Veterinary Research Communications
https://doi.org/10.1007/s11259-022-10017-6

RESEARCH

Epidemiology of Aujeszky disease in wild boars (*Sus scrofa* L.) in Croatia

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Received: 12 June 2022 / Accepted: 7 October 2022

Abstract

Aujeszky disease (AD) or pseudorabies is a viral disease of domestic and wild animals caused by the Suid alphaherpesvirus 1. In wild boar infection usually undergo latent phase but under certain conditions reactivation of the virus can result in a disease. Seroprevalence in wild boars ranges from 0.8 to 100%, and is among other influenced by region, type of management, age and sex of the studied animals. In this study we analyzed blood, lungs, olfactory bulbs and spleen from 222 free-living wild boars from different localities in Croatia and compared results obtained by ELISA with PCR, sex, age and locality. Total seroprevalence was 33.78%, ranging from 25.26% in males to 40.15% in females ($p = 0.0346$; $\chi 2 = 4.47$). According to the age categories prevalence was 10% in offspring, 27.53% in subadults, and 66.75% in adults. Seroprevalence in adult males (66.66%) and females (65.30%) was almost identical. In males, significantly lower seroprevalence was detected in offspring compared to subadults ($\chi 2 = 4.07$, $p < 0.05$) and adults ($\chi 2 = 31.04$; $p < 0.05$), and in subadults compared to adults ($\chi 2 = 15.13$; $p < 0.0001$). Among females, adults had a significantly higher prevalence compared to offspring ($\chi 2 = 19.27$; $p < 0.0001$) and subadults ($\chi 2 = 8.62$; $p < 0.01$). Analysis between counties revealed Sisačko-moslavačka county as a hot-spot for AD. None of the samples was positive for ADV antigens. The observed trend in prevalence points to the fact that the main transmission occurs during one part of the year (most probably the mating season). Also, triggers for virus reactivation might be more complex than previously thought, since none of our samples, collected during the mating and hunting season, was PCR positive. Finally, we can conclude that adult males represent the main transmission link between different wild boar groups.

Keywords Wild boar · Males · Aujeszky disease · Epidemiology

Introduction

In 1813 a new disease named ''Mad Itch'' was observed in the United States. Almost a hundred years later it was described and reproduced by credits of the Hungarian scientist Aladar Aujeszky, and was therefore named Aujeszky disease (Aujeszky 1902). Aujeszky disease (AD), or pseudorabies, is an acute infection of domestic and wild animals caused by the Suid alphaherpesvirus 1 (SuAHV-1), also called the Aujeszky disease virus (ADV) or pseudorabies virus (PRV). Natural hosts and reservoirs of this virus are members of the *Suidae* family (Kit 1999; Mettenleiter et al. 2012; Freuling et al. 2017). However, beside pigs, ADV can infect a wide spectrum of hosts, including rodents, ruminants and carnivores. Following its entrance into the organism, the virus will replicate in the mucosa of the upper respiratory tract, tonsils and the olfactory epithelia (Kit 1999). Further development of the disease depends on the virulence of the viral strain and the age of the affected suids (Card and Enquist 1995; Gortázar et al. 2002; Mettenleiter et al. 2012). If developed, clinical symptoms in pigs are characterized by diarrhea, vomiting, nervous system disorders (such as tremor, ataxia, and lethargy), accompanied with high morbidity and mortality in the case of piglets less than two

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Table 1 Sample structure according to the age and sex of animals

Locality		Total number	Sex		Estimated age category		
			Males	Females	< 1 yr	1–2 yr	> 2 yr
1	Zagrebačka	20	7	13	9	4	7
2	Zagrebačka	38	16	22	6	18	14
3	Sisačko-moslavačka	6	4	2	2	3	1
4	Sisačko-moslavačka	6	2	4	3	3	0
5	Sisačko-moslavačka	8	2	6	1	0	7
6	Sisačko-moslavačka	29	10	19	1	15	13
7	Karlovačka	21	13	8	10	5	6
8	Varaždinska	15	3	12	8	5	2
9	Osječko-baranjska	31	18	13	20	2	9
10	Osječko-baranjska	33	14	19	17	6	10
11	Grad Zagreb	15	6	9	3	7	5
Total		222	95	127	80	68	74

