AFRICAN SWINE FEVER

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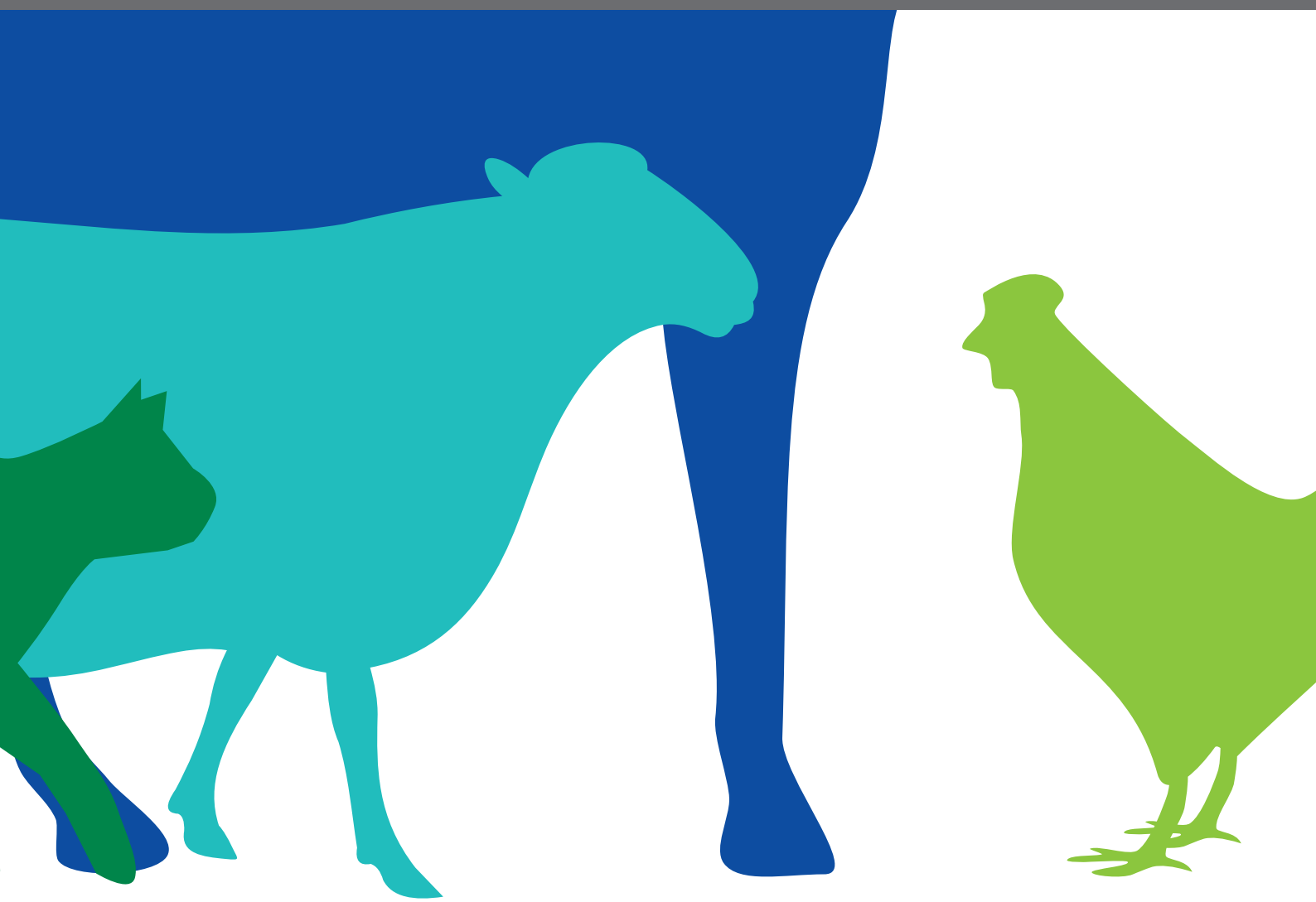

frontiers Research Topics

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AFRICAN SWINE FEVER

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EDITORIAL
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Editorial: African Swine Fever

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Keywords: ASF, vaccine, diagnosis, wild boar-domestic pig interface, epidemiology and control

Editorial on the Research Topic

African Swine Fever

The African swine fever (ASF) virus' third and deadliest tour outside Africa began in 2007, on a ship from the east coast of Africa bound for the port of Poti in Georgia (1). Once again, contamination of local pigs with food residues from the ship produced the first outbreak, which quickly spread among the pig and wild boar population from South to North and from East to West. ASF is currently present in four continents, affecting more than 50 countries, and causing millions of dead pigs.

More than seven different epidemiological scenarios are observed with different risk factors involved in each of them. This epidemiological situation has greatly changed the international pig market. The enormous losses of pig population affected Asia and mainly China (which represented 50% of the world pig population), in particular in its backyard and family population (50% of total Chinese production) which has been the worst affected by ASF (2). Due to this situation, China does not currently produce the necessary number of pigs to meet the country's needs, a situation that will continue for a while. This demand for pig meet has been supplied to date with important exports from the EU, USA, and Canada, and, to a lesser extent, Latin America (Brazil, Chile, and Mexico) (3). The recent infection of ASF in Germany and of Brazil with classical swine fever (CSF) (4) may change export flows and livestock movements, as well as the risk of ASF entry into other countries.

There are a few examples where ASF control has been possible despite infection in wild boar: Sardinia (Italy), affected since 1978 with a three-host epidemiological cycle in which free-ranging pigs played the most important role as virus source and reservoir, is now close to eradication (5). The last domestic pig outbreak in Sardinia was reported in September 2018 and the last finding of ASF virus in two wild boar carcasses was in April 2019 (6). Spain and Portugal were also able to achieve ASF eradication with a localized epidemiological scenario that included extensive pig production, ticks, and wild boar (7). On the other hand, the difficulty to eradicate the disease in other areas of Europe where the infection is very widespread and wild boar is the main virus host has been confirmed by the re-emergence of ASFV in Estonia last August (6), where no virus had been detected in the previous 18 months (8).

This Research Topic brings together 10 articles with updated knowledge on ASF pathology, diagnosis, vaccine development, epidemiology, and control and eradication.

ASF can cause different clinical courses, from peracute to chronic, depending on virus virulence, infective doses, or exposure route among others. Salguero reviews key clinical signs and lesions in domestic and wild pigs (Eurasian and African) infected with virus of different virulence. The acute form of the disease was observed in the first outbreak of ASF in Vietnam in 2019, as described by Nga et al., with high mortality and case fatality rate involving 3 farms. The first farm took longer to suspect ASF so clinical signs were observed for a month. Pig farming in Vietnam has low to no biosecurity measures to prevent the disease and feeding pigs with leftovers from cooking is common. CSF and PRRS are also present in Vietnam, both of which produce similar clinical signs to ASF, making the differential diagnosis more difficult. In this sense, the assay with high sensitivity and specificity developed and validated by Aira et al. to simultaneously detect ASF and

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CSF antibodies could help the timely recognition of ASF which is one of the most powerful tools to prevent its spread across huge geographic areas.

In effect, so far there have been no vaccines available to prevent or control ASF worldwide, and attempts to develop a safe vaccine have historically failed. Sang et al. review the key studies that have evaluated major approaches for the development of ASFV vaccines (live attenuated virus, inactivated virus, subunit vaccines, and live-vectored and DNA-based subunit vaccine candidates). The best vaccine candidates so far seem to be naturally attenuated viruses or produced by targeted gene deletions.

Despite the absence of vaccination or treatment, eradication has been possible in different epidemiological contexts. Risk factors together with the specific surveillance and intervention strategies to tailor them are reviewed by Danzetta et al. in each of the 11 countries that were able to eradicate this challenging disease, which in occasions has lasted for decades. The Italian island of Sardinia is one of the latest examples where, after more than 40 years of endemicity, is on its way to eradication after launching a risk-based plan adapted to the local situation which considered a three-host epidemiological cycle (wild boar, illegal free-ranging pigs, and domestic pigs) (Loi et al.). Frequent interactions between free-ranging pigs and wild boar populations and for long periods of time, particularly at water points, were observed with camera-traps by Cadenas-Fernández et al. in Sardinia.

Unfortunately, ASF in most of the European countries currently affected is nowhere near eradication. One of the challenges the European Union (EU) is facing is the high rate of infection in wild boar. Camera-traps were also used by Morelle et al. in a Polish National Park forest to assess the impact of ASF and hunting to control the disease in wild boar populations. Their study indicated that the intense hunting actions to control population during an acute ASF epidemic alone has a low additional impact on population decline compared with to the high mortality caused by the disease alone. Croft et al. reach a similar conclusion in their study about the hypothetical spatial and temporal patterns of ASF in wild boar following a hypothetical introduction of ASF in an abundant but low density wild boar population area of England. In isolated limited populations of wild boar, the model results show ASF fails to produce a self-sustaining disease. Finally, the epidemiological analyses of wild boar surveillance data by Martínez-Avilés et al. reveal that the expected increase in antibody detection is not always correlated with the time ASF has been present in an area, a potential explanation of which could be the circulation of less virulent strains in certain areas.

In summary, the articles in this Research Topic discuss important features of ASF and approaches to prevent and control it, which work best when adapted to the local situation, together with the latest developments and innovations in ASF research, advancing our understanding of this challenging disease. We trust readers will find these articles as stimulating to read as they were to edit.

AUTHOR CONTRIBUTIONS

JS-V wrote the introduction and final version of the editorial. MM wrote the articles' summaries and final remarks. AL amended and revised the final version. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Bead-Based Multiplex Assay for the Simultaneous Detection of Antibodies to African Swine Fever Virus and Classical Swine Fever Virus

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African swine fever (ASF) and Classical swine fever (CSF) are both highly contagious diseases of domestic pigs and wild boar. In the last years, several cases of both diseases have been reported in the Caucasus, Russian Federation and Eastern Europe. Thus, the probability of encountering these two viruses in the same area is increasing. Since differentiation by clinical or post-mortem examination is not possible, laboratory tools for differential diagnosis are required. In the present work, we have developed a triplex bead-based assay using some of the most immunogenic antigens of each virus, for the simultaneous detection of antibodies; i.e. the VP72 and VP30 of ASF virus (ASFV) and the E2 protein of CSF virus (CSFV). The assay was firstly set up and optimized using well characterized reference serum samples specific for each pathogen. Then, a panel of 352 sera from experimentally infected animals with either ASFV or CSFV were analyzed in the multiplex assay. A collection of 253 field negative sera was also included in the study. The results of the multiplex analysis were compared to those obtained by two commercially available ELISAs for detection of antibodies against ASFV or CSFV, and considered in this study as the reference techniques. The data obtained showed values of 97.3% sensitivity and 98.3% specificity for detection of antibodies to ASFV and 95.7% of sensitivity and 99.8% specificity for detection of antibodies to CSFV. This multiplex assay allows the simultaneous and differential detection of antibodies against ASFV and CSFV, providing a valuable tool for surveillance studies. Moreover, this method is rather versatile, offering the possibility of increasing the panel of antigens from other swine diseases that could be of interest for a differential diagnosis along with ASF and CSF.

Keywords: African swine fever, classical swine fever, multiplex, diagnosis, antibody

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INTRODUCTION

African Swine Fever (ASF) is a highly infectious disease in swine population, caused by an enveloped double-stranded DNA virus, the ASF virus (ASFV), which is the only member of the Asfarviridae family (1). ASFV is composed of more than 68 structural proteins, many of which are highly immunogenic (2). Among them, the structural viral proteins (VP) VP72 and VP30 are commonly used for diagnostic purposes (3–5). ASFV infection causes a strong humoral immune

response that persists for long periods of time, although neutralizing antibodies have consistently been described (6). There are no commercially available vaccines at the moment and therefore, the presence of antibodies in serum is a definitive indicator of infection. ASF control is based on early diagnosis and the enforcement of strict sanitary measures (7). Infection with ASFV correlates with a wide range of clinical syndromes from almost unapparent disease to a hemorrhagic fever with high fatality rates (95–100%) depending on the strain virulence and the immunological characteristics of the host (8, 9).

ASF was first described in Kenia in 1921 (10) and spread to other African, European, Caribbean and South American countries (11). The disease was successfully eradicated from all these territories, except for Sardinia and Sub-Saharan countries where the disease is still endemic (12). In 2007, ASF was introduced into Georgia, and since then it was spread into several Trans-Caucasian countries, the Russian Federation, Belarus, and Ukraine (13). Since 2007 to date, new outbreaks are continuously being reported in Eastern Europe and Russia (4, 14). During the last year, ASF has first been reported in China, Mongolia, Vietnam, Cambodia and spread to other countries in Asia is considered likely by the FAO (14, 15).

Classical Swine Fever (CSF) is also a highly contagious disease of pigs, caused by the CSF virus (CSFV), which is an enveloped singled-stranded, positive sense RNA virus belonging to the genus *Pestivirus* within the Flaviviridae family (16). CSFV has four structural proteins: the core protein (C) and three envelope glycoproteins: E1, E2, and E^{rns}. E2 has been shown to be the most immunogenic protein of CSFV, inducing production of neutralizing antibodies and protection against lethal virus challenge (17, 18) what makes it a good candidate for diagnosis of CSF. CSFV infection presents different clinical manifestations which can vary from unapparent to peracute courses ending in the death of the animal, depending on virulence of the virus strain and host factors (19).

CSF was first reported in Ohio, USA in 1833 (20) and was widespread into Europe and America within a few years (21). After implementation of strict control measures, which include appropriate vaccination programs, several countries succeeded in eradicating CSF, including the United States, Australia and New Zealand; however, it continues to have a severe impact on Asia, Eastern Europe, and most of South and Central America as well as the Caribbean (22, 23). New outbreaks in the European Union keep occurring due to the viral introduction via wild boar, causing huge economic losses (14, 19, 24). Last year, CSF has also remerged in Japan and an ongoing case has been notified in the east coast of Russia (14, 25). This fact together with the spread of ASF from the Caucasus, increase the probability to encounter CSF and ASF in the same region and increase the necessity for fast differential diagnosis.

Since ASF and CSF cannot be differentiated by clinical nor post-mortem examination, laboratory tools for differential diagnosis of the two diseases are essential. Currently, there are some available tests for the simultaneous detection of ASF and CSF based on the direct detection by RT-PCR (26, 27) or in the indirect diagnosis by detection of specific antibodies by immunochromatography tests (28). These assays are of great value for immediate implementation of control measures to prevent further spread of the diseases.

A useful approach developed during the last decades for the multiplex diagnosis, are the bead-based multiplex assays (BBMAs). These are an alternative to planar microarrays, using colored code polystyrene microspheres as the solid support for the capture molecule, which are mixed in a single microtiter plate well to create a microarray in suspension. BBMAs reduce time, labor and sample volume requirements, allowing the testing of many samples for multiple targets simultaneously (29). The xMAP technology (Luminex) combines fluorescent-dyed microspheres, lasers, and digital signal processing up to 500 individual analytes within a single sample. This technology is widely applied in human health for different applications, such as strain identification in infections, immune response characterization (humoral and cellular), or biomarkers identification as well as other uses (30, 31). However, less work has been carried out using this technology in the veterinary field (32–38) and there are only a few commercial kits available. Moreover, when compared to conventional ELISA, previous results have shown that xMAP formats can be more sensitive and reproducible (35).

In this work, we have developed a triplex assay for detection of antibodies to ASFV and CSFV, using immunogenic antigens of each virus: VP72 and VP30 of ASFV and E2 of CSFV, as an approach for the simultaneous detection and differential diagnosis of both diseases. This approach could be a very useful tool in surveillance scenarios, preventing, or at least reducing, substantially economic losses to the swine industry.

MATERIALS AND METHODS

Viral Antigens

The VP72 of ASFV was semi-purified by affinity chromatography with the monoclonal antibody 17LD3 (M.11.PPA.I17LD3; INGENASA, Madrid, Spain) from an inactivated extract of infected cells with ASFV strain (BA71). The VP30 of ASFV (BA71 strain) was produced with a 6X histidine tag in insect cells infected with a recombinant Baculovirus and further purified from the insoluble fraction under denaturing conditions. The glycoprotein E2 of CSFV (Brescia strain) was produced also in insect cells with a 6X histidine tag and purified from the culture media (secreted protein) by affinity chromatography with copper stabilized sepharose.

Serum Samples

Reference serum for ASFV and CSFV, have been used for assay optimization. The ASFV-positive reference serum was provided by the European Union reference laboratory for ASF (EURL) and previously characterized by the OIE ELISA against the BA71 strain. The CSFV-positive reference serum was provided by the National and FAO reference laboratory for CSF at the Friedrich-Loeffler-Institut (FLI) and characterized by VNT (virus neutralization) against CSFV strain Alfort/187 with a 50% neutralization dose (ND50).

Two panels of well-characterized swine sera were included in the present study. For detection of antibodies to ASFV, a

panel 333 serum samples from pigs used in vaccination/challenge experiments at BSL3 facilities at PIR, were included in this study. Briefly, 29 pigs were immunized with an attenuated Benin strain and serum samples were collected at 0, 2, 4, 7, 10, 15, 21, 28, 38, 43, 47, and 59 days post infection (dpi). The animals were boosted 21 days later with the same virus and on day 40 they were challenged with virulent Benin 97/1. A total of 115 samples were collected between 0 and 7 dpi, 57 samples between 8 and 15 dpi, 58 samples between 16 and 28 dpi and 103 samples taken after 1 month pi. (39). For detection of antibodies to CSFV, 30 experimental serum samples from pigs infected at FLI facilities were used (28). Briefly, 23 positive samples collected from pigs experimentally infected with the strain Alfort/187 of CSFV and 7 negative samples. Among these negative samples, one of them was an experimental negative sample and the other six were obtained from pigs infected with other serologically related *Pestivirus*: Border disease virus (BDV) and Bovine viral diarrhea virus (BVDV) (40). Finally, a collection of 253 negative field serum samples from Spanish farms free of both diseases were also evaluated.

In order to prepare pooled samples, each positive sample was spiked in negative serum to analyse a total of 5, 10, and 20 different sera per well. Negative sera were prepared by mixing equal volumes of 4, 9, and 19 negative field serum samples, respectively. This procedure was performed for one ASFV weak positive sample, one CSFV weak positive sample and a negative sample for both diseases. Pools were serially diluted in assay buffer, and the assay was performed as described for the triplex assay.

Coupling of Target Antigens to Beads

The three viral target antigens were covalently coupled to different carboxylated magnetic bead regions (Luminexcorp, Austin, USA) with the xMAP® Antibody Coupling Kit following manufacturer's indications (ref. 40-50016, Luminexcorp, Austin, USA). Briefly, one million carboxylated magnetic microspheres, identified individually by a unique fluorescence ratio (regions #12, #15 and #25, MagPlex® Microspheres, Luminex) were activated according to the NHS/EDC protocol (41), based on a two-step carbodiimide reaction. Activated beads were incubated with different amounts of VP72, VP30, and E2, respectively, ranging from 2.5 to 10 μg per one million beads, in a final incubation volume of 500 μl, and incubated for 2 h with rotation in dark. After washing steps, supernatant was replaced with 1 ml of storage buffer (PBS, 1% BSA, 0.05% azide). Beads concentration after coupling was determined by counting on a Neubauer plate. The coupled microspheres were kept in storage buffer at 4°C in the dark until use, as recommended by manufacturer. The beads were used within the next 3 months after coupling.

A coupling confirmation assay was performed using serial dilutions of monoclonal specific antibodies to each protein: 18BG3 (INGENASA, Madrid, Spain) for VP72, anti-6X His tag (MA1-21315; Invitrogen, Carlsbad, CA) for VP30 and 14E11 (INGENASA, Madrid, Spain) for E2, in order to assess the coupling efficiency.

Bead-Based Assay for Antibody Detection in Swine Serum

To perform the triplex assay, individual antigen-coupled microspheres were sonicated and vortexed for homogenization. A microsphere mixture was prepared mixing the three bead regions in assay buffer (PBS, 1% BSA) to a final concentration for each region of 25 beads/μl. Fifty microliters of this bead mixture was added over fifty microliters of individual pig serum samples diluted at 1/200 in assay buffer. Mixture was incubated for 1 h at room temperature (RT) and 650 rpm in a mini-shaker PSU-2T (Biosan). For this assay, 96-well plates (Stripwell™ Microplate Medium binding Polystyrene, Costar) previously stabilized for 15 min, were used. The plate was protected from light during all the incubation process. After every incubation step, the plate was washed twice with washing buffer (PBS, 1% BSA, 0.05% Tween 20) using a magnetic washer. Each well was incubated with 50 μl of a polyclonal anti-swine antibody labeled with biotin (SAB3700436; Sigma-Aldrich, Kawasaki, Japan), at a final concentration of 4 μg/ml in assay buffer, for another hour in the same conditions. Then, 50 μl/well of Streptavidin R-phycoerythrin (Molecular probes®, life technologies) were added at a final concentration of 2 μg/ml in assay buffer and incubated for 30 min at the same conditions. The beads were then resuspended in washing buffer and the results were read out in a MAGPIX® dispositive (Luminex). The signal was measured as median fluorescence intensity (MFI) of at least, 50 events of each bead region.

Two wells per assay were incubated in absence of sample, only with assay buffer, as a blank signal, which is subtracted from the sample signal. Positive and negative controls were included in all assays to confirm the performance of the test.

Statistical Analysis

Data were statistically analyzed by a ROC curve analysis using the MedCalc® 10 software to establish the optimal cut off value for each antigen.

For the statistical evaluation, samples were classified into positive or negative based on two commercial ELISAs which were used as the reference techniques in this study: INgezim 11.PPA.K3 for detection of specific antibodies against ASFV and INgezim 11.PPC.K3 for detection of specific antibodies against CSFV.

Statistical significance and 95% CI have been calculated for ASFV samples classified according to days post infection. For the statistical significance determination between ELISA and bead-based assay, a McNemar test has been performed.

RESULTS

Development and Optimization of the Multiplex Bead-Based Assay

Optimal coupling amount was established as the minimum quantity of protein that gave a saturation signal of MFI in the titration curve. Thus, the following concentrations were used for each bead region: 10 μg of the VP72 (region #12), 5 μg of the

VP30 (region #15), and 2.5 μg of the protein E2 (region #25) per one million beads.

Next, well-characterized reference swine serum samples for each pathogen were evaluated to establish the optimal conditions for screening purposes. Positive reference sera for ASFV and CSFV, respectively, and a serum from an animal free of both diseases, were included as positive and negative controls in this assay. Serial dilutions of each serum sample were incubated with the mix of the 3 bead regions and the assay was further performed as described in M&M. **Figure 1A**, shows the result of the ASF reference serum, giving a strong signal with VP72 and VP30, respectively, while no signal was detected against the E2, corresponding to the target antigen of CSFV. On the other hand, on **Figure 1B**, the reference serum for CSFV showed a strong signal with E2 antigen, while no significant reactivity with VP72 and VP30. Finally the negative serum showed no reactivity with neither of the antigens (**Figure 1C**). A 1/200 dilution of serum was selected as the optimal dilution for screening purposes. This dilution showed the highest responses to ASFV and CSFV antigens while no cross-reactivity to the non-target antigens in each case.

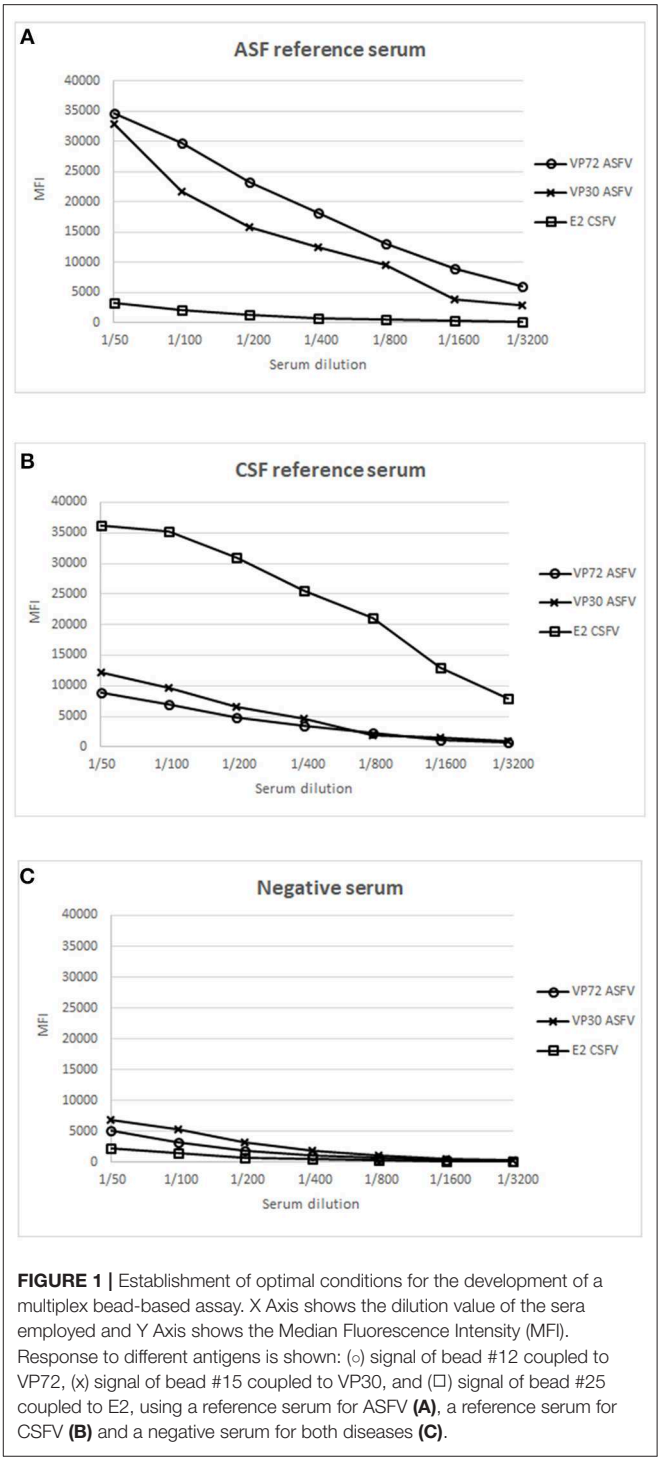

FIGURE 1 | Establishment of optimal conditions for the development of a multiplex bead-based assay. X Axis shows the dilution value of the sera employed and Y Axis shows the Median Fluorescence Intensity (MFI). Response to different antigens is shown: (○) signal of bead #12 coupled to VP72, (x) signal of bead #15 coupled to VP30, and (□) signal of bead #25 coupled to E2, using a reference serum for ASFV **(A)**, a reference serum for CSFV **(B)** and a negative serum for both diseases **(C)**.

Analysis of Experimental and Field Sera in the Multiplex Assay

Once the screening conditions were established, a collection of 605 swine sera were assessed in the triplex assay. A total of 333 experimental serum samples for ASF, 30 experimental serum samples for CSF and 253 field negative samples were included in the analysis. Out of the 333 experimental ASF sera, 185 were classified as positive by the 11.PPA.K3 and 11 as doubtful, so these were not included in the statistical analysis. Out of the 30 experimental sera for CSF, 23 were classified as positive by the 11.PPC.K3. The rest of the serum samples gave negative signals in both assays (**Tables 1A,B**).

In regard to ASFV, a cut off value was established for each antigen according to the Medcalc software: 3500 and 3700 MFI for VP72 (#12) and VP30 (#15), respectively. With the developed assay, a sensitivity of the 96.2% for both antigens and a specificity of 99.0% and 98.6% for VP72 and VP30, respectively, were reached (**Figures 2A,B**). Particularly, more than 96% (178/185) of the samples classified as positive with the reference technique gave also a positive signal with the VP72 (bead #12) in the multiplex assay, and more than the 99% of the negative samples (416/420) gave also a negative signal in the developed multiplex assay. Four samples gave a false positive result when compared to the reference technique. Among these; three samples were obtained from sera at early days post-infection and the other sample corresponded to a positive serum to CSFV. Seven samples classified as positive with the reference technique, gave a negative signal for the VP72 antigen coupled to the region #12 (**Table 2**). For the detection of antibodies to the VP30 of ASFV similar results were obtained. More than 96% of the serum samples classified as positive by the reference technique were detected with the VP30 in the multiplex assay (178/185), and more than 98% of the serum samples classified as negative by the reference ELISA were also negative in the multiplex assay (414/420). Six samples that gave a negative result with the reference technique used in this study were positive to the bead #15 coupled to the VP30 antigen. All of these false positive samples were obtained at different days post-infection. Moreover, seven positive samples by the reference technique were not detected as positive in the multiplex assay (**Table 2**).

TABLE 1A | Sera characterization by INgezim 11.PPA.K3.

Sample classification	Experimental ASF sera (PIR)	Experimental CSF sera (FLI)	Negative field samples	Total analyzed sera
Positive	185	0	0	605
Negative	137	30	253	

TABLE 1B | Sera characterization by INgezim 11.PPC.K3.

Sample classification	Experimental ASF sera (PIR)	Experimental CSF sera (FLI)	Negative field samples	Total analyzed sera
Positive	0	23	0	605
Negative	322	7[†]	253	

[†] *Six out of these seven sera were obtained from animals infected with border disease virus (BDV) and Bovine viral diarrhea virus (BVDV), other Pestivirus serologically related to CSFV.*

Taking together the reactivity of a given serum against VP72 and VP30, the values of sensitivity and specificity were slightly increased (**Table 2**). More than the 97% (180/185) of the serum samples classified as positive were detected by, at least, one of the antigens. And more than the 98% (413/420) of the negative samples gave a negative signal to both antigens. By the combination of both antigens, only five false negative samples were obtained with the multiplex assay. The sensitivity parameter increased to 97.3% with a specificity of 98.3%. Additionally, a stratified analysis of the positive samples to ASFV according days post infection is shown in **Figure 3**. Within the first 7 dpi no positive results were observed in any of the techniques used. Between 8 and 15 dpi the bead-based assay gave a higher proportion of positive samples (68%) in the inoculated group than the technique used as reference (58%). The same observation was obtained in the 16–28 dpi group, in which bead-based assay exhibited 97% of positive samples, while a 90% was obtained with the reference technique. Samples collected a month after infection, gave similar results with both assays.

According to the cut-off value established by the ROC analysis (5000 MFI) for the E2 antigen (bead #25), the performance characteristics of the multiplex assay for CSF showed a good correlation with the reference technique, reaching a sensitivity of 95.7% and a specificity of 99.8% (**Figure 2C**). Negative samples for CSF, including disease-free animals and ASFV-infected pigs, gave clearly negative results showing no cross reactivity with the E2 antigen. The six serum samples obtained from animals infected with other related *Pestivirus* (BVDV or BDV), gave negative results in this assay format. Only one weak positive sample for CSF was not detected with the bead-based assay (**Figure 2C**, **Table 2**).

Analysis of Pooled Samples for Surveillance Purposes

To increase the high throughput screening possibilities of the assay, the capacity of analyzing samples from up to 20 animals per well was analyzed as described in M&M. **Figure 4** shows the results of the weak positive sera for both viruses in order to detect the pooling effect over the sensitivity of the test. Results of pooling 20 different sera did not give good results for the antibodies to ASFV nor to CSFV detection, since weak positive signals were under the cut off established value (Data not shown).

Figure 4A shows the titration curves for the weak positive serum to ASFV spiked in the 4 and 9 negative sera, making the 5- and 10-pool, respectively. The reactivity against the three target antigens were assessed. A higher response can be observed to ASFV-antigens in all the pools when compared to the E2 antigen. The highest difference appears in the 5-pool sample, where the signal does not decrease in the first dilutions. The 10-pool sample also exhibits a good difference between target antigens (VP72 and VP30) and non-target antigens (E2). Preliminary results show that for the detection of antibodies to ASFV, pools of 5 and 10 different samples can be done maintaining good signals of weak positive samples and with no cross-reactivity between antigens.

In a similar way, the **Figure 4B** shows the titration curves for the weak positive serum to CSF pooled in the 4 and 9 negative sera. For the 5-pool assay, the response of the non-target antigens was over the cut off value (5000 MFI) whereas, in the case of the 10-pool sample, the difference between the E2 signal and the non-target antigens was high enough and negative signals were under the cut off value (**Figure 4B**). Thus, the selected conditions for the pooled assay would be a 10-pool sample diluted 1/10 in assay buffer.

DISCUSSION

African swine fever (ASF) and Classical swine fever (CSF) are two clinically indistinguishable diseases that cause high economic impact worldwide and, thus, both are included in the World Organization for Animal Health (OIE) list (42). In recent years, several outbreaks of both diseases have been detected in Eastern Europe, what increases the probability of encounter these two viruses in a same area (4, 13, 19, 24) what leads to the necessity of having fast and reliable tools for the differential diagnosis. In this study, a triplex assay has been optimized for the simultaneous detection of antibodies against both etiological agents, based on the xMAP Technology.

For the detection of antibodies against ASFV, both VP72 and VP30 antigens, showed a similar behavior against the experimental sera with rather good rates of sensitivity and specificity (**Figures 2A,B**). Three out of the four false positive results obtained with the VP72 coupled to the bead region #12 and the six obtained with the VP30 coupled to the bead region #15 (**Table 2**) were obtained from pigs at different days post-infection, mostly within days 10 and 15 post-infection. This observation may mean that the newly developed multiplex test is more sensitive than the ELISA used as reference techniques, being able to detect the infection at earlier times post infection. If these sera were considered positive instead of false negative samples, the newly developed assay would exhibit an increase in sensitivity and specificity values. Both antigens, as shown

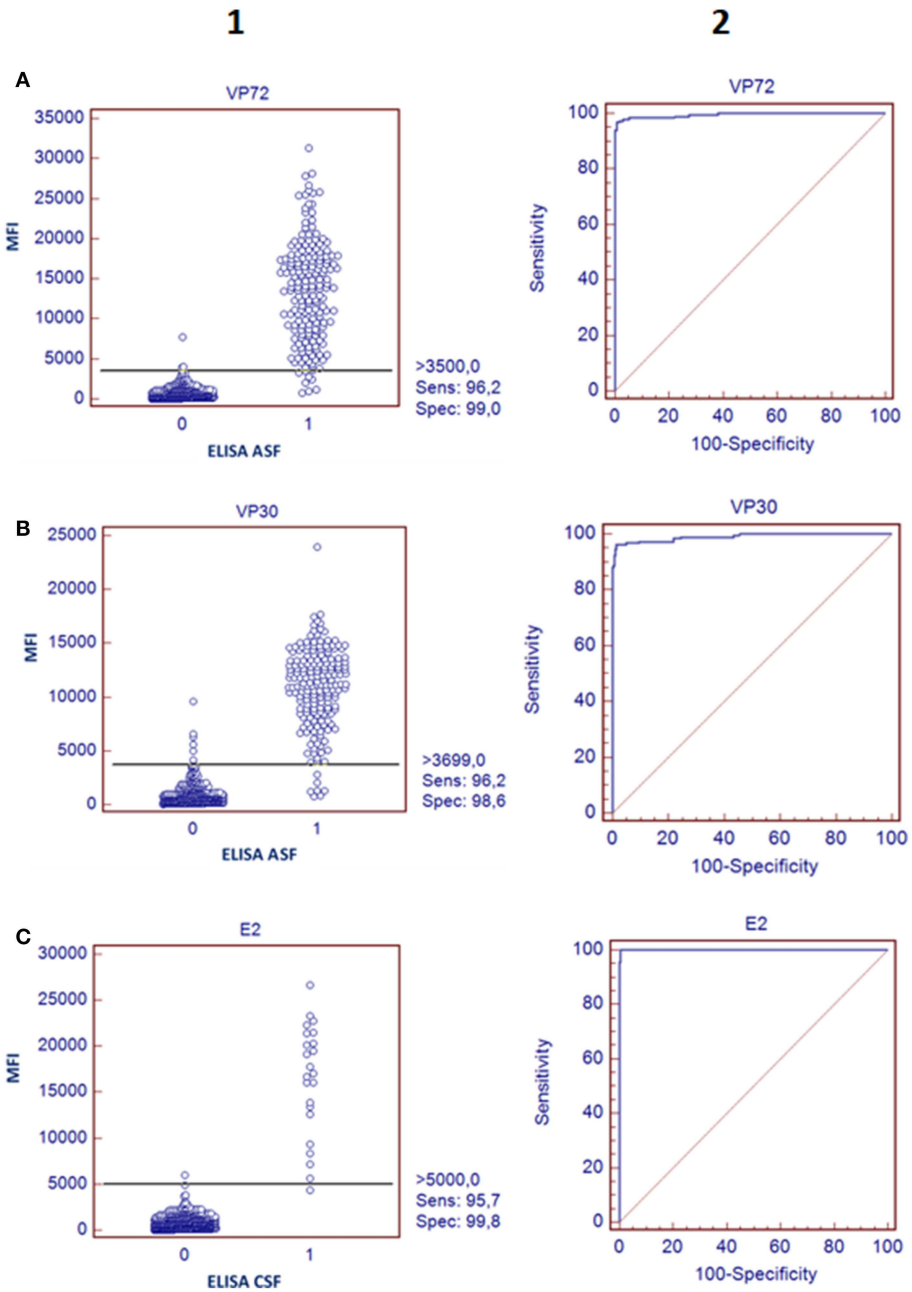

FIGURE 2 | Validation of the bead-bead assay. The left panels represent a dot diagram where each dot represents an individual sample: results obtained for VP72 coupled to bead #12 **(A)** VP30 coupled to bead #15 **(B)**, and E2 antigen coupled to bead #25 **(C)**, The horizontal solid line corresponds to the cutoff values in each assay, according to the Medcalc software. X Axis shows the positive (1) or negative (0) classification of samples according to the ELISA used as reference technique in this study and Y Axis shows Median Fluorescence Intensity (MFI) obtained in the developed assay The right panels show a ROC curve analysis based on the data obtained in the bead-bead assay.

in **Table 2**, can detect the same ratio of positive and negative samples separately. However, VP30 appears to be a good antigen for ASF diagnosis, half the amount of protein is needed to reach the same results when compared to VP72 and more positive samples are detected. This observation has also been described in previous studies (5).

Taking together the reactivity of a given serum against VP72 and VP30, the values of sensitivity were slightly increased to 97.3% with a 98.3% specificity (**Table 2**). By the observation of these results, including both antigens in the multiplex assay seems to be the best strategy for ASF diagnosis, since it increases the sensitivity value of the

TABLE 2 | Correlation between bead-based assay and the ELISAs used as reference for different antigens.

No. of serum samples with ELISA	No. of serum samples with VP72 (bead #12)			No. of serum samples with VP30 (bead #15)			No. of serum samples with E2 (bead #25)			No. of serum samples with VP72 (#12) + VP30 (#15)		
	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total	Pos.	Neg.	Total
Pos.	178	7	185	178	7	185	22	1	23	180	5	185
Neg.	4	416	420	6	414	420	1	581	582	7	413	420
Total	182	423	605	184	421	605	23	582	605	187	418	605
Sensitivity	96.2			96.2			95.7			97.3		
Specificity	99.0			98.6			99.8			98.3		

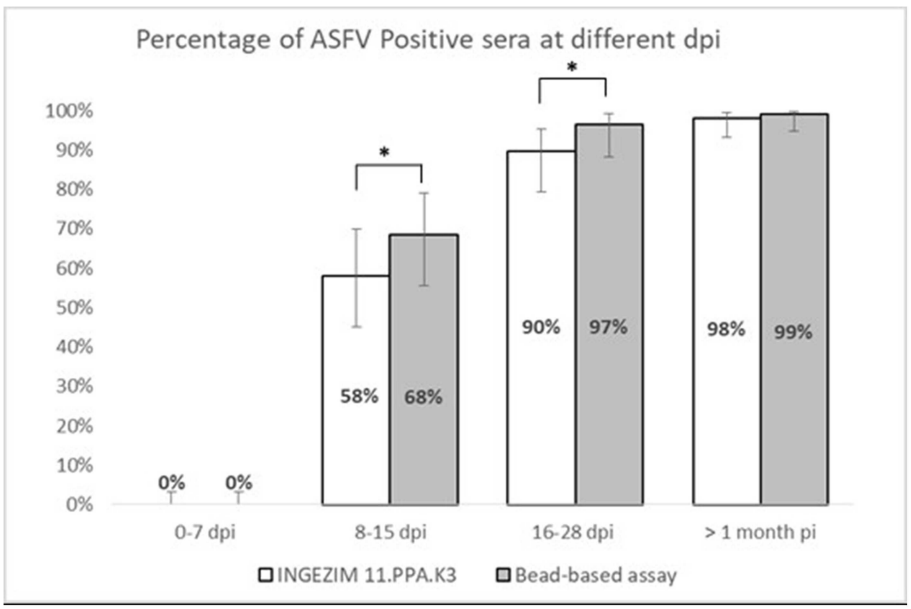

FIGURE 3 | Stratified analysis of positive samples to ASFV according to days post-infection. X axis shows the percentage of positive samples within each group. Y axis shows different days post infection clustered as follows: 0–7 dpi, 8–15 dpi, 16–28 dpi, and > 1 month pi. Error bars show the 95% confidence interval for each bin of data. Statistical significance has been calculated according to a McNemar test, $^*p < 0.05$.

assay. This could be especially of interest when analyzing field samples, where animals can react differently to viral exposure presenting diverse levels of antibodies to each of the virus proteins.

Moreover, if we consider that six out of the seven false positive samples came from animals at different days post-infection the developed test can bring an increase on the assay sensitivity, that specificity parameter would be also increased to 99.8% with a sensitivity of 97.4%. This hypothesis is strengthened by the observation in **Figure 3**, where the newly developed test can detect a higher percentage of positive samples in the 8–15 dpi group (from a 58% to a 68%) as well as for the 16–28 dpi group, in which the percentage is increased from 90 to 97%. When we analyzed samples after 1 month of infection, the percentage of positive samples is almost the same for both techniques. This would mean that the bead-based assay is slightly more sensitive than the reference technique used in this study, detecting infection at earlier dpi.

For detection of antibodies to CSFV, even though more positive sera from CSFV-infected animals should be analyzed to have a statistically representative value of sensitivity and specificity, a great correlation between positive and negative samples is observed, reaching a sensitivity of 95.7% and a specificity of 99.8% (**Figure 2C**). Moreover, the highest MFI signals observed in the negative samples were obtained from animals infected with BDV or BVDV, two *Pestivirus* related to the CSFV whose differentiation is complicated because they are highly cross-reactive antigenically (43).

By the combination of the three antigens, the developed multiplex assay shows great sensitivity and specificity parameters for the differential diagnosis of animals infected with ASFV or CSFV.

Surveillance studies are a priority when talking about high economic impact diseases such as the ones described (ASF, CSF), and it may therefore be beneficial to use pooling of samples to analyse the greatest number of animals per assay. The pooling

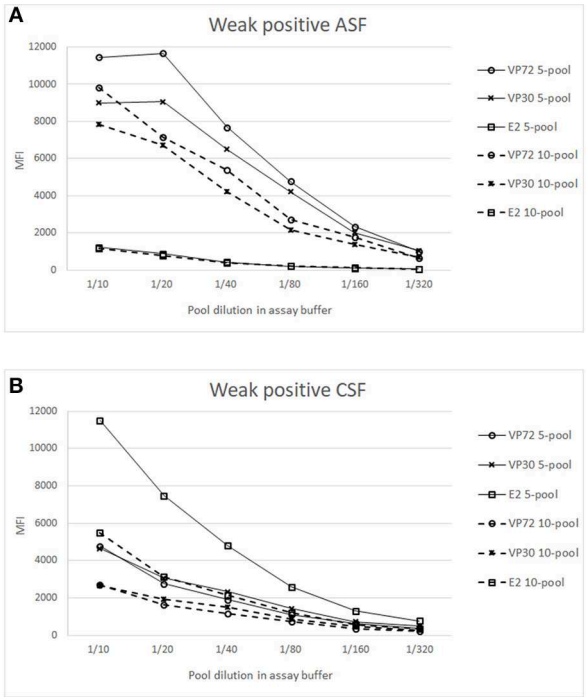

FIGURE 4 | Analysis of weak positive samples in pooled conditions. X axis shows the dilution of the whole pool in assay buffer and Y axis the Median Fluorescence Intensity (MFI). Signals of weak positive sample for ASFV **(A)** or CSFV **(B)**, spiked in a 4 (—) or 9 (- - -) negative sera matrix are represented for (○) bead #12 coupled to VP72, (x) bead #15 coupled to VP30 and (□) bead #25 coupled to E2. Cut off values for different antigens were established at 3500 (VP72), 3700 (VP30) and 5000 (E2) MFI.

of samples from several individuals for a single test has long been advocated as a way of reducing the cost and effort of diagnostic testing. In the veterinary field it has been used for the identification of infected individuals and populations (44, 45), and even the OIE recognize the utility of pooled samples, although it will require the determination of their own sensitivity and specificity parameters (46). Results obtained in this study indicate that pooling of 10 different sera is a good alternative to increase the high throughput screening options of the developed test, since it allows the detection of antibodies to both pathogens in the same conditions. Best conditions were established at the 1/10 dilution of the whole pool in assay buffer, which showed no cross-reactivity between target antigens and promising values of MFI for weak positive samples (**Figure 4**). A more in depth analysis must be done to establish the sensitivity and specificity of the assay in pooled conditions, since previous studies described an increase in specificity of pooled sera and a decrease in sensitivity when changing from unique to pooled sample analysis. This was due to the cut off readjustment for pooled samples analysis (47, 48).

The maintenance of animal health in production species and, particularly in swine, includes the control of a wide range of infectious diseases affecting both, economic and public health aspects. To date, these health evaluations are done with individual assays, and this forces the application of control plans centered in one unique pathology. The use of multiplex assays would dramatically help in those surveillance plans, by allowing the development of one unique plan for a complex infectious disease panel. Moreover, analysis of multiple analytes at once, instead of running several tests in parallel, presents several advantages compared to traditional methods, including saving labor, time and reducing user error and variability between independent assays.

It must be taken into account that this study only included positive samples experimentally obtained, in which animals were inoculated with high viral doses and trough clear inoculation routes. Real samples that reflect field conditions needs to be analyzed to determine the accuracy of the newly developed test and its diagnostic parameters.

This triplex assay would be the starting point for the development of a multiplex assay that include other diseases of special interest in swine. This multiplex assay can be of great interest and application in prevention, control and even eradication plans development.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by UK Home Office under the Animals (Scientific Procedure) Act UK 1986.

AUTHOR CONTRIBUTIONS

CA performed the experiments and drafted the manuscript. TR produced the recombinant proteins used in this study. PS and PR contributed to the design of the study and analysis, interpretation of data, and also critically revised the manuscript. SB provided CSF positive and negative experimental samples. LD provided ASF positive and negative experimental samples. All authors read and approved the final manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Standardized Risk Analysis Approach Aimed to Evaluate the Last African Swine Fever Eradication Program Performance, in Sardinia

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From more than 40 years African swine fever (ASF) is endemic in Sardinia. Historically, areas at higher risk are located throughout some inland parts of this island where domestic pigs are still illegally kept in semi-wild conditions, living in contact with the local wild boar population, thereby creating perfect conditions for disease endemicity. A new eradication plan (EP-ASF15-18) has been ongoing for the past 3 years, based on a comprehensive strategy adapted to the local situation and focused on strong actions on domestic pig farms, wild boars (WB), and the third Sardinian typical involved population [illegal free-ranging pigs (FRPs)]. A fundamental aspect of the plan is the classification of pig farms as "controlled" or "certified," based on clinical, structural, and biosecurity characteristics. The eradication plan also provides for strong action against illegal farms and pig meat marketing channels. In addition, this plan establishes specific control measures for WB hunting and ASF checks. Each control strategy is specifically based on municipality risk level, to focus actions and resources on areas at higher risk of endemic or re-emerging ASF. Thus, precise risk classification is fundamental to this goal. The aim of the present work was to establish an ASF risk index, to provide a summary measure of the risk level in the Sardinian municipalities. This synthetic measure can express the different aspects of a multidimensional phenomenon with a single numerical value, facilitating territorial and temporal comparisons. To this end, retrospective data (years 2011–2018) were used. The ASF risk index is the result of the algorithmic combination of numerical elementary indicators: disease prevalence in the suid populations, WB compliance with EP-ASF15-18, domestic pig compliance with EP-ASF15-18, and presence of FRPs. A negative binomial regression model has been applied and predictors calculated to obtain a risk index for each municipality. The result of the risk analysis was discussed and considered according to expert opinion and consensus. The results of this study, expressed as risk score and classified into five risk levels, can be used to help define actions to be carried out in each Sardinian municipality, according to the risk assessment for the territory.

Keywords: African swine fever, negative binomial regression model, risk analysis, epidemiological cycle, Sardinia, eradication program

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INTRODUCTION

African swine fever (ASF) is one of the most serious infectious diseases affecting domestic and wild pigs, responsible for serious economic and production losses (1). ASF is caused by a large icosahedral DNA virus (family Asfarviridae, genus *Asfivirus*), and characterized by up to 100% mortality (2). The considerable economic losses caused by the disease are even more serious considering the absence of an effective vaccine (3). The quarantine of the affected area and the slaughter of confirmed and suspected infected and contaminated animals (stamping out) in an outbreak, actually are the available methods of disease control, according to European legislation (Directive 2002/60/EC 27/06/2002). In 1921, Montgomery described the first ASF case in Africa and since then the disease is endemic in the African continent with a complicate sylvatic cycle (4, 5). At the end of the 1980s, several countries in Western Europe experienced ASF that were quickly eradicated. However, after its first notification in 1978, ASF persisted in Sardinia involving dense populations of free-ranging domestic pigs (DPs), with occasional incursions in wild boars (WBs) species (6). Since 2007, the disease has been reported in multiple countries including the Russian Federation, Belgium, Hungary, Bulgaria, Latvia, Moldova, Poland, Romania, Russia, and Ukraine, in both domestic and wild pigs (7, 8). Starting in August 2018, the disease has been spreading and having a considerable impact on the pig population of the Asian continent, primarily in China. It should be noted that China has over 50 per cent of the world's pig population, and continue to report outbreaks to date (9). More recently, ASF notifications have been reported from Mongolia in January 2019, Vietnam in February 2019, Cambodia in March 2019, and Hong Kong (SAR-PRC) in May 2019 (10). Recently, new outbreak in Slovakian backyard has been reported (11).

Geographical Distribution of ASF in Sardinia From 1978 to the Present

Forty years have passed since ASF entered Sardinia, probably owing to the upon arrival of processed meat contaminated by African swine fever virus (ASFV) from the Iberian Peninsula (12). The consequence of the first notification of ASF in southern Sardinia (March 1978) was the loss of more than 10,000 pigs. Consequently, serious concerns arose about the difficulties of disease control owing to the specific way that free-ranging pigs (FRPs) were kept in the island's inland areas. The most probable cause for the spread of this disease across the island are the uncontrolled movement of infected pigs which may survive infection, and consequently their introduction into healthy herds and the feeding of waste food containing meat from infected pigs. (13, 14). As soon as the disease spread to central Sardinia (June 1978), it became clear that disease control measures were not being practiced by the local population and that residents had not abandoned local cultural traditions of free-ranging and breeding (15, 16). In addition, the disease spread to the local WB population, creating an even more complex picture. Recently (2015–2018), strict measures have been implemented in Sardinia, aimed at fighting this disease by focusing on hunting management and eliminating illegal FRPs in the latest ASF eradication plan (EP-ASF15-18). The efficacy of this plan is reflected in an evident decrease of disease prevalence over the past 6 years, from 0.61% (95% CI = 0.51–0.74) to 0.007% (95% CI = 0.003–0.1) on DP farms, from 0.32% (95% CI = 0.22–0.46) to 0.04 (95% CI = 0.01–0.09) of ASFV positivity and from 6.23% (95% CI = 5.62–6.89) to 1.12% (95% CI = 0.84–1.49) of seropositivity in the WB population. Detailed spatiotemporal distribution of ASF over the years was provided by Mur et al. and an overall picture of outbreaks from 2011 to 2016 was described by Cappai et al. (17). In Sardinia, the disease is confined to the central part of the region (**Figures 1**, **2**), except for one isolated case near Cagliari in the south (2017), where ancient habits steeped in tradition persist and the disease has become endemic (17–19). No evidence of ASFV has been found in DPs since September 2018.

