# EPIDEMIOLOGICAL MODELS TO INFORM THE DESIGN AND EVALUATION OF OFFICIAL PLANS FOR RISK-BASED SURVEILLANCE OF FOREIGN HEMORRHAGIC FEVERS OF SWINE

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# Research Abstract

This project focused in two notifiable diseases to the World Organization for Animal Health (OIE), which are African swine fever (ASF) and classical swine fever (CSF), and how to calculate the risk of introduction, as well as measures for early detection in case of incursions in free areas.

This document is divided into chapters. Chapter 1 includes the introduction of the thesis, where the characteristic of the diseases of concern are presented, also improvements and novelties in diagnostic and surveillance for ASF and CSF.

Chapter 2 approached the risk assessment for introduction of ASF in Kazakhstan, with the objective of identifying high risk areas of possible incursions, in a country level perspective. We used Kazakhstan as a model because it is an ASF free country, surrounded by an infected neighborhood. To develop our model, we used conjoint analysis, which is a marketing tool for assessing and scoring clients preferences, linked to ordinal logistic regression to create a proxy-risk score for ranking the areas at higher risk of introduction of ASF in Kazakhstan, generating risk maps.

Chapter 3 continued with the same focus of risk prediction; however, it was for the introduction of CSF in Mato Grosso, a State in Brazil, which has borders with CSF non-free areas in Brazil, and extensive dry border with Bolivia, which also has unknown status for CSF with episodes of this disease some years ago. With that, the approach for risk prediction model was developed to be applied in a state level of surveillance, where we combined stochastic quantitative risk assessment for commercial pig farms, and the methodology developed for Kazakhstan to assess the risk of CSF introduction through

wild boars, which is related to backyard pig farms due to poor biosecurity. The results were displayed also in risk maps, but with more capillarity since we worked with states and their municipalities.

In chapter 4, we worked with a surveillance approach to aid the early detection of ASF or CSF at farm-level. To accomplish this project, we developed a scoring system for enhanced passive surveillance for ASF and CSF, where through this surveillance activity, farms would be able to trigger alerts to get pigs tested in an early detection, since the scoring was built in a weekly basis, which allows an objective and adaptable surveillance. This protocol was piloted in pig farms in the Dominican Republic and in the United States.

It is utmost to work with different perspectives of surveillance actions, where it is possible to have a macro vision of a disease-free area and being able to address particularities to the epidemiological model, increasing details, and being more specific in a dynamic and connected process. This Ph.D. project worked in different layers of surveillance and different data availability, being national, state, and farm-level, however with the same objective of calculating the risk of introduction ASF or CSF in free areas, and with that, we could deliver important material to subside Official Veterinary Services in actions related to early detection surveillance and disease control programs.

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# Abbreviations

ASF - African swine fever

ASFV – African swine fever virus

CSF – Classical swine fever

CSFV – Classical swine fever virus

TAD – Transboundary animal disease

RA – Risk analysis

FAO – Food and Agriculture for the United Nations

OIE – World Organization for Animal Health

INDEA – OVS from Mato Grosso/ Brazil - Instituto de Defesa Agropecuaria do Estado de Mato Grosso

OVS – Official Veterinary Service

DCP – Disease control program

MAPA – Ministry of Agriculture, Husbandry, and Food Supply of Brazil

USDA – United States Department of Agriculture

MaxEnt – Maximum Entropy

DNA – Desoxyribonucleic acid

RNA – Ribonucleic acid

qPCR – Real-time polymerase chain reaction

RT-PCR – Reverse transcription polymerase chain reaction

ELISA – Enzyme-linked immunosorbent assay

Lamp – Loop-mediated isothermal amplification

# CHAPTER 1 - Introduction

African swine fever (ASF) and classical swine fever (CSF) viruses are hemorrhagic fever disease viruses that infect only members of the S*uidae* family. Countries affected by these diseases experience far-reaching economic losses with massive decreases in the herd size leading to a disruption of protein sources. Consequently, there is typically an increase in the price of pork products, generating a negative impact on animal and human health. Both diseases are notifiable to the World Organization for Animal Health (OIE) and share clinical signs; therefore, diagnostic tests are required to identify and differentiate the pathogens [1].

Because of the magnitude of losses, countries that are free from these diseases, in total or part of their territories, should constantly pursue methods or surveillance approaches for measuring the risk of introduction, and to minimize the time before the first introduction and first detection of an outbreak occurs.

Animal health surveillance provides evidence to protect animal health, facilitate trade, and also protect public health [2]. Surveillance is important for supporting disease control activities by detecting cases and delimitating the extent of disease outbreaks. It is expected that any incursion will eventually be detected; however, time to first detection is the critical factor influencing the fate of an epidemic. Failure to rapidly detect the occurrence of a new or exotic disease could result in extensive disease spread before detection [3].

Prior to an outbreak (i.e., when a country is free from a disease), the objective of surveillance activities is to detect potential incursions as quickly as possible. Surveillance

sensitivity is defined as the probability of detecting an outbreak of a disease of concern, given that an outbreak of this disease has occurred. In this scenario, an emphasis should be placed on the temporal sensitivity of surveillance systems, which refers to the probability  disease given that an outbreak has occurred within a specified time frame [4]. Early detection also is a function of the temporal sensitivity of the system and its ability to accurately identify an agent at any given time in a population [5]. The ability to detect infectious pathogens or other causative agents early, and take steps to prevent their spread, is an essential first step in animal health surveillance [6].

The goal of early detection systems is to detect a disease incursion before it has spread beyond a focal area outbreak. It is very important to detect infected farms as early as possible after ASF or CSF virus introduction to avoid further spread, minimizing the losses to the pig sector, and reducing governmental costs associated with outbreak eradication [7].

The incursion of transboundary animal disease (TAD) in a free country or region is a big concern for a disease control program (DCP) from an Official Veterinary Service (OVS) at the state or federal level. OVSs tailor many actions, first, to avoid the introduction, second, to early detect any incursion of this type of disease as fast as possible, and third, to mitigate the outbreak with as less of an impact as possible, in case of failure in the two previous actions.

Specific objectives and indicators aiming for the success of a DCP, like availability of technical tools, control measures, and socioeconomic factors, should be established and improved by the OVS [8]. These indicators and objectives might consider risk factors and risk-based surveillance for a better monitoring and control of diseases.

Animal health risk analysis (RA) is an evolving tool that can be performed in qualitative or quantitative ways, and that is usually applied to inform decisions related to trade of animal, animal products, feedstuff, or genetic material. It is used to facilitate bilateral relations between countries guaranteeing safe trade at acceptable levels of risk. There is not a single format or method to perform RA, and this is an advantage of this tool because study of this nature can be performed with different degrees of data availability without compromising the credibility of results [9].

According to the OIE, the of risk is defined as the likelihood of the occurrence and the magnitude of the biological and economic consequences of an adverse event or effect to animal or human health [10].

The goal of risk-based surveillance approaches is to look or search for disease where it is most likely to be present. It is common that different group of animals (different populations) may show different levels of risk to be infected by a specific disease in association with the presence or absence of certain factors that may prevent or promote disease. Hence, surveillance activities might be most effective if efforts are concentrated to those specific populations that are at a highest risk. Clearly, examining the high-risk groups, there is a greater chance of finding the disease (if it is present) than by examining animals that are at lower risk compared to others [11].

## 1.1 – Diseases characterization studied in this project

### 1.1.1 - African swine fever - Etiology, hosts, transmission, clinical signs, and pathogenesis

African Swine Fever (ASF) is caused by a large, icosahedral, double-stranded DNA enveloped arbovirus of 170–190 kbp that contains between 151 and 167 open reading frames (ORFs). African Swine Fever Virus (ASFV) is the sole member of the family Asfarviridae. ASFV has 24 different genotypes described so far, and outside the African continent only genotypes I and II have been found [1], [12], [13]. The main target cell for ASFV is the macrophage, however, ASFV may also replicate in hepatocytes, renal tubular epithelial cells, neutrophils, and endothelial cells. ASFV can also replicate in soft ticks from the genus *Ornithodoros*, including *O. moubata* in Africa, and *O. erraticus* in the Iberian Peninsula [14]. In Europe, the observation of seasonal peaks of ASF cases in pig farms during summer months was attributed to mechanical vectors such as *Stomoxys* or *Tabanidae*, however, studies to confirm this role are necessary [15].

Transmission of ASFV may take place through three alternative cycles (sylvatic, tick–pig, and domestic), involving soft *Ornithodoros* spp. ticks, wild African pigs (mainly warthogs), domestic pigs, and pig-derived products such as pork. Recently, a wild boar cycle was included in the ASF transmission pathway, where wild boar habitat and carcasses play a role in the maintenance of ASF in Europe and Asia through environmental contamination with a resistant ASFV [16], [17]. In some countries, feral swine play a role similar to that played by the European wild boar free ranging, contributing to transmission and maintenance of the disease and its endemicity.

Although ASF is associated with high lethality (most infected animals die), it may not be as infectious as some other relevant transboundary animal diseases (TADs) such as foot-and-mouth disease. ASF usually spreads slowly within a herd, and some animals may not be clinically affected [18]–[20]. The incubation period has a range of 4 to 19 days in natural infections [13]. ASFV is shed in saliva, tears, nasal secretions, urine, feces, secretions from the genital tract, and mostly in blood, where a large amounts of virus is found [18].

ASFV is quite resistant, it can be viable at pH levels between 4 and 13. Undercooked and salted pork, as well as blood, and pig carcasses can be infective if fed to pigs (swill) or discarded in dumpsters sites where pigs or wild boar may feed. ASFV is inactivated after 30 minutes in a cooking process under 70°C. It may remain infective for at least 11 days in feces, for 15 weeks in chilled meat, for months in bone marrow or cured hams and sausages, and at least 1000 days in frozen meat [1], [18].

Clinical signs of ASF are highly variable and depend on the virulence of the strain, along with the age and immune status of hosts. ASF causes not only peracute and acute diseases resembling hemorrhagic fever, but also chronic and subclinical infections. In naïve pig population, with moderate virulent strains, the acute form can be present, with pigs showing high fever, lethargy, anorexia, and inactivity. Affected animals tend to huddle. Cyanosis in ears, snout, limbs, abdomen, tail, and perianal areas may be seen, as well as respiratory distress, with pulmonary edema. Petechial hemorrhages or ecchymosis in the skin, vomiting and diarrhea, and abortion in pregnant sows due to the high fever may also occur [1], [12], [14]. Low virulent strains are often related to subclinical and chronic courses, with unspecific symptoms and low mortality [12], [21].

In the necropsy findings, the most characteristic lesion of acute ASF is hemorrhagic splenomegaly, with enlarged spleen, dark in color and friable at sectioning, occupying a large space within the abdominal cavity. A multifocal hemorrhagic lymphadenitis is also commonly seen, with particular attention to the gastrohepatic lymph node, which can show signs of hemorrhage too. Petechial hemorrhages are often observed in the kidney, bladder and stomach wall [12], [14], [21].

### 1.1.2 - Classical swine fever - Etiology, hosts, transmission, clinical signs, and pathogenesis

The causative agent of Classical Swine Fever (CSF) is a small (approximately 12.3 kb), enveloped single-stranded RNA virus of the genus pestivirus, within the Flaviviridae family, referred to as CSF virus (CSFV). The genome is closely related to bovine viral diarrhea virus (BVDV) and border disease virus of sheep (BDV), which are also able to infect pigs causing cross-reaction with herd diagnostic tests like enzyme-linked immunosorbent assay (ELISA) [22]–[25].

CSFV strains can be divided into three genotypes, with three to four sub-genotypes. A link between genotype and geographic origin have been demonstrated, where group 1 isolates are present in South America and Russia; most viruses belonging to group 2 were isolated from outbreaks in Western, Central, or Eastern Europe and some Asian countries. More recently, group 2 was reported in Colombia, South America. Group 3 viruses are apparently confined to Asia. CSFV replicates in monocyte/ macrophage cells and vascular endothelial cells [23], [26].

Wild and domestic pigs are the only natural reservoirs of CSFV. In general, CSF signs are similar to those described for ASF, and other diseases like porcine reproductive and respiratory syndrome (PRRS), post weaning dermatitis, nephropathy syndrome (PDNS), salmonella, or coumarin poisoning [23], [26]. Neither arthropod nor rodents or birds are reliably related to serve as vectors for CSFV, however, airborne transmission was demonstrated experimentally for short distances (~ 1km) [1], [26].

CSFV infection may result in three different forms of clinical presentation, referred to as acute, chronic or sub-clinical, and congenital infection (also known as late onset). Clinical manifestation of the disease is related to the virulence of strains, immune status of the herd, and age of the pigs. In the most severe form, the mortality rate can be as high as 100%, although recovery may occur [23], [24], [27]. Young pigs suffer more from CSF, with losses averaging 80%. In older pigs the milder or sub-clinical symptoms can delay diagnosis, or it may even go unnoticed. However, in naïve populations, the mortality tends to be high [25], [28].

The incubation period ranges between 4 to 7 days, and, although no frequent, clinical signs may start as late as 10 days post. The acute form of CSF manifests initially with anorexia, lethargy, conjunctivitis, fever, respiratory signs, constipation followed by diarrhea. The acute lethal form can be accompanied by severe cutaneous hemorrhage or cyanosis, and neurological signs. In the chronic form, the same clinical signs are observed, although pigs survive for 2 to 3 months before dying [1], [23], [26]. In general, CSF infected sows show mild clinical signs, when the infection occurs between 50 and 70 days of gestation, an immunotolerance phenomenon might be induced and persistently

infected offspring are born. These piglets will be shedding virus for 2 to 3 months, when they probably die [23].

Under natural circumstances, the primary routes of transmission are oral and oronasal by direct or indirect contact with infected wild or domestic pigs. Secretions, excretions, blood, and semen are the main sources of CSFV infection. The ingestion of contaminated foodstuffs (swill) is one of the sources of contamination, especially in backyard pig farms, due to poor biosecurity [26], [29].

Although CSFV is an enveloped virus, it survives for prolonged periods under cool, moist, protein-rich conditions. In liquid manure, CSFV can survive for 2 weeks at 20°C, and more than 6 weeks at 4°C. Survives three days at 50°C and from 7 to 15 days at 37°C. Survives in meat during salt curing and smoking for 17 to more than 180 days depending on the process used. Virus persists 3 to 4 days in decomposing organs and 15 days in decomposing blood and bone marrow. CSFV is relatively stable over pH ranging from 5 to 10. The rate of inactivation under pH 5 is dependent on the temperature [26], [29].

At the necropsy, it can be seen enlarged or hemorrhagic lymph nodes or tonsils, which can be necrotic. Ecchymoses in the skin, hemorrhages and petechiae on lungs, kidneys, intestines, and urinary bladder, lymph nodes, larynx, also at the ileocecal junction are often described. Splenic infarctions can occur, and are considered characteristic for CSF, however this condition is not always present. In chronic forms, button ulcers in the cecum or large intestine may be present [23], [26].

## 1.2 - Similarities between African and classical swine fevers

Because of the similarities in ASF and CSF clinical signs, it was expected that epidemiology and disease spread would be similar. However, the recent spread of ASF epidemics through the Baltic EU Member States and Poland showed that the ASF dynamics did not follow the pattern for CSF [1].

A major problem of ASF and CSF is the absence of pathognomonic clinical signs, resulting in a high-risk period of several weeks of spread before an outbreak is detected [30]. In the case of moderate or low virulence strains of these swine fever viruses, not all the animals on a premise become infected at the same time and therefore windows of detection for the herd could be significantly longer than reported from experimental studies, making this situation even more challenging [31]. Table 1.1 shows a brief comparison between ASFV and CSFV.

## 1.3 - Current Situation of ASF and CSF

The recent spread of ASF through Asia and Europe with continuous cases of ASF in wild boars and domestic pigs reflect the lack of success of control programs and the potential threat for the worldwide swine industry due to the absence of an effective vaccine for the disease [32]. Since the re-introduction of ASF in Europe through Georgia in 2007, this disease has been a challenge not only for the European continent, but also for the entire world with the slow but systematic spread. This situation became more severe after August 2018, when ASF also reached China, and is now spreading in several Asian

countries. The latest affected countries were Papua New Guinea, India and Germany in 2020 [12], and in 2021, ASF arrived in the American continent, with outbreaks in the Dominican Republic in July, and Haiti in September of the same year [33]. In 2022, ASF appeared in wild boars in Italy, outside from Sardinia and caused by genotype II, the same genotype circling in Asia and Europe [34].

Outbreaks of CSF have been reported in the last decades in Asia (Bhutan, Cambodia, China, India, Indonesia, the Republic of Korea, Lao PDR, Mongolia, Myanmar, Nepal, the Philippines, Thailand, Timor-Leste, and Vietnam), Europe (Latvia, Lithuania, the Russian Federation, Serbia, and Ukraine), Africa (Madagascar), the Caribbean (the Dominican Republic, Guatemala, and Haiti), and Latin America (Bolivia, Brazil, Colombia, Ecuador, and Peru) [35], [36]. Generally, endemicity is driven by moderate or low virulence strains, and like ASF, wild boar or feral swine can play an important role in the establishment and chronic infection with CSFV [23], [25], [37]. In September of 2018, after 26 years without any notification, Japan had an outbreak of CSF, suggesting that the reintroduction of the virus was from outside Japan [38], [39].

Due to the increase in animal movement worldwide, and tourism, the risk of introduction of TAD in free areas is high, especially through infected pigs, contaminated pork products, or fomites.

## 1.4 – Economic impact related to ASF and CSF outbreaks

The U.S. was the first country to have CSF, appearing in the 1830s in Ohio [22]. The disease was eradicated from the US in 1978, after 16 years of eradication actions.

Eradication costed approximately $140 million US dollars (equivalent to $640 million at the U.S. dollar value in 2020) [32], [40], [41]. The CSF outbreak eradication in 1997/98 in the Netherlands had a total estimated cost of over US$ 2 billion [42].

Brazil has a CSF non-free zone, and in 2018, when some outbreaks in Brazil began to be detected again since 2009, the Confederation of Agriculture and Livestock of Brazil (CNA) estimated an impact of US$ 230 to US$ 790 million if the infection reached the CSF free zone [36].

ASF continues to spread in the Russian Federation, despite surveillance and control measures, and the economic consequences were estimated to be 0.8–1 billion US dollars until October 2009 [43]. The ASF outbreak in Cuba, after the introduction of the disease in 1980, led to a total cost, including the eradication program, of US $9.4 million. In Spain, the final 5 years of the eradication program alone were estimated to have cost US $92 million [44].

In a study, projecting a hypothetical ASF outbreak in the US, revenue losses would be up to US $15 billion in a 2-year scenario, and around US $50 billion in a 10-year scenario [45].

Control and eradication are costly not only for the Official Veterinary Service (OVS), but also for the producers and industry since the outbreaks of TADs promotes a business break. When a country is free of these diseases and has a valuable pig and pork production system, it is economically essential to maintain the status of freedom from these diseases.

## 1.5 – Diagnostic tests for detection of ASF and CSF

Many techniques are available and approved by the OIE and relevant organizations to confirm the suspected cases. Currently, the OIE-recommended tests for virus detection include virus isolation, fluorescent antibody tests (FAT), and both real-time and conventional polymerase chain reaction (PCR) assays [46]. Once a suspect case is identified, the time to confirm cases using these methods is up to 10 days, with the ability of PCR test results in less than 24 hours. Thus, reducing the time between the first introduction of a disease and the first suspected case provides the greatest opportunity for surveillance to impact the size of an outbreak.

There are diagnostic techniques established as a gold standard for detection ASFV and CSFV, the PCR is being used to confirm the first infection detected in a free area, it is a fast and reliable test for detecting these diseases and it is recognized and accepted by the OIE [47], [48]. Although PCR (conventional or real-time) is a highly sensitive method for the detection of ASFV and CSFV, its application relies upon the use of a thermocycler, making it an expensive technique that requires specialized implementation in a laboratory environment [49].

Certain modifications to PCR protocols have been evaluated to improve the system. For example, the use of lyophilized powder reagents (LPR) can decrease the time of the assay by 2 hours versus the regular protocol for ASFV detection in blood samples, by using less reagents. This is an example which could save time and costs during an outbreak investigation. The qPCR-LPR showed high sensitivity (92.61%) and specificity (90.48%) compared to the qPCR recommended by the OIE and conventional PCR. The analytical

sensitivity of the qPCR-LPR assay was 100 copies/µl, the analytical sensitivity of qPCR recommended by OIE was 1,000 copies/µl, and the analytical sensitivity of conventional PCR assay was 106 copies/µl [50]. Improvements to well-established techniques is one way of achieving balance between sensitivity and costs of tests. It creates a more efficient workflow in the laboratory, using less reagents, making this routine more economical. Because ASF and CSF cannot be differentiated clinically and may also be confused with other diseases like PRRS, Salmonellosis, and porcine dermatitis and nephropathy syndrome (PDNS) associated with PCV-2, there is a need to also consider the application of differential diagnostics. The use of multiplex PCRs should be encouraged in Veterinary Diagnostic Laboratories (VDLs) routinely, where required tests are generally for commonly seen swine production diseases. A multiplex PCR would allow earlier detection of TADs if applied to submissions for endemic disease with similar clinical presentations. A multiplex reverse transcription – PCRs (RT-qPCR) for simultaneous detection of antigens of swine production diseases (PRRS, PCV-2) and TADs (FMD, ASF, CSF, pseudorabies (Herpesvirus type 1)) is available for use [30], [51].

One study developed a multiplex reverse transcription real-time PCR (mRT-qPCR) for ASFV, FMDV, and CSFV to assess the feasibility of detecting these three pathogens in swine oral fluids (OF) collected from chewing ropes. During the study, ASFV was detected as early as 3 days post infection (dpi), 2 to 3 days before the onset of clinical signs; CSFV was detected at 5 dpi, coincident with onset of clinical disease; and FMDV was detected as early as 1 dpi, 1 day before the onset of clinical disease. In analytical sensitivity testing, the 4-plex RT-qPCR showed a minor reduction in CSFV and FMDV detection sensitivity as compared to the 2-plex RT-qPCR; however, regarding ASFV, no

loss in sensitivity was noticed. The use of the optimized OF nucleic acid purification and mRT-qPCR workflow allowed the detection of more positive samples than the USDA-NAHLN tests for ASF, CSF, and FMD, which can be assumed that the USDA-NAHLN tests were not optimized for use on OF samples, while this mRT-qPCR is optimized for OF [51]. Surveillance efficiency and utility can be increased by assays that simultaneously test for multiple agents [5]. Using multiplex PCRs routinely in VDLs can provide agility to differentiate common swine production diseases from notifiable diseases like ASF and CSF. Being able to rapidly differentiate notifiable TADs from common diseases would allow fast TAD response actions. However, any new or improved technique should rely on high sensitivity and specificity. The impacts of false positive and false negative samples can hamper the swine production of a country, and it will take time to have international credibility of buyers.

There are some discussions within the scientific community regarding the efficacy of cotton ropes for collecting OF for TAD surveillance. The reason for concern would be that sick animals may not be interested in chewing activity. Results from Grau et al. (2015) however, indicated that the time to genetic material detection for ASFV, CSFV and FMDV using OF and PCR is efficient and would be a good procedure for early detection in commercial farms routinely using this sampling procedure [51]. Although further evaluation of the costs of sampling and the sensitivity of this surveillance system is necessary, Beemer et al. (2019) assessed the costs of this type of surveillance and concluded that the best approach is not economic viable [52]. However, there is still an intermediate approach that assures both sensitivity and economic viability. This is discussed more later in the review.

The isothermal gene amplification-based assay has become an appealing alternative to PCR for molecular diagnosis of diseases because of the possibility of performing tests in low-equipped laboratories or in the field as a pen-side test. The recombinase polymerase amplification (RPA) is an isothermal DNA amplification technology, where the reactions are carried out at a constant temperature. This eliminates the need for an expensive thermocycler and can be completed in about 20 minutes. J. Wang et al. (2017) used the RPA for detection of VP72, the structural protein of ASFV DNA genome, with high analytical sensitivity, detecting 100 copies in only 10 minutes. This was the same detection limit found by these authors when they validated the test using real-time PCR. However, the RPA was tested with serum spiked with ASFV and not field samples. Therefore, further investigation with field samples is necessary [53].

Other methods using isothermal gene amplification, like loop-mediated isothermal amplification (LAMP) assays, have been developed yielding reliable sensitivity and also reduces the use of expensive laboratory equipment. The ASF outbreaks in Vietnam (2018) and Timor-Leste (2020) allowed the piloting of this new technology for detection ASFV [54], [55]. These pilot tests demonstrated that it is possible to detect ASFV using LAMP without extracting the DNA from samples as a first step, working directly on crude clinical samples. This allows diagnosis in an environment outside of a laboratory.

Overall, the results of assays using RPA are quite promising, showing to be a reliable technique for antigen detection. They also have the advantage of faster performance and are less complex than PCRs. Many pen-side tests use RPA, mostly LAMP, in their methods to produce a lab result in a shorter time and in the field.

The diagnostic tests that are being developed to identify ASFV and CSFV are not only using new technologies like RPA and LAMP to optimize the use of pen-side tests. They are also improving the well-established tests, like PCRs. The goal in an outbreak situation is to decrease the cost of testing and increase time efficiency, because sample numbers will increase and the wait time from sample taking to analysis will be crucial for releasing pigs from quarantine. Strategies for business continuity should be made, while keeping diagnostic tests effective so OVS can make fast and accurate decisions.

Enzyme-linked immunosorbent assay (ELISA) is a well-established and recognized test for antibody detection using primarily serum samples. The innovation in techniques for antibody detection for screening herds, described in the literature, is considering not only different sample matrices, but also improvements in diagnostic laboratory techniques. Techniques other than ELISAs are also described in the literature for antibody detection showing good sensitivity and time savings.

Bead-based multiplex assays (BBMAs) promote reduction of time, labor, and sample volume requirements because this technique allows antibody searches for different pathogens in a single process. This technology is widely applied in human health for strain identification in infections, immune response characterization (humoral and cellular), and biomarkers identification among other uses. However, in the veterinary field, there are only a few commercial kits available. Aira et al. (2019) tested this methodology to detect simultaneously ASF and CSF antibodies in serum, in a triplex assay, where the antigens proteins VP72 and VP30 of ASFV, and E2 of CSFV were used as the target. The study estimated sensitivity (97.3%) and specificity (98.3%) for detection of antibodies against ASFV and sensitivity (95.7%) and specificity (99.8%) for

CSFV antibody detection. Results indicated that pooling of 10 different sera was a good alternative because it allows the detection of antibodies to both pathogens in the same conditions. Although not measured specifically, previous studies described an increase in specificity of pooled sera and a decrease in sensitivity when changing from unique to pooled sample analysis [56]. A combination of pooling and joint assessment of ASF and CSF could optimize the use of resources.

