

***SALMONELLA* IN WILD BOARS (*SUS SCROFA*): CHARACTERIZATION AND EPIDEMIOLOGY**

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The large study on *Salmonella* spp. in the population of wild boars from twelve hunting estates in the South-West Vojvodina, Serbia was conducted with the aim to investigate the prevalence of *Salmonella* spp. in wild boars and to trace *Salmonella* sources. The hunting estates had similar epidemiological characteristics, i.e. lowland regions with an intensive management system of wild boars. The prevalence of *Salmonella* in wild boars was determined and the examination of molecular similarities of strains isolated from wild boars and domestic animals (pigs and poultry from nearby farms) was performed. The total number of 425 wild boars (25.3% of total population), shot on official hunts, were sampled (425 feces and 425 mesenteric lymph nodes samples) and examined by standard ISO protocols. Subtyping of the isolates was performed and compared by Pulsed-field gel electrophoresis (PFGE). The *Salmonella* prevalence in the fecal samples was 3.1% and in the lymph nodes was 0.2%. *Salmonella* Enteritidis was the most dominant serotype. A high molecular similarity was found between *Salmonella* isolates from wild boars and domestic animals. The proximity of communities and domestic animals, as well as improper removal of animal waste were identified as important epidemiological factors which significantly affect the epidemiology of *Salmonella* in wild boars from lowlands.

Keywords: wild boar, *Salmonella*, prevalence, hunting estates

INTRODUCTION

There are many potential wildlife reservoirs of *Salmonella* (game meat species, birds, rodents, foxes) and it is recognized that wild boars carry enteric pathogens more frequently than the wild ruminants [1]. Large differences in the prevalence of different *Salmonella* serotypes in game have been reported between particular regions of Europe,

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from 0-31.8% [2-5], but also between different regions in one country, 1.7-19.3% [6]. In our previous findings from Serbia a prevalence of 1.4% was reported [7]. Apart from some data from Spain on the prevalence of *Salmonella* in individual hunting estates [8,9], there are no other detailed data on comparative analysis of the difference in prevalence between fenced and open hunting estates. Furthermore, information on source tracking of *Salmonella* infection is also lacking.

The aim of this study was to determine the prevalence and distribution of *Salmonella* in wild boar populations in open and fenced intensively managed hunting estates. This was performed by examining the extent of *Salmonella* carriage in wild boars and tracking the source of *Salmonella* infection by examination of molecular similarities of strains isolated from wild boars and domestic animals (pigs and poultry) from nearby farms. By limiting research to a specific geographic region (South-West Vojvodina) which is a lowland region, where hunting estates have intensive opened/fenced management, and domestic animals live in close proximity, the results may be applied to other, geographically similar hunting estates.

MATERIAL AND METHODS

Study area and animals

The Vojvodina region is positioned in the north part of Serbia and with a very flat terrain. The study was carried out in the period between October and December, during two consecutive hunting seasons (2013-2014) as part of a large project on pathogens occurring in wild boars. Research on the prevalence of *Salmonella* was supposed to continue but had to be stopped due to the outbreak of African swine fever [10]. Animals originated from twelve different hunting estates in the region of South-West Vojvodina (10 large fenced and two open areas). Intensive management of wild boars for hunting purposes had been applied in all twelve hunting estates, which included: habitat management which facilitates the preservation of the natural ecosystem, supplementary feeding and intensive predator control. An important epidemiological fact is that all hunting areas are in close proximity to populated communities and farms (<5 km). The exact number of animals in the hunting area is a variable category, so the related data were collected from the hunting estate staff at the time of the hunt. The total number of wild boars in all twelve hunting estates in the course of the study was estimated to 1677 and in total 425 wild boars, shot on official hunts, were sampled (25.3% of the total population).

The sampling procedures and microbiological procedure

The fecal samples from a total of 425 recently shot wild boars were collected in this study. The fecal samples were taken directly from the rectum (n=425) and the mesenteric lymph nodes (n=425) were aseptically excised during evisceration, 850 samples in total. All samples were transported in a cool box at 4°C to the laboratory

within 3 h. Isolation and serotyping of *Salmonella* was performed according to the ISO 6579:2002 (Annex D) [11] and the ISO/TR 6579-3:2014 [12] standards. Confirmation was performed by API 20 *Enterobacteriaceae* (API 20E) strips.