Sardinian ASF Epidemiological Cycle

A unique and particular ASF epidemiological cycle has been present in Sardinia since 1978. Both the ticks of the genus *Ornithodorus* and other natural reservoirs, such as the warthog (*Phacochoerus africanus*) (20–22), which constitute the well-established "natural host–vector–pathogen system" or "sylvatic transmission cycle" of ASF (23, 24), are absent in Sardinia. In Sardinia, the disease occurs mainly as a result of interaction between the three suid populations, i.e., DPs, WB, and FRPs. On this island, ASFV is characterized by a more anthropogenic cycle in which FRPs (rather than warthogs) assume the role of epidemiological reservoir and act as the link between the other two suid populations, without the involvement of *Ornithodorus* ticks. The involvement of insect vectors other than *Ornithodorus* in disease transmission has not been excluded and is the object of ongoing studies in Sardinia. The three suid populations involved interact with each other in a more or less intensive manner, depending on the management of pig farms (biosecurity), hunting management, and observance of rules governing animal identification and registration. Given that the spread of ASFV in DPs is facilitated by human activities and animal movement (i.e., live infected animals or contaminated meat and other by-products), as demonstrated in many studies (17, 25–27), the consequent spread of disease is related to the growing human population and increasing number of DPs. Furthermore, human activities are the primary cause of long-distance ASF transmission (28). An exclusive and primary role of WBs in the persistence of this disease on the island has never been recognized (17, 29), and the irrelevant role of WBs in the maintainance of disease endemicity in absence of continuous source of virus has been demonstrated (30). Notwithstanding, the contribution of WBs in ASFV maintenance is owing to contact with the FRP population via live or dead animals (carcasses). As shown in **Figure 1**, illegal FRPs are distributed throughout high-density areas of WBs; thus, contact between these populations is estimated to be frequent and intensive. In contrast to consolidated active surveillance (i.e., during hunting season), passive surveillance aiming to locate and test WB carcasses is in place on Sardinia. During the past 2 years (2017–2018), a total of 278 WBs (i.e., hunted or found dead) have been collected and tested for

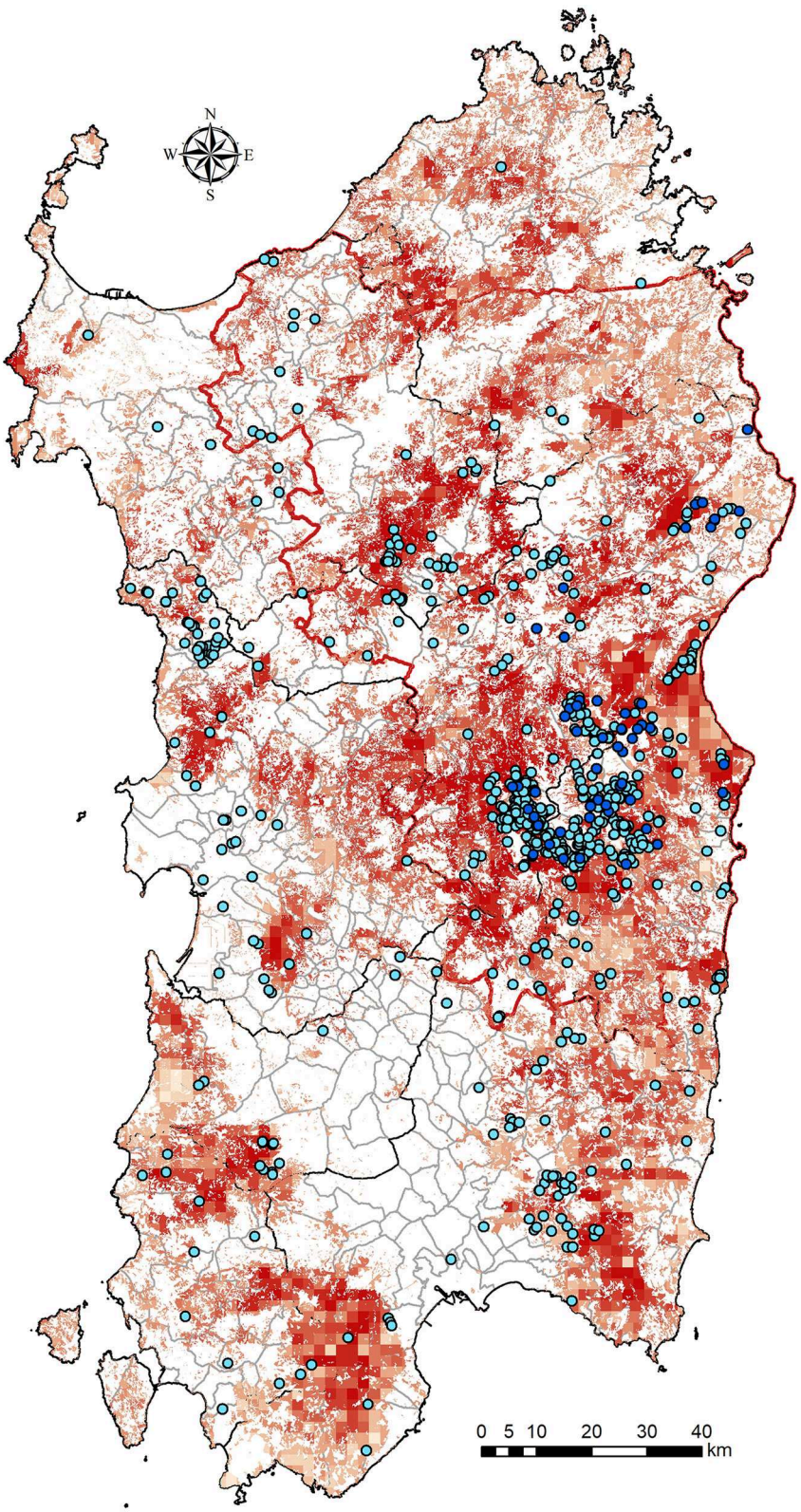

FIGURE 1 | Wild boars density distribution (red squares) in Sardinia and localization of free-ranging pigs (blue dots) during the 2013–2015 years (clear dots) and during 2016–2018 (dark blue dots).

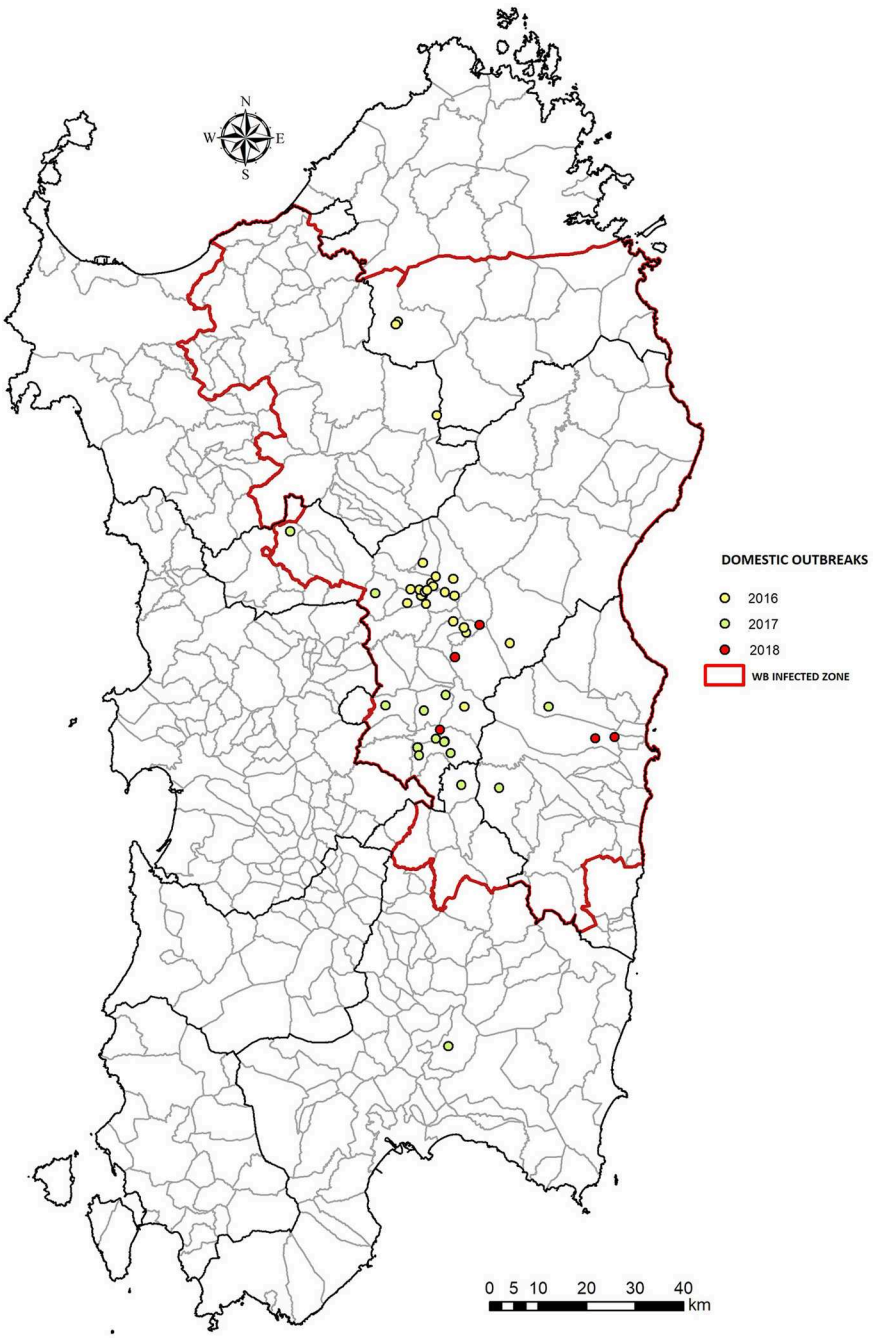

FIGURE 2 | Number of ASF outbreaks in domestic pig farms, between 2016–2018 in Sardinia (Italy). The WB Infected Zone is delimited by the red line.

ASFV, with dead animals showing similar but slightly lower prevalence than hunted animals (2.1%, 95% CI:). However, a significantly higher prevalence has been detected in FRPs for both seroprevalence 53.4% (95% CI: 50.6–56.3) and virus prevalence (2.6%; 95% CI: 2.1– 3.0) (18). Although these prevalence values have decreased with increased culling actions in the same area, these findings seem to confirm the key role of the FRP population in the persistence ASFV in Sardinia over the past 40 years.

Role of Illegal Free-Ranging Pigs (FRPs) in Disease Persistence

From the first ASF notification in Sardinia several eradication plans have been put in place at regional level, with special focus on DPs and WBs populations. From the first eradication program in 1982, many others have been carried out, with widely varying results. Some of these were able to came close to the ASF eradication, but none was able to solve the problem presented by FRPs, which in Sardinia have a key role in the spread and

persistence of disease (17–19, 31). The breed of few pigs in small backyard is common ancient practice in Sardinia. This manner of keeping pigs in free-ranging conditions is inherent to the cultural traditions of their owners; thus, pig owners refuse to change their habits because this would mean losing their cultural identity (13, 14, 32). The old practice has become a problem when the number of illegal FRPs drastically increased using free common land allocated to agriculture (18). Furthermore, illegal FRPs constantly come into contact with WBs, favoring the spread of disease and hindering its control. The role of FRPs in virus persistence has been previously suggested by many researchers (13, 14, 31); however, this issue has only recently been fully elucidated, thanks to the more stringent measures of EP-ASF15-18 to combat FRPs and any kind of illegal activity in the swine sector (18). These illegal unregistered animals have been defined as a virus reservoir that is out of the control of official channels, acting as a virus link between the other two pig populations: legal pigs kept on backyard farms and WBs. Up to the present, 3,800 FRPs have been culled in various parts of central Sardinia. To date, many studies have contributed to better understanding and quantifying the role of the most common factors involved in the persistence of ASF. However, many issues, such as the role of illegal FRPs and socioeconomic status of pig farmers, remain unclear and need to be studied in depth. In the present work, we aimed to perform a quantitative risk assessment based on all suid populations involved in the endemic persistence of ASF in Sardinia, as well as social factors, which could help to identify farms or municipalities at high risk for ASF occurrence or persistence. The result of this analysis is to create a band risk map in which the ASF risk for each Sardinian municipality has been calculated. On the basis of our results, subsequent actions of the EP-ASF15-18 can be planned and implemented, toward the goal of ASF eradication on Sardinia.

MATERIALS AND METHODS

Study Area

Sardinia island has an average area of 24,000 km^2, located in the middle of the Mediterranean Sea (40°03′N 9°05′E). The island characterized by various ecosystems, mounts, woodlands, lowlands, largely uninhabited areas, rivers, long sandy beaches, and rocky coasts. Sardinia is administratively divided into 377 municipal territories, covered by eight different Local Socio-Sanitary Areas (ASSL).

The coexistence of a modern economy within a vast unspoilt territory makes Sardinia one of the few examples in Europe of an integrated rural and modern society. Despite the vastness of its territory, Sardinia is characterized by largely uninhabited areas, that make it the third Italian region in terms of population density (33) Given the sparse population (69 inhabitants/km^2) and the presence of pristine areas, 48% of the island is used for pastoral and agricultural activities; of this proportion, 60% is used for breeding sector, 35% for planting, and the remainder for wood cultivation (34). Although pig livestock in Sardinia dates back to the 6th century BC, swine farming has always been secondary source production, limited to self-consumption. On the other hand, sheep and goat husbandry has always been primary production in Sardinia. Indeed, the culture of breeding one or a few pigs is still a very common practice, mostly in mixed farms where swine and sheeps are commonly breeded (13, 14, 18, 35, 36). As establish by the EP-ASF15-18 ("VI measure concerning the fight against ASF in WB population," Regional decree n.9, 07/06/2017), an inner Sardinian area of a total of 9,000 km^2, named "Infected Zone," has been adopted to apply stronger measure against the disease in sylvatic populations, and includes 121 municipalities (**Figures 2**, **3**).

Data Collection

This retrospective study covered an 8-year period of analysis (2011–2018) and included data regarding to the three suid populations involved in ASF persistence: DPs, WBs, and FRPs. Each of the 377 Sardinian municipalities was considered as epidemiological unit, arranged by study year and linked to all 59 variables collected (**Table S1**). For the purposes of this work, all ASF outbreaks occurred in DP farms between 2011 and 2018 constituted the outcome of this study. Based on official data recorded in the Italian National Information System for the Notification of Infectious Animal Disease (SIMAN) database, an ASF outbreak was defined as a diagnosed disease event in DP farm, in accordance with the World Organization for Animal Health (OIE) Manual of Diagnostic Tests (37). Several characteristics of the infected pig farms such as province and municipality, data of suspected diagnosis, data of confirmed diagnosis, and including both extensive and backyard pig farms, were collected. Because the outcome was the number of ASF outbreaks in the year and municipality of reference [considering both seropositive and virus-positive domestic pig farms (SVDPs)], the dependent variable SVDP follows a count data distribution rather than a normal distribution. We conducted an extensive review of the existing proven risk factors for ASF occurrence, to ensure completeness of this study (17–19, 26, 38–40). An *ad-hoc* database was created to collect detailed and complete information from various sources, based on municipality level data. Data related to DP farms (category A) were as follows: the number of SVDPs for ASF; the number of registered and active farms, including those active throughout the year (activity start date January 1 or later and end date not before December 31); the number of pigs, using data from March 31 as this is the date of the official census; data of animal movement (number of animals introduced to/removed from farms from one municipality to another). These data were collected from the official veterinarian databases: the Italian Veterinarian National Database (BDN), Veterinary Information Systems of the Italian Ministry of Health (VETINFO), and SIMAN. All data collected have been verified on the globally official site for animal health disease (https://www.oie.int/en/animal-health-in-the-world/wahis-portal-animal-health-data/), taking into account the possible inconsistencies due to different update time between Italian national database and OIE international database. The number of official veterinarian checks on pig farms was determined, to calculate the percentage of compliance among DPs. From 2015, this measure is largely used in Sardinia to evaluate the performance of DP farms in terms of ASF management (17). This measure is defined as the proportion

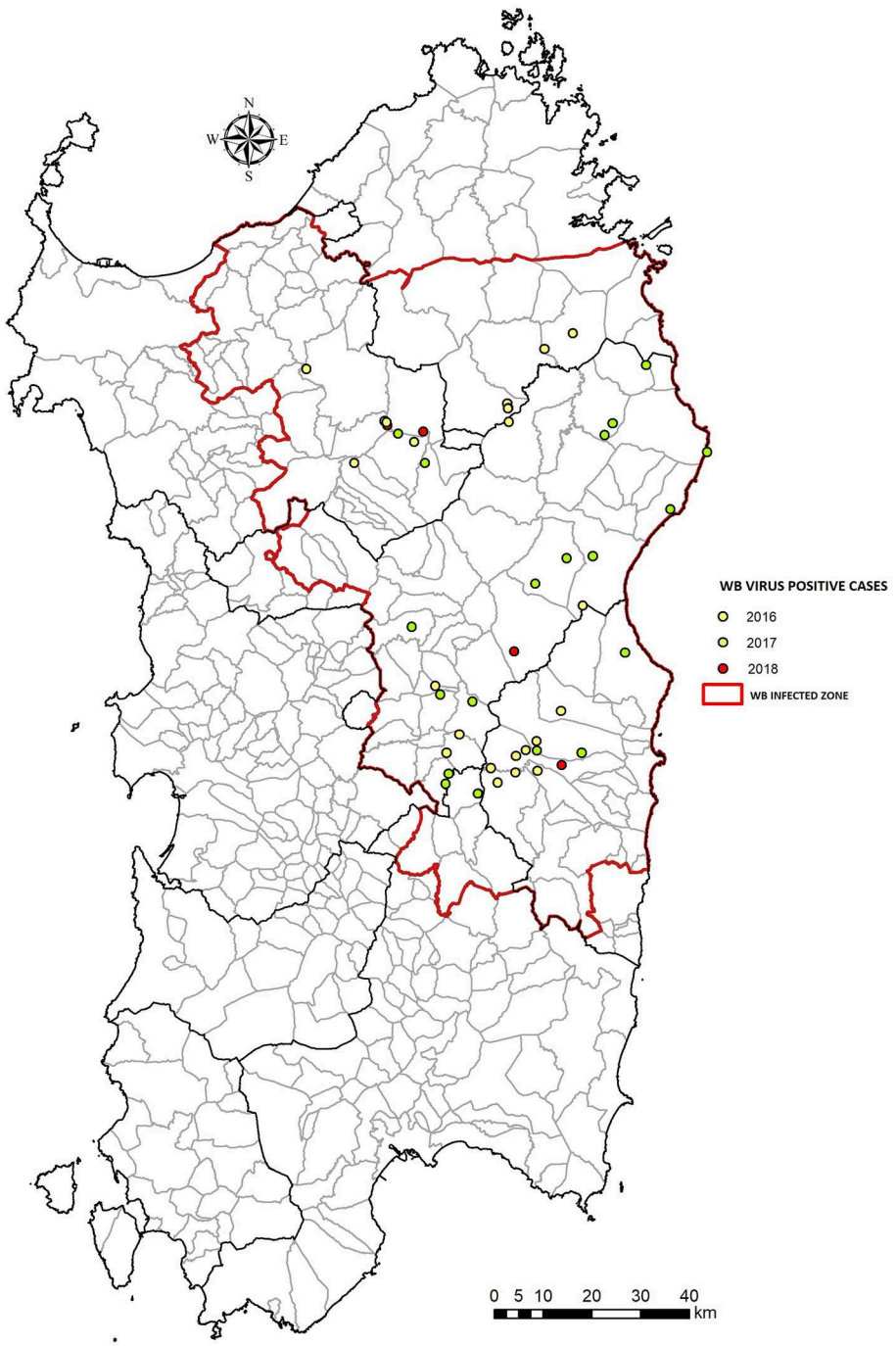

FIGURE 3 | Number of wild boars ASFV positive from 2016 to 2018 in Sardinia (Italy). The WB Infected Zone is delimited by the red line.

of farms complying with EP-ASF15-18 regulation over the total number of farms in the same municipality (reported as a percentage) during the previous year of reference, considering that farmers had a minimum of 6 months and a maximum of 1 year to solve nonconformities found during the previous check (17). Using data on confirmed outbreaks from SIMAN, the present work used the following variables to describe the WB population (category B): areas with WBs, estimated number of WBs living in each municipality, number of hunted and conferred WB, number of WBs tested for the presence of ASFV or ASF antibodies, number of WBs positive for ASFV, number of ASF-seropositive WBs, sex (male or female) and age (older or younger than 6 months) of ASFV-positive WBs, percentage of male ASFV-positive WBs (calculated over all

TABLE 1 | Description at baseline of all variables involved in the African swine fever risk analysis, according to municipalities with zero/one or more cases, related to domestic pigs and wild boars during 2011–2018.

Variable	Municipalities with zero cases (n = 2889)	Municipalities with one or more cases (n = 127)
N farms	34 [18–55]	55 [33–100]
Pigs censed	240 [121–463]	475 [271–843]
Seropositive farms	0 [0–0]	0 [0–1]
Virus positive farms	0 [0–0]	1 [1–2]
Farms checked	17 [8–32]	25 [11–47]
Movements	159 (77)	265 (190)
Compliance DP	87 [61–96]	80 [63–91]
Estimate living WB	177 [88–353]	494 [259–777]
Estimate hunted WB	80 [40–159]	222 [116–350]
Hunted WB	7 [0–34]	36 [15–61]
Sex WB		
Male	4 [0–11]	7 [1–17]
Female	4 [0–7]	5 [1–9]
Age WB		
< 6 months	3 [0–8]	6 [1–8]
≥ 6 months	3 [0–6]	5 [1–7]
WB virus tested	26 [9–56]	39 [21–50]
WB sero tested	27 [9–58]	38 [15–50]
Virus positive WB	0 [0–0]	0 [0–1]
Seropositive WB	0 [0–0]	4 [0–7]
Virus positive WB_M	0 [0–0]	0 [0–2]
Virus positive WB_F	0 [0–0]	0 [0–0]
Virus positive WB_Y	0 [0–0]	0 [0–1]
Virus positive WB_O	0 [0–0]	0 [0–0]
Seropositive WB_M	0 [0–1]	2 [0–3]
Seropositive WB_F	0 [0–1]	1 [0–4]
Seropositive WB_Y	0 [0–2]	3 [0–6]
Seropositive WB_O	0 [0–0]	0 [0–1]
Compliance WB	25 [11–50]	18 [10–27]
FRP presence (yes)	107 (4%)	24 (19%)
FRP culled	85 [15–292]	90 [21–173]
FRP_tested	46 [5–99]	49 [15–195]
FRP virus tested	39 [10–85]	42 [16–187]
FRP sero tested	41 [12–92]	45 [15–194]
Virus positive FRP	0 [0–1]	1 [0–4]
Seropositive FRP	0 [0–32]	18 [2–30]
Sex of the farmer		
Female	37221 (30%)	2009 (25%)
Male	86850 (70%)	6360 (75%)
Age (by 5 years old)	54 [52–57]	55 [49–56]
Educational level (1 = pre-primary, 5 = university)	4 (3,4)	3 (2,3)
Related		
Yes	14888 (12%)	1674 (20%)
Not	109183 (88%)	6695 (80%)
Human population	4957 [1230–10855]	2099 [974–3213]
Q_MDI		
1–very wealthy	623 (22%)	16 (13%)
2– wealthy	410 (14%)	14 (11%)
3–medium	678 (23%)	42 (33%)
4 –deprived	422 (15%)	18 (14%)
5 –very deprived	747 (26%)	37 (29%)
Roads (m^2)	52,692 [30,660–81,258]	72,333 [53,487–108,929]
Water (km^2)	24 [3–220]	37 [31–238]
Tourism	0.87 [0.75–1.14]	0.94 [0.91–1.13]
Flood risk population	5.2 ab / km2	6.1 ab / km2
Thefts	16.8 [15.4–25.4]	19.2 [17.5–24.7]
Robberies	0.33 [0.29–0.37]	0.38 [0.37–0.46]
Forest	2795 [1891–7806]	3564 [2411–9952]
Waste	49.5 [46.6–52.1]	44.3 [37.7–49.4]
Energy production	6.8 [6.0–8.2]	6.7 [5.7–7.8]
Roads (m^2)	52,692 [30,660–81,258]	72,333 [53,487–108,929]
Water (km^2)	24 [3–220]	37 [31–238]
Employment	52.3 [50.4–52.7]	50.7 [50.5–52.8]

Data expressed as mean and standard deviation (SD), median and quartile (I–III), frequency (n) and percentage (%), by municipality.

ASFV-positive WBs), percentage of young ASFV-positive WBs (calculated over all ASFV-positive WBs), sex (male or female) and age (older or younger than 6 months) of ASF-seropositive WBs, percentage of male ASF-seropositive WBs (calculated over all ASF-seropositive WBs), and percentage of young ASF-seropositive WBs (calculated over all ASF-seropositive WBs). The WB density estimation of the Faunal Vocation Chart of the Sardinian Region performed by Apollonio in 2012 was used to calculated the number of WB for each Sardinian municipality (41). Furthermore, it was necessary to identify those parts of the territory that could support the habitat cycle of these populations and to define macro areas within which there are about 1,000 WBs, according to current EU regulations (2003/422/EC approving an ASF diagnostic manual, Chapter IV(H)). Alternatively, sufficiently separated parts of the territory were distinguished, in which specific WB metapopulations are present. The overlap of these areas with the administrative limits of municipalities makes possible a correct representation of the wild populations per municipality. According to EP-ASF15-18 rules (42), all WBs hunted inside an infected zone should be tested for the presence of ASFV antibodies. Based on this, supposing that 45% of the total estimated number of live WBs are hunted during the hunting season, the percentage of WB compliance inside an infected zone is calculated as the proportion of WBs hunted over the total estimated WBs hunted in the same municipality during the year of reference. However, in Sardinian regions unaffected by ASF, a total of

58 WBs for each area must be serologically tested, upon which calculation of compliance is based. Based on Regional Wildlife Agency reports and data collected from ongoing actions of FRP depopulation, we collected information about the presence/absence of FRPs and their number, the number of culled FRPs and FRPS laboratory-tested for ASF, and virological and serological prevalence of ASF in these populations, to describe the illegal FRP population (category C). Given that recent studies suggest that socioeconomic status of farmers is strictly related to livestock disease risk (17, 31, 32, 38, 43) and a comprehensive and in-depth knowledge of relevant risk factors is basic requirement for disease prevention (44, 45), we collected a large number of covariates (category D) from the Italian Statistician National Institute database, specifically AgriISTAT (46). To describe the actual situation of pig breeding in Sardinia, data for the characteristics of farm owners, such as sex, age, level of education. and type of farm were collected using fiscal codes recorded in the BDN. A series of social indicators, called territorial indicators for development policies (47) were collected at municipality level and included in the present analysis, given their previously demonstrated contribution to describing the risk of ASF in DPs and WBs (32); these indicators included the Material Deprivation Index (MDI), employment rate, cultural demand, micro-criminality index, rate of tourism in low season, proportion of the population at risk of floods, rate of reported thefts and robberies, forest surface, amount of energy produced from renewable sources, and amount of differentiated waste. Areas (in square kilometers) of asphalted road and water bodies were collected from the Regional Geographical Service (Servizio Informativo e cartografico Regionale, Regione Sardegna, 2011) at municipality level, and 216 these were considered as potential covariates.

Statistical Analyses

An *ad-hoc* database has been created using Microsoft Office Access system and all information collected was double blinded and password-protected stored to ensure privacy. Extensive data checking was performed to evaluate the consistency and accuracy of the data collected and any disagreement was analyzed and corrected. Considering epidemiological, experimental, and statistical issues (i.e., non-collinearity), several putative and potentially relevant predictors were detected. The baseline distribution of each explanatory variable was summarized and described, according to municipalities with zero or more/equal to at least one case of ASF (**Table 1**, **Figure 4**). Most collected variables were quantitative and expressed as mean and standard deviation (SD) or median and interquartile range (IQR). Frequency (n) and percentage (%) were used to describe categorical data. Considering both experimental and statistical requirements, the features in **Table 1** were evaluated as potential covariates in our analyses. The outcome SVDPs represents non-negative count data, and regression techniques cound be used to estimate the mean value distribution. Poisson regression and negative binomial (NB) regression are among the most popular count data regression methods used in epidemiology (48–50). Although Poisson model is suitable for count data with mean equal to its variance, whereas the NB model is more appropriate in condition of overdispersed data with an excessive presence of zero values (51, 52), such as those in our dataset (**Figure 5**). Thus, the final developed model was a negative binomial regression model (NBRM). This model assumed that the outcome variable Y is the total number of events occurring in a specific space-time interval (here, the number of ASF-positive farms, for both the presence of ASFV or ASF antibodies, in each specific municipality). The earliest definition derived from the binomial distribution characterizes NBRM as the number of failures before the (1/α)th success. Recently, parametrizations have been used to describe the NBRM as derived from a mixture of gamma and Poisson distributions (50, 53). A mixed-effects NBRM was applied (Equation 1), including random effects (year and municipality), assuming not independent observations between years and municipalities and to control this level. Considering a series of M independent clusters, and conditional on the latent variable ζij and a set of random effects *u*j,

$$y_{ij}|\zeta_{ij} \sim Poisson\,(\zeta_{ij})$$

and

$$\zeta_{ij}|u_j \sim Gamma\,(r_{ij}, p_{ij})$$

and

$$u_j \sim N\,(0,\ \Sigma)$$

where y_{ij} is the count response of the ith observation, i = 1,…, n_j, from the jth cluster, j = 1,…, M, and r_{ij} and p_{ij} were parameterized using the mean overdispersion:

$$r_{ij} = \frac{1}{\alpha} \quad \text{and}\, p_{ij} = \frac{1}{1+\alpha\mu_{ij}}$$

The random effects u_j are M realizations from a multivariate normal distribution with a mean of 0 and q×q variance matrix Σ. The probability that a random response y_{ij} takes the value y and can be modeled by Mixed-effects NBRM is then given by:

$$\Pr\left(y_{ij} = y|\mathbf{u}_j\right) = \frac{\Gamma\left(y + r_{ij}\right)}{\Gamma\left(y+1\right)\Gamma(r_{ij})} {p_{ij}}^{r_{ij}} \left(1 - p_{ij}\right)^{y} \qquad (1)$$

Univariable NBRM was developed to initially tested each of the explicative variables and to quantify the association between these factors and the distribution of the number of SVDPs. Statistically significant risk factors with $p \leq 0.20$ in the univariable analysis, were considered for inclusion in the multivariable analysis. Irrelevant risk factors with likelihood ratio test results of $p \geq 0.05$ were deleted from the multivariable model, based on a stepwise selection procedure (54). Before inclusion into the multivariable model, collinearity presence was evaluated for all those variables with a *p*-value ≤ 0.20 in the univariable analysis, to ensure a variance inflation factor (VIF) <10 (55–57). Interaction therms considered in the multivariable model were between the number of FRPs and ASF-positive WBs. Based on the lowest Akaike information criterion (AIC), if multicollinearity

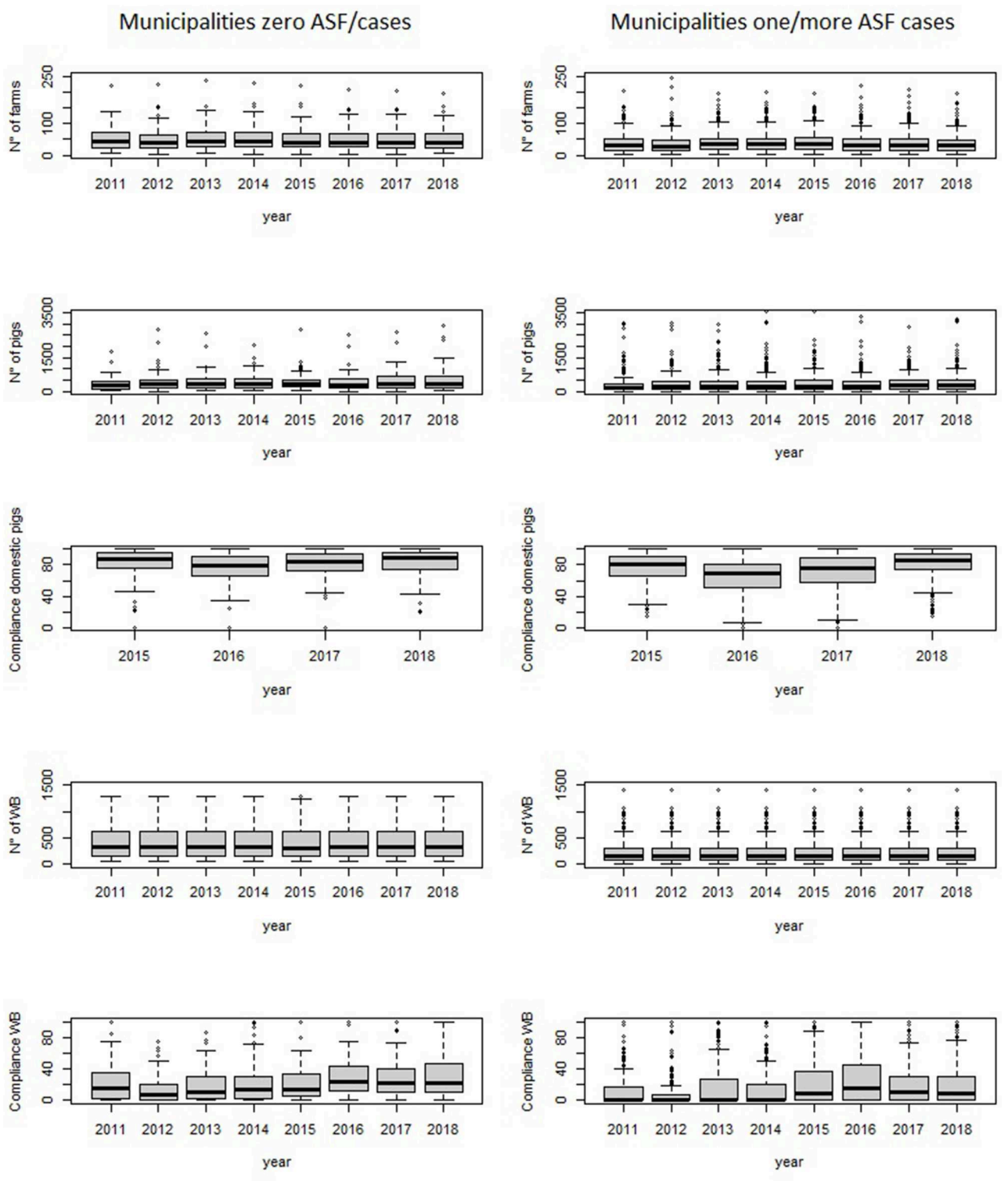

FIGURE 4 | Baseline distribution of the number of farms and number of pigs, domestic pigs compliance with ASF-EP15-18, number of wild boar and compliance with ASF-EP15-18 rules for hunting season management, from 2011 to 2018, according to municipalities with zero ASF cases and one or more cases.

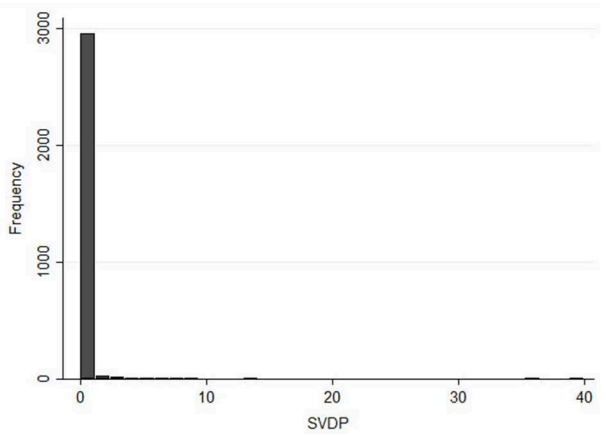

FIGURE 5 | Histogram distribution of NBRM's outcome, number of SVDP in all Sardinian municipalities during the 2011–2018 years.

was detected, the predictors involved were identified and one or more was removed (58–61). The final model was assessed using a "training dataset" (years 2015–2018) for internal validation, against a "test dataset" (year 2011–2014) that was not used to create the model, but rather for external validation (62). An assessment of goodness-of-fit of the model between the predicted and observed values was applied, to understand if the data were well-modeled by the NBRM, based on a residuals analysis and the Spearman correlation coefficient. The results of the NBRM are presented in **Table 2** as the adjusted odds ratio (ORadj), calculated as proposed by Gardner in 1995 (63). The municipality risk profile was generated, based on values obtained from the NBRM, and the predicted values for each municipality were calculated. To apply EP-ASF15-18 disease control measures, which lay out different actions based on the risk band (from 1 to 5), the predicted values were sorted in ascending order and divided into five equal parts (quintiles, Q1 … Q4). A quintile is one of five values that divide a data range into five equal parts, each being 1/5th (20%) of the range. Given N, the ordered population value (here, the 377 predictor values for each municipality), each quintile is calculated as:

$$Q_j = \frac{j^*(N+1)}{5} \quad j = 1, \ldots, 4$$

Figure 6 shows the different risk levels for each Sardinian municipality, and the different type of control measures for each risk band are illustrated in **Table 3**. All the tests were two-sided and a *p*-value level of 0.05 or less has been considered significant. The statistical analyses were made with R Version 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) and Stata 13 Release 13 (StataCorp LP, College Station, TX, USA).

TABLE 2 | Negative binomial regression model results used to obtain the number of ASF positive farms in relation to all known factors related to domestic pigs, wild boars, illegal free-ranging pigs, and farmer sociodemographic characteristics, using data collected in Sardinia 2011–2018.

Variable	OR_{adj} [95% IC]	*P*-value
N farms	1.013 [1.007–1.025]	< 0.0001
Pigs censed		
< 120	1.00	
≥ 120	2.581 [1.314–5.067]	0.006
Compliance DP	0.821 [0.803–0.867]	< 0.0001
Estimated living WB	1.001 [1.001–1.002]	0.007
Virus positive WB	1.198 [1.042–1.378]	0.011
Virus positive WB_M_perc	1.009 [1.002–1.016]	0.011
Virus positive WB_Y_perc	1.021 [1.001–1.045]	0.039
Sieropositive WB	1.152 [1.049–1.264]	0.003
Seropositive WB_M_perc	1.017 [1.012–1.022]	< 0.0001
Seropositive WB_Y_perc	1.023 [1.015–1.034]	< 0.0001
Compliance WB		
<21%	1.00	
≥21%	0.604 [0.398–0.916]	0.018
FRP presence	5.067 [3.068–8.368]	< 0.0001
FRP presence * WB positive	1.918 [1.872–1.966]	0.001
Age (by 5 years old)	0.851 [0.740–0.973]	0.019
Sex		
–Female	1.00	
–Male	1.304 [1.176–1.453]	<0.0001
Human population		
< 5000	1.00	
≥ 5000	0.470 [0.299–0.738]	0.001
Q_MDI		
1–very wealthy	1.00	
2– wealty	1.441 [0.593–3.492]	0.420
3–medium	2.402 [1.173–4.919]	0.017
4 –deprived	1.706 [0.743–3.922]	0.208
5 –very deprived	1.864 [1.385–2.551]	< 0.0001
Roads		
< 70.000	1.00	
≥ 70.000	1.227 [1.031–1.450]	0.023
Employment	0.955 [0.917–0.974]	0.002
Micro-criminality	1.432 [1.418–1.469]	< 0.0001
Tourism	1.196 [1.081–1.325]	0.001
Forest	1.164 [1.038–1.316]	0.013

RESULTS

The present risk analysis, conducted to create a risk score, included data related to all Sardinian pig farms, hunting season management, and data from FRP culling actions, based on the 8-year study period (2011–2018). Data were collected by year ($n = 8$) and municipality ($n = 377$). The *ad-hoc* database contained a total 3,016 records. Only 127 records represented municipalities with SVDP cases; all others were equal to a zero value (**Figure 5**). Descriptive baseline characteristics were divided according to municipalities with zero cases and those with at least one case (**Table 1**). As expected, most features were different between these two categories. In municipalities with at least one case, factors hypothetically associated with higher risk of ASF spread were more present. Generally, most

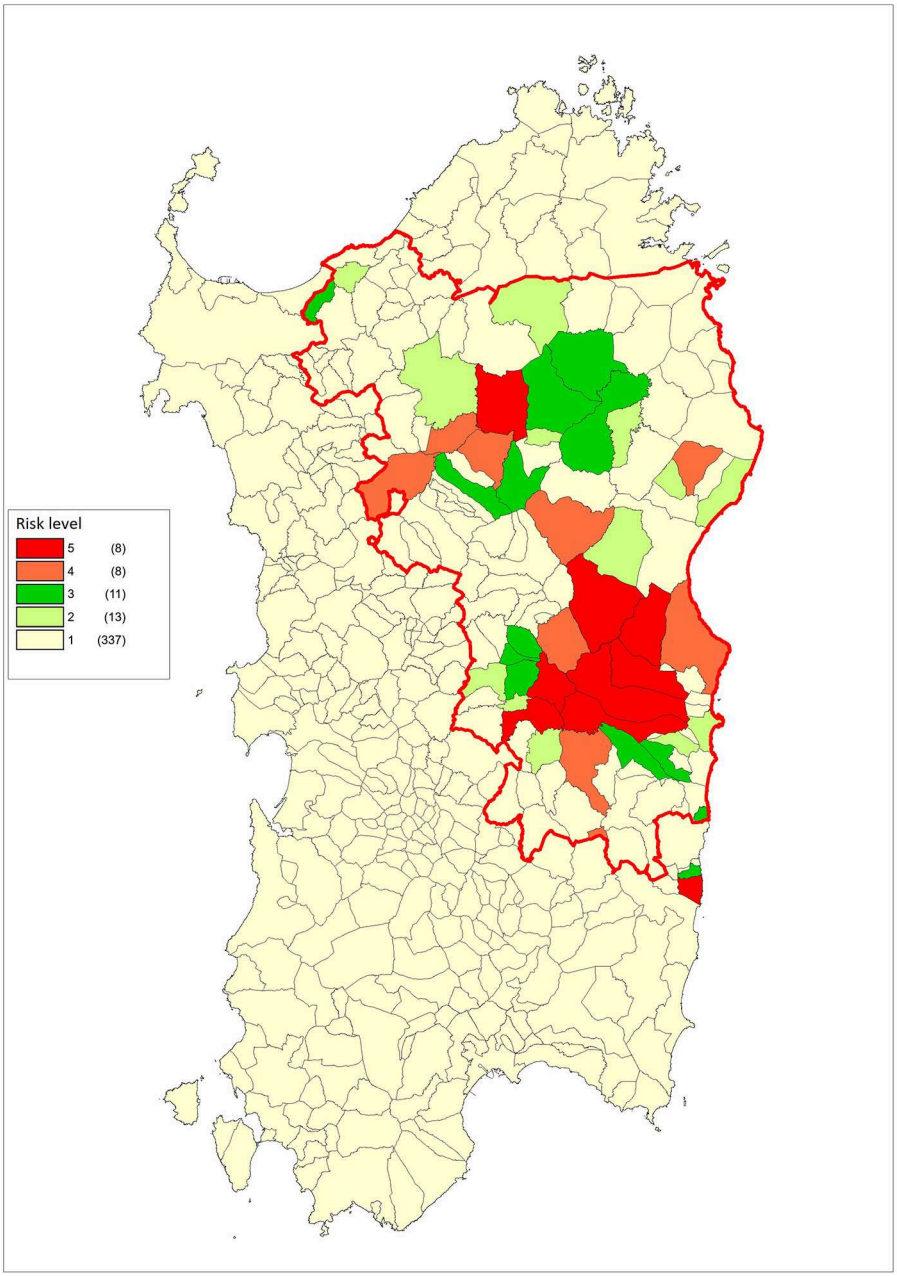

FIGURE 6 | Choropleth map of the different risk levels for each Sardinian municipality.

of the features assessed showed higher levels in municipalities with ASF cases, except for DP and WB compliance, human population, farmer educational level, and employment rate. We tested several potential interactions reported in previous works or according to veterinary experience (i.e., presence of illegal FRPs and WBs, total animal movements and road surface, number of pigs and road surface). However, only the interaction between infected WBs and the presence of FRPs was significant and gave a better-fitting model (AIC: 1126.7 vs. 1171.5). All non-relevant covariates were excluded, as planned in the model approach.

Negative Binomial Regression Model Results

Overall, 50 variables collected for the period of interest, were considered for inclusion into the final mixed-effects NBRM (**Table 1**). Totally, 29 were excluded from the final model because of multicollineraity (VIF $>$ 10 and/or Spearman test statistically

TABLE 3 | Guidelines for domestic pig farm's control measures, defined by the Sardinian Eradication Plan 2015–2018.

Risk band	Certified farm	Checked farm	Not-checked farm (during 12 months before)	Illegal free-ranging pig breeding
1–2–3	Clinical check Anagraphical check Biosecurity check Welfare check Serological control **only if** identified risk of disease introduction ($P \geq 10\%$;CI95%)	Follow-up and non-conformities verification: i. Clinical check ii. Anagraphical check iii. Biosecurity check iv. Welfare check Serological control **only if** identified risk of disease introduction ($P \geq 10\%$;CI95%)	i. Clinical check ii. Anagraphical check iii. Biosecurity check iv. Welfare check v. Serologicalcontrol	Contrasting activities to clandestine breeding e illegal handling Including the sanctions/actions of depopulation
4–5	2 × Clinical check 2 × Anagraphical check 2 × Biosecurity check 2 × Welfare check Serologicalcontrol			

significant), or owing to a non-significant association with outcome in the univariate analyses ($p > 0.20$). Twenty one of these were finally included, The results obtained by the analysis of the 21 variables are presented in **Table 2** and expressed as the ORadj and 95% CI, with p-values. The number of farms and pigs in each municipality revealed a role of significant risk factors favoring ASF outbreak, with ORadj 1.013 (95% CI: 1.007–1.025), $p < 0.001$ and ORadj of 2.581 (95% CI: 1.314–5.067), $p = 0.006$ with $\geq$ 120 pigs on the farm. However, increased farm and veterinary check compliance with the EP-ASF15-18 the previous year significantly decreased the probability of counting one case or more in the same municipality the following year (ORadj = 0.821; 95% CI: 0.803–0.867). The final results of the NBRM showed that a total of eight different features related to the WB population that was live and/or tested positive in the previous hunting season significantly contributed to the risk of ASF cases ($p < 0.05$). In particular, the effect was equal to 1% greater risk (ORadj = 1.001; 95% CI: 1.001–1.002) for each WB estimated to live in the same municipality, and 20% (ORadj = 1.198; 95% CI: 1.042–1.378) and 15% greater risk (ORadj = 1.152; 95% C: 1.049–1.264) if hunted and tested WBs were ASFV positive or ASF-antibody positive, respectively. In addition, an increasing percentage of male WBs that were virus positive and seropositive increased the risk of new SVDPs in the same municipality the following year by 1% (ORadj = 1.009; 95% CI: 1.002–1.016; $p = 0.011$) and 1.7% (ORadj = 1.017; 95% CI: 1.012–1.022; $p < 0.0001$), respectively. Likewise, the probability grew about 2% (ORadj = 1.023; 95% CI: 1.015–1.034; $p < 0.0001$) if positivity (virus or seropositivity) was found in young animals (between age 0 and 6 months). As well as the effect found for DP compliance, compliance with hunting season management rules was a protective factor against the risk of SVDPs the following year. In particular, when WB compliance was greater than 20%, the risk was significantly lowered by 40% (ORadj = 0.604; 95% CI: 0.398–0.916; $p = 0.018$). As hypothesized, the presence of FRPs in the same municipality increased the risk of SVDPs fivefold (ORadj = 5.067; 95% CI: 3.068–8.368; $p < 0.0001$), and about twice if a positive WB was found the previous year in the same municipality as FRPs (ORadj = 1.918; 95% CI: 1.872– 1.966; $p = 0.001$). Older age of farmers seemed to be protective upon an increased number of outbreaks (ORadj = 0.851; 95% CI: 0.740–0.973; $p = 0.019$) whereas the opposite effect was seen for male vs. female sex of the farmer (ORadj = 1.304; 95% CI: 1.176–1.453; $p < 0.0001$). Comparison between the first MDI level (lower deprivation) and others, suggested an increased probability of ASF outbreaks on farms, with statistically significant results between MDI level-1 and MDI level-3 (medium deprivation level) (ORadj = 2.402; 95% CI: 1.173–4.919; $p = 0.017$) or MDI level-5 (very deprived level) (ORadj = 1.864; 95% CI: 1.385–2.551; $p < 0.0001$). Regarding to ISTAT socioeconomic indicators, a low probability of outbreaks on farms located within municipalities with high employment rates (ORadj = 0.955; 95% CI: 0.917–0.974; $p = 0.002$) has been highlighted by the NBRM. Higher counts of outcome variables were observed with higher rates of micro-criminality and tourism in the low season: ORadj = 1.432; 95% CI: 1.418–1.469; $p < 0.0001$ and ORadj = 1.196; 95% CI: 1.081–1.325; $p = 0.001$, respectively. Finally, regions with asphalted road area of more than 70,000 m^2, high forest surface coverage, and a human population of < 5000 people showed a significantly higher risk of SVDPs ($p < 0.05$). The results of the likelihood ratio test (LR, $X^2 = 262.55$, probability $> X^2 = 0.0001$) supported the choice of the mixed-effects NBRM against the mixed-effects Poisson regression model. Based on internal validation criteria (residual mean = $4.18^{*}10^{-5}$, SD =

$2.05*10^{-6}$, Spearman correlation coefficient = 0.846, $p < 0.0001$) and external validation criteria (residual mean = $3.99*10^{-3}$, SD = $7.82*10^{-4}$, Spearman correlation coefficient = 0.793, $p < 0.0001$), it is possibile to affirm that the NBRM could properly predict the correct outcome with a strong goodness-of-fit.