Location number corresponds to numbers on Fig. 2

weeks old, and manifesting as dyspnea and retarded growth in fattening pigs, and reproductive disorders in adults. In wild boars the infection is usually asymptomatic, but occasionally can include clinical signs of the nervous system and weight loss, mainly in younger age categories (Gortázar et al. 2002; Schulze et al. 2010). In the case of prolonged infections or low virulence strains, the virus can establish a lifelong latent infection (Vannier 1986) that may be reactivated under special circumstances. Beside the potential implications for domestic pig production, the role of AD for conservation of endangered carnivores such as the Iberian wolf, Eurasian and Iberian lynx, European brown bear, as well as for hunting dogs, has been discussed (Zanin et al. 1997; Banks et al. 1999; Cay et al. 2009; Keros et al. 2015). In Croatia, AD in hunting dogs was rarely reported (Župančić et al. 1992; Keros et al. 2015). Active surveillance on the National level revealed yearly fluctuations in the number of seropositive domestic pigs. Seroprevalence showed a progressive increase from 2018 (2.04% of positivity) to 2019 (3.16%), reaching a peak of 6.82% in 2020. After that, however, the prevalence dropped drastically to 0.40% in 2021. Seroprevalence in wild boars ranges from high in Mediterranean countries to low in Central Europe and Scandinavia. In the Republic of Croatia, two studies have reported seroprevalence of 54.5% in the area of Moslavačka Mountain (Župančić et al. 2002) and 38.5% in wild boars collected at 4 different localities (Roić et al. 2012).

The aim of this cross-sectional study was to detect the seroprevalence and presence of the ADV sequences (using PCR) in different age categories of wild boars originating from different localities, and to compare them using epidemiological methods.

Materials and methods

Sampling

Study was performed during the winters of 2018, 2019 and 2020. Blood, olfactory bulbs, parts of the cranial lung lobe and spleen were collected from 222 free-living wild boar (Table 1). The sample size was estimated using a formula for prevalence detection.

$$n = \frac{z^2 P\exp\sqrt{1 - P\exp}}{d^2}$$

(Thrusfield 2007), with CI at 95% (z), expected prevalence of 50% (*P* exp), and 0.05 error margin (d), and adjusted for a known population based on the basic (spring) fund of wild boars (n_{adj}= (N x n)/(N+n)) in the respective hunting grounds. Presumptive numbers of wild boars in spring stock are obtained from Central Hunting Record (https://sle.mps.hr). Expected prevalence of 50% was chosen based on the previous results (Župančić et al. 2002; Roić et al. 2012). Samples were collected via non-probability convenient sampling (through regular execution of game management plans). Sampling was conducted in 10 different hunting grounds and one Nature Park, belonging to 6 counties, with a total sampling area of 101,817 ha. Such selection of localities enabled inclusion of different habitats, from the point of quality (based on the evaluation of five factors (food and water, vegetation, soil characteristics, disturbance, general quality) necessary to sustain wild boar population, and altitude (lowland, hilly and mountain) (Grubešić et al. 2006). Lowland habitats are in altitudes between 0 and 200 m.a.s.l. (Zagrebačka, part of Sisačko-moslavačka, Osječko-baranjska and Varaždinska county), hilly habitats are from 200 to 800 m.a.s.l. (Grad Zagreb, part

Table 2 Primers and probes used for the real-time PCR analysis and generation of genome fragments of ADV

Primer/probe name	Orientation 5'-3'	Target gene	Amplicom (bp)	Reference
gB1_F	ATGGCCATCTCGCGCTGC	gB gene	334 bp	Mengeling et al. (1992); Ruiz-Fons et al. (2007)
gB1_R	ACTCGCGGTCCTCCAGCA			
gB2_F	ACGGCACGGGCGTGATC		195 bp	
gB2_R	GGTTCAGGGTACCCCGC			
gB785_TM	ACGTCATCGTCACGACC	gB gene	94 bp	Ma et al. 2008
gB718_F	ACAAGTTCAAGGCCCACATCTAC			
gB812_R	GTCYGTGAAGCGGTTCGTGAT			
gE708_TM	TTCGACCTGATGCCGC	gE gene		
gE694_F	CTTCCACTCGCAGCTCTTCTC			
gE765_R	GTRAAGTTCTCGCGCGAGT			

