Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Monitoring of African Swine Fever in the Wild Boar Population of the Most Recent Endemic Area of Spain

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Summary

Wild boars are natural hosts for African swine fever (ASF). The ASF virus (ASFV) can persist for long periods in the environment, such as in ticks and contaminated products, which may be sources of infection for wild boar populations. African swine fever was eradicated in domestic pig populations in Spain in 1995, after 35 years of significant effort. To determine whether ASFV can persist in wild boar hosts after it has been eradicated from domestic pigs and to study the role of wild boar in helping ASFV persist in the environment, we checked for the presence of ASFV in wild boars in Doñana National Park, one of the largest natural habitats of wild boar in Spain and one of the last areas where ASF was endemic prior its eradication. Samples from 158 animals collected between 2006 and 2010 were analysed using serological and nucleic acid-based diagnostic techniques recommended by the World Organization for Animal Health (OIE). None of the samples was found to be positive. These results confirm the absence of disease in wildlife in what was once one of the areas most affected by ASF in Spain, and they suggest that wild boars play a limited role in ASFV persistence. These results confirm that ASFV cannot persist in isolated wild boar populations for long periods of time without the interaction of other factors such as re-infection by contact with domestic pigs or by feeding on contaminated swill.

Introduction

African swine fever (ASF) is a haemorrhagic infectious disease that occurs in swine herds and causes significant economic and associated losses in affected countries. The disease first appeared in 1957 in Portugal, apparently introduced from West Africa. After a disease silence of 2 years, ASF re-emerged in Portugal in 1960 and spread to neighbouring Spain, where it remained endemic until the mid-1990s, affecting domestic pigs and Eurasian wild boar (*Sus scrofa*). Following tremendous economic losses, Spain implemented an effective and well-coordinated eradication programme in 1985 with $72 million of support from the European Community (EC) (Bech-Nielsen et al., 1995). Within the first 5 years, the programme achieved great success in confining the disease to southwest Spain (Andalucía and Extremadura) (Fig. 1; panels a and b). The virus persisted in these areas primarily because of (i) inadequate sanitary and biosafety conditions in outdoor pig production facilities, (ii) the presence of soft ticks (*Ornithodoros erraticus*), which serve as medium- and long-term reservoirs of the disease (Boinas et al., 2011); and (iii) the presence of an uncontrolled wild boar population, as was the case in Doñana National Park (DNP). Doñana National Park is one of the largest national parks in Spain and it is located in the Huelva province in the southwest of the country, one of the last regions to eradicate ASF because of the complex