The “Biochip array” is a protein biochip, and it is a novel application of the sandwich-type antibody-capture assays such as ELISA. The “Biochip array” allows screening of thousands of samples simultaneously. Using this method, a study was performed in China, with the objective of establishing a screening test to simultaneously detect four proteins, namely, the E2 protein of CSFV, VP2 of porcine parvovirus (PPV), domain III of the E protein of Japanese encephalitis virus (JEV), and the N protein of porcine reproductive and respiratory syndrome virus (PRRSV). Compared to the ELISA assay, the biochip assay required very low levels of proteins and showed great sensitivity and specificity. The coincidence rates of the positive samples were 95.8 to 100% and the coincidence rates of the negative samples were 86.2 to 100% [57]. The great advantage of this technique is the high capacity of test samples per time. In active surveillance this can improve the speed of testing, allowing more samples and animals to be tested at the same time as other technologies. This should result in faster disease detection.

The use of antibody detection as a screening is important for demonstrating the free status of a specific disease in a country or region. However, if the objective is early detection, antibody detection tests might not be suitable. This is due to the latency from infection to the presence of antibody levels high enough for detection. This can make it difficult to

determine precisely when infection occurred. To determine the approximate date of infection and identify all infected and carrier animals, OVS in free areas should consider performing diagnostics with parallel detection of antigen and antibodies for complete understanding of the epidemiological situation in the affected area [58]. The integrated surveillance plan for hemorrhagic fevers from USDA corroborates testing sick pigs to increase the sensitivity with targeted surveillance [28].

The concept of early detection should not only apply to detection before the onset of clinical signs. Because diseases like ASF and CSF can vary with acute and subclinical forms, early detection should be seen as the detection of the outbreak before it spreads, allowing fast containment.

### 1.5.1 - Portable PCRs/ Lateral Flow devices

The goal of fast diagnosis and detection of foreign animal disease (FAD) is what OVS always seeks. One problem with diagnostic testing is the logistics of sending samples to VDLs which are sometimes located far from farms. To meet this gap in diagnostics, investigators are trying to create portable and easy access to laboratory tests. However, regulations regarding the use of these assays for notifiable diseases should be given careful consideration by OVS, to retain control over test results until effective actions can be taken. Decentralization of laboratories may be a powerful tool for early detections of TADs, and speed, simplicity, and low cost are desirable assets.

Some assays are being developed skipping parts of protocols that are followed in conventional tests in laboratories. Thinking about how to proxy field surveillance with

laboratory diagnostic techniques is the key for timely detection of introduction of exotic diseases. Devices that are being developed for use in field conditions should be considered as a possible tool in active and passive surveillance activities.

Portable PCR devices allow for mobility to perform diagnostic tests and pen-side (PS) tests can give a test result using the "naked eye". Rapid serological and virological tests, such as PS tests, are easy to use under field conditions, and there have been promising results in the sensitivity of detection of antigens and antibodies against ASF and CSF.

A portable real-time PCR thermocycler T-COR4 (Tetracore, Inc., Rockville, MD, USA) was evaluated regarding the feasibility and reliability of a duplex real-time ASFV/CSFV PCR assay, as a field diagnostic tool for the detection and differentiation of CSF and ASF in clinical samples. Preliminary results indicated that the portable equipment has the capacity to serve as a new tool for field diagnosis. However, the performance was more moderate when testing clinical samples compared to reference materials or experimental samples. Therefore improvements and optimizations were necessary to improve the functionality of this device [59]. Lihong Liu et al. (2019) later developed a pen-side technique skipping the DNA extraction step from the process for ASFV molecular diagnostic, utilizing ambient temperature, and performed the test in a portable real-time PCR machine (battery-powered) T-COR8 (Tetracore, Inc., Rockville, MD, USA) to mimic field conditions. Under these conditions, the portable PCR T-COR8 protocol showed similar results when using the Universal Probe Library (UPL) probe as described in the recommended OIE assay, which was chosen as a reference in this study. The extracted samples showed 10/11 positives for the UPL assay and 11/11 for the assay using the T-COR8 protocol [60].

The previously mentioned LAMP assay is a diagnostic technique which is gaining attention due to its versatility and speed of diagnosis. There are different approaches to LAMP, and by blending LAMP with a lateral flow device (LFD) may be able to perform ASF and CSF diagnostic tests outside of a laboratory. In 2014, a LAMP assay coupled with LFD for the detection of CSFV was developed and tested, giving results in 2 to 5 minutes. The performance of this RT-LAMP-LFD assay was similar to real-time RT-PCR. Both assays were able to detect CSFV RNA at the same dilution levels of the nine samples belonging to six genotypes of CSFV tested in the study. No cross-reactivity to non-CSFV pestiviruses was observed, which shows a strength of this technique [61].

In 2016, a novel assay was developed for detection of viral antigen by an immunochromatographic test using a LFD, based on a monoclonal antibody (MAb) against VP72, the capsid protein of ASFV. This pen side test was developed to detect ASFV antigen with results read in 10 minutes after adding the sample (whole blood without anticoagulant) into the device. When testing both experimental and field samples from known infected pigs and selecting the UPL-PCR as the reference method, the sensitivity and specificity of LFD assay was 67.86% and 97.97%, respectively. However, compared to Antigen ELISA, this LFD assay showed almost perfect agreement (Kappa values of 0.92 [CI 95 % = 0.86–0,98]) [62]. This tool can assist local veterinary services (OVS from local veterinary units) when attending the first notification, where in many cases first evidence of the disease is based only on clinical symptoms.

The same moderate sensitivity was found in a study performed in Sardinia, Italy, where the use of two PS tests kits (INGENASA, Spain) was evaluated to establish the specificity of detecting ASFV from carcasses of wild boars. In this case the objective was

increasing the efficiency of passive surveillance by testing hunted carcasses of wild boar before releasing the negative carcasses for movement, the positives need posterior confirmation. The two PS tests were applied simultaneously to detect antibodies (Ab) and antigens (Ag) specific to ASFV to improve diagnosis under field conditions, using also LFD as a form of visualization of the results. The entire test procedure was completed in 10 min, which is highly efficient. Although it was not possible to calculate the sensitivity of ASFV Ag detection (due to the absence of positive cases in this study), antibody specificity was 97.5%, and antigen specificity was 98.1%, showing similar results for both PS tests. However, the sensitivity of Ab detection (66.7%) was considered moderate. When these tests were performed simultaneously (in parallel), the global specificity decreased to 95.5%, while global sensitivity remained 66.7%. The costs of keeping the surveillance how it is, currently implemented in Sardinia for wild boars, using PCR and ELISA, are at least 40% higher than the costs of using the PS tests for detection of both antigen and antibody against ASFV. If PS tests were used as a routine for wild boar surveillance, it would cost less than ELISA and PCR assays [63].

Lu et al. (2020) developed a test named “Cas-gold”, which is currently in a patent process in China for rapidly detecting ASFV with high specificity and sensitivity by integrating Cas12a based detection and a gold nanoparticle-based lateral flow strip. The authors of this study compared it to the qPCR method using samples taken from ASF-infected swine. The detection limit of the two assays were comparable, with positive animals being detected by Cas-gold and by the qPCR, and the same results for the negative animals. Blood, oral, and anal swabs of pigs were the matrices tested for virus detection, demonstrating a versatility of samples that can be used with the Cas-gold test [64].

The K205R gene is a conserved and specific gene of ASFV that appears in early infection from 4 h post-infection onward with high antigenicity, although there are few studies on this protein (ASFV K205R). These features have made it a focus of ASFV research, which makes it a powerful and available indicator for rapid detection of ASFV [65], [66]. Because this protein shows good antigenicity in the early, middle and late diagnosis of ASFV infection, an RPA-LFD was developed for detecting this protein (K205R) from kitchen waste, swill samples, environment samples from meat stalls at farmers markets, and pork product samples collected in China. The test may be completed in 15 min, without cross-reaction to other viral genomes. The samples were heated to 70°C for 30 min to inactivate ASFV, and then the DNA was extracted. All samples were first analyzed by OIE recommended techniques, including traditional PCR and real-time PCR (qPCR), before being tested by RPA-LFD. The same analytical sensitivity (100 copies per reaction) was found with the new test (RPA-LFD) and the real-time PCR recommended by OIE as reference. The RPA-LFD was quite specific, and the target viral protein (K205R) of this test can be a good target, since this viral protein is present hours after pigs get infected by ASFV. For this reason RPA-LFD may be a future tool for detection ASF from pork products environmental matrices, and for testing feed supplies before entering feed mills or farms, allowing an increase in the biosecurity actions [67].

Zuo et al. (2020) also developed a LAMP combined with LFD to detect ASFV, however the target was the ASFV B646L gene, which encodes the VP72 protein (capsid protein). PCR assays, including conventional PCR, qPCR and nested PCR (nPCR), and LAMP monitored by electrophoresis targeting the same conservative region of B646L gene were used as validation of the novel pen-side test. DNAs from 52 clinical samples, collected in

a province of China, were used for testing the utility of the assays. LAMP and LAMP-LFD showed the highest positive rate (16/52), and the positive ratios in OIE-PCR, PCR, nPCR, and qPCR were 13/52, 13/52, 14/52 and 15/52, respectively [68].

There is an ongoing project, started in 2017 where a portable device is being developed to diagnose production diseases of swine (PRRS, PCV2, SIV, PPV (porcine parvovirus)), as well as notifiable diseases (ASF, CSF). This technology depends on antigen recognition by antibodies directed against selected swine viruses. This portable device will have the oral fluid as the main matrix, however, samples from blood serum, feces, and nasal swabs will be suitable for diagnosis using this device [69].

In the future, the combination use of the antibody PS tests and the antigen PS tests could be a useful tool during outbreaks as well as during regular surveillance activities [70]. Cases of ASFV infection with a moderate or low virulent strain usually induces high or medium viremia levels at the beginning of the infection, and high antibody titers from the second week post infection. In these cases, both virus and antibody tests must be used to obtain a reliable diagnosis [62].

With the early detection of ASF and CSF as the main goal of a surveillance system in free areas, diagnostic tests that provide fast detection of positive pigs in the field should be discussed between OVS and stakeholders. Defining a better approach to establish the use of these tests, once the portability of these assays may allow measures at farm level of containment of spread of diseases while the confirmatory sample is processed in a reference laboratory. Table 1.2 brings a summary for the diagnostic techniques approached in this section.

## 1.6 - Precision Livestock Farming – use of non-invasive tools in real-time to aid animal health surveillance

The use of computers programs, cameras, environmental thermometers, software, and other real-time measurement devices on farms is getting attention because these tools may detect changes in animal behavior that may be associated with disease. Systems using cameras, microphones, and sensors to enhance the farmers' eyes, ears and nose in everyday farming have been developed to monitor animal welfare and production. These technologies for remote monitoring of livestock, termed Precision Livestock Farming (PLF), provide the capability to automatically track individual livestock in real time generating data for evaluation of welfare, productivity as well as behavior and healthy conditions [71], [72] are now being assessed for animal health surveillance purposes.

According to Henry Berger, the Global Head of Strategic Partnerships and Pipeline Innovation – Integrated Health Management GSM Swine, Animal Health, the definition of PLF "is the continuous, automated, real-time monitoring of animals to maximize the individual contribution to the benefit of the whole herd's productivity, health, management and welfare" [73]. PLF is getting attention in the swine production in the US. Currently on-farm, pig production systems rely on human caretakers to observe animals daily to detect feed, water, and air issues, and to identify compromised animals.

The success of a PLF system depends on being relatively inexpensive to implement and execute, simplicity for users to operate on the farm, and on delivering meaningful data that allows evaluation and analysis [74]. The largest challenge for this technology are the structures of farms and connectivity of rural areas [73]. The economic factor is also a challenge because producers will only embrace this idea if the technology has a viable

cost. A US$ 1 million USDA-NIFA Agriculture and Food Research Initiative grant to study the advancement of precision farming in the U.S. swine industry was awarded to a group of researchers from Michigan State University, which will work in collaboration with North Carolina State and Iowa State. This project began in June 2021, and the group is exploring precision livestock needs, public perceptions and the willingness of farmers, producers and consumers to pay for new technology [75].

Studies using PLF and monitoring symptoms for infectious diseases are being developed, mostly in Europe. Siewert et al. (2014) used infrared cameras (IR) to detect fever in pigs, with the objective of early detection of infections, in a non-invasive method of surveillance [76]. The framework of the European Union research project Rapidia Field named Real-Time Monitoring System Online (RTMS-ON) was tested by Martínez-Avilés et al. (2017) having ASF as a model disease. Here differences in the movement of pigs could be noticed starting at 3 to 4 dpi [77]. Fernadéz-Carrión et al. (2017) also tested the use of video monitoring for early detection of diseases, using a low virulent strain of ASFV as a model. At 4 days post infection with ASFV, a decrease in movement could be seen, and 3 days before the onset of other clinical signs [78]. This technology can be efficient for monitoring disruptions in behavior, production values, and or other health standards before severe clinical signs or death of the animal, which can take up to 23 days. Relying on death only would be a delay in detection of ASF in a herd. Remote monitoring systems could be used as a first layer of traditional testing to enhance current surveillance systems by providing an objective, non-invasive way to measure multiple non-specific indicators of infection (i.e., increased body temperature, reduced movement).

PLF is not invasive, and is considered a low-cost individual animal sensing tool, with high fidelity activity recognition. There are three main capability needs for visual precision tools; they must achieve individual pig recognition (often done by the use of ear tag); the capability to distinguish between the behavior of the identified pig and pen mates, and remotely sensed alterations in pig behavior must be linked to health state (disease and/or growth) [79].

Although PLF may sound like a distant solution because of cost or lack of internet connectivity, this format of surveillance, using devices like cameras, infra-red thermometers, etc., is gaining attention and the technology is becoming less expensive. The USDA created a program called “Rural Development Broadband ReConnect Program” which has the goal of funding the costs of construction, improvement, or acquisition of facilities and equipment needed to provide internet service (broadband service) in eligible rural areas [80].

The quality of information generated by PLF can be useful to farms, helping producers collect information in an organized way using on farm computers and software for data generation and analysis. Considering the surveillance actions focused on early detection of TAD, PLF may be a great support for monitoring herds and detecting any alteration of health status. Because this technology relies on machines, we can expect 24/7 surveillance in place, allowing producers to fast notifying any suspicious cases to OVS. Thinking of a chain reaction, it would allow a faster response at the farm level in case of triggers or indicators are started. Another positive factor this technology brings is the decrease of stress in pigs regarding screening for any adverse effect, since the animals are remotely monitored.

## 1.7 - Sample matrices, sampling methods and approaches to increase surveillance sensitivity

Samples traditionally collected for ASF and CSF suspicion or surveillance include blood, serum, and tissues such as spleen, tonsils, lymph nodes. There are many interesting approaches being studied to improve cost-effectiveness or reduce the risk of disease dissemination when conducting disease surveillance in domestic pigs and wild boars. Some of these also have the potential to increase the chances of detecting disease sooner.

### 1.7.1 - Non-invasive samples

Non-invasive sampling methods are getting attention in the scientific community because they may facilitate surveillance, promote animal welfare, and avoiding spread of diseases caused by spillover of contaminated blood at the time of sampling. Use of techniques that allow performing surveillance with good sensitivity and low levels of animal disturbance should be encouraged.

The use of feces as a herd-level test would promote an easier way to collect samples in pig farms, since collecting blood from pigs can be time-consuming, and is not an easy task requiring trained people. Nieto-Pelegrín et al. (2015) detected antibodies against ASFV in feces starting at the same time as in serum, at 9 days post infection for experimentally infected pigs with low to moderately virulent ASFV strains. Feces kept at room temperature (23°C or 37°C) showed low titers of antibodies, compared to feces stored at -20°C. Therefore, lack of care in sample preservation can alter the diagnostic results [81].

Carvalho Ferreira et al. (2014) tested the detection of ASFV using feces as a sample matrix, where DNA virus could be detected in feces starting at 4 days post infection [82]. With that, the use of feces for monitoring the introduction of ASF in pig farms could be an option as a screening test instead of using blood/ serum. The best use of this would be in high genetic and economic value farms, where the less animal stresses, the better, avoiding any loss.

Systematic herd screening tests can be done as a complementary action for early detections, if fecal samples were used as an indicator of the presence of the virus in the herd (genetic material or antibodies) it would be well accepted by producers. Also, the OVS would get the samples in an easy and faster way than bleeding animals, which would be less work with similar sensitivity compared to conventional samples.

Oral fluids (OF) have been used for detection of swine production diseases for many years, and this approach is getting attention for use in surveillance of TADs, such as ASF and CSF, because of many advantages, including affordability and simplicity.

In OF samples, ASFV antibodies were first detected at 11 dpi [83] while the average onset of ASF clinical signs varies from 4 to 19 days in field conditions [18]. Giménez-Lirola et al. (2016) developed an ASFV dual-matrix serum/oral fluid indirect ELISA capable to detect antibodies against the target protein VP30 in either serum or oral fluid specimens. OF antibodies were detected by that assay as early as 8 dpi, which is equivalent to the performance reported for the OIE indirect serum antibody ELISA. Overall, the results showed that the VP30 indirect ELISA detects ASFV antibodies at early stages post-exposure in either OF or serum samples [84]. This ELISA may be

highly useful for ASF surveillance using both matrices, OF and/or serum, depending on samples availability.

With the same goal of using OF and/or serum, Panyasing et al. (2018) developed an indirect ELISA to detect antibodies against classical swine fever virus (CSFV) E2 and Erns proteins in OF and serum antibody in pigs inoculated with a CSFV moderately virulent field strain or with modified live virus vaccine strain, the serum was used as validation for the OF detection. The results showed that OF can be used for detection of E2 and Erns antibodies with good sensitivity and specificity (above 95% for both). Detection of CSFV E2 and Erns antibodies in OF was consistent with serum antibody testing, starting detectable after 10 dpi [85], however the clinical signs of CSF generally start around 4 to 7 days [23]. Therefore, this ELISA would be detecting positive cases as the same time as pigs with clinical signs. In terms of seeking for a technique that would be able to detect sick pigs before the onset, this would not be the most appropriate choice.

In 2017, a Canadian/American team reported a final project with multiple studies with the intention of using OF for detection of TADs, like FMD, ASF, CSF. The authors infected pigs with FMD, ASF and, CSF to see if, and when, antigens and antibodies would be detected in OF, comparing to oral and nasal swabs. They utilized quantitative real time PCR (qPCR) for ASFV, and quantitative transcriptase reverse real time PCR (rRT-PCR) for FMDV and CSFV. For antibody detection commercial and in-house ELISAs were used. As a result, CSFV genome was detected in OF at 10 to 14 days after the animals were inoculated with virus, likely after the onset of clinical signs. CSFV antigen was detected in sera earlier than in OF, starting at day 6 to 7 after inoculation of the pigs. Antibodies against CSFV were detected in OF starting at 14 to 21 dpi. For the ASFV

Malta ’78 strain, the genome was detected in OF starting at 6 dpi to 21 dpi. Detection of ASFV in oral and nasal swabs mirrored detection in OF. However, whole blood was the best sample type for ASFV detection, becoming positive at 4 dpi. Positive antibody response to ASFV in OF was only detected at 10 dpi. FMDV genome was detected in OF as early as one day after the animals were either injected with virus or exposed to infected animals and 21 days later FMDV genome could still be detected in OF when tested by a rRT-PCR. In addition, FMDV-specific IgA was detected in OF using an isotype-specific indirect ELISA starting at 14 dpi. Contrary to the vesicular disease agent (FMDV), the hemorrhagic disease agents (CSFV and ASFV) detection in OF was delayed and weaker in some cases compared to serum/blood [86].

While the described studies have shown that OF may be used for detection of swine hemorrhagic fevers, many results indicate that detection via OF might not occur until after the typical onset of clinical signs. However, as described in section 1.5, a multiplex reverse transcription real-time PCR (mRT-qPCR) for ASFV, FMDV, and CSFV using OF collected from chewing ropes, in experimental conditions, started detecting ASFV (antigen) as early as 3 dpi, 2 to 3 days before onset of clinical signs; CSFV (antigen) was detected at 5 dpi, coincident with onset of clinical disease; and FMDV (antigen) was detected as early as 1 dpi, 1 day before the onset of clinical disease [51].

Dietze et al. (2017) tested the use of ropes in a bait (pSWAB) for CSFV detection in domestic pigs to see the applicability of these pSWAB mostly in backyard pigs or scavengers pig farms, where the sampling of individual animal is hard to perform. CSFV nucleic acid was detectable in the blood starting from 2 dpi and was evident in all animals on 5 dpi, which is the average onset of clinical signs. The positivity detection of OF

started to be consistent after day 9 post infection, and the oropharyngeal swabs vary in the positive results between the animals, however, the earliest positive results for oropharyngeal swabs were 7 dpi [87]. Therefore, due to different results for different studies using OF and CSFV, improvement in detections assays is necessary for this type of matrix.

In the US, researchers from USDA/APHIS assessed the applicability of rRT-PCR diagnostics for detect ASF, CSF and FMD from OF. They considered not only the test accuracy, but also economic arguments for surveillance. In scenarios evaluating outbreak situations, OF sample testing showed advantages comparing to individual animal sampling, like reduction of number of samples required, decreased disruption of farm activity, and trained producers can submit the sample without the official veterinarians entering the farm. All these advantages would reduce testing costs. While testing every 3 days showed the best sensitivity for detection, this was the highest cost surveillance assumption in the study. Weekly surveillance was less effective than the sampling every 3 days, however it costs less 89% than testing every 3 days and is still better than passive surveillance. A common benefit to these scenarios is that OF sample testing for surveillance could contribute to emergency preparedness and response efforts directed toward ASF, CSF, or FMD in swine [52]. Studies for increasing the diagnostic sensitivity of OF assays for pathogens should be encouraged.

### 1.7.2 - Tissue types

For ASF and CSF detection, there are well-established types of samples to be submitted for diagnosis in the case of sick or dead pigs. In this sub-section, studies are reviewed evaluating the performance of detection these diseases in tissue samples. To see what a good approach would be, these studies discussed both ideal sampling situations and when the samples are found degraded in the field, with the purpose of increase the sensitivity of detection testing all samples that could be available, regardless their condition.

A recent study comparing different matrices and ASFV, detected lower amount of viral load in tonsils than in spleen or EDTA-blood, but there wasn't statistical difference between samples nor if they were from wild boar or domestic pig [88]. These results were in accordance with de Carvalho Ferreira et al. (2014), where the ASFV titers were also significantly higher in the spleen, compared to retropharyngeal lymph node, tonsil, and liver [82]. Therefore, due to some variation in detection levels found in published studies, sampling only tonsils may not be the best choice for surveillance of hemorrhagic swine fevers (ASF/CSF) and other tissues, such as spleen or lung should also be included.

A study with high, moderate, and low strains of CSFV performed by USDA in Plum Island Facility concluded that viral RNA was better detected by real-time reverse transcriptase PCR (rRT-PCR) in blood, tonsil tissue, and tonsil scrapings than virus isolation. Nasal swabs proved to be an inadequate sample. Both blood and tonsil scrapings proved to be reliable samples by rRT-PCR during the early stages of infection. Depending upon sample type, CSFV was detected between days 3 to 8 dpi for CSFV high virulent strain infected pigs before dying, 3 to 28 dpi for CSFV moderate virulent strain infected pigs, and 5 to 70 dpi for CSFV low virulent strain infected pigs. Regardless of

the virulence, rRT-PCR showed great sensitivity detecting close to 100% of positive samples from experimentally infected pigs starting at 3 dpi. Virus isolation technique was not as sensitive for early days post infection, with detection of 80% for samples from infected pigs [31]. This corroborates with current protocols for CSF surveillance in the US, which rely mainly on PCR tests for testing sick pigs, with the tonsils and blood as the sample types recommended for CSF and ASF diagnosis [28].

The quality of samples is barrier for ASF and CSF detection in the field, regarding surveillance in wild boar or feral swine. Sometimes an animal is found dead, in various states of decay, and the quality of diagnostic samples like blood and tissue is poor. For testing the viability of CSFV in samples after tissue degradation, mimicking wild boar found dead in the field, Weesendorp et al. (2010) studied the possibility of detecting CSFV virus from carcasses in decomposition. Tissue samples (tonsil, mesenteric lymph node, spleen, and kidney) were stored at room temperature (20- 24°C) from 1 to 21 days later than the experimentally infection of pigs with moderate strain of CSFV. The results showed that RT-PCR is more sensitive than virus isolation (VI) on fresh tissue samples, and less vulnerable to sample degradation. Tonsils and spleen were shown to be the most appropriate organs for the detection of infectious virus and viral RNA, in both fresh and degraded samples [89]. Donahue et al. (2012) also concluded that blood and tonsils (scraping and tissue) were better samples for CSFV detection than nasal swabs, which showed strong positivity concurrent with the start of clinical signs (after 5 dpi on average) [31].

Trying to innovate by using different matrices that may increase the sensitivity of early detection, and providing versatility regarding the type of samples, Flannery et al. (2020)

tested different matrices for detection ASFV. This study included bone marrow, ear biopsies, nasal, oral, and rectal swab samples. Those are different from the traditional matrices chosen by diagnostics of hemorrhagic fevers in swine (ASF and CSF) like spleen, tonsils, serum, gastrohepatic or renal lymph node, lungs, kidneys, and whole blood (heparin for virus isolation and EDTA for PCR). Although there was no statistical difference among the matrices, bone marrow yielded the highest concentration of ASFV, which can be a good option for sampling autolyzed or decomposed carcasses. An interesting finding of this study was the identification of ear biopsy as a suitable matrix to detect ASFV in infected pigs providing a much quicker alternative sampling matrix than EDTA blood and should allow for higher throughput sample collection in the field [90]. Pikalo et al. (2021) also tested ears biopsies for ASFV detection comparing them to splenic samples. The genome loads were higher in spleen than in ear biopsies, which showed low to moderate genome amounts [88]. Therefore, ear biopsies and swabs (oral, nasal, rectal) which can be obtained easily from pigs, causing minimal discomfort, overcoming of the difficulties of animal handling, effort, and time associated with sampling blood in this species.

Tonsil, spleen, and blood are the most standard samples collected in a suspicious case of ASF or CSF, and the studies confirmed that them are good and reliable choices for sampling, guaranteeing a high sensitivity of detecting positive samples. However, there are good studies showing that other types of samples can be collected, like ear biopsies, blood swabs, bone marrow swabs, among others. The most important is to have options to collect the type of sample that may be available and convenient for collection, without losing sensitivity. The next sub-section discusses blood swabs in more detail.

### 1.7.3 - Blood swabs and cold-free samples

To optimize the surveillance and promote early detection, all innovation and simplicity of methods and sampling are welcome. If samples can be collected in an easy way, and/or stored without the need of refrigeration until laboratory testing, it is a great gain in the surveillance system. This can optimize diagnostic processes, facilitation of testing, leading to early detection in a surveillance program. Fast identification and avoiding the spread of a FAD to other farms is the main objective of early detection.

Blood swabs may prove to be a valuable tool allowing sampling from not only recently dead or slaughtered animals, but also from carcasses of feral pigs. This would provide more opportunities for sampling and improve the likelihood of ASF and CSF antigen and antibody detection. Bleeding pigs is time consuming. Therefore, if the detection of a pathogen is not compromised by the use of blood swab, this sample type may simplify sampling for a number of surveillance objectives, especially when it may be necessary to rely on caretakers or hunters (in the case feral swine surveillance) for sample collection. By allowing testing of carcasses just collecting swabs from bone marrow, for example, will expand the number of animals samples. This would increase the coverage of surveillance for hemorrhagic fevers in the free areas. Fast-drying swabs are an alternative for ASF and CSF detection since they are easy to handle and can be store for long periods. A plus of using swabs for sampling is that they can be taken from blood, organs, and bone marrow, allowing diversity of samples. They can also be a good option for collecting samples from feral swine found dead.

