Pseudorabies virus in North-West Italian wild boar (Sus scrofa) populations: prevalence and risk factors to support a territorial risk-based surveillance

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Summary
Although the eradication of Pseudorabies virus (PrV) in domestic pigs is ongoing, the circulation of this virus in wild boars remains a threat in the currently unprotected, 'low prevalence', pig population. In this study, we reported PrV prevalence data and the influence of possible risk factors in 2 North-West Italian wild boar populations (free and enclosed) with the goal of supporting the implementation of a risk-based AD surveillance system. Sera from 1,425 wild boars were collected between 2011 and 2015 and tested by ELISA for the presence of PrV antibodies; the overall raw seroprevalence was 30.39% (433/1,425; CI 95% 28.01-32.85%). A significant difference was however observed between the prevalence rates of the free range population (9.98%; 90/902; CI 95%; 8.10-12.12%) and the enclosed population of La Mandria park (Piedmont, Italy) (65.58%; 343/523; CI 95%; 61.51-69.65%). In both populations a significantly higher number of adults and females were found positive to PrV ELISA. Specific territorial data on PrV circulation in wild boars should be acquired from other regions for guiding risk-based measures in order to reduce the threat of AD re-infection in a more cost-effective manner.

Il virus della pseudorabbia nei cinghiali italiani: prevalenza e fattori di rischio a supporto di un sistema di sorveglianza territoriale in Piemonte

Parole chiave
Età,
Fattori di rischio,
Genere,
Malattia di Aujeszky,
Piemonte.

Riassunto
Il virus della Pseudorabbia (PrV) rimane una minaccia per la popolazione di cinghiali non protetti nonostante sia in corso la sua eradicazione nei suini domestici. In questo studio sono riportati i dati sulla prevalenza e l'influenza di possibili fattori di rischio in 2 popolazioni (stato libero e in area protetta) di cinghiali italiani del Nord-Ovest, con l'obiettivo di supportare l'implementazione di un sistema di sorveglianza basato sul rischio, utile a determinare le aree in cui è più probabile la ricomparsa della malattia di Aujeszky. Dal 2011 al 2015 sono stati raccolti sieri di 1.425 cinghiali selvatici; la sieroprevalenza complessiva è stata del 30,39% (433/1.425, IC 95% 28,01-32,85%). Il tasso di prevalenza, invece, si è dimostrato significativamente differente tra la popolazione in condizione libera (90/902; IC 95%; 8,10-12,12%) e quella nel parco La Mandria in Piemonte (343/523 CI 95%; 61,51-69,65%). In entrambe le popolazioni è risultato positivo un numero significativamente maggiore di adulti e di femmine. Sull'AD dovrebbero essere acquisiti, tuttavia, dati territoriali specifici da altre regioni che possano indirizzare le misure basate sul rischio al fine di ridurre la minaccia della reinfezione da AD nella modalità economicamente più efficace.

Aujeszky's disease (AD) is an economically important disease affecting wild and domestic pigs. The disease is caused by *suid herpesvirus type 1*, which is also known as Pseudorabies virus (PrV), and belongs to the subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Verpoest *et al.* 2014). The natural hosts for PrV are members of the family *Suidae*, in which infection results in clinical or subclinical disease as well as latent infection with the possibility of viral reactivation. Strict control measures and eradication programs including DIVA (Differentiating Infected from Vaccinated Animals) strategy were successful in many European Union (EU) member countries. In other countries the PrV prevalence in pig farms has drastically decreased (Decision 2010/434/EU). In AD-free countries, the vaccination of domestic pigs is forbidden. In some regions, the persisting PrV circulation in wild boars (*Sus scrofa*) is regarded as a possible threat for the currently unprotected pig population. Since distinct infections and molecular differences between PrV strains isolated from wild boars/hunting dogs and domestic pigs have been demonstrated, the risk of PrV re-emergence and spillover from wild boar to domestic pigs is considered to be of very low concern (Caruso *et al.* 2014, Moreno *et al.* 2015). However, recent outbreaks in France and USA have been related to PrV circulating in wild boars (Hahn *et al.* 2010).