F: forward; R: reverse orientation; TM: TaqMan probe

of the Sisačko-moslavačka county) and mountain habitats are above 800 m.a.s.l. (Karlovačka county). The ages of the wild boars were estimated using body weight characteristics, tooth eruption pattern, dental wear and canine characteristics in the case of males, with subsequent categorization into offspring (< 1 year), subadult (1–2 years) and adult (> 2 years) classes, in order to minimize potential bias related to age estimation (Briedermann 2009; Merta et al. 2015).

Immediately after capture, blood was sampled directly from the heart or large blood vessels. All collected samples were properly labelled and stored at + 4 ˚C for up to maximum 24 h until analysis. Study was approved by the Committee for Ethics in Veterinary Medicine, Veterinary Faculty University of Zagreb (Class: 640-01/20 – 17/02; No.: 251-61-44-20-11).

ELISA test

The collected sera were analyzed using a commercial ELISA test (IDEXX ADV/ADV gI AB test) in accordance with the manufacturer's instructions. The test is based upon the detection of specific antibodies to the glycoprotein I (gI) of pseudorabies virus. The specificity of the test is 99.9%, whereas the sensitivity of the test was shown by the positive reaction of reference sera's even in dilutions of 1:10, while dilutions of 1:14 were defined as suspect reactions. The 1:8 dilution (S/N value was 0.54, positive) satisfies the EU standard requirement. Post challenge, antibodies were detectable 7 to 9 days after virus inoculation. The microtiter intraplate percent coefficient of variation (%CV) was 3.92–4.87%. On the basis of the recommended cut-off value (0.70) test results were presented as positive or negative. No suspect reactions were observed.

Molecular analysis – real-time and nested polymerase chain reaction (nested PCR)

Samples of the olfactory bulbs, lungs and spleen were homogenized by a Microdismembrator device (Sartorius, Gottingen, Germany). Prepared supernatants were used for DNA extraction using QIAmp Mini (250) Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. DNA amplification was carried out in a Gene Amp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, USA) using Platinum Taq Polymerase Kit (Invitrogen, Carlsbad, USA). As described by Mengeling et al. (1992) and Ruiz-Fons et al. (2007), we used two sets of primers to amplify a fragment within the highly conserved gB gene. Primers and probes used for PCR analysis and generation of genome fragments of ADV are shown in Table 2.

Statistical analysis

Differences in positive results among males and females were analyzed using the $\chi 2$ test, the Fisher exact test, and the McNemar $\chi 2$ test. The border value of the $\chi 2$ test with 1 df at the level of 5% was 3.842. Data were analyzed using Statistica 14.0.0.15 (TIBCO Software Inc. 2018). Odds ratio was calculated using free MedCalc online calculator.

Results

The total sample consisted of 95 males and 127 females, of which 80 were categorized as offspring, 68 as subadults and 74 as adults. The results of the ELISA test are presented in Fig. 1. Serological analysis revealed total seroprevalence of 33.8% positive animals. According to the sex, the prevalence ranged from 25.2% in males to 40.2% in females, which is statistically significant ($p = 0.0346$; $\chi 2 = 4.47$). This gives an odds of a 1.9 times higher possibility that females will be positive compared to males (OR = 1.9852, CI 95% 1.1080–3.5568). Tables 3 and 4 show an analysis of ELISA results in accordance with the age and sex of the wild boars. According to the age categories prevalence was 10% in offspring (7.5% in males and 12.5% in females), 27.5% in subadults (16.2% in males and 36.8% in females), and 65.75% in adults (66.6% in males and 65.3% in females) (Fig. 1).

