Research Article

Seroprevalence and risk factors of leptospirosis in wild boars (*Sus scrofa*) in Northern Italy

Mario Chiari, Bianca Maria Figarolli, Silvia Tagliabue, Giovanni Loris Alborali, Marco Bertoletti, Alice Papetti, Mario D'Incau, Mariagrazia Zanoni, Maria Beatrice Boniotti*

National Reference Center for Animal Leptospirosis (NRCL), Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna "Bruno Ubertini", via Bianchi 7/9, 25121 Brescia, Italy

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 the 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 to detect antibodies against *Leptospira interrogans sensu lato*. Culture isolation and *Leptospira* DNA detection by PCR were carried out using 244 kidney and 245 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 Copenhageni (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, includ-

ing 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; Żmudzki 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* (*L. interrogans s.l.*) 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 col-
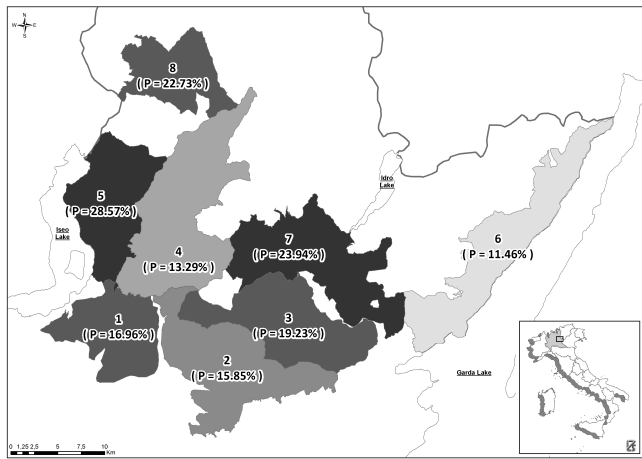
Figure 1 – Geographical distribution of seroprevalence in wild boars in the eight (1–8) hunting districts included in this study.

lected 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 Zooprofilattico 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 ⩾ 1:100 against one or more serovars were considered positive.

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 Quantifast (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 CFX96 System (BIO-RAD, Hercules, California, USA) 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 $35 \leqslant Ct_{lipL32} \leqslant 40$ were repeated.

Isolation and typing

Isolation was performed using Ellinghausen-McCullough-Johnson-Harris (EMJH) semisolid selective medium (Ellinghausen 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) (Boonsilp 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 $MgCl_2$ 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[TM] Post-reaction Clean-Up 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 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 km^2, 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`).
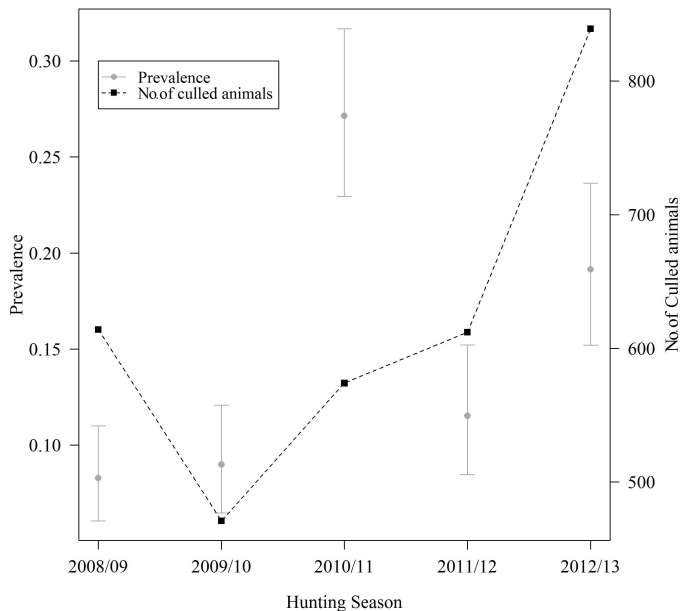
Figure 2 – Number of wild boars culled during five hunting seasons, and the overall prevalence of *Leptospira* spp. during each hunting season.

Results

In total, 321 out of 2101 sera samples were positive for anti-*Leptospira* antibodies (MAT cut-off $\geq$ 100), with an overall prevalence of 15.28% (95% CI: 13.2–16.6). The prevalence values were computed according to sex and age class. In males, the seroprevalence was 15.5% (95% CI: 13.4–17.9), while in females it was 14.8% (95% CI: 12.6–17.1). Seroprevalence values were 12.2% (95% CI: 9.2–15.8), 16.7% (95% CI: 13.7–19.9) and 16.3% (95% CI: 14.0–18.8), in "young", "sub-adults" and "adults", respectively. Seroprevalence varied from 11% and 22% in the eight hunting districts included in this study (Fig. 1).