Characterization of isolated *Salmonella* strains

In order to investigate the possible relation between *Salmonella* isolates from wild boar feces (n=13) and domestic animals (n=7) (Table 1), subtyping of the isolates was performed and compared by the Standardized Laboratory Protocol for Molecular Subtyping of *Salmonella* by PFGE method [13], (Figures 1 and 2). The macrorestriction of the genomic DNA was done with the *SpeI* and *XbaI* restriction enzymes. The macrorestriction of the *Salmonella* Braenderup H9812 strain was used as a molecular size standard. The obtained profiles were statistically analyzed by using Ward's linkage of correlation coefficients between PFGE patterns of different genotypes by using the SPSS cluster analysis software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

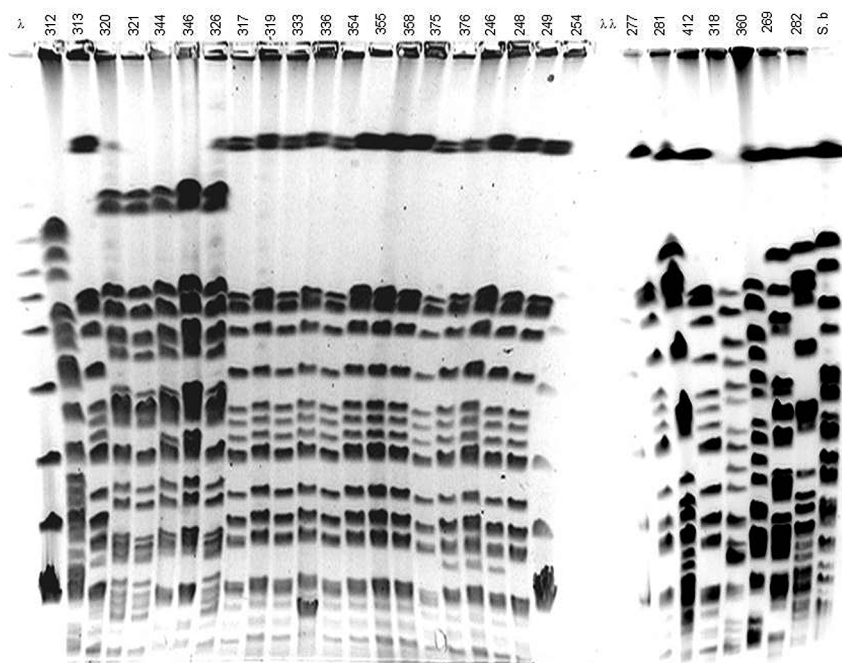


Figure 1. Pulsed-field gel electrophoresis (PFGE) macro-restriction fragment patterns of *Salmonella* sp. digested with *SpeI* enzyme