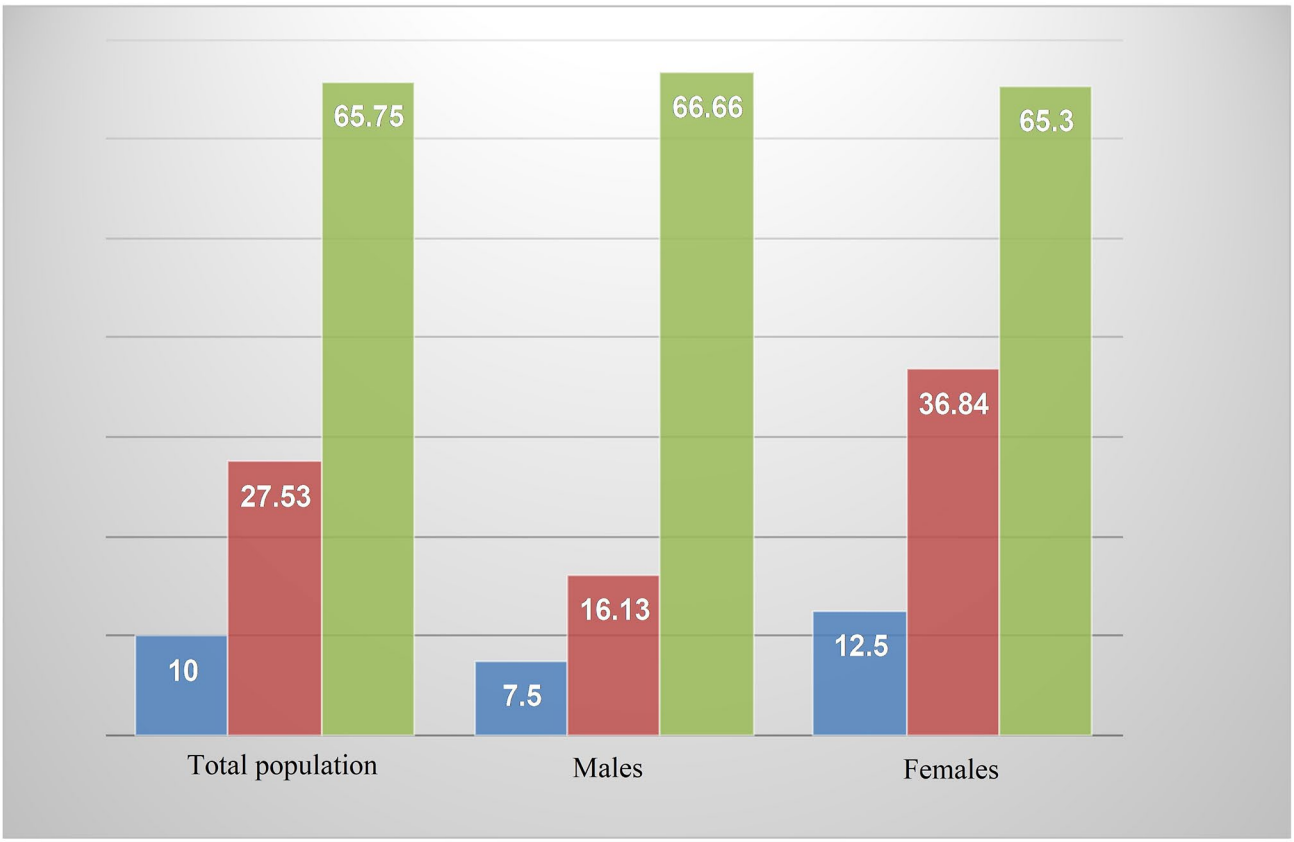

Fig. 1 Seroprevalence against ADV in wild boars, blue columns represent piglets (< 1 year), red columns subadults (1–2 yrs) and green columns adults (> 2 yrs)

Table 3 Analysis of differences in results obtained by ELISA test among age categories (numbers in bold indicate statistically significant difference, $p < 0.05$)

Age	Expected frequencies of seropositive animals (%)		Observed frequencies of seropositive animals (%)		χ^2 value	p
	Males	Females	Males	Females		
offsprings	2.95	3.05	1.64	8.20	3.11	0.078
subadults	7.20	9.80	8.48	20.34	1.64	0.20
Adults	13.71	30.30	24.59	47.54	0.64	0.4245
Total	**27.76**	**39.24**	**11.60**	**25.41**	**4.47**	**0.0346**