Among 321 positive samples, 285 (88.8%) were positive against a single serovar, while 36 (11.2%) showed positive titres against multiple serovars. The MAT titres were generally low (1/100 or 1/200) for all of the serovars detected, except for serovar Bratislava, which showed a high antibody titres $\geq$ 1:400) (Tab. 1). Bratislava was the serovar most often identified (14.6%; 95% C.I. 13.1–16.2%), followed by the serovar Copenhageni (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%). No positive serological reactions were found for Ballum, Sejroe or Tarassovi (Tab. 1).

The seroprevalence of Australis Bratislava showed a variable increasing pattern over the years (χ^2, p<0.05), with values increasing from the first season to 2010–11 and then another increasing trend after 2011–12. The recorded seroprevalence was 8.3% (95% CI: 6.1–11.0) in 2008–2009, 9% (95% CI: 6.5–12.1) in 2009–2010, 27.1% (95% CI: 22.9–31.7) in 2010–2011, 11.5% (95% CI: 8.5–15.2) in 2011–2012, and 19.2% (95% CI: 15.2–23.6) in 2012–13 (Fig. 2). A significant difference over the years was also observed for the Copenagheni serovar (p<0.05), with a prevalence equal to 0.96% in 2008–09 and a maximum value of 3.1% in 2012–13. The GLMM, based on Australis Bratislava seropositivity, showed a significant age-dependent increase of 44% underlying the increasing risk of being positive [OR=exp(0.37)=1.44] in "adult" or "sub-adults" subjects compared with younger wild boar (Tab. 2). The variable "year" resulted to be significant, confirming the variation in the prevalence of Australis over the years, as obtained also by the χ^2 test (Tab. 2). No effects attributable to the relative wild boar abundance or sex were detected. The increase due to the age effect was not dependent on the year of sampling since interactions between these terms were not significant.

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 (Boniotti 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 (Figarolli et al., 2012), Emilia Romagna (Tagliabue et al., 1996) and Tuscany (Ebani et al., 2003), Tarassovi in Campania (Montagnaro et al., 2010), and Pomona and Grippotyphosa in Sardinia (Piredda et al., 2011).

The titres 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., 2010; Żmudzki 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 (Żmudzki et al., 2016). A common occurrence in all of these studies is the prevalence of low titres, while high titres are not as frequent. In the present research, the high titres ($\geq$ 1/400) recorded in some animals could indicate recent infections, while low titres (1/100–1/200) may be explained by the wild boar having previous contact with leptospires present in the environment. Active infections, confirmed by the detection of *Leptospira* DNA, were found in 11% of the urine samples. Among these animals, 89% were males, but sample size is not large enough to assess different risk of infection for males than females. However, based on seroprevalence, both genders showed the same probability of having been exposed to *Leptospira*, and no clinical signs or typical lesions were observed in the necropsied animals.

Moreover, PCR positive results in seropositive animals against Bratislava and against multiple serovars, including Bratislava, may reflect an active excretion of leptospirosis through the urine of wild boars. This was supported by the isolation of Bratislava from the kidneys of a sampled animal. Since the identification of serovars is essential for epidemiological studies and the development of appropriate prevention strategies (Faine et al., 1999; Levett, 2001), the strain was characterized as genotype Bratislava by a combined MLST and MLVA approach.

As already reported by other authors (Vale-Gonçalves et al., 2015), and revealed by the GLMM in the present work, age class was an important risk factor for the seroprevalence of leptospirosis, since the possibility of direct or indirect contact with environmental leptospirosis 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 increas-

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

Serogroup	Serovar	Titres						
		1/100	1/200	1/400	1/800	1/1600	1/3200	?1/6400
Australis	Bratislava	120	79	50	28	19	13	5
Grippotyphosa	Grippotyphosa	7	1	2	-	-	-	-
Icterohaemorrhagiae	Copenhageni	22	9	1	-	-	-	-
Pomona	Pomona	9	-	1	-	-	-	-
Canicola	Canicola	-	1	-	-	-	-	-
Ballum	Ballum	-	-	-	-	-	-	-
Sejroe	Hardjo	-	-	-	-	-	-	-
Tarassovi	Tarassovi	-	-	-	-	-	-	-

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 (*) Conditional Coefficient of determination of GLMM (variance explained by both fixed and random factors) (**) Pearson residuals sum of square/residual degrees of freedom.

