

## Detection of *Leptospira* spp. serovars in wild boars (*Sus scrofa*) from continental Croatia

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### ABSTRACT

Over a period of five years (2002-2007) a total of 351 wild boar (*Sus scrofa*) serum samples were collected during regular hunting procedures. All samples were tested by microscopic agglutination test (MAT) for the presence of specific antibodies (AB) to 12 *Leptospira* spp. serovars. At different sera dilutions (ranging from 100 to 3200) 112 samples (31.9%) were found positive to at least one pathogenic serovar, and AB to nine *Leptospira* spp. serovars were detected: Australis, Pomona, Tarassovi, Sejroe, Grippotyphosa, Icterohaemorrhagiae, Ballum, Saxkoebing, and Bataviae. In total we recorded 147 positive reactions of which the majority (N = 101; 68.7%) were found positive at basic dilution (BD = 100), whereas 31.3% (N = 46) reactions were detected positive at higher dilutions. The most frequent serovar was Australis (33.3%), followed by the serovars Pomona (21.8%) and Tarassovi (14.3%), whilst the serovars Australis and Pomona recorded the highest AB titer (3200) and showed statistically significant frequency in cross-reactions (recorded in 23.8% cases). We found significant differences between AB prevalence in different age groups, with the highest distinction between detected positive samples in piglets (21.5%) and adult specimens (50.8%). According to the aerial distribution, the highest percentage of positive wild boar samples originated from lowland habitats in regions of Posavina (Novska - 46.8%, Kutina - 45.3%) and Slavonija and Baranja (Tikveš - 43.6%). Considering the fact that the

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wild boar population in Croatia is showing a constant tendency to grow, further research is indispensable to clarify the role of wild boar in the transmission of leptospirosis to other wild and domestic species.

**Key words:** *Leptospira* spp., wild boar, *Sus scrofa*, serology, Croatia

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## Introduction

Leptospirosis is a widespread zoonotic disease with a broad spectrum of clinical presentations, caused by the spiral bacteria *Leptospira* spp. (LANGSTON and HEUTER, 2003). Pathogenic serovars of *Leptospira* spp. could infect diverse species of domestic and wild animals (ATHANAZIO et al., 2008) and among the wide range of game species wild boars (*Sus scrofa*) have been pointed out as an epidemiological problem in various European countries (ELBERS et al., 2000; DEUTZ et al., 2002; EBANI et al., 2003; KRAWCZYK, 2005; VENGUST et al., 2008). Leptospirosis has also been declared as the most common occupationally acquired infectious disease, as well as very important public health issue (BLACKMORE and SCHOLLUM, 1982), especially for hunters (DEUTZ et al., 2003a), veterinarians and workers in the meat industry (BLACKMORE and SCHOLLUM, 1982; DEUTZ et al., 2003b). Many serovars of the *Leptospira* spp. have adapted to a particular host species (known as the maintenance host) in which an almost symbiotic relationship had been formed (THIERMANN, 1981). Other affected species different than the maintenance hosts could be more resistant to infection (MARSHALL, 1976), but once infected they become more susceptible to disease (these species are known as the accidental hosts). Domestic pigs were known as maintenance hosts for Australis (ELLIS et al., 1986) and Pomona (JANSEN et al., 2007) serogroups, while wild boars were declared as accidental hosts for serovar Grippotyphosa (TREML et al., 2003) and maintenance hosts for serovars Pomona and Bratislava (JANSEN et al., 2007). Because of their genetic relationship to domestic swine, wild boars play an important role in the transmission of leptospirosis among free living and domestic species (KRAWCZYK, 2005) and could be identified as an potential source of infection for domestic pigs (BAKER et al., 1989; TREML et al., 2003; WITMER et al., 2003; JANSEN et al., 2007), as well as humans (VINETZ et al., 1996; DEUTZ et al., 2002; SARKAR et al., 2002; JANSEN et al., 2006).