Table 4 Analysis of differences between results obtained by ELISA test among sexes (numbers in bold indicate statistically significant difference, $p < 0.05$)

	Males				Females			
	Expected frequencies	Observed frequencies	χ^2 value	p	Expected frequencies	Observed frequencies	χ^2 value	p
offsprings	**5.92**	**1.79**	**4.07**	**0.0436**	12.45	7.81	2.83	0.0922
subadults	**4.78**	**8.93**			14.11	18.75		
offsprings	**19.84**	**2.00**	**31.04**	**0.0000**	**19.68**	**6.94**	**19.27**	**0.0000**
adults	**12.16**	**30.00**			**27.55**	**40.28**		
subadults	**25.83**	**11.36**	**15.13**	**0.0001**	**24.13**	**15.79**	**8.62**	**0.0033**
Adults	**19.63**	**34.09**			**29.81**	**38.16**		

A statistical difference was observed between age categories within sexes separately, between all categories in males, while in females it was only observed in the category of adults. In males, significantly lower seroprevalence was detected in offspring compared to subadults ($\chi 2 = 4.07$, $p < 0.05$) and adults ($\chi 2 = 31.04$; $p < 0.05$), and in subadults

Table 5 Odds of being positive according to the age and sex category

Category	Comparison	Odds ratio	CI 95%
Offspring's	♂ vs. ♀	0.5676	0.1261–2.5542
	♀ vs. ♂	1.7619	0.3915–7.9291
Subadults	♂ vs. ♀	0.3297	0.1031–1.0539
	♀ vs. ♂	3.0333	0.9489–9.6967
Adults	♂ vs. ♀	1.0625	0.3783 – 2.9840
	♀ vs. ♂	0.9412	0.3351–2.6432
Total	♂ vs. ♀	0.5037	0.2812 – 0.9025
	♀ vs. ♂	1.9852	1.1080–3.5568

compared to adults ($\chi2 = 15.13$; $p < 0.0001$). Among females, adults had a significantly higher prevalence compared to offspring ($\chi2 = 19.27$; $p < 0.0001$) and subadults ($\chi2 = 8.62$; $p < 0.01$) (Table 4). Comparing males and females according to the age categories, odds of being positive are slightly higher for females except in the category of adults (Table 5). In the category of subadults the odds ratio indicates that there is 3 times higher odd that a female will be positive compared to a male.

The distribution of positive results according to the geographical location is presented in Fig. 2. The highest prevalence (%) was detected in Sisačko-moslavačka County (55.1, n = 49), followed by Zagrebačka (35, n = 60) and Osječko-baranjska County (28.1, n = 64) (Fig. 3). Analysis between counties with the largest sample size revealed that the prevalence in Sisačko-moslavačka County was significantly higher than in Zagrebačka County ($\chi2 = 4.531$, $p = 0.033$), and Osječko-baranjska County ($\chi2 = 8.428$, $p = 0.0036$). Among counties with a smaller sample size, the highest prevalence (%) was detected on the area of the City of Zagreb (30.8, n = 13), followed by Varaždinska County (20, n = 15) and Karlovačka County (4.8, n = 21).

Along with the blood samples, samples for molecular analysis were collected from each animal. None of the samples was positive for ADV sequences. However, since trigeminal and sacral ganglia were not sampled we leave the possibility that some animals might be positive.

Discussion

In Europe, several serological studies have yielded different prevalence's of antibodies against ADV in wild boars. For example, in Italy seroprevalence ranged from 7.9% in