Australis Bratislava	Estimate	Std. Error	Z-value	P-value
Intercept	-2.4652	0.2176	-11.33	< 0.001
Age (1)	0.3699	0.1948	1.90	0.058
Age (2)	0.3645	0.1793	2.03	0.042
Year	0.2485	0.482	5.15	< 0.001
AIC	1612.2			
R^2 approx(*)	6%			
Overdispersion parameter(**)	0.9735884			
Discarded variables:				
Year x Age (1)	-	-	-	0.967
Year x Age (2)	-	-	-	0.533
AIC	1615.4			

ing 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 and Varuzza, 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 Icterohaemorragiae), 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 prevalance 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 leptospiral 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.

References

Birtles R., 2012. Leptospira Infections. In: Gavier-Widén D., Duff J.P., Meredith A. (Eds.) Infectious diseases of wild mammals and birds in Europe. Wiley-blackwell, Oxford pp. 402–408.

Boniotti M.B., Papetti A., D'Incau M., Tagliabue S., 2015. Leptospiral infections in pigs from Northern Italy during the period 2002–2014. 2nd ELS meeting, Amsterdam p. 87.

Boonsilp S., Thaipadungpanit J., Amornchai P., Wuthiekanun V., Bailey M.S., Holden M.T., Zhang C., Jiang X., Koizumi N., Taylor K., Galloway R., Hoffmaster A.R., Craig S., Smythe L.D., Hartskeerl R.A., Day N.P., Chantratita N., Feil E.J., Aanensen D.M., Spratt B.G., Peacock S.J., 2013. A single multilocus sequence typing (MLST) scheme for seven pathogenic *Leptospira* species. PLoS Negl. Trop. Dis. 7(1): e1954 doi:10.1371/journal.pntd.0001954

Boqvist S., Bergström K., Magnusson U., 2012. Prevalence of antibody to six *Leptospira* serovars in swedish wild boars. J. Wildl. Dis. 48(2): 492–496.

Chiari M., Ferrari N., Bertoletti M., Avisani D., Cerioli M., Zanoni M., Alborali L.G., Lanfranchi P., Lelli D., Martin A.M., Antonio L., 2015. Long-Term Surveillance of Aujeszky's Disease in the Alpine Wild Boar (*Sus scrofa*). EcoHealth 12(4): 563–570. doi:10.1007/s10393-015-1064-x

Cvetnic Z., Margaletic J., Toncic J., Turk N., Milas Z., Spicic S., Lojkic M., Terzic S., Jemersic L., Humski A., Mitak M., Habrun B., Krt B., 2003. A serological survey and isolation of leptospires from small rodents and wild boars in the Republic of Croatia. Vet. Med.-Czech 48(11): 321–329.

Ebani V.V., Cerri D., Poli A., Andreani E., 2003. Prevalence of *Leptospira* and Brucella antibodies in wild boars (*Sus Scrofa*) in Tuscany, Italy. J. Wildl. Dis. 39(3): 718–722.

Ellinghausen H.C. Jr., McCullough W.G., 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: a serum-free medium employing oleic albumin complex. Am. J. Vet. Res. 26: 39–44.

Espí A., Prieto J.M., Alzaga V., 2010 Leptospiral antibodies in Iberian red deer (*Cervus elaphus hispanicus*), fallow deer (*Dama dama*) and European wild boar (*Sus scrofa*) in Asturias, Northern Spain. Vet. J. 183(2): 226–227.

Faine S., Adler B., Bolin C., Perolat P., 1999. *Leptospira* and Leptospirosis. MediSci, Melbourne, Australia.

Figarolli B.M., Gaffuri A., Alborali G.L., D'Incau M., Tagliabue S., 2012. Survey on leptospirosis in wild boars (*Sus scrofa*) in Lombardy, Northern Italy, 1°ELS meeting, Dubrovnik pp. 63–64.

Hartskeerl R.A., Terpstra W.J., 1996. Leptospirosis in wild animals. Vet. Q. 18(3): S149–150.

Hartskeerl R.A., Collares-Pereira M., Ellis W.A., 2011. Emergence, control and re-emerging leptospirosis dynamics of infection in the changing world. Clin. Microbiol. Infec. 17(4): 494–501.

Jansen A., Luge E., Guerra B., Wittschen P., Gruber A.D., Loddenkemper C., Schneider T., Lierz M., Ehlert D., Appel B., Stark K., Nöckler K., 2007. Leptospirosis in Urban Wild Boars, Berlin, Germany. Emerg. Infect. Dis. 13(5): 739–742.

Jansen A., Nöckler K., Schönberg A., Luge E., Ehlert D., Schneider T., 2006. Wild boars as possible source of hemorrhagic leptospirosis in Berlin, Germany. Eur. J. Clin. Microbiol. Infect. Dis. 25(8): 544–546.

Krawczyk M., 2005. Serological evidence of leptospirosis in animals in northern Poland. Vet. Rec. 156(3): 88–89.

Levett P.N., 2001. Leptospirosis. Clin. Microbiol. Rev. 14(2): 296–326.