DISCUSSION

Sardinia is the European area that has been affected by ASF the longest, since its first notification in 1978. Furthermore, Sardinian territory is the only region "where the epidemiological situation has become stabilized and the disease has become endemic," such that the region is the only one on the European continent included in Part IV (highest risk) of the European Commission Decision on ASF control measures (Decision 2014/709/EU). Despite the rapid spread of ASFV across Europe and parts of the Asian continent, excluding an isolated and quickly resolved case in northern Italy in 1983 owing to illegal introduction of pork from Sardinia, there is no evidence of disease spread from Sardinia to other countries (6). As demonstrated by many studies on the Sardinian ASFV genotype, Sardinian isolates are included in a cluster of genotype I (64–66), whereas genotype II circulates in other European countries, Transcaucasia, Russia, and China (67). This very low genetic variability determines the placement of strains into one of two clusters depending on the temporal distribution: subgroup III, including viruses isolated up to 1990, and subgroup X, including isolates identified from 1990 to 2009 (68, 69) A total of 11 outbreaks occurred during the first 8 months of last year whereas from September 2018 to the present, no virus evidence has been reported on DP farms in Sardinia. Nevertheless, in the previous hunting season (1 November 2018 to 31 January 2019), a total of four WBs were found to be ASFV positive and 106 presented antibodies against ASF. The prevalence of the disease during the past 7 years has decreased drastically in Sardinia, among both wild and domestic populations. During the 40 years of control and eradication efforts against ASF, different regionaleradication plans have been implemented, many of which are similar to those applied in countries where the disease has been eradicated, such as Spain and Portugal (70, 71) and some countries have been able to almost entirely eradicate ASF. However, the last eradication plan in Sardinia achieved the most striking results in terms of significant decline in disease among all the suid populations involved. The EP-ASF15-18 addresses not only improved target veterinary measures but also measures to eliminate FRPs to better manage the hunting season and animal movements, and providing greater general incentives toward good biosecurity practices. In particular, this plan is focused on checks and measures to be applied on DP farms, planned by year. As reported in **Table 3**, different timetables are planned based on municipality risk level. Until now, classification of each municipality's risk level was performed using qualitative analysis (39). Ours is the first work to describe the risk level based on the results of multivariable predictors, with external and internal data validation. From numerous previous studies as well as endemicity of the disease for more than 40 years, it is now known that the situation of ASF in Sardinia is very different from that of all other countries. It is almost as if the virus has found its perfect conditions for thriving within the unique Sardinian epidemiological cycle (6, 13, 14, 17, 18, 27, 31, 43, 72, 73). As described by Laddomada et al., in 2019 (18), the strong measures applied against illegal FRP populations have marked a turning point in the story of the fight against ASF in Sardinia, with record results in terms of declines in the disease. The typically Sardinian epidemiological cycle, described earlier in the present work, involves the three Sardinian suid populations, generating a virus transmission cycle that is very difficult to control, given the role of FRPs as a link between the WB population and DPs. For these reasons, the quantitative risk analyses performed here has taken into account many different features related not only to pigs bred in backyard farms, but also the local WB population and pigs that are illegally bred in a free-range manner, as well as the role of socioeconomic and demographic factors. Some results found in previous studies have been confirmed in this work for DP farms, such as the contribution of the number of farms and recorded number of animals to new ASF outbreaks in the same municipality (17, 27, 43). As demonstrated by the FAO (26), the key roles of both animal density and low biosecurity in disease maintenance are evident. The results of our study underline this relationship, showing a statistically significant increased risk with increased DP population and WB density, as well as the presence of the third population (FRPs). Furthermore, the simultaneous presence of FRPs and infected WBs doubles the risk of observing ASF infections on farms the following year. Thus, the close coexistence of domestic and wild pig species makes disease management more difficult, as underlined by Pastoret et al. (74). The problems related to this situation are many and complex because the geographical, ecologic, and economic conditions that permit transmission among populations are different and extremely variable, as is surveillance. Whereas, the situation may be relatively simple for domestic animals, the same consideration may not be applied to wild species, given the differences in their variety and population density. The Sardinian situation is complicated even more by related social and cultural issues that hinder ASF eradication. First is the cultural identity of pig farmers and resistance to respecting control measures, particularly those regarding elimination of FRPs, an ancient practice that is culturally rooted in the central Sardinian region (6, 17, 18, 38, 72, 73). The Sardinian context is that of small communes with very few inhabitants and almost no services that follow ancient and time-honored cultural traditions, in contrast with larger cities with very crowded areas and a capital defined as a metropolis. The need to take into account socioeconomic status has been suggested by the World Organization for Animal Health Guidelines in 2014, which have affirmed that animal disease management should consider several non-financial factors (i.e., social, cultural, and religious) affecting the livelihood and well-being of animal owners such as pastoralists, farmers, and small-scale backyard producers. These factors can be important incentives in participation or non-compliance and can ultimately impact the success of sanitary programmes (75). In Sardinia, the need to include social and economic factors in risk analysis is particularly pertinent, since animal farming has always been

one of the main economic resources. With reference to these particular Sardinian conditions, according to expert opinions and previous studies, the greatest risk for ASF spread and persistence has been determined to be located in smaller countries and rural contexts (17, 32). All social features included in the present work contribute to describing the typical Sardinian situation where high-risk areas are identified in deprived municipalities (Q_MDI = 5) with very few inhabitants, low employment rates, and high levels of micro-criminality. Furthermore, farmers at high risk of being associated with SVDPs were found to be young males with low educational levels, as also reported by Loi et al. for many different diseases in Sardinia (32). Although these factors are not directly associated with ASF development and spread, they could help to create conditions under which the disease can spread. Numerous limitations of the present work are related to data traceability, accuracy, and underreporting data. However, the checks carried out before at the beginning of the analysis may have been at least partly limited by problems related to registration in the BDN, leading to possible generation of selection bias. Furthermore, the present study is not exempt from the typical limitations of risk analysis with the use of proxies, which may give a reflected measure, characterized by evident less accuracy, of features not directly measured. For example, the significant role of tourism in the low season as an indicator of disease occurrence should not be interpreted as a direct effect but rather as a proxy for a low biosecurity context. During the previous autumn and winter seasons in particular, different traditional popular festivals take place one after the other in central Sardinia, during which pig meat products are elaborated and sold, sometimes without permission in an illegal context and without veterinary controls, favoring contamination by and spread of ASFV. These events are typical of inland areas, where a higher number of farms are recorded and where the epicenter of the disease has been identified in many studies conducted over the last 40 years (6, 13, 18, 31, 71, 76, 77). Although the results of the present work were obtained using data of Sardinia and are specific to this context, and despite the use of specific social variables using an Italian database (ISTAT), our findings can be considered a point of departure for future investigation. Furthermore, the present risk analysis reveals many new and unique details regarding the Sardinian ASF cycle (i.e., the interaction between infected WBs and FRPs and their association with ASF risk on DP farms, and the valid and effective use of social factors to describe at-risk areas). Further confirmation of our results, together with previous knowledge about this disease, could be useful to understanding the disease cycle in countries with similar conditions such as Ukraine and other parts of Eastern Europe (78). The implementation of the latest eradication plan and its effectiveness throughout Sardinian territory (described by the two compliance measures) contributed to the large observed decrease in ASF during the past 6 years, although the region remains endemic. As outlined previously, active surveillance conducted in endemic areas with decreasing prevalence is generally the most suitable approach, which includes monitoring the effect of interventions on the prevalence of infected animals. However, the EFSA's suggestions for countries where the disease is endemic in WB, such as Sardinia, define the passive surveillance as the most effective and efficient method for early detection of ASF in WBs, particularly in areas where ASF has not been detected for several time (79, 80). The decrease of serological and virological findings, indicating levels of disease activity, and accompanying improvement of the situation in DPs and WBs suggest the need to continue this strategy through the final phase of the eradication program.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript/**Supplementary Files.**

AUTHOR CONTRIBUTIONS

FL, SC, AC, and SR: conceptualization, investigation, writing—original draft, review, and editing. FL: data curation, formal analysis, and validation. FL and SR: methodology. SR and AC: project administration, resources, and supervision. SC and FL: visualization.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00299/full#supplementary-material

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Free-Ranging Pig and Wild Boar Interactions in an Endemic Area of African Swine Fever

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African swine fever virus (ASFV) is spreading throughout Eurasia and there is no vaccine nor treatment available, so the control is based on the implementation of strict sanitary measures. These measures include depopulation of infected and in-contact animals and export restrictions, which can lead to important economic losses, making currently African swine fever (ASF) the greatest threat to the global swine industry. ASF has been endemic on the island of Sardinia since 1978, the longest persistence of anywhere in Eurasia. In Sardinia, eradication programs have failed, in large part due to the lack of farm professionalism, the high density of wild boar and the presence of non-registered domestic pigs (free-ranging pigs). In order to clarify how the virus is transmitted from domestic to wild swine, we examined the interaction between free-ranging pigs and wild boar in an ASF-endemic area of Sardinia. To this end, a field study was carried out on direct and indirect interactions, using monitoring by camera trapping in different areas and risk points. Critical time windows (CTWs) for the virus to survive in the environment (long window) and remain infectious (short window) were estimated, and based on these, the number of indirect interactions were determined. Free-ranging pigs indirectly interacted often with wild boar (long window = 6.47 interactions/day, short window = 1.31 interactions/day) and these interactions (long window) were mainly at water sources. They also directly interacted 0.37 times per day, especially between 14:00 and 21:00 h, which is much higher than for other interspecific interactions observed in Mediterranean scenarios. The highly frequent interactions at this interspecific interface may help explain the more than four-decade-long endemicity of ASF on the island. Supporting that free-ranging pigs can act as a bridge to transmit ASFV between wild boar and registered domestic pigs. This study contributes broadly to improving the knowledge on the estimation of frequencies of direct and indirect interactions between wild and free-ranging domestic swine. As well as supporting the importance of the analysis of interspecific interactions in shared infectious diseases, especially for guiding disease management. Finally, this work illustrates the power of the camera-trapping method for analyzing interspecific interfaces.

Keywords: free-ranging pig, wild boar, camera trapping, interactions, critical time window, African swine fever

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INTRODUCTION

African swine fever (ASF) is a viral disease of swine, affecting both domestic pigs, and wild boar (*Sus scrofa*) of all ages and sexes (1). There is no vaccine nor treatment available to fight ASF. Therefore, the control strategy is based on the implementation of strict sanitary measures (2, 3). These measures include depopulation of infected and in-contact susceptible animals, based on the specific contingency plans for ASF of each affected country, and export restrictions, which can lead to important economic losses. These devastating economic consequences suffered by affected countries along with the unprecedented spread through Eurasia since 2007 (4, 5), make ASF the current greatest concern to the global swine industry. ASF was first detected outside Africa in 1957 on the Iberian Peninsula, from where the virus spread throughout many other countries in Europe and Central and South America. These outbreaks have been effectively controlled except in Sardinia (Italy) (6), where the disease has remained endemic since 1978 (7).

The four-decade endemicity of ASF in Sardinia has led to substantial efforts to identify factors responsible for the failure of eradication programs of the island (7–12). When ASF was endemic on the Iberian Peninsula, the presence of soft ticks of the genus *Ornithodoros* (*O. erraticus*) proved to be one of the greatest challenges in controlling the spread (6). However, *Ornithodoros* ticks are not present in Sardinia (13). Instead, the likely endemic factors appear to be lack of farm professionalism including limited biosecurity conditions, high densities of wild boar in the area and local practices such as raising non-registered domestic pigs (free-ranging pigs) in communal lands (7–9, 11, 12, 14). Within these factors, several studies suggest that the most important is the presence of non-registered domestic pigs, which is related to socioeconomic, cultural and traditional aspects (7–9, 14–17).

These animals are domestic pigs bred under free-ranging conditions for their entire life span, although they are occasionally fed by their owners during winter and summer seasons, when food is scarce in the natural environment (18). This practice is strongly rooted in tradition because it costs little to feed the pigs and their meat can fetch high prices on the local market. Sardinian authorities forbade the practice of raising free-ranging pigs in 2012 (19), and this ban was reiterated in the latest ASF eradication plan (PE-ASF15-18; Regional Decree Number 5/6, 6 February 2015), which also called for rapid eradication of cases when they occurred on registered holdings and incentivized good swine breeding practices (20, 21). However, no information on the sanitary status of free-ranging pigs was available up to 2019, and it showed higher ASF prevalence in free-ranging pigs than in wild boar and registered domestic pigs (14).

Susceptible pigs in direct and indirect contact with infected wild boar with ASF virus (ASFV), strain Armenia08, became infectious (22). This suggests that ASFV can be transmitted via direct between wild boar and domestic pigs, but also by environmental contamination [indirect; (22)] Free-ranging pigs share habitat with wild boar and can serve as a virus reservoir in Sardinia that provides a route of transmission between domestic pigs kept in backyards and wild boar populations (14). In fact, a recent study identified the combination of estimated wild boar density and mean altitude above sea level as one of the most significant risk factors, and free-ranging pigs commonly inhabit in mountainous areas (8). These considerations support the hypothesis that interaction between free-ranging pigs and wild boar was substantial to maintain ASF in Sardinia, yet we are unaware of published analyses of these interactions. Studies in other contexts have shown that intra- and interspecific interactions are socially, spatially and temporally structured, and their variations can influence the magnitude of outbreaks and the endemicity of infectious diseases (23–28). Different approaches have been taken to study animal interactions, such as questionnaires (26, 29), direct observations (30, 31), and telemetry (24, 25, 28). Another method is camera trapping, which provides a non-invasive way to collect direct and visual evidence of interactions (23, 32–34).

The current study, based on camera trapping, provides perhaps the first detailed insights into the frequency of direct and indirect interactions between free-ranging pigs and wild boar in an ASF-endemic area. The results support the importance of direct and indirect interactions between wild and free-ranging domestic pigs in ASF endemicity.

MATERIALS AND METHODS

Study Area

The study was carried out in two Sardinian provinces, Nuoro and Ogliastra, located in the central-east part of the island, where illegal breeding of free-ranging pigs is especially common (8, 15). This region has a Mediterranean climate with a mean temperature of 14°C year-round, 12.4°C in the spring, and 20.5°C in the summer (35). These provinces are traditionally considered the ASF-endemic region in Sardinia, because there the disease has persisted longer, and recent outbreaks have occurred more frequently, than elsewhere on the island (7, 11). The three ASFV hosts on the island coexist in this area: registered domestic pigs, free-ranging pigs, and wild boar.

Within this endemic area, we collected data at the border between these two provinces, in the National Park of the Bay of Orosei and Gennargentu (**Supplementary Material 1**), where data from the Istituto Zooprofilattico Sperimentale della Sardegna indicate high wild boar density (8). This area is wooded and mountainous, and it is surrounded by many pig holdings. More than 88% of these holdings contain fewer than 11 pigs and conduct non-professional pig production under limited biosecurity conditions (7).

Camera Trapping

Camera trapping surveys were conducted with heat- and motion-triggered infrared cameras (Model Ltl−6210 M, Little Acorn Outdoors, Denmark, Wisconsin, USA) left in the field at 15 different sites between April and August 2014, during spring and summer, to continuously monitor the area and recording images of animals. This non-invasive method did not require ethical approval. The date and time of each exposure was recorded. Cameras were placed to cover water sources and pasture areas as likely sites of animal congregation.

Two researchers independently analyzed the camera images manually. The following data were entered in an Excel 2007

spreadsheet: camera identifier, date (dd/mm/yyyy), start time of each animal observation (h:min:sec), animal subspecies (free-ranging pig or wild boar), animal age class (piglet, young, adult) and animal activity (moving, drinking, pasturing, inspecting, resting, washing). The different activities carried out by the animals observed have a great interest from a sanitary point of view, since activities which differ from movement, such as drinking or resting, imply a higher risk of ASFV transmission. In this sense, if a pig had several different behaviors, we have considered the most risky activity (Washing > Drinking > Pasturing > Resting > Inspecting > Movement).

Data were logged for each individual animal observation in a visit, which was defined as one or more images of the same subspecies until consecutive images were captured at least 10 min apart. This interval cut-off was chosen because an earlier study with ear-tagged wild boar in two areas of England indicated that animals rarely returned to the same area within 10 min (36). For each visit, the maximum number of animals from the same subspecies simultaneously present in any of the images was recorded. Since animals were not individually tagged, we assumed that animals in separate visits were distinct.

Interaction Rates

We wanted to define the risk of ASFV transmission associated with each visit. To do so, we defined critical time windows (CTWs) during which ASFV could remain viable in the environment and be transmitted to other animals. We reviewed the literature for ASFV survival and infectious times in the environment by searching Web of Knowledge and PubMed databases from 1980 to December 2018 using the following topic search terms: African swine fever virus AND environment AND (survival OR transmission OR inactivation).

Direct interactions were defined as the simultaneous presence of free-ranging pigs and wild boar in the same image (**Figure 1**). Indirect interactions were defined as the presence of either free-ranging pigs or wild boar in one or more images, followed by the presence of the other subspecies within a specific CTW (**Figure 2**). Indirect interactions were determined based on the start date and time for each individual observation and counted using a MySQL database and PHP scripts (**Supplementary Material 2**).

Data Analysis

Microsoft Excel 2013 and R 3.5.0 were used to analyze camera trapping data (37). Daily activity profiles were generated for free-ranging pigs and wild boar based on the proportion of animal observations that occurred in each hour of the day and in each season (23). Generalized linear mixed-effects models were conducted to identify factors influencing direct and indirect interaction rates. The models were specified with a negative binomial distribution because of the counting data and over dispersion (38).

The following potential predictors were considered because of their biological relevance for explaining free-ranging pig-wild boar interactions (**Table 1**). The categorical variables were the following: season, hour range (categories selected based on the observed daily activity profiles), direction of the interaction, age, animal activity, water source, and pastureland. The continuous variable was altitude. Direct interactions did not have a direction, so this variable was omitted from the model. In order to control

FIGURE 1 | Example of a camera trapping image showing direct interaction between a free-ranging pig and wild boar.

FIGURE 2 | Example of a camera trapping image showing indirect interaction between a free-ranging pig and wild boar.

TABLE 1 | List of explanatory variables included in the generalized linear mixed model (negative binomial distribution and log link function) as risk potential factor for free-ranging pigs and wild boar interactions.

Variable	Risk type	Categories
Season	Temporal	Season 1: Spring
		Season 2: Summer
Hour range	Temporal	Hour range 1: 06:00–13:00 h
		Hour range 2: 14:00–21:00 h
		Hour range 3: 22:00–05:00 h
Direction of the interaction	Social	Direction 1: Wild boar followed by free-ranging pig
		Direction 2: Free-ranging pig followed by wild boar
Age class	Social	Age 1: Juvenile
		Age 2: Adult
Animal activity	Social	Activity 1: Moving
		Activity 2: Other activity
Water source	Environmental	Water source 1: Absence
		Water source 2: Presence
Pastureland	Environmental	Pastureland 1: Absence
		Pastureland 2: Presence
Altitude	Environmental	*Continuous variable*: 900–1,350 m

TABLE 2 | Observations of free-ranging pigs and wild boar, stratified by season, and age class.

		Free-ranging pig	Wild boar	Total
Season	Spring	162	67	229
	Summer	272	235	307
Age class	Juvenile	118	152	270
	Adult	316	150	466
Total		434	302	736

the spatial correlation among observations, a variable identifying eight proximity area groups, from the 15 sites of camera trapping, was included in all models as a random factor.

A data exploration followed by a backward stepwise model selection based on the Akaike information criterion was performed (39), and the Bayesian information criterion was also taken into account in order to obtain the most parsimonious model (40). The final generalized linear mixed-effects models for the negative binomial family were performed using the *glmer.nb* function from the R-package MASS (41). The overdispersion of residuals was checked by the sum squared Pearson residuals and the degrees of freedom. The differences associated with $p < 0.05$ were considered statistically significant.

RESULTS

During 375 trapping days, 434 observations of free-ranging pigs and 302 of wild boar were recorded (**Table 2**). Adult free-ranging pigs were more frequent than juveniles (chi-squared test, $p < 0.01$), whereas adult and juvenile wild boar were balanced. Observations of pigs and wild boar were significantly more frequent in summer than spring, and this seasonal difference was greater for wild boar (chi-squared test, $p < 0.01$).

Free-ranging pigs were diurnal, showing a peak of activity between 15:00 and 20:00 h (**Figure 3**). Wild boar were mainly crepuscular/nocturnal, showing prolonged night-time activity. Some diurnal activity of wild boar was observed, which was more frequent in spring than summer.

Direct Interaction Rate

We observed 0.37 direct interactions per day (SD = 1.31; n = 140). The model to explain direct interaction between free-ranging pig and wild boar contained season, hour range, age, water source and pastureland as variables (**Table 3**). Direct interaction rate was positively associated with the hour range from 14:00 to 21:00 h, and negatively associated with adult animals (**Figure 4**), in other words, interactions occurred mainly among juveniles.

Indirect Interaction Rates

Our literature search for ASFV survival and infectious times in the environment identified 34 publications, but none reported survival times in the environment under field conditions. Therefore, we considered to define two CTWs based on the latest studies on survival time in excretions (feces and urine): a long CTW based on one estimate of survival time (42), corresponding to 7 days in spring (12°C) and 5 days in summer (21°C); and a short CTW based on the empirically short time window of 1 day for ASFV transmissibility (43).

Based on the short CTW, our results indicated 1.31 indirect interactions per day (SD = 6.64; n = 489). The corresponding model to explain indirect interactions contained season, activity, water source and pastureland as variables (**Table 4**). Indirect interaction rate based on short CTW was positively associated with movement (**Figure 4**).

Based on the long CTW, our results indicated 6.47 indirect interactions per day (SD = 26.21; n = 2418). In this case, the corresponding model to predict indirect interactions contained season, direction of the interaction, age, activity, and water source as variables. Also, the final model identified the interaction between season and direction as significantly associated with indirect interaction rate (**Table 5**). Indirect interaction rate based on long CTW was also positively associated with movement. These indirect interactions usually occurred in the presence of a water source, and they involved adults more often than juveniles (**Figure 5**). In the summer, indirect interactions occurred more often in the direction of wild boar followed by free-ranging pig than in the opposite direction.

DISCUSSION

This study provides the first evidence of interactions between free-ranging pigs and wild boar in the east-central part of Sardinia, and such interactions may help explain the endemicity of ASF. We observed higher rates of direct and indirect interactions between free-ranging pigs and wild boar than

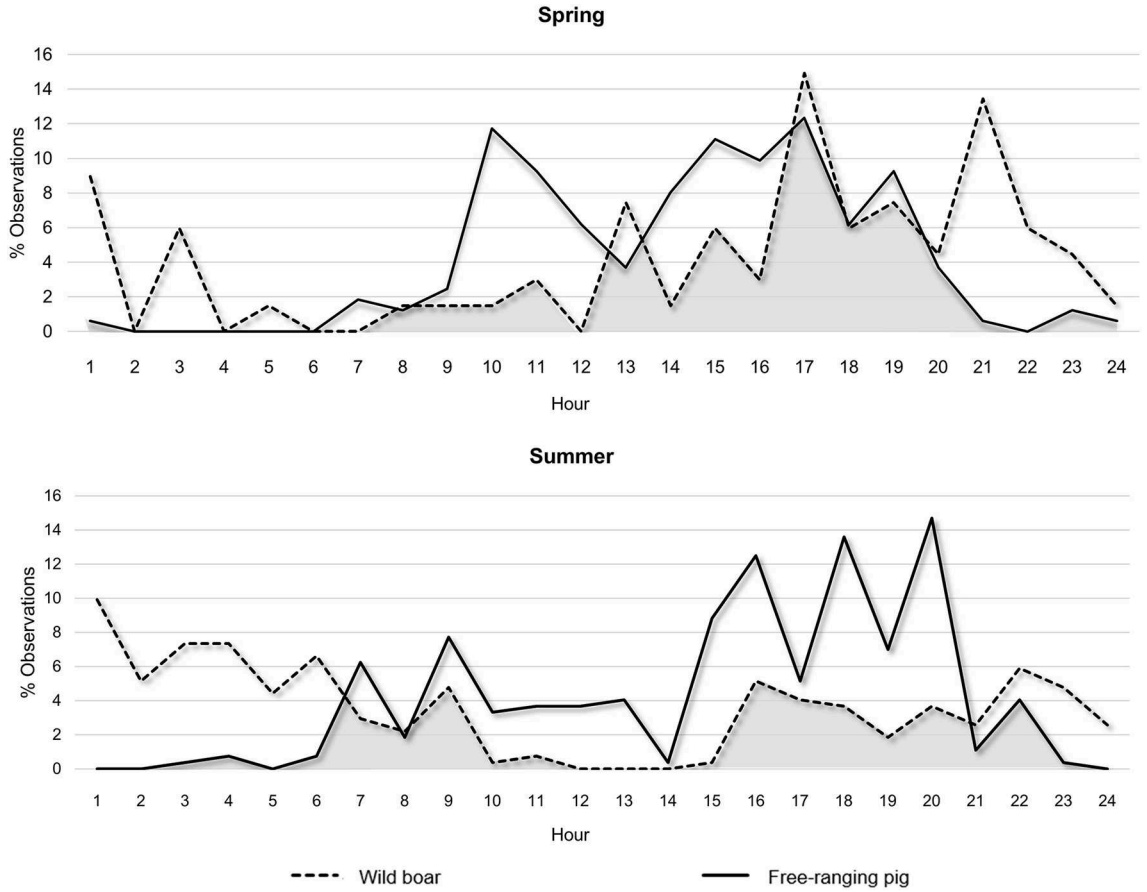

FIGURE 3 | Daily activity profile of free-ranging pigs and wild boar, expressed as the percentage of total observations by hour of day and season (spring or summer). The overlap in the profiles for the two subspecies is represented in gray.

TABLE 3 | Results of the best-fitting generalized linear mixed model (negative binomial distribution and log link function) to predict the rate of direct interaction between free-ranging pigs and wild boar.

		Estimate	Std. error	*Z* value	*P*-value
(intercept)		−16.90	38.46	−0.44	ns
Season 2	Summer	−0.28	1.83	−0.15	ns
Hour range 2	14–21 h	1.00	0.31	3.22	**
Hour range 3	22–5 h	0.67	0.44	1.51	ns
Age 2	Adult	−0.93	0.23	−4.04	***
Water source 2	Presence	1.26	2.66	0.47	ns
Pastureland 2	Presence	12.48	38.46	0.32	ns

P-values: $p > 0.1$ "ns"; $p < 0.05$ ""; $p < 0.01$ "**"; $p < 0.001$ "***".*
Coefficients are relative to Season 1 (Spring), Hour range 1 (6–13 h), Age 1 (Juvenile), Water source 1 (Absence), Pastureland 1 (Absence).

camera trapping studies on wildlife-domestic interface in other Mediterranean ecosystems (23, 32, 44), implying the relevance of this interaction in the epidemiology of ASF. Our study also confirms the usefulness of camera trapping for studying interspecific interactions more generally.

In our study, more animals were observed in the summer ($n = 307$) than in the spring ($n = 229$), and this increase in observations during summer was especially stronger for wild boar: 78% of all wild boar observations occurred in summer, compared to 63% of all free-ranging pig observations. The increase in observations during summer may be due to fewer food and water resources, reducing the home-range around natural resources (45). Reduction in home-range of free-ranging pigs may also occur if pig owners, to compensate for the shortage of natural resources during summer, supplement their animals' feed or even keep them on farms. Supplementing feed not only reduces the home-range size of free-ranging pigs but may attract wild boar. The increase in wild boar and free-ranging pig activity around natural resources in the summer may mean higher risk of contact with ASFV in the environment and therefore higher transmission risk.

Our rate of direct interactions in this area of Sardinia was considerably higher than the scarce or even undetectable rates reported in camera trapping studies of interactions between other wild ungulate and livestock species (23, 32, 44), and much higher than anecdotal direct interactions between wildlife and livestock in studies using other interaction-tracking methods

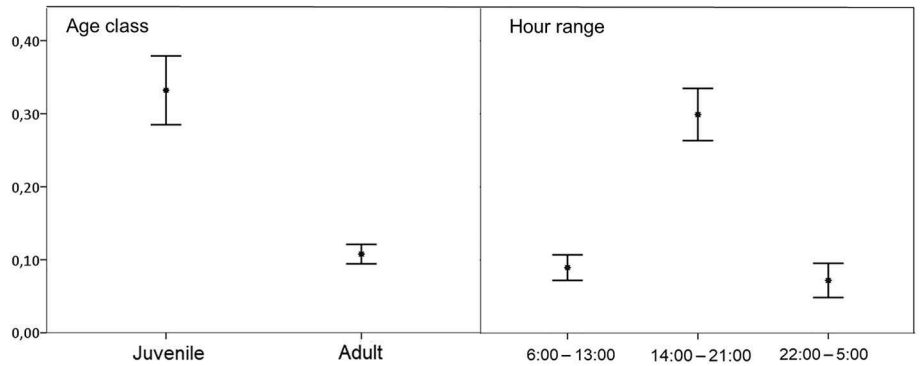

FIGURE 4 | Average predicted number of direct interactions between free-ranging pigs and wild boar per animal observed based on statistically significant variables in the best-fit model. Error bars show the 95% confidence interval.

TABLE 4 | Results of the best-fitting generalized linear mixed model (negative binomial distribution and log link function) to predict the rate of indirect interaction between free-ranging pigs and wild boar assuming a short critical time window of 1 day for transmissibility of ASFV.

		Estimate	Std. Error	*Z* value	*P*-value
(intercept)		−2.62	0.80	−3.27	**
Season 2	Summer	0.11	0.46	0.24	ns
Activity 2	Moving	0.61	0.16	3.88	***
Water source 2	Presence	0.47	0.64	0.73	ns
Pastureland 2	Presence	−0.60	0.64	−0.93	ns

*P-values: p > 0.1 "ns"; p < 0.01 "**"; p < 0.001 "***".*
Coefficients are relative to Season 1 (Spring), Activity 1 (Other: drinking, pasturing, inspecting, resting, washing), Water source 1 (Absence), Pastureland 1 (Absence).

TABLE 5 | Results of the best-fitting generalized lineal mixed model (negative binomial distribution and log link function) to predict the rate of indirect interaction between free-ranging pigs and wild boar assuming a long critical time window of 7 days in spring and 5 days in summer for transmissibility of ASFV.

		Estimate	Std. Error	Z value	*P*-value
(intercept)		−1.78	1.03	−1.73	.
Season 2	Summer	−0.27	0.35	−0.77	ns
Direction 2	Free-ranging pig followed by wild boar	0.45	0.28	1.65	.
Age 2	Adult	0.23	0.10	2.39	*
Activity 2	Moving	0.28	0.09	3.01	**
Water source 2	Presence	0.76	0.38	1.99	*
Season 2: Direction 2	Summer: Free-ranging pig followed by wild boar	−0.87	0.29	−2.96	**

P-values: p > 0.1 "ns"; p < 0.1 "."; p < 0.05 ""; p < 0.01 "**".*
Coefficients are relative to Season 1 (Spring), Direction 1 (Wild boar followed by free-ranging pig), Age 1 (Juvenile), Activity 1 (Other: drinking, pasturing, inspecting, resting, washing), Water source 1 (Absence).

(26, 28). Thus, our results provide a clear indication that wild boar and free-ranging pigs interact directly to a significant extent, highlighting the need to include this interface in epidemiological assessments of infectious swine pathogens, especially in extensive pig production systems.

Furthermore, our measured rates may underestimate direct interactions because we did not include the reproductive season from autumn to early winter, when most direct interactions occur between domestic pigs and wild boar (29). These reproductive interactions may have an important implication for understanding ASFV transmission, since the virus has been detected in semen and can be transmitted during mating (46). This lack of information on reproductive season may influence our finding that juveniles interacted directly more often than adults did, so this observation should be confirmed in further studies. The basic social organization of wild boar and free-ranging pigs is represented by male adults living singly and groups of females with juvenile offspring (47, 48). Males maintain greater distances with the rest of the adults than those maintained among female and juvenile groups (48), this behavior may explain the higher direct interaction rate observed in juveniles. Direct interactions between juveniles may have an impact on ASFV transmission and endemicity on the island, since young wild boar has previously been shown to be more likely to ASF seropositivity and virus positivity (49).

The frequency of direct interactions was significantly higher between 14:00 and 21:00 h (**Figure 4**), reflecting overlap in wild boar and free-ranging pig activity patterns (**Figure 3**). Overall, free-ranging pigs showed diurnal activity, while wild boar showed primarily nocturnal activity with sporadic diurnal activity, consistent with previous work in south central Spain (23). Domestic pigs on extensive or semi-extensive farms also show diurnal activity (23), so they may easily come into contact with free-ranging pigs in the absence of preventive measures, such as fencing fields where animals range free (50).

Our indirect interaction rate may also underestimate reality, since we had to define these interactions based on ASFV survival

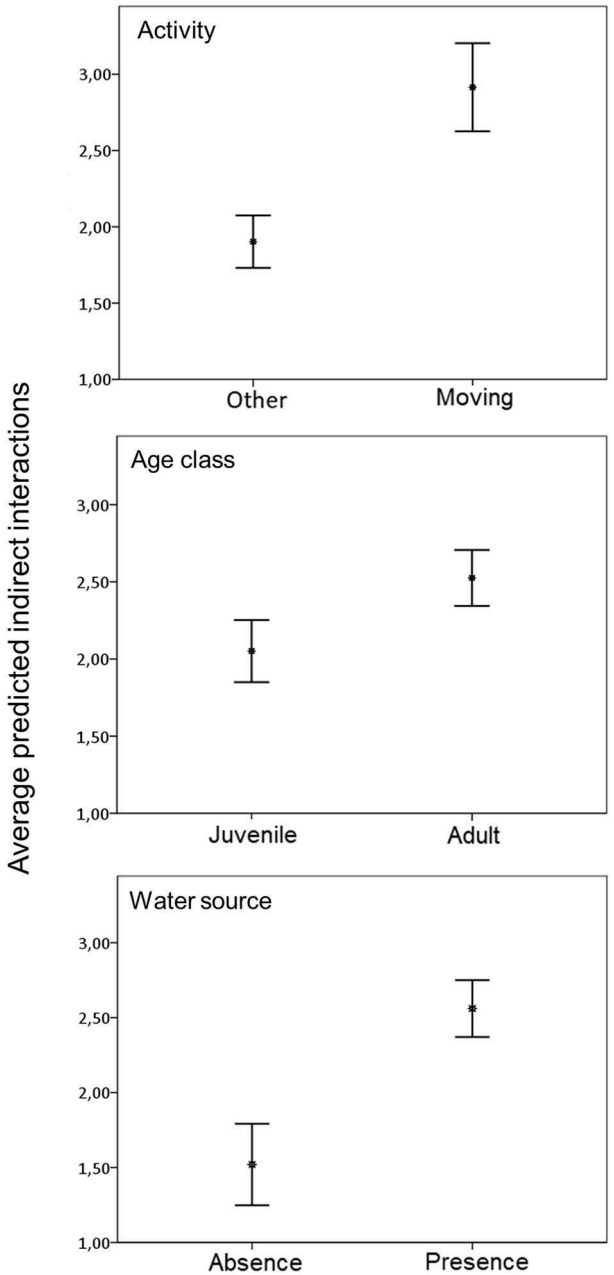

FIGURE 5 | Average predicted number of indirect interactions between free-ranging pigs and wild boar per animal observed assuming a long critical time window of 7 days in spring and 5 days in summer for transmissibility of ASFV, based on statistically significant variables in the best-fit model. Error bars show the 95% confidence interval.

times in feces and urine because of a lack of studies on virus survival time in the environment. Viruses are likely to survive in feces and urine for less time than in blood, where they can persist for up to 15 weeks (51). Interaction between wild boar and carcasses has been described to occur frequently (52), which contributes to ASFV transmission and might also occur among free-ranging pigs. However, we did not capture carcasses of wild boar or free-ranging pigs on cameras.

Most indirect interactions in our study involved animals in movement, suggesting that wild boar and free-ranging pigs do not share resting areas. Overall, indirect interactions were much more frequent near water sources. These findings are similar to those for interactions between other species in the Mediterranean basin (23, 32, 33, 53). Animal congregation around water sources is considered one of the most important factor for pathogen transmission between wildlife and livestock (28, 54). While ASFV survival time at natural water sources is unclear, infectious titers are considerably lower when the virus is transmitted in liquid than in feed (55). In addition, a recent study has shown the potential role of leeches to harbor ASFV, where the virus could remain active up to 140 days (56). Therefore, control measures should target water sources, as proposed for other infectious diseases (28, 50).

Another additional factor to take account when modeling direct and indirect interaction rates is the population density or abundance. Theoretically, we expect an increasing in contact rates (higher risk of pathogen transmission) with higher density but saturates upon reaching a threshold of population density (57). However, in the present study, we could not consider this factor due to the lack of availability of abundance and density data of wild boar and free-ranging pig populations at suitable spatial scale, but it would be greatly recommended for further studies.

CONCLUSION

Our results provide the first conservative estimates of interactions between free-ranging pigs and wild boar interactions in Sardinia. The likelihood that our data underestimate actual interactions further underscores the importance of this interface for understanding ASFV transmission. Such interactions may therefore quite reasonably account for the longstanding ASF endemicity on the island of Sardinia, and they support the need to eliminate free-ranging pig breeding practices. More broadly, we consider the control of free-ranging pigs as an important measure against ASFV transmission, taking especial attention during summer, at water sources and between 14:00 and 21:00 h. The findings of this study may help to model the spread of ASFV in the context of the domestic-wild swine interface, but it should be assessed in other epidemiological scenarios. Finally, we conclude that analysis of interactions between free-ranging pigs and wild boar has great potential for guiding effective prevention policies and evaluating disease management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

JS-V, AP, and JV designed the study. JS-V, AP, JV, DD, MC, and JB carried out the field work. EC-F and CJ collected the data. EC-F and JB performed the analyses. EC-F, JS-V, CJ, and JB wrote the

manuscript. All authors revised the manuscript and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2019.00376/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Progress Toward Development of Effective and Safe African Swine Fever Virus Vaccines

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African swine fever is a major concern due to its negative impact on pork production in affected regions. Due to lack of treatment and a safe vaccine, it has been extremely difficult to control this devastating disease. The mechanisms of virus entry, replication within the host cells, immune evasion mechanisms, correlates of protection, and antigens that are effective at inducing host immune response, are now gradually being identified. This information is required for rational design of novel disease control strategies. Pigs which recover from infection with less virulent ASFV isolates can be protected from challenge with related virulent isolates. This strongly indicates that an effective vaccine against ASFV could be developed. Nonetheless, it is clear that effective immunity depends on both antibody and cellular immune responses. This review paper summarizes the key studies that have evaluated three major approaches for development of African Swine Fever virus vaccines. Recent immunization strategies have involved development and *in vivo* evaluation of live attenuated virus, and recombinant protein- and DNA-based and virus-vectored subunit vaccine candidates. The limitations of challenge models for evaluating ASFV vaccine candidates are also discussed.

Keywords: ASF, vaccine, attenuated virus, subunit vaccine, live vector

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INTRODUCTION

African swine fever is caused by a DNA virus classified in the *Asfarviridae* family, genus *Asfivirus* (1). The pathogen is an arthropod-borne highly complex enveloped double-stranded DNA virus which primarily replicates in the host cell cytoplasm (2, 3). The virus is easily transmitted since it is extremely stable and persists under a variety of environmental conditions, for up to several months, thus creating a requirement for implementation of strict biosecurity measures to prevent transmission (4). The virus causes a highly contagious hemorrhagic disease in pigs that produces a wide spectrum of clinical syndromes ranging from rapid lethality to relatively mild symptoms. The internal lesions closely resemble those of the unrelated classical swine fever virus but with higher morbidity and mortality rates (5). ASF is an economically important disease that is currently enzootic in sub-Saharan Africa (24 genotypes described based on the sequence of the c-terminus of the p72 surface antigen) and Sardinia (p72 genotype 1). In 2007 a genotype II virus from Southeast Africa reached the Caucasus region and subsequently Russia and Eastern Europe (6, 7). Multiple outbreaks almost certainly originating from the single index case in the Caucasus have recently (from August 2018) been reported in China, Vietnam, Cambodia, Laos, North and South Korea,

Philippines, and Timor-Leste (OIE, December 2019). The consequences for the 450 million pigs in China are already devastating. Given the level of global interconnectivity of the world economy and the stability of the virus, there is a high risk of spread to ASFV-free large scale pork producing countries, such as U.S.A, Germany, Denmark, and Brazil (7).

As the causal agent of one of the most severe diseases of domestic pigs that spreads easily, in the case of the major genotype II pandemic facilitated by the movement of wild boar in which the disease is lethal, ASFV has many sanitary and socio-economic consequences which significantly impact the national and international trade of animals and animal products (8). At present, mass slaughter of infected and in-contact pigs with proper disposal and disinfection is the only way to manage outbreaks. The host cell entry and replication mechanisms utilized by the virus, the strategies it uses to evade host defense systems, identity of viral proteins that are important in causing an effective host immune response, and the protective immune mechanisms involved, are gradually being discovered (9). Since completion of sequencing of the first entire virus genome (10), a concerted effort has been made to analyze the genomes and predicted proteome of multiple isolates to generate knowledge that is vital for designing innovative disease control strategies, which include an effective vaccine against various ASFV genotypes (11–14).

Attempts to develop a safe vaccine for protection of pigs against ASFV have continued without significant success from the time ASFV was first isolated (15). Without a safe and efficacious vaccine, pig farmers in the affected areas are venerable to the disease whose prevention depends exclusively on ensuring that infected pigs, contaminated feeds and materials, or fomites (for example virus on the clothes or shoes of pig workers) are not introduced into areas that are ASFV-free (16). All eradication programs that have proven successful involved the prompt diagnosis, quarantine, slaughter, and properly discarding all animals in infected sites (17–19). Subsequently, surveillance of all pig farms within a specific region must be conducted to ensure maintenance of disease-free zones.

The focus of this review is the historical progress made so far in regards to the efforts directed at development of safe and effective vaccines for protection of swine against ASF virus. Several prospective vaccine candidates have been evaluated and some novel candidates are being developed and tested. The development strategies for the vaccine can be divided basically into these broad categories; live attenuated ASF viruses, inactivated ASF virus, live-vectored subunit, mammalian expression plasmid DNA-based, recombinant protein-based-subunit candidates, and a combination of the above (20). Live attenuated virus can be generated by deletion of genes encoding virulent factors for safe induction of protective immunity (21, 22). Some ASFV antigens have been identified and used to generate recombinant proteins for evaluation of protein-based candidate immunogens (23). Direct delivery of viral nucleic material into host cells can result in *de novo* gene expression and the expressed antigen can elicit immune responses. Live-vectored vaccines are similar to nucleic acid-based vaccines except that the genes encoding target antigens are delivered into the host cell by employing non-pathogenic attenuated virus or bacteria. There are constraints to all of these approaches that have prevented rapid progress in development of safe and cost effective vaccines to control the virus.

LIVE ATTENUATED ASFV VACCINE CANDIDATES

A range of mutant viruses have been either isolated from the field or experimentally generated and tested for their ability to safely induce protective immunity in pigs and wild boars. Attenuated viruses can be either naturally occurring low-virulence isolates or virulent strains attenuated by deletion of defined DNA sequences encoding virulence factors. Whole virus-based vaccines can be sub-divided into two categories: live attenuated viruses and inactivated or killed viruses.

Live Attenuated Vaccine Candidates

Live attenuated ASFV vaccine candidates can induce protective immunity, but the use of naturally attenuated strains of ASFV has the potential to cause post-vaccination reactions and side effects. Although it has previously been demonstrated that following subclinical infections of domestic pigs with low virulent strains of ASFV, immunity against homologous, but not heterologous, challenge was conferred (24). A Portuguese group was the first to demonstrate subclinical infections of domestic pigs with low virulent strains of ASFV (20). They found that pigs immunized with the naturally occurring ASFV NH/P68 virus, which was isolated subsequent to the introduction of a genotype I virus into that country from Angola, were protected against challenge with virulent ASFV L60 and this correlated with increased NK cell activity (20). Immunization of pigs with low virulence ASFV isolates provide varying levels of protection against challenge with virulent virus. For instance, pigs immunized with naturally attenuated ASFV strains NH/P68 or the *Ornithodoros erraticus* tick-derived OURT88/3 were protected following challenge with closely related ASFV strains and those challenged with heterologous strains were partially protected (20, 25–27). The level of protection in both cases varied from 60 to 100% (26–32). These outcomes provided useful data concerning immune parameters involved in protection. Both antibodies and cytotoxic $CD8^+$ T cells were demonstrated to play important roles in conferring protection (25, 33–35).

Despite the ability to induce protective antibody and T cell responses, naturally attenuated isolates have been associated with adverse side effects and safety concerns (29). To improve safety, mutant viruses have been generated with deletions of genes involved in virulence and progress of clinical disease (DP96R and DP71L) and inhibition of IFN-γ (A276R) (23, 36). However, varying levels of protection were observed in immunized pigs. Virulent virus isolates can be attenuated by deletion of rationally selected genes encoding virulence factors to obtain attenuated virus that can safely induce protective immunity. However, deletion of some genes has been shown to significantly reduce the virulence of the virus in pigs, whereas deletion of others had no apparent effect (37). In one study, deletion of virulence genes

DP96R and DP71L from the ASFV OURT88/3 isolate reduced its ability to protect against challenge with virulent virus OURT88/1 isolate, whereas in another study, 60–100% protection was observed following challenge with heterologous virulent ASFV Armenia 07 (23, 29). It has been shown that deletion of IFN-γ inhibitor genes DP148R, MGF360, and 530/505 genes from ASFV Benin97/1 isolate induced protective immune responses against challenge (38, 39). By contrast, deletion of the early virus protein L83L from the ASFV Georgia 2007 isolate did not reduce viral virulence in experimentally infected swine, and no challenge studies were performed (40). Recently, immunization of pigs with a naturally attenuated genotype II ASFV Lv17/WB/Rie1 isolated from wild boars in Latvia conferred protection upon challenge through contact with animals infected with virulent ASFV (41).

Immunization with attenuated virus, rather than with selected antigens, is advantageous since it elicits immune responses against all the viral antigens that are normally encountered by the host during the course of an infection, and it may therefore be more effective. Several attenuated viruses have been tested for their ability to induce immune protection (**Table 1**). Among the genes that have been deleted in these attenuated viruses are; EP402R (a homolog of CD2), B119L, DP71L, K169R, DP96R, E165R, EP153R, MGF360/530, A224L, A238L, and E269R (46). Many of the proteins encoded by the deleted or inactivated genes in these attenuated constructs have predicted functions based on sequence identity, and biological observations. The product encoded by EP402R is involved in mediating hemadsorption of RBCs to infected host macrophages and extracellular virus particles; DP71L exhibits similarity to a Herpes simplex virus (HSV) neurovirulence factor; KI69R encodes Thymidine kinase; E165R encodes a dUTPase; EP153R encodes a C-type lectin; A22L is an IAP apoptosis inhibitor that presumably prevents host programmed cell death; A238L is an inhibitor of host cell transcription; and E296R encodes an AP endonuclease Class II (47). The function of the MGFs, including families 360 and 530 is unknown, although some of the proteins contain predicted signal peptides, suggesting secretion and interaction with host proteins (47). B119L has sequence identity to several yeast proteins including ERV1 which functions in oxidative phosphorylation (4).

Deleting certain genes from the genome of a virulent ASFV isolate affects pathogenesis in pigs (48). For example, when the EP402R gene was deleted, there was reduction in virus dissemination through tissues (49). However, recent studies showed that deletion of the EP402R gene from the genotype I BA71 isolate attenuated the virus and the mutant conferred protection against challenge with homologous virulent BA71 virus, and also heterologous E75 (Genotype 1) and Georgia 2007/1 (Genotype II) viruses (30). Surprisingly, deletion of the DP71L and DP96R genes from the ASFV strain OURT88/3 decreased its protective capacity in pigs following challenge with virulent virus (23). Recent studies have also shown that deletion of the B119L, DP71L/NL, and DP96R/UK genes from the ASFV Georgia 2007/1 strain reduced its replication efficiency, but the mutant did not protect immunized pigs against challenge with parental virus (45).

Deletion of MGF 360, MGF 505, or B119GL genes attenuated the ASFV Georgia 2007/1 isolate and the respective mutant virus elicited immune responses that protected immunized pigs against homologous virulent challenge. However, protection was not observed when both MGF 360/505 and B119GL genes were deleted, indicating that deletion of multiple genes can sometimes significantly reduce protective capacity of the resulting mutant (42, 43, 50). However, by contrast, improved protection and safety was observed when the DP96R/UK and B119GL genes were simultaneously deleted from the ASFV Georgia 2007/1 isolate (44). In the case of other specific virulence genes, such as Thymidine Kinase (TK), although less pathogenic viruses were generated, the performance of the resultant mutants was not consistent. Notably, deletion of the TK gene in Georgia 2007/1 and Malawi strains attenuated the viruses, however the Malawi strain, but not the Georgia 2007/1 strain, induced protective responses in immunized pigs (30, 51, 52). The outcome suggests that the effect of gene deletions on the ability of the virus to elicit immune protection is strain-specific (52). Thus, additional new knowledge is required for rational development of live attenuated ASFV candidate vaccine and that evaluation has to be on a case by case basis.

Although attenuated ASFV is currently the most promising vaccine candidate, there are still major challenges that need to be addressed. These include safety concerns because the viruses are not sufficiently attenuated, requirement for high biocontainment for production of the attenuated virus, availability of suitable cell lines and optimization of culture conditions for vaccine virus scale up which remains a key constraint (53).

Inactivated ASFV Vaccines

Efforts to generate inactivated or killed ASFV vaccines capable of conferring protection have been unproductive (54–57). One recent study showed that although an inactivated preparation of the ASFV Armenia08 formulated with contemporary adjuvants elicited ASFV specific antibodies, there was no protection upon challenge with homologous virulent virus (11). This outcome raises serious questions regarding the role of antibodies in protection against ASFV, but it is possible that the antibodies elicited by this particular immunogen failed to confer protection. Although antibodies have been implicated in protection against ASFV, the antibody target(s), the actual effector mechanism(s) or the isotype(s) involved, remains unknown (16).

SUBUNIT VACCINES

Subunit vaccines utilize a defined pathogen structural, non-structural or unassigned proteins as antigens to elicit protective immune responses (58). This is accomplished by using a gene encoding a candidate antigen to generate recombinant antigen that is formulated with an adjuvant. Alternatively, the gene can be used to generate a live-vectored recombinant construct for *in vivo* antigen expression. Several antigens, including p12, p30, p54, and p72, have been evaluated for their protective potential as recombinant proteins. Antibodies against p12 and p72 have been shown to hinder binding of the virus to the host cells, while antibodies against p30 protein prevents the

TABLE 1 | Live attenuated ASFV vaccines.

Strain	Vaccine virus	Protection	References
Naturally attenuated OURT88/3	OURT88/3	Homologous OURT88/3 strain	(23)
		Heterologous OURT88/1 strain	(28)
		Heterologous Benin 97/1, Uganda 65 strains	(28)
NH/P68	NH/P68	Heterologous L60, Armenia 07 strains	(20, 32)
Gene-deletion OURT/88/3	OURT/88/3ΔDP71L ΔDP96R	Homologous OURT/88/1strain	(23)
NH/p68	NH/P68ΔA238L	Homologous L60 strain	(32)
		Heterologous Armenia 07 strain	(32)
	NH/P68ΔEP153R	Homologous L60 strain	(32)
	NH/P68ΔA224L	Homologous L60 strain	(32)
		Heterologous Armenia 07 strain	(32)
Benin97/1	Benin 97/1ΔMGF	Homologous Benin 97/1 strain	(38)
	Benin 97/1ΔDP148R	Homologous Benin 97/1 strain	(39)
Georgia 07/1	Georgia 07/1Δ9G L	Homologous Georgia 07/1 strain	(42)
	Georgia 07/1ΔMGF	Homologous Georgia 07/1 strain	(43)
	Georgia 07/1Δ9GL	Homologous Georgia 07/1 strain	(44)
	ΔDP96R/UK		
	Georgia 07/1ΔB119/	No protection	
	ΔDP71L/ΔDP96R		(45)
Ba71	Ba71ΔEP402R	Heterologous E75 and Georgia 07/1 strains	(30)

TABLE 2 | Protein subunit candidate vaccines.

ASFV proteins	Expression system	Protection	References
CD2v	Baculovirus expressed	Partial protection	(37)
p54, p30	Baculovirus expressed	Protection	(48)
p54, p30, p72	Baculovirus expressed	Partial protection	(60)
CD2v and C-type Lectin	Baculovirus expressed	Protection	(27)

virus from entering cells (37, 46, 48, 59). However, p12-specific antibodies induced in both natural infections and in animals inoculated with inactivated virus or recombinant p12 protein, do not block virus binding to the host cell or neutralize virus infectivity (59).

The p30 and p54 proteins mediate interactions between ASFV and host cells and simultaneous interference with the interactions of these two proteins with the host cells has a complementary effect in antibody-mediated protection (48). Some preliminary vaccination experiments using these recombinant proteins gave promising results and these could be followed up with other combinations of recombinant proteins, either as purified proteins, or recombinant live-vectored virus constructs. For instance, baculovirus-expressed p30 and p54 elicited antibodies that protected pigs against challenge with ASFV E75CV1-4 (48). However, in another study, antibodies elicited against p30, p54, and p72 were not sufficient to confer protection against challenge with the ASFV Pr4 isolate (60). Another study showed that immunization of pigs with baculovirus-expressed EP402R antigen, a viral transmembrane protein, elicited hemadsorption inhibition antibodies and conferred partial protection against lethal challenge (37). Moreover, immunization of pigs with a combination of baculovirus-expressed EP402R and C-type Lectin, induced a significant level of protection following challenge with homologous ASFV (**Table 2**) (27).