The efficiency of three types of swabs in detecting ASF and CSF, a routine cotton swab (COPAN), a flocked swab (FLOQSwabs, COPAN), and a forensic livestock swab

(GenoTube, Prionics) were studied by a European group to test different sampling strategies. The resulting blood swabs were stored for three days (ASF) or overnight (CSF) at room temperature to mimic sample transport without cooling. The forensic swabs (GenoTube) showed advantages over the others because it does not need another recipient for transportation, such as a plastic bag or a test-tube, and it was easier for fragment preparation and further storage, which are lab procedures regarding the samples to be analyzed. The results from q-PCR showed no qualitative differences among EDTA blood samples vs swab samples. Regarding swab sample performance, they were quite similar, with slightly better results for flocked and forensic swabs. A good level of detection was reported for both ASF and CSF, showing that blood swabs were suitable for reliable ASF and CSF virus detection. [91].

In a continuation of the performance evaluation of GenoTube in detecting ASFV and antibodies, the forensic swabs showed a great sensitivity and specificity compared with serum and EDTA blood. The idea of using Whatman FTA® cards and filter papers would be the same for the GenoTubes, however, GenoTubes have their own receptacle and are safe to handle, avoiding cross-contamination in the moment of sampling. GenoTube swabs had a lower viral genome load compared to the original EDTA blood in qPCR assay, however, sensitivity (98.8%) and specificity (98.1%) were still good. In comparative studies using the commercial kit antibody ELISA, serum samples and GenoTube swabs dipped in whole blood had similar results, with sensitivity (93.1%) and specificity (100%) when comparing GenoTube to serum samples [92].

Another option for sampling pigs for the diagnosis of ASF or CSF, is the use of Whatman 3MM-filter papers soaked with whole blood, which can work similarly to swabs. This

type of filter papers does not contain additives used for lyses of proteins, as some specific filter papers used for PCR (FTA ® cards). Because of that, the Whatman 3MM-filter paper can preserve infectivity, and theoretically be used for further pathogen amplification. Another advantage is that they do not contain PCR inhibitor and can be directly used in conventional PCR without previous nucleic acid extraction. This Whatman 3MM-filter was assessed for ASF diagnosis with a series of currently available tests (conventional and real-time PCR, viral isolation, and antibody -ELISA). The results showed that the use of this specific filter paper is a good option for diagnostic sample collection, as the procedure for collecting blood on 3-MM filter paper is easy (blood droplets obtained by scarifying the skin of the pig's ear), with high sensitivity and specificity for all techniques performed [93].

In a short communication, Sauter-Louis et al. (2020) reported methods of sampling used for detecting an ASF outbreak in Germany, where the use of swabs and bone marrow were the target procedure for German surveillance. After extensive validation studies under experimental and limited field conditions showed reliable results, blood swab suspensions were included into the official method collection in Germany [94]. This work demonstrated blood swabs are accurate not only in experimental conditions, but also in field conditions, optimizing sampling collection, and possibly reducing the risk of spreading virus during a necropsy or from blood sample collection. Because the amount of blood released during a necropsy is far higher than collecting blood to soak a swab, which can be done in a skin scratch in the ears. The practicality of blood swabs yielding reliable results can encourage sampling in the field, improving the number of assays, hence the likelihood of early detection of ASF or CSF.

Having the option of sampling animals in any circumstance is essential for early detection of ASF and CSF. Therefore, techniques which allow for the easy conservation of samples, ease of collection, and maintain high sensitivity, should be encouraged. Also, the use of blood swabs may increase the number of feral swine tested, increasing the sensitivity of the surveillance system. Table 1.3 summarized the matrices used for detection of ASF and CSF.

## 1.8 - Surveillance strategies to aid early detection

Surveillance strategies for TADs should be regularly evaluated seeking improvements. Also, surveillance should base approaches on risk, including a broad view of probable sources for introduction of TADs, which will increase the likelihood of early detections. Early detection of the first outbreak of a disease in a previously free population is a very demanding surveillance objective, and even more demanding for emerging, previously unrecognized diseases [95]. For example, in areas where PRRS is an endemic disease, it can impose a serious delay in detection of ASF or CSF because the first differential won't be for these diseases.

Because ASF and CSF infections may start in a herd with unspecific symptoms, syndromic factors like mortality should be a trigger for investigating what is happening in the herd. An ASF model for spreading in large commercial farms was performed using the mortality rate threshold as a trigger for sampling dead pigs. Mortality rate threshold in pens, rooms, and barns was evaluated to identify the best surveillance strategy for

sensitivity of early detection and sampling costs due to the number of false alarm cases. The results of this study showed that using a mortality threshold of 4 dead pigs per room (400-head room) within a 7-day rolling observation window, the time to detection was 8 dpi for a median number of 9 false alarms per year, with a total of 45 dead pigs tested per year. Using a pen- mortality threshold of 2 dead pigs per pen (40-head pen) within the same observation window of 7 days provided the same time to detection (8dpi) and a similar number of dead pigs tested per year (48 dead pigs) while raising 24 false alarms per year. Similar results could be achieved using a barn-mortality threshold of 11 dead pigs (3,200-head barn) or a pen-mortality threshold of 2 dead pigs. Using a barn mortality threshold, results showed to achieve the same time to detection (8 dpi) would be necessary a higher number of dead pigs to be tested per year. This would result in more pigs being sampled per year. For example, using a mortality threshold of 11 dead pigs in the barn (3,200- head barn) within a 7-day rolling observation window, 72 dead pigs would be tested per year per barn. In this model, mortality thresholds at the room level provided the optimal balance between rapid detection and lowest rate of false alarms [96].

Although modelling of surveillance considering dead pigs is an interesting approach, the sick pigs should be included in the sampling protocol to guarantee a low rate of possible spreading. This approach focusing on sick animals is also recommended in the OIE at the Terrestrial Animal Health Code [97], [98], and also in the Integrated Plan for Surveillance of ASF and CSF from USDA [28].

A reported experience with ASF in Latvia showed that to facilitate the early detection of outbreaks in their country, regular sampling and testing of sick and dead pigs for ASF

virus were required as a new measure. Under this plan at least the first two deaths each week, including post weaning pigs or pigs older than two months in each production unit, had to be sampled and tested. With a case fatality around 90%, almost all ASF infected pigs will become sick and die [7]. Therefore, any sick or dead animal would be a good candidate for ASF testing.

In Germany, CSF surveillance strategies were evaluated to bring the best suitable approach for demonstration of freedom of CSF in the wild boar population. To do this, an Epi tool called an Integrated Evaluation Framework (EVA tool) was used. Sixty-nine scenarios for surveillance of CSF in wild boars were assessed, considering active and/or passive surveillance, as well as the hunter acceptability of the surveillance scenario and the costs. The current surveillance in Germany turned out to be the best strategy in which 59 wild boars are sampled each year in each district, achieving detection at 5% prevalence threshold with 0.95 probability of detection. However, sampling only within sub-adults resulted in a better acceptability and timeliness than the currently implemented strategy. Strategies that were completely based on passive surveillance did not achieve the desired detection probability of 0.95 in Germany [4]. This evaluation of the Germany CSF surveillance system illustrated some important general principles about designing surveillance strategies. Evaluation showed that it is necessary to find a balance between costly active surveillance, and lower cost passive surveillance, to optimize detection with available resources. Stakeholders should be involved in developing the surveillance strategy. Enhanced Passive Surveillance (EPS) promotes more notifications and may be a solution to find this balance between passive and active surveillances. According to Cameron et al. (2020), the enhanced passive surveillance, which they termed “Farmer-

based clinical surveillance" and syndromic surveillance can affordably provide high population and temporal coverage achieving relatively high surveillance sensitivity. While periodic sample surveys or sentinel surveillance does not achieve full population coverage, which decreases surveillance sensitivity [95].

Biosecurity actions or measures present or absent on a pig farm can be used to indicate risk of introduction of diseases on that farm. If certain biosecurity measures are present on the farm with good compliance, the risk tends to be low. Conversely, if certain biosecurity measures are absent or in low compliance, the risk of introduction of diseases is higher. After ASF reached China in 2018, Tian et al. (2020) described a good set of biosecurity procedures for small-scale pig farms he called quadruple protection procedure (QPP) to increase the chances of a pig production cycle without ASF. These procedures were based on 4 aspects including the farm's construction, environmental disinfection, regular immunization, and feed quality. The authors PCR tested samples from the floor, waste system, feed through system, and the water dispensing system, where all these areas were positive for ASF before the disinfection procedure, with the waste system showing the highest percentage of contamination [99]. Using this approach, sampling pens and barns as critical points, such as the floor, waste system, feed through system, and the water dispensing systems, can be a good approach for surveillance and early detection. Once these points will show positivity in case positive animals are housed in the barns. Early detection should guide surveillance actions for a fast detection of the exotic agent, and thus surveillance should not be based on sampling animals only. Other samples must be included, like air, water, slaughterhouse fluids, manure, etc., depending on the specific disease agent, to enhance the likelihood of detecting the disease [5].

With that, surveillance strategies may include both active and passive surveillance, however, they should be based on risk. Therefore, for an effective and robust system, public and private partnerships (PPP) between OVS and other involved stakeholders (producers, private veterinarians, slaughterhouses, among others) should be developed. According to OIE, the establishment of PPP contributes to a more efficient and effective use of both public and private sector resources, finding synergies through an active and structured collaboration to bring, among other things, a well-structured and efficient surveillance system with active and passive surveillances [100]. For the passive surveillance component, most animals are observed by their owners quite frequently with almost complete population coverage. This far exceeds anything that can be afforded in terms of surveys or other forms of direct observation by the OVS [101]. Using the model of EPS to actively search to specific indicators (healthy and syndromic information) there is an opportunity to improve this passive surveillance, which will guide producers to test their sick and dead pigs [6].

## 1.9 – Objectives of this Ph.D. project

The primary objective of this Ph.D. project was to work with epidemiological methods to support Official Veterinary Services (OVS) aiding for early detections incursions of ASF and CSF, based on risk-based surveillance for these diseases, considering all nuances from different levels of actions (national, state/region, and farm-level), and also, considering different data availability.

With that, each chapter is referencing to a level of surveillance action. While reading this thesis, it is interesting to make an analogy with a microscope and its lens. First, in a lens with low resolution, a study to predict the risk of introduction of ASF in a country level was performed. There was a limited data, with that, a combination of conjoint analysis (marketing tool used to assess and score clients choices or satisfactions) with ordinal logistic regression allowed to create a proxy-risk for ASF incursion in Kazakhstan, a free country.

Thereafter, increasing the resolution of the lens, we worked with a project at a state level, with more data availability, performing risk assessment for the introduction of CSF in Mato Grosso, a state located in the CSF free zone of Brazil. With official commercial pig movement data and pig farms from Mato Grosso and other states that have trades with Mato Grosso, it was possible to perform a stochastic quantitative risk assessment using a scenario tree to depict the hazard pathway. However, to calculate the risk for backyard farms, which it is believed to have intrinsic relationship with the presence and contact with wild boars, it was use the same methodology developed for the risk prediction for the introduction of ASF in Kazakhstan. And combining these two pathways, it was possible to generate the risk assessment for each municipality of Mato Grosso, with more capillarity than at national level.

Increasing more at the resolution of the lens of the imaginary microscope and ending up inside of the farm, the method developed was able to score factors present at farm that could trigger awareness regarding the possible introduction of ASF and CSF at a farm level. And in a weekly base report, the method was able to detect fluctuations of the scoring system, therefore, showing an increase or not in the risk of introduction of

hemorrhagic fevers of swine based on three components intrinsic to the farm (i.e., biosecurity, syndromic surveillance, and necropsy findings). The idea of developing a protocol for enhanced passive surveillance, to aid early detection of these diseases in a farm was built because first, the producers are the ones that can notice sick animals at their farms; and second, the power of coverage a surveillance area is almost 100%, since the OVS cannot be visiting all the farms at the same time, with that, having an alert producer, or other stakeholder involved, increases the probability of early detection of these diseases and avoiding spread to large areas in the country. This enhanced passive surveillance protocol was tested in Dominican Republic, which is an infected country for ASF and CSF, also tested in pig farms of the Midwest of the United States, in order to assess the biosecurity component.

The idea of having methods and or epidemiological models able to cover these three levels of surveillance was the objective of this Ph.D. project, delivering a complete option for risk assessments and surveillance tool to support OVS surveillance actions, based on risk. Figure 1.1 is depicting a summary of the levels of surveillance impose to ASF and CSF risk of introduction in free areas, where the data availability can drive the lens of the microscope to a high or less resolution, however, the risk can be assessed and informed to OVS to be prepared and focus the surveillance to an early detection goal.

## 1.10 List of Figures

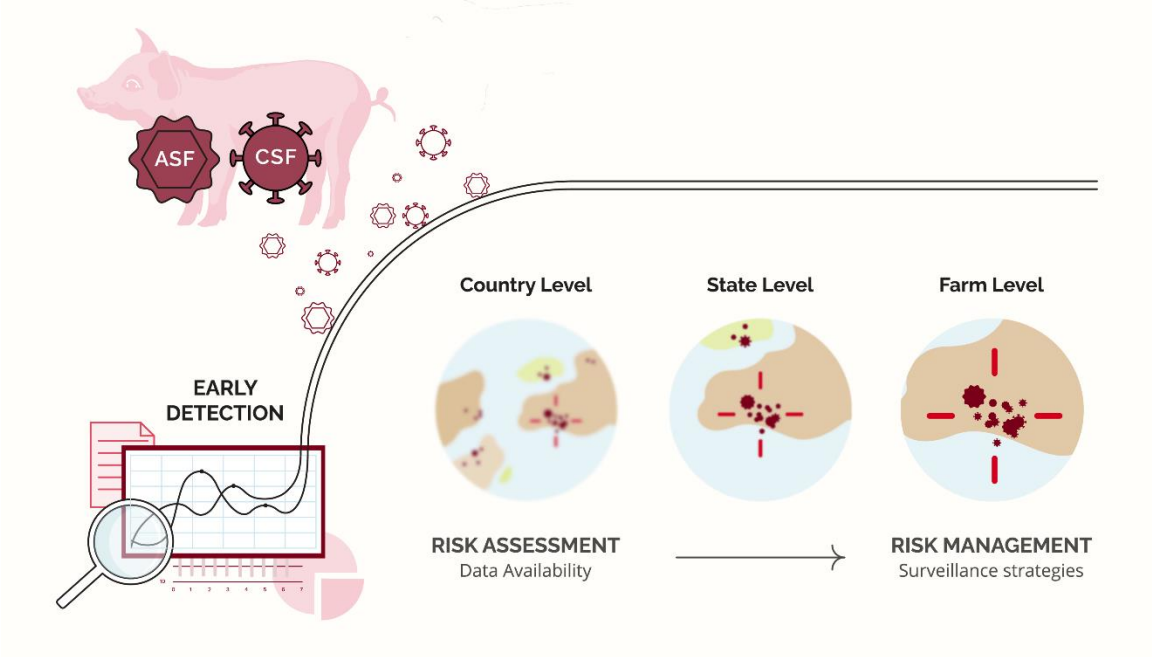


**Figure 1.1**- Schematic view for levels of risk assessment in this Ph.D. project

## 1.11 List of Tables

**Table 1.1 -** Differences and similarities of ASF and CSF

| | **African swine fever** | **Classical swine fever** |
|---|---|---|
| Virus Description | a double-stranded DNA, enveloped arbovirus, sole member of Asfarviridae family | a small, enveloped RNA virus, genus pestivirus, Flaviviridae family |
| Incubation period | usually 4–19 days, acute form 3–4 days, OIE considers 15 days | from 3 to 10 days, most common from 4 to 7 days |
| Forms of clinical presentation | peracute, acute, subacute, and chronic | acute, chronic, sub-clinical, and congenital infection |
| Clinical Signs | fever, anorexia, pigs huddle together, multifocal hyperemia and hemorrhagic skin, abortion, diarrhea with blood | starts with unspecific (fever, anorexia, constipation or diarrhea, weakness, conjunctivitis), then neurological signs and skin hemorrhages or cyanosis |
| Available Vaccine | There is no current vaccine | There is vaccine, but it is used only in endemic countries |
| Laboratorial identification | Virus isolation from 7 to 10 days by hemadsorption. Genome detection by PCR in 5- 6 hours | Virus isolation through fluorescent antibody tests after 24—72 hours or by Immunoperoxidase staining after 3–4 days' incubation. Genome detection by RT-PCR in 5- 6 hours |

Source: [21], [23], [25], [47], [48]

**Table 1.2 -** Diagnostic tests in a glance - Summary of tests described in the document

| Assay | Target | Matrix | Sensitivity (Se) | Specificity (Sp) | Notes | Reference |
|---|---|---|---|---|---|---|
| qPCR-LPR | Antigen (ASFV) | Whole blood | Analytical sensitivity 92.61% | Analytical specificity 90.48% | LPR stands for lyophilized powder reagents. Compared to qPCR recommended by OIE, and conventional PCR. | [50] |
| mRT-qPCR | Antigen (ASFV, CSFV, FMDV) | Swine oral fluids | 4-plex RT-qPCR exhibited 83.9–98.3% efficiency (R2 > 0.99) for the detection of 10-fold serial dilutions of each tar- get | no ASFV, CSFV, or FMDV target amplification was observed using a negative cohort panel of 82 independent swine OF field samples from free areas | Analytical sensitivity set up for detection 10-fold serial dilution of each target. Sensitivity was assessed in comparison to the respective single pathogen test. | [51] |
| Recombinase polymerase amplification (RPA) | Antigen (ASFV) | Tested in serum spiked with ASFV | Analytical sensitivity - was able to detect 100 copies of ASFV DNA in 10 minutes | Used 106 copies of PCV-2, PRV, PRRSV, CSFV, and FMDV were used for RPA reactions to calculate specificity, and none reacted positively, only ASFV was detected | Target to VP72 of ASFV DNA. It does not need thermocycler. | [53] |
| "Cas-gold"-RPA technolog | Antigen (ASFV) | Blood, oral, and | All positive samples were detected by | All negative samples were negative in | This assay used qPCR as a reference | [64] |

| | | | | | | |
|---|---|---|---|---|---|---|
| y (Cas12a based detection and gold nanoparticle-based on lateral flow device (LFD)) | | anal swabs | Cas-gold and qPCR. | Cas-gold and qPCR. | assay. It is in a patent processing in China. Pen side test. | |
| RPA with LFD – K205R gene | Antigen (ASFV) | Kitchen waste, swill samples, environmental samples from meat stalls at farmers market, and pork products | Analytical sensitivity of 100 copies per reaction | Only ASFV was detected, while CSFV, RVA, PEDV, TGEV, PRRSV and PRV did not, showing analytical specificity | Samples collected in China. Pen side test. It used qPCR as reference assay. | [67] |
| Loop-mediated isothermal amplification (LAMP) | Antigen (ASFV) | Crude serum and extracted genomic DNA of ASFV | Analytical sensitivity - near 100% | Analytical specificity - near 100% | This author compared this LAMP to commercial ASF qPCR kit. It does not need thermocycler | [54] |
| LAMP | Antigen (ASFV) | Serum and oral and rectal swabs | Substantial level of agreement (k 0.74; 95% CI 0.503—0.979) based on Cohan's kappa coefficient, between the LAMP and qPCR results for the detection of ASFV | Analytical specificity was further investigated through the testing of 160 pig bloods from an ASFV negative area, with no cross-reaction with this sample matrix detected | Used field samples. 37 pigs were sampled by both means (serum and rectal/oral swabs), only two additional positives were detected by serum sampling compared to swabs. It | [55] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | does not need thermocycler. | |
| LAMP combined with LFD | Antigen (CSFV) | RNA extracts, and infected sera | The analytical sensitivity was about 100 copies per reaction when testing two genotypes (1.1 and 2.3) | No cross-reactivity to non-CSFV pestiviruses was observed | It can be used as a pen side test. | [61] |
| LAMP combined with LFD | Antigen (ASFV – B646L gene) | DNA extract, and clinical samples of infected pigs | Analytical sensitivity of 100.6 copies per reaction | CSFV, RVA, PEDV, TGEV, PRRSV, delta-CoV, and 2 DNA viruses PRV PPV were used as[68] analytical specificity control (none were detected, only ASFV genome. | VP72 is encoded by B646L gene. Pen side test. Clinical samples from infected pigs were not specified in the study. PCRs (conventional, real-time, and nested) were used in the validation of this LAMP, with this test showing more | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | analytical sensitivity than the others. | |
| Immunochromatography test using LFD | Antigen (ASFV) | Whole blood without anticoagulant | 67.86% comparing to UPL-PCR | 97.97% comparing to UPL-PCR | Target VP72 protein. Compared to Antigen ELISA, this assay showed almost perfect agreement (Kappa value of 0.92). It can be used as a pen side test. | [62] |
| INGENASA® Pen side test | Antigen & Antibody (ASFV) | Blood and organs from wild boar | Tests in parallel showed global sensitivity of 66.7% | Tests in parallel showed global specificity of 95.5% | Pen side test for using at surveillance in wild boar. | [63] |
| Bead-based multiplex assay (BBMAs) | Antibodies (ASFV & CSFV) | Serum | Sensitivity (97.3%) for ASFV and sensitivity (95.7%) for CSFV | Specificity (98.3%) for ASFV, and specificity (99.8%) for CSFV | In veterinary medicine are few commercial kits available | [56] |
| "Biochip array" technology | Antibodies (CSFV, and other viruses – PRRS, Parvovirus, and Japanese Encephalitis) | Serum | Coincidence rates for positive samples: 95.8 to 100%, being 100% for CSFV and PRRSV | Coincidence rates for negative samples: 86.2 to 100%, being 100% for CSFV and PRRS. | This assay was compared to ELISA assay. It can be used to screen diseases for mixed infections. | [57] |

**Table 1.3 -** Type of matrices – Summary of some studies with other matrices than conventional ones for ASF and CSF diagnostics.

| **Matrix** | **Targeted disease** | **Notes** | **Reference** |
|---|---|---|---|
| Feces | ASF (antibodies) | Started detection at the same time as detection in serum (9 days post infection (dpi) in experimentally infected pigs with low virulent ASFV strain. | [81] |
| Feces | ASF (antigen) | Started detection at 4 dpi | [82] |
| Oral fluid (OF) | ASF (antibodies) | Target protein VP30, detected in serum and OF as early as 8dpi | [102] |
| OF | CSF (antibodies) | Starting detection after 10 dpi | [85] |
| OF | CSF, ASF, FMD | CSF – antigen detected at 10 – 14 dpi, antibodies detected starting at 14- 21 dpi; ASF – antigen detected at 6 to 21 dpi, positive antibody detection was only positive at day 10 post infection; FMD – antigen detected starting as 1 dpi, antibodies started at 14dpi | [86] |
| OF (ropes in baits) | CSF (antigen) | Positivity started being detected after 9dpi | [87] |
| Ears biopsy, bone marrow, nasal, rectal, and oral swabs | ASF (antigen) | Detected great amount of virus. Innovation for the use of ear biopsies as sample matrix | [88], [90] |
| Blood swabs | ASF & CSF (antigen) | Testing efficiency of detection of ASF and CSF using cotton swab, flocked swab, and GenoTube® swab. | [103] |
| Blood swabs | ASF (antigen/ antibody) | Great sensitivity (98.8%) and specificity (98.1%) compared blood swabs (GenoTube®) and | [23] |

| | | | |
|---|---|---|---|
| | | EDTA blood in qPCR assays. And sensitivity of 93.1% and specificity of 100% when compared blood swabs and serum using commercial antibody ELISA kit. | |

# CHAPTER 2 - Risk for African Swine Fever introduction into Kazakhstan

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## 2.1 Chapter Summary

African swine fever (ASF) is a disease of swine that is endemic to some African countries, and that has rapidly spread since 2007 through many regions of Asia and Europe, becoming endemic in some areas of those continents. Since there is neither vaccine nor treatment for ASF, prevention is an important action to avoid the economic losses that this disease can impose on a country. Although the Republic of Kazakhstan has remained free from the disease, some of its neighbors have become ASF-infected, raising concerns about the potential introduction of the disease into the country. Here, we have identified clusters of districts in Kazakhstan at highest risk for ASF introduction. Questionnaires were administered and districts were visited to collect and document, for

the first time at the district level, the distribution of swine operations and population in Kazakhstan. A snowball sampling approach was used to identify ASF experts worldwide and a conjoint analysis model was used to elicit their opinion in relation to the extent at which relevant epidemiological factors influence the risk for ASF introduction into disease-free regions. The resulting model was validated using data from the Russian Federation and Mongolia. Finally, the validated model was used to rank and categorize Kazakhstani districts in terms of the risk for serving as the point of entry for ASF into the country, and clusters of districts at highest risk of introduction were identified using the normal model of the spatial scan statistic. Results here will help allocate resources for surveillance and prevention activities aimed at early detecting a hypothetical ASF introduction into Kazakhstan, ultimately helping to protect the sanitary status of the country.

## 2.2 Introduction

African swine fever (ASF) is a viral disease of pigs, affecting members of the *Suidae* family (domestic pigs or wild boars) without differentiation of age or sex. ASF is caused by a large enveloped, double-stranded deoxyribonucleic acid (DNA) arbovirus that belongs to the *Asfivirus* genus of the *Asfarviridae* family and that is generically referred to as ASF virus (ASFV). ASFV infection has an impact on international trade in pigs and pork products, being a threat to global food security, hence, the disease is notifiable to the World Organization for Animal Health (OIE). ASF epidemics also represent a public health issue because they disrupt the value chain and access to international markets,

limiting food access to the population in affected regions and trade partners [104][1][17][105]. Control measures for ASF are based on biosecurity measures as neither a licensed vaccine nor any treatments are currently available [12].

The ASFV was introduced in 2007 into Georgia, from where the virus spread throughout the Caucasus region (Armenia and Azerbaijan) and the Russian Federation, where the disease became endemic. ASF was subsequently reported in Ukraine and Belarus in July 2012 and June 2013, respectively. In January 2014, ASF reached Eastern Europe, where it spread throughout Estonia, Latvia, Lithuania, Poland, Belgium, Bulgaria, Moldova, Czech Republic, Hungary, Romania, Slovakia, Serbia, Greece, and Germany affecting wild boars, and in some countries, domestic pigs [3]–[10]. In addition, since 2017, ASFV has rapidly spread eastwards, with the Russian Federation registering new cases in Eastern Siberia followed by China in 2018; in 2019 Mongolia, Vietnam, Cambodia, Democratic People's Republic of Korea (North Korea), Republic of Korea (South Korea), Lao People's Democratic Republic, Myanmar, Philippines, Timor-Leste, and Indonesia, and in 2020, India and Papua New Guinea also registered cases of ASF. Since 2018, more than 7,300,000 pigs were culled or destroyed in Asia, causing far-reaching economic losses to the region. The unprecedented ASFV spread through Asia and Europe has resulted in great concern for many free countries and regions worldwide [104][113].

