. Cultures were checked weekly, for up to 3 months, using dark field microscopy.

Multilocus sequence typing (MLST). To genotype the *Leptospira* isolate, we used a previously published MLST scheme based on the amplification of seven housekeeping genes (mreA, pfkB, pntA, sucA, tpiA, caiB, and glmU) (Boonslip et al., 2013). These loci were amplified using KAPA2G Robust HotStart PCR kit (Kapabiosystems Resnova, Roma, Italy) in a 25 µl total volume with 0.4 µM each primer, 5 µl DNA and MgCl2 at the following concentrations: 1.5 mM mreA, pfkB, pntA, caiB, glmU; 2.5 mM sucA; 3.5 mM tpiA. Temperature cycling was performed as follows: 1 cycle at 95 °C for 15 min, 35 cycles of amplification with 1 cycle consisting of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C, followed by a final elongation at 72 °C for 10 min. PCR products were purified using the NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). Cycle sequencing reactions were performed using the BigDye® Terminator Cycle Sequencing kit version 1.1 (Applied Biosystems, Foster City, CA, USA). Reactions were filtered through SigmaSpin™ and PCR reaction Cleanup Columns (Sigma, St. Louis, Mo, USA) and sequenced on an ABI PRISM 3130 Automated Capillary DNA Sequencer (Applied Biosystems) according to the manufacturer’s instructions. Nucleotide sequences were assembled with the Lasergene sequencing analysis software package (DNASTAR, Inc., Madison, WI, USA). Assembled sequences were trimmed and aligned to reference sequences downloaded from the leptospira.mlst.net website to assign allele numbers to all seven loci. For strain identification, allelic profiles were queried against the Leptospira MLST database.

**Variable-Number Tandem-Repeat (VNTR) analysis**

Five discriminatory loci (VNTR-4bis, VNTR-7bis, VNTR-10bis, VNTR-Lb4 and VNTR-Lb5) were used to characterize the isolate as described by Salaün et al. (2006). A total of 5 µl of DNA was added to 20 µl of the KAPA2G Robust HotStart PCR kit (Kapabiosystems Resnova) reaction mixture, which contained 10 pmol of each primer. The PCR was carried out at 95 °C for 15 min and by 35 cycles in three steps: 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min. An additional extension for 10 min at 72 °C was added to the end of the run. The PCR products were analysed on 1–2% agarose gel stained with ethidium bromide, and the molecular weights were estimated by comparison with a 100–bp DNA ladder (Invitrogen).

**Statistical analysis**

Confidence intervals for serovar prevalence were computed using the exact method and a binomial distribution.

Since Australis Bratislava showed the highest seropositive values, a further statistical analysis has been applied to this serovar. A chi-square test was used to assess the variability of Australis Bratislava seroprevalence over the years. The significance level was p<0.05.

A generalized linear mixed model (GLMM), with logit link function and binary data, was applied to the data to determine which factors could significantly influence the seroprevalence of Australis Bratislava. The response variable is the binary outcome for the serovar Australis Bratislava. The response variable is the binary outcome for the serovar Australis Bratislava. The response variable is the binary outcome for the serovar Australis Bratislava. The response variable is the binary outcome for the serovar Australis Bratislava.