LIVE-VECTORED AND DNA-BASED SUBUNIT VACCINE CANDIDATES

Gene expression vectors, either viral, bacterial, or plasmid-based have been used as antigen delivery platforms that can be tailored to elicit a desired immune response (**Table 3**). Only a few studies have been conducted to evaluate immunogenicity and protective efficacy of prototype vectored ASFV subunit vaccine candidates. Argilaguet et al. (49) showed that immunization of pigs with BacMam-sHAPQ, a baculovirus-based construct encoding p30, p54, and secretory hemagglutinin or sHA, induced antigen-specific T-cell responses in pigs. Following challenge, 4/6 of the immunized pigs, but not the negative controls, were free of the virus (49). A recombinant modified vaccinia virus Ankara (MVA) expressing the p72, EP402R, and EP153R antigens, induced T cell responses, but the animals were not challenged to determine whether the induced responses were protective (61). Alphavirus expressing ASFV p30, p54, or p72 were tested for immunogenicity in pigs and the results suggested that an attenuated live virus boost of an initial immunization of a vector-expressed antigen may broaden humoral epitope response (65). It has recently been shown that cocktails of adenoviruses expressing multiple ASFV (Georgia 2007/1) antigens [p32, p54, pp62, p72, A104R, K205R, B438L, EP402RΔPRR, B602L, B119L, and A151R], induced robust cellular and antibody responses (62, 63). Although highly immunogenic, the adenovirus-vectored ASFV antigen cocktail did not confer significant protection following intranasal challenge with ASFV Georgia2007/1 isolate (64), whereas in a sub-study, protection was observed in 5/9 of the vaccinated animals (64). This study further suggested that antibodies induced by one of these adenovirus vectored antigen cocktails may be counter-protective, since delivery using an adjuvant that induced lower levels of antibodies, resulted in enhanced protection of pigs following virus challenge (64). Moreover, recent studies has also shown that a cocktail of Adenovirus and Modified Ankara Virus expressing up to 18 antigens [I215R, I73R, CP530R [pp62], CP204L [p32], MGF110-5L, B646L [p72], MGF110-4L, M448R, L8L, E146L, C129R,

TABLE 3 | Live vectored and DNA sub-unit vaccine candidates.

ASFV proteins/genes	Expression system	Protection	References
Vectored p54, p30, sHA	BacMam-sHAPQ	Partial protection	(49)
p72, CD2v, and EP153R	Modified vaccinia virus ankara	No challenge study	(61)
7 and 12 antigen cocktails	Adenovirus vectored	No challenge study	(62, 63)
7 antigen cocktail	Adenovirus vectored	Partial protection	(64)
7 antigen cocktail	Adenovirus vectored	No protection	(64)
12 antigen cocktail	Adenovirus vectored	No protection	(64)
p30, p54, and pHA-72	Alphavirus vectored prime, Attenuated OURT88/3 boost	No challenge study	(65)
18 antigen cocktail	Adenovirus and MVA vectored	No protection	(66)
DNA sub-units DNA expression library	DNA constructs	Partial protection	(67)
p54/E183L, p30/CP204L	DNA constructs	No protection	(68, 69)
Ubiquitin-CD2v/pEP402R-p54/E183L-p30/CP204L	DNA constructs	Partial protection	(69)
DNA and vectored/protein 47 antigen pool	DNA constructs and vaccinia virus	Partial protection	(70)
p15, p35, p54, and ±p17 and p32, p72, CD2v, and ±p17	DNA and protein vaccine	No protection	(71)

A151R, MGF110-1L, L10L, K78R, E184L, E165R, and CP312R] used in a prime-boost strategy induced antigen specific immune responses but failed to protect against challenge (66).

DNA vaccination involves inoculation of expression plasmid constructs encoding defined target antigens for expression in mammalian host cells. Potential advantages of DNA vaccination over traditional approaches, include stimulation of B-cell, CD4, and CD8 T-cell responses, improved vaccine stability, the absence of any infectious agent and the relative ease of large-scale production, although production to GMP standard may be more expensive than adenovirus (72, 73). A DNA vaccine candidate, pCMV-sHAPQ, encoding ASFV p30 and p54 fused to hemagglutinin extracellular domain (sHA) improved humoral and the cellular responses in pigs, but provided partial protection against lethal challenge with the virulent E75 ASFV-strain (68). Similarly, immunization of pigs with a plasmid construct encoding p30, p54, and sHA genes fused to ubiquitin, elicited T cell responses but conferred partial protection against challenge with lethal E75 virus strain in the absence of neutralizing antibodies. In this study, protection correlated with presence of sHA-specific $CD8^+$ T cells (68, 69). A further experiment demonstrated that immunization of pigs with a DNA expression library of more than 4,000 plasmid clones, each one containing a random Sau IIIa restriction fragments derived from the viral genomic DNA fused to ubiquitin conferred 60% protection against lethal challenge with the virulent E75 strain (67).

More recent approaches have evaluated several heterologous prime-boost strategies in an attempt to improve protective efficacy of prototype subunit vaccines. Jancovich et al. (70) showed that pigs primed with DNA plasmids encoding 47 ASFV antigens and boosted with recombinant vaccinia virus expressing the same antigens, significantly reduced ASF viral load in the vaccines following challenge with ASFV Georgia 2007/1. However, the same group showed that immunization of pigs with 12 adenovirus constructs expressing selected ASFV antigens and boosting with vaccinia virus expressing cognate antigens, reduced viral loads but the immunized pigs were not protected against challenge with ASFV OURT88/1 (66). Another study has demonstrated that immunization of pigs with recombinant proteins [p15, p32, p54, and ±p17] and plasmid DNA constructs encoding [p32, p72, EP402R, and ±p17] in a prime and two booster doses induced cell mediated immune responses and antibodies that were shown to neutralize ASFV *in vitro*. However, the immunized pigs were not protected against challenge with Armenia 2007 strain (71).

IMMUNIZATION PROTOCOL

The route of vaccine administration is worthy of further research in the context of immunization protocols. For example, it was observed that the naturally tick attenuated genotype I OURT88/3 virus when administered at low to intermediate doses (10^3–10^4) pfu was protective against virulent wild type OURT88/1 challenge when administered intranasally, but not when administered intramuscularly at the same doses (74). Most of the ASFV vaccine candidates tested so far have been delivered by parenteral injection. Recent global consortia call for improved effective vaccine delivery systems, amongst others measures, as a roadmap for developing a vaccine (75, 76). An oral bait-based vaccine would be more attractive, particularly for immunization of wild boars and feral pigs. Oral bait-based vaccine delivery has been used for successful immunization of wild animals (77, 78). Notably, a vaccinia virus-vectored rabies vaccine [RABORAL] and an adenovirus-vectored oral bait rabies vaccine [ONRAB] have been used successfully to control rabies in domestic and wild animals in U.S.A and Europe (77, 79, 80). Recently, an oral ASFV vaccine candidate, attenuated genotype II ASFV (Lv17/WB/Rie1), was tested in wild boars and shown to confer 92% protection against virulent challenge with ASFV Armo7 isolate (81). The Lv17/WB/Rie1 mutant has potential to be used for ASFV management in domestic pigs and to control ASFV from spreading in wild boar populations. However,

further studies are needed before the vaccine can be approved for deployment.

CHALLENGE MODELS AND THEIR LIMITATIONS

Lack of knowledge on the appropriate challenge model relevant to the candidate vaccine limits the development of a safe and efficacious ASFV vaccine. Transmission of ASFV in domestic swine often occurs via direct contact between persistently infected and susceptible animals, via soft ticks in the genus *Ornithodoros*, or contaminated feed including other pigs that have been slaughtered or succumbed to the disease (82). ASFV epidemiology is complex since infection of domestic pigs typically results in mortality and morbidity, whereas wild suids including warthogs and bushpigs can be infected but they are asymptomatic. There are also different patterns of pathogenesis and clinical outcomes in domestic pigs across different regions of the world where ASFV is endemic. In addition viral pathogenicity may evolve over time and as the virus expands its range into new areas (1). Genetic variability amongst different breeds of swine, which originate from multiple independent domestication events, could be one factor explaining clinical disease why outcomes vary between different infected animals (1). Factors such as husbandry systems and the involvement of wild boar and tick transmission may also be important. Therefore, simulation of most common natural routes of infection and transmission is critical for evaluation of protective efficacy of vaccine candidates. Currently, live attenuated ASFV are the most promising vaccine candidates for eliciting protective immunity, but safety concerns combined with scale-up issues have delayed progress in deployment of these candidates in the field. The BA71ΔEP402R deletion mutant was shown to protect against lethal challenge with both genotype I strains, BA71 and E75 (30). Additionally, 100% of pigs immunized with the mutant survived lethal challenge with genotype 2 Georgia 2007/1 isolate (30).

The cross protection conferred by BA71ΔEP402R makes this most promising candidate vaccine developed to date. However, biosecurity and biocontainment concerns remain, as well as the requirement to ensure that pigs immunized with this vaccine and others can be differentiated from infected pigs.

Following immunization with candidate vaccines, protection levels vary from 0 to 100%, depending on the breed of pigs, vaccine dose, delivery route, and the virus isolate used for the challenge (30, 63, 64, 70, 81, 83, 84). As mentioned, ideal challenge models should closely resemble natural ASFV transmission in swine and the most common transmission route is likely to be via direct contact through mucosal surfaces (17, 85). Therefore, a novel challenge model, such as incorporating ASFV into feed/liquid for an oral and/or intranasal challenge post-vaccination, may be key to better understanding of the immune responses induced and obtaining protection following challenge. Therefore, to identify protective antigens needed for subunit vaccine development, there is a need to empirically define an appropriate ASFV challenge dose. This is important given that the correlates of protection are not yet available and the optimal antigen(s) for inducing protection have not yet been defined. Additionally, challenging animals immunized with a subunit vaccine candidate with a high dose of virulent ASFV that has been shown to work for evaluating efficacy of attenuated ASFV candidate vaccines may not be appropriate and hinder identification of antigen-specific immune responses that correlate with protection.

To date, the majority of ASFV immunization studies have used intramuscular administration of vaccine and the same route for challenge. Few studies aim to determine effective intranasal challenge doses of ASFV isolates that differ in virulence. The majority of immunization studies have used well-characterized domestic breeds, such as large white or landrace as the target animal for immunization studies (16, 27, 32, 67, 71, 74). To date, only a few groups have used indigenous breeds of pigs from ASFV endemic areas, such as Africa for vaccination research (83).

The high costs associated with BSL3 biocontainment laboratories and space constraints in such facilities have limited the number of challenge studies performed and hindered long-term monitoring of animals post-challenge. Studies have reported variable duration of monitoring post-challenge, ranging from 17 to 63 DPV and this does not provide consistent data for comparison of vaccine candidates (41, 64, 81). Thus, vaccine immunization and challenge protocols need to be standardized to allow uniform interpretation of outcomes.

ASFV CANDIDATE VACCINE-INDUCED DISEASE EXACERBATION

Vaccinated pigs can potentially develop chronic ASF or severe pathology either post-vaccination and/or post-challenge. Following vaccination and challenge more severe clinical disease, when compared to the non-vaccinated animals, has been observed. Jancovich et al. (70) showed that vaccine-induced antibodies correlated with increased viremia. This observation was also supported by outcomes reported in several other studies (64, 70, 71). In the 1960s, live attenuated vaccines were used to immunize pigs following outbreaks of ASF in Portugal, Spain and Dominican Republic (53, 86). Although there were reports of survival and protection from naturally attenuated ASFV used, the biggest concerns with deploying LAVs is safety and the ensuing persistence of chronic forms of ASF in pig populations. Such persistence of chronic clinical signs were observed during evaluation of the attenuated ASFV NH/P68ΔA276R, which failed to confer protection against Arm07 challenge (32). In another study, pigs immunized with the ASFV-G-ΔL83L mutant had severe ASF clinical symptoms, similar to pigs inoculated with the parental ASFV-G virus, and either died from the infection or had to be euthanized (40).

The ASFV causes high mortality rates in domestic swine, regardless of gender and age (87). Another point to be considered is whether gender and sex differences have any effect on vaccination outcome (88). Netherton et al. (66) recently observed a variation in disease outcome between male and female immunized pigs. The authors reported that male immunized pigs

showed enhanced ASF clinical disease, while female pigs had reduced viremia compared to control pigs (66).

CONCLUSION AND FUTURE PERSPECTIVES

African swine fever virus causes acute hemorrhagic fever in pigs that results in high mortality and lack of a vaccine limits control to test and mass slaughter of infected and in-contact pigs. Sequencing genomes of attenuated and virulent strains, and targeted gene deletions from virulent strains have revealed genes encoding some of the factors involved in virulence and immune evasion, and with increasing spread of the disease, there is an impetus to sequence genomes of more isolates to identify relevant genes. It is clear that effective immunity depends on both antibody and cellular immune responses. Pigs immunized with naturally low virulence isolates or attenuated viruses produced by targeted gene deletions can induce protection against challenge by wild type virulent viruses. Virus antigens that are potential targets for inducing neutralizing antibodies have been identified and immunization with some of these antigens has been shown to confer partial protection. However, antigens that can elicit protective immunity, especially $CD8^+$ T cell targets, have yet to be identified. Although several live attenuated ASFV are currently the most promising vaccine candidates, further work is needed to address some limitations, in particular scale up, prior to approval for deployment. Importantly, definition of correlates of protection against ASFV will enable rational identification of protective antigens for development of DIVA subunit vaccine. Recent studies have sequenced the warthog (*Phacocherus africanus*) and bush pig (*Potamochoerus larvatus*) genomes to better understand mechanisms of tolerance to ASFV infection, and how the disease burden is reduced in these swine species compare to domestic swine (89). This data will support current and future vaccine development strategies by comparing susceptible to resistant pig species.

AUTHOR CONTRIBUTIONS

WM, SL, RB, and SW planned and wrote the paper. HS, GM, and NS conducted literature review and wrote the paper.

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Evolution of the ASF Infection Stage in Wild Boar Within the EU (2014–2018)

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African swine fever (ASF) is one of the most important emerging transboundary diseases of pigs, causing trade restrictions, and a health impact on susceptible pigs. Nine countries in the continental European Union (Estonia, Lithuania, Latvia, Poland, Czech Republic, Bulgaria, Belgium, Romania, and Hungary) have been affected by ASF from 2014 to 2018 and it keeps spreading despite the efforts to control it. For a number of years, we have witnessed high case-fatality rates in wild boar found dead particularly in new infected areas, which is typical of the peracute and acute forms of the infection at the beginning of an ASF epidemic. Experimental evidence with currently circulating strains indicates that some infected animals can remain asymptomatic and might even survive the infection. An increased presence of virus of moderate virulence can complicate ASF diagnosis as well as the mitigation and control of the disease. We analyze the ASF surveillance data in wild boar in the four EU countries where ASF has been present for longer, comparing the spatial density of antibody positive notifications with the time ASF has been present per region. Results indicate an increasing annual distribution of notifications based on antibodies over nucleic acid detection in hunted wild boar in Estonia, Latvia and Poland. Potentially, Lithuania, and Poland seem to have experienced more acute forms in 2017 and 2018 than Latvia and Estonia. Overall there was a positive statistical correlation between time with infection (TWI) and antibody positive density, with some variations in certain regions, particularly of Lithuania and Estonia. The increasing trend in potential survivors (hunted wild boar with confirmed PCR negative and antibody positive results) enhances the importance of surveillance design to sample and test shot wild boar. In conclusion, surveillance data based on ASFV detection by PCR and serology can be used to assess the status of the epidemic in wild boar.

Keywords: antibodies, epidemiology, surveillance, moderately virulent virus, survivor, African swine fever, wild boar

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INTRODUCTION

Wild boar in Estonia, Latvia, Lithuania, and Poland have been affected by African swine fever (ASF) since 2014, following the spread from other Eastern European countries where the disease had been expanding since its first occurrence in this part of the world in 2007. ASF continued spreading within the European Union (EU), affecting the Czech Republic and Romania in 2017, Belgium, Bulgaria, and Hungary in 2018, and reaching the backyard pig population of Serbia and Slovakia in 2019 (1, 2). Despite the surveillance and control actions taken in the EU, ASF has continued to

spread. ASF has since 2018 also quickly expanded in up to 10 countries in Asia including China, causing severe consequences within the pig industry. Of the 24 known genotypes of ASF virus (ASFV), only two have caused epidemics outside Africa: genotype 1 (1960–1990's, affecting mainly Spain and Portugal in Europe and reaching some countries in Central and South America) and genotype 2 (current epidemic in Europe and Asia).

Attempts to control the infection in wild boar in the current epidemic have only been successful in the Czech Republic (3). Wild boar is a challenge for ASF control since it is difficult to detect the infection early. The EU surveillance strategy in wild boar from 2015 and until its next review in 2021 is mainly based on the promotion of passive surveillance and active patrolling to find dead wild boar, with ASFV detection being the test of choice in the four epidemiological scenarios identified: free areas, free areas bordering infected areas, infected areas to control, infected areas to eradicate (ASF Strategy for the EU, SANTE/7113/2015-Rev 11). Antibody testing is recommended additionally for shot animals (sometimes referred to as culled and others as hunted) in the infected-to-control and infected-to-eradicate scenarios. The detection of antibodies is always indicative of infection since there is yet no safe commercial vaccine available (4) and should be used for the diagnosis of subacute and subclinical forms of ASF.

Moderately virulent ASFV are already currently circulating (5–8). These virulent viruses produce clinical signs and lesions that are compatible with the simultaneous occurrence of acute, subacute, and chronic forms of the disease. The incubation period is therefore variable and when it is longer than in acute infections, virus shedding is prolonged over time too, particularly since the percentage of animals that could survive the infection can oscillate between 50 and 75% of the population (6). The existence of survivor animals has been described in the current epidemic (8–10) but their role in ASFV spread is still under discussion within the scientific community.

It has been hypothesized that under stressing conditions, like hunting, drought, lack of food or concomitant infections, survivors that have apparently cleared the infection (negative to virus detection but antibody positive) can become infectious again (11). A prolonged shedding together with a higher percentage of survivors may therefore constitute a prolonged source of infection for other susceptible animals.

During the 1960–1980's, in the previous ASF epidemic outside Africa (with virus genotype 1), the disease was first detected in Portugal and subsequently in Spain. In <5 years since its introduction, increased numbers of subacute and chronic forms appeared (12). These modified forms spread insidiously and remained extremely difficult to diagnose. As a consequence, low and moderately virulent ASFV spread through the Iberian Peninsula and were introduced to other countries in Europe and Latin America, mostly through meat or meat products from pigs in which the infection was unnoticed and to which susceptible pigs were exposed to Mebus (13). The fall of pork prices in affected territories due to the restrictive control measures also contributed to spread ASF to neighboring countries (14). At the time, there seemed to be a higher awareness about the risk of ASFV spread through moderately pathogenic strains, since even in the presence of unspecific or contradictory clinical signs with a low mortality rate, samples were tested against ASF. The early laboratory confirmation together with hard but effective control measures like quick stamping out of the affected farm and all of its contacts, banning of transport and movements, and repopulation with sentinel animals previously quarantined, was sufficient to eradicate ASF in mainland Italy, France, Belgium, the Netherlands, and Cuba (15, 16). In the islands of Malta, Dominican Republic and Haiti, eradication was achieved when the whole swine population was destructed, but in Haiti the implementation of measures took longer and was a threat for other countries in the area (15, 17, 18). In Brazil, despite an early detection, ASF perpetuated through swill feeding, the presence of classical swine fever, and social factors that resulted in mistrust toward the situation of ASF in the country complicated control, that was finally achieved with the support of government and military police, the destruction in slaughterhouses of animals confirmed positive by the National Reference Laboratory with direct immunofluorescence and heamadsorption in leucocyte cultures for virus detection and indirect immunofluorescence and immunoelectrophoresis for antibody detection (19). Only Portugal, Spain and Sardinia remained endemic. The development of a sensitive and specific ELISA test in 1979 in Spain was one of the most important pillars to detect and eradicate ASF positive animals in an endemic situation. There was evidence of a small percentage (<5%) of survivors that were able to further transmit the virus, but their role in the maintenance of the disease in the population was not as frequent as other routes of transmission such as contacts among neighboring farms (17). Nonetheless, it was not until all survivors were eliminated, thus suppressing any possibility of any of them becoming carriers, that ASF was finally eradicated from the Iberian Peninsula in the 90's (20). A 2012 study also confirmed the absence of ASF in wild boar in the area that had been most affected (21).

Alternatively, other authors assume that survivors would not shed significant amounts of virus and would not represent a prolonged source of infection (10, 22, 23). These authors argue, among other reasons, that animals that survive ASF infection are rare in the current epidemiological situation. Schulz et al. (10) could only detect nine wild boar that were both ASF positive by PCR and serology by surveillance in an area in Estonia where seropositive animals dominate the epidemiological situation, possibly indicating the late phase of the epidemic. However, they recognize that ASF could become endemic instead of fading out. In any case, it is now clear that comprehensive surveillance and laboratory results based on ASFV detection by PCR and serology, can be used to assess the status of the epidemic in wild boar.

The aim of this study is to analyze the ASF surveillance data notified through the EU Animal Disease Notification System (ADNS) with the objective of characterizing the infection in wild boar in those areas in which ASF has been present for longer (Estonia, Latvia, Lithuania and Poland). Following ASF dynamics, one would expect to find a higher density of seropositive wild boar in those areas in which the infection has been present for longer.

MATERIALS AND METHODS

Each notification (confirmed ASF) in the ADNS database contains at least information on the host (wild boar/domestic pig), the location (latitude, longitude, region, and country), confirmation date, reference number, outbreak type (primary/secondary), and number of affected animals. There is space to add free text and countries generally include here other useful information in a non-systematic way: test results, found dead or hunted wild boar, age and gender, type of farm, location. We restricted the study to wild boar notifications.

From the free text, we were able to assign the category of dead/hunted for each wild boar notification. To do so, we searched for key terms like "hunted," "shot," "hunting," "executed," "killed," "shoot," to assign the "hunted" category, and "dead" or "found" for dead wild boar. The data were checked several times since, for example, some notifications included both the words hunted and dead in the text, and it was necessary to classify these on a one-by-one basis. When in the same notification there was information about both dead and hunted wild boar ($n = 62$), we favored the category "hunted" since our interest is primarily to analyze the evolution of infection when the disease might be unnoticed. However, if there was no information on whether the wild boar were either hunted or dead, we favored the category "dead" ($n = 1{,}213$).

Notifications were also classified according to whether the confirmation of infection had been performed by PCR, which we assumed represented the initial stages of infection (Stage 1); by PCR and an antibody test (ELISA and/or IPT), which we assumed would represent animals which had the infection for some time longer (Stage 2); or which were positive to the antibody test and the nucleic acid detection test was either not specified or negative, which we assume would represent the latest stage of infection, when ASFV detection decreases but immunity mounts, leading to an increased percentage of survivors (Stage 3). For 1,160 notifications (<10% of the total 12,661) with no information on whether the wild boar was hunted or found dead or on the test used, we assumed they were dead wild boar tested with PCR. Wild boar notifications estimated to be in Stage 3 of infection comprise those with a positive antibody result together with either those that specifically state that a negative PCR has been obtained or those in which we assume the PCR has been negative because this diagnostic test result is not specified.

Since only Estonia, Latvia, Lithuania, and Poland have confirmed the detection of antibody against ASF, we restricted the analysis of the evolution of ASF infection to these four countries. The temporal evolution of the notifications in dead and hunted wild boar and the diagnostic test/s specified in the notifications was analyzed descriptively for these four countries. Notifications comprised between 2014 and March 2019, but we restricted the analysis to complete years (2014–2018). Differences and trends were statistically analyzed in R Core Team (24).

For each administrative unit within a country, we estimated the time with infection (TWI) by subtracting the last from the first date in which ASF was notified to obtain the number of days ASF has been present in each unit. The assumption is that independently on whether the infection has remained or has been reintroduced, the probability of finding antibody will be higher the longer the infection has been present in that area (longer TWI). The administrative units used were "powiat" (second level, county or district) for Poland, "savivaldybe" (second level, municipality) for Lithuania, "aprinki|rajoni" (second level, district) for Latvia and "maakond" (first level, county) for Estonia, similar in size and publicly available for download at https://gadm.org (version 3.6, last accessed on June 2019). The estimated TWI was explored spatially by representing the distribution of natural breaks (Jenks) classification in a choropleth map in each administrative unit.

We explored whether there could be a correlation between the number of notifications in which antibodies were detected and the estimated TWI per administrative unit by computing Spearman's correlation coefficient Rho in R Core Team (24), where a $p < 0.05$ was considered statistically significant. The same analysis was also carried out with the proportion of notifications in which antibodies were detected and TWI.

The ASF wild boar notifications with positive serology were fitted a kernel density function in a map, using geodesic distances between points and an output cell size of 0.034 sq km. Both maps were developed in ArcGIS 10.2 (ESRI) and were compared qualitatively.

RESULTS

From the entry of ASF in Eastern EU in 2014 to December 2018 there have been 13,379 wild boar notifications to the ADNS from EU countries, of which 95% (12,661) have occurred in Estonia, Latvia, Lithuania and Poland, which have been the only countries in the EU infected since 2014. In these 4 countries, ASF has been detected in over 8,100 wild boar found dead (64%) and over 4,500 hunted (36%). The annual evolution of ASF positive wild boar found dead and hunted in Estonia, Latvia, Lithuania, and Poland from 2014–2018 is represented in **Figure 1**. Lithuania and Poland mainly notified ASF in wild boar from dead animals. Lithuania and Poland have increased the number of notifications in wild boar each year, while Estonia's notifications in wild boar peaked in 2016 and Latvia's in 2017.

The annual distribution of notifications by diagnostic test used and estimated stage of infection is shown in **Table 1**. The majority of notifications (78%, 9,882) were based on PCR results (Stage 1). The remaining 22% comprise 393 notifications that include both PCR and antibody positivity results (Stage 2) and 2,386 notifications based on antibody results only (Stage 3).

The apparent increase in notifications in Stage 3 can be better observed in **Figure 2**, where the proportion of notifications in each stage over the total ASF notifications in wild boar per year has been stratified by dead/hunted and by country. In dead wild boar, the predominant diagnostic result is obtained by PCR. In fact there are very few notifications ($n = 15$) in Stage 3 in dead wild boar: 2 from Latvia (one in 2015, the other in 2016) and the rest from Bialski, in Poland, in 2018. In hunted wild boar, there are some differences by country but not statistically significant according to a factorial ANOVA test. In general, the % of notifications based only on PCR results (Stage 1) has decreased

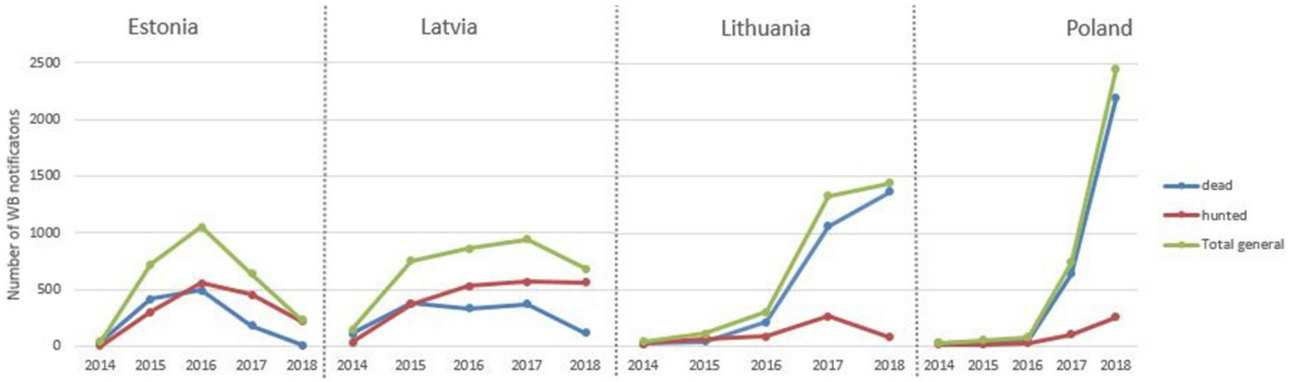

FIGURE 1 | Annual number of ASF notifications in wild boar.

TABLE 1 | Annual distribution of ASF notifications in wild boar by diagnostic test/s used and estimated stage of infection.

Year	Stage 1 (PCR+, AB−)[a]	Stage 2 (PCR+, AB+)[b]	Stage 3 (PCR−, AB+)[c]
2014	243	19	2
2015	1,284	70	285
2016	1,596	122	582
2017	2,813	127	713
2018	3,946	55	804
Total	9,882	393	2,336

[a] *Includes combinations with ELISA– or not specified, and IPT– or not specified.*
[b] *Includes the combinations ELISA+ and IPT+, –, or not specified, and ELISA– or not specified but IPT+.*
[c] *Includes the combinations PCR– or not specified, ELISA+ and IPT+, –, or not specified, and PCR– or not specified, ELISA– or not specified and IPT+.*

since 2014 to give rise to the notifications based on antibody detection (Stage 3). A factorial ANOVA test for hunted wild boar in Stage 3 showed statistically significant differences by year, particularly from 2016 onwards (Tukey's honest significant test, confidence level = 0.99). Only Lithuania has not increased the % of antibody notifications by year. In hunted wild boar, 1,218 Stage 3 notifications are truly PCR negative, antibody positive and in 1117 PCR is not mentioned, but they are antibody positive. Out of the 1,218, 1,106 are from Latvia and exhibit an annual increasing trend (2015 = 158; 2016 = 282; 2017 = 297; 2018 = 369), 4 are from Lithuania (2015), and the remaining 108 are from Poland (2016 =1; 2017 =19; 2018 = 88). A Poisson regression model on the apparent annual increase of notifications in Stage 3 in Latvia indicates that it is statistically significant ($p < 0.01$). The correlation test indicated an overall strong positive statistical association between ASF serology notifications and TWI by administrative unit (rho = 0.77, $p = 2.2 \times 10^{-16}$). Although still statistically significant, the correlation was weaker when considering the proportion of notifications based on antibody detection (rho = 0.35, $p = 2.2 \times 10^{-4}$).

The spatial representation of the TWI in each administrative unit per country can be found in **Figure 3**. The areas bordering Belarus and those between Latvia and Estonia, have had ASF for longer. The kernel density map of notifications in either Stage 2 or Stage 3 (**Figure 4**) showed that in some instances, a higher density of antibody positive notifications is present in those areas where ASF has been present for longer, particularly in Latvia. The density of antibody positive notifications is very low in the border of Lithuania with Belarus, where ASF has been present for more than 3 years. In contrast, in certain areas relatively recent in their acquisition of the infection, like the Estonian island of Saarema or the southwestern notifications in Poland, there is a higher density of serological notifications. We have represented in both maps the location of the virus that were characterized as moderately virulent by the EURL (5, 6). Both Estonian virus were isolated from 2015 outbreaks, one in Valga and the other in Tartu. In both regions ASFV has continued to circulate since 2015, since Valga is classified in the longest TWI category (3.5–5 years) and Tartu in the second longest (2.5–3.5 years). Both fall in an area corresponding to the second highest seroprevalence density category. In Latvia, the virus recovered from a 2017 outbreak in Engures was non-hemadsorbing (non-HAD). This area has a TWI of only 1.5–2.5 years, however it also falls into an area with the second highest seroprevalence density category.

DISCUSSION

The analyses of the evolution of wild boar ASF notifications to the European Union (EU) surveillance database (ADNS) in Estonia, Latvia, Lithuania and Poland, the four countries which have had ASF since its introduction in the EU in 2014, reveal a progressive and statistically significant increase in the percentage of notifications based on antibody positive results with either negative or assumed negative PCR result in the period 2014–2018 in hunted wild boar (Stage 3), even if the number of notifications in hunted wild boar has remained relatively stable and much lower than notifications of wild boar found dead across the whole period. The annual increase in "truly" Stage 3 (PCR negative, antibody positive) notifications was tested only for Latvia since it was the only country with consistent data across the study period.

For the analyses of ASF wild boar evolution with ADNS data, we have had to make certain assumptions. We cannot

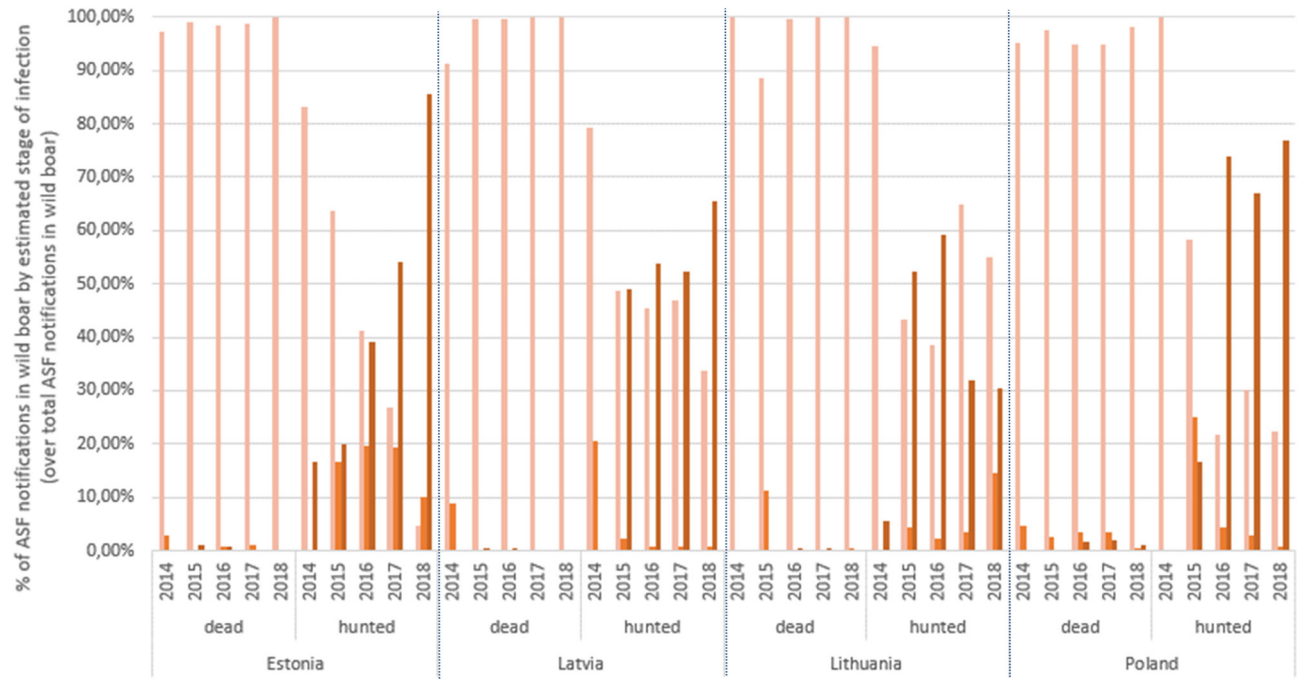

FIGURE 2 | Proportion of ASF wild boar notifications by estimated stage of infection over total ASF annual wild boar notifications.

control the way the data was recorded into the system, and consequently any bias derived from data collection or entry will be accumulated. While the EU Regulation and the ADNS system ensures certain harmonization, our analysis was based mainly on classifications made from the "free text" and thus subject to our interpretation and assumptions as explained in the Materials and Methods section. For example, there is a notification in Estonia in February 2015 with 10 hunted wild boar in which ASF was confirmed by ELISA and immunoblotting. There is no information on PCR results so it has been classified as Stage 3. If correctly classified, one could interpret that as soon as that early in the epidemic there were potential survivors. Latvia also confirmed 6 hunted wild boar PCR negative and ELISA positive in a single notification. Further analyses could be performed if, in addition, information on the antibody titers were included with these type of notifications, reducing the potential bias derived from our classification method. Similarly, we are assuming that the animals classified in Stage 3 could be potential survivors. They remain potential since with the information provided it is impossible to estimate the uncertainty regarding their status. The dynamics of ASFV, widely studied in the scientific literature, show that antibodies are detectable from 1 week onwards after infection, peaking between days 10–20 post infection and then maintained at high levels over time if the animal survives (10, 11). Gallardo et al. (11) also summarize in their article that viremia has experimentally been detected by PCR as early as 3 days post-infection (dpi) in acute infections and at an average of 8.5 ± 3.6 dpi in subacute infections. Also, that in pigs surviving acute or subacute infections, viral DNA has been detected in blood for up to 78 days, but with several peaks, similarly to the excretion pattern.

ASFV has circulated for almost 12 years in Eastern Europe, of which the last nearly 5 years correspond to spread in the EU (mainly in wild boar). The probability of co-circulation of virus with different virulent degrees is higher than at the beginning of the epidemic, as is the probability of prolonged "high risk periods" (time between infection and field detection) that would allow a "silent" spread of infection. The "high risk period" was estimated to be between 7 and 20 days in domestic pig farms in Estonia between 2014 and 2017, where all antibody positive animals were also PCR positive (25). In wild boar, since there has been up to now an active component of surveillance for hunted boars in infected areas, this offered an opportunity to evaluate the likelihood of ASFV spread by "healthy" animals. In terms of laboratory results, more antibody positive and PCR negative field samples are to be expected if the surveillance design still contemplates hunting to test wild boar for control and eradication purposes at least. This is because antibodies for ASF are assumed to remain for life, but viremia, when it persists, it is with intermittent peaks and therefore easier to miss under surveillance conditions.

The representation of the time with infection (TWI) per administrative unit is a quick and easy way to capture the evolution of ASF spread, particularly when prevalence data cannot be measured adequately because of a changing and often imperfect denominator data. The wild boar population density in the affected areas has changed over the last years probably due to ASF deaths and to the application of drastic

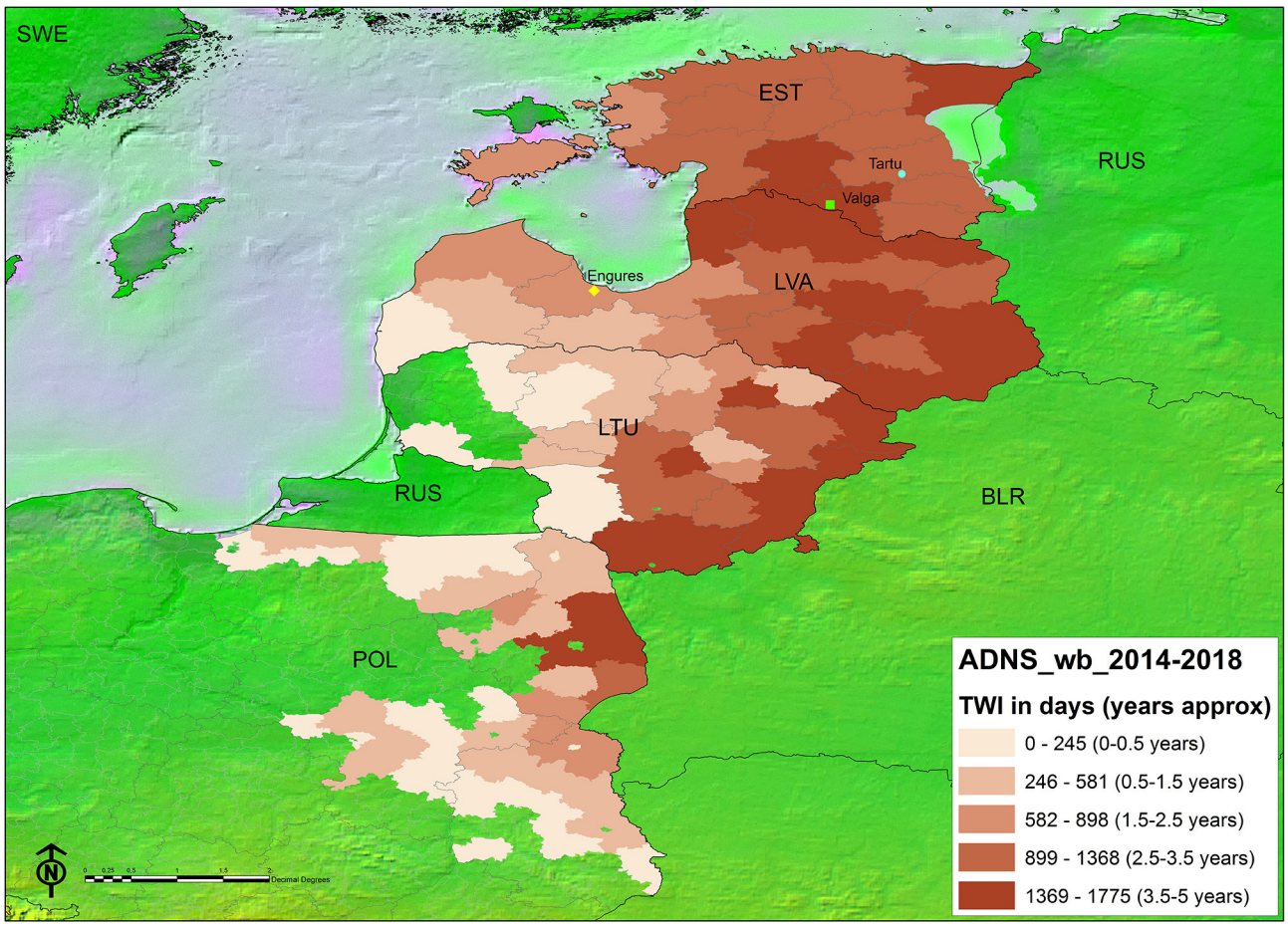

FIGURE 3 | Map of the time with infection (TWI) distribution (natural breaks) by administrative unit (Data source: EU Animal Disease Notification System). Country acronyms as per ISO 3166-1 alfa-3). Points indicate the location of ASF virus of attenuated virulence characterized at the EU Reference Laboratory for ASF, from top to bottom: circle: ES15/WB/Tartu14 (5); square: ES15/WB/Valga6 (5); diamond: LV17/WB/Rie1 (6).

depopulation measures to fight ASF (26). Other analytical studies on surveillance data have used wild boar density estimates dividing the hunting records per year by the sum of the hunting grounds [(10) for Estonia] or by reconstructing a numerical value per map cell based on habitat suitability maps combined with abundance data based on hunting records [(27) for Poland]. For our study, we preferred to use the TWI since our primary interest was to analyze surveillance results assuming that, in the light of ASFV dynamics, an increase in time of PCR negative and antibody positive results could reflect a higher probability of animals surviving the infection. However, if the population of wild boar has indeed decreased, rising percentages of notified seropositive animals would also be expected naturally if the number of animals surviving the infection remained constant in time. So far the survival rate is not known. Similarly, if further field observations reveal that there is a difference in incidence between age groups as was evidenced with classical swine fever (28), the interpretation of TWI and seropositive findings should also take this difference into account. There are higher TWI values along the Belarusian border with EU countries. Belarus was infected before the EU (in 2013) and, together with Russia, it was the suspected origin of wild boar notifications in the EU (29). The TWI also indicates the direction of spread, east to west, since in the latter ASF has appeared later. Finally, the TWI also allows further epidemiological investigation into areas in which the infection could be perpetuating. In this sense, we would expect to find more ASF potential survivor wild boar, particularly if the virus circulating in those areas correspond to strains of attenuated virulence. A potential increase in the number of animals surviving the infection could also reflect a balance in the host-virus interaction either because of a possible attenuation of ASFV virulence, a higher immunity of the host or a change in the routes of transmission of ASFV that lead to lower viral infection doses. The evidence of circulation of attenuated strains is scarce for the moment and is mainly restricted to experimental observations. The experimentally identified attenuated virus (5, 6) were obtained from areas in which ASF has been present as soon as 1.5–2.5 years with the infection. All three identified ASFV attenuated strains correspond to areas with a high density of antibody positive notifications. It is hard to expect that these

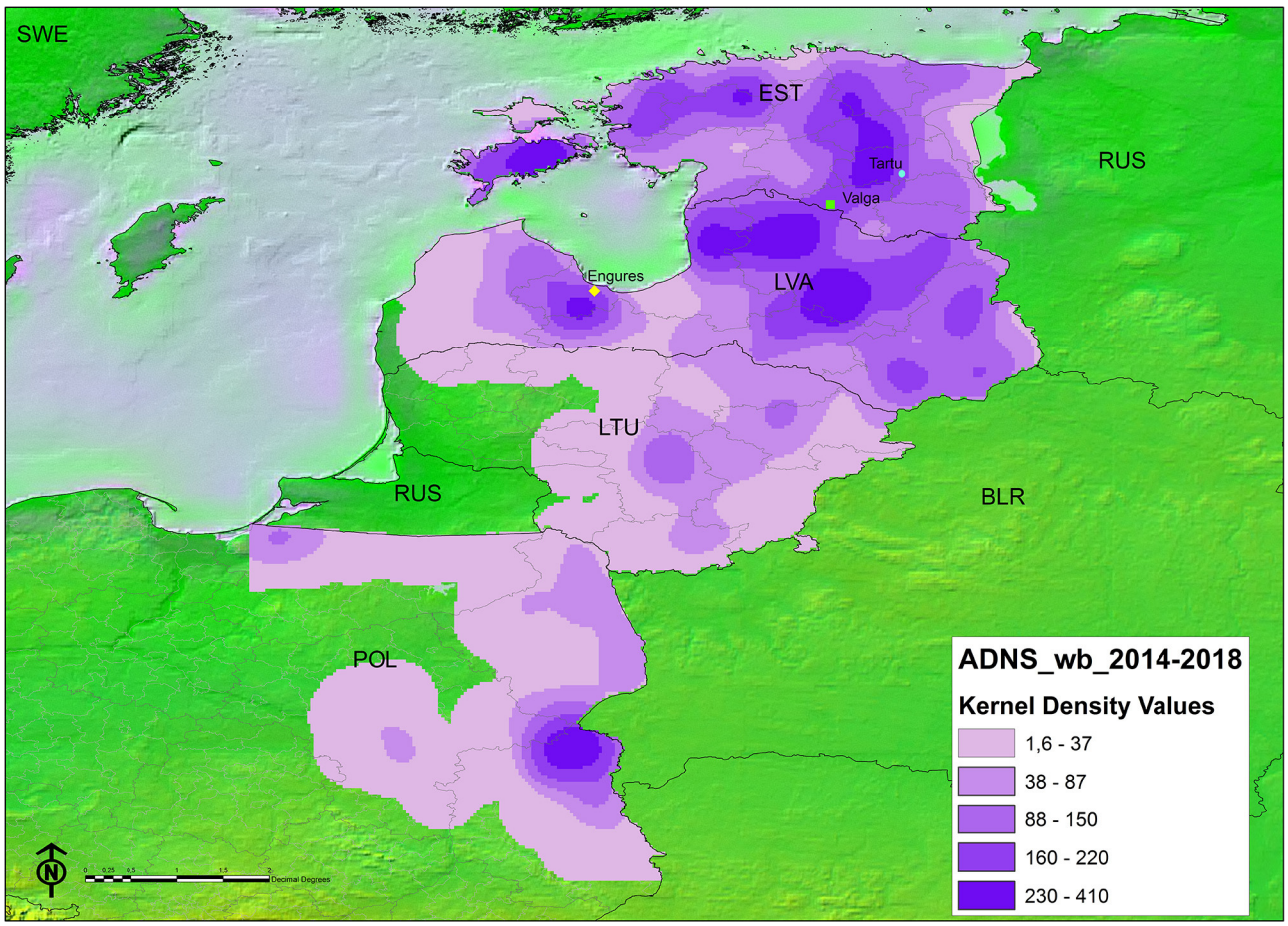

FIGURE 4 | Kernel density map of antibody-based ASF notifications in wild boar classified by natural breaks (darker color indicates higher density). Points indicate the location of ASF virus of attenuated virulence characterized at the EURL, from top to bottom: circle: ES15/WB/Tartu14 (5); square: ES15/WB/Valga6 (5); diamond: LV17/WB/Rie1 (6).

strains be identified in the field if the surveillance design does not contemplate their potential detection. So far, the EU legislation (Council Directive 2002/60/EC) intends a 100% sampling of the whole hunting bag in restricted areas, applying an optimal strategy for their identification. In Latvia, results have showed an annual increasing trend of potential survivors: 1,106 notifications of hunted animals in Stage 3 were PCR negative and antibody positive (2015 = 158; 2016 = 282; 2017 = 297; 2018 = 369). This fact strengthens the importance of enhancing testing of shot wild boar for surveillance purposes because, opposite to what Schulz et al. (10) stated, the probability of finding more animals surviving the infection should not be considered a rare event in the current epidemiological situation. Finding dead wild boar can be hard, particularly if there are only a few hours of light like is the case in the Baltic countries in winter. In addition, it can also be difficult to find dead animals under harsh weather conditions, like snow or rain. Wild boar surveillance data is imperfect by nature and its epidemiological interpretation is of utmost importance to understand the extent of the infection in the field.

The main area in which a high TWI does not correspond with a high density of antibody positive notifications is in the Lithuanian border with Belarus. Lithuania and Poland have fewer wild boar notifications from hunted animals than Estonia and Latvia. Assuming a similar surveillance effort for hunted wild boar across countries, one could interpret that Poland and Lithuania are experiencing more recent and acute infections, while in Estonia and Latvia more moderately pathogenic forms could have started to occur. Lithuania experienced outbreaks in commercial hunting grounds densely populated during 2017, and from 2016 there was also a compensation scheme to notify wild boar found dead (30). Both aspects could explain the increase in ASF wild boar found dead. In Estonia, there has been an increase in the proportion of antibody positive notifications, and the latest area to be infected, the island of Saarema, concentrates a high number of notifications with serology. Nonetheless, overall, there is a strong statistical correlation between the number of notifications with an antibody positive result and TWI per administrative unit, which is what was expected given

the common regulatory framework that harmonizes surveillance efforts among countries.

In addition to the current information provided by countries in the official notifications, it would be extremely useful to include the quantitative result of the antibody titration. Antibody titration allows to estimate the time since infection, which would provide further insight on the epidemiological situation by allowing to identify recovered and asymptomatically infected animals. The most commonly test used for ASF antibody detection is the ELISA but it is only suitable for serum or plasma (31). ASF antibodies persist for many months and even years (20, 32) and serological assays are the most efficient way, due to their simplicity and relatively low cost to detect animals with unspecific signs of disease due to infection with moderately virulent strains (11). For antibody detection in blood, exudate tissues or body fluids, IPT is the test of choice (11). IPT has a higher sensitivity than the ELISA and is used as a confirmatory test for ELISA positive sera from ASF free areas or when doubtful ELISA results are obtained from endemic areas or serum samples are poorly preserved (31). However, IPT requires specific expertise and training to interpret the results and they are not commercially available. For this reason, the availability of a commercial confirmatory serological assay has been identified as a priority for the near future (11). The continuous presence of ASF in certain areas together with the never-ending threat of reintroduction from endemic areas or with a tendency to become endemic should be considered to update the surveillance and control plans.

In conclusion, the TWI provides a relatively fast and easy tool to assess the evolution of ASFV infection by geographical area even with limited population data. Surveillance based on ASFV detection by PCR and serology is a powerful source of data to assess the status of the epidemic in wild boar despite its imperfect nature, and allows to follow up the evolution of further potential survivors.

DATA AVAILABILITY STATEMENT

Data were from the EU Animal Disease Notification System database. The authors don't have permission to share the datasets. Requests to access these datasets should be directed to Marta Martínez-Avilés, marta.sanidadanimal.info@gmail.com.

AUTHOR CONTRIBUTIONS

All authors contributed to the design, analyses, interpretation of results, and writing of the manuscript.