Massei G., Kindberg J., Licoppe A., Gačić D., Šprem N., Kamler J., Baubet E., Hohmann U., Monaco A., Ozoliņš J., Cellina S., Podgórski T., Fonseca C., Markov N., Pokorny B., Rosell C. , Náhlik A., 2015. Wild boar populations up, numbers of hunters down? A review of trends and implications for Europe. Pest. Manag. Sci. 71(4): 492–500.

Montagnaro S., Sasso S., De Martino L., Longo M., Iovane V., Ghiurmino G., Pisanelli G., Nava D., Baldi L., Pagnini U., 2010 Prevalence of antibodies to selected viral and bacterial pathogens in wild boars (*Sus scrofa*) in Campania region, Italy. J. Wildl. Dis. 46(1): 316–319.

Mori E., Menchetti M., Bertolino S., Mazza G., Ancillotto L., 2015. Reappraisal of an old cheap method for marking the European hedgehog. Mamm. Res. 60(2): 189–193.

OIE. 2008. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Chapter 2.1.9.

Pedersen K., Pabilonia K.L., Anderson T.D., Bevins S.N., Hicks C.R., Kloft J.M., Deliberto T.J., 2015. Widespread detection of antibodies to *Leptospira* in feral swine in the United States. Epidemiol. Infect. 143(10): 2131–2136.

Piredda I., Palmas B., Noworol M., Canu M., Fiori E., Picardeau M., Tola A., Pintore A., Ponti N., 2011. Indagine sulla prevalenza della leptospirosi nei cinghiali del centro-nord Sardegna. Atti del XIII Congresso Nazionale S.I.Di.L.V., Trani, Italy pp. 63–64.

Ruiz-Fons F., 2015. A Review of the Current Status of Relevant Zoonotic Pathogens in Wild Swine (*Sus scrofa*) Populations: Changes Modulating the Risk of Transmission to Humans. Transbound. Emerg. Dis. 2015 May 8.

Sáez-Royuela C., Gomariz R.P., Telleria J.L., 1989. Age determination of European wild boar (*Sus scrofa*). Wildl. Soc. Bull. 17(3): 326–329.

Salaün L., Mérien F., Gurianova S., Baranton G., Picardeau M., 2006. Application of multi-locus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. J. Clin. Microbiol. 44(11): 3954–3962.

Santilli F., Varuzza P., 2013. Factors affecting wild boar (*Sus scrofa*) abundance in southern Tuscany. Hystrix, the Italian Journal of Mammalogy. 24(2): 169–173.

Stoddard R.A., Gee J.E., Wilkins P.P., McCaustland K., Hoffmaster A.R., 2009. Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. Diagn. Microbiol. Infect. Dis. 64(3): 247–255.

Tagliabue S., Raffo A., Foni E., Candotti P., Barigazzi G., 1996. Serologic survey in wild boars (*Sus scrofa*) for antibodies against *Leptospira interrogans* in the Appennines of the Parma province (Italy). Suppl. Ric. Biol. Selv. XXIV: 631–635.

Treml F., Pikula J., Holešovská Z., 2003. Prevalence of antibodies against leptospires in the wild boar (*Sus scrofa* L., 1758). Vet. Med.-Czech 48(3): 66–70.

Vale-Gonçalves H.M., Cabral J.A., Faria M.C., Nunes-Pereira M., Faria A.S., Veloso O., Vieira M.L., Paiva-Cardoso M.N., 2015. Prevalence of leptospira antibodies in wild boars (*Sus scrofa*) from Northern Portugal: risk factor analysis. Epidemiol. Infect. 143(10): 2126–2130.

Vengust G., Lindtner-Knific R., Zele D., Bidovec A., 2008. *Leptospira* antibodies in wild boars (*Sus scrofa*) in Slovenia. Eur. J. Wildl. Res. 54: 749–752.

Vicente J., León-Vizcaíno L., Gortázar C., Cubero M.J., González M., Martín-Atance P., 2002. Antibodies to selected viral and bacterial pathogens in European wild boars from Southcentral Spain. J. Wildl. Dis., 38(3): 649–652.

Vijayachari P., Sugunan A.P., Shriram A.N., 2008.Leptospirosis: an emerging global public health problem. J. Biosci. 33(4): 557–569.

WHO. 2011. Leptospirosis: an emerging public health problem. Weekly Epidemiological Record. 86(6): 45–52.

Żmudzki J., Jabłoński A., Nowak A., Zębek S., Arent Z., Bocian Ł., Pejsak Z., 2016. First overall report of *Leptospira* infections in wild boars in Poland. Acta Vet Scand. 58 3. doi:10.1186/s13028-016-0186-7

Associate Editor: N. Ferrari