In Italy, the national PrV-monitoring programme began in 1997 (Decreto Ministeriale 01/04/1997). The programme includes the application of direct prophylaxis, biosecurity measures, and vaccination; to date, even if the spread of the virus has been considerably reduced, AD has not been eradicated from pig herds yet (Chiari *et al.* 2015). Over 80% of the Italian pig production is concentrated in Lombardy, Emilia-Romagna, Piedmont and Veneto. The Piedmont region (45.2500° N, 7.9167° E) covers an area of 25,399 km^2; pig industry is one of the most important agricultural sub-sector of the region. With a census of 1,164,095-reared animals and 2,895 farms concentrated in the provinces of Cuneo and Torino, it accounts for 11% of the entire Italian production. Pig production in this region is mainly dedicated to breeding 'Italian heavy pigs' in order to produce Protected Designation of Origin ham (Prosciutto di Parma, Prosciutto di Cuneo).

The wild boar is the most common ungulate in Italy in terms of distribution and population size. Within the alpine and the prealpine areas, the presence of wild boars extends up to Western Italy, including the Piedmont region. In the absence of an effective census methodology, the estimation of wild boar populations relies on indirect indices with an estimated regional wild boar population of about 32,000 animals (Regione Piedmont, Piano faunistico venatorio)[1] and no less than 600,000 wild boar throughout Italy (Pedrotti *et al.* 2001).

In order to expedite their Aujeszky-free status and thus attain inclusion in annex 2 of the EU Decision 2008/185/EC, all the Northern Alpine regions (including Piedmont, Lombardy, and Veneto) implemented strategies and strengthened sanitary measures. With the implementation of its regional control plan for AD (Nota Regionale 2192 DB2017), Piedmont region decreased PrV seroprevalence from 22.10% in 2012 to 9.84% in 2015 in pig farms, although an active serological and virological monitoring demonstrated PrV circulation in wildlife (Caruso *et al.* 2014). According to Presidential Decree 607 of 17/10/96, since 2003, the Piedmont region also established a sanitary plan for the surveillance of diseases in wildlife. This plan also ensures permanent monitoring of occurrence, distribution, and evolution of PrV in wild boars and carnivores (the latter as dead-end hosts). In accordance with this final stage of eradication, an updated analysis of the PrV infection in wild boars due to contact with wild reservoirs may help to assess the potential re-incursion of PrV in naive herds or AD-free areas.

In this study we reported epidemiological data on the PrV seroprevalence rate and its associated risk factors among wild boar populations under different management regimes: i) free-range population hunted in the Piedmont region; ii) enclosed population living in a Regional Park La Mandria, which includes 36 km^2 of fenced area located in the proximity of the alpine chain, and is mainly characterised by grazing meadows, cereal fields, and deciduous forests (27% of the surface), with an estimated boar density of 18 animals/km^2. Overall, 1,425 wild boar serum samples were collected during 4 hunting seasons (season 1 from 2011 to 2012, season II from 2012 to 2013, season III from 2013 to 2014, season IV from 2014 to 2015). In total, 902 sera were from free-range wild boars while 523 were from the enclosed population. Antibodies (Abs) against PrV were detected by ELISA (Enzyme Linked Immunosorbent Assay), which was produced and provided by the Italian National Reference Center for Aujeszky disease. In order to detect Abs against anti-glycoprotein B (gB), a blocking standardised ELISA method was employed. Briefly, this method evaluates the ability of the tested sera to inhibit specific monoclonal-labeled antibodies (2C7) to bind PrV-antigen. The method works as a sandwich ELISA; plates are pre-coated with a monoclonal Ab specific to capturing the PrV antigen that is provided in the kit and, after an incubation step, serum is added. After 3 washing steps, monoclonal-labeled Ab 2C7 is added to the plate and a reaction developed with

[1] http://www.sistemaPiedmont.it/fedwossfa/elenco.jsp.

TMB (tetramethylbenzidine); results are expressed as a percentage of inhibition.