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Modelling Spatial and Temporal Patterns of African Swine Fever in an Isolated Wild Boar Population to Support Decision-Making

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African swine fever (ASF) is a highly contagious disease affecting all suids including wild boar. As the disease can damage commercial pig production and its circulation can threaten international trade, understanding the risks produced by free-living wild boar (as a wildlife reservoir) is important to ensure proportionate policies to exclude the disease, as well as an effective contingency response. The recent spread of the virus into Western Europe has produced concerns in many stakeholders including pig producers and national governments. Unlike in mainland Europe, where wild boar are widespread, in Britain, free-living populations have only recently re-established, and whilst these are still relatively small and isolated, they may provide a sufficient reservoir capable of sustaining disease and may thus present a continual source of infection risk to domestic pigs. This study focuses on one component of the risk produced by wild boar, specifically the distribution and persistence of virus in a landscape produced by the natural circulation of disease within wild boar. We used a spatial individual-based model run across a representation of a real landscape to explore the epidemiological consequences of an introduction of ASF into the Forest of Dean, currently hosting the largest population of wild boar in England. We explore various scenarios including variations in the prophylactic management of boar, as well as variations in reactive management (contingency response) following the detection of disease to evaluate their value in reducing this specific risk (presence of ASF virus of wild boar origin in the landscape). The abundance and distribution of wild boar is predicted to increase across our study extent over the next 20 years. Outbreaks of ASF are not predicted to be self-sustaining, with the median time to disease "burn-out" (no new infections) being 14 weeks. Carcass removal, as a tool in a package of reactive management, was of limited value in reducing the duration of outbreaks in this study. We suggest that useful predictions of some of the risks produced by ASF might be possible using only the distribution of the boar, rather than more difficult abundance or density measures.

Keywords: individual-based, real-world landscape, UK, wildlife management, contingency planning

INTRODUCTION

African swine fever (ASF) is a highly contagious and virulent disease of suids, known to severely damage commercial pig production and infect free-living wild boar in Europe (1). It is recognized by the World Organization for Animal Health (OIE) as a notifiable disease and its circulation has an impact on international trade. There is, therefore, considerable interest in detailed assessments of the risks posed by this disease to help countries prepare for its incursion, and the required contingency responses. These responses include those affecting commercial pig farms, as well as back-yard producers rearing small herds and some interventions produce an economic burden *per se*; limiting the duration of these controls is of economic interest. Where the disease may circulate in wild hosts, the risks this produces to commercial pig production must be also be considered. The description of these risks includes the character of outbreaks in wild boar (i.e., duration, intensity, geographical extent) where free-living populations overlap with commercial or back-yard pig premises. Predicting what an outbreak of ASF in wild boar might look like can also help inform the design of policies to address these risks. Such policies should be proportionate and practical (2), following available guidelines (3, 4) and should be based on the best available science (5, 6).

The spread of ASF westwards across Europe has been thoroughly described and analyzed to support both local action and to adhere to international agreements on actions and trade (7). There have been several studies using simulation models to explore the relative benefits of a variety of management responses to the detection of ASF in wild boar [e.g., (8, 9)]. All these studies assume a widespread distribution of wild boar across extensive regions of Europe, which are often densely forested and/or sparsely inhabited.

The current guidance produced by EFSA for the management of ASF in wild boar lists actions to contain the infection to a geographically discrete population of wild boar until the infection "burns-out" (defined here to mean a situation with zero infected animals) within this prescribed zone, using intensive focal hunting and a non-hunted buffer to prevent the propagation of disease across the wider landscape (7). In parallel, active searches for wild boar carcasses must be carried out to reduce onward infection. This policy has proven effective in one of the more recent outbreaks in the Czech Republic (Zlín area), where the infection in wild boar has now been declared absent (https://europa.eu/rapid/press-release_MEX-19-1431_en.htm).

The situation in England differs sufficiently from that of continental Europe to suggest that additional predictive modeling is required to inform decision-making. In particular, differences include the character and ownership patterns of the landscapes supporting boar, along with attitudes toward them. The first of these (patterns of landscape) influences the demographic and spatial dynamics of the wildlife host, potentially altering the course of an unmanaged outbreak of ASF from that anticipated in Europe; whilst the latter (attitudes) may affect the acceptability or effectiveness of potential management tools. In England, landscapes commonly host fewer, smaller and more fragmented forested areas than those typically found in Europe, and are dominated by a fine-scaled mosaic of arable land and pasture interspersed with small woodlands, people, and infrastructure (e.g., settlements, major roads, canals), all of which are likely to modify the movement behavior of individual boar as well as their population processes (population dynamics). The recent reintroduction of boar and the landscapes available to them have to date restricted them to a few discrete populations. The most significant of these, in the Forest of Dean, is thought to have only established relatively recently (10) and appears to still be spreading and increasing in density in its core.

To better understand the risks to commercial pig production posed by the incursion of ASF into a free-living population of wild boar in England (high density but spatially isolated) we developed a spatially explicit individual-based model (IBM) and used it to predict the course of disease in wild boar and explore the value of different prophylactic and reactive management actions to mitigate the risk. Specifically, we wished to test the hypothesis that the risks produced by ASF would be similar to those identified for foot-and-mouth disease (FMD) (11), and required a detailed prediction of the duration of disease circulating exclusively within the population of free-living wild boar (and the consequent continuous presence of uncontained or unmanaged ASF virus in the landscape). We then wished to evaluate the significance of population size (boar abundance and distribution) and the value of prophylactic population control in dictating the severity of an outbreak, and compare this to a number of reactive tools (after the discovery of disease; increased levels of culling and carcass retrieval) to most quickly, or effectively achieve disease elimination.

METHODS

Study Site

The Forest of Dean (e.g., 51.80°N, 02.52°W) is located in south-western England. It includes an extensively forested core area owned by the Forestry Commission (hereafter the FC estate) comprising a fragmented 75 km^2 of mixed woodland surrounded by a mosaic of arable, pastures and smaller woodlands largely in private ownership (**Figure 1**). Our study extent is defined by a 25 km buffer around the FC estate in the Forest of Dean and describes an arena of ∼4,500 km^2.

Feral wild boar was first detected on the FC estate during the 1990's and has been monitored annually since 2013. Population management using targeted culls (restricted to the FC estate) has been ongoing since 2008 (13). Latest figures published by Forest Research suggest that the current population is 1,635 (14); an increase of 60% from figures reported in 2015 (15), despite removal of ∼500 individuals per year (14). Information about the distribution or abundance of feral wild boar in the wider landscape is unavailable, as is any empirical description of the hunting effort or hunting bag size.

Model Framework

Overview

The model was adapted from that outlined in Croft et al. (11), previously developed to explore management of foot-and-mouth disease (FMD) in wild boar. Written in the

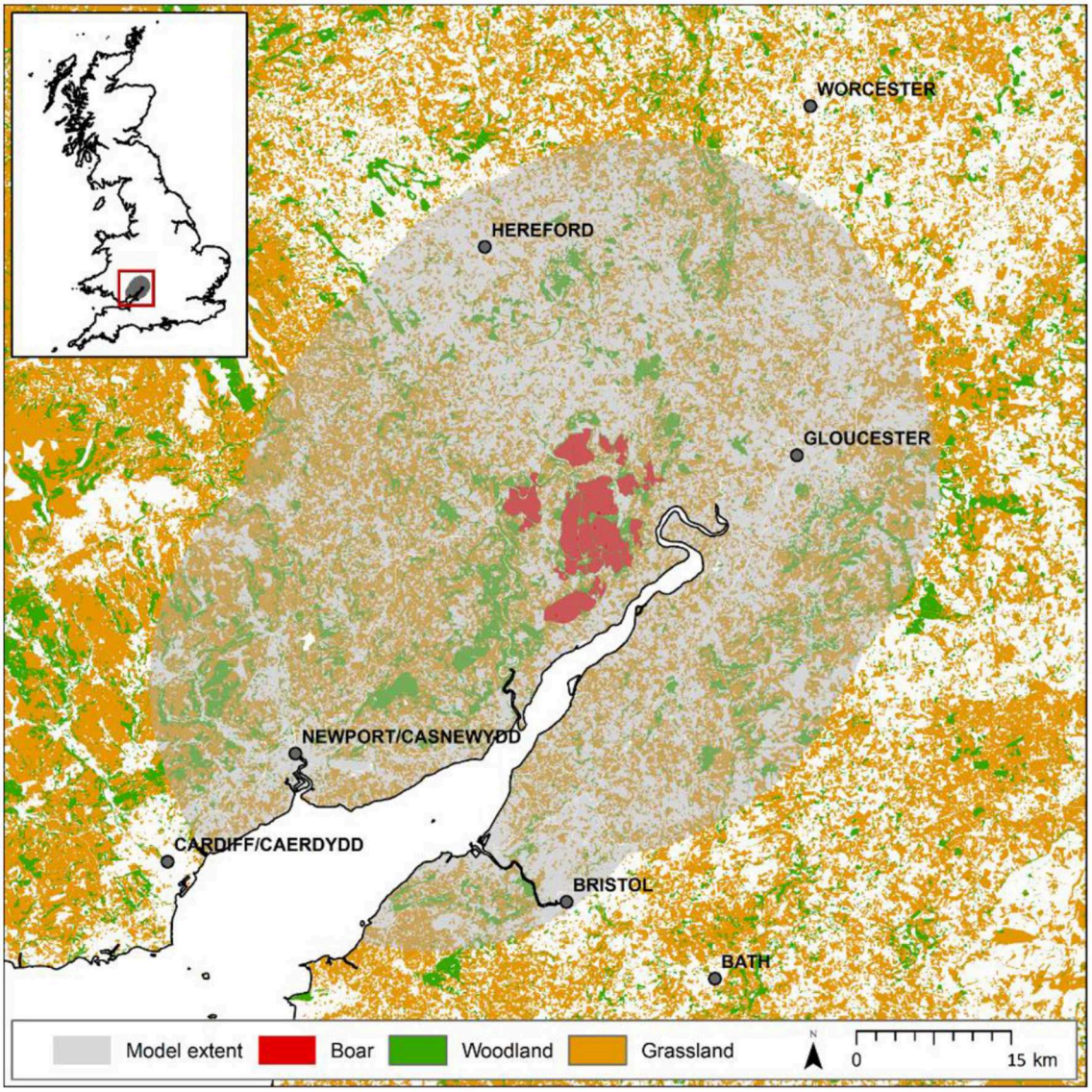

FIGURE 1 | Map of study extent around the Forest of Dean. Model considers a 25 km buffer around the forest estate separated into two regions: that currently monitored (red), where boar are known to be present and are controlled, and that unmonitored (shaded) where anecdotal evidence suggests boar are yet to establish possibly due to hunting activities. Presumed habitats suitable for boar (woodland and grassland) are shown demonstrating the quality of the wider landscape to support species expansion. The map shown in this figure contains data (GB coastline) obtained from the OS Strategi™ dataset 2016. This data is freely available under an open government license (Crown copyright and database rights 2016). Land cover data (woodland and grassland) is based upon LCM2007 NERC (CEH) 2011 made available to Defra under license. LCM data also contains Ordnance Survey data 2007. For full details of the LCM dataset [see Mortan et al. (12)].

Python 2 programming language (16) (code files provided in **Supplementary Material**), this model applied an individual-based approach with agents (individual wild boar) operating across a simulation of a real-world landscape. Agents were distinguished by their demographic class (e.g., age, sex) and exhibited defined stochastic behaviors (e.g., survival, reproduction, dispersal) in order to emulate a realistic population and spatial dynamics. The avoidance of a grid-based model has been shown to reduce bias (17, 18). In addition, the model also included processes to represent management activity applied to remove boar within the FC estate (removal through variable effort—culls to achieve fixed target quotas) and the wider landscape (unregulated hunting without descriptions of effort or effect). In the UK, both culling and hunting rely on shooting free ranging animals and are functionally identical. We distinguish between them here for clarity of inference and discussion by defining culling as the organized and coordinated activity in one area to achieve a policy objective, and hunting as the unmanaged, unregulated and unmonitored *ad-hoc* activity across the wider study arena. To simulate these activities, animals were removed

TABLE 1 | Epidemiological parameter values used in the model.

Parameter	Value
Probability of infection (conspecific)	0.05
Probability of infection (carcass)	0.15
Group overlap distance (km)	1.35 km
Period from infection to death	1 week
Persistence of maternal antibodies	15 weeks
Disease-induced mortality (individual)	0.95
Disease-induced mortality (pre-natal mortality)	0.5
Disease-induced fertility reduction	0.625

ASF specific parameter values adopted from Lange and Thulke (8).

from the model with fixed weekly probability (hereafter referred to as p. culled and p. hunted, respectively). Full details of the core host model are available in Croft et al. (11).

The main modifications relate to the model's epidemiological component to simulate the key characteristics of ASF. Following Lange and Thulke (8), we consider direct transmission through contact with infected conspecifics within the same social group and through contact with infected carcasses distributed across the landscape. To simulate carcass mediated transmission, individuals that die as a result of disease are retained in the environment as a source of infection with which living conspecifics in the deceased individual's current social group (patch) and up to one neighboring social group (determined according to the relative proportions of range overlap) can interact (8), becoming infected according to a fixed probability. Carcasses remain in the environment for a fixed period after which they are no longer considered a source of infection and are removed. The small number of boar which survive ASF are considered to become immune for life; females beginning lactation within 15 weeks of their initial infection can convey maternal immunity to their dependent offspring until the end of this period (**Table 1**), though these subsequently become susceptible once independent.

Parameterization

The boar components of the model were parameterized as described in Croft et al. (11), based on values from existing literature, empirical studies and other models. Epidemiological parameters were adopted from Lange and Thulke (8) and are detailed in **Table 1**. All were applied directly, with the exception of the probability of disease-induced death in the core areas of a patch, i.e., distinct non-overlapping area where conspecifics in neighboring social groups will have no contact with carcasses.

Lange and Thulke (8) define this factor based on an interaction between 3 × 3 km resolution cells as the central 1% or 300 × 300 m of each cell (or patch). Here, we generalize this representation by computing the corresponding width of overlap between neighboring social groups (1.35 km either side of a boundary line) which, when applied to the irregular polygons we use to portray our real-world landscape (mean area 3.9 km^2), can be used to derive the probability that contact with a carcass will remain exclusively within a social group.

Simulations

Populations were initialized according to a fixed distribution of boar approximating that reported for the FC estate in 2015 (15). Initial demographics were applied to match the stable structure achieved following a 5 year burn-in period (running the model prior to the main simulation including disease and management) (11). For each parameterization, we performed 100 unique repetitions [10 repetitions for each of 10 different randomized representations of boar social grouping across the landscape; refer to (11)] from which summary statistics were produced. Specifically these were: change in total population (boar), area occupied (km^2), density (boar/km^2) and, for simulations with ASF introduction, the time to disease freedom (weeks) and change in the cumulative size of infection; the latter represented by both the linear distance between the centroids of the patch hosting the focal case and the most distant patch of cumulative infection (km) and the cumulative area infected (km^2).

Disease Scenarios

Using our model we simulated different outbreaks of ASF, infecting an individual selected at random, assuming various timings of release (after 0, 3, 5, 10, 15, and 20 years), which produces a range of simulations run across populations of differing size and distribution. In this study, infection always occurred on the same day of the year (representing the 15th April), producing a seasonally fixed response. For each of these scenarios we tested the efficacy of different management options combining both removal (culling and hunting) and environmental decontamination (carcass retrieval). Initially, we varied hunting rate (p. hunted) with a fixed rate of culling (p. culled) within the FC estate equivalent to that applied in 2015 [p. culled = 0.0065; weekly probability of removal (11)] considering: (i) where hunting is completely absent (p. hunted = 0); (ii) where its rate matches the rate within the FC estate (p. hunted = 0.0065); (iii) where hunting is so efficient it immediately removes any boar that appear in the wider landscape (p. hunted = 1). We also considered scenarios exploring intensified culling rates within the FC estate, specifically a 50 and 100% increase in weekly removal probability (p. culled = 0.01 and 0.013, respectively), assuming a fixed rate of hunting (p. hunted = 1).

We then considered the addition of carcass retrieval to control an outbreak by shortening the time that carcasses remain in the model. For each management scenario using removal alone, we tested reducing carcass persistence from 8 weeks [reflecting the average time mainly invertebrate scavengers take to completely consume a carcass (19)] to either 4 weeks or 2 weeks to reflect moderate and intense search and removal efforts.[Based on the time to disease eradication (zero infected individuals), we compared results to assess the efficacy of both prophylactic (long-term population control prior to disease introduction) and reactive control options (increased removals and environmental decontamination). It should be noted that in all simulations disease detection occurs quasi-simultaneously with release. As a consequence of this choice, the efficacy of reactive control options tested should be interpreted as an absolute "best case" intervention when an outbreak is at its weakest.

RESULTS

The levels of culling and hunting affect the size and distribution of the population of wild boar over time (**Figure 2**). If no hunting were to occur outside of the FC estate (p. hunted = 0) then within 20 years the wild boar population could reach around 8,000 individuals (more than 7 times the population in 2015) occupying 500 km^2 (more than double that of 2015). With moderate hunting (p. hunted = 0.0065), equal to the estimated culling rate in the FC estate [i.e., ∼45% of the population removed per year (15)], our results showed that wild boar populations would still grow but at a slower rate, reaching nearly 4,000 individuals occupying 300 km^2 in the same 20 year period. With intense and continuous hunting (immediate removal of animals in the landscape beyond the FC estate i.e., p. hunted = 1) wild boar populations would continue to grow within the FC estate until reaching a self-regulating carrying capacity at a mean density of 15 boar/km^2 (∼3,000 boar occupying the 200 km^2 of the FC estate and immediately adjacent land) but would not establish in the wider landscape. Extending the latter scenario to consider increased culling rate within the FC estate successfully reduced population size and distribution, albeit with declines occurring slowly over the 20 year course of the simulations (**Figure 2**). Eradication of wild boar within 20 years might be achieved if populations were contained and the culling quota within the FC estate were doubled, although we cannot specify the effort that would be required to sustain such a high rate of removal at low densities of boar.

Considering the time to disease elimination for each of these management scenarios (**Figure 3**), we observed a notable positive relationship between population size (abundance and distribution; **Figure 2**) and the persistence of disease in the landscape. For a wild boar population similar to that estimated in 2015 (∼1,500 individuals), our results predicted a median time to elimination of disease of around 15 weeks. If populations were allowed to grow uncontrolled across the wider landscape (hunting = 0) for 20 years, an outbreak of ASF could last nearly 3 times longer (median 40 weeks) with the un-managed disappearance of disease (no reactive controls such as carcass retrieval) becoming very unlikely to ever occur within 10 weeks (<5% probability). It is only in populations of wild boar hunted into decline that burn-out is predicted to occur in <10 weeks, i.e., scenarios (a) (iv) and (a) (v) (**Figure 3**) where culling was increased within the FC estate whilst preventing dispersal to the wider landscape (p. hunted = 1). However, such measures would not prevent the outbreak of disease unless boar populations were very small (<100 boar) at the point of introduction. Comparing the impact of carcass retrieval (**Figures 3B,C**), our results showed only marginal shortening of the time to disease elimination, except in the case when initial populations are largest where this environmental decontamination could nearly halve outbreak length when retrieval is within 4 weeks (from 40 to 20 weeks). Increasing effort further to ensure retrieval within 2 weeks showed no additional benefit. Whilst carcass retrieval did not impact median times to disease elimination it did limit the likelihood that outbreaks would last substantially longer than the median duration.

Comparing scenarios with identical initial conditions, we assessed the effect of reactive population control rather than prophylactic reductions prior to disease introduction (**Table 2**). These results showed that increasing the rate of management (culling or hunting) in response to ASF has no effect. As already stated, carcass retrieval does not reduce median outbreak length but does reduce the likelihood of more extreme events (i.e., exceptionally long outbreaks). This result was supported by examining the relationship between time to ASF elimination and initial population size, distribution and density across all management options (**Figure 4**).

Finally, we evaluated how management, specifically hunting, alters disease spread by comparing rates of spread (**Figure 5**) in the absence of hunting [scenario (a) (i), p. culled = 0.0065 and p. hunted = 0, with release after 20 years; **Table 2**]

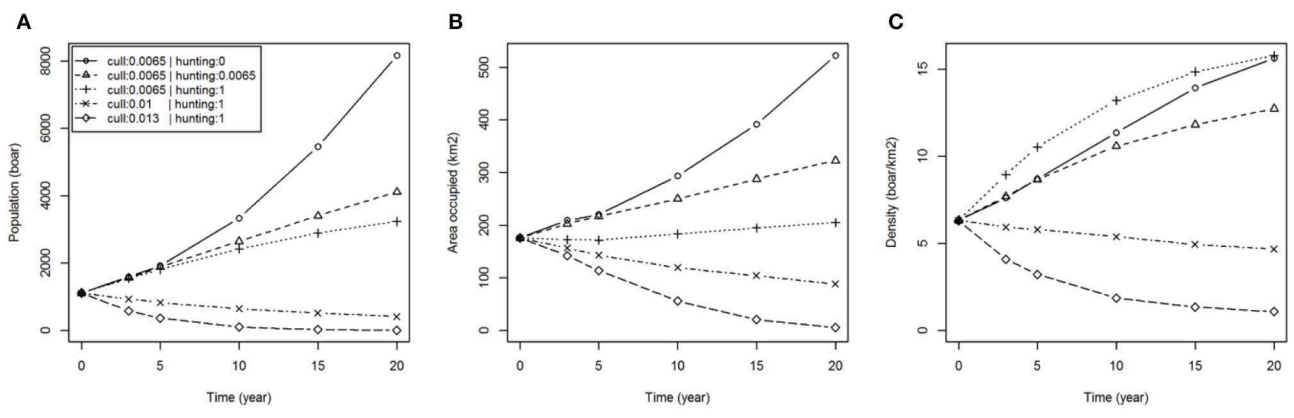

FIGURE 2 | Boar population dynamics under various hunting and culling scenarios. Plots show: **(A)** total population (boar); **(B)** area occupied (km^2); **(C)** density (boar/km^2); over time for different hunting scenarios (none: p. hunted = 0, equal to current culling: p. hunted = 0.0065 and immediate removal: p. hunted = 1) with fixed culling (p. culled = 0.0065) and different culling scenarios (50 and 100% increase in culling rate: p. culled = 0.01 and 0.013, respectively) assuming containment (immediate removal from hunting: p. hunted = 1).

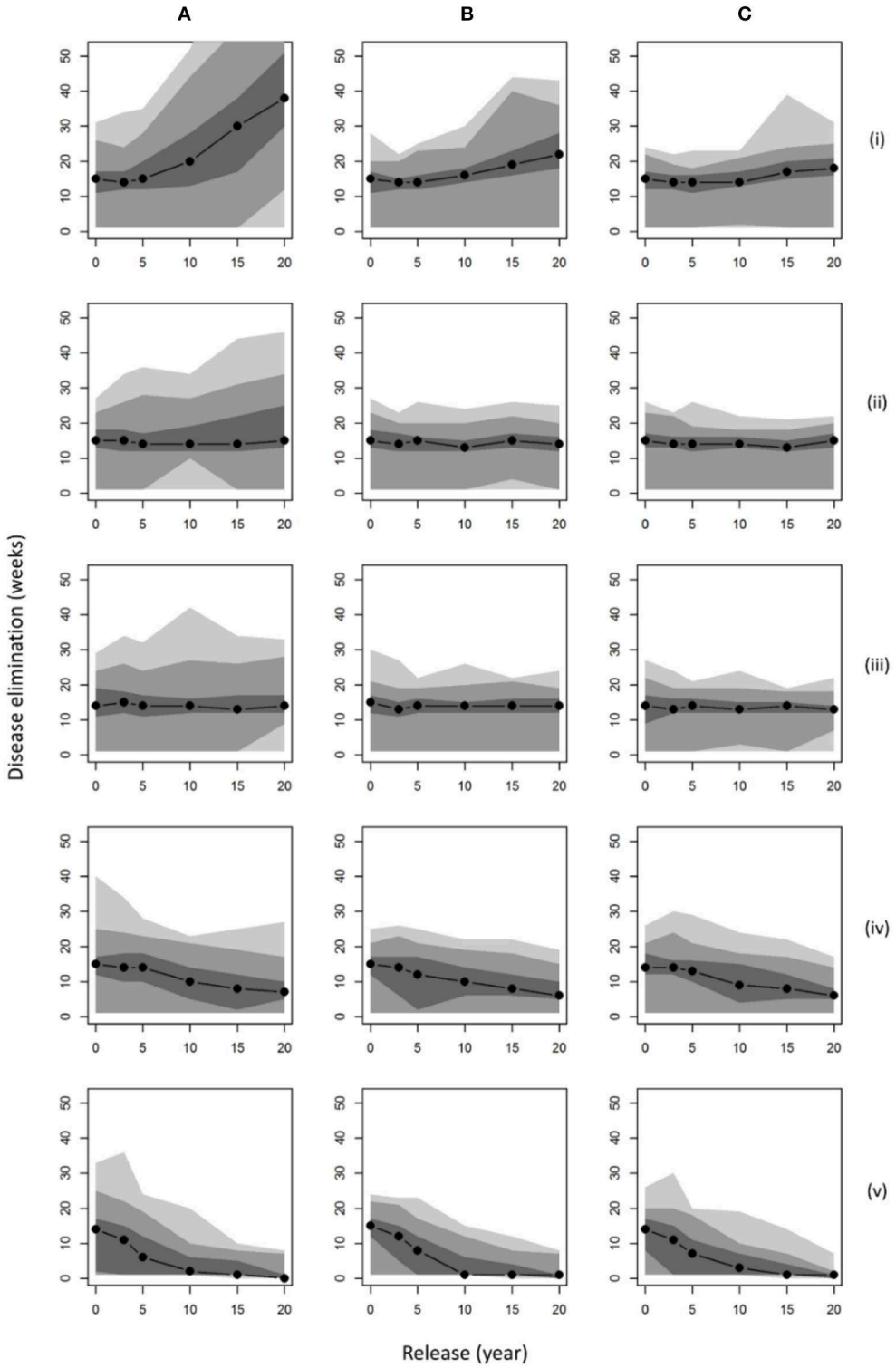

FIGURE 3 | Disease persistence under various management scenarios. Plots show the median time to disease elimination (zero infected individuals) given different points of initial release assuming: **(A)** no carcass retrieval (8 week persistence); infected carcasses retrieved within **(B)** 4 weeks; **(C)** 2 weeks; and population control applying: current culling (p. culled = 0.0065) on the FC Estate with (i) no hunting (p. hunted = 0); (ii) identical hunting (p. hunted = 0.0065); (iii) immediate removal from hunting (p. hunted = 1); on surrounding land; (iv) 50% additional culling (p. culled = 0.01); (v) 100% additional culling (p. culled = 0.013); both with immediate removal from hunting (p. hunted = 1). Shaded regions denote smoothed ranges centered on the median containing (from darkest to lightest): 50, 90, and 100% of model repetitions.

against, and with, moderate hunting pressure [scenario (a) (ii) where p. hunted = 0.0065; **Table 2**]. Patterns of spread were similar with expansion at an initial rate of ∼1 km/week by distance, 20 km^2/week by area, before rapid reduction to zero as infection reached the limit of the boar distribution. Hunting did appear to slow disease spread but only marginally, perhaps as a result of a lower boar density (13 compared to 15 boar/km^2 when no hunting was applied). Similarly, toward the edge of boar distributions, where densities were closer to that observed in Europe (5 boar/km^2), the rate of spread reduced to ∼0.5 km/week, half of that of the core, where densities were substantially higher.

TABLE 2 | Results of potential responsive control options.

Culling (p. culled)	Hunting (p. hunted)	Elimination (weeks)	Scenario (Figure 3)
Responsive hunting			
0.0065	0	15 (1, 20)	(A) (i)
0.0065	0.0065	15 (1, 21)	(A) (ii)
0.0065	1	14 (1, 22)	(A) (iii)
Responsive culling			
0.0065	1	14 (1, 22)	(A) (iii)
0.01	1	15 (1, 23)	(A) (iv)
0.013	1	14 (1, 23)	(A) (v)
Carcass retrieval (2 weeks)			
0.0065	0	15 (1, 24)	(C) (i)
0.0065	0.0065	15 (1, 21)	(C) (ii)
0.0065	1	14 (1, 24)	(C) (iii)
0.01	1	14 (1, 24)	(C) (iv)
0.013	1	14 (1, 25)	(C) (v)

Median time to disease elimination (zero infected individuals) for various responsive management options simulating the release of disease in 2015 (year 0). Brackets denote 5 and 95% CI.

DISCUSSION

In this study we have outlined a spatially-explicit individual-based model of wild boar, incorporating a novel description of the underlying model landscape used to simulate our real-world example. We applied this model to predict the epidemiological consequences of introducing ASF to a wild boar population and extended this to explore a wide variety of prophylactic and reactive management strategies. Here we suggest that ASF is unlikely to persist and circulate within this wildlife host indefinitely (i.e., become a self-sustaining endemic disease in wildlife), though we note that our prediction is specific to a discrete and limited population of wild boar in the Forest of Dean. This stands in contrast with the prediction made for FMD in the same landscape (11). In all of our simulations here, even those where boar is projected to have spread for 20 years, ASF is likely to spontaneously disappear, limiting the duration of the risk this produces to commercial pig production. However, in the scenarios in which the population of boar is most extensive, disease might continue to circulate for up to 40 weeks, which compares to burn-out in a median of 15 weeks for more contemporaneous simulations of disease.

Unlike previous modeling studies [e.g., (8, 26)], which have focused on mainland Europe, where wild boar are ubiquitous (25), we consider an isolated and expanding population typical of that found in England, and estimated to contain ∼1,500 individuals at densities up to 20 boar/km^2 (10). However, without near eradication of the host population (geographically restricted populations with densities below 2 boar/km^2) few of the tested reactive management responses shorten the length of an outbreak to <14 weeks. Reactive controls, including increased culling and carcass retrieval, have negligible impact in this context, only showing notable improvements in the most extreme scenarios (halving the time to elimination in the largest populations from 40 weeks to 20 weeks). Similar to previous work (11) on foot-and-mouth disease (FMD), our results suggest that an important factor predicting the severity (duration) of an outbreak, and

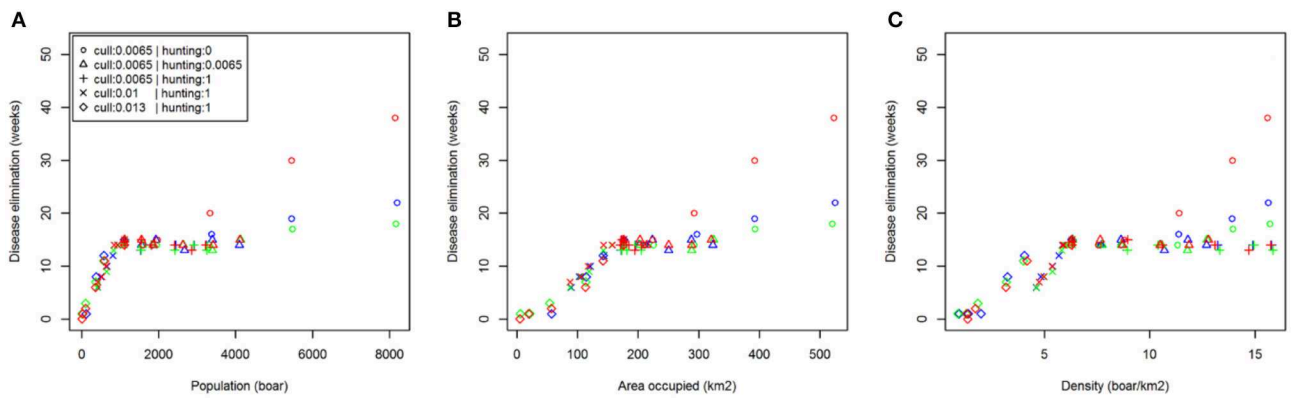

FIGURE 4 | Relationship between disease persistence and population structure. Plots show median time to disease elimination (zero infected individuals) against: **(A)** total population (boar); **(B)** area occupied (km^2); **(C)** density (boar/km^2); at the time of initial disease introduction. The color of symbols denotes different carcass retrieval strategies: (red) none; (blue) 4 weeks; (green) 2 weeks.

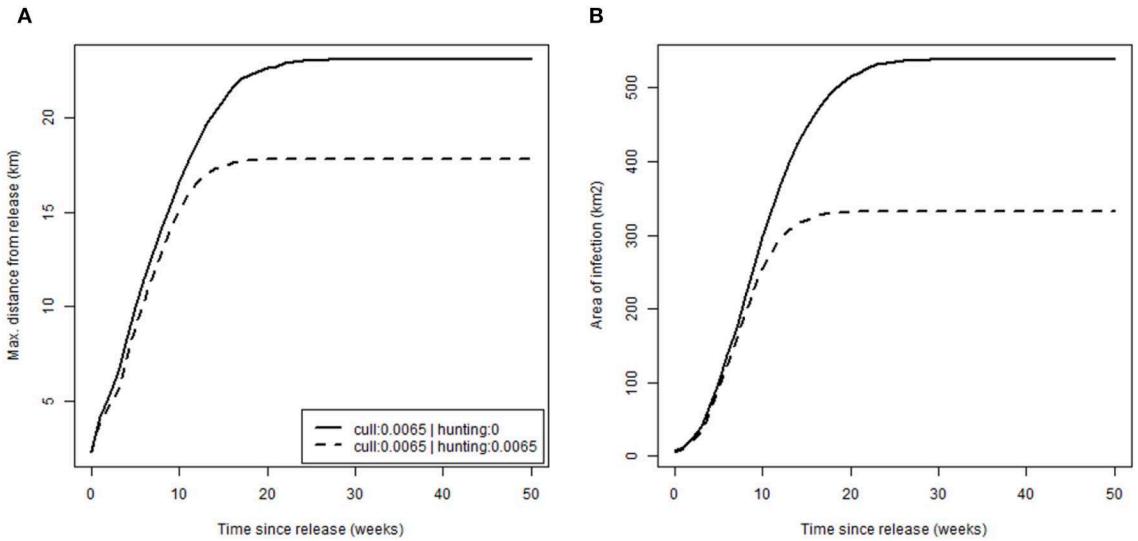

FIGURE 5 | Rate of disease spread. Plots show cumulative disease spread based on: **(A)** the maximum distance from release (km); **(B)** the area of infection (km^2); over time for different hunting scenarios (none: p.hunted = 0 and equal to current culling: p.hunted = 0.065).

therefore the key to reducing it, is not population density as may be expected, but the extent of the wildlife host distribution (occupancy across the landscape). However, we also note a potential relationship between density and the rate of disease spread, which if reduced sufficiently may inhibit the progress of outbreaks in some instances.

Model Validation

Other than our description of ASF, which for this study follows the relatively deterministic approach of studies supporting the current EFSA guidance (26), there are considerable uncertainties in simulating the demography and behavior of wild boar in a real-world context. The limited quantity and quality of available data or similar examples of focal outbreaks in isolated wild boar populations makes this approach very difficult to validate. Evidence of the utility of our model, therefore, rests on the quality of its component elements (i.e., representation of the landscape, the host wildlife population process, the simulation of host wildlife movement, epizootiological rates) and their coherent integration into the model system. We achieved this by selecting approaches known to minimize the introduction of bias and which simulate, as directly as possible, the biology and behavior of the wildlife host in real landscapes. Unlike other models (8, 26), which use a grid-based structure, our model landscape (a continuous mosaic of patches of irregular geometry scaled precisely to socially coherent sub-populations wild boar; sounders) allows us to represent real-world locations without bias (17), capturing the fine scaled variation in land-use (composition and configuration of habitats) and the heterogeneity of their value to boar, their movement (accounting for any natural barriers such as major roads, rivers, and canals), and as a consequence, the spatial heterogeneity in contact rates. Likewise, our choice to use an IBM approach allows us to capture individual variation in movement (e.g., dispersal of juveniles, stasis of boar in favorable locations), as well as permitting us to consider population processes in small populations without bias. For example, in our simulations, sub-populations in each patch are often small or very small, either because their local environment (patch) may not support more boar, or because they are at the spreading edge of an expanding population. Similarly, ASF is so infectious that variations in outbreak outcomes can be dictated by the fate of individual boar; such as stochastic mortality inhibiting disease spread, or relatively rare long-distance movement by individuals promoting disease spread.

Despite the lack of data on ASF in English wild boar, we are able to compare an emergent property of our epizootiological model with descriptions produced in other studies. The model identified a rate of spread of ASF of ∼4 km/month within the core population at densities around 15 boar/km^2, reducing to nearer 2 km/month toward the population edge where densities were closer to that observed in Europe (5 boar/km^2). This compares well with an empirical description of 1.5 ± 1.3 km/month for the same disease in boar in Poland (27), 1–2 km/month in a number of eastern European countries (24), and a broader range of estimates for the unassisted spread of ASF cited in (7) between 8 and 25 km/year, though most fall between 10 and 17 km/year (0.8–1.4 km/month). Whilst not a definitive validation of our model, the similarity of our result with that of others suggests that our representation and parameterization of our model system is of some value and is free of substantial consistent bias.

Disease Predictions

In all of our scenarios, even those simulating relatively large and extensive populations of boar [e.g., scenario (a) (i) after 20 years; **Table 2**], ASF fails to produce a self-sustaining disease. Infection spreads rapidly wherever boar occur in our simulated landscape,

causes substantial and rapid mortality, and is predicted to eventually disappear ("burn-out"). Given our choice to model a virulent strain of the virus (95% mortality) and a geographically isolated population, the duration of ASF in the landscape represents the time it takes disease to physically spread to every occupied patch. This was illustrated by the close correlation of outbreak duration to a measure of distribution (**Figure 4B**). We anticipate that once the virus arrives at a patch, the virulence of the disease and the social behavior of the boar will reduce its sub-population in a relatively short time. Model assumptions and the rapid progress of disease make it unlikely that recruitment has introduced a substantial cohort of new susceptible boar into our system during an outbreak (excepting a few scenarios), and immigration can also be discounted in this study. These latter processes must partly explain the apparent endemicity of ASF at large scales in extensively forested European landscapes. Some scenarios in this study [i.e., scenario (a) (iii) where p. culled = 0.0065 and p. hunted = 1] replicate the consequence of the current EFSA recommendation to create and maintain a buffer around an infected core zone in landscapes where boar are widespread (1). Our model suggested that if activities that might induce long movements by potentially infective wild boar are avoided, ASF should eventually disappear from small core zones (e.g., $< 500\,km^2$).

Both the demographic and spatial dynamics of boar vary seasonally, mainly in relation to food availability, in ways that might alter the course of disease outbreaks. For example, plentiful food in autumn might reduce or delay density-dependent dispersal between patches (temporarily dampening the source-sink spatial dynamic) (21) or temporarily reduce ranging within patches, lowering inter-patch contact rates. Conversely, the presence of taller crops in arable fields might temporarily promote movement within- and between- patches (enhance the spatial dynamic). Outbreaks of disease, which persist throughout seasonal variations in movement may produce distinct epizootiological dynamics. The fixed annual date of the disease scenarios applied here may have failed to catch some of this variation, as the disease in our simulations will have systematically removed most of the birth pulse, and burnt-out before most juveniles were considering dispersal (22). Future work could explore varying the date of the focal infection, to explore the effect of recruiting susceptible individuals during an outbreak (likely to lengthen outbreaks), seasonal variation in the spatial dynamics of boar, seasonal variation in the removal of boar, or a seasonal variation in the virulence of the disease in boar. However, given the short duration of the epidemic in most circumstances, we do not believe this is likely to change the probability of ASF becoming endemic.

Implications for Risk Assessment and Contingency Planning

In agreement with Croft et al. (11), this study suggests that the distribution of boar is a useful predictor of the duration of an outbreak of a highly contagious disease in a closed population. The utility of this finding recognizes that establishing the distribution for a large ungulate prone to leaving obvious activity signs is relatively inexpensive and quick compared to the effort required to measure abundance or density. We suggest that risk assessments of the impact of ASF might be possible using the distribution of a population, potentially informed by citizen scientists; although the quality of that description and the risk assessment it underpins would be substantially improved by extending the data to include quality assured observations and some element of geographically targeted and/or systematic descriptions of distribution (23).

We explored the importance of differing cull and hunting rates and the effect these have on the duration of an ASF outbreak. Our implementation of this (a per capita risk of removal) simulates the removal of boar in direct proportion to their density homogenously across the landscape. The culling and hunting rates do not describe the effort needed to remove wild boar. Thus, we do not consider the additional effort necessary where densities of boar are low or the terrain difficult (e.g., dense forests or areas close to people). Whilst the additional costs and complexities of delivering an effective annual cull across the difficult terrain of the FC estate is borne by its state owner, there is likely to be substantial spatial variation in the rate of hunting by private landowners. This heterogeneity is likely to perturb natural source-sink dynamics between patches, either directly through disturbance or indirectly by stimulating movement produced by density-dependent dispersal, and consequentially leading to increased spread of disease. This principle has been recognized in the advice given to EFSA on the control of ASF in areas of Europe where wild boar are widespread, for member states to ban hunting close to the focus of disease (1). Unlike some previous studies [e.g., (9), we did not associate high rates of removal with an increased rate of dispersal despite some evidence that this can occur (20, 28, 29), as our choice recognizes that removal in England will almost certainly be restricted to shooting. We also make an additional important distinction between the functionally similar culling and hunting. Culling, as we use it here, encapsulates not only the coordinated and targeted action to achieve a pre-defined target, but also the professionalism of contracted specialists who can be required to fulfill complex directions in official guidance (1) and remove animals with minimal disturbance. This contrasts with our use here of the term hunting, to describe an un-managed activity where variations in the interests of landowners may drive variations in hunter behavior (e.g., for sport, to protect property, for commercial gain), some of which may be problematic. For example, private hunters shooting free ranging boar commonly use sites baited with supplementary feed; an activity which might promote boar population growth because bait points are overstocked (30), produce conflicts with other biodiversity (31), or may act as points of enhanced disease contact (30). Importantly the heterogeneity in hunting rate will also be conditional, with some landowners objecting in principle to any hunting, effectively providing refuges for boar; across our study arena we estimate there to be at least 6,200 separate landowners (mean ownership of $0.55\,km^2$ ranging up to $130\,km^2$). In reality, it is unlikely that the perfect hunting we simulate here (hunting = 1) could ever be realized in landscapes with complex fine-scale patterns of private ownership and variable densities of boar, and that target rates

for hunting would need to be set appropriately high to realize any policy to prophylactically control wild boar. This highlights a critical gap in our knowledge of the costs (efficiency per hunting day) and consequences (disturbance) of hunting boar in England, how this is affected by the variation in method of hunting, mode (intensity and frequency), or the varying interests of landowners.

The model suggested that in all scenarios median disease burn-out occurred around week 14–15. The only benefits of an ongoing contingency response (the removal of boar beyond the FC estate, doubled culling rates within the FC estate and carcass removal within 2 weeks) are a reduction in the likelihood of long outbreaks (worst case) lasting more than 20 weeks. However, in this context, we note that our estimates of culling rates or their integer multiples, whilst "realistic" and derived from FC estate cull operations (10, 11), are still small when applied on a weekly basis and are likely to produce little effect as a response within the short duration of most disease outbreaks.

Similarly to other authors (26, 32), we show that the removal of carcasses as a contingency response to the outbreak of disease has little value in shortening the median duration of the infection in wild boar for many of our scenarios. Only where boar have become relatively widespread in our simulations [scenario (a) (i) vs. (a) (iii); **Table 2**] does the considerable effort of retrieving carcasses within 2 weeks appear to be of substantial value in changing the course of an outbreak. However, removing carcasses may have other benefits and might still be considered as a prudent tool in response to disease. For example, the considerable persistence of virus in decaying boar produces a number of risks resolved by the collection of carcasses, such as the accidental movement of contaminated fomites into commercial pig units or backyard pigsties by man or wildlife. Our choice not to simulate the short-term intensive culling, such as that applied within the Czech outbreak (https://europa.eu/rapid/press-release_MEX-19-1431_en.htm), was motivated by the observation that disease "burn-out" is relatively rapid and is likely to overtake the sum of delays produced by the detection and confirmation of disease, the deployment of assets to manage the disease in wild boar (financial, staff and equipment, legal permissions), and the efficient operation of an intense responsive cull.

CONCLUDING REMARKS

This study predicts that ASF introduced to the free-living wild boar is very unlikely to become endemic in the Forest of Dean for some time, in contrast to the predictions made for FMD. Consequently, the duration of the risks to commercial pig production resulting from uncontained and unmanaged ASF virus circulating in this English landscape is limited. The last new infection of wild boar is likely to be around 15 weeks after the focal infection, and active disease in wild boar rarely persisted for more than 25 weeks in any individual simulation. This outcome is likely to be consequent on the spatially discrete (isolated) and limited size of the population of wild boar, even in scenarios projected after 20 years of population growth and natural spread. This and earlier work (11) both suggest that the persistence of an exotic disease in an isolated population is dependent on the total distribution of the population, rather than the population size *per se*. We suggest that measures of the distribution of boar (e.g., occupancy) are the easiest measures by which the duration of disease circulating in wild hosts can be quickly assessed. Here, all of the reactive management responses appear to have limited value in reducing the duration of ASF in wild boar, though in part that is a consequence of the rapid burn-out of the disease in free-living boar even without intervention. We also explore the potential value of prophylactic management of boar populations, and outline some of the issues which may need to be considered if this is to be adopted as a disease management strategy. These include the distinctions between organized culls undertaken as a professional activity and voluntary hunting in its varied forms. The potential for mismatches between the local density of boar and the spatial heterogeneity in management effort, as well as how management itself may affect the behavior of boar are identified as substantial gaps in knowledge.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

GS, JA, and SC conceived the main research idea. SC designed the methodology with contributions from JA. All authors contributed critically to the analysis and interpretation of the results, to the writing of the manuscript, and contributed to sourcing and collating of key input data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00154/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparative Pathology and Pathogenesis of African Swine Fever Infection in Swine

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African Swine Fever (ASF) is a viral disease that affects animals of the *Suidae* family, and soft ticks from the genus *Ornithodoros* can also be infected by the ASF virus (ASFV). The disease was first described in Africa at the beginning of the twentieth century as an acute disease characterized by high mortality and fatal hemorrhages. ASF has caused outbreaks in numerous countries and it continues to be devastating nowadays for the porcine sector in those countries affected, and a massive threat for those free of the disease. ASF can follow clinical courses from peracute to chronic in domestic pigs (*Sus scrofa*) depending on a variety of factors, including the immune status of the animals and the virulence of the ASFV strain. The key features of the pathogenesis of the disease in domestic swine are a) a severe lymphoid depletion including lymphopenia and a state of immunodeficiency, and b) hemorrhages. However, African wild swine like bushpigs (*Potamochoerus larvatus*), red river hogs (*Potamochoerus porcus*), and warthogs (*Phacochoerus africanus*) can be infected by ASFV showing no clinical signs of disease and acting as natural reservoir hosts. In this article we review the key features of the gross and microscopic pathology together with a description of the pathogenesis of ASFV infection in domestic pigs following the different clinical courses. The pathogenesis of ASF in wild and domestic swine is also described, what can provide important information for the design of control strategies, such as vaccines.

Keywords: African swine fever, pathology, pathogenesis, virus, swine

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INTRODUCTION

African swine fever (ASF) is the most important infectious disease of swine and has proven to be devastating for the pork industry worldwide. ASF was first observed in the early 1900's in East Africa, when European domestic pig breeds were introduced in the Kenya Colony and animals developed a form of hemorrhagic disease with high morbidity and mortality (1). ASF was confined to African countries until 1957 when it reached Portugal via contaminated waste containing infected pork products that were used to feed local pigs. This outbreak was quickly controlled, but ASF re-entered Portugal in 1960 and spread rapidly to the Iberian peninsula (2) and produced sporadic outbreaks in several European countries, including Belgium, the Netherlands, Italy, Malta, and France (3–6). ASF spread to the Americas, with sporadic outbreaks in Brazil, the Dominican Republic, Haiti, and Cuba (7–11). ASF was eradicated from all these countries out of Africa, except the Italian island of Sardinia, where the disease has persisted since 1978 (2, 12–14). The disease

continued to persist and spread within Africa (15) and entered the Republic of Georgia in 2007 through the port of Poti (16), most likely via contaminated food used to feed domestic pigs (17). ASF spread rapidly within the Caucasian region and neighboring countries and continues to spread to West, including European Union countries (18, 19) and to the East, with the disease causing abundant outbreaks and affecting dramatically the pork industry in China, Vietnam, Cambodia, Philippines, Laos, and East Timor (20–23).

ASF is caused by a large, complex, enveloped DNA virus (ASFV), from the family *Asfarviridae* (24). ASFV is composed of more than 50 structural proteins and can produce more than 150 proteins in the infected cells (17, 25–27), many of which are highly immunogenic. The main target cell for ASFV is the monocyte/macrophage in both domestic and wild swine (28–30), but infection in lymphocytes has not been reported (30). ASFV may also replicate in other cell types, including hepatocytes, renal tubular epithelial cells, neutrophils, and endothelial cells (31–33). The ASFV replication and the immune responses from the host induce different clinical courses and pathology in swine species. ASFV can also replicate in soft ticks from the genus *Ornithodoros*, including *O. moubata* in Africa and *O. erraticus* in the Iberian peninsula (34–37), which are involved in the epidemiological cycles of ASF (38, 39). Other soft tick species have also been reported to be susceptible to ASFV infection and may play a role in the epidemiology of ASF in other countries.

ASF has produced a high economic cost to the pork industry and it is the most important porcine disease nowadays, mostly due to the difficult prevention and control as no vaccine is available and other strategies must be used to control the disease from different territories. In this review article, we describe the different clinical and pathological features of ASF in domestic and wild suids together with the key pathogenic mechanisms that induce the disease in the host species.

CLINICAL PRESENTATION AND GROSS PATHOLOGY OF ASFV INFECTION IN DOMESTIC PIGS

The clinical presentation and the gross pathological lesions of ASF in domestic pigs may vary depending on the virulence of the virus isolate, the route, and dose of infection and host characteristics (17). ASFV isolates can be classified as highly virulent, moderately virulent, and low virulent (40). The clinical courses observed in ASF in domestic pigs can be described as peracute (or hyperacute), acute, subacute, or chronic.

Peracute ASF: Clinical Signs and Lesions

Highly virulent strains are typically responsible for this clinical course, characterized by a very rapid clinical course, with high fever (up to 42°C), anorexia, lethargy, and sometimes sudden death without signs of disease. This is often observed when the virus enters a naïve farm causing death of some animals before the explosion of clinical cases. Some animals can show respiratory distress due to the high fever, but no gross lesions are usually found at the *post mortem* examination.

Acute ASF: Clinical Signs and Lesions

This clinical form is cause by highly or moderately virulent isolates, and it is the typical course observed in naïve farms very quickly after the first fatal cases are reported. The clinical course is characterized by high fever, with temperatures of 40–42°C, lethargy, anorexia, and inactivity (**Figure 1A**). The affected animals tend to bunch up together. Many affected animals show a centripetal cyanosis, easily found in the ears (**Figure 1B**), snout (**Figure 1C**), limbs (**Figure 1D**), abdomen, tail, and perianal area. Respiratory distress is usually observed, with severe pulmonary oedema in animals affected by highly pathogenic isolates (41, 42). Skin lesions are frequent, with presence of petechial hemorrhages or ecchymosis (**Figure 1E**). Other clinical signs may include nasal discharges, sometimes stained with blood (epistaxis), vomiting, and diarrhea, that can be also blood-stained (melaena) (17, 43–45), causing black-colored stains in the perianal area of the animal (**Figure 1F**). Abortions may occur in pregnant sows and the mortality rates may reach up to 100% in affected farms within 7 days of the onset of the disease.

At the *post mortem* examination, the most characteristic lesion of acute ASF is the hemorrhagic splenomegaly (28, 46, 47), with a very enlarged spleen, dark in color and friable at sectioning, occupying a large space within the abdominal cavity (**Figures 2A,B**). The second most important lesion described in acute ASF is a multifocal hemorrhagic lymphadenitis. Lymph nodes can have multifocal or extensive hemorrhages that can produce a marbled appearance (**Figure 2D**). The most affected lymph nodes are the gastrohepatic (**Figure 2E**), renal (**Figure 2F**), and other abdominal lymph nodes as ileocaecal (**Figure 2G**), and mesenteric (**Figure 2H**). Hemorrhages may also be observed with less frequency in other lymph nodes, such as submandibular, retropharyngeal, or inguinal. Petechial hemorrhages are often observed in the kidney surface (**Figure 3A**) and at sectioning. Other lesions can also be observed, mostly hemorrhages in the mucosa or the serosa of other organs, as the large (**Figure 3E**) and small intestine (**Figure 3F**), the epicardium in the heart (**Figure 3G**), or the urinary bladder (**Figure 3H**) (17, 43, 44, 48–51).