In Croatia the potential role of wild boars as reservoirs for leptospirosis has been evaluated in a few studies (KOVAČIĆ et al., 1984; BORČIĆ et al., 1989; KOVAČIĆ et al., 2001) and recent research has analyzed the presence of leptospires in wild boars and small rodents (CVETNIĆ et al., 2003), while the latest studies described the incidence of leptospiral antibodies in wild boars from north-west (MILAS et al., 2007) and central (SLAVICA et al., 2008) parts of Croatia. The wild boar is one of the most important large game species in Croatia, whose population density has increased substantially over the last decade. According to data from the Central Bureau of Statistics (ANONYM., 2007) 5986 wild boars were shot in 2000, in 2005 the number of hunted wild boars reached 9827, while in 2006 the number of shot specimens rose to over 10,000. As evidence of the

rising trend, a rapid increase in wild boar hunting quotas in the last two years (17,920 in 2007, and over 25,000 in 2008) has been registered (KONJEVIĆ et al., 2008). Higher wild boar population density has resulted in boar migration to suburban areas and subsequently closer contact with domestic animals and humans. In this study our objective was to detect the prevalence of specific antibodies (AB) to pathogenic serovars of *Leptospira* spp. in the wild boar population from the continental part of Croatia and to assess the potential role of wild boars as reservoirs of leptospirosis in their natural habitats.

### Materials and methods

Blood samples from shot wild boars (N = 351 /164 females/187 males) were collected during regular hunting activities over a five year period (2002-2007). Samples were collected at 34 different locations from the continental part of Croatia (1. Varaždin, 2. Ludbreg, 3. Koprivnica, 4. Đurđevac, 5. Virovitica, 6. Podravska Slatina, 7. Valpovo, 8. Beli Manastir, 9. Tikveš, 10. Kneževo, 11. Osijek, 12. Vinkovci, 13. Županja, 14. Đakovo, 15. Slavonski Brod, 16. Slavonska Požega, 17. Novska, 18. Kutina, 19. Sisak, 20. Vrbovec, 21. Dugo Selo, 22. Pisarovina, 23. Jastrebarsko, 24. Karlovac, 25. Slunj, 26. Vrbovsko, 27. Bjelolasica, 28. Mrkopalj, 29. Fužine, 30. Delnice, 31. Brod na Kupi, 32. Risnjak, 33. Gerovo and 34. Klana), in coordination with field veterinarians, foresters and hunters. The sampling area was divided into five main regions: Podravina (from locations No. 1 to 6); Slavonia and Baranja (from locations No. 7 to 14); Posavina (from locations No. 15 to 21); Kordun (from locations No. 22 to 25) and Gorski kotar (from locations No. 26 to 34). The micro locations were chosen to include all three types of habitats in Croatia (lowland, hilly-karst and karst), with the emphasis on lowland habitats as the most important areas in maintaining leptospirosis (BORČIĆ et al., 1982).

The researched wild boars were classified on the basis of age as: piglets (<1 year old), yearlings ( $\geq 1$  to  $\leq 2$ ) and adults ( $\geq 2$ ). Age groups were determined according to tooth eruption and exchange pattern, as well as characteristics of canines (WAGENKNECHT, 1984). Collection of blood samples was performed aseptically directly from heart of shot animals using a long syringe (POLJIČAK-MILAS et al., 2004), as modification of the method described by SLAVICA et al. (2000). Blood samples were transported to the laboratory within 12 hours and all sera were tested by the microscopic agglutination test (MAT) for 12 leptospiral antigens (serovars: Australis, Pomona, Tarassovi, Sejroe, Grippotyphosa, Icterohaemorrhagiae, Ballum, Bataviae, Saxkoebing, Hardjo, Canicola and Poi). Minimum sera dilution was 100 (BD - basic dilution) and positive samples were examined for particular leptospira serovars up to a final dilution titer of 50% agglutination (MD - maximum dilution). Moreover, the differences between positive reactions and the number of positive samples were noted, due to the high possibilities of cross-reactions (co-agglutinations) among several antigens of *Leptospira* spp. (KRAWCZYK, 2005). If one sample was positive for two or more *Leptospira* antigens, the serovar with the highest titer

(HT) was considered as an indicator of earlier infection (MILAS et al., 2006). The obtained results were analyzed (data distribution, Pearson's  $\chi^2$  test) using the standard statistical package, Sigma Stat for Windows. The value of  $P < 0.05$  was considered significant.

### Results

Out of 351 tested wild boars serum samples 112 (31.9%) were found positive to at least one pathogenic serovar, and the presence of specific AB to nine *Leptospira* spp. serovars were detected: Australis, Pomona, Tarassovi, Sejroe, Grippotyphosa, Icterohaemorrhagiae, Ballum, Saxkoebing, and Bataviae (Table 1).