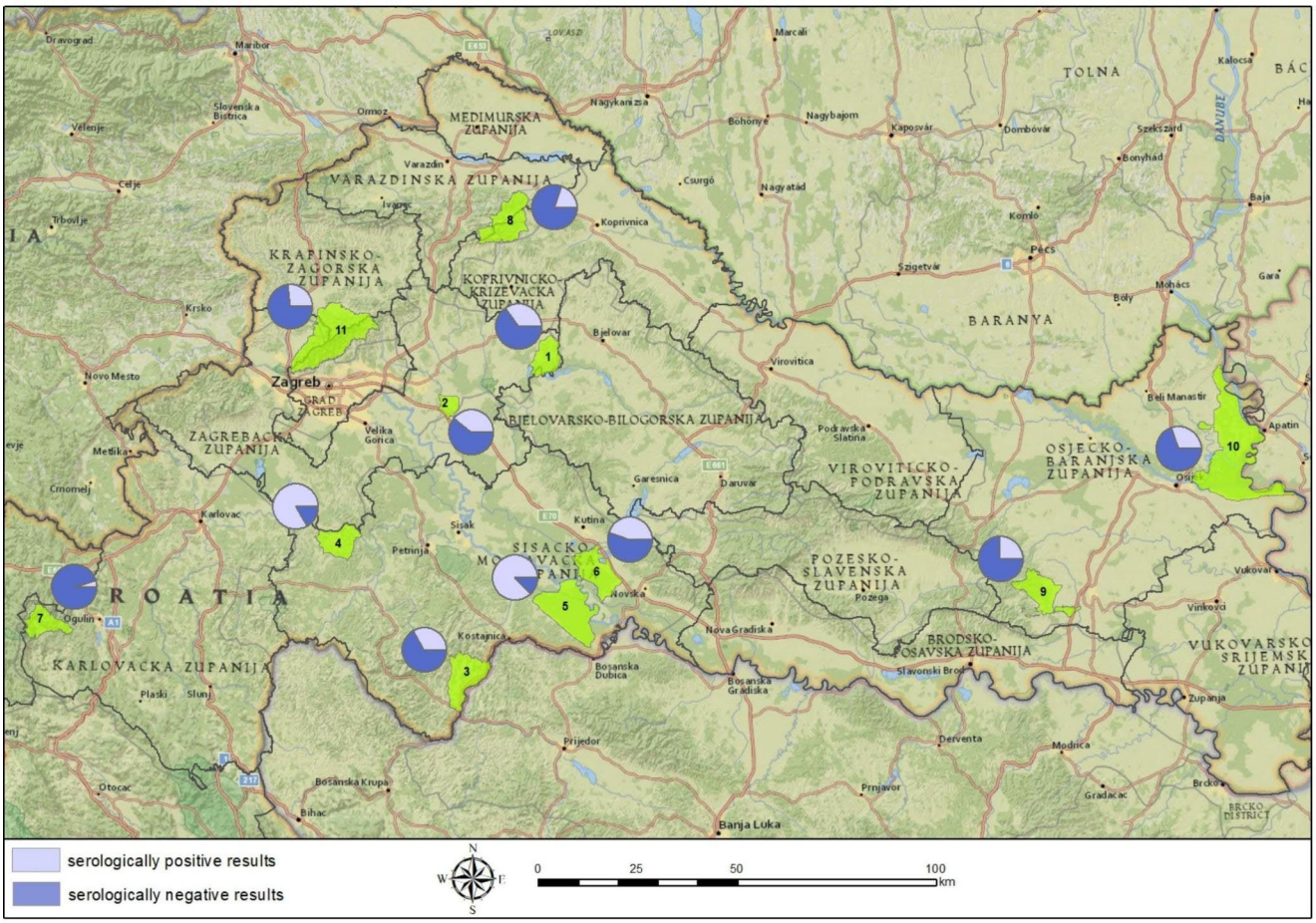

Fig. 2 The distribution of positive results according to the geographical location