Kazakhstan is a land-locked country located in the transition of Eastern Europe and Central Asia, sharing extensive borders with three countries (Russian Federation, Mongolia, and China) that have been infected by the ASFV. The Kazakh domestic pig sector is relatively small, with approximately 936,300 pigs and an average density of 0.34 pigs/km$^2$ [114]. Nevertheless, there is still a potential for increasing the exporting of pork

products in association with bans imposed to ASF-infected countries and the consequent increase in demand in importing markets. For those reasons and given that Kazakhstan is still free from the disease, there is an urgent need to increase preparedness for enhancing the chances of early detecting and mitigating a hypothetical ASFV introduction into the country. Because ASF has never been reported in Kazakhstan, there is no information on the socio-economic or environmental factors associated with the disease spread in the country. For that reason, the allocation of resources in preventive measures that are effective in minimizing the risk of disease incursion is particularly challenging in Kazakhstan.

ASF may be introduced into free areas through different pathways, such as trade of live pigs and pork products, wild boar transboundary movements and contacts with free-ranging pigs, fomites, and vehicles. The objective of this paper was to identify the areas of Kazakhstan that are most likely to serve as port of entry for a hypothetical ASFV incursion into the country. Results will help the public veterinary authority of Kazakhstan to selectively allocate financial and human resources to target surveillance activities in districts with highest predicted risk for disease introduction. Additionally, the methodological approach applied here may be used for ranking regions in ASF-free countries located in affected regions worldwide, with the ultimate goal of designing and implementing surveillance programs to prevent and mitigate the impact of the disease [111] [115].

## 2.3 Material and Methods

### 2.3.1 Data sources

Because data on the distribution of the susceptible swine population at the district level in Kazakhstan were not available, a country-wise survey was undertaken, aimed at the creation of a national database of pig-related operations. The survey was conducted in 2018-2019 as a series of trips in close collaboration with regional authorities and veterinary services. Location of all facilities related to the swine industry were georeferenced and relevant attributes were recorded. The work resulted in the construction of a unique national database of pig holdings as well as slaughterhouses, meat storage, and processing facilities and retail stores. Additionally, data on other relevant variables, as number of pigs per farm and type of pig production based on the ownership of the farms were compiled and organized in *ad hoc* databases. The database enabled the calculation of pig density and backyard farming share for Kazakhstan used in the present study. Additionally, the estimated wild boar density of Kazakhstan was retrieved from the "Forestry and Wildlife Committee Ministry of Ecology, Geology and Natural Resources of the Republic of Kazakhstan" website [116]. The sources of other data used here are provided in Table 2.2.

### 2.3.2 Analytical approach

Conjoint analysis, which is a marketing research tool used in surveys aimed at capturing the best fit decision of costumers and determining tradeoffs [117], was used in the current study. Districts in a hypothetical ASF-free country located in an ASF-infected region

were designed using a factorial design to balance the distribution of epidemiological features hypothesized to influence the risk for ASF introduction. Subsequently, ASF experts were asked to rank those hypothetical districts in terms of the likelihood of serving as port of entry for the disease into the country. An ordinal logistic regression model was run to estimate the relative weight that the experts implicitly gave to each of the variables, as approximated by the value of the regression coefficients. The regression coefficients were then validated using data from the Russian Federation and Mongolia. Finally, the model was used to project the risk in Kazakhstani districts, and high-risk clusters were identified using the spatial scan statistics, to help inform the regionalization of surveillance activities in the country.

### 2.3.3 Conjoint analysis - questionnaire and selection of variables

A hypothetical ASF-free country was divided into 10 districts using a combination of epidemiological factors hypothesized to influence the risk for ASFV introduction. The 10 districts were designed so that 8 of them were created using a factorial design to balance the distribution of epidemiological factors, and 2 of them represented the scenarios of best and worst possible combination of factors, in terms of their expected risk for the disease (Table 2.1). A factorial design considers input variables as a factor, where they are combined, and different "treatments" are generated, allowing comparison of the effect of these factors in the independent variable (here, the introduction of ASF) [118]. The selection of factors hypothesized to influence the risk was based on previous experience of the authors and supported by a literature search. Pig density, estimated wild boar density, and backyard farming were chosen with the objective of capturing the influence

associated with the distribution of the susceptible population. During the ASF outbreaks in Russian Federation, for example, pig population density was identified as an important risk factor for the disease [115], [119]. Wild boars can also be responsible for transboundary ASF spread due to their natural dispersal ecology in search of new territory [107], [111], [120]. Swill feeding is considered a relatively common practice in many backyard farming systems, which, in addition to limited biosecurity in those types of farms, has been associated with a high risk for the disease [105], [121]. Shared border (yes/no) and border length with an infected territory were included because of the risk for movement of infected animals, illegal trade or movement of infected pork, and infected vehicles and other fomites. Finally, human density and road density were included as a proxy for the movement of people, given that travelers can carry contaminated or infected goods and because ASFV can survive for extended periods of time in the environment and in pork products [112], [119]. (Table 2.2)

### 2.3.4 Selection of experts

The questionnaire listing the hypothetical scenario described above was shared with three OIE Reference Laboratories Centers for ASF (South Africa, Spain, United Kingdom), and the National Reference Laboratory of the Russian Federation in Pokrov, which was selected due to its regional experience on ASF both in wild boars and domestic pigs. The four Reference Centers for ASF were asked to provide names for individuals that would have sufficient knowledge and experience to rank the hypothetical districts in terms for their risk for an ASFV introduction. Snowball sampling [122] was used to designate experts, defined as those individuals that were mentioned at least by two reference

centers. A list of 12 experts was identified and were invited to rank the 10 hypothetical districts in terms of the risk for an ASFV incursion, so that #1 and #10 denoted the districts with the lowest and highest risk of becoming ASF-infected, respectively. A table with some definitions and reference values was provided to the experts for helping them understand the values that were used for categorizing the variables (Table 2.2). Most (n=11, 92%) experts accepted the invitation and answered the questionnaire, which was de-identified prior to data introduction into a master database for analysis.

### 2.3.5 Predictive model

An ordinal logistic regression (OLR), proportional odds model was fitted to the answers provided by the experts so that

$\ln = \frac{p(Y \geq j)}{p(Y < j)} = \beta_0^{(j)} + \beta_J X$, where

Y was the dependent variable "score", as provided by the experts, and so that score had J categories with j designating categories from 1 to J (i.e. j= 1, …, 10). $\beta_0$ was the intercept, and $\beta_j$ denoted the coefficients for the independent variables X, which were the epidemiological factors used to characterize each of the hypothetical districts [123]. Variables were screened for collinearity prior to their introduction as candidate predictors in the model, and the final model was selected using Akaike's Information Criterion (AIC).

### 2.3.6 Model validation and predictions for Kazakhstan

ASF-infected countries in Central Asia and Eastern Europe (n=14 at the time when this manuscript was written in December 2020, Table 2.3) were considered as initial candidates for the validation of the model because countries in those regions are culturally, socially, and politically, relatively similar to Kazakhstan, compared to countries in other regions [124][125][126]. Because Kazakhstan is a large country (9th largest in the world) and because the size of the units at which data are aggregated may influence results, the five largest countries (Poland, Germany, Ukraine, Mongolia, and the Russian Federation) from the initial pool of fourteen were subsequently selected as candidate countries for validation. Values for the variables used as risk factors in the model were collected for the five countries at the sub-national level and compared with those observed in Kazakhstan [127]–[130] (Table 2.4). Poland, Germany, and Ukraine were eliminated as candidate countries for the validation because they are substantially smaller (ranking #69, #63, and #45 in globally size countries, respectively) and also because of the differences in the distribution of values for all assessed variables compared to Kazakhstan --i.e., in general, districts in Ukraine, Germany, and Poland have a higher share of backyard farming, and have a higher density of human, domestic pigs, and estimated wild boar density than Kazakhstan. Subsequently, only Mongolia and the Russian Federation were considered adequate for the validation, even acknowledging the differences that exist between those countries and Kazakhstan.

For the validation, the regression coefficients obtained from the OLR model were used as weighting factors for the data collected in both Mongolia and the Russian Federation to identify the three districts (regions or oblasts) predicted to be at the highest risk for

introduction of ASFV when those countries were free from the disease (Table 2.5). The results, which indicated the districts that would have been identified by our model and the expert opinion elicited here at the highest risk for ASFV introduction into the Russian Federation and Mongolia, were compared to the districts through which the disease was introduced into those countries when they first-became ASF-infected, as recorded by OIE's World Animal Health database (WAHID) [131], [132].

Finally, the OLR model was applied to the district-level data (pig density, estimated wild boar density, backyard farming, shared border (yes/no), human density, and road density) collected in Kazakhstan to predict the districts at highest risk for disease introduction. Figure 2.1 depicts the categorization of these district-level data in Kazakhstan. Results were allocated to each of the district centroids and the normal model of the spatial scan statistic was run to identify clusters of districts in which the predicted risk of introduction of ASFV was significantly ($P<0.05$) higher than the expected under the null hypothesis of even distribution of risk. The normal model of the spatial scan statistic has been described elsewhere [133]. Briefly, circles of variable radius are alternatively imposed over the centroids and candidate clusters, including groups of neighboring districts, are identified. The average risk for ASF introduction was computed for each candidate cluster and compared with the expected under the null hypothesis that all observations come from the same distribution. Significance of the deviation of the observed risk, compared to the expected, was estimated for each candidate cluster using Monte Carlo simulation. Results for Kazakhstan were plotted in choropleth maps.

### 2.3.7 Modeling environment

The SPSS software [134] was used for the factorial design of the 10 hypothetical districts. The RStudio Team (2019) version 3.5.3 [135] was used for performing the OLR model, using the packages MASS, tidyverse and ggbeeswarm. The SaTScan v.9.6 software was used to identify clusters of predicted risk in Kazakhstan [136]. ArcGIS 10.5.1 was used for spatial data processing and mapping data and results [137].

## 2.4 Results

The data collection process led to the registration of 2,021 pig farms throughout Kazakhstan. Based on the legal property form of the farms, most operations (n=1612, 79.5%) were considered privately owned (i.e. belonging to a single stakeholder) farms, with swine population sizes ranging between 1 and 6,110 pigs (median of 107 pigs). The remaining operations (n=409, 20.5%) were classified as farms belonging to commercial associations, with 1 to 50,775 pigs (median of 47 pigs) (Figure 2.2). This categorization only reflects the legal property type, as no biosecurity-based classification is currently effective in Kazakhstan. For the purposes of data analysis, we only used pig population per farm to categorize all holdings in “small”, conventionally treated as backyards (less than 100 pigs) and “large” (more than 100 pigs), consistently with the Food and Agriculture Organization of the United Nations (FAO) definition of backyard production systems as those in which pigs are confined in very simple pens, are dependent for their keeper for feed, and the herd is usually small (1-100 animals raised per year) [138].

Because there was a high level of collinearity between border length (km) and shared border (yes/no) with an infected country, the former variable was considered redundant and removed from the model. The factor that experts considered most important in driving the risk for introduction of ASFV into a free district was a high density of backyard farming, followed by high density of pigs and high estimated density of wild boars (Table 2.5).

Despite road and human densities were not significantly associated with the score provided by the experts, inclusion of those variables in the final model resulted in the lowest AIC value recorded for any combination of variables (AIC: 343.4), and for that reason, all variables listed in Table 2.5 were retained in the final model.

The three Russian Federation districts predicted to be at highest risk for introduction of the disease were the Republic of North Ossetia-Alania, Bryansk Oblast, and the Orenburg Oblast, respectively. Although ASFV was first reported in Chechen Republic in November 2007, which would not have been predicted by our model, the second massive incursion of ASFV into Russian Federation was reported in June 2008 in the Republic of North Ossetia-Alania, followed by cases in Orenburg Oblast in July 2008, coincidentally with the model predictions. For Mongolia, the three districts predicted to be at highest risk of the introduction of ASF were Ulaanbaatar, Bulgan, and Selenge. Coincidently, the three districts had the first occurrence of ASF in January 2019. Subsequently, the resulting model was used to predict the risk for ASFV introduction into Kazakhstan, and two clusters of significantly ($P<0.05$) high risk for introduction of ASFV in that country were detected using the spatial scan statistic. High risk clusters were located in the

Almaty (southern Kazakhstan) and Kostanay (northern Kazakhstan) regions, and included seven and nine districts, respectively (Figure 2.3).

## 2.5 Discussion

Following the fall of the Soviet Union and given that the majority of the population of Kazakhstan is Muslim, the number of swine operations in the country has substantially decreased. However, the swine industry of Kazakhstan still supplies the demand for >25% of the population of the country that is not Muslim. Furthermore, the geographical proximity of Kazakhstan with China has increased the country's interest in promoting the production of pork to supply the emerging demand in China associated with the ASF epidemic. The Kazakh Ministry of Agriculture has signed a memorandum of understanding "on inspection, quarantine and veterinary-sanitary requirements for pork exported from Kazakhstan to China" with the Chinese State Technical University, which was considered a first step to promote pork exports into China [139]. In order to protect the status of the Kazakh swine industry, it is critical to understand the distribution of the susceptible population and characterize the risks associated with disease status. For the first time, we have conducted here a comprehensive survey of the distribution of swine farms in Kazakhstan, showing its selective concentration in the northern and southern regions of the country (Figure 2.2).

The relative isolation of Kazakhstan, along with the small size of its pig industry, may have helped the country to avoid the introduction of ASFV, despite the unprecedented spread of the disease through Europe and Asia. However, given that a number of neighboring

countries have become ASF-infected, there is a need for supporting Kazakhstan preparedness through the identification of areas at highest risk for ASFV introduction. The results here may help to target surveillance activities to those districts identified at highest risk for disease introduction to increase the sensitivity of the national surveillance system and support the early detection of a hypothetical ASF introduction into the country (Figure 2.3). The characterization of districts within those clusters as at highest risk for ASFV was driven by the presence of a number of factors that have influenced disease introduction into free regions. Those factors include the size of their domestic pig population and the estimated wild boar population, and their close proximity to ASF-infected countries, which are important to inform the design of targeted surveillance efforts [140]. Most importantly, many risks predictions studies suggest wild boars as the highest risk factor involved in ASF transmission [107], [120], [141] for countries in Europe, the Caucasus region, and Central Asia.

Because Kazakhstan has never been infected by the ASFV, there is no historical information that could help the country to categorize districts in terms of their risk for the disease. In the absence of such information, we gathered expert opinion on the factors that have driven the introduction of the disease in free countries of Europe and Asia. The highest risk oblast in the Russian Federation, the Republic of North Ossetia, was the second district infected in the country. Noteworthy, the failure of our model to identify Chechen Republic (the district through which the disease was first introduced into the Russian Federation) was likely due to the social disruption associated with the constitutional war suffered by the region at the time of the epidemic. Such social disruption may have resulted in an unexpected frequent movement of people and contaminated products or food that could

not have been predicted by the formulation of our model. Noteworthy, cases in the Chechen Republic were very limited. In contrast, the next affected region, the Republic of North Ossetia, which was predicted at the highest risk for introduction by our model, suffered a large number of cases in domestic pigs and actually may be considered a starting point of the consequent spread of ASF in the Russian Federation. The Republic of North Ossetia is also closely connected with the neighboring regions of Georgia, and ASF transmission was certainly expected here. Similarly, the first introductions of ASF into Mongolia took place at one of the districts identified at highest risk by our model. For those reasons, and in the absence of a prior history of ASF in Kazakhstan, the results of the validation process suggest that the model may help to accurately predict the expected risk for ASFV introduction into the country.

The study here did not assess the likelihood of disease introduction into Kazakhstan. Instead, we ranked the districts through which the disease was most likely to be introduced into the country, given that an incursion effectively occurs. This information is important to inform the design of targeted surveillance efforts in the country. One limitation is that epidemics are typically low probability events, and the realization of those processes are susceptible of being affected by random events, such as the social disruption in Chechenia. For that reason, the risk predicted here would be accurate only if the modeled conditions, reflected by the epidemiological factors weighted by the experts, remain constant in the future. Any variation in those conditions, or if those assumptions would not hold truth for Kazakhstan, may result in a variation of the predicted risk for the country.

In conclusion, the study here provided updated information on the spatial distribution of swine operations in Kazakhstan, along with the prediction of areas at highest risk for

introduction of ASFV into the country. Results have been shared with the government of Kazakhstan to support the development of recommendations on prevention and control measures for ASF in the country. This document will define a national strategy to prevent the introduction of ASFV from neighboring countries and it is intended to become mandatory for implementation at all pig farms in Kazakhstan under the supervision of the national veterinary authority. For those reasons, ultimately, the results will help to sustain the ASFV-free status of Kazakhstan and support the country's vision and efforts to supply international markets.

## Acknowledgements

The authors would like to thank the collaboration of the OIE reference centers in the Russian Federation, South Africa, Spain, and the United Kingdom that helped with the identification of experts, and the 11 experts that provided input for the conjoint analysis model.

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## 2.6 List of Figures

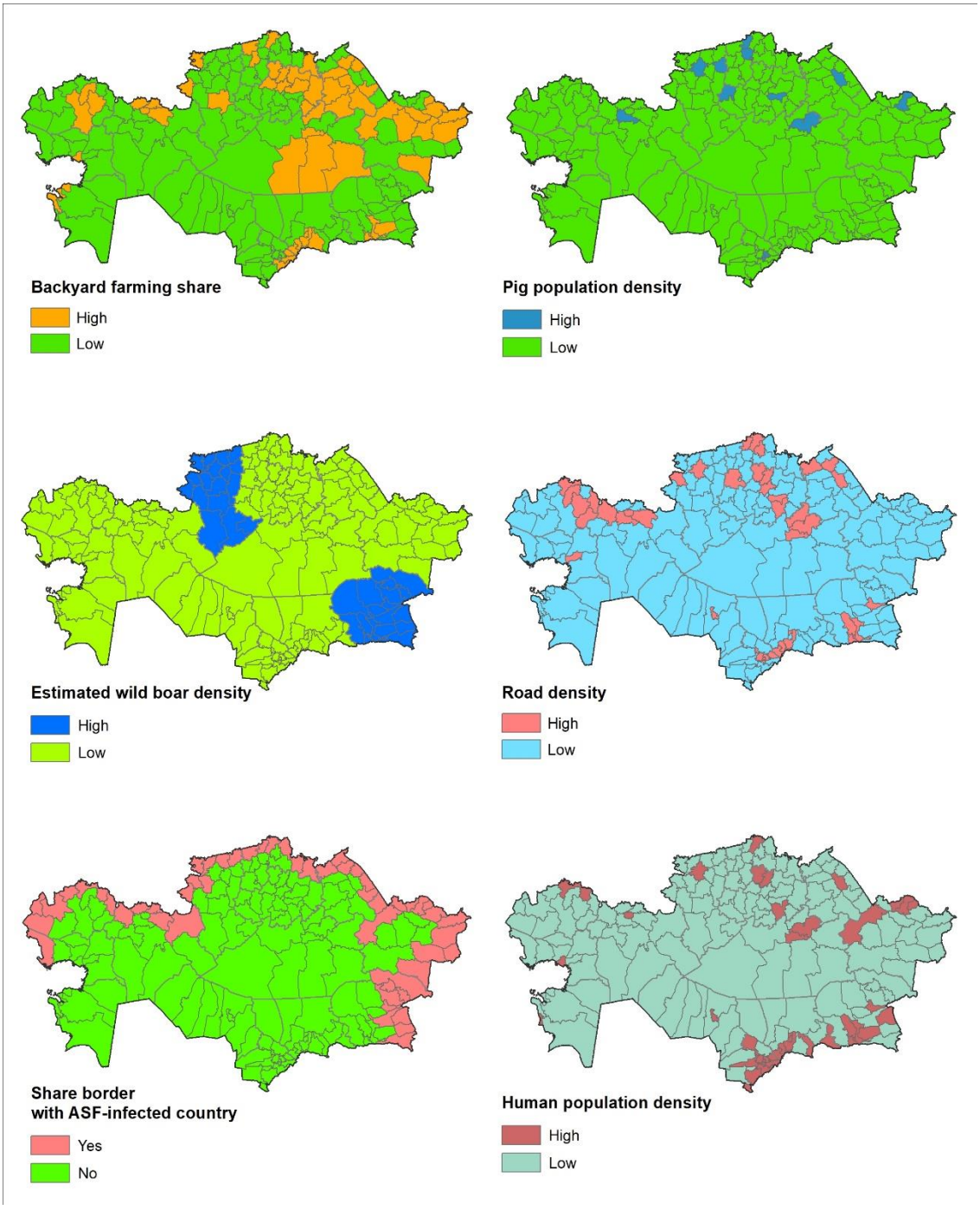


**Figure 2.1** - Kazakhstan district-level data for the variables used in the model (backyard farming share, domestic pig density, estimated wild boar density, share-border with ASF-infected country, human population density and road density).

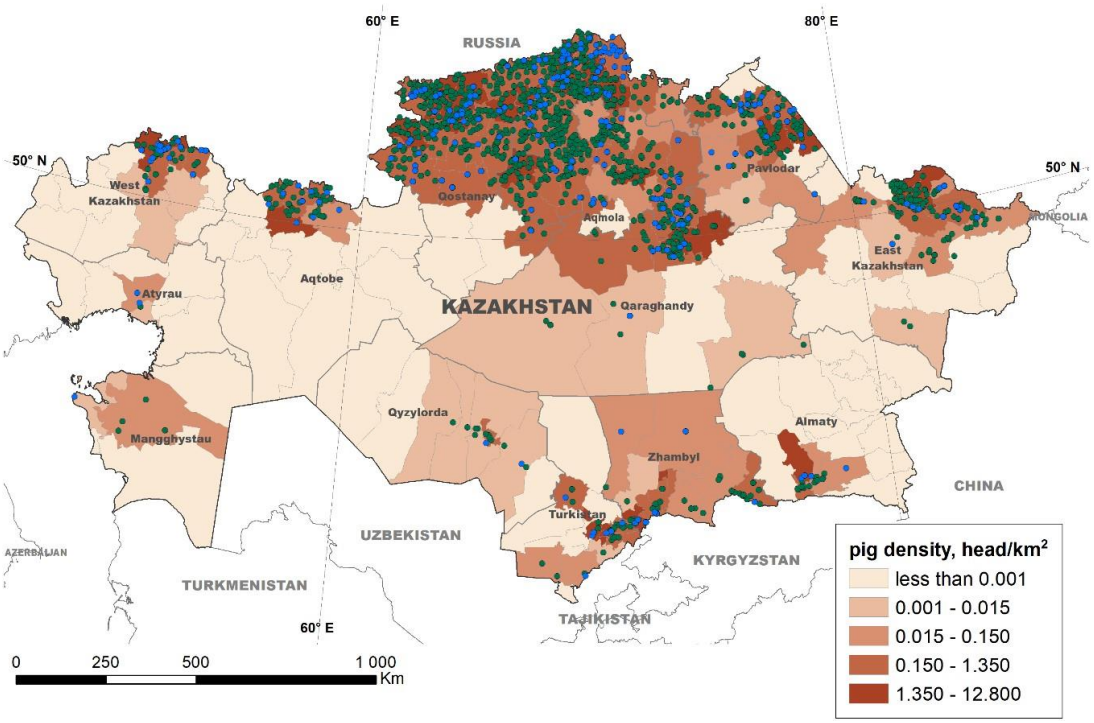


**Figure 2.2 -** Distribution of swine farms in Kazakhstan. The location of swine operations is indicated and categorized as single owner farms (green dots) and commercial associations owned (blue dots) farms. The color gradient denotes the pig density (head/sq km) estimated at the district level.

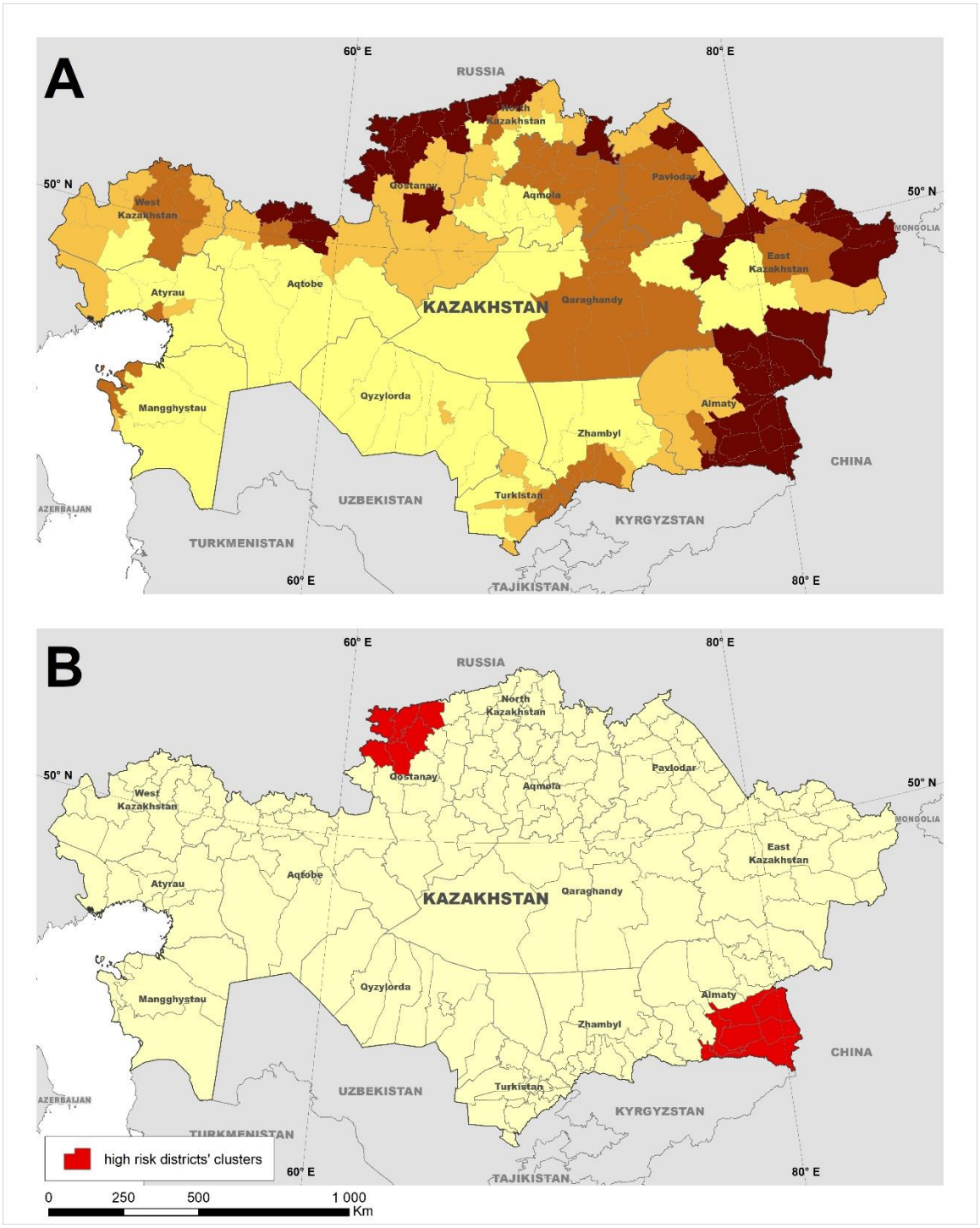


**Figure 2.3 -** Risk for introduction of African Swine Fever (ASF) into Kazakhstan estimated using a conjoint analysis model. The map on the top (A) depicts districts grouped into four quantiles based on the predicted risk (the darker the shade, the higher the risk), whereas the map on the bottom (B) illustrates the location of clusters of high risk for the introduction of ASF into the country detected using the normal model of the spatial scan statistic.