Table 1. *Leptospira* serovars tested by MAT with percentage of positive reactions and titers

<i>Leptospira</i> spp. serovars	Positive reactions	%	Titer 100	Titer 200	Titer 400	Titer 800	Titer 1600	Titer 3200
Australis	49	33.3	37	5	3	2	1	1
Pomona	32	21.8	25	3	1	1	1	1
Tarassovi	21	14.3	12	4	3	1	1	-
Sejroe	15	10.2	9	3	1	1	1	-
Grippotyphosa	12	8.1	6	3	2	1	-	-
Icterohaemorrhagiae	10	6.8	6	2	1	1	-	-
Ballum	4	2.7	3	1	-	-	-	-
Saxkoebing	2	1.4	1	1	-	-	-	-
Bataviae	2	1.4	2	-	-	-	-	-
Hardjo	0	0	0	0	0	0	0	0
Canicola	0	0	0	0	0	0	0	0
Poi	0	0	0	0	0	0	0	0
Total	147	100	101	22	11	7	4	2
%	100	-	68.7	14.9	7.5	4.8	2.7	1.4

In total we recorded 147 positive reactions, and in 46 (41.1%) of 112 positive sera we detected AB to more than one serovar - ranging from two to four serovars per sample. The majority (N = 101) of all positive serological reactions (N=147) were recorded at BD (68.7%), whereas 22 positive reactions (14.9%) were recorded at dilution 200, 11 positive reactions (7.5%) were recorded at 400, seven positive reactions (4.8%) were recorded at 800, four positive reactions at 1600, and two positive reactions were recorded at dilution 3200. Out of all positive reactions, we found the highest AB frequency ratio for the serovar Australis (49/147, 33.3%), followed by Pomona (32/147, 21.8%) and Tarassovi (21/147, 14.3%). Co-agglutinations between different serovars occurred in 35 sera (31.3%), mostly between serovars Australis and Pomona ( $P < 0.001$ ), followed by serovars Tarassovi, Sejroe, Icterohaemorrhagiae, and Grippotyphosa. No positive

serological reactions were recorded for serovars Hardjo, Canicola and Poi. According to age groups we found the highest difference ( $P < 0.001$ ) between detected positive samples in piglets (34/158, 21.5%) and adult specimens (61/31, 50.8%), while in yearlings we recorded 47 (35.6%) positive sera out of 132 tested samples (Table 2).

Table 2. Number of tested wild boars over the research period (2002-2007) with number and percentage of positive individuals according to age and sex classes

Age class	Tested boars (N)	Positive samples	%	N° of females (F)	Positive F	%	N° of males (M)	Positive M	%
Piglets (<1 year)	158	34	21.5	87	21	24.1	71	13	14.1
Yearlings (1-2 y)	132	47	35.6	68	22	32.3	64	25	39.1
Adults (>2 years)	61	31	50.8	9	4	44.4	52	27	51.9
Total	351	112	100%	164	47	28.6	187	65	34.7

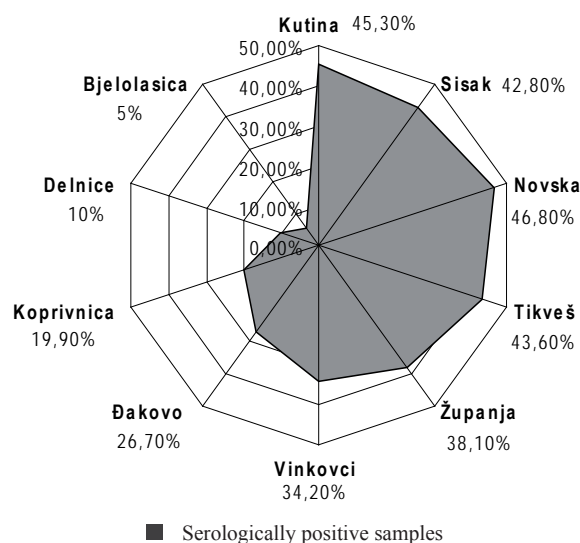


Fig. 1. Percentage of wild boar positive samples according to geographic origin

Comparing the sex classes we recorded no significant differences ( $P > 0.05$ ) in AB prevalence between males (M - 58%) and females (F - 42%), but in the adult group of

animals we found much more positive samples in males (N = 27) than females (N = 4). According to aerial distribution (Fig. 1) the highest percentage of positive wild boar samples originated from lowland habitats in the regions of Posavina (Novska - 46.8%, Kutina - 45.3%), Slavonija and Baranja (Tikveš - 43.6%) and Podravina (Koprivnica - 19.9%), while the lowest percentage of positive wild boars was recorded in the regions of Gorski Kotar - Delnice (10%) and Bjelolasica (5%).