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A generalized linear mixed model (GLMM), with logit link function and binary data, was applied to the data to determine which factors could significantly influence the seroprevalence of Australis Bratislava. The response variable is the binary outcome for the serology (Pos=1/Neg=0) whereas age, year of sampling, sex and relative abundance for each hunting district were used as independent variables. For the relative abundance estimation based on the official hunting activity data provided by the local hunting office (data not shown), we assumed similar and constant hunting efforts among the hunting districts and years. For this reasons, we used the total number of wild boar hunted per year as an approximation of the wild boar abundance. To take into account the different sizes of the hunting districts, a relative index of abundance was calculated, scaling the animal abundance to its district’s area, expressed in km², as already described in Chiari et al. (2015). The hunting area was considered a random factor. The significant model was selected through a step-by-step procedure based on the Akaike information criterion (AIC) values. All of the analyses were performed with the software R 3.2.0 (lme4 and AER).
Leptospiral DNA was detected in 27 out of 245 urine samples: 24 male and 2 female (1 not recorded), and 18 “adult”, 5 “sub-adult” and 2 “young” (2 not recorded). MAT results on the sera from five PCR positive samples showed reactivity against Bratislava in four cases and against multiple serovars (Bratislava, Grippotyphosa and Pomona) in one sample. Unfortunately, culturing failed to isolate Leptospira, except for one kidney sample (ID: 259801/1, 2010). The strain was identified as *L. interrogans* serogroup Australis by MLST, showing Sequence Type 24 (ST 24). Using this technique Bratislava, Munchen and Jalna cannot be distinguished. The MLVA analysis of five VNTR loci (4bis, 7bis, 10bis, Lb4 and Lb5) identified a genetic profile (1, 11, 9, 5) that was identical to that of the reference strain Jez Bratislava.

### Discussion

The present study showed an overall antibody prevalence of 15.28% (95% CI: 13.2–16.6), characterized by an increasing trend of seropositivity against the serovar Bratislava, which is identified most often. This confirms the importance of Bratislava as the serovar that is becoming prevalent among wild boar and domestic pigs (Bonotti et al., 2015). Anti-Leptospira antibodies were found both in females and males, and in all age categories, suggesting that *Leptospira* infections among wild boars is endemic in the Lombardy region and reflects the epidemiological situation observed in other Italian regions. In fact, even though data on leptospirosis in wildlife in Italy are limited, seropositivity to *Leptospira* has been demonstrated in wild boar, and different distribution of serovars were observed in different geographic areas. In particular, Bratislava was the most detected serovar in Lombardy (Figarellet et al., 2012) and Emilia Romagna (Tagliabue et al., 1996) and Tuscany (Ebani et al., 2003), Tarassovi in Campania (Montanaro et al., 2010), and Pomona and Grippotyphosa in Sardinia (Piredda et al., 2011).

The titre detected against serovars Pomona, Grippotyphosa and Copenhageni, even if at a low prevalence, indicate that different *Leptospira* serovars are present in the environment. The detection of antibody reactions against different serovars in wild boar indicates that they are susceptible to *Leptospira* strains circulating in the environment and/or in other natural sources, even if there is no evidence of the clinical disease.

In Europe, the distribution of *Leptospira* serovars is not homogeneous, as well as in Italy. Pomona is prevalent in Germany, Croatia, Spain and Poland (Vicente et al., 2002; Cvetnic et al., 2003; Jansen et al., 2006, 2007; Espí et al., 2009; Žmudzki et al., 2016); Bratislava is frequently detected in Sweden (Boqvist et al., 2012); Tarassovi in Slovenia (Vengust et al., 2008) and Northern Portugal (Vale-Gonçalves et al., 2015); Grippotyphosa in the Czech Republic (Treml et al., 2003) and Hardjo in Poland (In Available in other Italian regions. In fact, even though data on leptospirosis in wildlife in Italy are limited, seropositivity to *Leptospira* has been demonstrated in wild boar, and different distribution of serovars were observed in different geographic areas. In particular, Bratislava was the most detected serovar in Lombardy (Figarellet et al., 2012) and Emilia Romagna (Tagliabue et al., 1996) and Tuscany (Ebani et al., 2003), Tarassovi in Campania (Montanaro et al., 2010), and Pomona and Grippotyphosa in Sardinia (Piredda et al., 2011).
increases over time. In the current study, we observed no effect of wild boar abundance on seroprevalence. This could be a consequence of the lower wild boar densities in the study area compared with other areas of Europe. In the study area, even though there has been an increasing trend in the wild boar population in the last decade, wild boar is not specifically managed for hunting (i.e. supplementary feeding) and it is completely free-living (i.e. not restricted to fenced areas). As a consequence, similar to in other central Alpine areas, the population densities are lower and are characterized by a discontinuous and fragmented distribution (Santilli et al., 2013).