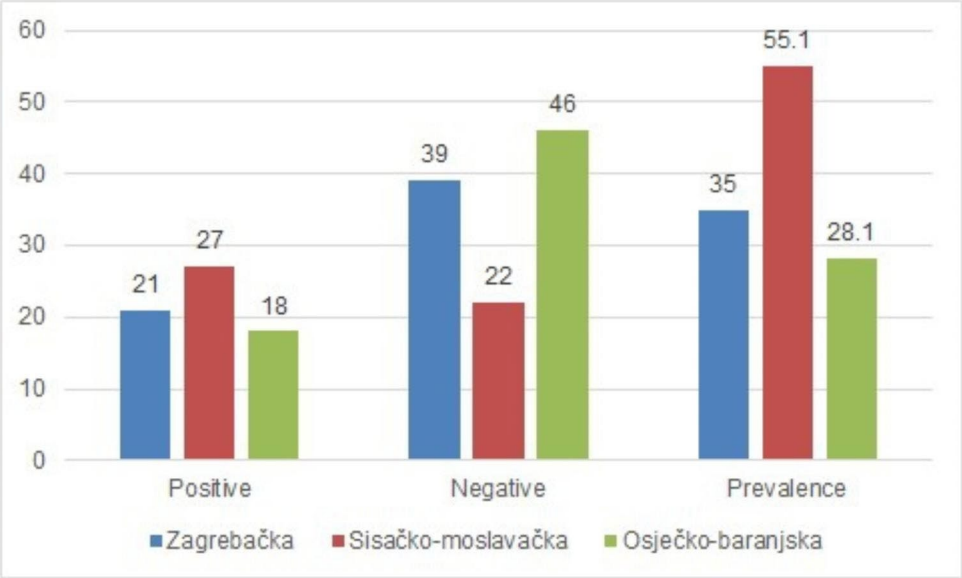

Fig. 3 Results of the ELISA test in counties with largest sample size. Blue columns represent Zagrebačka county, red columns Sisačko.moslavačka county and green columns Osječko-baranjska county

free-living animals sampled in the northern part of the country to 23.85% in southern parts, 30.9% in central Italy, to as much as 65.58% in animals kept in fenced areas (Montagnaro et al. 2011; Verin et al. 2014; Caruso et al. 2019; Ferrara et al. 2021). During one outbreak in Spain, Gortázar et al. (2002) reported seroprevalence of 56%. In general, seroprevalence in Spain ranged between 0.8 and 44%, or even 100% (Vicente et al. 2002; Vicente et al. 2005; Ruiz-Fons et al. 2006, 2007; Closa-Sebastia et al. 2011; Boadella et al. 2012; Cano-Manuel et al. 2014). In Germany, Denzin et al. (2020) analysed 108,748 wild boar sera and detected overall seroprevalence of 12.09%. Similarly to our results (P = 33.8%, n = 222), Vengušt et al. (2006) and Ruiz-Fons et al. (2008) detected seroprevalence of 31% (n = 178; Slovenia) and 36.63% (n = 1714; Spain), respectively. In Steinrigl et al. (2012) reported seroprevalence of 38%. Similar findings were reported for the Czech Republic (P = 30%) and Poland (32.2%) (Sedlak et al. 2008; Lipowski et al. 2017). On the other hand, seroprevalences in Switzerland, The Netherlands and Sweden were up to only 4% (Dekkers and Elbers 2000; Elbers et al. 2000, 2001; Köppel et al. 2007; Leuenberger et al. 2007; Meier et al. 2015; Lindberg 2018). In Croatia, Župančić et al. (2002) analyzed wild boar sera collected in the Moslavačka Mountains and confirmed a prevalence of 54.54%. Similarly, our study samples from the same County had seroprevalence of 55.10% (Sisačko-moslavačka County). Roić et al. (2012) analyzed wild boar sera for the presence of antibodies against several viral pathogens, including ADV. Interestingly, seroprevalence against ADV showed similar trends as in our research, with overall seroprevalence of 38.5% while, geographically, Sisačko-moslavačka County had a higher seroprevalence compared to Osječko-baranjska County (36% vs. 29.6%). These differences between counties are in correlation with the findings of Denzin et al. (2020) who found differences in seroprevalence at district level in Germany. The cause of these differences is still unclear, since the population density of wild boars in the three counties with the largest sample size is similar, and all are lowland to hilly habitats. Among the counties with a smaller sample size, a slightly larger population of wild boars in the area of the Medvednica Nature Park (City of Zagreb) can explain the higher seroprevalence. From all that we observed in our study and on the basis of previous studies in Croatia, we can point out Sisačko-moslavačka County as a hot spot of AD in wild boar in Croatia.

Further, in our study the highest prevalence was detected in adult categories (P = 66.75%) which corresponds to the 60.60% detected in sows from Spain (Ruiz-Fons et al. 2006). Similarly, statistical differences were found between age categories by Ferrara et al. (2021) but not between sexes.