Explanatory variables included in the study were: age (juveniles < 12 months *vs* adult > 24 months) according to Caruso and colleagues (Caruso *et al.* 2015), gender (male *vs* female) and, in the free-range population, geographic districts (Piedmont Alpine districts of Cuneo, Torino, Biella, Vercelli, and Verbania). A multivariate analysis was performed using logistic binomial regression model of fixed effects by proc logistic procedure of SAS® v 9.2 (SAS 2004). The likelihood ratio test was used to assess the overall significance of the model (two-tailed significance level $p \leq 0.05$). The significance of each term in the model was tested by Wald's χ^2. Estimated odds ratios (OR) and 95% Wald's CI were obtained as measures of predictor effect. Hosmer-Lemeshow test was performed to assess the model's goodness-of-fit (Hosmer and Lemeshow 1989).

Descriptive analysis of the 2 samples (free range/ enclosed) showed a similar structure according to age and sex. The free range population was 51.5% female and 70% adult while the enclosed population was 57% female and 74% adult, with no statistically significant difference among the 2 populations for both variables ($\chi^2 = 2.91$, $p < 0.08$; $\chi^2 = 1.41$, $p < 0.23$, respectively). Considering age and sex, the free-range population consisted of 71% adult females and 76% adult males, while the enclosed population consisted of 69.9% adult females and 71.2% adult males.

The overall raw seroprevalence for the entire period of study was 30.39% (433/1425; CI 95% 28.01-32.85%). These results were not unexpected, since in Italy the PrV prevalence in wild boar populations is variable, ranging from 4% to 30%, with 2 emerging scenarios: Alpine, in Northern Italy, where the density of wild boar population is low, and Appennine, in Central-Southern Italy where the density of wild boar population is high. The overall prevalence rate (30.39%, including free-range and enclosed population) was therefore consistent with the data reported in Central and Southern Italy (Montagnaro *et al.* 2010, Lari *et al.* 2006, Guberti *et al.* 2002).

The prevalence rates of free-range and enclosed populations were significantly different ($\chi^2 = 483.90$, $p < 0.0001$). In the free-range population, prevalence was 9.98% (90/902; CI 95%; 8.10-12.12%), while in the enclosed population living in La Mandria park was much higher (343/523; 65.58%; CI 95%; 61.51-69.65%) and consistent with the prevalence (56%) found by Boadella and colleagues (Boadella *et al.* 2012). These results underline the fact that artificial conditions such as fencing and feeding lead to a significant increase of wild boar abundance and aggregation, which in turn promote interaction and transmission of PrV .

Our study was set in Piedmont region, whose territory is mostly characterised by the presence of Alpine mountains and the seroprevalence rate in the free-range population is actually in-line with that (4.97%) found by Chiari and colleagues in a low-density free-range Alpine wild boar population hunted in 6 districts in the Brescia province (Chiari *et al.* 2015). Unfortunately, our data were limited and fragmented, and thus we were not able to quantify a comprehensive index of abundance of wild boar population in the Piedmont region district. It has recently been reported that, in contrast with other Northern regions, in Piedmont the wild boar population characteristics are similar to Appennine populations (constant, permanent, and diffuse) (Guidelines on 'Wild boar management', Ministero Politiche Agricole e Forestali 2003).

Seroprevalence data according to sex and age, and relative OR esteems are summarised in Table I. In the free-range population, a statistically significant difference was found between prevalence rates of juveniles and adults, with the latter being 3 times (OR: 3.24, 95% CI 1.57-6.66) more probable to have Abs anti-PrV. These findings confirm those reported in literature (Muller *et al.* 1998, Vicente *et al.* 2005, Panwitz *et al.* 2011) In this study, females were nearly twice as probable to be PrV-seropositive than males (OR: 1.67; 95% CI 1.06-2.63). As suggested by Vicente and colleagues, this is likely due to different

Table I. *Aujeszky's disease seroprevalence stratified by variables and Odds Ratio (OR) (wild boar, Piedmont, Italy, 2012-2016).*