The general increase in seropositivity recorded during the five years of the study, underlined by the increase in Bratislava (serogroup Australis) and Copenagheni (serogroup Icterohaemorrhagiae), could be related also to factors such as the habitat and the climate (i.e. small mammal reservoir population dynamics). Further studies are needed to clarify the influence of these variables on the inter-annual variability found in the present study. This knowledge can contribute to understanding the ecology of leptospirosis. Additionally, infections in the kidney renal tubules of reservoir species can persist for months or longer, leading to the spread of Leptospira for a long time period. Susceptible hosts can acquire the infection directly from infected animals, but also indirectly by coming into contact with a contaminated environment. As a consequence, the transmission of leptospirosis is influenced not just by the reservoir population dynamics, but also by abiotic conditions, such as climate and hydrology, that can influence the survival rates of the bacteria outside the host (Birtles, 2012). Based on the limited home range of wild small rodents and hedgehogs (Erinaceus europaeus), which are considered reservoir species (Mori et al., 2015; Birtles, 2012), locally and geographically limited foci of infection could influence the presence of Leptospira in other hosts, such as wild boar. In our study, the relative high prevalence of Leptospira (15.28%), the occasional presence of high antibodies titers, the detection of active excretion in urine samples, and the isolation of the pathogen, could suggest a potential role of wild boar as reservoir of Leptospira. However, further studies are needed to clarify the epidemiological role of wild boar in the transmission of leptospirosis.

In accordance with other authors, our results indicate that wild boars are a potential source of leptospirosis for domestic animals, wild animals and humans, which could be exposed to infected materials. For this reason, the presence of leptosomal serovars should be monitored in wild boar and reservoir species to verify possible changes in their diffusion and to confirm the role of the different species in the epidemiology of the frequently underestimated leptospiral diseases. €%

Table 1 – Antibody titres detected against one or more serovars of Leptospira in 321 wild boar sera samples.

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Serovar</th>
<th>1/100</th>
<th>1/200</th>
<th>1/400</th>
<th>1/800</th>
<th>1/1600</th>
<th>1/3200</th>
<th>?/6400</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australis</td>
<td>Bratislava</td>
<td>120</td>
<td>79</td>
<td>50</td>
<td>28</td>
<td>19</td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>Grippotyphosa</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>Copenagheni</td>
<td>22</td>
<td>9</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pomona</td>
<td>Pomona</td>
<td>9</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Canicola</td>
<td>Canicola</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ballum</td>
<td>Ballum</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sejroe</td>
<td>Hardjo</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tarassovi</td>
<td>Tarassovi</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2 – GLMM results for Australis Bratislava. The best fit (lower Akaike information criterion (AIC)) includes age class and year of sampling as significant variables. The interactions among these variables did not add any useful information (higher AIC values and non-significant p-values), and for this reason they were discarded from the model.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Estimate</th>
<th>Std. Error</th>
<th>Z-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>-2.4652</td>
<td>0.2176</td>
<td>-11.33</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Age (1)</td>
<td>0.3699</td>
<td>0.1793</td>
<td>2.03</td>
<td>0.042</td>
</tr>
<tr>
<td>Age (2)</td>
<td>0.3645</td>
<td>0.1793</td>
<td>2.03</td>
<td>0.042</td>
</tr>
<tr>
<td>Year</td>
<td>0.2485</td>
<td>0.482</td>
<td>5.15</td>
<td>0.001</td>
</tr>
<tr>
<td>AIC</td>
<td>1612.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discarded variables:
- Year x Age (1) - - - 0.967
- Year x Age (2) - - - 0.533

| Overdispersion parameter | 0.9735884 |
| R² approx (*) | 6% |

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