From the epidemiological point of view, an extremely important characteristic for the spread and maintenance of the disease in wild boars is their social structure. The main groups (herds) are formed of adult females, subadults and piglets. Subadult males are forced to leave the group at the age of two. At that time, they form small bachelor groups that will eventually be replaced by a solitary way of life (Tack 2018). Therefore, the detected lower prevalence in male offspring (7.5%) and subadults (16.3%) should have resulted in a lower prevalence in solitary adult males, which, however, was not the case. This could be explained by the fact that subadult males, as previously mentioned, leave the groups at the age of two, a period when seroprevalence in these animals is still quite low. Their separate life could keep them, at least partially, away from potential

infection. However, as they grow up, males will approach different groups and have conflicts with other males in adulthood, which results in increasing seroprevalence in adult males. A similar conclusion was drawn by Fernández-Llario and Møller (2019) who found that seroprevalence for Aujeszky disease was higher in males with larger tusks, which in turn were more sexually active and approached more females and groups during mating seasons. Another important fact here is previously mentioned potential of ADV to form latent infections, with possible reactivation under certain circumstances, such as stress and immunosuppression, which leads to the shedding of the virus and maintenance of the infection at the group level (Howarth 1969; Davies and Beran 1981; Alemañ et al. 2001). This explains the relatively high seroprevalence, while none of the samples from the same animals was PCR positive. This intermittent latent and shedding phase can also explain the increasing seroprevalence with the age of the wild boar. In this study, the seroprevalence on the herd level was 29.8%, mainly due to the lower seroprevalence in younger age categories (particularly young males). In the case of wild boar groups, it is also important that different groups avoid mixing with each other. Therefore, a link for pathogen transmission between groups is needed. It is known that solitary males approach females during the mating season, which is a highly stressful time (Eggermann et al. 2013). In addition, the mating season coincides with the main part of the hunting season, mainly in the form of driven hunts, which represents additional stress (Güldenpfenning et al. 2020). Precisely the stress caused by the mating and hunting seasons has already been pointed out as an important factor in the spread of Aujeszky disease (Vicente et al. 2005). It is also necessary to mention that results of studies performed on smaller areas cannot be transferred directly to the state levels. It was shown again that besides the age and sex of the animals, seroprevalence is related to geographical location. From our research, we can also conclude that this is not simply a result of a population density, which was similar in the Counties with the highest and yet statistically different seroprevalence.

The observed increasing prevalence with age and almost identical prevalence in adult males and females points to the fact that the main transmission period occurs during one part of the year (most probably the mating season), and less throughout the whole year, when ADV probably undergoes a latent phase. Also, triggers for virus reactivation might be more complex than previously thought, since none of our samples, collected during the mating and hunting season, was PCR positive for ADV antigens. On the basis of all that we have observed, we can conclude that adult males represent the main link for ADV transmission between different wild boar groups. Recently, due to habitat fragmentation and altered age and sex structure, we are facing a prolonged mating season in wild boars. Furthermore, increased wild boar populations and threats of the spread of African swine fever have led to increased measures to reduce populations, and have resulted in unrestricted hunting all year round. Both are resulting in prolonged and elevated stress levels and may potentially result in the increased prevalence of Aujeszky disease among wild boars.

Abbreviations

AD	Aujeszky disease
ADV	Aujeszky disease virus
PRV	pseudorabies virus

Acknowledgements Authors would like to thank Croatian Forests and hunting associations for their help during the sampling period.

Author contribution DK made study design, wrote first draft of the manuscript; IS and MB performed field work; TK, JP and LJ performed laboratory work; KK, NT, LjB and DK analyzed data, all authors contributed to the final version of the manuscript and approved it.

Funding This study received no external funding.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations Study was ethically approved by the Committee for Ethics in Veterinary Medicine, Veterinary Faculty University of Zagreb (Class: 640-01/20 – 17/02; No.: 251-61-44-20-11). All samples were obtained following regular game management operations, and no animals were killed for the purpose of this study.

Competing interests The authors declare that they have no competing interests.

Consent to participate All authors agreed with participation in this study.

Consent for publication All authors agree with the publication of the manuscript.

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