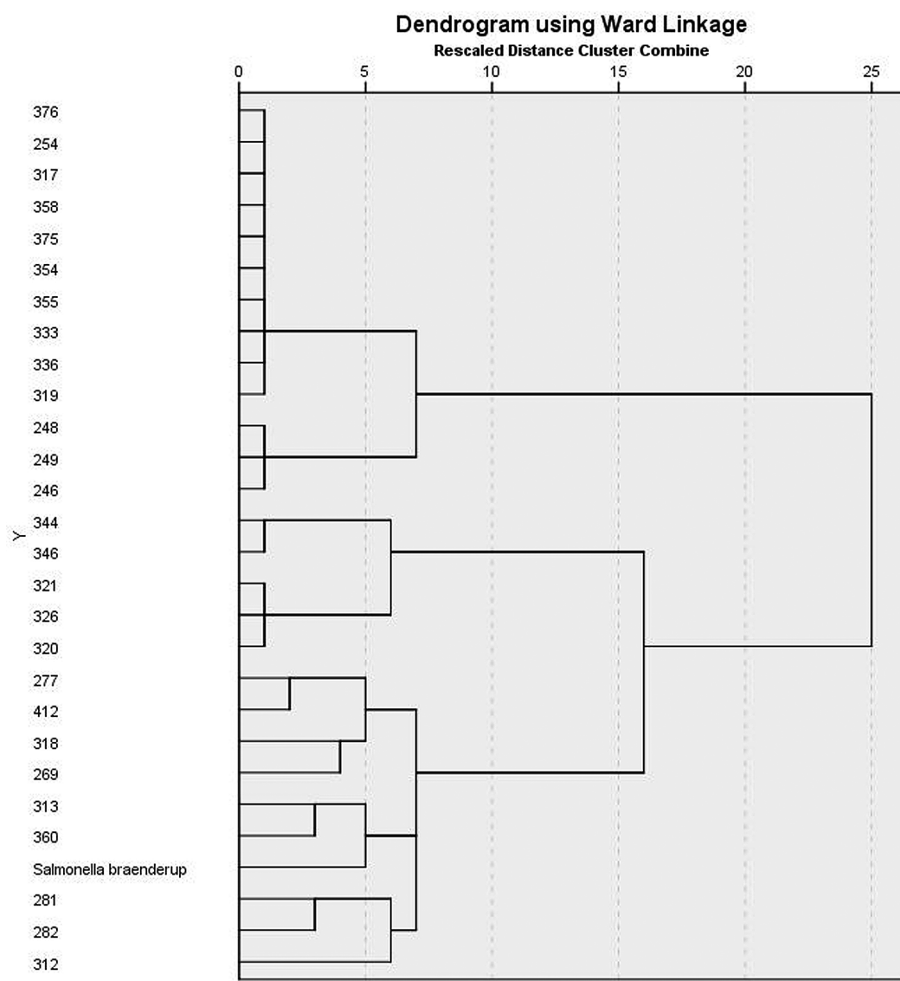


Figure 2. Dendrogram derived from the Ward linkage coefficient of correlation between the obtained PFGE SpeI macro-restriction profiles

Data analysis

The prevalence of *Salmonella*, χ^2 square test and p value were calculated using IBM SPSS Statistics 20 (IBM, Armonk, NY, USA). The results of the statistical tests were considered significant for $p < 0.05$.

Table 1. List of isolates examined by PFGE

Isolate Sign	Sampling site	Hunting estate	Group	Salmonella
312	Feces	C	B	S. Typhimurium
320	Feces	I	B	S. Typhimurium
344	Feces	D	B	S. Typhimurium
346	Feces	D	B	S. Typhimurium
326	Domestic pig	farm	B	S. Typhimurium
317	Feces	G	D	S. Enteritidis
333	Feces	D	D	S. Enteritidis
336	Feces	D	D	S. Enteritidis
354	Feces	D	D	S. Enteritidis
355	Feces	D	D	S. Enteritidis
358	Feces	D	D	S. Enteritidis
375	Feces	E	D	S. Enteritidis
376	Feces	E	D	S. Enteritidis
277	Poultry	farm	D	S. Enteritidis
281	Poultry	farm	D	S. Enteritidis
412	Poultry	farm	D	S. Enteritidis
318	Feces	E	C	S. Infantis
360	Domestic pig	farm	C	S. Infantis
269	Domestic pig	farm	C	S. Infantis
282	Poultry	farm	C	S. Infantis

RESULTS

Prevalence of *Salmonella* spp. in wild boars

As shown in Table 2, the presence of *Salmonella* spp. was confirmed in 13 wild boars' feces samples, with an overall prevalence of 3.1%. *Salmonella* was detected in eight hunting estates (66.7% of the total number), with the prevalence ranging from 1.7% up to 33.3% (in D and K estates). *Salmonella* was detected in only one mesenteric lymph node (0.2% prevalence). *Salmonella* was also detected in the feces of the same animal so this result did not affect overall prevalence. In four hunting estates *Salmonella* was not detected. *S. Enteritidis* was isolated in 1.9% fecal samples, *S. Typhimurium* in 0.9% and *S. Infantis* in 0.2% out of all examined samples. *S. Enteritidis* was isolated in one lymph node (0.2%).

Table 2. Prevalence of *Salmonella* in each hunting estate (HE)

Hunting estates	No of animals in HE per year	Examined animals (% in HE)	<i>Salmonella</i> positive animals/ Prevalence (%)
A	180	63 (35.0)	2 (3.2)
B	160	26 (16.3)	0
C	210	59 (28.1)	1 (1.7)
D	82	12 (14.6)	4 (33.3)
E	340	66 (19.4)	1 (1.5)
F	210	57 (27.1)	1 (1.8)
G	150	48 (32.0)	1 (2.1)
H	220	26 (11.8)	0
I	55	32 (58.2)	1 (3.1)
J	20	10 (50.0)	0
K*	35	15 (21.4)	2 (13.3)
L*	15	11 (73.3)	0
TOTAL	1677	425 (25.3)	13 (3.1)

* “K” and “L” are open hunting estates and all other are fenced areas

Influence of different factors on *Salmonella* findings

Salmonella was significantly more commonly detected in animals from open hunting estates compared to fenced estates ($\chi^2=28.11$, $p=0.00001$). The prevalence of *Salmonella*, with statistically significant higher findings in female animals ($\chi^2=8.25$, $p=0.02$) and the prevalence of *Salmonella* in females heavier than 75 kg and older than 36 months was significantly higher ($\chi^2=8.07$, $p=0.018$) compared to all other categories.