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**Table 2.1 -** A hypothetical African Swine Fever (ASF)-free country was divided into 10 districts that were characterized in terms of the risk for an ASF introduction using a list of epidemiological factors hypothesized to influence the risk and a factorial design. The values used to categorize the variables are described in Table 2.2.

| Region | Pig density | Wild boar density | Backyard farming share | Share border with ASF-infected country | Border length | Road density | Human population density | RANK (1-10)* |
|---|---|---|---|---|---|---|---|---|
| **A** | Low | Low | Low | No | N/A | High | High | |
| **B** | Low | High | High | Yes | Long | High | Low | |
| **C** | Low | Low | Low | No | N/A | Low | Low | |
| **D** | High | High | Low | No | N/A | High | Low | |
| **E** | Low | Low | Low | Yes | Short | Low | Low | |
| **F** | High | Low | High | No | N/A | Low | Low | |
| **G** | High | High | High | Yes | Long | High | High | |
| **H** | Low | High | High | No | N/A | Low | High | |
| **I** | High | High | Low | Yes | Long | Low | High | |
| **J** | High | Low | High | Yes | Short | High | High | |
| *** Where 1 means the LOWEST Risk and 10 the HIGHEST Risk** | | | | | | | | |

**Table 2.2 -** Epidemiological factors hypothesized to influence the risk for African Swine Fever (ASF) were categorized as dichotomous variables considering the values observed in selected countries and regions.

| **Risk factor** | **Categories and explanations** | **Reference values** | **Data source** |
|---|---|---|---|
| Pig density (heads/km$^2$) | ≤2 – LOW<br>>2 - HIGH | a. Mongolia: from 0.02 to 0.1 with a mean of 0.05±0.03<br>b. Russian Federation: from 0 to 168.6 with a mean of 9.5±19.4<br>c. China: from 0 to 363 with a mean of 133±103 | Gridded Livestock of the World (GLW 3).<br>Gilbert et al., 2018. [142] Available at: https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/33N0JG |
| Estimated wild boar density (heads/km$^2$) | ≤0.03 – LOW<br>>0.03 – HIGH | a. Mongolia: from 0.01 to 0.05;<br>b. Russian Federation: from 0 to 0.3 with a mean of 0.04±0.05<br>c. China: from 0 to 2.3 with a mean of 0.2±0.4<br>d. Most of European countries: from 0.5 to 10 | Mongolia, Russian Federation and Europe: Pittiglio et al., 2018 [129]<br>China: Hongxuan, 2014 [143] |
| Backyard farming share (share of the pig population kept in backyards) | ≤10% - LOW<br>>10% - HIGH | a. Russian Federation: 16.5%;<br>b. China: 35%<br>c. Georgia: close to 100% | Russian Federation: Federal State Statistic Service (https://eng.gks.ru/) [144]<br>China: Cheng et al., 2011 [145]<br>Georgia: Beltran-Alcrudo et al., 2018 [146] |

| | | | |
|---|---|---|---|
| Border length with an ASF-infected country (km) | ≤200 – LOW<br>>200 – HIGH | a. between Belgium and Germany ~110 km<br>b. between Ukraine and Poland ~400 km;<br>c. between Russian Federation and China ~3000 km; | Data: Esri Data and Maps, 2020. [147] Computed with ArcGIS |
| Road density – density of major automobile routes ($km^{-1}$) | ≤0.1 – LOW<br>>0.1 – HIGH | a. Mongolia: from 0.003 to 0.037<br>b. Russian Federation: from 0.001 to 0.183<br>c. China: from 0.05 to 0.31<br>d. USA: from 0.002 to 0.45<br>e. Poland: from 0.07 to 0.53 | Data: Esri Data and Maps, 2020. [147] Computed with ArcGIS |
| Human population density ($persons/km^2$) | ≤10 – LOW<br>>10 – HIGH | a. Mongolia: from 0.28 to 9.3<br>b. Russian Federation: from 0.37 to 345<br>c. USA: from 0.4 to 409<br>d. Poland: from 46 to 806<br>e. China: 198 to 5597 | Gridded Population of the World (GPW), v4.10 (Center for International Earth Science Information Network – CIESIN, 2018) [148] |

**Table 2.3 -** Comparison of number of oblasts/districts, and country extension (area and world rank) between Kazakhstan and ASF infected countries in Eastern Europe and central Asia. Kazakhstan uses districts and the other countries uses regions/oblasts.

| Country | Number of admin 2 units (oblasts/districts) | Area (sqkm) | Area (world rank) |
|---|---|---|---|
| Bulgaria | 28 | 110,993 | 103 |
| Estonia | 15 | 45,227 | 129 |
| Germany | 38 | 357,114 | 63 |
| Hungary | 20 | 93,036 | 108 |
| Latvia | 119 | 64 589 | 122 |
| Lithuania | 10 | 65,301 | 121 |
| Moldova | 47 | 33,846 | 135 |
| Poland | 16 | 312,696 | 69 |
| Romania | 42 | 238,391 | 80 |
| Russia | 82 | 17,098,246 | 1 |
| Serbia | 25 | 88,361 | 111 |
| Slovakia | 8 | 49,034 | 127 |
| Ukraine | 27 | 603 549 | 44 |
| Mongolia | 22 | 1,564,110 | 18 |
| Kazakhstan | 173 | 2,724,900 | 9 |

Source: United Nations Statistics Division [124]

**Table 2.4 -** Distributions of district and regions/oblasts for the countries considered as candidate countries for model validation for predicting the risk of introduction of ASF virus in Kazakhstan.

| Variables (Risk factors) | Value | Kazakhstan | Ukraine | Russia | Mongolia | Germany | Poland |
|---|---|---|---|---|---|---|---|
| Backyard farming share | High | 58 | 25 | 55 | 20 | 22 | 16 |
| | Low | 115 | 2 | 27 | 2 | 16 | 0 |
| Human pop. Density | High | 41 | 27 | 56 | 3 | 38 | 16 |
| | Low | 132 | 0 | 26 | 19 | 0 | 0 |
| Estimated wild boar density | High | 34 | 25 | 41 | 9 | 36 | 16 |
| | Low | 139 | 2 | 41 | 13 | 2 | 0 |
| Domestic pig density | High | 12 | 25 | 43 | 1 | 34 | 16 |
| | Low | 161 | 2 | 39 | 22 | 4 | 0 |
| Road density | High | 40 | 3 | 4 | 0 | 38 | 12 |
| | Low | 133 | 24 | 78 | 22 | 0 | 4 |
| Share Border with ASF infected country | Yes | 52 | 8 | 7 | 8 | 5 | 3 |
| | No | 121 | 19 | 75 | 14 | 33 | 13 |

**Table 2.5 -** Association between selected epidemiological factors and risk for introduction of African Swine Fever (ASF) into a free country located in an infected region, as suggested by elicitation of expert opinion through a conjoint analysis model.

| | **Coefficient** | **CI (95%)** | **Odds ratio** | **Std. Error** | **p-Value** |
|---|---|---|---|---|---|
| Pig density (high) | 3.39 | 2.27, 4.52 | 33.3 | 0.58 | <0.01 |
| Estimated wild boar density (high) | 3.4 | 2.28, 4.52 | 33.3 | 0.57 | <0.01 |
| Backyard farming (high) | 4.16 | 3.00, 5.33 | 50 | 0.59 | <0.01 |
| Share border (yes) | 2.34 | 1.49, 3.19 | 10.4 | 0.43 | <0.01 |
| Road density (high) | 0.67 | 0.08, 1.44 | 2 | 0.39 | 0.083 |
| Human density (high) | 0.55 | 0.2, 1.3 | 1.8 | 0.38 | 0.148 |

# CHAPTER 3 - Risk for introduction of Classical Swine Fever into the State of Mato Grosso, Brazil

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## 3.1 Chapter summary

Classical Swine Fever (CSF) is considered one of the most important diseases of swine because of the far-reaching economic impact the disease causes to affected countries and regions. The State of Mato Grosso (MT) is part of Brazil's CSF-free zone. CSF status is uncertain in some of MT's neighboring States and countries, which has resulted in the perception that MT is at high risk for the disease. However, the risk for CSF introduction into MT has not been previously assessed. Here, we estimated that the risk for CSF introduction into the MT is highly heterogeneous. The risk associated with shipment of commercial pigs was concentrated in specific municipalities with intense commercial pig production, whereas the risk associated with movement of wild boars was clustered in certain municipalities located close to the State's borders, mostly in northern and southwestern MT. Considering the two pathways of possible introduction assessed here, these results demonstrate the importance of using alternative strategies for surveillance that

target different routes and account for different likelihoods of introduction. These results will help to design, implement, and monitor surveillance activities for sustaining the CSF-free status of MT at times when Brazil plans to expand the recognition of disease-free status for other regions in the country.

## 3.2 Introduction

Classical swine fever (CSF), also referred to as hog cholera, is arguably one of the most important viral disease affecting domestic and wild swine, and for that reason, the disease is notifiable to the World Animal Health Organization (OIE). CSF's impact on the swine industry is associated with the mortality and reduction of productivity caused by the disease and, most importantly, with disease-related trade restrictions, which results in important economic and social consequences for infected areas [23][149][150]. CSF is caused by an enveloped RNA virus of the genus Pestivirus of the family Flaviviridae referred to as CSF virus (CSFV). The most common routes for CSF spread include oronasal transmission through direct or indirect contact with infected pigs, consumption of pig meat infected with the virus, and vertical transmission from an infected sow to her offspring [151][22][152].

Sixteen (15 States and one Federal District) of the 27 administrative units of Brazil have been recognized by the OIE as CSF-free since May 2016; those 16 administrative units constitute the majority of the country's national pig production. The State of Mato Grosso (MT) is the fifth largest pig producer in the country, with 2,590,872 head corresponding to approximately 8.7% of the Brazilian pig herd and is located in the CSF-free zone of

Brazil. Most (n=1,933,248 pigs, 74.6%) of MT's pig population is concentrated on 1.3% (n=550) of the premises registered as commercial pig farms in the State, whereas the remaining 657,624 (25.4%) pigs are located in 43,398 backyard (subsistence) farms [153]. There are also 7 multiplier farms in MT, and commercial operations are divided into farrow-to-finish, sow, and finishing farms. Commercial pig farms are highly concentrated in municipalities at the central-northern region of the state. Although CSF has never been reported in MT, the State is adjacent to the non-CSF-free zone of Brazil in the north (States of Amazonas and Para) and Bolivia (where the CSF status in uncertain) in the southwest. For that reason, there is a perception among MT swine producers that the State is at high risk for the introduction of CSFV. Additionally, the last CSF outbreaks reported in Brazil (2009, and 2018/2019/2020) affected backyard pig farms in the non-CSF-free zone [33], increasing concerns among swine farmers in the CSF-free area.

Free roaming of CSF-infected wild boars, which are considered an exotic and intruder species in the Brazilian territory [154], may result in the introduction of CSF into MT. Additionally, although the movement of pigs and pork products is only allowed between states in the CSF-free zone, the CSF-free zone is quite extensive and includes a number of Brazilian States. For that reason, if an outbreak occurs in a State other than MT, there are chances that infected pigs may be moved into MT prior to the time of outbreak detection, when animal movements would be banned.

Risk assessment is an epidemiological tool frequently used by countries to assess the risk for transboundary animal diseases (TADs) such as CSF, African swine fever (ASF), and foot and mouth disease (FMD). Many studies have been developed to assess the risk of

introduction of these diseases into free areas, mostly through movement of live animals or animal products as part of international trade, which is one of the reasons for performing risk assessments according to the OIE. In the early 2000s, most published risk assessments were related to FMD and CSF and considered pathways such as pig movement, pig products (semen, pork), and fomites [149], [155]–[157]. After the incursion of ASF into Georgia in 2007, many risk assessments were performed for ASF introduction into free areas, and wild boars started to be included as potential pathways [107], [111], [158], [159]. Risk assessments are most frequently performed at the national-level to propose risk mitigation actions associated with international contacts, but for countries in which regulations are implemented by States rather than federal governments, such as Brazil, there are also benefits in estimating the risk at the subnational level [157].

The objective of the study here was to rank MT municipalities in terms of their risk for CSFV introduction either through wild boar movements or through legal movement of commercial pigs, and to compare those ranks to evaluate the correlation at the municipality-level risk of entry through those two pathways. The results will help to inform the design of surveillance strategies and allocation of resources in MT with the ultimate objective of preventing or early detect a hypothetical introduction of CSFV into the State.

## 3.3 Material and Methods

### 3.3.1 General approach

The risk for CSFV introduction into MT through two alternative pathways, namely i) the movement of live pigs assuming a hypothetical CSF outbreak in the CSF-free zone of Brazil, and ii) free ranging of wild boars, described in the following sections, was assessed at the State and municipality levels in MT. Municipalities were subsequently ranked in terms of the risk associated with each pathway, and ranks were compared to evaluate the correlation between pathways.

### 3.3.2 CSFV introduction through movement of live pigs - assessing risk for commercial farms

#### 3.3.2.1 Animal data sources

Official data from MT's Official Veterinary Service (INDEA/MT) regarding the legal movement of pigs into MT from 2016 through 2018 were used [153]. All shipments originated from CSF-free States, given that pig movements from non-CSF-free States are banned, were retrieved from the INDEA/MT database. Movements for slaughtering and/or fair purposes were screened-out because slaughter is a dead-end for disease transmission and pig fairs are rare in MT. Subsequently, only between-farm movements were considered for the analysis.

### 3.3.2.2 Analytical framework

A stochastic risk assessment model was fitted to estimate the probability of introduction of CSFV into MT via movement of live pigs during a 1-year time period, which was assessed both at the State and municipality levels. For the estimate of risk at the State level, we considered the total number of pigs that were shipped to MT from States that are part of CSF-free zone and, hence, allowed to trade with MT, given the hypothetical scenario of one undetected epidemic on the CSF-free zone of Brazil. For the probability of introducing the disease into any municipality of MT, we considered the number of animals that were shipped into each municipality of MT. The annual risk for CSF introduction into MT farms through pig movements (Rpm) was quantified assuming a binomial model of the form

$$\text{Rpm} = 1 - (1 - Psm)^{Nsm}$$

where N*sm* was the number of pigs shipped from the CSF-free zone into each municipality *m* of MT before detection of the outbreak in the free zone; for the estimates at the State level, the total number of pigs shipped into MT was used. P*sm* was the probability of introduction of one infected animal. The value of P*sm* was the same for each municipality *m* and for the state of MT, and it was computed as the product of four conditional probabilities (P1 to P4) describing the nodes of the risk pathway, which were modeled in a scenario tree [158][160][155]. Nodes were parameterized (Table 3.1) following principles explained in detail elsewhere for selecting distributions [161], and the approach was similar to risk assessments done for introduction of CSF [156] and FMD [149] in Spain, and ASF [158] in European Union.

The first node (P1) of the scenario tree [162] was the probability of importing an infected commercial pig from the CSF-free zone during the silent phase of the epidemic, i.e., before detection of the Official Veterinary Service (OVS) in the origin (the CSF-free zone of Brazil). A Beta distribution was used to calculate this probability, of the form α1 =NI+1 and α2= NT-(NI+1), where NI was the "*Number of pigs, expected to be infected in the free-zone before the detection of the outbreak*", and NT was the "*Population of commercial pigs in the CSF-free zone (NT)".* The calculation of these parameters is described later in this section.

The second and third nodes, denoted as P2 and P3, were the probabilities that the infected pig survived infection and shipment, respectively, for which we used a Pert distribution parameterized with data extracted from the literature [156] [149].

The last node of the scenario tree (P4) represented the probability that an infected imported pig established contact with a susceptible pig in a farm in MT, causing a CSF outbreak, i.e., assuming a failure of quarantine and detection by OVS at the municipality of destination *m*. This probability was calculated as 1-Pq*Pd, where Pq was the probability of quarantining the animal at the destination, and Pd was the probability of detecting the disease during that quarantine [156] [163].

For the calculation of the "*population of commercial pigs in free zone (NT)"* variable, we used a normal distribution (Normal μ, σ), considering as mean (μ) the total number of pigs in commercial pig farms at the CSF-free zone in 2017, except MT, and σ was the standard deviation of the total number of commercial pigs at the CSF-free zone during the period of 2014 – 2017. This input was one of the components used to calculate P1.

Data required to estimate the parameter NT was obtained from the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA/BR) [164].

Values for the "*total number of commercial farms - herd number (NH)*" variable were calculated using a normal distribution (Normal μ, σ), considering as mean (μ) the total number of commercial pig farms at the CSF-free zone in 2017, and σ was the standard deviation of the total number of commercial pig farms in the period of 2014 – 2017. This input was used to calculate the average herd size (H). Data required to estimate the parameter NH was obtained from the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA/BR) [164].

The variable *"Number of pigs, expected to be infected in the free-zone before the detection of the outbreak (NI)"* was calculated by the equation IP * H* EO, where intra-herd prevalence (IP), the average herd size in the CSF- free zone (H), and the number of expected undetected outbreaks at the origin (EO) were multiplied to generate the number of pigs expected to be infected at the CSF- free zone during an outbreak in the silent phase (NI), that is, before the detection of the index case of CSF by the OVS in the origin [156]. The parameters *average herd size (H), intra-herd prevalence (IP), and expected undetected outbreaks (EO)* were calculated as explained in the following paragraphs.

Because states in the CSF-free zone have the same sanitary status regarding CSF, and they are allowed to trade pigs between them, we assumed the CSF-free zone as one single unit, whereas the risk for introduction of CSF was stratified for each municipality of destination *m*. The *average herd size (H)* was approximated as the *NT/NH* ratio in the CSF-free zone.

The "*intra-herd prevalence (IP)*" was calculated using a Pert distribution; although the incubation period of CSF is generally 4-10 days, under field conditions, CSF is expected to show unspecific symptoms at the beginning of an outbreak, which can delay the detection of infected herds in 2-4 weeks, increasing the intra-herd prevalence at the moment of detection by the OVS [1], [24].

The "*expected undetected outbreaks (EO)*" was defined as the number of herds that would be infected by the time when a hypothetical epidemic in the CSF-free zone was detected and pig movements into MT banned. EO was assumed to follow a Pert distribution with a minimum of 1 undetected outbreak (the index case), and the most likely and maximum equal to the number of herds that were affected before the detection of the CSF epidemics in Spain in 2001, and in The Netherlands in 1997, respectively [156].

To adjust the number of pigs that would be sent to MT between the beginning and detection of the outbreak in the CSF-free zone, we estimated the *Time-to-detection (Td)*, i.e., the length in days before the epidemic is detected and movements into MT are banned. Under field conditions, the detection is expected to take longer than the incubation period. A Pert distribution was used for modeling *Td*, with the minimum, most likely, and maximum values being those reported in Colombia, in Ceará (a State of Brazil in the CSF non-free zone in which outbreaks occurred in 2018), and the recommendation of the European Union on the parameter that should be used when there is no information available, respectively [33], [155], [165].

The number of pigs that were shipped from the CSF-free zone was estimated considering the number of pigs that came from States *s* into MT and into each municipality *m* of MT

during the years 2016 – 2018. For each municipality of destination *m* during the period of the study, we grouped the movement from 2016 to 2018 and computed the mean (μ) and standard deviation (σ) of the total number of pigs that were sent to each municipality *m* from States of origin *s*, during this period [153]. Then, the number of pigs annually shipped into MT and into each municipality m (n) from the CSF-free zone was assumed to follow Poisson -LogNormal distributions, with mean (μ) and standard deviation (σ) estimated for MT and for each municipality *m*, respectively. The number of pigs that each municipality *m* of MT received is listed in the Supplementary material. The number of pigs that would be shipped from the CSF-free zone before detection of the outbreak in the free zone (N*sm* ) was subsequently computed for MT and for each municipality *m* as the number of pigs shipped per day (n/365) multiplied by the time-of-detection (in days) of an outbreak in the CSF-free zone (Td), so that:

N*sm*= (*n*/365) *Td.

A spider graph was generated in Excel to evaluate what parameters (Table 3.1) mostly contributed to changes in the mean risk for the introduction of CSF into MT, i.e., assessing the sensitivity of the model to the uncertainty and variability associated with its parameterization. For that sensitivity analysis, we selected the first (Q1), second (Median – Q2), and third quartile (Q3) for the distribution of each parameters evaluated, i.e., P1, P2, P3, P4, Td and *n*. The median was the measure of central tendency, and Q1 and Q3 were measures of dispersion. We systematically calculated the final risk probability with different situations for each parameter at a time when the others were kept fixed in the median (second quartile) as the central tendency.

#### 3.3.2.3 Computational environment and software

The stochastic model was implemented in the @Risk 8.0 software [166] and run through 10,000 iterations. Results were spatially visualized using Arc GIS version 10.5.1. [137].

### 3.3.3 CSFV introduction into MT through wild boars– assessing risk for backyard farms

#### 3.3.3.1 Animal data sources

Pig farms registered in the INDEA/MT database by July 2019 were retrieved, including data on type of farms (subsistence, commercial), their geographic location, and the total number of pigs per farm [153].

Additionally, data regarding active surveillance activities for CSF in MT pig farms from 2016 to 2018 were retrieved to determine the presence or absence of free-range wild boars at those premises. Records of visits were organized in a dataset, and records repeated on any given farm were removed manually. Presence of wild boars was reported in 1,688 (24.7%) of the 6,827 visited farms [153]. Data were used to estimate the distribution of wild boars fitting a Maximum Entropy (MaxEnt) model and procedures described elsewhere [167]. Briefly, farms in which wild boars were reported were geolocated. Then, data on 27 environmental layer variables assumed to influence the presence of wild boars population in MT were retrieved, including 19 rasters from the WorldClim online database for the period 1970-2000 at a spatial resolution of 5 arc-minutes (~ 10 km). These variables (WorldClim) are derived from records of temperature and precipitation. Consequently, it is possible for at least some of those 19 variables to be

highly correlated with each other, potentially leading to issues with collinearity; for those reasons, there is a need to remove highly correlated variables from the final model [168]. The human influence or anthropogenic impact was approximated using the human footprint raster obtained from the Socioeconomic Data and Applications Center from Wildlife Conservation (WCS) and Center for International Earth Science Information Network (CIESIN) – Columbia University. The global footprint raster is a global dataset of 1-kilometer grid cells, created from nine global data layers covering human population pressure (population density), human land use and infrastructure, and human access [169]. The variable altitude/elevation data (referred to as SRTM) was extracted using DIVA-GIS, which is a free computer program for mapping and geographic data analysis with ready-to-use downloading raster. SRTM stands for Shuttle Radar Topography Mission (SRTM) and it is a 3 arc, i.e., 30 seconds of resolution, raster created with data from the National AeroSpatial Agency (NASA) representing a near-global set of land elevations [170]. For the variables land cover, vegetation, crops, temperature, and isothermality, raster data were extract from IPUMS Terra – Integrated Population and Environmental data, which is a global-scale framework data that allowed extraction data by country-level (Brazil) [171]. The vegetation index was extracted as a product of MODIS Land Cover, which is produced by NASA, and from this was selected the specific vegetation index for MT with a 250 m of resolution [172]. The global total irradiation was acquired by downloading a raster data from Global Solar Atlas, which is published by the World Bank Group, and prepared by Solargis, with resolution of 250 m [173]. Our choice of final variables was ultimately determined by the procedure of reducing multicollinearity but keeping variables that make sense for the purpose of

detecting the wild boar population distribution in MT. Thus, a collinearity diagnostic was performed to screen-out highly correlated environmental variables. The redundant variables were identified by the Raster package in R studio [135] and removed from the model if the meaning of the variable would not hamper the final model. Subsequently, only 15 environmental variables were used in the model (Table 3.2). The prediction value generated by each geographic coordinate was summed by each polygon, which were the 141 municipalities of MT. Then, these set of values were separated by the median, and the values were set as 50% high and 50% low density. This final information regarding the high/low density for wild boar population per each municipality of MT was included in the model generated for the risk calculation of introduction of CSF in MT via wild boars and explained in detail in the analytical framework – section 3.3.3.2.

#### 3.3.3.2 Analytical framework

The assumption here was that wild boars in Bolivia and in Brazilian states outside of the CSF-free zone may carry the CSFV and pass freely through the MT borders. We also assumed that the risk at the municipality level would be influenced by the domestic pig density, wild boar density, backyard farming share, shared border with CSF-infected zone or Bolivia, road density, and human population density in the State. The values of those variables were dichotomized (high/low, or yes/no). Specifically, (a) pig density was calculated as the number of pigs in each municipality divided by the area in $km^2$ and dichotomized using the median value (50% high, 50% low). The number of commercial pigs in the municipality was included in the computation, in addition to backyard pigs, to account for the probability of contact between backyard pigs and commercial pigs, and

because backyard farming was specifically included as a separate variable, thus, accounting for that factors in the computations [174]. (b) Backyard farming share was calculated as the number of backyard farms divided by the number of farms per each municipality and dichotomized using the median value (50% high, 50% low); this risk factor can play an important role in the dynamic of CSF due to low biosecurity and little interaction with veterinary services [25]. Values for calculation of (a) and (b) were extracted from the database of the MT OVS [153]. (c) Human density was calculated as the population estimated in the last national census conducted in 2010 [175] for each MT municipality, divided by the area ($km^2$) of each correspondent municipality of MT, and dichotomized using 5 habitant/$km^2$ as the threshold (high, low); the 5 habitant/km threshold was set up because it was the approximate midpoint between the median (2.29 habitant/$km^2$) and mean (6.76 habitant/$km^2$ ) densities and that resulted on an acceptable 1:3 ratio for the classification of districts as high or low density –alternatively, the use of the mean and median as cut-off values for the classification did not affect the results of the regression model (data non shown). Human density was included as a proxy for the movement of people (tourists or workers) that can carry contaminated food that can be disposed and accessed by wild boars [36], [165], [176], [177]. (d) Road density was calculated using ArcGIS, considering the layers of municipalities and layers of roads of MT and dichotomized using the median (50% high, 50% low); road density was included because the introduction and spread of the disease may be influenced by human activities that could increase the risk for contacts with wild boars [25]. (e) Wild boar density was estimated aggregating the results of the maximum entropy (MaxEnt) (described at Section 3.3.3.1 of this manuscript) prediction model at the municipality level and

dichotomized using the median (50% high, 50% low). Wild boar density is important not only because of the susceptibility of wild boar to CSF infection, but also because if infected, populations of wild pigs may be the primary source for CSF introduction in domestic pig herds [36]. Dichotomization of the variable was required to incorporate it in the regression model and also, to increase accuracy of the Maxent predictions. (f) Shared border with a non-CSF-free state or country was estimated using ArcGIS and dichotomized (yes, no). The relative contribution of each variable to the final risk was assumed to be similar to the weight estimated by a panel of experts for the risk of introduction of African swine fever (ASF) into a free region from a neighboring infected country described in detail elsewhere [177]. Briefly, the model approach was based on a factorial design to identify 10 representative scenarios of the combination of parameters hypothesized to influence the risk for introduction of ASF (domestic pig density, wild boar density, backyard farming share, share border to a country that is infected with ASF, road density, and human density). Each scenario was referred to as hypothetical Region A to hypothetical Region J (n=10) representing different epidemiological conditions. International experts, which were chosen by snowball sampling technique after consultation with the OIE reference laboratories for ASF in Spain, the UK, and the National Reference Laboratory of the Russian Federation, were requested to rank the 10 hypothetical scenarios in terms of their likelihood of serving as port of entry for ASF into the country, where 1 meant the lowest risk, and 10 meant the highest risk for introducing the disease in districts of a free country, and the hypothetical scenarios were categorized by a combination of dichotomized (high/low -yes/no) risk factors listed before. An ordinal logistic regression model was fitted to estimate the relative weight that the experts

implicitly gave to each of the variables (pig density, backyard farming share, human density, road density, wild boar density, and share border with a non-CSF-free region), as approximated by the value of the regression coefficients. A risk score of the introduction of CSF through wild boar (Rbm) was subsequently computed assuming an increase by factors of Rbm = $\beta_0$ + 3.39*pig density + 4.16*backyard farming share + 0.55* human density +0.67* road density + 3.4* wild boar density + 2.34* share border with non-CSF-free region for municipalities categorized as high (or yes), compared to those categorized as low (or no). Rbm was computed for each of the 141 municipalities in MT as the sum of the dichotomized values of the risk predictors weighted by increase in risk assumed for each of the factors. Finally, municipalities were ranked in terms of the computed value of Rbm.