Subacute ASF: Clinical Signs and Lesions

This clinical form is usually observed in animals infected by moderately virulent isolates, with similar clinical signs as those observed in acute ASF, although normally less marked (17). Affected pigs show moderate to high fever and the mortality rate ranges from 30 to 70% (17), with pigs dying at 7–20 after infection.

The vascular changes, mostly hemorrhages and oedema, in the subacute form of the disease can be more intense than the acute form (45, 52).

The death of affected animals may happen at two different stages: (a) during an initial thrombocytopenia and leukopenia (53–55), or (b) during a "recovery" phase, observed in young animals, causing erythrodiapedesis induced by vasodilation (53, 56).

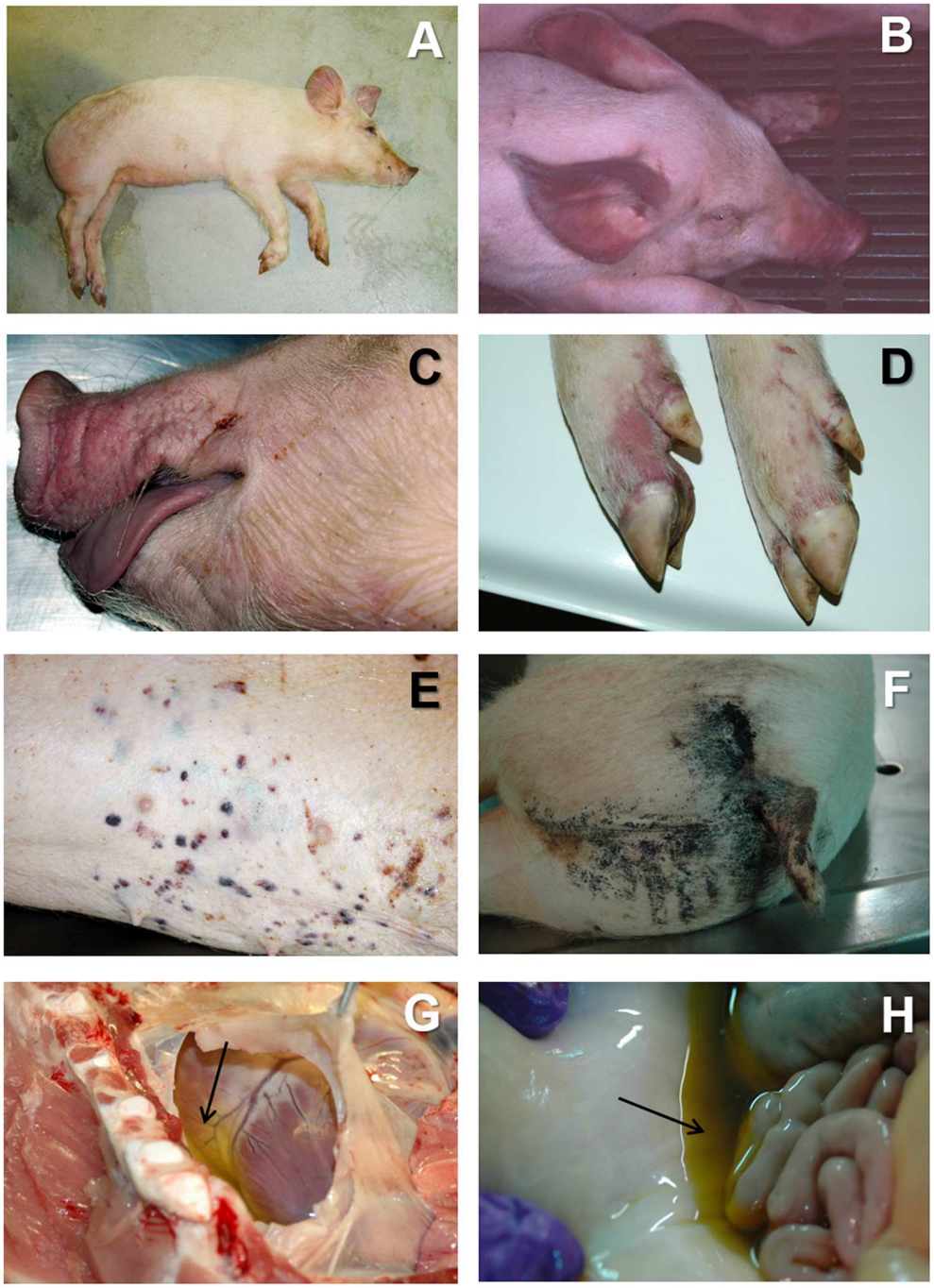

FIGURE 1 | (A) Lethargic animal in acute ASF. The animal show cyanosis ion the ears abdomen and limbs. **(B)** Severe cyanosis in an animal suffering from acute ASF, associated to very high hyperthermia (41–42°C). **(C)** Cyanosis in the snout and lips in acute ASF. **(D)** Cyanosis in the limbs in acute ASF. **(E)** Multifocal petechiae and ecchymosis in the skin in acute ASF. **(F)** Blood-stained perianal area in a pig affected by subacute ASF. **(G)** Severe hydropericardium (arrow) in subacute ASF. **(H)** Moderate to severe ascites (arrow) in subacute ASF.

At the *post mortem* examination, animals show hydropericardium (**Figure 1G**), ascites (**Figure 1H**), and multifocal oedema, very characteristic in the wall of the gall bladder or in the perirenal fat (**Figure 3B**) (17). Some animals may show hemorrhagic splenomegaly as described for the acute form of the disease, but many animals will show partial splenomegaly, with patches of spleen affected and other areas unaffected (**Figure 2C**). A multifocal hemorrhagic lymphadenitis can also be observed with multiple lymph nodes in all areas of the body showing the hemorrhages and the "marble"

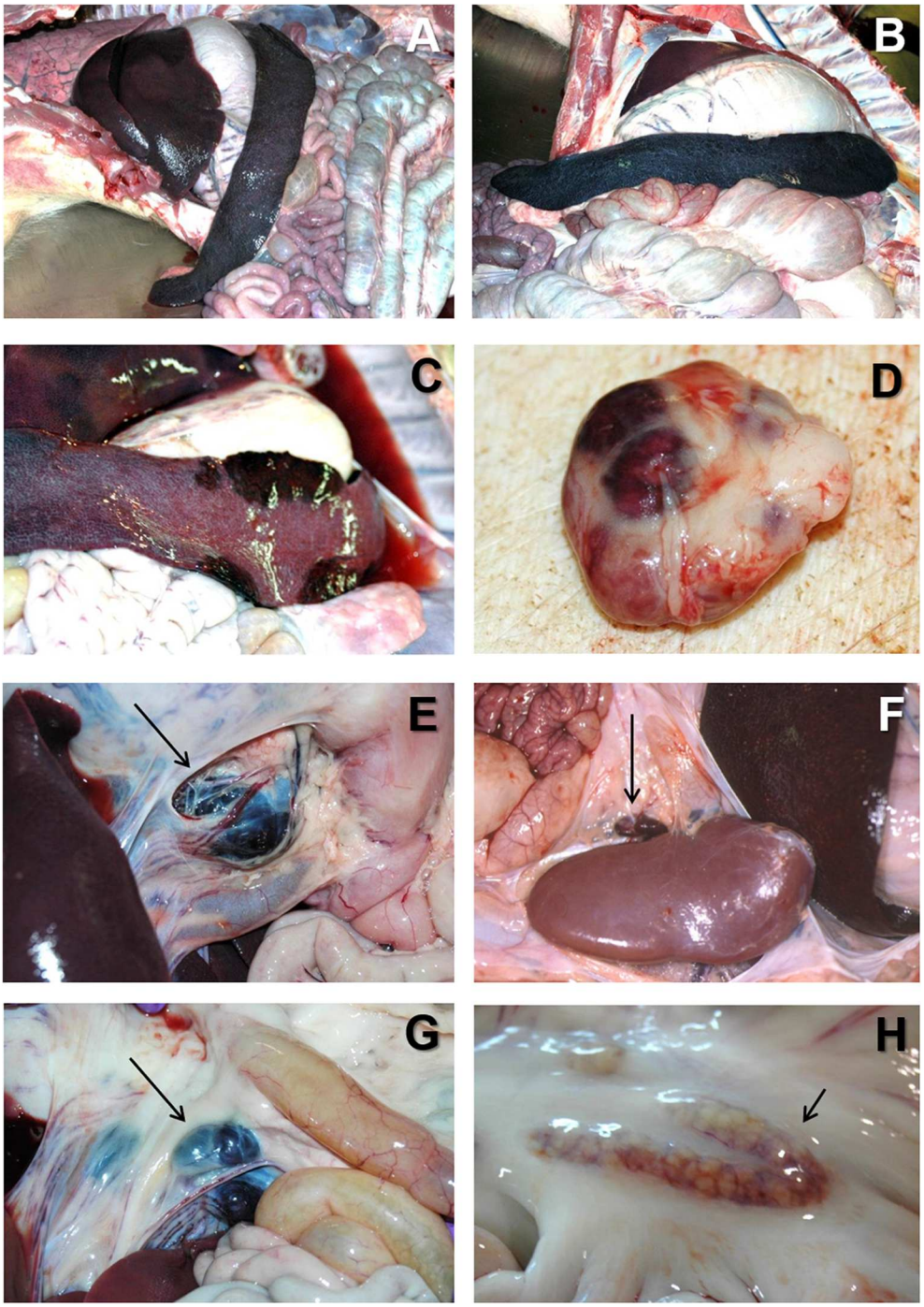

FIGURE 2 | (A) Severe hemorrhagic splenomegaly observed at the opening of the abdominal cavity of an animal with acute ASF. The liver is severely congested. **(B)** Very large, dark colored spleen with rounded edges (hemorrhagic splenomegaly), and occupying a large volume of the abdominal cavity in acute ASF. **(C)** Multiple areas of partial hemorrhagic splenomegaly in the spleen from an animal with subacute ASF. **(D)** Multifocal hemorrhages in a lymph node with a marbled appearance in acute ASF. **(E)** Severe hemorrhagic lymphadenopathy in the gastrohepatic lymph node (arrow) in acute ASF. **(F)** Severe hemorrhagic lymphadenopathy in the renal lymph node (arrow) in acute ASF. **(G)** Severe hemorrhagic lymphadenopathy in the ileocaecal lymph node (arrow) in acute ASF. **(H)** Moderate hemorrhagic lymphadenopathy in the mesenteric lymph node (arrow) in acute ASF.

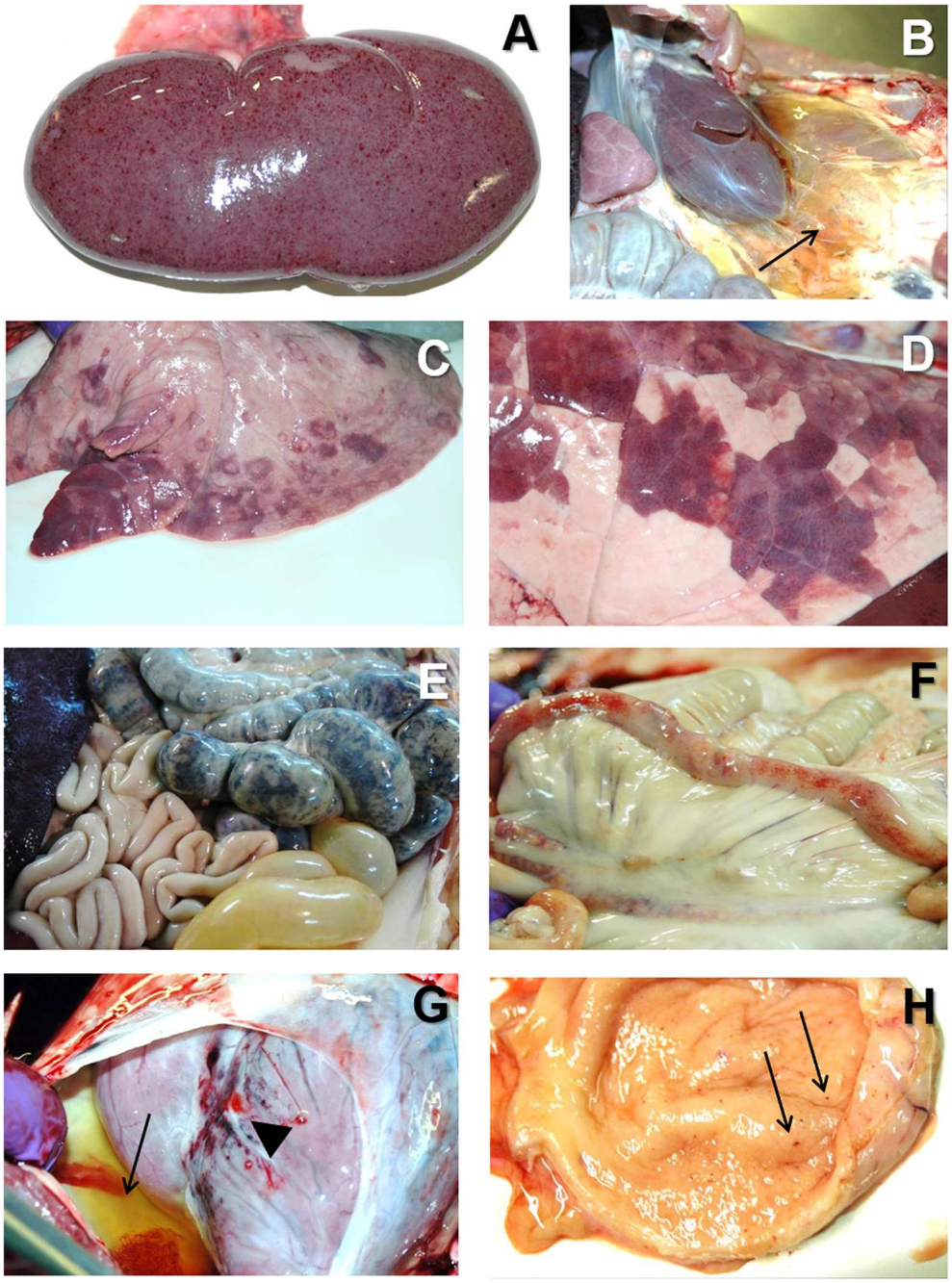

FIGURE 3 | (A) Multiple petechial hemorrhages in the cortical surface of the kidney in acute ASF. **(B)** Severe perirenal oedema (arrow) in a pig with subacute ASF. **(C)** Multifocal areas of lung consolidation and pulmonary oedema in subacute ASF. **(D)** Multifocal pneumonia with dark color areas in the diaphragmatic lobe of the lung in subacute ASF. **(E)** Severe extensive hemorrhagic colitis in subacute ASF. **(F)** Multiple petechial hemorrhages in the serosa of the small intestine in acute ASF. **(G)** Multiple petechial ad ecchymotic hemorrhages in the epicardium (arrowhead) together with severe hydropericardium (arrow) in subacute ASF. **(H)** Multiple petechial hemorrhages in the mucosa of the urinary bladder in acute ASF.

appearance (45). Petechial hemorrhages can also be observed in the kidney (50, 51). Multifocal pneumonia is also observed with patches of consolidation and dark color in the lung (**Figures 3C,D**). This lesion can also be attributed to secondary infections due to the state of immunosuppression induce by ASFV (45, 57, 58).

Chronic ASF: Clinical Signs and Lesions

This clinical form is caused by the infection of low virulence isolates and has been observed, quite infrequently, in the Iberian Peninsula and the Dominican Republic (17, 54). It has been hypothesized that this low virulence isolates, and the associated chronic form, has evolved from ASFV isolates employed in early

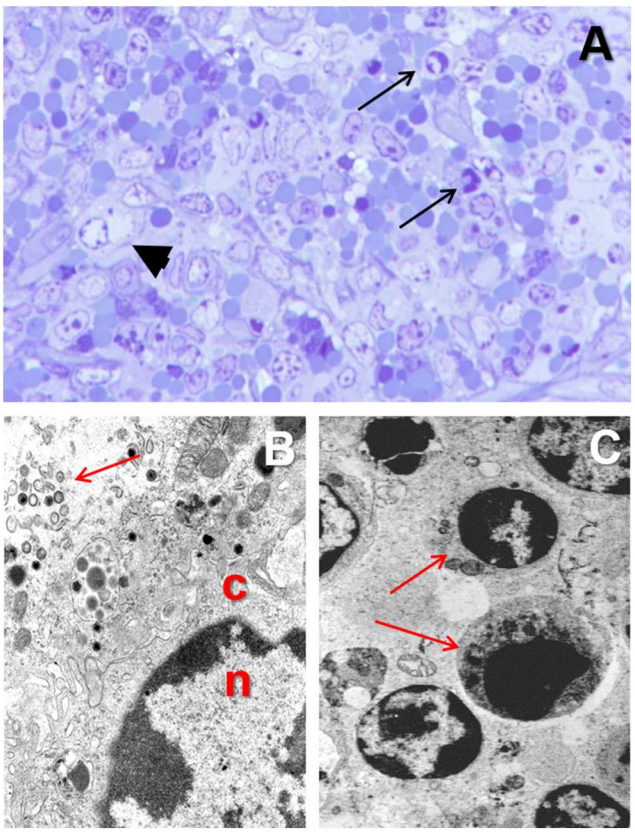

FIGURE 4 | (A) Toluidine blue stained semithin (1 μm) section showing a macrophage with margination of the nuclear chromatin and a juxtanuclear clear intracytoplasmic inclusion body (arrowhead) in the spleen from a pig experimentally infected with acute ASF (3 dpi). **(B)** Transmission electron microscopy image of the nucleus (*n*) and cytoplasm (*c*) of a macrophage in the spleen from a pig infected with ASFV showing margination of the nuclear chromatin and a viral factory within the cytoplasm (arrow). **(C)** Apoptosis of lymphocytes (arrows) in the spleen of from a pig experimentally infected with acute ASF (5 dpi).

vaccine trials carried out in the Iberian Peninsula in the 1960's (17). The evolution of highly and moderately virulent isolates in other areas where the virus has been present for long periods of time has not produced this chronic form of the disease (17, 59).

This clinical form is characterized by multifocal necrosis in the skin and arthritis, growth retardation emaciation, respiratory distress and abortion (60, 61). No vascular changes are observed in the chronic form of ASF, and many observed lesions are associated with bacterial secondary infections, inducing fibrinous polyserositis, necrotic, or chronic pneumonia, necrosis of the skin, tongue, and tonsils (17, 43, 60).

PATHOGENESIS OF LYMPHOID DEPLETION

ASF is characterized by severe leukopenia, mostly associated with lymphopenia, and a general state of immunodeficiency (58, 62). Initially, the virus enters the pigs following an oral-nasal route of after the bite of an infected soft tick. The virus replicates initially in the tonsils or regional lymph nodes (63, 64), spreading through the lymph and blood to secondary organs of replication within 2–3 days (65), and then spreading to the rest of the organs, where virus can replicate in a variety of cells (56, 66).

Monocytes and macrophages are the main target cell for ASFV (28, 42, 45). ASFV is a DNA virus, but the replication occurs within the cytoplasm and not in the nucleus (67–69). The infected monocyte-macrophage appears swollen, with margination of the nuclear chromatin (**Figures 4A,B**) and showing an intracytoplasmic juxtanuclear inclusion body, identifiable by its pale color when semithin (1-micron) sections are stained with toluidine blue dye (**Figure 4A**). These inclusion bodies show viral factories when studied under transmission electron microscopy (**Figure 4B**). The virus replication induce necrosis in the infected cells and virions are released by budding, and can be observed free in the blood, lymph, and the interstitial tissue (31, 70–72).

The destruction of monocytes-macrophages in ASF has been attributed to apoptosis (73) or necrosis (74) due to the action of ASFV (75). ASFV genome contain genes involved un programmed cell death both in an inhibitory or an inducing manner (64, 76–85). Some of these genes may promote the survival of the infected cells, and apoptosis has been described as the less likely cause of cell death in the infected monocyte-macrophage population (52, 58, 86).

ASF is characterized by a massive destruction of the lymphoid organs and tissues, including spleen, lymph nodes, thymus, and tonsils (58, 86, 87). There is a large proportion of B and T lymphocytes and macrophages undergoing cell death in acute ASFV infection (58, 78, 86, 88).

The virus replication in the monocyte-macrophages (**Figures 5F–H**) induces an activation in this cell population and an increase in the secretion of proinflammatory cytokines have been observed at the early stages of the disease (28, 42, 58). The upregulation in the expression of proinflammatory cytokines, including IL-1, TNF-α, and IL-6, and described as a "cytokine storm" (89), is the responsible mechanism for the massive induction of apoptosis in lymphocytes (**Figure 4C**) neighboring the activated/infected monocyte-macrophages in tissues (58).

PATHOGENESIS OF VASCULAR CHANGES

ASF can be considered a hemorrhagic fever, with some pathogenic mechanisms similar to those described for hemorrhagic fevers affecting humans, as Ebola or Marburg filovirus infection (90, 91). Among the typical vascular changes observed in acute ASF, we can include petechial and ecchymotic hemorrhages in multiple organs, hemorrhagic, or hyperaemic splenomegaly, pulmonary oedema, and disseminated intravascular coagulopathy (D.I.C.). In subacute ASF, we can also observe these vascular changes together with a more marked oedema, ascites, and hydropericardium.

The most typical lesion in ASF is the hemorrhagic or hyperaemic splenomegaly (44, 46). The severity of this lesion will vary depending on the virulence of the isolate. The

histopathological appearance of the spleen will include a hyperaemic red pulp, that can be completely filled with red blood cells (**Figure 5A**), platelet thrombi and cell debris, producing a disruption of the normal architecture of the organ (47, 58). The porcine splenic red pulp contains a mesh of fibers and smooth muscle cells surrounded by a population of macrophages fixed in the splenic cords (92). The necrosis of the macrophages in the red pulp is followed by a loss of intercellular junctions with the smooth muscle cells and the exposure of the basal lamina, inducing the activation of the coagulation cascade, platelet aggregation, and fibrin deposition, giving rise to the accumulation of red blood cells within the splenic cords (56, 93).

Hemorrhages are very common in the late phases of the disease, mostly in organs without a fixed vascular macrophage population, as the renal and gastrohepatic lymph nodes or the kidney (**Figures 5B,D**) (56). Even though ASFV can replicate in endothelial cells, this phenomenon has not been observed in all the organs showing hemorrhages (**Figure 5C**), and more importantly, this virus replication has only been reported in endothelial cells in the last phases of the disease, while hemorrhages may occur at earlier stages (33, 48). A different pathogenic mechanism has been observed and proposed as one of the main factors contributing to the hemorrhages in the early phases of the disease: the phagocytic activation of capillary endothelial cells, followed by endothelial cell hypertrophy that may lead to the total occlusion of the capillary lumen and a severe increase in the intravascular pressure (56). The subsequent loss of endothelial cells results in the exposure of the capillary basal membrane to which platelets can adhere, prompt the activation of the coagulation system and induce the D.I.C. (54–56).

An intense transient thrombocytopenia is frequently observed during subacute ASF, when hemorrhages are very frequent and severe (54, 55). This phenomenon may play an important role in the development of hemorrhages in the middle stages of the disease and is associated to structural changes of megakaryocytes in the bone marrow, with the presence of frequent denuded megakaryocytes (94), a feature also observed in relationship to hemorrhages in Classical swine fever (95).

The pathogenesis of the pulmonary oedema starts with the severe infection of pulmonary intravascular macrophages (PIMs), that is the main target cell for ASFV in the lung (31). Infected and non-infected PIMs tend to be enlarged and show signs of secretory activation. The production of proinflammatory cytokines such as IL-1α and TNF-α induce chemotactic activity and increase the endothelial permeability, leading to the leakage of fluid into the interalveolar septa and the alveolar spaces (42).

The marked anorexia in infected animals reduces dramatically the food/protein intake and accelerate the presence of hypo-oncotic oedema leading to internal fat consumption, ascites, hydrothorax, and hydropericardium, very typical in subacute ASF. Moreover, the liver of infected animals show a marked congestion, but also histopathological lesions, including multifocal periportal inflammatory infiltrates (**Figure 5E**), infection of Kupffer cells, which show severe secretory activation, and hepatocytes in the late stages of the disease (32, 49, 70, 96, 97). Hepatic malfunction may also contribute to the development of the multifocal oedema.

ASF IN THE EURASIAN WILD BOAR

The Eurasian wild boar (*Sus scrofa*) is a native suid species of most of Europe and Asia and Northern Africa, but has also been introduced in other continents, including many islands. It is considered the natural ancestor of the domestic pig and both are classified as the same species. At present, the wild boar play a very significant role in the spread of ASF infection in Europe, and probably also in Asia, being also considered the main source of infection in the recent outbreaks in Central and Eastern Europe (98–102).

Due to the close taxonomic relationship between Eurasian wild boar and domestic pigs, many similarities in terms of immune responses to infections can be observed. However, even though they are the same species (*Sus scrofa*), they belong to different subspecies (101). Moreover, domestic pigs, and in some instances also wild boar, are managed with a close control on the health, reproduction and nutrition, whereas free-ranging wild boar are subjected to many natural variations on reproductive, sanitary, and nutritional conditions (101).

Before the outbreak of ASF in Georgia in 2007 and its further expansion, several studies were conducted to study the pathology and pathogenesis of ASFV infection wild boar, both in natural and experimental conditions [reviewed by Sanchez-Cordon et al. (101)]. No significant differences were found in the clinical presentation of ASF in wild boar compared with the domestic pig, with very similar acute, and subacute clinical courses, and associated lesions (17, 24, 103, 104). After 2007, a major emphasis has been put on the study of ASF in wild boar after the reports of infected individuals in relationship to the spread of the virus (105–109).

Several studies have been carried out in wild boars with low and high virulent isolates, in different settings and conditions. Highly pathogenic isolates from genotype II (110) induce hemorrhagic/hyperaemic splenomegaly, hemorrhagic lymphadenitis, pulmonary oedema, and multifocal petechial hemorrhages (64, 107, 111), sometimes described as even more severe than in the domestic pig (101). The mortality in is also very high (90–100%) in these infected animals. However, there are attenuated variants of the genotype II circulating in some parts of Europe (112–114). Infected wild boar with low virulent isolates and surviving the infection may transmit the virus to naïve contact animals for months, although current non-haemadsorbing genotype II isolates do not induce long-term carriers as a major outcome for recovery pigs isolates (111).

ASF IN AFRICAN WARTHOGS AND BUSHPIGS

In East Africa, ASFV is maintained in an ancient sylvatic cycle involving the common warthog (*Phacochoerus africanus*) and the arthropod vector (soft tick), *Ornithodoros moubata*, that inhabit their burrows (24, 85).

Since very early experimental studies, it was demonstrated that warthogs were very resistant to ASFV infection (1, 115), showing no clinical signs of the disease, except in young animals, which

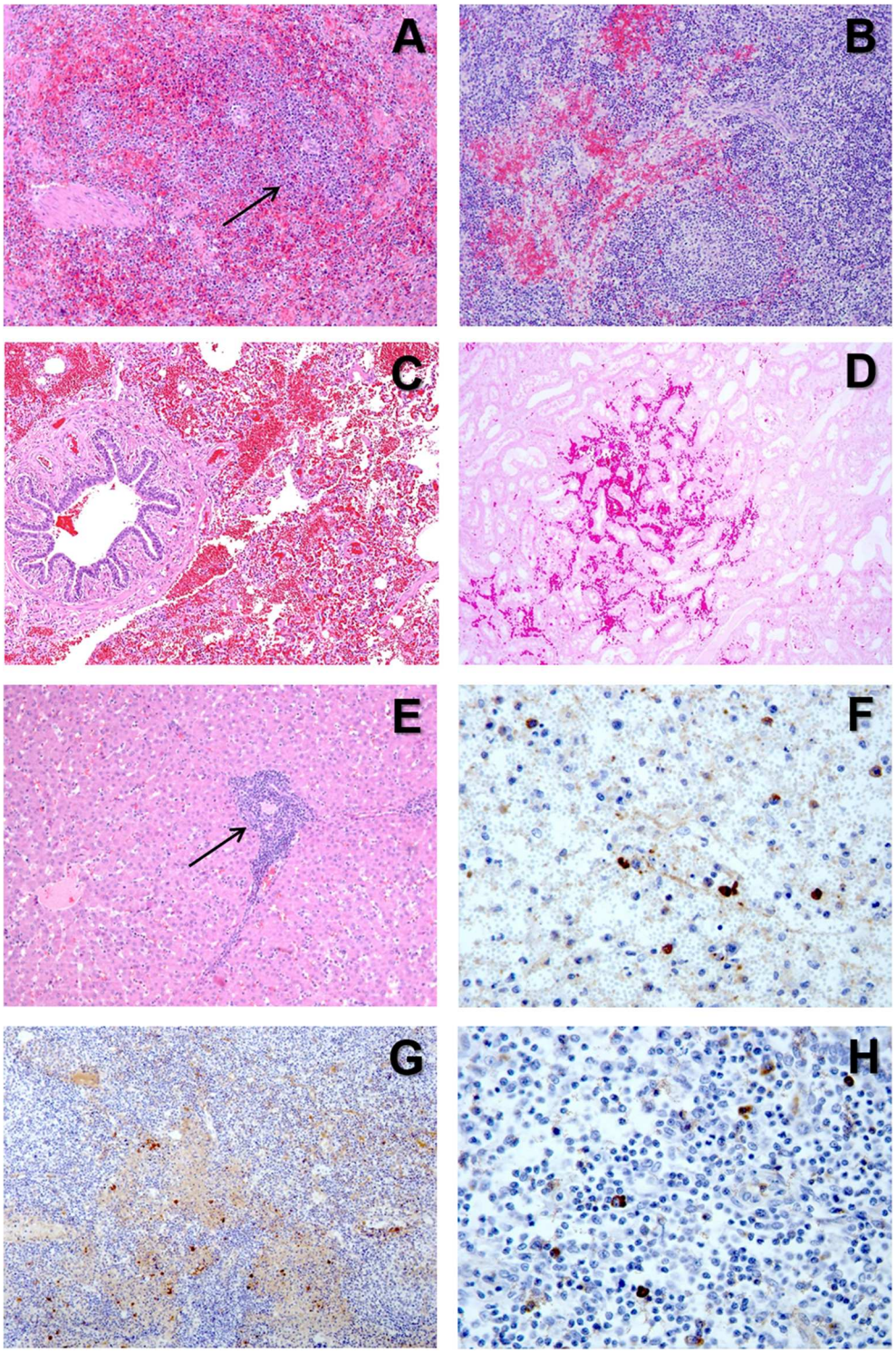

FIGURE 5 | (A) H&E stain of the spleen from a pig with acute ASF showing abundant red blood cells within the red pulp and severe lymphoid depletion, with very small lymphoid follicles (arrow) in the white pulp. **(B)** H&E stain of the gastrohepatic lymph node from a pig with subacute ASF showing hemorrhages in the perifollicular lymphoid tissue and the medulla, together with a moderate lymphoid depletion. **(C)** H&E stain of the lung from a pig with subacute ASF showing severe hemorrhages in the septa and the alveolar spaces. **(D)** H&E stain of the kidney from a pig with acute ASF showing interstitial hemorrhages within the renal cortex. **(E)** H&E stain of the liver from a pig with acute ASF showing periportal inflammatory infiltrates (arrow) composed of lymphocytes, macrophages and plasma cells. **(F)** IHC detection of ASFV p72 in the spleen showing strong positive reaction in macrophages in the red pulp and cell debris within the necrotic areas. **(G)** IHC detection of ASFV p72 in the gastrohepatic lymph node showing strong positive reaction in macrophages within the perifollicular areas and the medulla. **(H)** IHC detection of ASFV p72 in the tonsil showing strong positive reaction in macrophages within the perifollicular areas.

develop a transient viremia (116, 117). Viremia in adult warthogs is very rare with infectious virus mostly restricted to lymph nodes (85). The infectious ASFV may persist in warthog tissues for up to 25 weeks post infection, but is cleared by 56 weeks (118), what could explain the repeated re-infection of warthogs by ticks with the same virus strain (85).

Several genetics differences have been described between warthogs and domestic pigs (85). A difference between tolerance to infection and severe pathology may be due to a polymorphic RELA (p65; v-rel reticuloendotheliosis viral oncogene homolog A) variant found in warthogs (119).

ASFV has also been isolated from bushpigs (*Potamochoerus larvatus*) and red river hogs *(Potamochoerus porcus*), wild suid species found in sub-Saharan West and Central Africa (85, 116, 120, 121). ASFV infection does not induce clinical signs in these species, but moderate viremia can be observed (118, 120). ASFV can replicate in tissues without causing histological lesions, and mostly restricted to the B cell areas of the lymph nodes (85). Infected animals may transmit ASFV to feeding ticks but also to in-contact domestic pigs, although the role in the epidemiological maintenance of ASFV as a reservoir in unclear since these species do not inhabit burrows like warthogs and they are not in close contact with the *Ornithodoros spp.* ticks (85).

CONCLUSIONS AND FUTURE CONSIDERATIONS

ASF is spreading very rapidly worldwide, and current control strategies rely on rapid detection, strict biosecurity, and implementation of quarantine and slaughter policies, in the absence of a commercial secure, and efficacious vaccine. These measures are not always implemented correctly or are insufficient, leading to culling large numbers of animals. The rapid detection is very important when ASF enters a new territory, and education, and communication are crucial tools to detect the first cases of the disease and follow up the official measures implemented to control the outbreaks. The clinical course and associated lesions of the disease may vary, and farmers and veterinarians must be always aware of the different presentations of ASF.

The pathogenesis of this disease is very complex, and more research is required to understand some of the pathogenic mechanisms, including how ASFV modulates the host immune responses and the role of the multiple proteins encoded by the virus. Several research groups are developing prototype vaccines mostly based on subunits or live attenuated isolates. More information is also needed to understand the correlates of protection to help with the development of these vaccines.

Finally, the presence of wild suids in the epidemiological cycles in Africa and Eurasia, makes the control of the disease very complicated, with the added problem of soft tick species as potential arthropod reservoirs in different countries. Moreover, the population of wild boar is increasing dramatically in Europe, but also in some parts of Africa and America, adding more problems to the control of ASF when outbreaks are reported. The rapid expansion of ASF in South Asia also raises the concern about the possibility of transmission into local wild suid species and the establishment of potential new epidemiological cycles in this and other areas of the world.

AUTHOR CONTRIBUTIONS

FS is the sole author of this manuscript, and conceived the idea of this review article after discussing ASF pathology with many colleagues in Asia during 2019, trying to produce a review focused on the pathology of ASF that could be useful to support veterinarians working in government and academic institutions, with abundant images and briefly discussing the main features of the disease in wild suids.

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SYSTEMATIC REVIEW
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African Swine Fever: Lessons to Learn From Past Eradication Experiences. A Systematic Review

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Prevention, early detection, prompt reaction, and communication play a crucial role in African swine fever (ASF) control. Appropriate surveillance capable of early detection of the disease in both domestic and wild animals, and the implementation of consolidated contingency plans, are currently considered the best means of controlling this disease. The purpose of this study was to understand the lessons to be learned through the global disease eradication history. To establish which strategies were successful for prevention, control, and eradication of ASF, and which errors should not be repeated, we conducted a systematic review. A query was defined to search for surveillance and control strategies applied by countries worldwide for ASF eradication in the past. Inclusion and exclusion criteria were defined. Decisions on study eligibility and data extraction were performed by two independent reviewers and the differences were resolved by consensus or by a third reviewer. From 1,980 papers, 23 were selected and included in the qualitative analysis. Reports from Belgium, Brazil, Cuba, the Dominican Republic and Haiti, France, mainland Italy, Malta, Portugal, and Spain were included. Despite the economic resources allocated and the efforts made, eradication was possible in only eight countries, between the 50s and 90s in the twentieth century, in different epidemiological and cultural contexts, in some instances within <1 year, and in others in about 40 years. Classical surveillance strategies, such as active and passive surveillance, both at farm and slaughterhouse levels, targeted surveillance, together with conventional biosafety and sanitary measures, led to eradication even in countries in which the tick's epidemiological role was demonstrated. Historical surveillance data analysis indicated that eradication was possible even when technological tools either were not available or were used less than they are currently. This emphasizes that data on surveillance and on animal population are crucial for planning effective surveillance, and targeting proper control and intervention strategies. This paper demonstrates that some strategies applied in the past were effective; these could be implemented and improved to confront the current epidemiological wave. This offers encouragement for the efforts made particularly in Europe during the recent epidemics.

Keywords: African swine fever, data sharing, emergency preparedness, eradication, risk factors, surveillance, systematic review

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INTRODUCTION

The causative agent of African swine fever is a unique member of the *Asfarviridae* family, the *Asfivirus* (ASFV) (1); a genetically complex double-stranded DNA virus that contains a series of genes related to virulence, immune evasion, and cell process modulation (2). Twenty-three genotypes have been described based on the partial sequences of the p72 gene (3, 4). All 23 genotypes are present in Africa, whereas only genotypes I and II have been found outside of that continent. ASF is a notifiable disease in the European Union (EU) and should be reported to the World Organization for Animal Health (OIE). Due to the related impact on international trade in live animals and swine products and the socio-economic consequences on individuals' livelihoods, the disease remains a major concern for infected and non-infected countries, as there is no effective treatment and effective vaccines are not still available (5, 6).

The virus can affect species of the Suidae family (both wild and domestic) of all breeds and ages. Virulent ASFV strains cause peracute or acute hemorrhagic fever in infected animals, with up to 100% mortality (7). Generally, clinical disease can manifest in multiple ways, ranging from death, with no signs (peracute, mortality nearing 100%), to an asymptomatic infection; however, most isolates of ASFV cause acute hemorrhagic fever in domestic pigs and result in mortality nearing 100% (8, 9). European wild boar (*Sus scrofa*) is highly susceptible to the disease and shows similar clinical signs and lethality as domestic pigs (*Sus scrofa domesticus*). In contrast, infected wild African suids usually have occult infections and develop subclinical and asymptomatic long-term persistent infections, acting as ASFV reservoirs in Africa.

ASFV is primarily transmitted via direct and indirect contact between animals, through infected swine and their products, and via contaminated fomites or uncooked meat from infected animals. Its ability to persist for a long time in the environment or in infected biological samples makes eradication difficult once the disease has become established. Additionally, some arthropods that may have acquired ASFV during preceding years (up to 5 years) can transmit the virus (10). Soft ticks of the *Ornithodoros* spp. can be an effective reservoir of infection (8, 11), with documented trans-stadial, trans-ovarial, and sexual transmission (12). However, these tick species have not been shown to be involved in transmission of ASFV in Eastern Europe, Russia, or the trans-Caucasus region (13), whereas potential sources of infection in Europe are represented by infectious domestic pigs (*Sus scrofa domesticus*) and wild boar (*Sus scrofa*), contaminated carcasses, food waste, and vehicles or equipment. Furthermore, in Sardinia (Italy), where the disease has been persisting for more than 35 years, recent studies have reaffirmed the absence a role of *O. erraticus* ticks in the ASF cycle, despite strong climatic and ecological similarities with the Iberian Peninsula, where this tick was involved in ASFV transmission and the persistence of ASF (14, 15).

In addition to the presence of carrier animals (16), there are several other mechanisms that can lead to long-term circulation of ASFV in pig or wild boar populations. The most important are human-induced factors, such as illegal movement of infected pork and swill feeding (16–23), as well as free-range pig management systems as it was observed in some regions of Russia (18, 21).

ASF was confined to Africa until the end of the 1950's, when Genotype I ASFV strains first appeared in Portugal, in 1957, probably via a single-source introduction from Angola (24). This epidemic wave involved different countries in Europe and then also in some Central and South American countries. After the virus introduction into the Russian Federation in 2007 (20), in order to mitigate the risk of ASFV spread toward the EU, the EU Member States bordering the Russian Federation implemented specific protection measures. Despite this, in 2014 ASF entered Estonia, Latvia, Lithuania, and Poland, where the disease has become endemic in the wild boar population (25), whereas the sporadic outbreaks occurring in domestic pigs have been efficiently controlled, thus preventing extensive secondary spread (26). However, in 2016 ASFV spread into Moldova and in 2017 it was reported for the first time in Czech Republic, Romania (27), Bulgaria, and Hungary (28). In September 2018 the virus made a big leap, infecting hundreds of wild boars in Southern Belgium, in a well-limited and confined area of the Walloon region (28). There were also large outbreaks in Asia, starting in China, where a wide part of the territory has been infected since August 2018. In July 2019 the disease was notified for the first time in Slovakia and a month later, in August 2019 (28), it appeared for the first time in Serbia (28).

Currently, the disease is present in more than 20 sub-Saharan African countries (29), in some islands of the Indian Ocean (Madagascar and Mauritius), and from 2007 in some Eastern, Central European countries and in eight countries belonging to the European Union (Lithuania, Polonia, Latvia, Estonia, Romania, Belgium, Slovakia, the island of Sardinia in Italy). In this alarming context, the positive resolution of an outbreak that occurred in a wild boar population resident in a restricted area of the Czech Republic should be considered (30). Nevertheless, there is great concern about the spread of ASFV infection in Asia: after the first occurrences of the disease in China, a number of bordering countries notified many outbreaks and the epidemiological situation appears far from being effectively controlled (31).

The sole European territory where ASF Genotype I (vp72) has been present for a long time is the Italian island of Sardinia (32). The same genotype has been present in Spain and Portugal from 1960 to 1995, and caused outbreaks in some other European countries [France (1964, 1967, and 1977), Belgium (1985), Italy (1967, 1980) Malta (1978), and the Netherlands (1986)] (33). This genotype was also responsible for several outbreaks in the Caribbean and South America (from 1971 until 1981) (34). Since 1995, all affected European and south American countries had successfully eradicated the disease (32), with only Sardinia being the exception. On the other hand, all ASFV isolates circulating in Azerbaijan, Armenia, the Russian Federation, in other Eastern and Central European countries since 2007, are all clustered within Genotype II (29).

ASF epidemiology is thus very complex, determining different epidemiological patterns of infection when considering Africa or Europe. From an epidemiological point of view, three independent epidemiologic cycles (sylvatic, tick–pig, and

domestic) have been described (35) until recently in literature. After the ASF epizootic occurred in Central and Eastern EU Member States, the researchers could consider a fourth cycle in addition to the three already recognized: the "wild boar–habitat cycle" (36). This cycle focuses on the wild boar population and its habitat as a virus reservoir (37). Different epidemiological scenarios can be outlined according to the geographical area, the species involved, the transmission route, and the risk factors identified for ASF persistence and spread (**Table 1**).

All the current applicable control and eradication measures at local level are based on classical disease control methods, including surveillance (active/passive, targeted to domestic/wild species), epidemiological investigation, pig tracking, and stamping out the virus in infected holdings. All these measures are combined with strict quarantine and biosecurity measures in domestic pig holdings and by the control of animal movement. Early disease detection both in wild and in domestic pigs is considered to be crucial to maintaining an ASF-free health status and is the most complex facet of effective disease surveillance.

The main purpose of this review was to study the ASF eradication history, in order to highlight effective strategies applied for ASF surveillance, control, and eradication in countries that succeeded in stamping out the disease, and to identify what are possible gaps currently hampering ASF control and eradication.

MATERIALS AND METHODS

Literature Sources and Search Strategy

The literature search was performed by querying PubMed, Web of Science, and Scopus databases to retrieve all papers ("primary sources of information") that could be included in the process of identification, screening, and final eligibility. Additional papers were found by manual searching or by screening the primary sources of information. The platforms were queried by means of Boolean operators, including the search terms (African swine fever OR ASF virus) AND (epidemiology OR spatial pattern* OR temporal pattern* OR trend* OR "control measures" OR control* OR eradication*).

The query was searched in "all fields" to allow the retrieval of articles in which the terms appeared in the titles, abstracts, or keywords. Moreover, a filter on the geographical area/territories/countries was applied to exclude the African continent, and the time frame selected was from 1st January 1960 to 31st October 2019.

Inclusion and exclusion criteria were defined on the systematic review aims and objectives. A PRISMA flow chart was used to map out the number of records identified, included, and excluded, and the reasons for exclusions in each step of the screening process were described (**Figure 1**).

Studies were initially selected through a search of the titles and abstracts (first screening), and then by reading the full texts (second screening). Decisions on study eligibility and data extraction were performed by two independent reviewers, using electronic forms, and differences between the reviewers were resolved by discussion to consensus or by consulting a third reviewer. References were managed in RefWorks.

During the reading of the title and abstract, the papers were judged ineligible for further screening in full text if they were clearly referring to diseases other than ASF, or at least one of the exclusion criteria was clearly met, in which case, the paper was eliminated.

Each paper identified and included in the previous step was considered eligible for data analysis during the second screening step if fulfilled at least one inclusion criterion.

Information was collected on the dates of first occurrence and eradication of ASF, the type of intervention strategies implemented and the surveillance strategies applied for each country, the risk factors contributing to ASF appearance and its persistence before the eradication goal was met.

Secondary Sources of Information

Additional information was considered if new papers (in addition to the primary sources) were retrieved by reading the primary sources of information or by manual searches. Secondary information sources were considered in the analysis to ensure inclusion of all available past literature by including additional papers not directly found by the primary searches. The additional papers found as supplementary source of information were used if they met the eligibility criteria or if they complemented some information already achieved through the primary source of information. They were included as "other sources" within the PRISMA Flow Diagram in the identification section (**Figure 1**).

Inclusion and Exclusion Criteria

Papers were included in the screening process if they dealt with control and surveillance strategies applied by specific countries to eradicate ASF; if they described control-eradication measures put in place to face and then to eradicate ASF; if they were epidemiological studies and/or studies aimed at designing surveillance and control strategies; studies on transmission dynamics aimed at designing and improving control measures and surveillance in countries where the disease was eradicated; studies aimed at defining or suggesting surveillance and control strategies in countries where the disease was eradicated; or were reviews on surveillance and control strategies applied by countries that achieved eradication. All the articles dealing with ASF surveillance and control measures in countries where the disease was eradicated, they were included. Articles written in English, French, Spanish, and Italian were included.

Studies were excluded if they were performed in the African continent, were outbreak notifications, prevalence studies, description of clinical disease, were studies on pathogenicity and diagnosis, experimental infections in animals and ticks, described research on vaccine development, genome sequencing, if not relevant to the surveillance purposes of ASF; were reviews, if not dealing with surveillance/control and eradication measures, or if dealt with, these were not focused on ASF or were not described in detail; were qualitative and quantitative risk assessments, if these did not target ASF eradication, or papers for which full text was not available. All the articles dealing with ASF surveillance and control measures in countries where the disease was not eradicated, they were excluded. All the articles written

TABLE 1 | [a]Epidemiological scenarios, by geographical area.

Geographical area	Species involved	Route of transmission	Risk factors for persistence or spread	Other areas with an overlapping scenario
Eastern and Southern African countries (currently)	Wild suids (asymptomatic *Phacochoerus* and *Potamochoerus* spp.), Soft ticks (*O. moubata* as reservoir) Domestic pigs (34)	Sylvatic warthog–tick cycle and/or domestic-tick or domestic pig cycle (38). Transmission to domestic pigs through the **bite of infected ticks** and the ingestion of tissues from acute-infected warthogs. Movement of infected pigs and products (38).	**Low biosecurity in pig farms**, marketing of infected pigs and products, cultural constrains (38), human behavior (8). Relevant role of soft tick and wild pigs in the maintaining of the disease.	N.A.
West African countries (currently)	Domestic pigs. Ticks suspected not to be involved A sylvatic cycle has never been demonstrating (34, 39).	**Direct contact** between domestic pigs (infected-not infected) **Indirect contact** between not infected pigs and infected pork products	Socioeconomic factors: **lack of compensation** to farmers (underreporting); **lack of veterinary services, low biosecurity farms** with home slaughter with indiscriminate disposal of pig viscera**, swill feeding, illegal selling** of infected pigs and pork products, **cultural practices** (39).	The same as in some areas of the Caucasus and the Russian federation
Russian Federation and trans-Caucasian countries (currently)	Domestic pigs and wild suids (*Sus scrofa*)	Movement of infected/carrier animals (**direct contact between wild boars and domestic pigs**) Transmission **within wild boar population**. Movement of **infected products**.	**Lack of compensation** for slaughtered animals; lack of resources for adequate control measures; **lack of traceability; delays in identification** of new cases; **non-compliance** with **movement bans**; farms with **poor biosecurity.**	N.A.
Sardinia (currently)	Domestic pigs, and wild suids (*Sus scrofa*) No ticks found	Movement of infected/carrier animals (**direct contact between domestic pigs and wild boars/non-registered domestic pigs).**	Arduous natural **habitats** (hard access). Traditional breeding practices (free ranging pigs or "brado" illegally maintained in demanial areas) (40).	N.A.
Baltic Republics[b]	Mainly wild suids (*Sus scrofa*) Domestic pigs	Uncontrolled movement of infected pigs, pigswill with ASFV. Spread through the continuous wild boar population habitat. **Direct/indirect contact between domestic pigs and wild boars** (41).	Contamination of wooded areas where **infected carcasses of dead wild boars lied for several months**. Association between the number of settlements, the human population size as well as the number of domestic pigs and pig farms, roads, forest cover percentage, and the presence of ASF in wild boar (26). **Long jumps** spread in wild boars likely **by human activity** (38) Lithuania: lack of biosecurity in the non-commercial pig farms (41). Estonia: contaminated fomites, vehicles, or clothing of farm workers (41).	N.A.
Eastern Europe[c]	Mainly domestic pigs Wild suids (*Sus scrofa*) No ticks found		**Small/backyard pig farms** (21, 38). Involvement of humans in the disease spread in Poland, Bulgaria (41).	N.A.

[a] *The table was created by the use of information (modified and updated) provided by Sánchez-Vizcaíno et al. (7).*
[b] ***Baltic Republics:*** *Latvia, Lithuania, Estonia.*
[c] ***Eastern Europe:*** *Belarus, Bulgaria, Hungary, Moldova, Ukraine, Slovakia, and Poland (belonging to Central Europe).*

in languages other than English, French, Spanish, and Italian were excluded.

Term Definition

Surveillance strategies were defined as all strategies aimed at collecting, collating, and analyzing information related to animal health and the timely dissemination of information so that action could be taken, according to the Terrestrial Animal Health Code's definition (43). For the purpose of this study, all these strategies aimed at detecting ASF outbreaks and demonstrating freedom from ASFV circulation were considered under the "surveillance strategies" umbrella.

Intervention strategies were defined as all the actions put in place to prevent or reduce the likelihood of ASFV introduction and spread (within and between farms, after having identified the index case) and those aimed at eliminating (eradicating) the sources of virus, according to the definition provided by Guinat et al. (44). They also included biosecurity measures (segregation, cleaning, and disinfection).