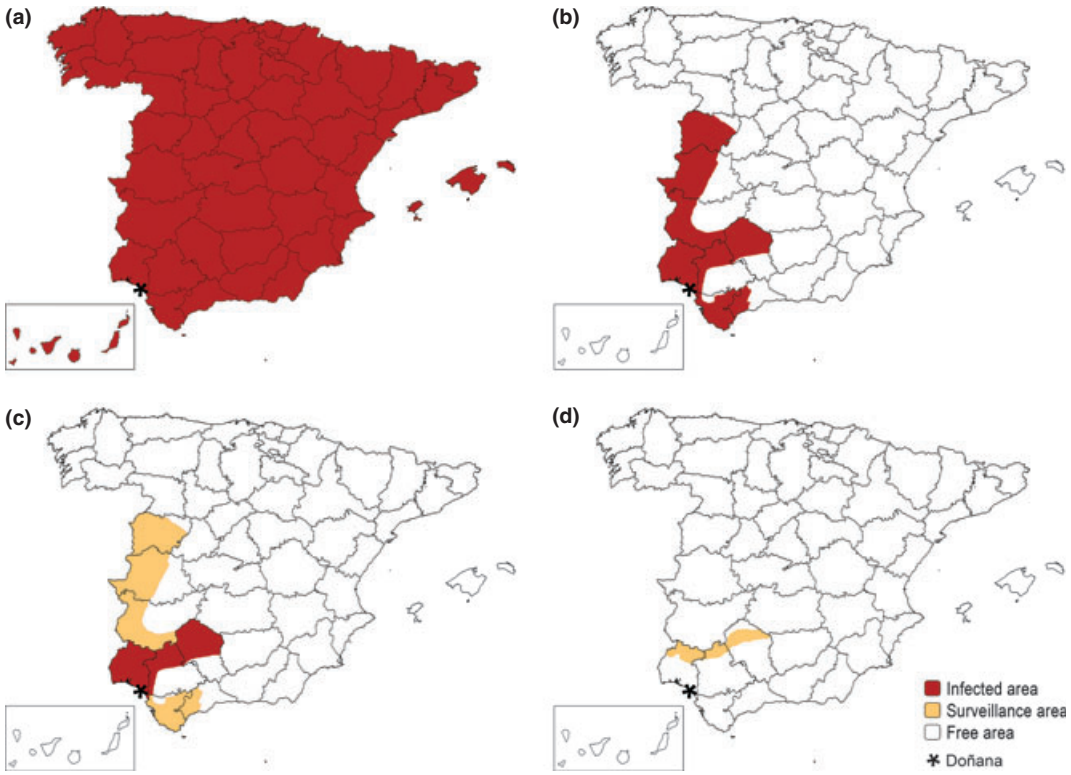

Fig. 1. Occurrence of African swine fever in Spain during the national eradication programme. (a) 1985, (b) 1989, (c) 1994 and (d) 1995.

epidemiology arising from the interaction between wild and domestic animals (Fig. 1). Serological monitoring carried out during the 1990s in the wild boar population in this region showed seroprevalence levels of 10% in areas where domestic pigs remained infected (Pérez et al., 1998). Moreover, 5.8% of ASF outbreaks in domestic pigs in Spain until 1981 were associated with potential contact between domestic pig populations and infected wild boars (Ordas, 1983). Within the DNP, in contrast to surrounding areas, there is no contact between domestic pig populations and wild boar populations, making this region suitable for testing for ASF virus (ASFV) persistence in natural hosts following the eradication programme in pigs.

No ASF outbreaks have been reported in Spain since 1994, confirming the effectiveness of eradication efforts. Nevertheless, ASFV can persist for long periods of time in the environment, soft ticks and contaminated products (EFSA, 2010). In particular, wild boars are a natural host for ASFV, as shown in studies in the Iberian Peninsula (Pérez et al., 1998), Sardinia (Firinu and Scarano, 1988) and in the currently affected areas of the Russian Federation and trans-Caucasus countries (TCC) (Beltrán-Alcrudo et al., 2009). However, their role in the persistence of the disease still remains unclear. Several studies have demonstrated that ASFV tends to disappear in wild boar populations when the interaction with infected domestic or free-ranging semi-domestic pigs is limited (Laddomada et al., 1994; Manelli et al., 1997, 1998; Rolesu et al., 2007; Jori and Bastos, 2009), suggesting that the virus does not persist for long in wild boars in the absence of other factors. This hypothesis should be tested more extensively, to gain a better understanding of the role of wild boars in disease persistence.

To confirm the ASFV-free status of a formerly endemic region in Spain and assess the role of wild boars in ASFV persistence, we conducted a wild boar surveillance programme in the DNP. Given that no ASF outbreaks have been reported in DNP or other parts of Spain since the eradication campaign ended, and based on studies of ASF in wild boars described previously, we hypothesized that even the large and dense wild boar population in the DNP, where ASF was widely present and persisted longest during eradication efforts, would be unable to maintain ASFV circulation. This hypothesis predicts that wild boars alone are unable to maintain the virus and do not interfere significantly with ASF control and eradication in affected countries as long as their contact with domestic pig populations is restricted.