### Discussion

The results of our survey confirmed the presence (31.9%) of specific AB to different serovars of *Leptospira*, proving the fact that these leptospiral agents are present in the wild boar population over most of Croatian territory. In the last 25 years the epidemiological status of wild boars as sources of different leptospiral serovars has changed (SLAVICA et al., 2008) in Croatia, as well as in other European countries (JANSEN et al., 2007). In neighbouring countries the serovars Bratislava (DEUTZ et al., 2002), Grippotyphosa (TREML et al., 2003) and Tarassovi (VENGUST et al., 2008) recorded the most frequent serological reactions. In Croatia past surveillance of leptospiral serovars incidence and distribution in large game species (BORČIĆ et al., 1989) revealed wild boars as the main source of *Leptospira* spp. The most prevalent serovars at that time were Ballum (KOVAČIĆ et al., 1984) and Grippotyphosa (BORČIĆ et al., 1989). Serological surveys conducted in first decade of the 21<sup>st</sup> century revealed Pomona (CVETNIĆ et al., 2003) and Australis (KOVAČIĆ et al., 2001; CVETNIĆ et al., 2003) as the most prevalent serovars. Recent studies have pointed out the Australis serovar (MILAS et al., 2007; SLAVICA et al., 2008) as the most frequently recorded leptospiral serovar in the Croatian wild boar population. The results from this study confirmed the highest AB prevalence (33.3%) and the highest recorded titer (3200) for serovar Australis in wild boars from continental parts of the country. In Croatian lowland areas the epidemiological importance of the serovar Australis was confirmed more than 50 years ago by its isolation from humans (ZAHARIJA, 1955). After that the serovars Australis and Pomona were isolated from four different species of small terrestrial mammals (BORČIĆ et al., 1982) and those four species - *Apodemus agrarius*, *A. sylvaticus*, *A. flavicollis* and *Clethrionomys glareolus* were declared to be maintaining hosts for leptospiral serovars. Small rodents as potential sources of wildlife leptospiral infections with serovars Australis, Pomona, Sejroe and Grippotyphosa have been pointed out by many authors (MODRIĆ and HUBER, 1993; MILAS et al., 2002; TURK et al., 2003; MILAS et al., 2006) and CVETNIĆ et al. (2003) found high prevalence of specific AB for serovar Australis in small rodents, feral pigs ("Turopolje swine") and wild boars in Posavina region. Small rodents and wild boars share the same natural habitats with large fresh water areas, which are the best reservoirs for leptospiral serovars, especially for wallowing wild boars and feral pigs (WITMER et al., 2003; SLAVICA et al., 2008). Carrying and hiding leptospire long-term in the environment in urine is well known for rodents

(MARSHALL, 1976; BORČIĆ et al., 1982; ATHANAZIO et al., 2008), while ELLIS et al. (1986) found leptospire of the Australis serogroup in the urine of farmed domestic swine with an abortion problem. Concerning the fact that wild boars are declared as a possible source of infection for domestic swine (BAKER et al., 1989; MASON et al., 1998; ELBERS et al., 2000; TREML et al., 2003) and humans (VINETZ et al., 1996; DEUTZ et al., 2002; SARKAR et al., 2002; JANSEN et al., 2006) we can conclude that wild boars together with small rodents play an important role in the perpetuity of wildlife leptospiral in Croatia. It is necessary to declare wild boars as maintaining hosts for serovars Australis and Pomona with renal isolation of leptospiral agents, although we can say that our results proved the fact revealed in earlier research (SLAVICA et al., 2008) which pointed out wild boars and foxes as the most important game species in the leptospirosis chain of transmission.

In contrast to VENGUST et al. (2008) who did not find any difference in AB prevalence between wild boar age groups in Slovenia, we found a considerable difference of AB prevalence between piglets (21.5%) and adults (50.8%). Our results are in accordance with TREML et al. (2003) who detected the lowest prevalence in the youngest (<1 year) boars and the highest prevalence in adult wild boars (>2 yrs) from the Czech Republic. Differences in AB prevalence between juveniles and adults have to be expected, because piglets are very probably protected by non specific maternal immunity and have a much shorter period to come in contact with leptospiral antigens than the adult specimens. The higher prevalence of AB detected in older wild boars may indicate previous exposure to leptospire, but not necessarily current infection or reservoir status, which is a problematic issue when we try to assess the role of wild boars in the transmission and maintenance of leptospirosis. Statistical analyses revealed no significant differences in AB prevalence between males (M) and females (F) boars, while differences recorded in the number of specimens (M = 27, F = 4) in the adult group of animals were the result of hunting regulations, which protected females during the maternity period. Other authors (MASON et al., 1998; TREML et al., 2003; VENGUST et al., 2008) also did not record differences in AB prevalence associated with sex classes, but some authors (EBANI et al., 2003; WITMER et al., 2003) found an association between sex and leptospirosis as a consequence of wild boar migration, as males are more migratory than females in the mating period.