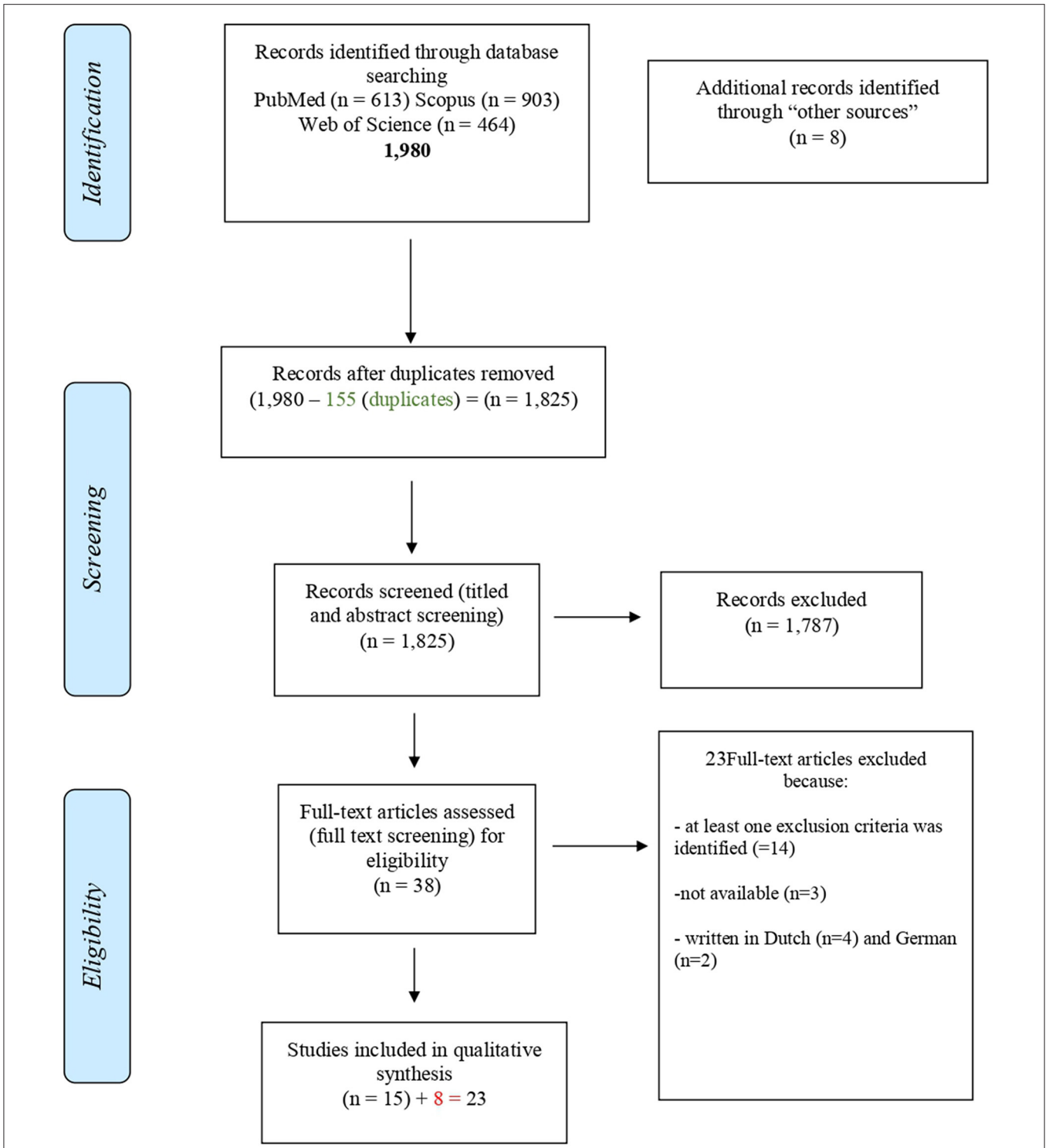

FIGURE 1 | PRISMA flow chart schematizing the process for paper identification, screening, and eligibility determination (42).

RESULTS

A total of 1,980 papers were found in the databases searched as primary sources of information. After the duplicates were removed (n = 155), 1,825 papers were selected for the first screening of titles and abstracts. Of these, 1,787 were excluded by the following criteria: dealing with diseases other than ASF (n = 729), type of publication (studies on ASF pathogenicity

and diagnosis, experimental infections in animals and ticks with ASFV, communications on clinical findings, $n = 1{,}058$).

Thirty-eight studies were selected for the second screening by reading of full texts. After the application of the eligibility criteria, 23 papers were excluded because:

- Fourteen met the exclusions criteria: studies on pathogenicity and diagnosis, $n = 9$; papers not dealing with surveillance and control strategies applied for eradication, $n = 5$,
- Three full texts were not available
- Six were written in languages other than the included languages: Dutch ($n = 4$), German ($n = 2$).

Fifteen studies were selected for eligibility from primary sources of information and eight studies from secondary sources of information were added. Finally, 23 papers dealing with the surveillance and control strategies applied for eradication of ASF by specific countries in the past were considered as "eligible" (**Figure 1**; **Table 2**) (44).

The 23 selected papers described historical approaches to ASF eradication and were included in the qualitative analysis (defined as "qualitative synthesis"). Three of these originated from Cuba, 1 from Belgium, 4 from Brazil, 3 from Spain (1 of the three papers retrieved for Spain [ref **Table 2**, Arias and Sánchez-Vizcaíno (67), was also considered as eligible for Portugal, and was therefore counted once in the methodological approach, but is listed twice in **Table 2**), 3 from Portugal, 4 from mainland Italy, 1 from Malta, 2 from France, and 2 from the Dominican Republic and Haiti. **Table 3** summarizes the literature analysis according to surveillance and intervention strategies.

Each country's eradication history is described below following the chronological order of ASFV appearance.

ASF Eradication From Portugal

The first outbreak of ASF outside the African continent was notified in Portugal, and probably arose from Angola in May 1957. The spread of ASFV to Portugal was thought to have taken place via contaminated food waste from African airline flights and/or ships docking at seaports, which was fed to pigs (33, 68). This outbreak was effectively controlled and eradicated in June 1958. After 2 years of epidemiological silence, a new outbreak occurred in April 1960 near Lisbon (62), probably caused by the improper use of food waste and waste originating from an infected dead pig whose carcass was not well-buried. From the 1960 epizootic, ASFV spread to many other areas of the Iberian Peninsula (Spain and other areas of Portugal), where it remained endemic for decades until 1994. In 1999, ASF appeared again in the Antalejo region, but it was successfully eradicated. The man-mediated transmission was considered as the most common cause of infection, via the uncontrolled movement of infected animals or the transport of infected animal products from contaminated sites. The uncontrolled movement of animals was probably closely related to the marketing circuits for live animals, as well as the decision-making mechanisms at farm level affecting production and marketing, and which in turn, were affected by the economic environment (64). Furthermore, the complex cycle of the disease, involving probable interaction between wild and domestic suids in the grazing areas (wild boar was considered to represent a potential virus reservoir), and the role of *O. erraticus,* made the eradication very difficult, particularly in outdoor swine production areas where pigsties were used to shelter the free-range pigs (54). In these types of areas, *O. erraticus* was the cause of disease re-emergences, even after disease eradication, as it was the case of the single outbreak in Portugal in 1999 (10). Studies were performed to find *O. erraticus* in the usual resting places of wild pigs; these suggested that the link between soft ticks and wild pigs was not important in the epidemiology of ASF in the wild pig population (69). After tremendous efforts, eradication was finally achieved, jointly with Spain, and specific programs were applied, including the detection of anti-tick antibodies in domestic and wild boars, as well as the destruction or isolation of the pigpens where ticks were present (67).

ASF Eradication From Spain

The first time ASF was reported in Spain was in 1960 where the disease remained endemic for decades until 1995. The disease spread within the pig sector when the family-type production system was characterized by low-level biosecurity. Extensive husbandry methods used in the management of Iberian pigs made ASF eradication extremely expensive and difficult. In fact, an analysis of the effort to control ASF in Spain in the year 1983 alone estimated costs at 11.4 million Euros (67). After ASF introduction, the pig production system structure was modified to industrial production. Therefore, a specific plan for eradication providing new restrictive policy measures, as compared to the previous plan, was adopted in 1985 (and remained in force until 1995). From 1985 to 1990, the disease was completely confined to southwest Spain. The virus persisted in these areas for several reasons: primarily because of inadequate sanitary and biosafety conditions in outdoor pig production facilities, but also because of the presence of soft ticks (*O. erraticus*), which served as medium and long-term reservoirs of the disease (11), and the presence of an uncontrolled wild boar population, as was the case in Doñana National Park (70). The application of this plan made it possible to divide Spain into an ASF-free region and an ASF-infected region, through a regionalization approach. Afterwards, in 1991, the infected region was divided into a surveillance area (with no acute outbreaks and very few seropositive animals for at least 1 year) and an infected area (66).

During the eradication plan, after outbreak confirmation a protection (with a radius of at least 3 km) and surveillance zone (with a radius of at least 10 km) were established and their radius was adapted according to epidemiological investigations. Movement of live pigs within the two zones was forbidden for 30 days; however, if serological tests proved that the area was negative, movements were allowed within the zones while movements of live pigs outside of the zones were forbidden. All pigs within the protection zone were serologically screened and further screenings were performed in the 3 and 10 km zones, not sooner than 30 days after the preliminary cleaning of the infected holding was completed (67). For holdings that were known to be infested with *O. erraticus*, specific measures were applied, such as no restocking unless special arrangements were made after consultation with the Central Veterinary Administration (67). At the beginning of the program, diagnosis was made

TABLE 2 | Papers included in the review process (PS, primary sources; SS, secondary sources).

Country	Title	Platform searched	Source type	References
Belgium	"An epizootic of African swine fever in Belgium and its eradication"	PubMed	PS (article)	(45)
Brazil	"The eradication of African swine fever in Brazil, 1978–1984" (article in Spanish)	PubMed; Web of Science	PS (article)	(46)
	"Eradication of African swine fever from Brazil"	By analyzing PS	SS (article)	(47)
	"Epizootiology, laboratory and virulence analyses during the emergency phase of the African swine fever eradication program in Brazil in 1978: a historic account"	PubMed	PS (article)	(48)
	"An analysis of the 1978 African swine fever outbreak in Brazil and its eradication"	PubMed	PS (article)	(49)
Cuba	"Preliminary Report on the African Swine Fever Epizootic in Cuba Methods of diagnosis and control"	PubMed	PS Communication by the Director General—National Institute of Veterinary Medicine	(50)
	"Status of African swine fever"	PubMed	PS (article)	(51)
	"Eradication of African Swine Fever in Cuba (1971 and 1980)"	By analyzing PS	SS (chapter in a book)	(52)
Dominican Republic and Haiti	"Experiences with Fever in African Swine in Haiti"	By analyzing PS	SS (article)	(53)
	"African swine fever. New developments"	By analyzing PS	SS (article)	(54)
France	"Identification en France· métropolitaine de la peste porcine africaine ou maladie de Montgomery" (article in French)	By analyzing PS	SS (article in Academic University Bulletin)	(55)
	"Peste porcine africaine isolement et identification en France métropolitaine. Données épidémiologiques, cliniques, anatomopathologiques et de laboratoire" (article in French)	By analyzing PS	SS (article in Academic University Bulletin)	(56)
Mainland Italy	"African swine plague. Diagnosis and interventions in the territorial jurisdictions of the Experimental Zooprophylactic Station of Mezzogiorno" (article in Italian)	PubMed	PS (Proceedings of the Conference held in Naples the 1st of March, 1968)	(57)
	"The outbreak of African swine plague in Italy" (article in Italian)	PubMed	PS (article)	(58)
	"African swine plague. Spread, losses and preventive measures in Naples" (article in Italian)	PubMed	PS (Proceedings of the Conference held in Naples the 1st of March, 1968)	(59)
	"Genome Analysis of African Swine Fever Virus Isolated in Italy in 1983"	PubMed	PS (article)	(60)
Malta	"African swine fever in Malta, 1978"	PubMed	PS (article)	(61)
Portugal	"Réapparition de la Peste Porcine Africaine (P.P.A) au Portugal" (article in French)	By analyzing PS	SS (article)	(62)
	Epidemiological research of African swine fever (ASF) in Portugal: the role of vectors and virus reservoirs"	PubMed	PS (Proceedings of the 5th International Symposium on Veterinary Epidemiology and Economics, 1988)	(63)
	"Persistence of African swine fever (ASF) in relation to the economic environment"	PubMed	PS Proceedings of the 5th International Symposium on Veterinary Epidemiology and Economics, 1988	(64)
Spain	"Relationship between the persistence of African swine fever and the distribution of *O. erraticus* in the province of Salamanca, Spain"	PubMed	PS (article)	(65)
	"A case study of an outbreak of African swine fever in Spain"	PubMed	PS (article)	(66)
	"African swine fever eradication: The Spanish model"	By analyzing PS	SS (article)	(67)

TABLE 3 | African swine fever (ASF) surveillance and intervention strategies for ASF eradication.

Country	YFO/YLO[a]	Epidemiological cycle	Risk factors for spread or persistence	Intervention strategies	Surveillance strategies[b]
Belgium	March 1985/May 1985	Pig to pig	Improper use of infected syringe needle	1. Slaughtering and destruction of animals within the infected farm and culling of infected and not infected traced animals 2. Cleaning and disinfection of farms	1. Syndromic and surveillance on sentinel piglets (AS and PS of pigs at farm) to demonstrate freedom
Brazil	May 1978/Dec 1984	Pig to pig	Contaminated food used to feed pigs	1. Ban of swine movements within and from the affected areas; ban of vehicle and human movements; ban of shows and markets; ban of feeding pigs food waste 2. Inspection at ports, airports, and post offices with more attention to at risk areas 3. Culling and incineration of all swine living in the affected areas 4. Cleaning and disinfecting of vehicles, buildings, and contaminated objects 5. Training campaigns	1. AS at slaughterhouses (serological tests); AS at animal level (special surveillance plan for trade in some at risk regions; test at the origin and destination); AS at herd level (herd certification for trade toward shows and fairs)
Cuba	May 1971/1980	Pig to pig	Contacts between different compartments of pig production characterized by different levels of biosecurity	1971 and 1980 epidemics: 1. Quarantine and movement ban, ban of swill feeding 2. Culling of all infected pigs and in-contact healthy pigs, slaughter of all pigs in neighboring herds (5-km), slaughter of all privately-owned pigs with partial compensation 3. Cleaning and disinfection of buildings, transport vehicles, and personal protective equipment 4. Training in diagnosis 5. Control of entry and departure via railways, roads, ships, and aircraft 1971 epidemic: Radius of 10–15 km around the infected place: 1. Compensation for all culled pigs 2. Transport with high biosecurity measures 3. Movement restrictions of all pigs, commodities, people, and vehicles 4. Complete census of all pigs	1. RBS: division into risk zones based on geographical and political characteristics and density of pork production 2. PS (syndromic surveillance and of pig mortality) 3. AS of pigs at sentinel farms and sentinel abattoirs (specific area) 4. Eradication phase: AS and PS of sentinel pigs at farm level. Test and slaughter approach. 5. Repopulation phase/recovering plan in affected areas: AS on sentinel pigs to demonstrate freedom
Dominican Republic and Haiti	Dominican Republic: 1978/1981 Haiti: 1978/1982	Pig to pig	N.A.	Dominican Republic: 1. Total pig depopulation Haiti: 1. Culling with compensation through Military Army 2. Cleaning and disinfection 3. Training and public education to different stakeholders and cooperation with rural population	Dominican Republic: 1. AS with sentinel pigs for repopulation Haiti: 2. AS with sentinel pigs
France	1st outbreak: 1964/1964 2nd outbreak: 1974	Pig to pig	N.A.	N.A.	1. PS with thermal exploration and blood sampling of positive animals
Mainland Italy	1st epidemic: 1967/June 1967 1969 1983	Pig to pig	Feeding of swine with infected food waste	1. Biosafety and sanitary measures 2. Stamping out in infected farms	N.A.

(Continued)

TABLE 3 | Continued

Country	YFO/YLO[a]	Epidemiological cycle	Risk factors for spread or persistence	Intervention strategies	Surveillance strategies[b]
Malta	March 1978/April 1978	Pig to pig	1. Feeding of swine with infected imported swill 2. Time elapsed between introduction and disease notification	1. Slaughter policy rigorously applied (ban of slaughtering) with compensation; 2. Stamping-out; pig movement restrictions, quarantine of infected and uninfected animals and premises, carcass removal and incineration; 3. Tracing of outbreaks; 4. Prohibition of pork's sale and swill feeding ban	AS at slaughterhouse (serum surveillance) and at farm level.
Portugal	1st epizootic May 1957/June 1958 2nd epizootic April 1960/November 1999	Pig to pig Tick-pig Wild-domestic	1. Transport and improper use of contaminated food waste 2. Uncontrolled movement of animals	1. Stamping-out within infected farms with compensation 2. Cleaning and disinfection of farms, transports, and Personal Protective Equipment 3. Movement restrictions of pigs and pig products from the infected zones or under surveillance; movement ban of pigs and pig products or pig by-products from the infected zone 4. Market and exhibition ban in the infected zones and suspected to be infected; Ban of swill feeding and repopulation	Compulsory notification of suspected and confirmed cases
Spain	1960/September 1994	Pig to pig Tick-pig Wild-domestic	1. Contacts between infected pigs 2. Intimate association between *O. erraticus* and pigs	1. Stamping out in infected farms with compensation 2. Biosafety and sanitary measures: fences, safe disposal of manure, sanitary enclosure 3. Cleaning and disinfection	1. Eradication phase: AS at slaughterhouse and at farm level 2. Repopulation phase: AS in pigs

[a] *YFO, Year of first occurrence; YLO, Year of last occurrence.*

[b] *AS, active surveillance; PS, passive surveillance; RBS, risk-based surveillance.*

through indirect ELISA, which was selected as the best assay for obtaining a rapid and reliable diagnosis (71) for screening, and indirect fluorescent antibody (IFA) assay for confirmation. In the final stages of the program, the National Reference Laboratory developed an improved ELISA containing all the ASFV proteins for better recognizing carrier animals (71). After 35 years of hard work, a key role in disease eradication was played by application of proper biosafety measures, together with a coordinated eradication program conducted with Portugal.

ASF Eradication From France

In April 1964, the disease appeared in France, with the notification of five outbreaks: one in the southwest, three in the southeast area bordering Spain, and one in the Bretagne region. The disease entered France through the illegal introduction of infected pigs from Spain (55), but it was eradicated in May 1964 (56). No surveillance and control measures were described in literature. A second outbreak was notified in 1967, and a last outbreak in 1974 in the southwestern part of France, in the Atlantic Pyrenees region (56). In this last case, the movement of infected animals traded from Spain probably caused the outbreak. Classical surveillance on clinical suspects was applied together with thermal exploration, followed by blood sampling in case of positivity (56). The outbreaks observed in France were characterized by low virulence both from an epidemiological and a clinical point of view (55, 56).

ASF Eradication From Mainland Italy

In Italy, an extensive outbreak was recorded in Rome, in the Lazio region (58), during the first month of 1967. The disease appeared because of the practice of animal feeding of raw urban food waste (58). This first epizootic affected 28 provinces with 205 outbreaks and was contained through the culling of 99,458 pigs. This intervention of the veterinary services was severe and immediate, so that the wild boar population located in the area surrounding the outbreaks remained free from the infection (58). After the first outbreak confirmation in 1967, an infected zone (Municipality of Rome) (58) and a protection zone (the entire province of Rome) were established (57, 58). A strong collaboration was set up among different Italian ministries, the national authorities, the OIE, and the veterinary services.

Afterwards, the disease spread to Naples through illegal commerce of infected pigs and swill feeding (57). Italy experienced a recurrence in 1969 and then in 1983, when ASF was lastly reported on a farm near Turin (57, 59). All these outbreaks were controlled by a rapid slaughter policy and each time the disease was eradicated. The disease was swiftly controlled and eradicated from mainland Italy through the interdiction of swill feeding and the massive stamping out of all infected holdings (57, 59), with compensation (59) and proper cleaning and disinfection measures. Repopulation was done after 6 months from the date of the culling of the last animal. During the post-eradication phase, no particular surveillance measures were described in literature.

The situation in Sardinia is not described here, because eradication has not yet been achieved. Since 1978, this Italian island has been the only European ASF-infected area (14).

ASF Eradication From Cuba

The disease was never been diagnosed in Central America until 1971 when the virus was introduced to Cuba and then spread within the country through privately-own pigs, private vehicles and transport, or by swill-feeding (50). Although firstly reported in May 1971, the authorities admitted its presence only in late June 1971. The length of time that elapsed between the actual occurrence and the notification was due to the time required for diagnostic support provided by Russia and Canada (51). The first epizootic occurred in a fattening holding in the province of Havana, which received animals mostly from the State's swine units (specialized porcine farms) and from some privately-owned pigs (farms in which the number of pigs per unit is limited and pigs are only for personal consumption). The late diagnosis allowed ASF to spread throughout the whole province of Havana and was confined to the province (51). The success of disease confinement was likely attributable to the involvement of several technical working groups (National Institute of Agrarian Reform, Ministry of Public Health with different Epidemiology groups, the Ministry of Home Affairs, the Ministry of Industrial Feeding, and the University of Havana) with different skills, and clear and defined tasks in the command chain (51). Furthermore, the Cuban authorities set up a dedicated Control Commission with national and international bodies (51).

On 26 January 1980, a second epidemic occurred in the eastern region of the island, in the municipality of Barcoa, in proximity to the Republic of Haiti (52). Initial analysis indicated that the disease entered Cuba by means of food products brought by Haitian immigrants arriving in an uncontrolled immigration (52). The overall loss was estimated to be 9,359,414 US Dollars. Surveillance on sentinel pigs to prove freedom from ASF started at the end of September 1980 (52).

Various control measures were applied for eradication both in the first and in the second epidemic. In infected premises, several measures were applied: strict quarantine, culling of all sick pigs and in-contact healthy pigs, or pigs suspected to be infected; disinfection of both infected premises and the area surrounding the outbreak; killing of rats, dogs, cats, and other animals that could have been mechanical vectors of the virus; treatment of the herbage and the soil with calcium hypochlorite; wood burning in buildings that could not be properly disinfected, and finally repopulation activities (51). Around the infected premises, in an area with a radius of 10–15 km, compensation was provided for all culled pigs, and special transport, with high biosecurity measures, was arranged for these pigs to official slaughterhouses; all the equipment used in the pig units were cleaned and disinfected. Moreover, movement restrictions of all the pigs and related commodities, both in the private and in the state sectors, people, and vehicles entering swine establishments, in addition to a complete census of all pigs in Cuba, were enforced (52).

ASF Eradication From the Dominican Republic and Haiti

In the Dominican Republic, ASF entered in February 1978, and subsequently it entered Haiti in December 1978, with the classical form characterized by high mortality. The disease probably

entered the Dominican Republic through infected pork scraps from an international flight from Spain and spread rapidly throughout the country (54). When the disease was confirmed in the Dominican Republic in July 1978, an agreement was reached between the two countries to slaughter all swine within 15 km on both sides of the border (53). With the cooperation of the Food and Agriculture Organization (FAO), the United States, and the International Development Agency, all outbreaks were eradicated from the Dominican Republic and total depopulation was achieved. In July 1980, in an effort to detect the residual virus, sentinel pigs were introduced for repopulation Up until September 1981, no cases of clinical disease were recorded, and all serological tests of newly introduced pigs were negative (54).

While the Dominican Republic endorsed an eradication program, Haiti took no actions at the beginning of the outbreak, either because of lack of funds or appropriate animal health infrastructure. With the support of four countries, the U.S. Animal Health Association, the U.S. National Pork Producers Council, and the National Association of State Department of Agriculture, an eradication program was drafted and started in Haiti in April 1981. It comprised 4 phases: (I) Six months of planning and information/public education; (II) Slaughter/compensation; (III) Cleaning and disinfection and raking; and (IV) The establishment of pig sentinels. Eradication was possible through the elimination of the swine population with the support of the Haitian Army, but the public information program was considered crucial for gaining the cooperation of the rural population. Haiti declared eradication on 28 April 1982 (53). Furthermore, the Pan American Health Organization (PAHO) and FAO defined emergency measures and training activities for field and laboratory, for the early identification of cases, and a specific program was established to coordinate ASF control for Latin America and the Caribbean. Together with the government of Jamaica, PAHO worked very closely with the veterinary services of Haiti to strengthen their capacities, quarantine measures, to review regulations governing entry of pigs and pork products into the country, to provide training involving customs, police, and animal health personnel, and to investigate deaths in pigs (53).

ASF Eradication From Malta

ASF was first notified in Malta in March 1978, after an outbreak involving infected imported waste illegally fed to animals. The first cases were notified in pigs in fattening premises, which had bought weaners from swill-fed premises where the disease was well-established, indicating that it had probably been in Malta for at least a month before diagnosis and notification. Therefore, ASF rapidly spread throughout the country affecting 25,100 pigs and 304 premises. In addition to the spread of virus in contaminated swill the movement of weaners from infected swill feeders was a key means of spreading the infection. In the early stages, farmers voluntarily depopulated their premises. A serum survey was carried out at slaughterhouse and at farm levels. By August, the pig population was reduced to one-third. A rigorous policy of slaughtering with compensation was applied in the island leading to the disease confinement and finally eradication. This result was achievable thanks to the restriction of pig movements and the elimination of the large number of infected pigs once the slaughter policy was adopted (61). After 10 months from the notification, at the end of January 1979, there were no pigs left in Malta (61).

ASF Eradication From Brazil

First notified in Río de Janeiro, in the municipality of Paracambi, in May 1978 (46, 49), Brazil experienced ASF due to tourism between Spain, Portugal, and Brazil, and the illegal trade in leftover food from flights landed in Río de Janeiro that was used for swine feeding (46, 49). Brazilian authorities declared an animal health emergency even before the laboratory results became available (49) and rapidly applied proper control measures. The disease spread due to contaminated food used to feed pigs housed on farms with low-level biosecurity (thus, the epidemiological determinant was a social factor), and through contaminated classical swine fever (CSF) vaccines that arrived in Paraná via the municipalities of Ourinhos and Jacarezinho in Sâo Paulo State (46, 49). During the emergency period (1978–1979), a federal level working group and an official laboratory for ASF diagnosis (ASFDL) were set up. The ASFDL was a paramount tool for the adoption of best eradication practices, providing information on ASFV heterogeneity (low- and high-virulence strains) (48). During the emergency period, all the actions were integrated between the Ministry of Agriculture, the Ministry of the Army, and the Military police. Several actions to control the disease were implemented, such as the immediate notification of cases to neighboring countries with which Brazil had bilateral agreements, and to the OIE and the FAO. Other measures applied included the destruction of clandestine deposits of food waste in the cities, with the removal and destruction of all food waste, the ban on sale of animals and pork products and on feeding of food waste; control of pig movements, with a ban on exhibitions, fairs, and other events of aggregation; the setting up of check-posts; census activities in the focal area; culling and immediate cremation of pigs within the affected areas; repopulation 6 months after the last eliminated case, and at least two rounds of disinfection of the affected premises, with the reintroduction of sentinel pigs free from ASF and vaccinated against CSF; active training and social programs related to preventive measures (farmers and veterinarians received phone numbers for free direct calling, so that they could notify the authorities as easily as possible).

In November 1980, a vast national program was launched which aimed at eradicating ASF and controlling CSF simultaneously in a joint effort. The program's activities had characteristics in common with the previous phase, with exception of vaccination against CSF (48). The technical and financial support for the program (from 1980 to 1987) and the establishment of diagnostic facilities for ASF surveillance were only possible jointly with the Federal University of Río de Janeiro, the financial support by the FAO and OIE and the Ministry of Agriculture (46, 49). The program was applied throughout the country, with selection of the Southern region as a priority area, due to its pig density. The program consisted of three stages of actions, namely, attack, consolidation, and maintenance stages.

The attack stage, applied between 1980 and 1984, consisted of targeted surveillance at ports, airports (mainly for flights coming from at-risk areas), control of internal movements, inspection comprising serological tests both at the place of origin and the destination, in addition to active surveillance both in pigs for slaughter at the slaughterhouses and in breeding centers associated with certification of the sanitary status of farms as ASF-free. Other actions, such as systematic vaccination against CSF, the restructuring of regional laboratories, training and awareness in animal health, and the implementation of a national information system, were also adopted.

The consolidation stage, which was in force between 1984 and 1986, aimed to identify new possible outbreaks through maintenance of the surveillance system and control of animal movements. The last stage, the maintenance stage, began in 1987 by way of the application of the general surveillance system set up for pig diseases (46, 47).

An activity named "garbage operation" within the eradication campaign was noteworthy; this was based on the registration and elimination of pigs kept in public garbage plants and slums performed with the help of the Ministry of Health and the Military Police (46–48). This action was responsible for the end of the transmission cycle of the disease within non-industrialized breeding programs (46, 47). Between November 1981 and September 1984, no new outbreaks were reported, and Brazil regained its status of ASF-freedom in December 1984. The prompt identification of the disease, the rapid notification, the swift implementation of actions, the social communication with farmers, the active participation of breeder associations in the democratic decision process, the government support (49), the financial compensation, the collaboration with international organizations (FAO and OIE), the stamping-out policy within the infected and suspected areas, the self-limiting nature of the disease in low-density pig farms, and the absence of soft ticks (Brazil has the advantage of an absence of complicating factors, such as wild hosts and vectors) (46, 49), led to successful eradication of the disease within 6 years (46, 47).

ASF Eradication From Belgium

The first case of the ASF in Belgium was reported in West Flanders in March 1985. The virus was probably introduced through infected pork from Spain that was fed to a wild boar. Afterwards the spread occurred through direct contact (trade) of infected animals and improper use of infected syringe needles (45). The disease was eradicated in all 12 infected farms within the country during 3 months after its first detection. The slow spread of the virus (due to epidemiological circumstances) together with the severe control measures applied led to eradication, which was declared in September 1985. The absence of viral circulation was confirmed by a large serological survey after the last confirmed case. The eradication goal was achieved by combining severe control measures with active and passive surveillance at farm level. Serological surveillance, aimed at eradicating the disease, was applied to both infected and not infected herds, and to several farms with indirect contact with those suspected to be infected. The interval between disease confirmation and eradication dates was short: for 5 of the 12 infected farms, the date of confirmation and the eradication date coincided, while, in other cases, a maximum of 5 days elapsed between confirmation and eradication. In the literature, no specific risk factors for maintenance were described given the fast eradication achievement (45).

ASF Eradication From the Czech Republic

The first ASF positive carcass was found in Příluky, Zlín district, in an inhabited area of the Czech Republic, in June 2017. This epidemic focal incursion of ASFV involved a limited wild boar population and progressed slowly in space. Since its first introduction until December 2017, the disease spread slowly at a rate of 0.5 km/month, despite the high wild boar density (8–10/km^2) (72). The infected area was located 30 km from the Slovak border and 80 km from both the Austrian and the Polish borders. From 2014 to March 2019, 4,296 wild boars found dead were tested for ASF, of which 211 tested positive. The last ASF-polymerase chain reaction-positive case in hunted wild boar was found in February 2018, and the last two positive cases in carcasses probably dead 4–5 months before discovery were identified in April 2018 (72).

Nationwide passive surveillance started in 2014 and was applied to all dead pigs found throughout the country. It proved to be a key factor in early detection of ASF that enabled an immediate and effective response (72). The strategy for successful eradication was based on the definition of different wild boar management zones according to a certain level of risk into three areas:

1. An infected area divided into (1a) zone with low risk and inside it a (1b) zone with high risk defined by a polygon of 159 km² estimated on wild boars' year-long home range. In addition, fences were built within the high-risk zone to delimit an area of 57 km² where the total depopulation with high biosecurity measures was performed by policy snipers specially trained for hunting in biosecurity;

2. An intensive hunting area of 8,500 km², excluding the Zlín district (72), on the outskirt of the low risk zone;

3. and the rest of the country.

After first confirmation of ASF in June 2017, hunting was regulated firstly through a ban within the infected area, then it was allowed only in infected area of the low risk zone, then it shifted from the trapping of wild boar in the high risk zone to individual hunting in the same zone in the infected area (73). The measures and approaches used after the outbreak's confirmation differed depending on the risk of infection. The success of ASF eradication in the Czech Republic relied on the management zones' demarcation, enhanced passive surveillance of dead wild boars through intensive and systematic searching and removal of carcasses, a ban on driven hunting, motivation for hunters through financial rewards and compensation, high biosecurity during hunting and sampling collection in the infected area, disposal of hunted wild boars from the infected area to/selected//definite rendering plants, effective hunting in the infected area by snipers, and awareness training campaigns and education of hunters, veterinary services, and the public (72).

DISCUSSION

The history of ASF is close to be one century long and in this period it was possible to collect several key elements from an epidemiological point of view. The disease was confined to Africa until the end of the 1950's when it appeared in Portugal in 1957. After 2 years' silence, the disease appeared again in Lisbon in 1960 and spread to the Iberian Peninsula and to other European countries: Spain in 1960; France in 1964, 1968 (74), and 1974; mainland Italy in 1967, with recurrences in 1969 and 1983; Malta in 1978; Belgium in 1985; and the Netherlands in 1986 (75). Between 1971 and 1980, ASF appeared in several American countries: Cuba, in 1971 and again in 1980; Brazil in 1978; the Dominican Republic in 1978 and Haiti in 1979 (67, 76). In the past, in both European and American countries the disease has been successfully eradicated, whereas in the current epidemics, only the Czech Republic managed to eradicate the disease in wild boar population (72).

Eradication was possible in different epidemiological contexts, with intensive or extensive swine breeding, and also in areas with the presence of or with an intimate association between *O. erraticus* and pigs, such as in Portugal and Spain (77). Nevertheless, it should be considered that eradication of *O. erraticus* ticks is extremely difficult (78) and epidemiological studies carried out in infected areas of Spain highlighted that, once ASF was eradicated from the domestic pig population, it also disappeared from the wild pig population. Therefore, most probably, the role of the wild boar population was not relevant in the spread of the disease (65) or in the persistence of viral circulation. Based on epidemiological data from the Spanish scenario, the role of carriers in virus dissemination seemed to be not so important when appropriate control measures were put in place (66).

Eradication was sometimes difficult, long-lasting, and costly, as demonstrated in Spain, where the disease was present for 35 years before its eradication (9) or in Portugal, where ASF was also present for decades. It was reached in a reasonable, or very short, period in Cuba, Brazil, Belgium, Malta, mainland Italy, France, the Dominican Republic, and Haiti due to the application of classical preventive and surveillance measures. Cases of particular interest were represented by France and Belgium. In France, the eradication was possible through the application of classical measures, but was facilitated by the presence of the Pyrenees (68), which acted as a natural barrier and minimized ASFV spread, leading to the occurrence of local epidemics (45) that were promptly eradicated. In Belgium, both the favorable epidemiological circumstances leading to slow viral spread and the short interval between the disease confirmation and eradication in most of the affected farms, enabled disease eradication in 6 months.

The recent experience of the Czech Republic was noteworthy, because it is the sole country officially declared free from ASF in recent years. Early detection and strict new measures in wild boar populations have been applied to prevent ASF spread, and containment efforts have recently met with success using different wild boar management zones; leaving wild boar in the infected area and by removing the carcasses, and depopulating around the infected zone (i.e., the fenced area, high- and low-risk areas, and intensive hunting area) (72). When the infection levels estimated from the carcasses decreased, depopulation was also put in place in the infected area. As a matter of fact, 10 months after discovery of the index case, ASF had been confined to a very small territory in the Czech Republic and has apparently not spread. Although eradication has not been achieved in the other involved EU countries, the Czech Republic experience can be considered to be a first successful attempt in disease control in an epidemiological scenario characterized by a small cluster of infections in wild boar population. As in the past, classical surveillance strategies and control measures continue to be valid tools for disease control and eradication. Also the experience of Belgium deserves special mention. In this country the disease was absent since 1985 but reappeared in a confined area on 13 September 2018 in wild boars, likely due to human activities (79). Even though Belgium has not yet been completely declared free from the disease, the control strategy applied was proving effective in limiting ASFV inside the affected area and confined to the wild population. This was possible thanks to preventive culling of all domestic pigs and captive wild pigs in the provisional "infected zone" extending over 630 km² along with a ban on the repopulation. In the rest of the country enhanced passive surveillance in all pig holdings, training of veterinarians, increased biosecurity measures and prohibition of assembly of pigs were assured. After the replacement of the provisional "infected zone" with zone II and I according to the Directive 2002/60/EC, specific additional and more stringent measures than those imposed by EU were applied within the three operational zones (an infected area bounded by two concentric peripheral zones called "reinforced observation area" and "vigilance area"). The ban of hunting and wild boars' feeding, the active and systematic searches for dead wild boar with immediate carcass removal and transport to the principal collection center then to the rendering plant jointly with soil disinfection were applied. Furthermore, a network of concentric fences was built with the dual purpose of slowing down the spatial diffusion of the disease and defining corridors aimed at collecting wild boars to be depopulated by avoiding their dispersal. The depopulation was carried in all the three zones by hunters who had received specific training on biosecurity procedures and compensation.

These results are sustained by a recent review (44), in which different surveillance and intervention strategies for ASF and their effectiveness were assessed, based on expert opinion. The authors identified surveillance and intervention strategies perceived as being the most effective. Among the 20 surveillance strategies identified, passive surveillance of wild boar and syndromic surveillance of pig mortality were considered to be the most effective for controlling ASFV spread, whereas culling of all infected herds and movement bans for neighboring herds were considered as the most effective intervention strategies. Regarding wild boar populations, active surveillance, and carcass removal were rated as the most effective surveillance and intervention strategies, respectively, but they were also considered the least practical, suggesting that more research is needed to develop more effective methods (44).

Currently, ASF is still present in some geographical areas of eastern and northern Europe and it is endemic in Sardinia (Italy) (76). In contrast to countries that achieved eradication, the Italian island of Sardinia is the only European ASF-infected area where the disease has been endemic since 1978 (14) as a consequence of the first European epidemic wave. In the past, the arduous habitat and the old practice of "brado" (free-range pig keeping, illegally maintained in public concession areas in traditional breeding practices) (40) on state-owned pastures represent an essential epidemiological link between the domestic pig and the wild boar population in the central-eastern part of the island (14). The overlap of these epidemiological conditions, together with other social and economic factors, represent the main obstacle to eradication. Recently, the fight against illegal breeding was intensified by mandatory culling and economic support to improve the farms' biosecurity levels, aiming to promote high quality pig products in compliance with local traditions (40). At present, the levels of infection in the population of feral pigs are decreasing and wild boars are considered a source of infection that is of secondary relevance to the presence of illegal wild pig breeding. Therefore, a hunting regulation plan, aimed at increasing the biosecurity level of hunting, as well as effective monitoring of the epidemiological situation were applied, and additional actions to limit wild boar population density were promoted.

Furthermore, the significant improvement of the epidemiological situation in domestic pigs in Sardinia (no disease outbreaks were registered from the beginning of 2018 until June 2019) was mainly attributable to improved control of illegal free-ranging pigs and better biosecurity on pig holdings (80, 81). On the whole, the significant progress in ASF control currently recordable (80, 81) demonstrated that it is not possible to control the spread of the infection underestimating the rules yet expressed in the EU legislation. A strict biosecurity approach on pig holdings, an effective animal registration as well as the contrast of illegal practices are all burdensome measures difficult to implement, but definitely essential. Actually, the application of this strategy includes a paradigm shift in traditional practices and in human behavior that are possible only by a great effort in informative campaigns.

It is noteworthy that only in one occasion ASF has spread outside Sardinia: in Piedmont, in March 1983 (60), affecting only three farms. This was due to wild boar meat imported from Sardinia. Strict quarantine and slaughter measures limited the spread of the disease in Piedmont and the outbreak was successfully eradicated (60). Therefore, the presence of ASFV in the island seems to pose a limited risk to the pig sector of ASF-free European countries (82, 83).

Similarly, as in Sardinia, humans' role was also considered to be relevant in the disease spread in the Northern European scenario (**Table 1**). Epidemiological analysis of ASF in the Baltic States and Poland, performed by the European Food Safety Authority (EFSA), aiming at estimating the relationship between the presence of ASFV in the wild boar population and environmental/biological factors, indicated that the human-mediated spread of ASFV played a critical role in the epidemiology of the disease. It was concluded that reduction of the wild boar population and carcass removal to stop the spread of ASFV in the wild boar population were more effective when applied preventively. The pressure exerted by outbreaks both in the domestic and in the wild population in the former Soviet Republics eventually involved European Union Member Countries, such as Poland and the Baltic Republics (Estonia, Lithuania, and Latvia) that were progressively affected from the beginning of 2014 to date (26). The analysis of available data regarding the incidence of ASF outbreaks in certain non-EU Countries authorizes the suspect of lack of information. In this context, it is quite impossible to properly investigate the relevance of multiple introduction of the virus in the epidemiology of this disease. However, ASFV does not recognize country borders and if considering the viral circulation in connected wild boar populations, progress of the virus in the border areas can be foreseen. On the other hand, it is pleonastic to remark that in the case of single introduction of the virus in a previously free territory or, better, in the case of focal spread in a very limited area, the chances to promptly reach the disease eradication are significant, especially if associated with an early detection and an efficient application of restriction measures.

Unlike the Eastern Europe scenario (**Table 1**), where the backyard network of farms with low-level of biosecurity was the main reason for the local ASF transmission, and the transfer of food products was the probable cause of long-distance infection (84), in the Northern Europe scenario, the wild boar population played the main epidemiological role (11, 85). The main risk factor facilitating the persistence of infection in Northern Europe was the contamination of the forest areas where the infected carcasses of dead wild boars lay for many months (23).

Results of this review also confirmed that the role of wild boar was generally supported by other factors (the presence of tick vectors in Portugal, human-mediated in the Baltic states, human factors in Sardinia, etc.). However, the density and population dynamics of wild boars currently represent a new challenge to solve. A scientific opinion was recently published by the EFSA (86), with the aim of providing an estimate of the wild boar densities in the EU, identifying thresholds in the wild boar density that do not allow sustaining the disease in different settings, and reviewing wild boar depopulation methods or population density reduction methods. They reported that passive surveillance on dead wild boars is the most effective and efficient method for early detection of ASF in free areas. Preventive measures for reducing and stabilizing wild boar density, before ASF introduction, will be beneficial both in reducing the probability of exposure of the population to ASFV, and the efforts needed for potential emergency actions (i.e., less carcass removal) if an ASF incursion were to occur.

History of ASF eradication indicates that this infection may appear in different ways, although the ASFV can shows very limited genetic diversity (87). In fact, in continents where only genotypes I and II have been circulating the genetic diversity among isolates collected over long time periods and from different geographical regions was very limited (87), in contrast to isolates from the sylvatic cycle in East and South Africa characterized by greater genetic diversity (34, 87, 88). Furthermore, large differences highlighted in the virus genome

(89) do not seem even to influence the ASF epidemiology in terms of mortality, morbidity, and resistance; if ever, the interaction with the hosts and the environment are more affecting the virulence expression: in fact, recent studies (89) indicate that the virulence may be modified as a consequence of the extended exposure of the host population to the infection.

As a matter of fact, ASF can occur as an epidemic, making long jumps, crossing borders, and even passing through continents; very often the first occurrence of the disease is a harbinger of rapid dissemination in naïve populations, whereas, in the past, certain outbreaks were immediately resolved by applying restrictive measures to the infected farms due to early detection. On the other hand, the viral spread could evolve in an endemic manner, in both the domestic and wild populations, due to its persistence in vectors or wild hosts, or due to human factors. In these cases, the eradication strategies are less effective and very expensive to apply in terms of direct and indirect costs. These lessons have been widely underestimated; nevertheless, we are learning that new sources of infection, which can create new scenarios, should be considered in risk analysis: the most important factor, which has been underestimated in the past, is the human factor. Probably, when early detection is applied along with strong awareness campaigns, this factor could have a limited effect. Nowadays, globalization, the movements of people, trades, and other similar factors, are currently contributing to increase the risk of ASF spread. Therefore, the most relevant lesson that should be considered is that the human role, human behaviors, social, cultural, and historical factors involved particularly in endemic areas, are crucial in any step of ASF control. Besides the wild boar population and habitat, the current European epidemiological situation also implicates humans as the main cause of both long-distance transmission and virus introduction to domestic pig farms (90). Therefore, in addition to biological aspects, it becomes crucial to include social science when planning prevention, control, or eradication measures (90). The countries that succeeded in eradicating the disease teach us that prompt eradication can be achieved only by applying early detection and proper control and intervention strategies, as foreseen by the EU legal framework for ASF. In fact, the prompt identification of cases allowed rapid eradication of the disease in the case of mainland Italy, Malta, and Belgium, and the epidemiology and laboratory networks played an important role in gathering data and providing epidemiological interpretation. Where a well-structured collaboration among different institutions of affected countries was put in place in the cases of Brazil, Cuba, the Dominican Republic, and Haiti, mainland Italy, Portugal, and Spain, successful eradication was achieved even in scarce economic contexts. Effective eradication was achieved when task forces of experts and appropriate communication skills, appropriate to that historical period, were applied. Instead, drastic measures applied for eradication of ASF in Cuba, such as killing of rats, elimination of dogs, cats, and other animals that could have acted as mechanical vectors of the virus, would be inapplicable in EU countries.

A final consideration of topical interest involves data collection on ASF at the European level. Linking outbreak information with surveillance and laboratory data, with the pathogen characteristics, would help in understanding the disease and its genetic dynamics in the spatial and temporal context and allow improvement of control and eradication strategies. At present, these data, if available, are usually collected at country level, with several information systems in place even in different regions of the same country, having different aims, and owned by different organizations. At EU level, data on the outbreaks of notifiable animal disease are currently registered into the Animal Diseases Notification System (ADNS) (91). However, the quality of data concerning each outbreak is currently poor, especially for data indispensable for evaluating the progression of the disease. Moreover, the information is often not linked to surveillance and laboratory data. The collection of data and information on ASF surveillance is fragmented even within a given country; this does not support the progression of control and eradication of the disease. Moreover, while data on farmed susceptible species and information on herds, densities, and locations (geographical coordinates) are stored in well-structured databases and information systems (92), densities and geographical distribution of wild susceptible animals are collected by the EU countries with different systems, each having their own specific characteristics with respect to the methodology used, the type of data acquired, the repository implemented, and data accessibility. This is of particular concern given the spread of ASF from Eastern Europe areas. In this framework, the ENETWILD EFSA funded project is attempting to develop standards for data collection, validation, and to create a data repository (81). Moreover, starting from 2019, EFSA has conducted a project with the support of volunteers EU Member States, aimed at building a harmonized and coherent platform for exchange of surveillance and laboratory information on ASF, lumpy skin disease, and Avian influenza (93). A coherent and harmonized data collection system would allow EFSA to perform proper risk analysis, with the aim of improving surveillance systems, and achieving eradication of the diseases.

CONCLUSIONS

We found documented reports for nine countries all over the world (Africa excluded) that had to manage ASF, as a whole, between 1954 and 1999 and they were able to reach the eradication. The eradication was achieved in few months or in more than 35 years.

The ASF infection demonstrated, over the years, to be really difficult to be eradicated. The sole continuous presence of viral circulation in Africa gives the evidence that the risk of new incursions of the disease are possible and the current epidemiological situation multiplies the chances of ASF virus spread all over the world.

The first epidemic wave started in the 50s', as such as the recent experiences of Czech Republic and Belgium, lead us to be optimistic: the virus first incursion is generally referable to an epidemic form that, in case of prompt and rigorous containment, can be kept under control or eradicate in a reasonable period of time.

Conversely, the disease, if not properly controlled, can easily turn into the endemization, as confirmed after the second epidemic wave began in the Caucasus region in the 2007, when the disease became endemic, involving also other countries.

African swine fever can be controlled and eradicated through classical surveillance and control measures, as demonstrated in the past experiences of countries worldwide if the main epidemiological target remains the domestic pig population. Classical measures are based on disease control methods, including surveillance strategies, epidemiological investigation, tracing and culling of pigs in infected holdings, in combination with strict quarantine and biosecurity measures on domestic pigs, holdings, and the control of animal movement. These measures are currently in force within the EU legal framework for ASF control, as laid down by Council Directive 2002/60/EC (94). The Directive also requires that Member States develop and implement plans for the eradication of the disease (95). These measures were effective in addressing a number of outbreaks, as exemplified in the Czech Republic's first experience of ASF. However, evidence also suggest that this strategy is difficult to sustain for a long period in endemic situations, such as in the Baltic States and Poland, where the disease affects larger areas. A successful strategy in this scenario has not yet been found.

In fact, the experiences collected in recent years demonstrated that the involvement of wild boar population in the viral spread hampers the eradication and, for sure, it is a relevant risk factor facilitating the virus spread across the country borders.

Therefore, an efficient strategy for ASF prevention or control should be based on deep knowledge of target domestic and wild population, of environmental conditions and type of swine sector. Nevertheless, all the strategies have to take in count that the disease knows no bounds and a common policy should be defined.

Finally, unlike in the past, considering the increase in globalization of animals and food products trade as well as of human beings, the effective collaboration among EU and non-EU neighboring countries would allow the definition of standards for data collection and validation, preventing new virus incursion.

AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript. FF helped to conceptualize the study. MD and FF designed the study and defined the research objectives. MM and MD defined the research methodology. MD and FF retrieved the papers, compiled all information, and wrote the manuscript. MD, FF, MM, PT, and SI contributed to writing the manuscript. PC and PT contributed to final revisions. All authors contributed to critical review of the manuscript and approved the final version.

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CASE REPORT
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Clinical and Pathological Study of the First Outbreak Cases of African Swine Fever in Vietnam, 2019

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African swine fever (ASF) is a devastating disease of swine and the most important disease for the pork industry. Since the outbreaks in 2007 in the Caucasian region, it has been spreading to the West and East quite swiftly. In this study we have analyzed the clinical signs and pathological features of the first outbreaks on ASF in Vietnam in 2019, caused by an isolate with 100% similarity to the genotype II (p72) isolates from Georgia in 2007 and China in 2018. The disease onset with a peracute to acute clinical course with high mortality. Some animals showed very unspecific clinical signs with other showing severe hyperthermia, respiratory distress, diarrhea, or vomit. Hemorrhagic splenomegaly and lymphadenitis were the main lesions observed at *post mortem* examination, with histopathological changes confirming the lymphoid depletion and multiorganic hemorrhages. Monocyte-macrophages were identified by means of immunohistochemical methods as the main target cell for the ASF virus in tissue sections.

Keywords: African swine fever, virus, pathology, pig, porcine

BACKGROUND

African swine fever (ASF) is a devastating hemorrhagic infectious disease that constitutes nowadays the major threat for the pork industry worldwide. ASF was first detected in East Africa in the early 1900s (1) and spread to Europe and South America in the 1950s and 1960s (2–7), where it was eradicated after many years and substantial effort (8–10). After the appearance of ASF in the Caucasian region in 2007 (11), it has been spreading quickly to neighboring countries (12–14) and beyond, making its first appearance in China in 2018 (15–18) and other Asian countries very quickly in 2019, including Vietnam (19–21). ASF is produced by the infection of ASF virus (ASFV), affecting domestic and wild suids (*Sus scrofa*) of all breeds and ages (22–26). The disease is characterized by hemorrhages and immunosuppression (27–34) leading to a high morbidity and mortality often up to 90–100% in naïve animals (23, 35).

The clinical and pathological manifestations of ASF are varied depending on the virulence of the ASFV strain, the route of exposure and the health status of the animals. The manifestation of the disease may evolve from the initial features after the invasion to an ASF free-region to the observations when the disease is established for longer time in a territory. Also, as Classical Swine Fever (CSF) and highly pathogenic Porcine Reproductive and Respiratory Syndrome (hpPRRS) are prevalent in Vietnam, it is important to clearly identify the clinical and pathological findings of ASF cases in Vietnam for differential diagnosis. In this study, we describe the clinical and pathological

presentation of the first two pig farms confirmed with ASFV infection in Vietnam at the beginning of 2019, before the disease spread to all provinces of the country in just a few months' time (19).

CASE PRESENTATION

Clinical Case #1

A breeding sow from a farm with 21 sows in Hung Yen city (Hung Yen province) suddenly stopped eating and displayed high temperature and disseminated cyanosis on the 29th of December 2018 (day #1). The animal was found dead on the 1st January 2019 (day #3) after a rapid non-specific clinical course. On day #5, another sow onset with the same clinical signs and was culled at day #9. The third and fourth sows followed a similar clinical course and were found dead or culled 4 days after the onset of the anorexia and hyperthermia. At day #22, two groups of piglets (23 animals of 4–8 weeks of age and 49 animals of 3–20 days of age) started showing lethargy and reduced appetite, following a very quick clinical course with anorexia, severe hyperthermia and death from 3 days after the onset of the clinical signs (day #25). Fatality rate was 100% among affected animals. At day #35, ASF was confirmed by the official laboratory and all remaining live pigs were culled.