Material and Methods

This study was conducted in the DNP (Fig. 1), where the estimated population of wild boar is 1700 animals (Equipo de Seguimiento de Procesos Naturales de la Estación Biológica de Doñana, 2011). This number was estimated by multiplying the animal abundance index obtained by DNP biologists, by the DNP surface (km^2). A total of 158 individuals were sampled during the hunting season from 2006 to 2010. Blood samples were collected from the heart or the thoracic cavity during field necropsies. Non-EDTA blood was allowed to clot, and serum was separated by centrifugation. Sera were sent to the ASF-OIE Reference Laboratory at the University Complutense of Madrid for serological and virological detection of ASF. The haemolytic or non-haemolytic status of sera was recorded before diagnostic procedures were performed.

Antibody detection against ASFV was performed using OIE-approved serological tests consisting of an initial screening of the 158 sera by indirect enzyme-linked immunosorbent assay (OIE-ELISA) followed by an immunoblotting assay (IB) to confirm doubtful and positive results. Briefly, the antigen in both conventional ELISA and IB assays was lysate from an MS stable monkey kidney cell line (ECACC, 91070510) infected with ASFV E70MS48, and the reporter system was protein-A conjugated to enzyme horseradish peroxidase (HRPO). Both procedures were carried out according to the OIE Diagnostic Manual (OIE, 2008) using ELISA plates, reference sera and IB strips supplied by the EU-ASF Reference Laboratory at the Animal Health Research Center in Madrid (CISA-INIA). A commercially available blocking ELISA (Ingezim PPA-Compac®; INGENASA, Spain) was also used to perform the initial screening.

African swine fever virus genome detection was performed on 146 sera, because the volumes of 12 samples were insufficient for DNA extraction. DNA was extracted directly from serum using the High Pure Extraction Kit® (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's instructions. DNA was amplified using OIE-recommended conventional and real-time PCR methods previously described by Agüero et al. (2003) and King et al. (2003).

FreeCalc v.2 software (Available at: http://www.ausvet.com.au/content.php?page=software) was used to test the alternative hypothesis that the wild boar population is free from the disease based on the results obtained with the sample in this study. The hypergeometric exact probability formula for imperfect tests and finite populations was used in the calculations (Cameron and Baldock, 1998). The chi-square test was used to determine whether an association existed between ELISA results and the haemolytic status of samples.

Results

Antibody testing was carried out on 158 sera, and 11 (7.0%) were positive by commercial ELISA and 38 (24%) by OIE-ELISA. These tests gave doubtful results for 6 (3.8%) and 29 (18%) sera, respectively. No positive samples were detected by IB assay. Because the OIE considers the IB assay as the confirmatory technique for detecting anti-ASFV antibodies, we concluded that no antibodies against ASFV were present in the analysed samples. None of the 146 samples analysed by PCR was positive by either the conventional or real-time method.

A total of 28 samples (18%) showed extensive haemolysis, while 49 (31%) showed moderate haemolysis. The chi-square test between OIE-ELISA results and haemolytic status of the samples showed a significant association between serum haemolysis and false positive results (P-value <0.001). This association was significant regardless of whether the extent of haemolysis was moderate or severe (data not shown). In contrast, the chi-square test for the association between haemolysis and INGENASA-ELISA results was not valid.

Discussion

This study aimed to verify the ASF-free status of the wild boar population in the DNP located in southwest Spain, the last region to achieve ASF eradication in the 1990s. All samples analysed were negative by both virus and antibody detection, suggesting the complete absence of the virus in the DNP wild boar population. Considering that (i) the total wild boar population in DNP has been estimated at around 1700 individuals; (ii) the sample size in this study was 158; (iii) the minimum number of wild boars to be sampled in the defined sampling area must allow for the detection of 5% seroprevalence with 95% confidence (Commission Decision 2003/422/EC); (iv) the sensitivity and specificity of the IB test are assumed to be at least 99% and 98%, respectively (Pastor et al., 1992); and (v) all samples tested were negative by IB and PCR methods, we can accept the alternative hypothesis that the population is free from disease at the 99.21% confidence level. Eradicating ASF in Spain was an extremely difficult and expensive process that required more than 30 years of efforts from the government, farmers and veterinarians. This tremendous work led to the development and improvement of the pig production sector in Spain, which has grown to 3.48 million tons per year, making the country the second largest pig producer in the European Union (Marquer, 2010). This massive growth could

never have been achieved with the production difficulties and international restrictions imposed on an ASF-infected country.