Salmonella characterization

The dendrogram carried out based on the Ward’s linkage correlation coefficient obtained between the *SpeI* (Figures 1 and 2) and *XbaI* PFGE macrorestriction profiles demonstrates the existence of 8 groups of isolates. Two groups of identical isolates were obtained after digestion with the *SpeI* and *XbaI* restriction enzymes: group 358, 354 and 333; group 321, 326 and 320. The four groups of highly related isolates (277 and 412), (318, 269), (317, 246, 248 and 249) and (254, 412 and 277) were also obtained.

DISCUSSION

Prevalence of *Salmonella* spp. in wild boars

A relatively low prevalence (3.1%), such as the one found in this study, was also reported in other published studies, as well as in our previous examinations [7]. Navarro-

Gonzalez *et al.* [4] determined the prevalence in the feces of 5% from wild boars in the suburban area of Barcelona. Somewhat higher values were 22% and 22.8% from fecal samples in Portugal and Italy, respectively [14,15]. In Spain, seroprevalence in wild boars was much higher, 19.3-25.7% [6,8]. Data about the seroprevalence are usually higher than data on the prevalence of isolated *Salmonella*. This has been confirmed from the data from Italy where 66.5% of serum samples were positive for *Salmonella* by ELISA, while microbiological assessment showed that 10.8% of the wild boars had *Salmonella* in their feces [16]. These differences are expected, as the presence of antibodies cannot be directly linked to the presence of the pathogen itself. The seroprevalence is a measure of historical exposure, which may or may not correlate to the microbiological burden at the time of sampling. Thus, serology helps to identify the infected population, but is not useful for determining the infection status of individual animals. Therefore, a combination of serological and bacteriological tests is important for monitoring infection in animal populations [16].

In this study *Salmonella* was detected in two third of sampled hunting estates. The prevalence of *Salmonella* was very variable, in four hunting estates the presence of *Salmonella* was not determined, six had a low prevalence (1-7%) and two hunting estates had a very high prevalence (33.3%). A variable prevalence between estates is in agreement with a previous study from Spain where *Salmonella* was isolated in 12% of the sampled hunting estates, with prevalence ranging from 5% to 33% between estates [9]. Also seroprevalence is variable 28.2-51.8% [6]. These data are in accordance with Gill [17], who also concluded that *Salmonella* has a frequency variation across populations.

Ortega *et al.* [6] speculated that *Salmonella* presence was more common in high density estates in Spain. In Vojvodina, a more noticeable presence of wild boars from open hunting estates was observed around waste sites, where usually a large amount of improperly removed carcasses of domestic animals were dumped (estate "K"). This is directly correlated to statistically significantly higher *Salmonella* prevalence in boars from open hunting estates compared to fenced estates, albeit open estates were usually associated with a low density population (<5 animals/km²).

Based on the findings from our study, the wild boars category mostly at risk of being infected with *Salmonella* were females heavier than 75 kg and older than 36 months. A higher prevalence determined in females was similar to the reports from Wahchek *et al.* [2] and Cano-Manuel *et al.* [8], although Ortega *et al.* [6] did not find significant differences in seroprevalence between male and female animals. While higher prevalence in adults is in contrast to the study by Closa-Sebastià *et al.* [18] where younger animals displayed a higher seroprevalence of *Salmonella* spp. antibodies. However, these authors also found higher seroprevalence in adults for other eleven viral and bacterial pathogens investigated and they concluded that adult wild boars have a higher probability of being exposed to pathogens.

High prevalence in older females fits well with the behavior of female animals, which was observed in the hunting estates in the course of this study. Older females have a great protein demand during the lactation season, they often roam in searching for food and cover large area often expressing scavenging behavior. They feed on corpses of deer, boars, wild birds, rats, domestic animals which contributes to the transmission of pathogens [19].