#### 3.3.3.3 Computational environment and software

The MaxEnt software [178] was used for computing the maximum entropy model of wild boar distribution. The correlation between environmental layers was conducted in RStudio Team (2019) version 3.5.3 [135] using “raster” and “rgdal” packages, the packages “MASS”, “tidyverse” and “ggbeeswarm” were used for performing the ordinal logistic regression to generate the proxy-risk for introduction of CSFV in MT considering the model developed by ASF risk prediction for Kazakhstan [177]. ArcGIS 10.5.1 [137] was used for spatial data processing and mapping data and results.

### 3.3.4 Correlation between pathways

The correlation between the two pathways for the risk of introduction of CSF into MT was computed using a Spearman correlation test ($R_s$) as

$$R_s = 1 - \frac{6\Sigma(R_i - S_i)^2}{n(n^2-1)}$$ [179]

where $R_i$ was the rank for the value $x_i$, which was the mean risk generated by risk assessment for the introduction of CSFV through movement of commercial pigs (Rpm), $S_i$ was the rank for the value $y_i$, which was the risk score generated by the assessment for the introduction of CSFV through movement of wild boars (Rbm), and n was the number of observations, i.e., the number of municipalities in MT (n=141). The correlation was implemented in the RStudio Team (2019) version 3.5.3 software [135] using the statistics base-package “cor.test (x, y, method = "spearman", exact=FALSE)”.

Additionally, municipalities were categorized as low or high risk for each of the two pathways assessed. For the risk of introduction through movement of live pigs we used 0.01 as the cut-off value because values lower than that would mean that, on average, one would expect one outbreak every 100 epidemics in the CSF-free zone, which is also relatively unexpected. For that reason, values < 0.01 were assumed to represent negligible risk for this pathway. For the risk of introduction through wild boars, the median was used as a cut-off value to be able to divide the municipalities of MT as the 50% low and 50% high proxy-risk, allowing a conservative comparison. Both dichotomizations were subsequently combined to group municipalities into four categories, representing high risk to both, either (two groups), or none of the pathways.

## 3.4 Results

The risk associated with the legal movement of pigs (Rpm) was heavily concentrated, with five (3.5%) municipalities accounting for 96% of the total risk and much of the risk clustered in the central districts of MT (Figure 3.1). The risk was higher than the threshold (0.01) in only 6 municipalities, but it was relatively high (>0.1) in 5 of those 6. In contrast, the risk was nil for most (n=89, 63.1%) municipalities (Figure 3.1, districts in white) because they did not receive any pigs from outside MT from 2016 through 2018. The mean risk of introduction into MT (0.763 - 95% CI [0.21 – 1.0]) suggests that, in the scenario of a hypothetical outbreak in the CSF-free zone of Brazil and assuming that time-to-detection of the first outbreak would be similar to those observed in other epidemics, it is likely that MT would suffer an outbreak. The model was most sensitive to variations in the probability of importing an infected pig (P1) and the time-to-detection of the outbreak by the OVS at the origin (Td), followed by the probability of the pigs survive the infection (P2) and the number of pigs shipped into MT (*n*), respectively (Figure 3.2).

The maximum entropy algorithm calculated the distribution of the wild boar population in MT using 1,048 observations of wild boar as a training data and 261 observations as a testing data from the total of 1,688 observations captured from active surveillance performed by OVS of MT from 2016 to 2018. 379 observations were excluded from the model due to issues with the geographic coordinates collected during the surveillance activity. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was 0.765 for testing, with 0.014 of standard deviation, which was considered an acceptable accuracy. Although wild boars were predicted to be distributed throughout the

state (Figure 3.3 – A), most of the risk associated with CSFV introduction through free roaming of wild boars (as approximated by the value of the risk score Rbm) was concentrated in northern and southern districts of MT (Figure 3.3 - B). Eight municipalities were estimated to be at the highest risk for introduction of CSF through wild boars and these municipalities are bordering the non- CSF- free zone in the north of MT, and Bolivia in the southwest (Figure 3.3- B, hatched areas).

The municipality-level risk for introduction of CSFV via movement of domestic pigs was poorly correlated (Spearman correlation coefficient, $R_s$=0.11, p-value=0.185) with the risk associated with free roaming of wild boars. Only five municipalities (four of them located in the central part of the State) were estimated at highest risk for introduction of CSF into MT through both pathways (Figure 3.4).

## 3.4 Discussion

The work here characterized the risk associated with, arguably, two of the most important routes for introduction of CSF into MT, Brazil. We used these results to generate maps that depicted the spatial distribution of risk and identify municipalities that are most vulnerable to each of the assessed routes. Movement of live animals is one of the main routes for disease introduction into free areas [158]. Other routes of introduction of CSF, such as legal or illegal contaminated pork products, contaminated trucks due to fecal contamination, genetic material from infected pigs such as semen, and human contact due to contamination clothing [155], were not specifically assessed here and these results were restricted to the risk associated with movement of live pigs and wild boars. For the

computation of the risk associated with wild boars, however, certain variables that may serve as proxy for unassessed routes, such as human and road density, were included in the model, which may have helped, in part, to account for that risk. If an outbreak occurs in the CSF-free zone, the economic impact will be devastating. In 2018, when some outbreaks in Brazil were detected in the CSF-non-free zone, the Confederation of Agriculture and Livestock of Brazil (CNA) estimated an impact of US$ 230 to US$ 790 million if the infection reached the free zone of CSF in Brazil [36]. For the risk assessment of introduction of CSFV in MT through movement of live pigs (Rpm), we considered a hypothetical scenario of an ongoing CSF outbreak in any other Brazilian State that is part of the CSF free-zone, with the intention to estimate the risk that MT would become infected were this to occur. The CSF-free zone is quite extensive and the OVS of each State has its own surveillance system, which can impose variations for the time of detection outbreak, and this is a factor out of the control of MT. Ultimately, these results will help to evaluate the implementation of surveillance activities in MT and the prioritization of surveillance activities in relation to the route that imposes the highest risk for any given municipality.

The legislation that MT follows regarding CSF surveillance is dictated by the Brazilian Federal government, by which active serological surveillance is conducted only bi-annually in random backyard pig farms, and in commercial farms only on months when the mortality rate exceeds the threshold for different ages or categories [180]. However, the legislation does not consider the spatial heterogeneity of the risk imposed by alternative routes of entry. In states like MT, in which there are more than 40,000 registered pig farms, but only 550 of those are categorized as commercial farms, there is

a need for specifying selective actions for municipalities, in alignment with the risk imposed by different routes, to complement the national regulation. For example, the correlation between the risk imposed by both routes was not significant ($R_s$= 0.11 , p-value=0.185), indicating that the districts estimated at highest risk for a given pathway were not at highest risk for the other route. However, because the risk for these two pathways was calculated using different methods, the raw values are not comparable. This finding is consistent with the need for enforcing different policy for districts regarding the design of surveillance and early detection strategies to prioritize practices associated with the routes that impose the highest risk to the municipality.

The Rpm, which was estimated assuming an undetected outbreak in the CSF-free zone of Brazil, was highly clustered in the central part of the State, where the largest pig farms in MT are located (Figure 3.1), with 5 municipalities concentrating 96% of the risk. A similar result was obtained in Spain, where risk was also concentrated in few provinces and in relation to those locations in which pig production is highly concentrated [156]. Similarly to a study conducted in Denmark, the risk associated with animal movements was relatively low, due to the small number of imported pigs [155]. Another study had similar results, with overall low risk probability for introduction of ASF/CSF into the US via legal import of pigs and pig products, and the highest values for the probability of introduction were concentrated in three US States traditionally associated with pig production [32]. In MT, only a few municipalities account for most of the pigs moved from out of the State, and only those municipalities showed high mean risk probability. Thus, targeting a relatively low number of farms in those specific municipalities, for example, through enhanced passive surveillance protocols, would help to design

surveillance strategies that account for most of the risk of introduction into MT through that route.

The sensitivity analysis showed that time-to-detection (Td) highly influences the risk. Because Td is expected to be the same for all municipalities, the variability of Td is not expected to affect the ranks estimated here. However, because the variability of Td may affect the likelihood of an outbreak, the sensitivity of results to the variability of the parameter contributes to highlighting the importance of coordination and collaboration between districts in Brazil, and the impact that early detection has in the mitigation of the impact of epidemics.

Although certain municipalities at the borders were found at highest risk for the introduction through wild boars (as approximated by the value of the risk score Rbm), we found that certain municipalities at the central region of MT were also at high risk, likely because of the combination of a number of factors such as high density of humans and pigs and presence of wild boars that would increase risk. The model used for the calculation of the risk in this pathway may outweigh the lack of a shared border with CSF- free areas, and the model did not require a shared border with CSF-free areas to have a negligible risk from this pathway. Certainly, some believe that the biggest challenge in maintaining a free or controlled area for CSF is for the OVS to be able to enforce control and eradication measures on subsistence pig farms [151]. In those municipalities, surveillance efforts may be directed towards education and outreach actions involving small holders. Those outreach and education actions may be particularly challenging in MT, given that informal reports and anecdotal evidence suggest some backyard pig owners let sows commingle with wild boars to generate a

strongest offspring, which increases the risk for CSF introduction. Wild boars play an important role in the environmental maintenance of CSF and its transmission to domestic pigs. In CSFV-infected regions in which there is a high density of wild boars, a situation of endemicity may be established [152]. Targeted and strategic hunting may be considered as an action to reduce wild boar population and support the implementation of a surveillance program using samples obtained from hunted animals.

Epidemic models have been increasingly used to evaluate and inform disease surveillance and control policies. For that reason, risk assessments are important tools that should be routinely incorporated by OVSs to support the design of risk-based surveillance activities [181]. Quantitative assessment of the risk for CSF introduction into a country or state may help the decision-making process to ultimately prevent and control disease introduction [149]. Risk assessments combining different routes of introduction broaden the scope of results, enhancing the availability of information for guiding surveillance actions [182]. Noteworthy, risk assessments are formulated considering a series of limitations and assumptions, and regular updates are required to evaluate if the conditions observed when formulating the models are still valid.

In conclusion, results here indicate that the risk for introduction of CSF into MT is spatially heterogeneous, suggesting that different approaches of targeted surveillance should be implemented in the state considering, at least, two primary objectives. On one hand, there is a need for increasing the number of OVS visits to commercial farms that receive animals from outside the state, inspecting and quarantining pigs as soon as they arrive at the farm, and considering the design of passive surveillance activities targeting the early detection of CSF-like signs in those particular farms and municipalities. On the

other hand, for districts in which risk was mostly associated with wild boars, actions like sampling hunted wild boars and implementation of surveillance and educational and outreach programs in backyard farms should be prioritized [25]. Results here will help MT to increase the efficiency of CSF surveillance, enhancing the federal rules for CSF surveillance actions, with the ultimate objective of preventing the introduction of the disease into the State.

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## 3.5 List of Figures

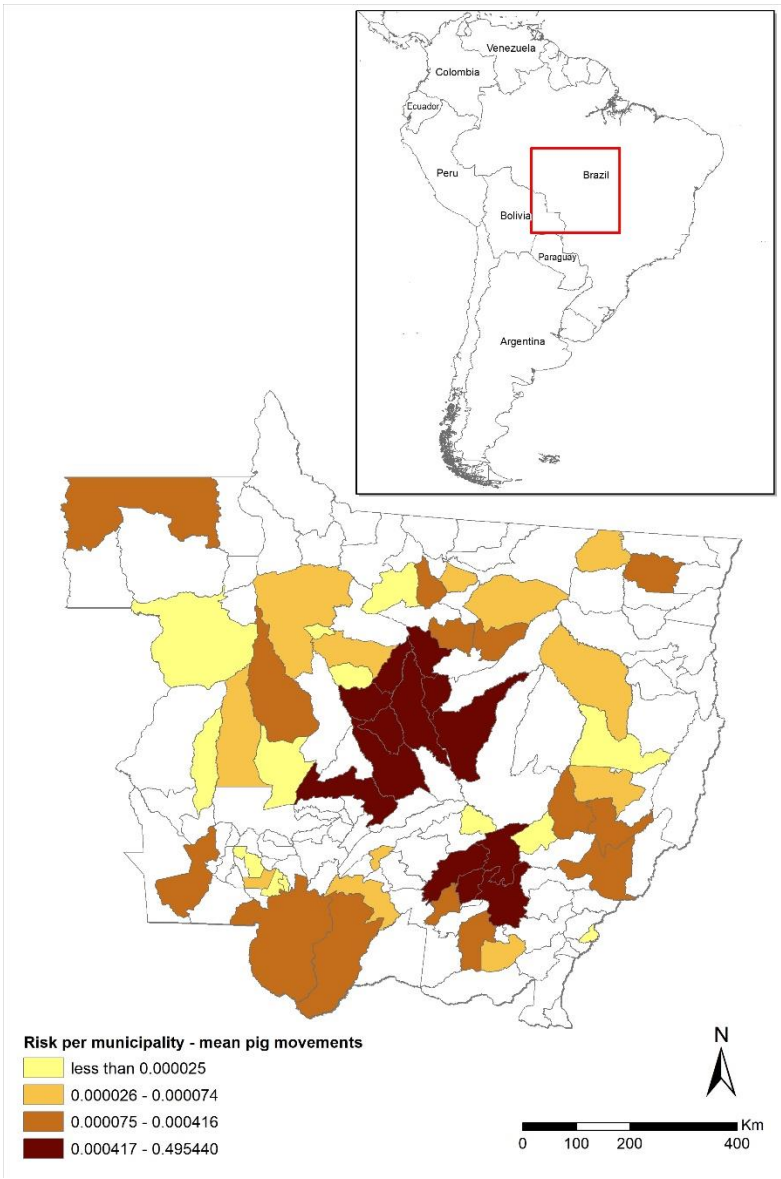


**Figure 3.1 -** Risk of CSF introduction into MT through movement of pigs (Rpm) stratified by municipality and assuming an undetected outbreak in states in the CSF-free zone that ship pigs to MT. The darker the shade, the higher the risk. Municipalities in white did not receive pigs from outside MT during the assessed three-year period. The red square shows the localization of MT in Brazil/ Latin America map.

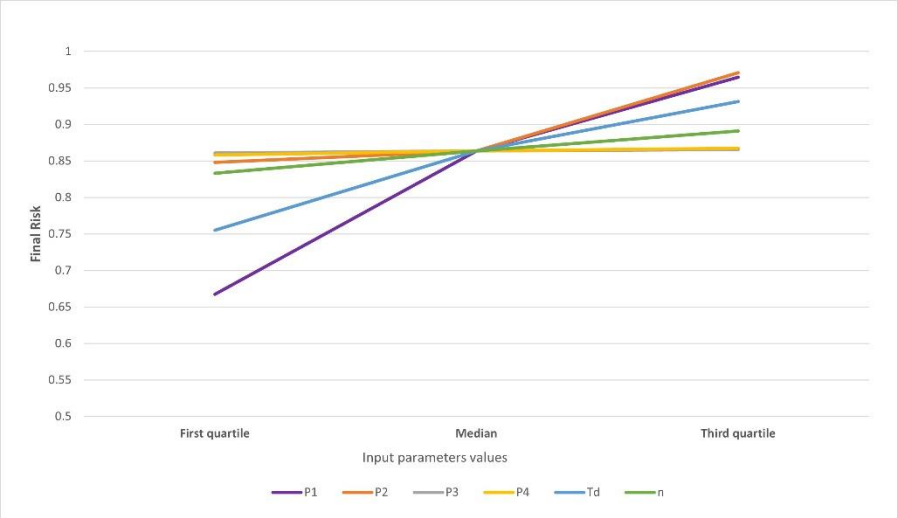


**Figure 3.2 -** Sensitivity to variations in the parameters of a risk assessment model for the introduction of CSF into MT. Model parameters are the probability of importing an infected pig (P1 - purple line), the probability that an infected pig survives the infection before the shipment to MT (P2- orange line), the probability that an infected pig survives the shipment to MT (P3- gray line), the probability that an infected imported pig established contact with a susceptible pig in a farm in MT (P4 - yellow line), the time-to- detect the outbreak (Td - blue line), and the number of pigs shipped into MT (n - green line).

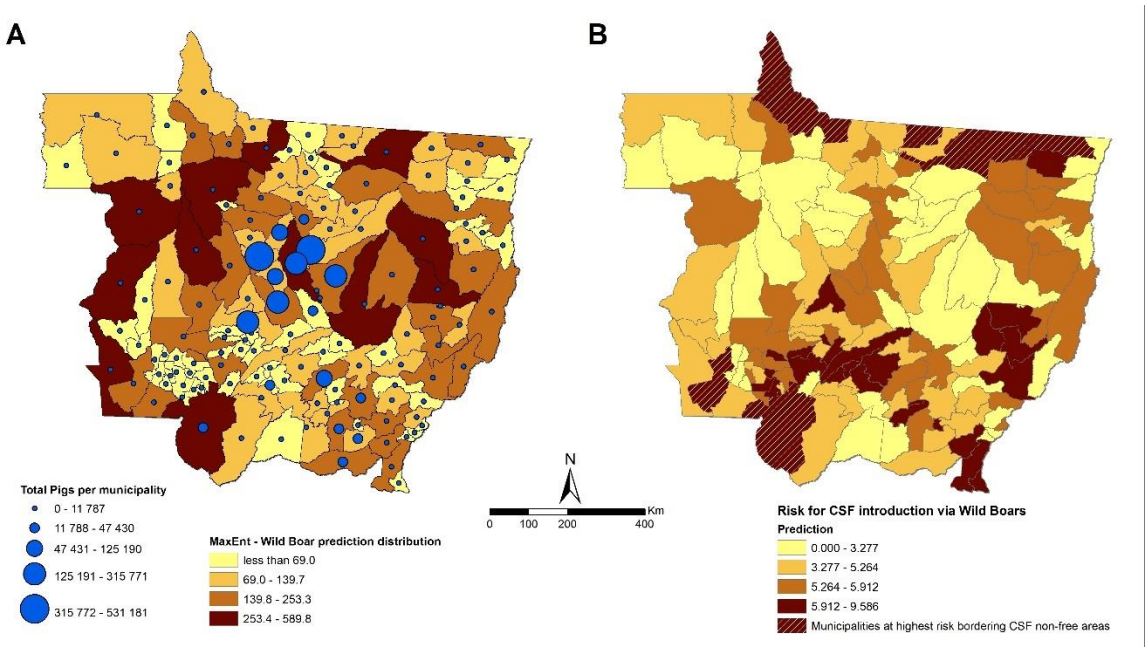


**Figure 3.3 - A** - Distribution of wild boars predicted by a maximum entropy model aggregated at the municipality level in MT (the darker the shade of the polygon, the higher the predicted value), and municipality-level number of pigs (the larger the size of the blue dot, the larger the number of pigs). **B** – Results of the model for risk scores of the introduction of CSF into MT through wild boar movement (Rbm) (the darker the polygon, the higher the risk). The hatched areas are the municipalities at highest risk bordering CSF non-free areas.

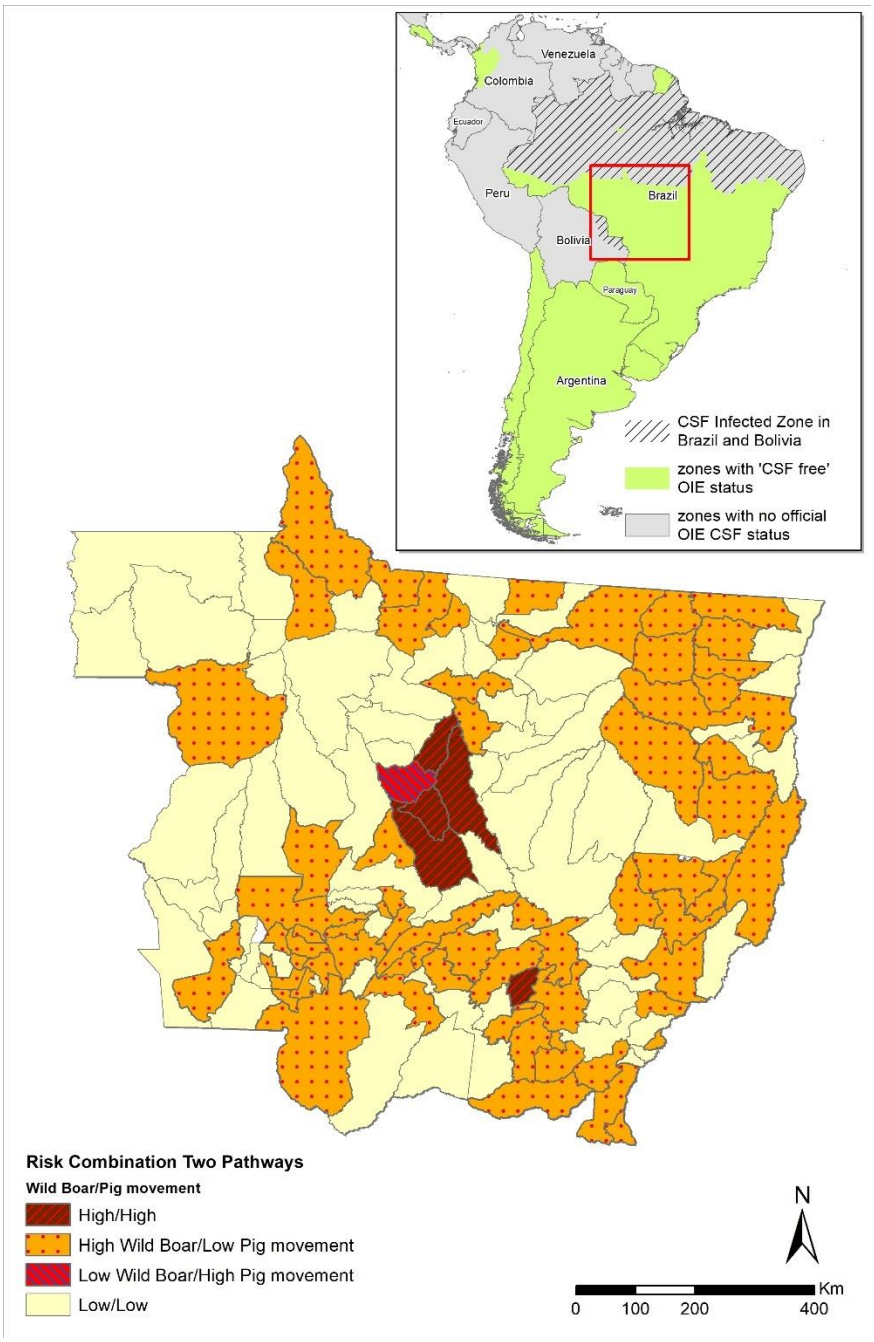


**Figure 3.4 -** Risk for the introduction of CSF into MT through legal movement of pigs and through free roaming of wild boars, estimated using a combination of risk analysis models. Municipalities were categorized as high risk for both pathways (brown with red hatch area), high risk for wild boars and low risk for commercial pig movements (orange with red dots), low risk for wild boars and high risk for commercial pig movements (pink with blue hatched area), and low risk for both pathways (light yellow). The green area in the Latin America map (up right corner) shows the CSF-free area recognized by OIE. The hatched gray area shows the non-CSF-free zone.