According to geographic origin, we found the highest percentage of positive wild boar samples in the lowland habitats of Posavina, Podunavlje and Podravina regions, known as the "historical natural foci" of leptospirosis (BORČIĆ et al., 1982; KOVAČIĆ et al., 1984; MILAS et al., 2002; CVETNIĆ et al., 2003). The highest AB prevalence of *Leptospira* spp. in the wild boar population was observed in the south-western districts of Novska, which are particularly rich in freshwater areas (Veliko and Malo Jezero, Velika, Srednja and Mala Toka) and their western parts are connected with territory of the Nature Park "Lonjsko Polje". Same habitats were already (BORČIĆ et al., 1982; MILAS et al., 2002; CVETNIĆ et

al., 2003) pointed out as primary or "archaic" focal points of leptospirosis, created a long time ago without human influence and moderate in terms of specific climate, hydrological and edaphic factors. On the other hand the lowest percentage of serologic positive wild boars was recorded in the Gorski Kotar region, originating from areas around Delnice and Bjelolasica. In these areas the predominant characteristics of the habitats are hilly-karst terrains with an almost total absence of swamp territory, and such conditions prevent the creation of primary leptospiral foci. Absence of primary foci in the Gorski Kotar region does not exclude the persistence of synanthropic leptospiral foci, which has been registered near garbage dumps (SLAVICA et al., 2008) in the suburban regions of central Croatia. The shift from the sylvatic to synanthropic appearance of wild boars might lead to the enhancement of leptospirosis in grazing species of domestic swine. In order to point out the importance of wild boars as a possible source of infection for domestic animals and humans, further research is needed, especially in lowland habitats of the Posavina and Podunavlje regions characterized by numerous "archaic" foci of leptospirosis.

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**SAŽETAK**

Tijekom perioda od pet godina (2002.-2007.) ukupno je pregledan 351 uzorak seruma divljih svinja (*Sus scrofa*) iz kontinentalnoga područja Hrvatske. Uzorci su prikupljeni tijekom uobičajenih lovnih aktivnosti te su bili pretraženi mikroskopskom aglutinacijom na prisutnost specifičnih protutijela za 12 različitih serovara *Leptospira* spp. Pri različitim razrjeđenjima seruma (u rasponu od 100 do 3200) u 112 uzoraka (31,9%) ustanovljena je pozitivna serološka reakcija na najmanje jedan patogeni serovar. Ukupno smo utvrdili protutijela za devet serovara *Leptospira* spp. kako slijedi: Australis, Pomona, Tarassovi, Sejroe, Grippotyphosa, Icterohaemorrhagiae, Ballum, Saxkoebing i Bataviae. Većinski udio od 68,7% (N = 101) ukupno utvrđenih pozitivnih reakcija (N = 147) registriran je pri osnovnom razrjeđenju (OR = 100), dok je preostali dio 31,3% (N = 46) pozitivnih reakcija zabilježen pri većim razrjeđenjima. Kao najučestaliji serovar u serumu divljih svinja izdvojili smo Australis (33,3%), a potom su slijedili Pomona (21,8%) i Tarassovi (14,3%). Serovarovi Australis i Pomona iskazali su najviši titar protutijela (3200), kao i statistički značajnu učestalost pojavljivanja u "međureakcijama" ko-aglutinacije, koje su zabilježene u 23,8% slučajeva. Utvrdili smo značajne razlike u prevalenciji specifičnih protutijela s obzirom na dobne razrede divljih svinja, tako da je najveća razlika između pozitivnih seroloških reakcija utvrđena pri komparaciji prasadi (21,5%) i odraslih jedinki (50,8%). S obzirom na prostornu raspodjelu najviši postotak pozitivnih uzoraka u kontinentalnoj populaciji divljih svinja ustanovljen je u nizinskim područjima Posavine (Novska - 46,8%, Kutina - 45,3%) te Slavonije i Baranje (Tikveš - 43,6%). S obzirom na činjenicu da brojnost populacije divljih svinja u Hrvatskoj iskazuje tendenciju konstantnog rasta, nužnost daljnjih epidemioloških istraživanja uloge divljih svinja u prijenosu leptospiroze nameće se kao budući imperativ.

**Ključne riječi:** *Leptospira* spp., divlje svinje, *Sus scrofa*, serologija, Hrvatska

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