This study was carried out with the most reliable OIE-recommended techniques for ASF diagnosis. Nevertheless, the ELISA tests did give some false positive results. Specifically, the OIE-ELISA gave a higher number of false positive results when haemolytic samples were analysed ($P < 0.001$), revealing a problem of specificity in this test when wild boar hemolysate sera- such as the ones in the present study- are analysed. These results confirm previous findings that the OIE-ELISA lacks specificity and sensitivity when applied to poorly preserved samples (Gallardo et al., 2006; Perez-Filgueira et al., 2006; Gallardo et al., 2009) and supports the OIE recommendation of routinely confirming diagnostic results using the IB test (OIE, 2008). These results also highlight the importance of proper sample collection and preservation to ensure correct diagnosis, which is sometimes difficult when working with wildlife (Boadella et al., 2011). If samples have not been properly collected in a way that guarantees a minimum of quality and quantity, they should not be analysed, to avoid non-specific reactions and incorrect interpretations.

Major factors complicating ASF eradication in Spain included the low biosecurity level of the outdoor pig production systems at that time (1980s), the absence of an adequate identification and traceability system for pig herds and pig movements, and continuous contact between infected and susceptible domestic pigs, wild boars and soft ticks (Arias and Sanchez-Vizcaino, 2002). The last two factors (wildlife and soft ticks) are not easy to control, but must be considered in the design and development of disease control and eradication programmes. Infected wild boars posed a problem for the ASF eradication programme, specifically in parts of southwest Spain where outdoor pig husbandry methods with low biosecurity were used in areas with high wild boar density. During the last three decades, wild boars have expanded their range and increased their densities throughout the Iberian Peninsula (Gortazar et al., 2000; Acevedo et al., 2006). In fact, wild boars are considered overabundant in some circumstances, such as in fenced hunting estates where they are fed artificially as part of intense management, and in some protected areas where hunting pressure is low. These overabundant wild boar populations raise concerns about aspects of environmental conservation and disease maintenance (Gortazar et al., 2006). Wild boar densities in such areas can be as high as 90 individuals per km^2 and have been shown to correlate with infectious disease prevalence (Acevedo et al., 2007). In DNP for instance, *Mycobacterium bovis* infection prevalence grew from 33% to 52% between 1998 and 2007 (Gortazar et al., 2008). However, although European wild boars are highly susceptible to the disease and shed ASFV in similar quantities as domestic pigs (McVicar et al., 1981), their role in ASF persistence seems to be limited in the absence of infected domestic or feral pigs. This was the case in DNP, where no contact with domestic pigs is possible. Our study confirms that even a highly dense wild boar population, such as the one in DNP, cannot maintain ASFV circulation in the absence of other sources of infection.

Several Spanish regions recently initiated a wildlife disease monitoring programme that in 2011 was formalized into a National Wildlife Disease Surveillance Scheme (MARM, 2011). This Scheme proposes the sampling of 2070 wild boars across the entire country for surveillance of numerous wild boar diseases. In this study, we sampled three times more animals than the sample sizes of 58 and 60 animals required, respectively, by the National Scheme and by the European Community Decision 2003/422/EC. The purpose of this intensive sampling was to demonstrate the complete clearance of ASFV from wild boars at the highest possible confidence level.

Conclusion

The negative results of an ASF study within a representative wild boar population in Doñana National Park (Spain) support previous studies confirming that wild boars by themselves are unable to maintain ASFV infection for long periods. These findings also suggest the need to further study the role of these animals in ASFV persistence in currently affected areas, such as the Russian Federation.

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Conflict of Interest

The authors declare that they have no competing interests.

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