## 3.6 List of Tables

**Table 3.1 -** Parameterization of a quantitative assessment for the risk of introduction of Classical Swine Fever (CSF) into the State of MT, via legal movement of pigs and assuming a CSF outbreak in the disease free-zone of Brazil.

| Input | Parameter | Distribution | Value | Source of Information |
|---|---|---|---|---|
| Population of commercial pigs in free zone (NT) | NT | Normal | $\mu^{*}$: 22758504<br>$\sigma^{**}$: 1529008.972 | Database MAPA-BR (2019) [164] |
| Total number of commercial farms - herd number (NH) | NH | Normal | $\mu^{*}$: 25902<br>$\sigma^{**}$: 784.621 | Database MAPA-BR (2019) [164] |
| Average herd size (H) | H | equation | NT/NH | Model equation |
| Intra-herd prevalence (IP) | IP | Pert | Min: 0.05<br>Most likely: 0.4<br>Max: 1 | Martinez-Lopez et al. (2009) [156] |
| Expected undetected outbreaks (EO) | EO | Pert | Min:1<br>Most likely: 6<br>Max: 39 | Martinez-Lopez et al. (2009) [156] |
| Number of pigs in free zone expected to be infected before the detection of the outbreak (NI) | NI | equation | IP*H*EO | Model equation |
| Probability of importing an infected commercial pig from free zone (assuming an outbreak before detection) (P1) | P1 | Beta | $\alpha1$= NI+1 and $\alpha2$= NT-(NI+1) | Adapted from Martinez-Lopez et al. (2009) [156]; Database MAPA-BR (2019) [164] |
| Probability of infected pig surviving the infection (P2) | P2 | Pert | Min: 0.63<br>Most likely: 0.78<br>Max: 0.932 | Martinez-Lopez et al. (2009) [156] |

| | | | | |
|---|---|---|---|---|
| Probability of infected pig surviving shipment (P3) | P3 | Pert | Min: 0.908<br>Most likely: 0.9973<br>Max: 0.9995 | Murray and Johnson (1998) [183] |
| Probability of quarantine in destination (Pq) | Pq | Beta | $\alpha1$= 130.71 and $\alpha2$= 15.41 | Martinez-Lopez et al. (2008) [149]; Martinez-Lopez et al. (2009) [156] |
| Probability of detection during quarantine (Pd) | Pd | Beta | $\alpha1$= 1.33 and $\alpha2$= 34.16 | Martinez-Lopez et al. (2009) ; Mur et al. (2012) [158] |
| Probability of non-detection of infected animal at destination and of animal establishing contact with susceptible in MT farm (P4) | P4 | Equation | 1-Pq*Pd | Martinez-Lopez et al. (2009) ; Mur et al. (2012) [158] |
| Time of detection in days (Td) | Td | Pert | Min: 11<br>Most likely: 40<br>Max: 127 | Bronsvoort et al. (2008) [155]; Pineda et al. (2020) [165]; OIE -WAHIS (2020) [33] |
| Number of pigs shipped to MT (and to each municipality m) | *n* | Poisson-LogNormal | $\mu^{*}$ and $\sigma^{**}$ of number of pigs sent from States s to MT (and each municipality of destination *m (*2016-2018)) | INDEA/MT database (2019) [153] |

* Mean, ** standard deviation

**Table 3.2 -** Environmental variables used to predict the distribution of wild boars in the State of MT, using a Maximum Entropy (MaxEnt) model.

| **Type** | **Variable Name** | **Description** |
|---|---|---|
| Human Influence | hfp | Human footprint. Represents the impact of human in the environment. |
| | | |
| Climate | bio 3 | Isothermality (BIO2/BIO7) (×100) |
| | bio 7 | Temperature Annual Range (BIO5-BIO6) |
| | bio 8 | Mean Temperature of Wettest Quarter |
| | bio 13 | Precipitation of Wettest Month |
| | bio 15 | Precipitation Seasonality (Coefficient of Variation) |
| | bio 18 | Precipitation of Warmest Quarter |
| | bio 19 | Precipitation of Coldest Quarter |
| | isotherm | Oscillations day-night temperature comparing summer/winter |
| | | |
| Altitude/Elevation | bralt | Shuttle Radar Topography Mission (SRTM) with 3 arc seconds (30 seconds) of resolution |
| | | |
| Vegetation | crop | Area used as a cropland |
| | landcover | Global land cover area reference |
| | veg | Cropland/Natural vegetation mosaic |
| | | |
| Vegetation index | sdat | The vegetation index variation from the years 2000- 2001 and 2003 – 2004, specific for Mato Grosso. |
| | | |
| Solar incidence | gti | Global total irradiation |
| | | |

# CHAPTER 4 - Development and implementation of an enhanced passive surveillance protocol for the early detection of African and classical swine fevers

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## 4.1 Chapter summary

African swine fever (ASF) and classical swine fever (CSF) are transboundary animal diseases (TADs) of pigs that have shown a potential for rapid spread through regions, countries, and continents. The swine industry is globally vulnerable to ASF and CSF, and much effort and many resources are regularly put into preventing disease introduction and spread in free regions and farms. Passive surveillance activities directly performed by producers, private veterinarians, or a third party bring the highest chances for the early detection of TAD incursions because they are routinely and widely conducted by the industry and because these activities focus on the time between introduction and the time the first sample is sent for diagnostic testing which makes up the greatest proportion of the time prior to detection. One of the challenges in the design of passive surveillance

programs is standardizing the collection and interpretation of data to prompt preventive actions that could be implemented on a large scale by the industry. Here, we proposed the implementation of an enhanced passive surveillance (EPS) protocol based on collecting data through participatory surveillance actions using an objective and adaptable scoring system to aid the early detection of ASF or CSF incursions at the farm level. The EPS protocol contains three components, namely, the biosecurity background of the farm, syndromic or clinical surveillance results, and necropsy findings. The protocol was piloted for 10 weeks in two commercial pig farms in the Dominican Republic, which is a CSF- and ASF-infected country. As a first step for piloting the project in the US, 10 commercial pig farms were assessed using the biosecurity background component of the EPS protocol. In the Dominican Republic, the EPS protocol detected substantial variations in the risk score and triggered testing in one of the farms based on that variation, although the test results were negative. In the US, variations in biosecurity practices were observed among farms despite relatively standardized production practices, suggesting that biosecurity may serves as a proxy for different levels of risk to hypothetical ASF or CSF introductions. Results suggest that standardized EPS protocols may contribute to the early detection of CSF and ASF introductions by triggering sampling activities to confirm or rule out suspects, with the ultimate goal of promoting public-private partnership to mitigate the impact of TAD incursions into free regions.

## 4.2 Introduction

Transboundary animal diseases (TADs), such as African swine fever (ASF) and classical swine fever (CSF), are highly contagious and transmissible diseases with the potential for rapid spread across national borders, typically causing far-reaching losses to affected countries and regions [184]. For that reason, notification of ASF or CSF outbreaks to the World Organization for Animal Health (OIE) is mandatory. Only swine (*Suidae* family) are affected by ASF and CSF and, collectively, ASF and CSF are sometimes referred to as foreign hemorrhagic fevers (FHF) of swine by disease-free countries. Although both diseases have similar names, and cause significant disruption to the pig industry, they are caused by unrelated pathogens.

CSF is caused by a small, enveloped RNA virus of the genus *Pestivirus*, which is a member of the *Flaviviridae* family, referred to as CSF virus (CSFV). Clinical signs of CSFV infection are related to the virulence of the strain, and in the most severe form, the mortality rate can be as high as 100%, mostly in naïve populations [23],[24]. Signs are most severe in young pigs, with mortality rates averaging 80%, whereas in adult pigs CSFV infection may be sub-clinical or with mild signs, which delay or prevent the diagnosis of the disease [25], [28].

ASF is caused by a double- stranded DNA, enveloped arbovirus, which is the sole member of the *Asfarviridae* family, and referred to as the ASF virus (ASFV). Although ASF is associated with high lethality in domestic pigs, it may not be as infectious as some other relevant TADs such as foot-and-mouth disease. ASF usually spreads slowly within

the herd, and some animals may not be clinically affected especially wild pigs such as warthogs, bush pigs, and giant forest hogs [18], [19], [20].

Since the re-introduction of ASFV into Europe, through Georgia in 2007, no country has been able to eliminate the disease after it reached its domestic pig population. The sustained ASF spread through Asia and Europe, highlights the lack of success of control programs [32]. In 2021, ASFV was also detected in Central America (Dominican Republic and Haiti), reaching pandemic proportions [185]. Similarly, CSF has recently re-emerged in Japan and other regions, many located near free areas with dense swine production such as in Brazil. Consequently, the swine industry is globally concerned about the increasing risk associated with FHF incursions and the associated impacts on animal health and economics.

Much work has been done to reduce the time to confirm an FHF incursion into a free area after a first suspect is identified. Generally outbreaks would be confirmed within hours or few days of sample collection, depending on the country and region [47], [48]. However, the duration of time between virus introduction and identifying the first suspect of the disease in a free region is quite uncertain and, in many cases, may be extended for weeks or months, resulting in secondary disease spread. Figure 1 shows some examples of the estimated number of undetected outbreaks that occurred before the first detection (index case) in the high-risk period (HRP), which is the period of time between the initial infection and official diagnosis and notification of the disease for selected ASF and CSF epidemics [7], [158]. Delays in identification of FHF incursions is in part explained by the absence of pathognomonic clinical signs, resulting in relatively broad case-definitions

for the diseases, wide range of presentations, and a relatively long period of undetected spread [24], [28], [30], [186], [187].

Consequently, there is a need for implementing actions for reducing the HRP as much as possible, which may be most effectively achieved by enhancing the industry capacity to early detect and report FHF incursions.

Early detection of a TAD incursion may be defined in terms of the temporal sensitivity of a surveillance system and its ability to accurately identify an agent at any given time in a population [5]. An effective early detection surveillance system is expected to detect a TAD incursion as soon as possible, preventing or mitigating its spread into other farms and regions [3]. Awareness and engagement among relevant stakeholders, including the industry, practitioners, and the regulatory sector, are important components of an effective early detection system. For example, official estimates suggesting that Vietnam discovered the first ASF case in 2019 within 5-10 days of its first introduction may be explained by the alert issued in the country soon after the detection of the disease in China in 2018 (Vo, C., Personal communication, 2022, [188]). In many western countries, where primary veterinary assistance relies on the private sector, producer and veterinary practitioner awareness is a key component of early detection and industry-led passive surveillance efforts tend to be more effective in the spontaneous detection of outbreaks than active surveillance. For example, when ASF was introduced into Latvia in 2014, 32 outbreaks were recorded, 31 of which were identified by passive surveillance activities following the initiation of awareness campaigns by the official veterinary services [189].

One may argue that, consequently, enhancing passive surveillance (EPS) actions through public-private partnerships is an effective strategy to support the goal of early detection of TAD incursions. However, implementing EPS systems on a large scale is challenging due to a lack of standardized methods, definitions, and procedures.

The objective of the study here was to develop and pilot an EPS protocol for FHF of swine to aid the early detection of ASF and CSF incursions into pig farms. The protocol presented here represents the first known standardized attempt to combine systematic and routine collection of data with anomaly detection systems to inform a public-private action with the ultimate objective of shortening the high-risk period of undetected spread after disease introduction and mitigating the impact of ASF and CSF outbreaks in free farms or regions. The concepts presented here may be easily adapted for implementation by farms, countries, and regions to enhance actions for early detection of FHF in their free populations.

## 4.3 - Methods

### 4.3.1- General approach

An EPS protocol was developed to characterize the risk for an FHF incursion into a farm. The system uses a scoring system that serves as a proxy for the risk to trigger sampling activities to confirm or rule out suspects. The EPS protocol was piloted in two populations. In the Dominican Republic, an ASF- and CSF-infected country, the protocol was used in two ASF- and CSF-free farms for 10 consecutive weeks to evaluate the

temporal variation in scores in a population potentially exposed to FHF. An anomaly detection algorithm was implemented to inform the decision to collect samples and support early detection of cases. In the Midwest region of the United States, the first component of the EPS, related to farm biosecurity, was computed in a group of 10 farms to assess the spatial variation of the background risk in a population believed to be relatively homogeneous.

### 4.3.2. EPS protocol and scoring system

The EPS protocol was built considering three components, namely, 1) the biosecurity background of the farm, 2) the routine observation of clinical or syndromic data, and 3) the result of necropsy findings (Table 4.1). Each of the three components included the assessment of presence or absence of certain factors, weighted by scores to model their relative importance. The final score was computed following an additive model, i.e., as the sum of the presence (1) or absence (0) of the factors and conditions, weighted by the relative importance of the factor. Both the selection of factors for each of the three components and the weights were estimated in consultation with three experts with extensive field and/or experimental experience with the disease gained through their work at the OIE reference laboratory for ASF in Madrid, Spain, the Plum Island Animal Disease Center, and the swine industry in Russia and Asia. Each of the experts identified the factors and weighed the factors independently. Factors and weights were compiled and shared again with the experts to reach final consensus on the values. The list of components and variables, along with the final weights and the references that support

the inclusion of the variables is provided in Table 4.1. Noteworthy, the weights used here are the reflection of consensus among the consulted experts and could be easily modified or adapted if deemed necessary.

A closed-question survey [123] was developed in an MS Excel spreadsheet, and transferred to a Qualtrics software [190]. This allowed data collection in a paper-free format using cellphone devices or laptops decreasing possible errors in transcriptions and giving options of accessibility for producers collect data during inspection of their pigs.

### 4.3.3. Study populations

Two surveys based on a participatory surveillance approach were developed [2], considering pig producers as the target audience of the protocol. Briefly, a participatory surveillance approach recognizes that farmers, practitioners, and farm workers are knowledgeable about issues that are important to them, such as diseases affecting their animals, and takes advantage of that knowledge and the activities that routinely conduct in the farm by incorporating them into a formal surveillance system [2], [191].

The first study was conducted in two ASF and CSF-free commercial pig farms in the Dominican Republic over a 10-week period, between December 13, 2021, and February 20, 2022, and anomaly detection algorithm was used to assessing the temporal variation of the score and informing the decision to collect samples. Those two farms, referred to here as farm A and B for confidentiality reasons, are located in the province of San Pedro de Macorís, a region that first reported ASF outbreaks in August 2021 [33]. Farm A is a finishing site with an average of 6,500 pigs, whereas farm B is an independent farm,

working in a farrow to finish system, with total average of 280 pigs. The biosecurity background of those two farms was assessed only at the first week of the study because practices remained stable for the entire period. For the second and third components (syndromic surveillance and necropsy findings) data were collected weekly. A composite score was computed for each farm on a weekly basis.

The second study was conducted in December 2021 in 10 farms located in the Midwest region of the United States. It only included the first component of the EPS with the objective of assessing the variation in the biosecurity background risk in a population expected to be relatively homogeneous in terms of management practices.

### 4.3.4. Anomaly detection for targeted surveillance activities

In the Dominican Republic, an anomaly detection algorithm was used to detect periods of time in which variations in the score would result in the recommendation for active sampling of FHF. Specifically, a purely temporal scan statistic model was performed to identify clusters of weeks with highest chances of being associated with an FHF incursion, as indicated by the value of the scores. Briefly, the scan statistics in a temporal analysis may be interpreted as a scanning window that moves across time. The window represented the number of weeks considered as candidate high risk clusters [192]. A Discrete Poisson purely temporal scan statistic model was performed for farms A and B separately, under the null hypothesis that the rate of observed-to-expected score was homogeneous through the study period, whereas the alternative hypothesis was that there were certain weeks in which the rate was significantly higher or lower than the expected

under the null hypothesis. The ratio of observed-to-scores within each candidate cluster was computed and their significance was tested using a Monte Carlo simulation approach with 999 iterations [192]–[194]. The SaTScan v.9.6 software was used to identify these temporal clusters of weekly scores [136]. The graphs were generated in MS Excel.

## 4.4 – Results

The mean scores over the 10-week period for farms A and B in the Dominican Republic were 95.6 [sd= 8.22] and 143.6 (sd= 1.78), respectively. The biosecurity background risk score remained constant through the study period and was 79 and 130 for farms A and B, respectively. The weekly fluctuation during the study period, associated with variations in the values estimated for the second (clinical surveillance) and third (necropsy findings) components of the EPS is depicted in Figure 4.2.

Although the background risk (associated with biosecurity practices) was higher for farm B, the highest weekly fluctuation was observed in farm A due to the presence of clinical signs and necropsy findings compatible with ASF.

The results of the anomaly detection algorithm showed that there was a 6% increase over the expected scores between weeks 5 and 9 for farm A and a 1% decrease in farm B (Figure 4.3). Although, those variations were not significant ($P>0.05$), acknowledging that absence of significance may be due to insufficient data collected, ASF testing was recommended for farm A at week 6 and whole blood was collected from randomly selected pigs. Polymerase chain reaction (PCR) results were negative.

Under the second study, the median biosecurity score computed for the 10 farms in the United States was 46.5. The farm with the highest recorded score (88) was almost three times higher than the farm with the lowest score (23) (Figure 4.4). Interestingly, two of the farms had a higher background risk score (80 and 88) than the background risk estimated for farm A in the Dominican Republic.

## 4.5 – Discussion

Results of the pilot study presented here suggest that EPS protocols may help standardize participatory surveillance methods, in a format of scoring system based on risk factors, aiding swine producers to detect early evidence of FHF incursions thus reducing the time between disease introduction into a free country and first suspect and reporting. Passive surveillance actions implemented at the farm level play a critical role in early detection of TAD incursions, and systems that can help identify early signs of infection are critically needed. Passive surveillance is highly dependent on the awareness and engagement of each individual producer. Consistency of passive surveillance systems increases when the industry follows the principles of a participatory process including systematic data analysis to trigger diagnostic testing. Results presented here show that the formal incorporation of an explicit scoring system to replace casual passive observations combined with data collection and analysis to identify a trigger for testing may help standardize actions implemented for early detecting FHF incursions.

Animal health organizations encourage the development of early disease detection systems using non-diagnostic information, often derived from electronic data [192]. One

advantage of the EPS protocol here is that it quantifies what are typically qualitative observations related to biosecurity, morbidity and necropsy findings. This protocol not only standardizes the data that were collected but it also allows a level of analysis not routinely performed on such observations. In disease surveillance, to guarantee an early detection of disease outbreaks computationally efficient methods must be designed [195]. As demonstrated in the pilot in Dominican Republic, the data collected with this protocol captured quickly and shared electronically. Incorporating such an algorithm into the IT systems already used by companies to collect health and production data is straightforward and would allow decisions to submit samples for testing to be made at the farm level. Additionally, if location of farms were incorporated into a regional database, it would be possible to explore the spatial relationship among results, in addition to their temporal scale [45-47]. The statistical techniques employed here was just one among many that could be considered and, regardless of the specific technique used, it highlights opportunities to further leverage data collected through this EPS protocol and inform early detection systems in a reliable and accurate way. Because the algorithm may be incorporated into the IT systems routinely used by swine companies to collect health and production data, the EPS may not necessarily increase the work already performed by farmers and farm workers. In addition to systematize the collection and interpretation of data, the approach proposed here may also help the design and implementation of risk-based testing to early detect FHF incursions, for example, through the use of point of care tests [70].

The ability to detect significant high-risk clusters is influenced by the number of observations assessed, which affects the power of the statistical test. Because the analysis

here was purely temporal, the sample size is then determined by the number of units of times (weeks here) through which data were collected. The limited number of weeks for which data were available is a potential explanation for the absence of significance in the detection of clusters and should be considered when implementing the system at a large scale. It is possible that at least one year of routine data collection, to incorporate seasonal fluctuations and to increase the power of detection in variations of the score, may be needed before the EPS protocol could be implemented at a fully operational scale in a country or region. Similarly, the first component of the EPS, focused on the assessment of farm biosecurity, should be regularly updated to reflect changes in the farm practices and conditions.

Certain diseases show clinical signs similar to ASF or CSF and that are not reportable, such as porcine reproductive or respiratory syndrome (PRRS), porcine multisystemic wasting syndrome (PMWS), or certain forms of salmonellosis. Presence of these disease may delay the diagnosis of an FHF because the FHF will not be the first suspicion by producers and veterinary practitioners. Consequently, in addition to knowledge about the clinical signs of the disease, awareness of the increase in risk for the incursion of a new disease is an important factor influence rapid detection and response. Having a participatory surveillance system in place may help identify an outbreak of one of these endemic diseases more quickly and may eventually provide a profile for these diseases to help distinguish between more common disease and an FHF. Additionally, the EPS protocol may aid the design of targeted surveillance efforts. For example, in the pilot study in the Dominican Republic, farm A showed lower scores than farm B, suggesting that the former was, a priori, less vulnerable to FHF introduction than the latter.

However, the relatively high variation in the scores observed in Farm A were suggestive of an introduction of disease and triggered the recommendation for testing. This observation highlights the value of systematic, long term surveillance efforts to detect early signs of disease incursions.

The relatively large variation observed the biosecurity component assessment in the Midwest region of the US, with an approximate 3-fold difference between the maximum and minimum score computed, was unexpected. These farms are in the same region of the country, an area with a long tradition in pig production. The small number of farms that answered the survey is considered a limitation for this part of the project. Even with this limitation, the wide differences in biosecurity practices reflected found may result in a large variation in terms of farm vulnerability to the entrance of pathogens. There is still work to be done in enhancing awareness and implementation of biosecurity in this swine densely populated and highly productive region. More activities in sanitary education, developed through public-private partnerships (PPP), to increase the receptiveness of pig producers to actions related to surveillance and monitoring should be stimulated.

The outbreaks of ASF and CSF in Dominican Republic increase risk of wider spread in the western hemisphere. Strong PPP are needed to support efforts for early detection of disease incursion in free areas. Both OIE and FAO highlight need for PPP to prevent and detect TADs including systems for early detection. The EPS protocol here promotes mechanisms that allows producers to perform surveillance, aiding the official veterinary services contributing to the faster action in case of introduction of any TADs. For that reason, the protocol may be applied as a bridge between public and private sectors and facilitating the communication between those sectors necessary to coordinate rapid

assessment of potential TAD incursions. Additionally, according to OIE, the establishment of PPP contributes to a more efficient use of public and private sector resources, finding synergies through an active and structured collaboration to bring, among other things, a well-structured surveillance system with active and passive surveillances very efficient [100]. According to the Global Framework for the Progressive Control of Transboundary Animal Diseases (GF-TADs) from OIE & Food and Agriculture Organization for the United Nations (FAO), a multi-sectoral approach, with the involvement of all actors at the national, regional and global levels are essential to the success of preventing, detecting and responding to TADs [196]. This same document also mentioned the importance of developing tools that advocate to TADs, focusing on many aspects of control programs, but also to warning systems for early detections of TADs.

## 4.6 – Conclusion

Shortening the time between incursion and first detection is critical to limit the impact of ASF or CSF incursions in free areas. Results here demonstrate the opportunity for standardizing data collection processes through the use of EPS protocols and participatory surveillance methods, and as part of effective PPP implemented with the objective of early detecting the incursion of FHF of swine and supporting the ultimate goal of reducing the time to first report of a suspect, and the impact of TAD epidemics in free countries and regions.

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## 4.7 List of Figures

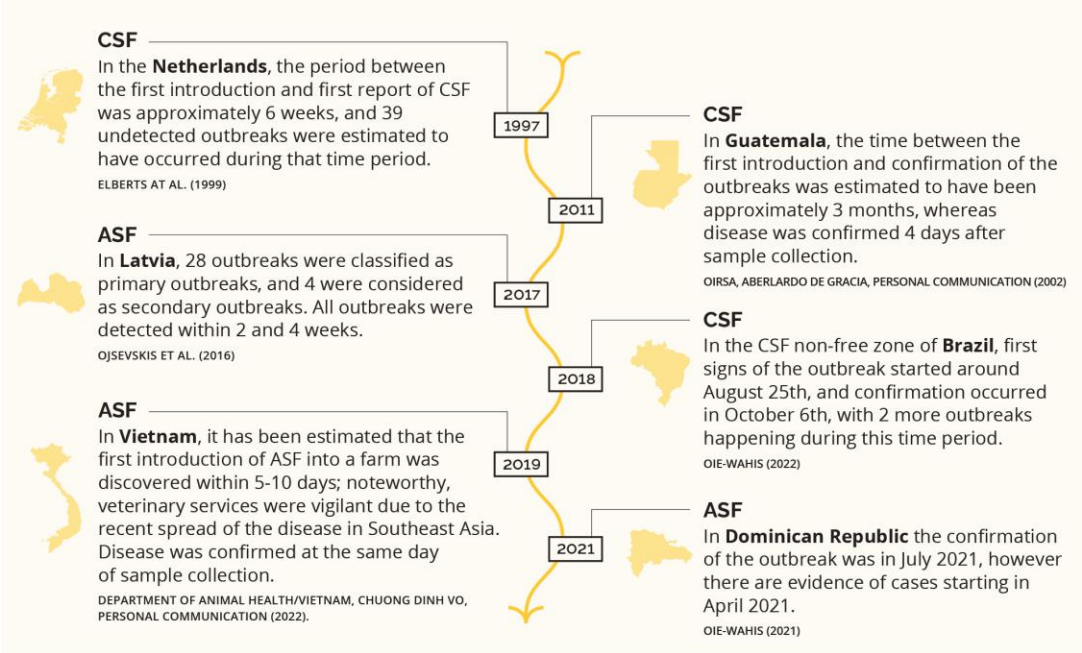


**Figure 4.1 -** Estimated duration of the time period between virus introduction and disease confirmation, and number of outbreaks that occurred over that period, for selected African swine fever (ASF) and classical swine fever (CSF) epidemics [33], [197]–[199]

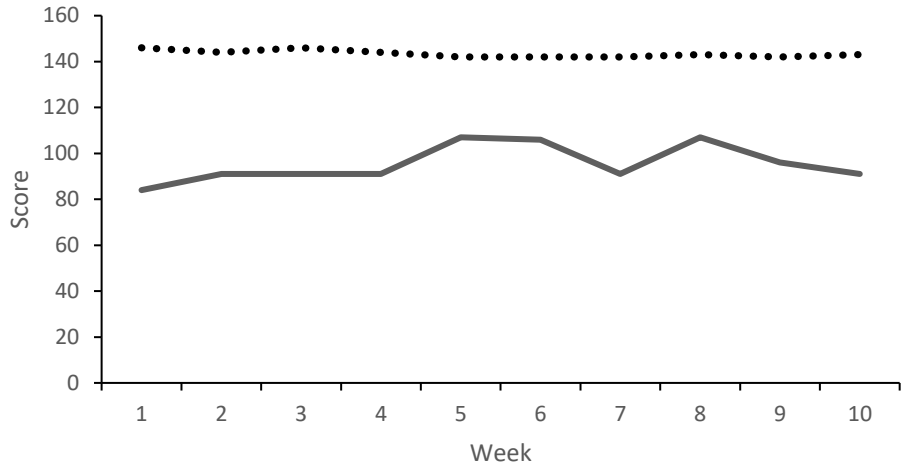


**Figure 4.2 -** Weekly variation in the risk score for African and classical swine fevers estimated using an enhanced passive surveillance protocol in two pilot farms (being farm A in solid line, and farm B in dotted line) in the Dominican Republic over a 10-week period.

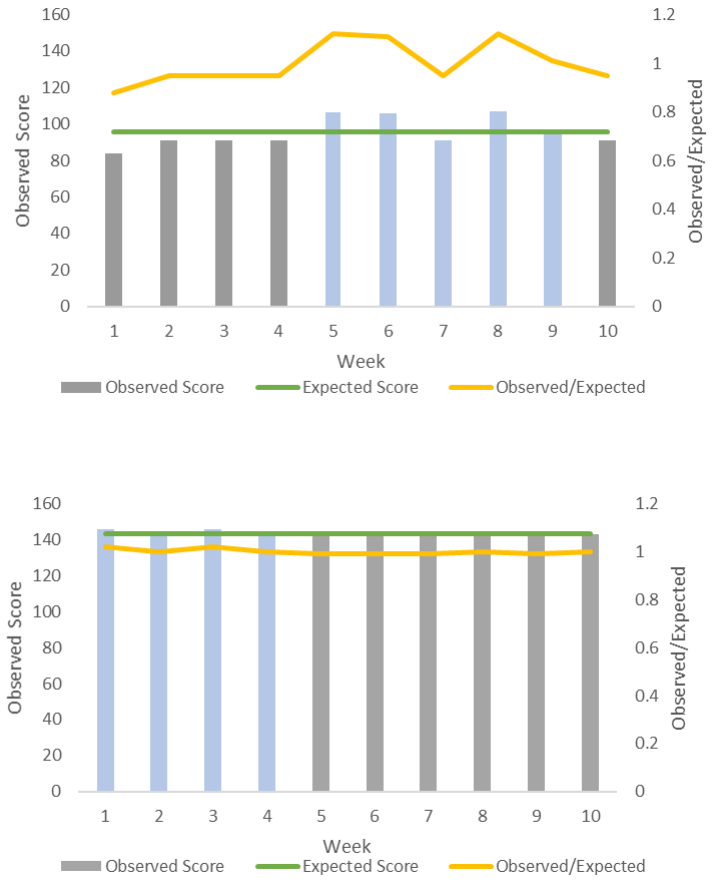


**Figure 4.3 -** High-risk periods (light blue) detected using a temporal scan statistic model on two pig farms in the Dominican Republic denoted as farm A (top) and farm B (bottom) using data collected during 10 weeks of application, and follow-up of an enhanced passive surveillance protocol.