Free range population			
Categories	**Prevalence (CI 95%)**	**Standard error**	
Female	58.16% (47.78-68.05%)	4.98%	
Male	41.84% (31.95-52.23%)	4.98%	
Adult	90.82% (83.28-95.71%)	2.92%	
Juvenile	9.18% (4.29-16.72%)	2.92%	
Factors	**Odds Ratio**	**95%CI**	
Adult *vs* Juvenile	3.236	1.573	6.659
Female *vs* Male	1.667	1.059	2.625
Entire district *vs* Cuneo	1.804	1.027	3.166
Enclosure population (La Mandria park)			
Categories	**Prevalence (CI 95%)**	**Standard error**	
Female	68.25% (61.10-74.82%)	3.39%	
Male	31.75% (25.18-38.90%)	3.39%	
Adult	78.84% (72.32-84.43%)	2.97%	
Juvenile	21.16% (15.57-27.68%)	2.97%	
Factors	**Odds Ratio**	**95%CI**	
Adult *vs* Juvenile	3.724	2.251	6.161
Female *vs* Male	2.290	1.425	3.681

social behavioural traits and/or to age differences in sexual maturation between males and females (Vicente *et al.* 2005). In female wild boars – already susceptible to higher prevalence rates than males during the breeding season – social gregariousness may favour direct routes of infection, such as the respiratory route (Ruiz *et al.* 2007). In other studies, however, males and females were equally likely to be infected (Boadella *et al.* 2012, Pedersen *et al.* 2013).

Although the highest number of farms (56%), domestic pigs (72.6%), and AD outbreaks in 2015 (57/80; 71.25% 95% CI 60.05-80.82%, data from Osservatorio Epidemiologico IZSPLV), the PrV seroprevalence in the wild boar populations hunted in the Cuneo district was lower than in other areas of the region (OR: 1.80; 95% CI 1.03-3.17). This finding, added evidence that PrV maintenance in wildlife is not linked to the livestock.

Significantly higher prevalence rates were also observed in adults and females of the enclosed population. Different management systems seem not to affect the influence of these variables on PrV seropositivity. However, if we look at the main transmission routes of PrV (venereal and oral/nasal excretion/infection) and consider the attenuated nature of wild swine PrV and the low concentration of individuals in sounders compared to domestic pig holdings, aerosol transmission over long distances (no direct contact) seems to be unlikely (Muller *et al.* 2011).

As a consequence of regional control plans, increased attention was given to AD in the Piedmont region and biosecurity measures were strengthened in pig farms. Even if the results of this study seem to confirm that the risk of PrV re-emergence and spillover from wild boar to domestic pigs could be considered of low concern, preventing direct contact between free-range wild boar and fared swine seems an appropriate risk-mitigating measure.

A higher seroprevalence rate (65.58%) in the enclosed population is offset by the fact that the possibility of direct contact with pigs is minimised by limited freedom of movement. However, the risk of spillover – through rats and mice, which are moderately resistant to the infections and could also serve as mechanical carriers – indicates that a 'zero risk' hypothesis is not possible in this context.

In this study, we did not provide any insight into what it is currently known to drive PrV transmission within and between wild boar populations and, as is also noted by Muller and colleagues (Muller *et al.* 2011), seroprevalence in populations of wild boar should be interpreted with care, since these findings may have been biased by sampling, or by investigation periods. Indeed, if we focus only on the free-range population in the whole Alpine region, our results are in compliance with the Northern Italy region, though the population density in Piedmont region is higher than in most other Alpine regions. Nevertheless, the PrV seroprevalence rate of enclosed populations drastically increased the average seroprevalence, and this fact should be carefully taken into account in the interpretations of AD epidemiological studies.

Our data reported a 4-year surveillance study and also included an acceptable number of serum sample. This study thus addressed the lack of data on PrV in north-western Italian wild boar populations and its associated risk factors.

As PrV could infect Alpine wolves, which are currently re-introducing in Northern Italy, this study might also be relevant to issues relating to wild boar health and conservation or for veterinary authorities involved in AD control and eradication campaigns in Italy and in Piedmont region. As a possible spillover cannot be completely ruled out, data and strategies to prevent the transmission of PrV from endemically infected wild boar to domestic pigs have to be investigated; additional epidemiological studies are needed, as well as studies relating in particular to the molecular characterisation of PrV circulating strains in order to determine whether wildlife and domestic animals share the same strains.

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