Clinical Case #2

Two farms in the Dong Do commune, Hung Ha district (Thái Binh province) started with clinical signs in January/February 2019.

Farm "A," with 20 sows, 50 fattening pigs, 50 growing, and 50 piglets started with a sow showing anorexia and vomiting for 3 days before dying. One week after the death of the first sow, 4 fattening pigs were found dead after a short clinical course with vomiting as the main sign. *Post-mortem* examinations were carried out and ASFV infection was suspected. Farm "B," with 30 sows and 30 piglets started showing anorexia on the 6th of February 2019. One sow was found dead after just 1 day with no other clinical sign. Five days after the onset, three piglets displayed hyperthermia, anorexia, and diarrhea. Post-mortem examination was carried out and ASFV infection was suspected. Mortality rate was 100% of sows and 90% of piglets.

DESCRIPTION OF LABORATORY INVESTIGATIONS AND DIAGNOSTIC TESTS

Some found dead or culled animals were subjected to a *post-mortem* examination to rule out possible infectious diseases. In the clinical case #1, samples were taken for the official veterinary diagnostic laboratory at day #35, when ASFV infection was confirmed. No post-mortem examination was carried out and gross pathology was not recorded for this case. In clinical case #2, ASFV infection was suspected very quickly and a thorough *post-mortem* examination was carried out in the initial cases of both farms. For histopathological analyses, samples were fixed by immersion in 10% buffered formalin and routinely processed for paraffin embedding. Five micron sections were cut and routinely stained with hematoxylin and eosin (H&E) for light microscopy examination. For immunohistochemical detection of ASFV antigen in tissue sections, viral protein p72 of ASFV was performed as previously described (32). Specific antibody was replaced by PBS or an IgG isotype control in negative control sections. For ASFV PCR and sequencing, blood and organ samples were submitted to the Vietnam National University of Agriculture for ASF diagnosis. Samples were homogenized and viral DNA was extracted (14). For molecular detection of ASFV nucleic acid, both conventional PCR a using specific primers as recommended by the *Office International des Epizooties* and qPCR were performed as described in a previous report (19). p72 and p54 gene sequences of ASFV were aligned using BioEdit v7.2 (Ibis Biosciences) with ClustalW (clustal.org) and calculated sequence identity MEGA7 software was used with the neighbor-joining method to analyse the phylogenetic information with 1,000 replicates.

The first affected farm showed quite unspecific clinical signs in the affected female breeders, including anorexia and moderate hyperthermia. Very few skin lesions were observed, such as cyanosis, with no presence of hemorrhages. Affected piglets showed similar unspecific clinical signs, with a quick course (peracute) and high mortality. The animals from clinical case #2 also displayed unspecific clinical signs with some animals showing gastrointestinal signs such as diarrhea and vomiting. At *post*-mortem examination of case 2, hyperemic or hemorrhagic splenomegaly was consistently found in affected animals, characterized by an enlarged spleen with intense dark color (**Figure 1A**). Lung showed areas of consolidation in different lobes, mostly in the cranial and medial lobes and multifocal hemorrhages (**Figure 1B**). Lymph nodes also showed hemorrhagic lymphadenitis, mostly affecting the renal, gastrohepatic (**Figure 1C**) and mesenteric (**Figure 1D**) lymph nodes. Multiple hemorrhages were found in different organs, including the kidneys (**Figure 1E**), gastrointestinal, and respiratory tracts or externally on the skin (**Figure 1F**). Histopathological lesions were found in multiple organs. Skin hemorrhages were observed in several animals.

Hemorrhages and lymphoid depletion was a common finding in different lymphoid organs as spleen, lymph nodes (**Figures 2A,B**), and tonsils from affected animals. Lymphoid depletion was particularly prominent in the splenic follicle within the white pulp (**Figure 2C**) or lymphoid follicles present in renal, gastrohepatic and mesenteric lymph nodes (**Figure 2B**) or tonsils (**Figure 2D**). The kidney showed extravasated red blood cells (hemorrhaging) in between the renal tubules within the cortex and mild to moderate lymphoplasmacytic inflammatory infiltrates (**Figure 2E**). Hemorrhages and periportal inflammatory infiltrates were observed in the liver, infiltrates composed of mainly macrophages and lymphocytes but also occasional plasma cells. Segmental transmural hemorrhages were observed in the small and large intestine. Lung showed moderate to severe multifocal hemorrhaging, alveolar and interstitial oedema and congestion, and multifocal severe catarrhal bronchopneumonia consistent with secondary bacterial infections. Viral antigen (p72) was found in multiple

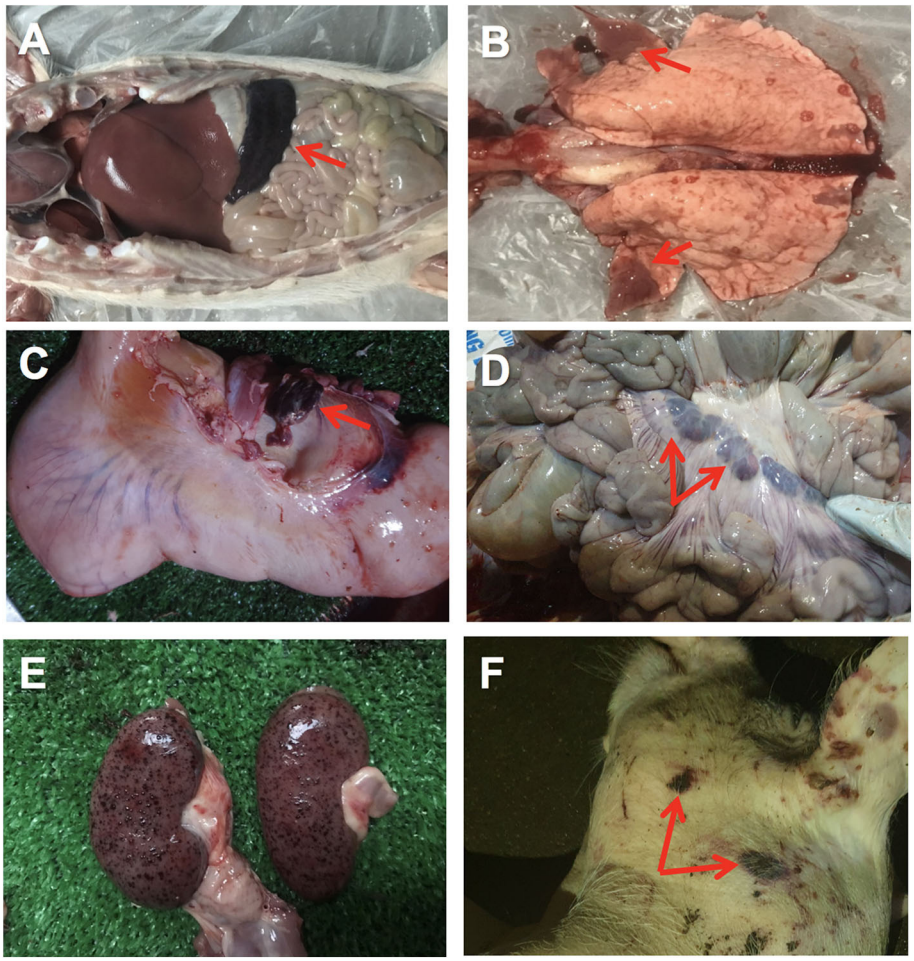

FIGURE 1 | Gross pathology of ASFV infected pigs in Vietnam, 2019. **(A)** Hemorrhagic splenomegaly (arrow) can be observed at the abdominal cavity inspection. **(B)** Multiple areas of lung consolidation in cranial lobes (arrows) and multifocal hemorrhages. **(C)** Hemorrhagic lymphadenitis in the gastrohepatic lymph node (arrow). **(D)** Haemorrhagic lymphadenitis in the mesenteric lymph nodes (arrows). **(E)** Multiple severe petechial hemorrhages in the renal cortex. **(F)** Multifocal hemorrhages on the skin (arrows) of the head and neck.

tissues and organs by immunohistochemistry. The main positive cell population was the monocyte-macrophage, with intense presence of positive immunoreaction in the cell debris associated to infection (**Figure 2F**). All affected animals showed qPCR positive results in blood, serum and the submitted organs, including, spleen, liver, lung, lymph nodes, tonsils, and kidney. Very low ct values were found in body fluids and tissues (**Table 1**). The genotype was determined by p54 and p72 gene characterization as previously described (36, 37). In the present study, the gene sequences of p72 and p54 of ASFV strains of VNUA/HY-ASF1 (accession no. MK554698 and MK554697) and VNUA/TB- ASF1 (accession no. MN793050 and MN793051) were deposited on GenBank. Phylogenetic trees revealed that the isolated strains from these two clinical cases belonged to the p72 and p54 genotype II (**Figure 3**) and were identical to ASFV strains isolates from China in 2018 and other genotype II isolates from Europe (Georgia/2007/1).

DISCUSSION

Pig population in Vietnam is about 30 million and about 49% of them are raised in small pig-raising farms and backyard household farming units. Pork accounts for three-quarters of total meat consumption in Vietnam where most of its farm-raised pigs are consumed domestically. ASF was first detected in Vietnam in February 2019 I Hung Yen province (19), just 5 months after it was reported for the first time in China in 2018 (18, 21, 38). By October 2019, the ASF has spread to all 63/63 provinces in Vietnam killing over 5 million pigs. The first reported ASF outbreak was detected in a small family farm and the onset of the disease was very unspecific. Once the mortality rate reached 50%, post mortem examinations and samples were sent to the official laboratory for diagnosis and confirmation of ASFV infection (19). Small pig-raising farms and households in Vietnam have low to no

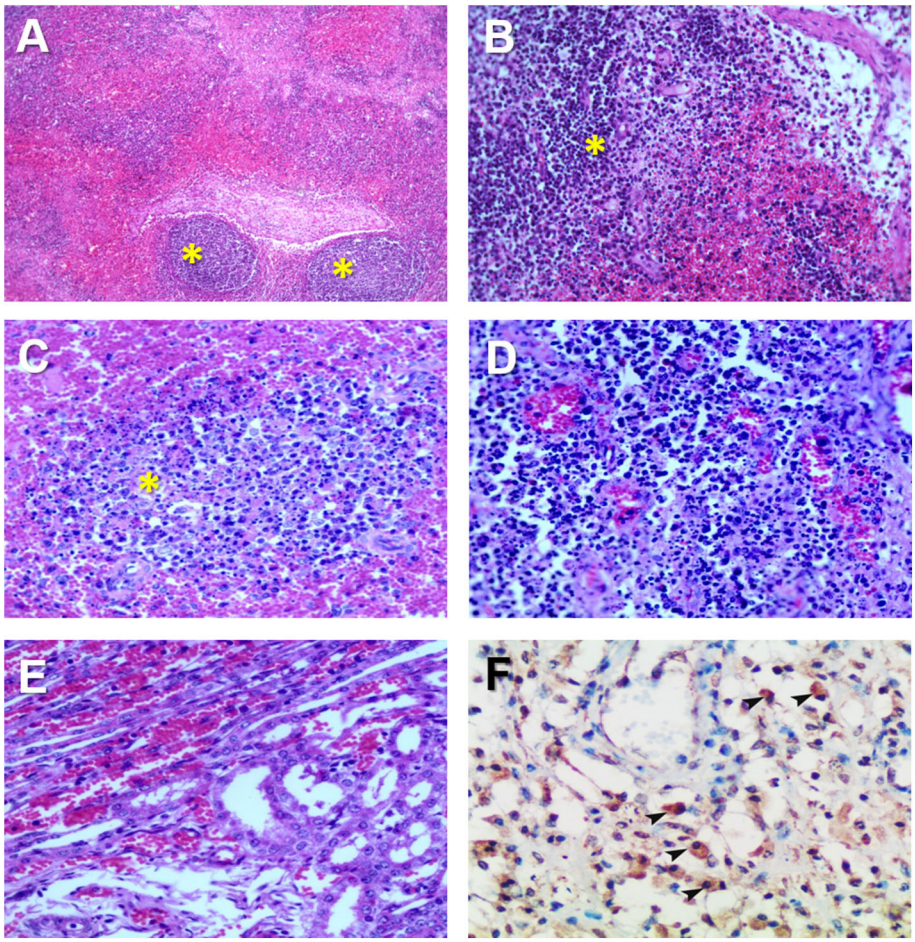

FIGURE 2 | Histopathological changes in ASFV infected pigs in Vietnam, 2019. **(A)** Lymph node: Severe hemorrhages within the lymph node medulla and lymphoid depletion in the follicles (*). H&E stain, 10X. **(B)** Lymph node: Severe hemorrhages within the lymph node medulla and lymphoid depletion in the follicles and parafollicular lymphoid tissue (*). H&E stain, 20X. **(C)** Spleen: Marked lymphoid depletion with the presence of pyknosis, karyorrhexis, and nuclear chromatin condensation within the splenic follicles (*) of the white pulp. H&E stain, 40X. **(D)** Tonsil: Lymphoid depletion, hyperaemia, and hemorrhages in the tonsil. **(E)** Kidney: Marked diffuse hemorrhaging within the renal cortex characterized by numerous extravasated red blood cells among renal tubuli. H&E stain, 40X. **(F)** Spleen: Immunohistochemical detection of ASFV p72 in abundant macrophages within the splenic red pulp (arrowheads). IHC stain (ABC technique), 40X.

biosecurity measures to prevent the disease, and many pig-raising households still use leftovers from cooking to feed their pigs. In many municipalities, pig farmers have not been able to properly dispose of infected animals and many pig farmers have culled their pigs themselves and dumped the carcasses into local rivers and bushes along the roadside further spreading the disease.

This may explain why ASF outbreaks were reported very quickly on household farms and rapidly spreading throughout the country in a short time. The pathway for disease transmission is very diverse, including ASF-infected fomites/vehicles, contaminated feed and/or pork products. A characteristic clinical manifestation in both cases described here was that the first signs of disease occurred in the sows. The reason why the outbreak started in the sows was unclear, but it might be related to differences in host susceptibility or to the entry site of the virus in the farms.

The clinical course of the disease recorded from the first ASF cases in Vietnam can be classified as peracute or acute, due to the lack of specific clinical signs and lesions in some of the animals. However, some animals showed the typical hemorrhagic splenomegaly at *post-mortem*, pointing out to a possible case of acute ASF, similar to previous reports (14, 39). Moreover, in this study other typical lesions associated to acute ASF were also identified during the *post-mortem* examination, including the hemorrhagic lymphadenitis, mostly affecting the renal, gastrohepatic and mesenteric lymph nodes (31), hemorrhages in the skin (40), lung (29), and gastrointestinal tract (41).

The presence of other diseases such as Classical Swine Fever (CSF) and highly pathogenic Porcine Reproductive and Respiratory Syndrome (hpPRRS) in the area makes the differential diagnosis more difficult as these diseases may have some similarities in the clinical course as well as the lesions at

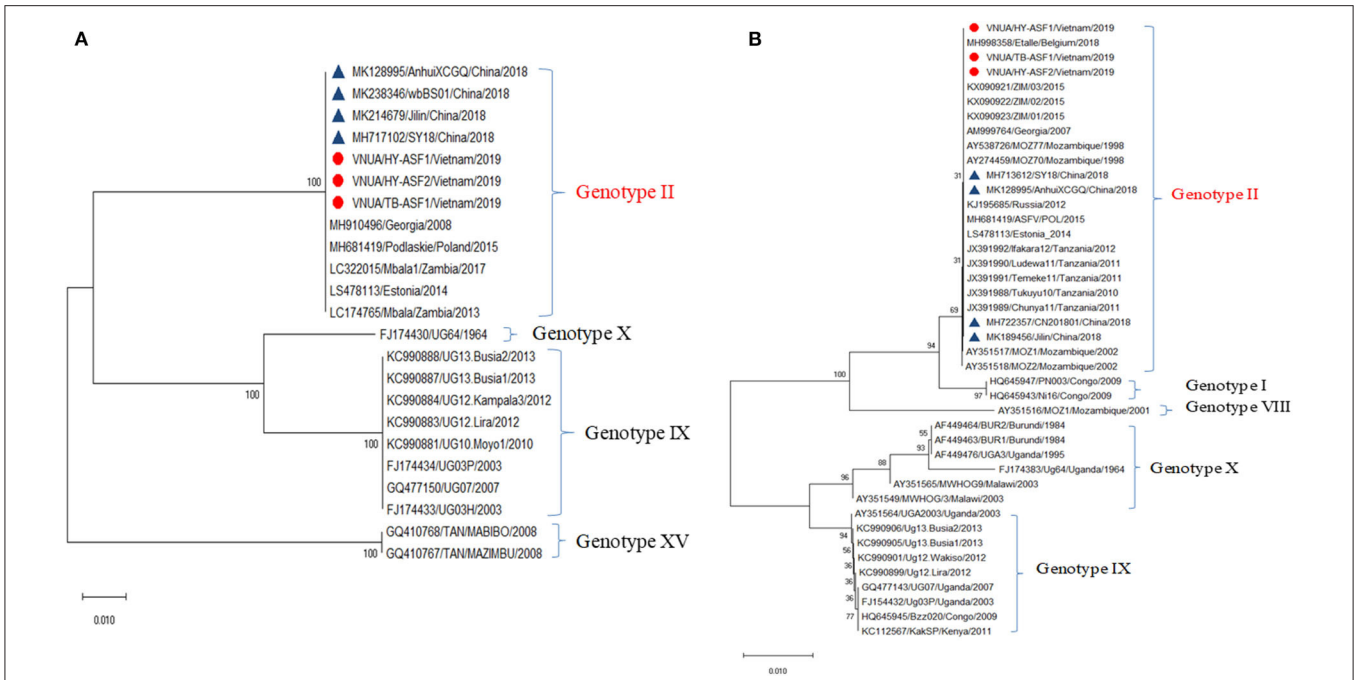

FIGURE 3 | Phylogenetic analysis of major structural proteins p54 **(A)** and p72 **(B)** of African swine fever virus isolated from case studies #1 and #2 (VNAU/HY-ASF1/Vietnam/2019; VNAU/HY-ASF2/Vietnam/2019; VNAU/TB-ASF1/Vietnam/2019) and reference isolates including recent ones from China/2018 (Δ).

TABLE 1 | Distribution of ASFV by qPCR (19) in different body fluids, organs, and tissues from the first 2 infected pigs detected in Vietnam, 2019.

Sample	qPCR-ct value		Mean qPCR-ct value
	Pig #1	Pig #2	
Whole blood	19.2	15.56	17.38
Urine	31.43	25.89	28.66
Spleen	15.29	11.88	13.585
Kidney	22.86	17.11	19.985
Lung	20.28	14.56	17.42
Liver	18.86	14.48	16.67
Submandibular lymph node	16.91	13.61	15.26
Inguinal lymph node	18.8	16.57	17.685
Mesenteric lymph node	19.54	15.86	17.7

post-mortem examination, with hemorrhagic lymphadenitis as a common lesion observed in the three diseases (42–44).

The histopathological lesions observed in the present study confirmed the severe immunosuppression during the typical acute ASFV infection (32). The lymphoid organs, including the spleen (31, 32), lymph nodes (31, 45), and tonsils (30) showed severe lymphoid depletion due to apoptosis of lymphocytes (32, 42, 46).

Multiorganic hemorrhages were also identified as in the acute clinical courses of ASF, including the typical petechial hemorrhages in the kidney (47) and multiple organs including the small and large intestines and the liver. Immunohistochemistry demonstrated to be a valuable tool to study the presence of the virus in different tissues and organs, mostly affecting monocyte/macrophages, the most important target cell of ASFV (48). The viruses isolated from the affected farms were identified genotype II from the similarity of the p72 and p54 genes. The similarity of the other genes has not been investigated. We suggest that the pathogenicity of the first isolate in Vietnam was similar to other ASF virus isolates prevalent in Europe or Asian countries from the clinical and pathological manifestation (49–51).

In conclusion, the first cases of ASF in Vietnam in 2019 were produced by a virus very similar to the one circulating in neighboring China and induced a clinical course from peracute to acute, with some difficulties to be identified at the early stages of the outbreak, but showing common signs and lesions of acute ASF in some of the animals, leading to the diagnosis of the disease and the confirmation in the official laboratory by molecular techniques.

DATA AVAILABILITY STATEMENT

In the present study, the gene sequences of P72 and P54 of ASFV strains of VNUA/HY-ASF1 (accession nos. MK554698 and MK554697) and VNUA/TB- ASF1 (accession nos. MN793050 and MN793051) were sequenced and deposited on GenBank.

ETHICS STATEMENT

This study was carried on naturally infected animals. Samples used for this study were diagnostic samples and no experimental procedures were carried out in any animal. Written informed

consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BN, BT, LN, and VL performed the initial investigations of the outbreaks in the farms and carried out the clinical examinations and gross pathology. BN, BT, LN, and FS carried out the pathological study. MO, KK, DS, and VL carried out the molecular analysis of the samples. BN and FS wrote the first draft of the manuscript that was reviewed and approved by all authors.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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BRIEF RESEARCH REPORT
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Disease-Induced Mortality Outweighs Hunting in Causing Wild Boar Population Crash After African Swine Fever Outbreak

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African swine fever (ASF) has been spreading in the Eurasian continent for more than 10 years now. Although the course of ASF in domestic pigs and its negative economic impact on the pork industry are well-known, we still lack a quantitative assessment of the impact of ASF on wild boar (*Sus scrofa*) populations under natural conditions. Wild boar is not only a reservoir for ASF; it is also one of the key wildlife species affecting structure and functioning of ecosystems. Therefore, knowledge on how ASF affects wild boar populations is crucial to better predict ecosystem response and for the design of scientific-based wild boar management to control ASF. We used a long-term camera trap survey (2012–2017) from the Białowieza Primeval Forest (BPF, Poland), where an ASF outbreak occurred in 2015, to investigate the impact of the disease on wild boar population dynamics under two contrasting management regimes (hunted vs. non-hunted). In the hunted part of BPF ("managed area"), hunting was drastically increased prior and after the first ASF case occurred (March 2015), whereas inside the National Park, hunting was not permitted ("unmanaged area," first detected case in June 2015). Using a random encounter model (REM), we showed that the density and abundance of wild boar dropped by 84 and 95% within 1 year following ASF outbreak in the unmanaged and managed area, respectively. In the managed area, we showed that 11–22% additional mortality could be attributed to hunting. Our study suggests that ASF-induced mortality, by far, outweighs hunting-induced mortality in causing wild boar population decline and shows that intensified hunting in newly ASF-infected areas does not achieve much greater reduction of population size than what is already caused by the ASF virus.

Keywords: disease ecology, camera trap, culling strategies, host-disease interaction, *sus scrofa*

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INTRODUCTION

In 2007, the African swine fever (ASF) virus reappeared in the Eurasian continent in Georgia (1, 2). From there, ASF further spread to the neighboring countries (3), entered the European Union in 2014 (4), and led most recently to local outbreaks in Western Europe (5, 6). Reported lethality rates induced by ASF were very high, reaching 95–100% in both domestic pigs and wild boar (7).

While concerns connected to this ASF outbreak focused mainly on threats to the pork industry and associated economic losses (8, 9), the impact of ASF on wild boar population size and the resulting consequences for ecosystem functioning has been so far neglected. Wild boar play a key role in the ASF cycle in Europe, facilitating virus transmission and survival in the environment (10). This wild boar–habitat cycle and its interaction with the domestic cycle is a major concern in Europe. Thus, understanding the impact of ASF on wild boar population is needed to better assess the dynamic of the wild boar–habitat transmission cycle.

To our knowledge, there are no published results on wild boar population mortality due to ASF under natural conditions. Considering that wild boar is one of the key species affecting structure and functioning of ecosystems globally (11–18), knowledge on how ASF affects wild boar populations is crucial to better predict ecosystem response and to gain knowledge to prepare a scientific-based wild boar management plan aimed to control ASF more effectively (19). The default policy in Europe consists in a drastic reduction of wild boar population before ASF incursion (20), and once the disease is present, an active carcass removal within the infected zone combined with intense hunting in buffered zones (21). However, host population and disease-management plans can interact and generate unexpected demographic and behavioral responses of the targeted populations (22, 23). In this respect, it is crucial to know the relative contribution of hunting actions and ASF in affecting wild boar population dynamics.

In this paper, we studied the dynamics of a wild boar population in the period 2012–2017 that overlapped with an ASF outbreak in 2015 in the Białowieza Primeval Forest (BPF, Poland). The BPF offers the unique opportunity to study wild boar population dynamics under two contrasting management regimes: a hunting-free area ("unmanaged area") and an area with intensified wild boar culling in response to the ASF outbreak ("managed area"). We hypothesized that, in the managed area, wild boar population decline will be stronger and faster due to the additive impact of hunting- and ASF-induced mortality compared to the unmanaged area.

METHODS

Study Area

The BPF, located in eastern Poland (52°450N, 23°500E) and western Belarus, is a large continuous forest composed of mixed deciduous stands. The BPF covers in total 1,450 km² and consists of a mosaic of forest types, which is dominated by deciduous oak-lime-hornbeam forest. The climate is continental with a mean temperature of 6.8°C and a mean annual precipitation of 641 mm. Five native ungulate species occur in the BPF (in decreasing order of abundance): red deer (*Cervus elaphus*), wild boar, roe deer (*Capreolus capreolus*), European bison (*Bison bonasus*), and moose (*Alces alces*). These ungulate co-occur with two large carnivores: the Eurasian lynx (*Lynx lynx*) and the wolf (*Canis lupus*) (24). Before the ASF outbreak, wild boar belonged to the most abundant ungulate species both in numbers and in biomass (25). For a more detailed description of the study area, see (26).

In the polish part of the BPF, where our study was carried out, the area is divided into two management regimes (**Figure 1**). The largest protected part is the Białowieza National Park, which is managed for biodiversity conservation. Hunting is not allowed inside the national park ("unmanaged area," area = 105 km²). The area outside the national park is managed for timber production (by the State Forest National Forest Holding), and ungulate numbers are regulated ("managed area," area = 600 km²). Wild boar hunting is conducted all year round with the main hunting season occurring in winter (October–February).

In the region, the first cases of ASF in wild boar were detected in February 2014 near Sokółka in the northeastern part of the country at a distance of c. 50 km from the BPF (4), and the first official cases of ASF in the BPF were reported in March 2015 (**Figure 1**). In the managed parts of the BPF, hunting followed the national policy aimed at drastically reducing wild boar numbers prior to ASF arrival. This led to a 4-fold increase in hunting bags in 2014/2015 when compared to the average hunting bag over the 2005–2014 period (**Figure 2C**). In the following hunting season of 2015–2016, when the first case of ASF had been officially confirmed within the BPF, intense hunting actions continued (3-fold increase in hunting bag compared to 2005–2014). Inside the unmanaged area, no hunting or any other wild boar–targeted management actions took place in reaction to the ASF outbreak.

Camera Trapping Design

We used available camera trap surveys taking place in the BPF between 2012 and 2017 to provide an objective estimate of wild boar population size. Because camera survey objectives varied over time, the study design (i.e., camera placement and timing) varied accordingly (**Supplementary Figure 1**). Specifically, between 2012 and 2014, camera traps followed a random placement design [see (26)] while between 2015 and 2017, cameras were placed along forest roads and trails to increase capture rates of large carnivores (26). We investigated the potential effect of this change in design (placement and timing) on wild boar population estimates in our analysis (see the section on *Detection Probability*). During the entire survey period, the same digital trail camera model (Ecotone SGN-5210A) was used. Cameras were triggered by passive infrared sensors with a detection angle of c. 35° and a maximal detection range of c. 20 m. After detection, with a time lag of 1 s, a photograph was taken and the camera recorded a 60-s video (26). During low-light conditions, cameras switched to a stealth infrared mode. Cameras were attached to a tree at a height of c. 1 m at locations with a clear view of at least 20 m [see (26)]. Camera trap surveys took place during summer and autumn (August–October), except for the 2014 survey (survey between January and March). Photographs and videos were manually analyzed and information on timestamp, the number of individuals, and when possible, age class (piglet, juvenile, or adult) and sex were recorded. The different camera trap surveys as well as the related data, photographs, and videos were managed using the open-source Trapper software (27).

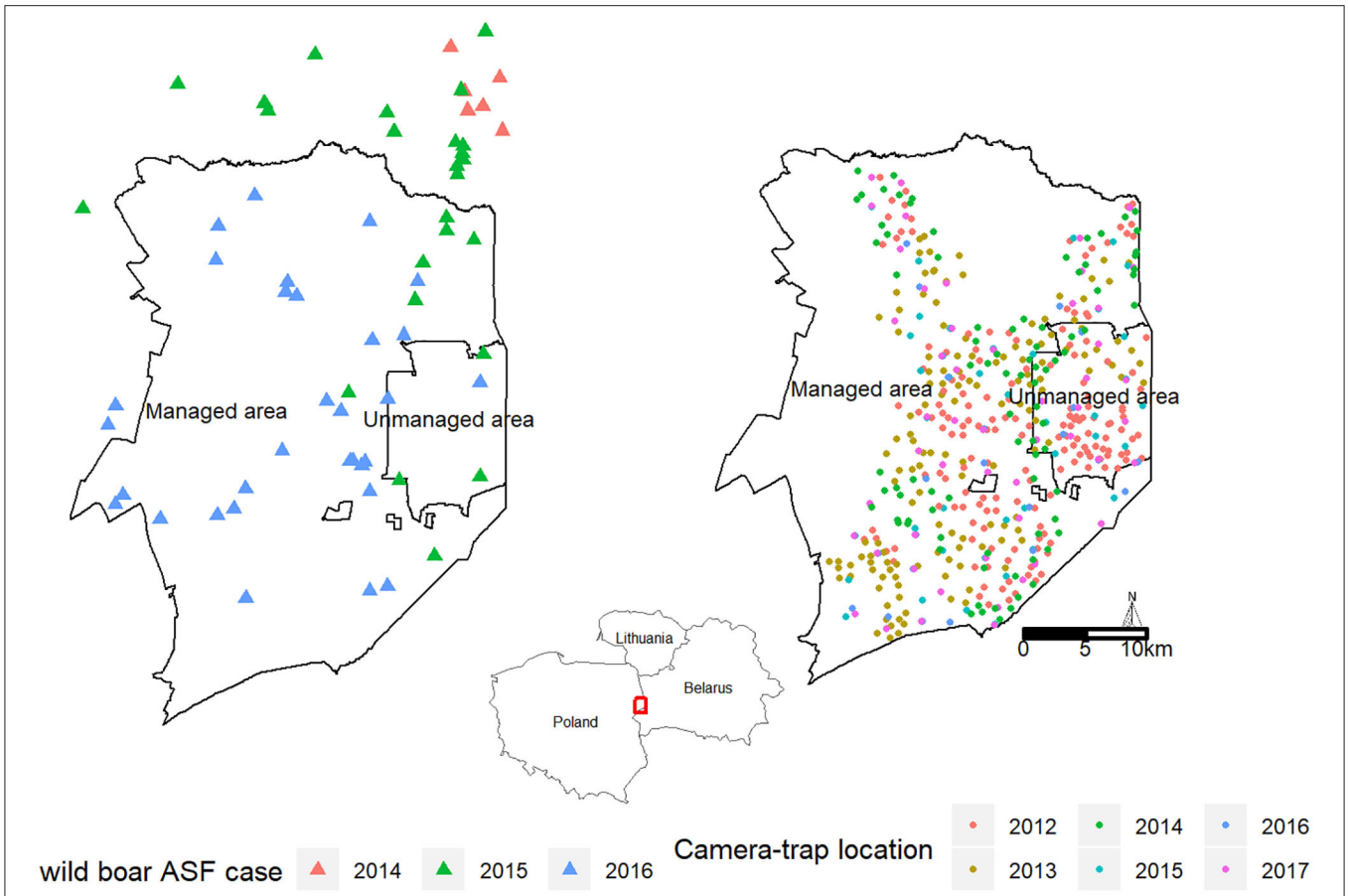

FIGURE 1 | Study area, the Białowieza Primeval forest (BPF), with two management regimes. In the "managed area," wild boar culling intensified as a result of the ASF outbreak, whereas, in the "unmanaged area," no wildlife management actions were taken. Camera placement is indicated in different colors during the survey periods in 2012–2017.

Data Analysis

Detection Probability

As the long-term wildlife monitoring in Białowieza contains changes in (i) camera trap placement (random vs. trail/road-based) and (ii) survey period (different seasons), we tested the effects of these two variables on the probability of detecting wild boar. To assess the impact of camera placement, we used the 2016 camera session, in which both methods of camera trap placement were used in a paired design. Specifically, 50 cameras were installed along the existing network of forest roads, and a paired camera was installed randomly ca. 200 m from the initial camera in the forest. To test for seasonal effects, we used the 2013 survey in which cameras were deployed continuously throughout the year (26). We pooled data on wild boar for each season (spring: March–May, summer: June–August, autumn: September–November, winter: December–February). We used a single-season occupancy model assigning the type of camera trap placement and the season as covariates (28). We used "camtrapR" (29) and "unmarked" packages (30) to prepare the dataset and to perform analysis within the R environment (31).

Camera Trapping Rate and Density Estimation

To quantify yearly changes in the wild boar population number, we used a relative index of abundance based on the wild boar trapping rates and a density estimate based on these figures. Camera trapping rate is defined as the ratio between the encounter rate, i.e., the total number of photographic events y and the camera trapping effort t, i.e., the number of 24-h periods each camera was deployed. To ensure independency between subsequent event records, we only used consecutive camera capture events (i.e., visiting individuals or groups of wild boar) with a minimum of 10-min interval between records (32). This resulted in the removal of 197 records from the full dataset comprising 2,089 records. For species, such as wild boar, that are difficult to individually recognize [but see (33)], methods considering the process of contact between animals and sensors have been developed. Here, specifically we used the random encounter model (REM), describing the rate of contact between moving animals and static cameras to estimate animal density (34). The REM requires information on the species number of encounter y, sampling effort (i.e., camera days) t, camera

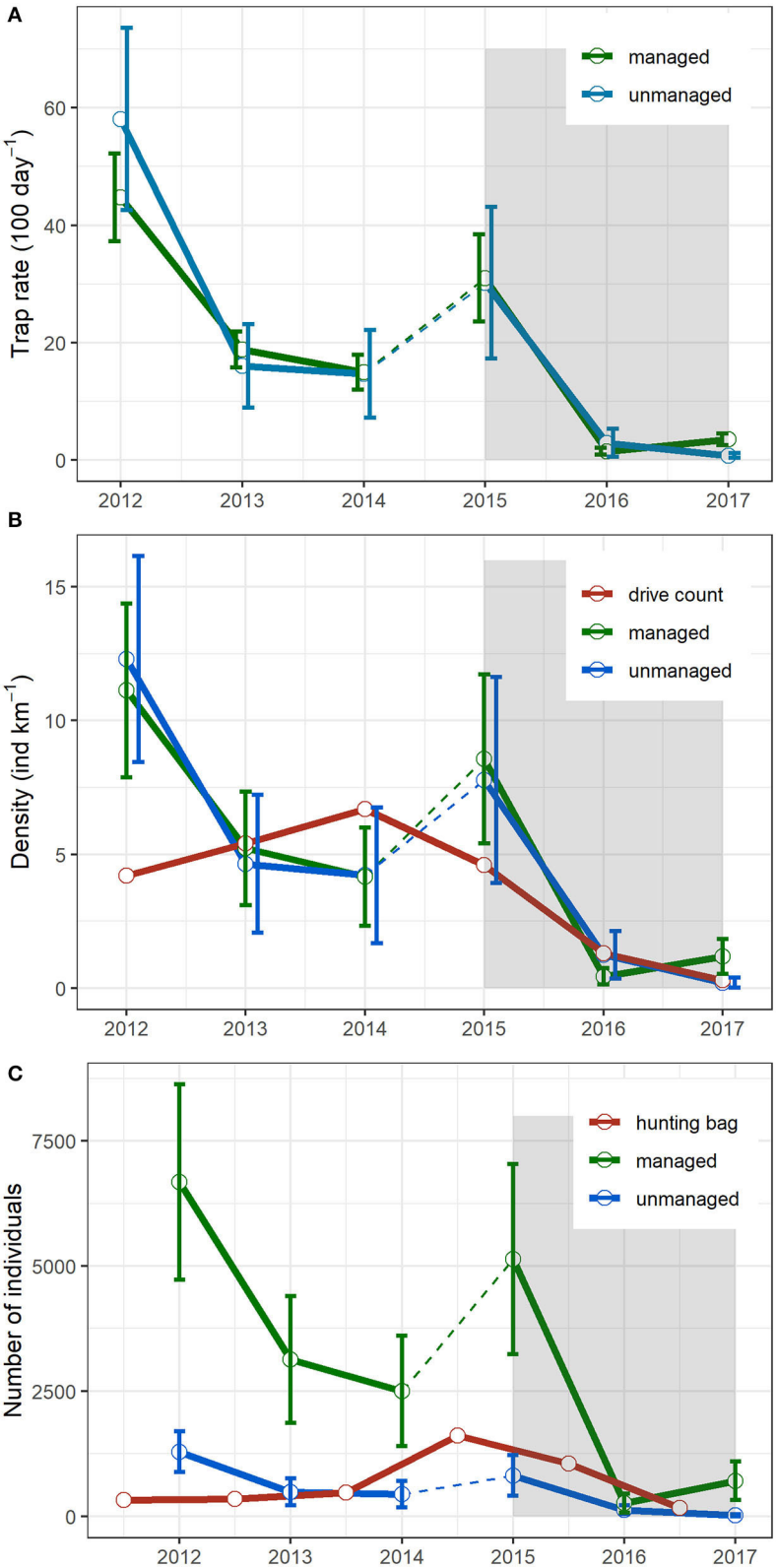

FIGURE 2 | Wild boar trapping rates based on camera trap surveys **(A)**, wild boar density estimation based on the random encounter model and comparison with independent drive count estimates **(B)**, and derived abundance and comparison with hunting bag in the managed and the unmanaged parts of the BPF **(C)**. The dotted lines in between census years 2014 and 2015 indicate a change of camera trap placement. The shaded area represents the period where ASF is officially observed in the BPF.

TABLE 1 | Parameters used in the random encounter model to estimate wild boar density.

Parameters	Description	Value	References
y	Number of independent photo-captures	–	This study
t	Camera effort (days)	–	This study
v	Daily range (km/day)	8.9 ± 3.4	Podgorski et al. (38)
r	Detection distance (km)	0.02	Bubnicki et al. (26)
theta	Detection angle (radian)	0.61	Bubnicki et al. (26)

detection zone specified by radius *r* and angle *theta*, and an estimated average speed of movement of the target species *v*.

In case of social species like wild boar, individual records can be considered as group records, in which case REM density is multiplied by unbiased independent estimate of average group size *g* (34). Because camera trapping estimates are sensitive to group size (35) and ASF and culling pressure might have impacted wild boar group structure and size, we decided not to include this parameters in our REM. Our view is analogous to that put forward in the context of distance sampling of clustered animals. The authors of (36) acknowledge that treating grouped individuals as independent values may sometimes be necessary if accurate group counts are not easily obtained, or if groups are not cohesive, as is the case for lions (37). In this case, variance connected to the REM estimates will be inflated, but estimates remain unbiased (36). We thus calculated wild boar density (*D*) according to

$$D = \frac{y}{t}\frac{\pi}{vr(2+\theta)}$$

where *y* and *t* are the same as for the camera trap rate. Estimation of average speed *v* was based on daily range estimations from collared wild boar in the same study area (38). Considering the large underestimation of daily range movement with telemetry methods (39), we applied a correction factor to improve the daily range estimate following (40). Camera detection radius *r* was based on (41), and the angle *theta* was based on camera model specifications (**Table 1**). We estimated uncertainty around y/t using non-parametric bootstrapping (42), resampling camera trap locations with replacement 10,000 times (34).

Further, to account for uncertainty due to other parameters (*v* and *r*), we used the propagate package in R (43). Propagate uses first-/second-order Taylor approximation and Monte Carlo simulation to calculate uncertainty propagation. We ran 10,000 simulations of these variables using the mean and standard deviations obtained from our data for *y/t*, *v*, and *r*, fixing all the other parameters. We compared our estimates of population density derived from camera trap analyses to drive count estimates, the method applied in the BPF to assess ungulates population (25). Drive count consists of a yearly census organized in the same day (in February) in the whole BPF (both managed and unmanaged parts). During these drive count, more than 200 people (divided into mobile pushers and stationary observers placed at the compartment limits) counted animals in randomly selected forest compartments [see (25) for more details], covering 10% of the entire Białowieza forest (including the managed and unmanaged parts).

From our density estimates for the managed and unmanaged areas, we derived the total wild boar population size by multiplying by the study area size, i.e., 600 km² for the managed and 105 km² for the unmanaged area, respectively. Observed population decline was then calculated for the two areas as the relative change (in percent) in abundance between 2015 and 2016 survey. Variation around the population decline was estimated by taking the average between the maximal (i.e., mean+$sd_{abundance,2015}$ to mean-$sd_{abundance,2016}$ relation) and the minimal (i.e., mean-$sd_{abundance,2015}$ to mean+$sd_{abundance,2016}$ relation) possible decline.

Finally, to assess the relative impact of ASF- and hunting-induced mortality on wild boar population size, we used two approaches. In the first one, we simply compared the decrease in abundance between the managed and the unmanaged populations, assuming that (i) populations are closed and (ii) population growth is equal in the two areas, so that the difference in population decline between the areas can be attributed mainly to hunting. The population closure assumption is congruent with telemetry study indicating very few movements of individuals between managed and unmanaged areas (38). The assumption of similar population growth is also reasonable considering the comparable resource and climatic conditions occurring in the two adjacent areas. Specifically, we assumed that the observed population decline inside the unmanaged forest is only due to ASF, following

$$decline_{unmanaged} = mortality_{ASF} = \frac{abundance_{unmanaged,2016} - abundance_{unmanaged,2015}}{abundance_{unmanaged,2016}}$$

Whereas, in the managed area, the total observed decline was due to both hunting and ASF.

$$decline_{managed} = mortality_{ASF} + mortality_{hunting} = \frac{abundance_{managed,2016} - abundance_{managed,2015}}{abundance_{managed,2016}}$$

In the second approach, we focused on the managed area only, investigating the relative share of hunting- and ASF-induced mortality. Specifically, we calculated the contribution of hunting-induced mortality to the observed decline in wild boar population since the first case of ASF according to

$$hunting_{percent} = \frac{hunting_{bag,2015-2016}}{abundance_{managed,2015}}$$

In this calculation, we make the assumption that available figures of hunting bags are accurate, i.e., that all shot wild boar have been reported and no animals died after a hunting event following shot wounds (and thus were not reported).

RESULTS

Wild boar detection probability (i.e., probability of detecting wild boar) was not influenced (*t*-test, $t_{42} = 3.15$, $p > 0.1$) by camera placement (randomly placed 0.15 ± 0.05 sd vs. camera traps placed on roads 0.17 ± 0.05 sd) (**Supplementary Figure 1**). This result indicates that the change in camera placement that occurred during our study period unlikely affected our density estimates. Detection probability of wild boar differed between seasons (one-way ANOVA: $F_{3,591} = 27.29$, $p < 0.001$) with increasing wild boar detections from spring to autumn and a decline in winter (**Supplementary Figures 2**, **3**). We therefore based our comparisons across years only on data collected in the same season, i.e., in summer–fall when wild boar numbers are highest for all years except 2014, for which only a winter survey was available. For the year 2014, we cautiously interpret the estimate when compared to other years.

Wild boar trapping rates in the managed and unmanaged areas followed the same pattern during the 2012–2017 survey period (**Figure 2A** and **Supplementary Table 1**). In both areas, the trapping rate decreased dramatically from 2015 onward and remained at a low level. Density estimates from the REM showed similar trends as the camera trapping rate. For both managed and unmanaged areas, the density dropped from 8.6 ± 3.2 (mean $\pm$ sd) and 7.8 ± 3.9 individuals km^{-2} in year 2015, to 0.4 ± 0.3 and 1.2 ± 0.9 individuals km^{-2} in year 2016, respectively (**Figure 2B**).

Comparing the population size between summer 2015 (just after the ASF outbreak in the BPF) and summer 2016, we observed a 94.8 ± 6.4% decline in the managed area and a 83.8 ± 25.5% decline in the unmanaged part (**Figure 2C**). This would indicate that hunting in the managed parts resulted in an 11% additional mortality to the ASF-induced mortality. When we compared hunting bags and abundance estimates of years 2015 and 2016 in the managed area, the relative share of hunting-induced mortality rose to 21.7 ± 11.2%, while ASF accounted for 78.3 ± 11.2%. This value is relatively close to the one observed for the unmanaged area (83.8% decline).

DISCUSSION

This is the first study to quantify the impact of ASF on the mortality of wild boar population under contrasting management conditions consisting of a hunted and a hunting-free area. After official presence of ASF within the borders of the BPF, we observed a population decline of 83.8 ± 25.5 and 94.8 ± 6.4% for the unmanaged and managed parts of this forest, respectively. This result only slightly corroborates our initial hypothesis that the wild boar population in the managed part will experience a stronger and faster decline due to the additive impact of hunting. Indeed, the observed difference (11%) between these two areas suggests that the intense hunting actions implemented during 2014–2015 and 2015–2016 had a relatively low additional impact on the observed population decline.

When investigating the relative hunting ASF share in the population decline using hunting bags and abundance estimates, we showed that the relative share of hunting- and ASF-induced mortality could be 21.7 and 78.3%, respectively. In our analysis, we assumed that hunting bag records are accurately reported and that all shot individuals have been retrieved and there is no additional delayed mortality following hunting events. Such underreporting of hunting bags could lead to an inaccuracy of the estimation of the hunting-induced mortality. Together, these two approaches suggest that ASF has a large impact on wild boar population, removing around 80% of the population in 1 year of disease presence. Furthermore, our results indicate that the increased hunting pressure during the ASF epidemic led to only a small additional impact on population decline.

Many lessons can be learned from the management actions implemented in the BPF in response to the ASF outbreak. The first management actions took place in 2014–2015 before the ASF presence in the BPF. It followed the Polish national emergency plan and EFSA recommendation to preventively reduce wild boar density before ASF introduction (44). To reach this aim, hunting pressure was increased dramatically (four-time increase in the number of wild boar shot compared to previous years' average) in the managed part of the BPF. The action apparently failed to reach its goal since the population density in the following year remained high (7.5 animals km^{-2}), and no difference in trends between the managed and unmanaged parts could be observed (**Figure 2B**). This result is congruent with previous work demonstrating that wild boar population can still increase even when hunting mortality is increased (45). It further suggests that other environmental factors, such as climate (46) and pulsed resources (47), could have played a greater role in driving wild boar population dynamics than the increased intensity of hunting. High hunting pressure might also have induced unwanted effects inducing compensatory population growth rate and accelerated generation time, i.e., higher juvenile female contribution to the reproductive set (48) and earlier reproduction (49). In the managed part of the BPF, the camera trap data suggest such a positive feedback, with an increased ratio of observation of piglets and juveniles in the year following the hunting actions (unpublished result). The second action took place in 2015–2016 (a 3-fold increase in hunting bag compared to the years before the ASF outbreak) after the first case of ASF was already observed in the BPF and continued through 2016–2017. The second hunting action might have had an unwanted effect on the spread of ASF in the area itself and outside, i.e., increased transmission and large movement of groups and individual wild boar (44, 50).

In the BPF, ungulates drive counts are annually performed (see the *Methods* section). In general, the trend based on drive count density estimates was similar to the camera trap estimates (**Figure 2B**). But for some specific years (2012, 2014, and 2015), there were clear differences illustrating the inaccuracy of the population index approach like drive count census to capture population changes for the following reasons. The timing of the drive count, taking place in February before wild boar reproduction peak, does not allow seeing potential positive feedback of management actions on population dynamics (such as discussed above). Furthermore, drive count census provides a snapshot of the population status at one particular day (the day of the census) in a part of the area (10% of the study area), thus inaccurately taking into account existing spatiotemporal patterns in wild boar presence in the

BPF [see (26)]. In comparison, camera trap surveys have been shown to be particularly efficient to monitor animal populations in various conditions (51). During the 2012–2017 survey period, we had a spatiotemporal coverage of the population of 0.07–0.50 camera km^{-2} deployed for a minimum of 3 months. The camera traps approach therefore provides a much more representative picture based on longer-term observations with a higher spatial resolution. We therefore argue that camera traps provide more reliable population size estimates, considering their higher spatial and temporal sampling resolution.

We are aware of some limitations of our study. Firstly, we assumed that only ASF and hunting influenced wild boar mortality. In the BNP, however, natural predators, lynx, and wolf are also present. The impact of these predators on wild boar is, however, moderate [predation from wolf and lynx has been estimated to account for 14% of mortality (24, 52)]. Since both wolf and lynx are not hunted in neither the managed nor the unmanaged area and both species occur in similar densities across the area (26), it is expected that predator-induced mortality rates are not largely different between the managed and unmanaged areas. Road casualties, another important cause of ungulate mortality, are not considered in our study. However, the road network in the BNP is very limited, and the number of casualties is negligible (25). Secondly, we used published parameters necessary for the computation of densities based on the REM. While daily range estimates come from the same study area, our density estimates would be improved if camera detection distance and angle parameters would be assessed specifically for our study.

Our study showed that the ASF outbreak led to a drop of 83.8 ± 25.5% and 94.8 ± 6.4% of the wild boar population in a non-hunted and a hunted area, respectively, within 1 year from the detection of the first ASF case. The observed wild boar decline was mostly due to ASF, and even a 3-fold increase in the hunting intensity during ASF outbreak had only minor additional effect (11–22%) on wild boar mortality in areas already affected by ASF. This fact has significant implications for management and disease control efforts. First, it appears reasonable to limit (or even ban) hunting activities in newly infected areas, at least during the first stages of epidemic, because the ASF virus appears to be more effective in reducing wild boar numbers, while intense hunting poses a high risk of virus spread, e.g., through fomites (53), disturbed animals (50), or hunters' movement (54). Effectiveness of such an approach is supported by its successful implementation in the Czech Republic and Belgium (55). Secondly, high ASF-induced mortality and subsequent abundance of infectious carcasses underline the critical importance of systematic carcass search and removal for effective disease control. This measure should help to reduce the viral load in the environment, enhance passive surveillance, and facilitate tracking of disease dynamics (56). To optimize resources use in ASF control, we suggest that hunting to reduce wild boar population size is reasonable only as a preemptive measure in anticipation of the disease and should be replaced by systematic carcass removal efforts once an epidemic breaks out.

While our results indicate that more than 80% of the wild boar population disappeared within 1 year of the ASF outbreak, one might wonder what happened with the remaining population. Do they get infected and recover, becoming carriers? The question has still no clear answer (57–59) but will need careful attention in post-infection areas (e.g., by means of hunted population surveillance) to ensure complete disease eradication. Another possibility is that the remaining population is made of individuals and/or groups of individuals that succeeded in avoiding the infection. In this case, we will need to know if there are specific traits favoring disease avoidance (e.g., age, sex, boldness)? These questions along with the relative impact of ASF on wild boar population structure and post-infection recovery will need careful attention in the coming time in order to improve our understanding of the ASF–wild boar system.

The drastic wild boar population decline observed in the BPF not only has important disease-management implications. It also has important implications in terms of ecosystem functioning, considering the fundamental roles played by wild boar (11–18). Pursuing monitoring of the population recovery along with forest dynamics will thus be of crucial importance in the coming years to better understand potential and so far unconsidered consequences of ASF on trophic cascades induced by wildlife diseases (60).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Our study was not subject to permission/authorization from an ethical commission, since we used a non-invasive method (camera trapping) which does not disturb the natural behavior of animal.

AUTHOR CONTRIBUTIONS

KM, MC, DK, JB, and TP conceived the analysis. MC, DK, and JB designed and implemented the camera trap survey and the data collection. KM performed data analysis and wrote the first manuscript draft. All authors discussed the results and contributed to the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00378/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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