***Salmonella* epidemiology**

PFGE subtyping of *Salmonella* Typhimurium from wild boars (isolate 320) and domestic pigs (isolate 326), showed that these isolates were identical. A high degree of similarity was also found in *S. Infantis* from wild boars (isolate 318) and domestic pigs (isolate 269). Although *Salmonella* Enteritidis is characterized by low genetic diversity, some similarities between isolates were found. *S. Enteritidis* isolate 254 found in wild boars (estate “K”) is highly related with the ones isolated from poultry farms (isolates 277 and 412). *S. Enteritidis*, *S. Infantis* and *S. Typhimurium* frequently found in domestic animals [20-22].

Hunting estates with the highest prevalence (33.3%) of *Salmonella* were “K” (open estate) and “D” (fenced estate). Sampling of twelve animals in estate “D” was performed during one hunting day and included eight female animals (among which six were older than 36 months), meaning that a half of the shot animals were from the high risk group. A relatively high number of hunted animals that belong to the risk group explains in part the high incidence of *Salmonella* in the hunting estate “D”.

In open hunting areas on mountains, usually lacking a relevant domestic pig population in the area, wild boars act as a true host for *Salmonella*, as well as some other food borne pathogens (*Mycobacterium*, *Leptospira*, *Erysipelotrix*), maintaining an active infection focus and pathogen circulation [8,19]. However, in lowland geographical areas (like in the current study), the situation is different. The identical and highly similar PFGE profiles found in wild boars and domestic pigs and poultry indicate an existence of a molecular and possible epidemiological link. Wild boars may easily come into contact with domestic animals due to the lowland nature of the Vojvodina terrain. In our studies [23,24], it was concluded that some zoonotic pathogens (*Trichinella spiralis*) on the territory of Vojvodina circulate from domestic pigs to wild boars and *vice versa*.

CONCLUSION

This study provides valuable data on the presence of *Salmonella* spp. in the wild boar population from the lowland regions (Vojvodina). The overall *Salmonella* prevalence in wild boars from hunting estates in Vojvodina region was 3.1%, with a dominating serotype being *S. Enteritidis*. Females weighing more than 75 kg (older than 36 months) were in the highest risk category for *Salmonella* carriage.

A very variable prevalence was determined between hunting estates and was particularly high in open hunting estates which directly relates to the proximity of human settlements. High molecular similarity found between *Salmonella* isolates from wild boars and domestic animals indicates a molecular and possible epidemiological link between these animals in lowland regions.

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Authors' contributions

JP carried out literature research, designed the study, did the microbiology, analyzed the results and drafted the manuscript, JM carried out the sampling, data collection and analysis and statistics, JB was involved in microbiology, MM was involved in the sampling and data collection, BB and JPR were involved in data analysis, DA designed study, data analysis and drafted the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SALMONELLA KOD DIVLJIH SVINJA (*SUS SCROFA*): KARAKTERIZACIJA I EPIDEMIOLOGIJA

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U cilju ispitivanja prevalence *Salmonella* spp u populaciji divljih svinja kao i utvrđivanja potencijalnih izvora *Salmonella*, izvršena je studija koja je obuhvatila populaciju divljih svinja iz 12 lovišta na području Jugozapadne Vojvodine, Srbija. Obuhvaćena lovišta imaju slične epidemiološke karakteristike odnosno ravničarski region sa intenzivnim sistemom menadžmenta divljih svinja. Izvršeno je određivanje prevalence salmonella divljih svinja i ispitivanje molekularne sličnosti sojeva izolovanih od divljih svinja i domaćih životinja (svinje i živina iz okolnih farmi). Primenom standardnih ISO metoda, izvršeno je ispitivanje uzoraka poreklom od 425 izlovljenih divljih svinja (25,3% ukupne populacije), odnosno 425 uzoraka fecesa i 425 uzoraka mezenterijalnih limfnih čvorova. Subtipizacija izolovanih sojeva je izvršena metodom elektroforeza u pulsi-rajućem polju (PFGE). U uzorcima fecesa utvrđena je prevalenca *Salmonella* 3,1% a u uzorcim limfnih čvorova 0,2%. Dominantni serotip je bila *Salmonella Enteritidis*. Utvrđene su visoke molekularne sličnosti između salmonella izolovanih kod divljih svinja sa sojevima izolovanim kod domaćih životinja. Epidemiološki, blizina naselja i domaćih životinja kao i nepravilno uklanjanje animalnog otpada su identifikovani kao značajni faktori koji utiču na epidemiologiju salmonella kod divljih svinja u ravničarskim regionima.