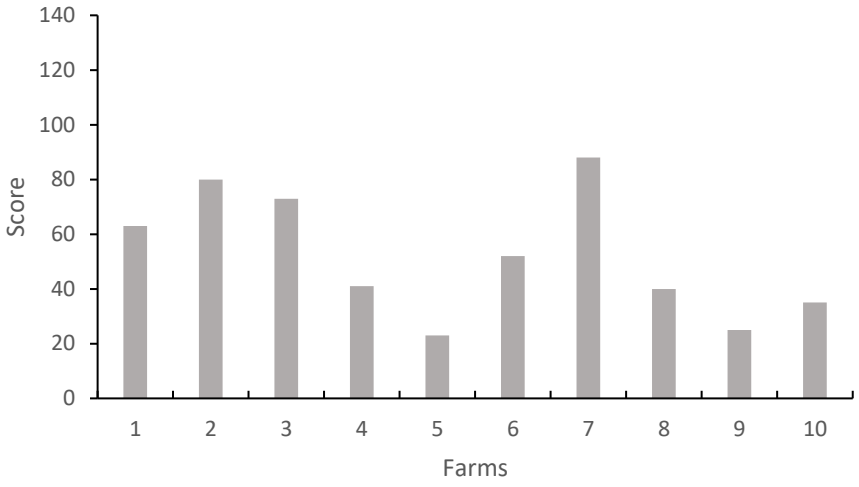


**Figure 4.4 -** Biosecurity scores from 10 assessed US Midwest pig farms.

## 4.8 List of Tables

**Table 4.1 -** Components and factors included in an enhanced passive surveillance protocol for scoring risk and supporting the early detection of African Swine Fever and Classical Swine Fever in swine farms.

| Score | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Probability of finding the sign/factor (qualitative) | Negligible | Low | | | Medium | | | High | | | Very high |
| Probability of finding the sign/factor (quantitative) | <0.1 | 0.1-0.4 | | | 0.4-0.7 | | | 0.7-0.9 | | | >0.9 |

| Components | Factors | Increase in Risk | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|
| | | Area with feral pigs | | | Area without feral pigs | | | |
| | | Commercial - sows | Commercial – finishers & nurseries | Small holders & outdoors | Commercial - sows | Commercial – finishers & nurseries | Small holders & outdoors | |
| Biosecurity background (Farm-level risk factors) | Use of feed with ingredients of foreign origin | 3 | 3 | 3 | 3 | 3 | 3 | [105], [108], [200], [201] |
| | Swill-fed | 5 | 5 | 5 | 1 | 1 | 1 | [22], [24], [108], [121], [138], [152], [202] |
| | Absence of double fencing | 7 | 7 | 8 | 4 | 4 | 4 | [203] |
| | Presence of flies and ticks | 7 | 7 | 8 | 7 | 7 | 7 | [106], [108] |
| | Presence of small and domestic mammals (rats, dogs, cats) | 7 | 7 | 9 | 5 | 5 | 8 | [18], [138] |
| | Absence of a protocol for changing clothes, of separate entries and exits, of disinfection of objects, introduction of food allowed, and external individuals allowed in the farm | 10 | 10 | 10 | 10 | 10 | 10 | [18], [138] |
| | Cars and trucks may enter premises | 8 | 8 | 8 | 8 | 8 | 8 | [18], [138] |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Non-closed herd with recent introduction of new animals, but no quarantine station or location within 0.5-1 mile from premises, or sharing personnel | 9 | 9 | 9 | 9 | 9 | 9 | [18] |
| | Dead animals disposed in a manner that does not prevent the attraction of wildlife, rodents, and scavengers | 9 | 9 | 9 | 9 | 9 | 9 | [18] |
| | Personnel (including Vets, Inseminators, Technicians) move between this farm and other farms with trusted biosecurity | 6 | 6 | 6 | 6 | 6 | 6 | [138] |
| | Personnel (including Vets, Inseminators, Technicians) move between this farm and other farms WITHOUT trusted biosecurity | 9 | 9 | 9 | 9 | 9 | 9 | [138] |
| Syndromic surveillance | Increase in mortality (sudden death) | 4 | | | | | | [12], [14], [21], [23], [25], [204] |
| | Drop in feed consumption | 1 | | | | | | [12], [14], [21], [23], [25], [204] |
| | Fever | 2 | | | | | | [12], [14], [21], [23], [25], [204] |
| | Erythema | 2 | | | | | | [12], [14], [21], [23], [25], [204] |

| | | | |
|---|---|---|---|
| | Cyanosis of ears and limbs | 3 | [12], [14], [21], [23], [25], [204] |
| | Abortion | 1 | [12], [14], [21], [23], [25], [204] |
| | Constipation followed by diarrhea | 1 | [12], [14], [21], [23], [25], [204] |
| | Hematochezia (Diarrhea with frank blood) | 6 | [12], [14], [21], [23], [25], [204] |
| | Reduced motility/movements or abnormal recumbence | 2 | [12], [14], [21], [23], [25], [204] |
| | Vomiting | 5 | [12], [14], [21], [23], [25], [204] |
| | Hematuria | 7 | [12], [14], [21], [23], [25], [204] |
| | Hematemesis | 7 | [12], [14], [21], [23], [25], [204] |
| | Bleeding from nose | 7 | [12], [14], [21], [23], [25], [204] |
| Necropsy/samples collected | Kidney hemorrhages | 7 | [12], [14], [21], [23], [25], [204] |
| | Lymphadenomegaly | 7 | [12], [14], [21], [23], [25], [204] |
| | Lymph node hemorrhage or necrosis | 9 | [12], [14], [21], [23], [25], [204] |
| | Splenomegaly | 9 | [12], [14], [21], [23], [25], [204] |
| | Hydropericardium | 7 | [12], [14], [21], [23], [25], [204] |
| | Hydrothorax | 7 | [12], [14], [21], [23], [25], [204] |
| | Shock lung / ARDS | 7 | [12], [14], [21], [23], [25], [204] |
| | Pneumonia | 5 | [12], [14], [21], [23], [25], [204] |
| | Hemorrhagic intestinal contents | 8 | [12], [14], [21], [23], [25], [204] |

# CHAPTER 5 - Final remarks and conclusions

Epidemiological methods are applied in the veterinary field to investigate the dynamics, frequency, and determinants of diseases in animal populations. Epidemiological models may be used to assess and evaluate surveillance strategies, interventions, and assess risk of introduction into naïve populations, with the ultimate objective of informing policy [205]. This Ph.D. project worked with different levels of assessment of the risk for ASF and CSF.

ASF and CSF are putting the global swine industry at risk, with severe economic consequences, therefore, for free countries or areas, is utmost important to develop prevention strategies, as also early-warning systems based on risk analysis to reduce the likelihood of introduction of these TADs [32]. Disease control programs (DCP) for ASF and CSF of free countries should consider the development of plans, which rely in early detection of these diseases.

The diagnostic techniques for ASF and CSF are evolving, with the development of tests with high sensitivity, and reliable as PCR tests for antigen, and ELISA tests for antibodies detections, used as gold standards for detection of these diseases. In Chapter 1, the innovations regarding the diagnostic detections for ASFV and CSFV were reviewed. As the studies in this field are evolving, recent research is pointing to point-of- care (also referred to as point-of-need) tests as an emerging opportunity to reduce the time to diagnosis of a foreign animal disease. This opportunity should be discussed between OVS and the private sector to promote effective public-private partnerships to promote disease detection. Also, different ways of sampling, with non-invasive methods that

guarantee reliable matrix for finding the virus, allied to animal welfare, offer promise to support early disease detection.

These laboratory advances may give agility and less time (hours) to get a confirmatory laboratory result. However, the interval between the introduction and the first detection of suspicious cases is the most sensitive link in the detection chain. The main challenge is the long time it may take to detect ASF or CSF in the field, or at least to suspect its occurrence, where it can take weeks, or even months specially in free countries/areas, where these diseases may not be the first clinical suspicious [21]. Early detections systems are challenging to be implemented, however, combination of different levels of risk-based surveillance can contribute for the robustness of the system.

Regarding the risk assessment of hypothetical introduction of FHF of swine into free areas, this Ph.D. project assessed alternative models to support risk-based approaches at the country, state, and farm-levels, aiding the design of surveillance actions that would target areas most vulnerable for the incursion of these virus. The models also helped showing the vulnerability regarding type of farms at the state level, and how the producer could implement a protocol for surveillance based on the own characteristic of each farm –ie, leading to the design of individual surveillance protocol for each pig farm.

Prevention of TAD incursions is complex and requires a dynamic management of potential incursions scenarios. Risk assessment can use quantitative data as a reliable and auditable source of information. However, such data are sparse in many situations. In these circumstances, expert opinion presents an alternative source of information [182]. We used this reasoning at this Ph.D. for developing risk assessments; when the data were available, we opted for a stochastic quantitative risk assessment, with the use of

conditional probability organized in a scenario tree. However, we also used expert opinions to create proxy-risk or scores to guide us in risk assessment models.

The ideal condition of data availability many times will not be achieved, nevertheless, Chapter 2 showed that reliable risk prediction can be done using tools combined to create a system for risk evaluation. The use of conjoint analysis based on expert opinions, combined with statistical methods, was able to create a reliable risk map at a country level. With that, the country can put efforts in ports and airports in those states/ departments that showed a higher vulnerability, i.e., high risk of the introduction of FHF of swine in the territory.

At Chapter 3, using the official pig movement that, plus data from farms (herd size and geographic coordination), we were able to develop a risk assessment with more capillarity, that is, this study revealed what municipalities at higher risk of introduction FHF were, considering the type of pig farm, if it was commercial or backyard farms. This project can aid the OVS to propose surveillance actions for different types of farms, and wild boar population control.

Control of ASF and CSF requires a surveillance system that detects the outbreaks as early as possible, regarding specifically to ASF control strategies, it is decisive for the outbreak duration because the virus survives for extended periods in the environment and in pork products [15]. Also, because of the slow morbidity, for an effective early detection surveillance, large pig farms will be able to detect ASF incursions within the first 2 weeks, only through testing regularly sick and dead pigs [206]. This passive surveillance approach focused on early detections and sampling pigs was proposed at Chapter 4 with an enhanced passive surveillance (EPS) protocol.

Regarding the EPS protocol proposed at Chapter 4, we expected to aid the OVS in early detection of FHF. Although the success of this surveillance type will be due to the engagement of producers. However, based on the assumption that people are knowledgeable about issues that are important to them, such as diseases affecting their animals [191], the model of EPS has strength foundation relying on producers being aware of health conditions of their herds, and having a fast response in suspicious cases of ASF or CSF.

The effectiveness of passive surveillance for early detection of FHF depends on the engagement of different types of actors involved in the pork food system, farmers are the ones most important actors in this value chain. They should be able to recognize any suspected cases of FHF, as early as possible, and be willing to report them immediately to OVS [15]. Maybe, clinical signs can be hard to catch, however if the herd is being followed regarding health parameters in a close approach, like weekly, as we proposed in our EPS protocol, farmers will be able to notice any alteration on the health status of their herd. Here is needed to point out the necessity of strong campaigns of sanitary education toward producers, and other stakeholders like private veterinarians, to not only show the importance of notification of suspicious cases, but also to assist producers in how to identify symptoms and/or lesions compatible to FHF and listed in the EPS protocol developed by this project.

Therefore, at Chapters 2 and 3, the spatial component of the risk was assessed, regardless the data availability, it was possible to generate risk maps, with methods that allow the risk assessment at national and state levels. In Chapter 4, the temporal component was included to the epidemiological model, with assessment of the risk in a weekly basis,

triggering indications for herd sampling after detections of observed farm score exceeds the expected scores (anomaly algorithm detection).

We expect that the models presented here may aid OVS performing a better allocation of resources for states that are at higher risk, considering the national level of surveillance. At a state level administration, it will be possible to perform surveillance tailored for specific type of pig farm (commercial or backyard), and also promoting actions linked to the risk of introduction FHF, but prioritizing funds for municipalities at higher risk. At a farm-level, application of the ideas presented here may, for example, lead to regulations that would allow for OVS give incentives for pig farmers that would enroll in the EPS program, enhancing the temporal sensitivity of the system. These farms would be performing systematically passive surveillance, with that, the financial and personal resources could be used for active surveillance in farms that would not be engaged in the early detection surveillance approach for ASF and CSF, developed by the EPS protocol.

In conclusion, the methods explore here demonstrate the role that routine application of risk analysis and risk-based surveillance approaches may play in the design of official programs aimed at the early detection and prevention of FAD incursions in free countries.

## 5.1 – Overall Limitations

During the development of this Ph.D. project, we could identify some limitations. In Chapter 2, the limitation was that our model for risk estimation for introduction of ASF in Kazakhstan did not capture the first introduction of this disease in Russia in 2007, however, we did not account for the civil war that district was undergoing. Possibly

happened an increase in movement of people to that region and it was not registered in the demographics that we used as risk factor (human density).

In Chapter 3, we only addressed two pathways in our model of risk assessment, however, movement of live pigs is the main source cited in the literature involving introduction of FHF in free settings. And wild boars are related to not with introduction, but also with the establishment of endemicity of these diseases in the environment.

The limitation in Chapter 4 is related with the small number of farms and the short period that we tested our EPS protocol. Also the design of testing animals and the workflow of sending samples to the laboratory in Dominican Republic were not under our control.

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# Appendix A

S.1 – Supplementary data – Chapter 3 – Number of pigs (mean and standard deviation) that were sent to each municipality of Mato Grosso during a period of 2016 - 2018, to subside the Poisson- LogNormal distribution.

| Municipality of destination (MT) | Mean of pigs (2016 - 2018) | Standard deviation (2016 - 2018) | Number of pigs for each municipality (Poisson-LogNormal) |
|---|---|---|---|
| Acorizal | 3.33 | 5.77 | 3 |
| Água Boa | 2.00 | 3.46 | 2 |
| Araputanga | 1.33 | 1.15 | 1 |
| Barra do Garças | 7.33 | 12.70 | 7 |
| Brasnorte | 8.67 | 9.29 | 9 |
| Cáceres | 19.33 | 31.75 | 19 |
| Campinápolis | 22.33 | 38.68 | 22 |
| Campo Novo dos Parecis | 0.67 | 0.58 | 1 |
| Campo Verde | 160.00 | 55.05 | 160 |
| Campos de Julho | 0.67 | 1.15 | 1 |
| Canarana | 0.33 | 0.58 | 0 |
| Cláudia | 13.33 | 23.09 | 13 |
| Colíder | 4.67 | 8.08 | 5 |
| Colniza | 5.33 | 9.24 | 5 |
| Confresa | 4.00 | 6.93 | 4 |
| Curvelândia | 0.33 | 0.58 | 0 |
| Diamantino | 31.33 | 8.08 | 31 |
| Dom Aquino | 2936.67 | 592.48 | 2937 |
| Ipiranga do Norte | 13580.67 | 12181.18 | 13581 |
| Itanhangá | 0.33 | 0.58 | 0 |
| Jaciara | 7.33 | 5.69 | 7 |
| Juara | 2.00 | 3.46 | 2 |
| Juína | 1.00 | 1.73 | 1 |
| Lucas do Rio Verde | 30009.00 | 1906.69 | 30009 |
| Marcelândia | 2.67 | 3.06 | 3 |
| Mirassol D'Oeste | 0.33 | 0.58 | 0 |
| Nossa Senhora do Livramento | 1.67 | 1.53 | 2 |
| Nova Canaã do Norte | 1.00 | 1.73 | 1 |
| Nova Mutum | 8916.33 | 8855.42 | 8916 |
| Nova Ubiratã | 59.67 | 39.27 | 60 |
| Nova Xavantina | 7.00 | 12.12 | 7 |
| Novo Horizonte do Norte | 0.67 | 1.15 | 1 |
| Pedra Preta | 3.33 | 3.21 | 3 |
| Planalto da Serra | 0.33 | 0.58 | 0 |
| Poconé | 4.33 | 5.86 | 4 |
| Pontes e Lacerda | 7.33 | 12.70 | 7 |
| Porto dos Gaúchos | 2.67 | 2.31 | 3 |

| | | | |
|---|---|---|---|
| Poxoréu | 54.33 | 43.39 | 54 |
| Primavera do Leste | 150.33 | 65.90 | 150 |
| Querência | 4.00 | 6.93 | 4 |
| Ribeirãozinho | 1.00 | 1.73 | 1 |
| Rondonópolis | 18.00 | 21.79 | 18 |
| Santa Cruz do Xingu | 3.67 | 1.53 | 4 |
| Santo Antônio do Leste | 1.00 | 1.73 | 1 |
| São José dos Quatro Marcos | 3.67 | 6.35 | 4 |
| Sapezal | 2.00 | 2.65 | 2 |
| Sinop | 43.33 | 53.43 | 43 |
| Sorriso | 35527.33 | 10652.16 | 35527 |
| Tapurah | 49717.33 | 7189.05 | 49717 |
| Terra Nova do Norte | 2.00 | 3.46 | 2 |
| União do Sul | 12.33 | 8.62 | 12 |
| Vera | 54.67 | 7.51 | 55 |

# Appendix B

S.2 – Supplementary data – Chapter 4 – Visit report from Dominican Republic Farms that piloted our protocol for EPS for ASF and CSF.

**Report of a visit to Dominican Republic on the week of April 4th to April 8th 2022.**

The purpose of this trip to Dominican Republic was to visit the two farms that we are applying a Enhanced Passive Surveillance protocol for early detect possible incursions of African swine fever (ASF) or Classical swine fever (CSF). The main idea is testing the feasibility of the protocol, in a pilot project, and in a future to offer the tool for swine farms in free areas.

We expect to detect anomaly algorithms in week scores that could trigger actions like sending samples to laboratory.

The first farm visited was Farm B, and after 2 days of downtime, we visited Farm A. This denomination follows the same we are using in the project to identify the farms and at the same time to protect the identity of them.

**Report of visit**

Farm B, Dominican Republic

Date: 04/04/2022

**Introduction:**

This farm is part of the pilot project for Enhanced Passive Surveillance (EPS) for Foreign swine Fevers (African Swine Fever (ASF)/ Classical Swine Fever (CSF)). This EPS aids early detection of these diseases at a farm level, being a great approach to disease-free areas.

This visit had the purpose of knowing the farm that we are collecting data from last December 2021 and proposing measures to improve biosecurity, allowing better health conditions for pigs raised on that farm.

At the end of the report are some pictures to illustrate the farm.

**List of possible hazards:**

- The farm has a gate that is kept closed, however, dogs and chickens can pass freely, and go to the two barns where the pigs are housed.

- Only the truck that is from the property comes inside the premise, however, it goes to markets to deliver carcasses. The owner's car also comes inside the farm perimeter without disinfection of tires.

- The path linking the entrance of the premise and the evisceration area is not cemented and this path surrounds the barns where the pigs are housed.

- The premise has fences separating the farm from the exterior, but it cannot avoid the movement of dogs and chickens, also the employee from the soccer camp can come and go freely, using the same shoes.

- In the perimeter of the farm has a hen house and a goats house.

- There are separated areas for store feed (basic corn, soy, and minerals that are mixed at the farm). They don't feed the pigs with swill feed. In this same area, the disinfectants are stored too.

- The exterior floor pavement of barns is made of ground and grass, there is no cemented or asphalt pavement.

- The interior pavement of barns is made of cement.

- At the barn where run the maternity and wean sectors are, there is a footbath with glutaraldehyde solution.

- At the finishing barn, there is not a footbath at the entrance.

- Both barns are less than 500 m of the distance between them.

- There is not a system of "all-in all-out" for the finishing barn, and the reason for that is the absence of high demand, the slaughter is to attend to small, local commerce.

- Although there are nets on the lateral of the maternity/wean barn, the entrances and roof have open spaces that allow entrances for birds, bats, and chickens, among other animals.

- There is not a specified area to bury the rest of the deliveries, dead, or rests of slaughtered pigs. They use the end of the property to bury them, however, the area is not isolated, or identified.

- There is not a corridor guiding the pigs from the barns to the area where they are eviscerated. So, they slaughter the pig outside the barn (close to the entrance), and with a wheelbarrow, they bring the slaughtered pig to the area where they make a toilet of the pig carcasses. The blood is on the ground, so, in an outbreak situation, mostly ASF, the virus would be easily spread to other areas, because of the truck that makes pork deliveries pass through the same grass area.

- The area where the evisceration occurs is an opened space with a cover, a water box, and an adapted tub that is used to clean the carcasses with hot water. Dogs are allowed to grab some pieces of offal or parts that won't be traded to markets. The last year's veterinarian student is responsible for the farm, and he inspects the carcasses.

- The blood and water waste from an eviscerate process drain to the ground in an "open" area, attracting flies, and being a source of ground contamination, once, the disinfection would not be efficient due to the presence of organic material and ground. Here, extrapolates the reality of an outbreak situation. The farm would take a long time to become free of resistant viruses like the ASF virus.

**Animal health-related aspects:**

- The pigs haven't shown signs of diarrhea, fever, reddish areas, or hemorrhages. But coughs and some with the difficulty of breading.

- This farm vaccinates pigs against CSF at 50 days of age.

- There is not a division or separate pen for sick pigs, like a "pen-hospital".

- There are two boars on the farm, one of them is kept in a small place and they are fed on the floor, there is not a feeder for them. (Welfare??)

- The litter size is an average of 6 to 7 piglets per sow.

- The purchases for replacement are few, and they already bought a pregnant sow from Farm A group (maternity / nursery site).

**Short-term suggestions for improvement of biosecurity and herd health:**

- Build a footbath for people coming outside of the farm perimeter, especially people that go to the soccer camp, and come back to the farm.

- Build a corridor from barns to the slaughter/ evisceration area

- Build walls in the slaughter/ eviscerated area, to avoid flies, dogs, and other animals having access to this area, also a sewer system for the slaughter/evisceration area, with ceramic allowing cleaning and disinfecting the area.

- Provide lids for the feed ingredient bins, instead of using cardboard boxes, to avoid the entrance of rodents.

- Create a system for disinfecting tires from owners' cars, and truck delivery.

- Create an effluent drain in a closed system mode, avoiding blood and wastewater (sewer) going to the ground, creating environmental contamination, also possibly reaching groundwater.

**Long-term suggestions for improvement of biosecurity and herd health:**

- Build one more barn, to separate animals by age- categories

- Create a cemented or asphalt pavement path for the truck that makes carcasses deliveries.

- Build a different gate for the entrance of the truck delivery, maybe using the open area beside the farm that today is used to eventually let goats or horse grazing. Separate the live animals from the slaughtered ones.

- Establish and identify a specific area for being the "cemetery", avoiding scavengers, and also groundwater contamination.

- Increase the flies' control.

- Avoid using wood separators on the weaning area, and everything made with wood, because of its porosity, hampers great disinfection.

- Avoid dogs and other animals having access to the pigs (try to finish the hen house and goats or move the pig barns away and to an isolated area).

**Feedback from Senior vet student and employee of the farm, who is performing the EPS protocol:**

- He said that the owner doesn't know who is, or where the OVS office, which is responsible for that region, is located. And if they have some suspicions, probably they will have a hard time figuring out who oversees the OVS there. So, in my interpretation, the OVS of the Dominican Republic is not visiting farms performing active searches. I know that probably they are swamped with the number of cases, but maybe some advertising with a free call for notifications might help the detection of new cases and hence, control them.

- I asked about his impression of the EPS protocol being performed by producers if it would be difficult or easy for producers to apply the protocol. And he said that overall, it is easy to follow, but the necropsy parts can be hard for producers. So, the idea of spreading a booklet with images of lesions and clinical signs that are suggestive of ASF or CSF should be included in an EPS protocol.

**Pictures registered in my visit to Farm B:**


Figure 1- Gate at the entrance of the farm.

Figure 2 – Area used as a storage for feed, disinfectants, tools, small feed mill.

Figure 3 – Bins with feed separated by production group.

Figure 4 – Inside the storage room.

Figure 5 – Ground corn – base ingredient of the feed, prepare at the farm.


Figure 6 – The disinfectants used at the farm (glutaraldehyde).

Figure 7 – View of outside barns

Figure 8 – Lateral view of finishing barn and the slaughter / evisceration area down there.

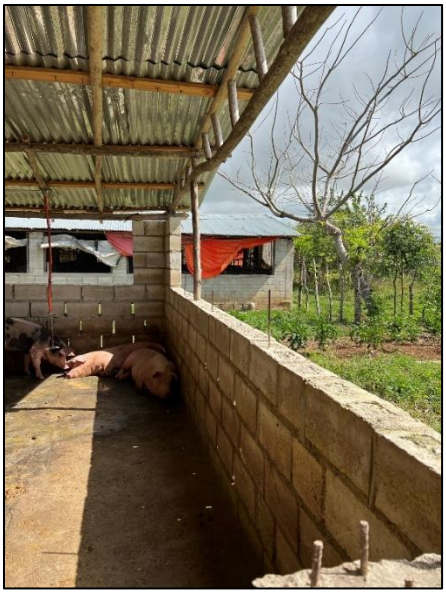
Figure 9 – blood on the grass, in front the entrance of finishing barn, the finished pig is bled (slaughtered) in front of the barn, and the animal is led to slaughter /evisceration area in a wheelbarrow.

**Sequence of images from inside maternity/wean barn:**

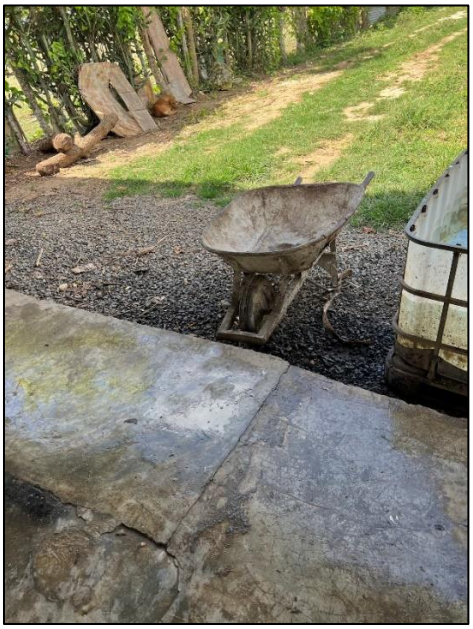




**Sequence of images from inside finishing barn:**

**Sequence of images from slaughter / evisceration area:**






**Conclusion:**

In terms of biosecurity, this farm is highly vulnerable to entry diseases, and mostly the access of people and trucks close to barns should be avoided and remodeling is of utmost necessity.

Also, the farm should be visited by Dominican Republic Official veterinary service.

**Report of visit**

Farm A- Dominican Republic

Date: 04/07/2022

**Introduction:**

This farm is part of the pilot project for Enhanced Passive Surveillance (EPS) for Foreign swine Fevers (African Swine Fever (ASF)/ Classical Swine Fever (CSF)). This EPS aids early detection of these diseases at a farm level, being a great approach to disease-free areas.

This visit had the purpose of knowing the farm that we are collecting data from last December 2021 and proposing measures to improve biosecurity, allowing better health conditions for pigs raised on that farm.

At the end of the report are some pictures to illustrate the farm.

**List of possible hazards:**

- The farm has a gate that is kept closed, any car cannot enter the farm without permission, however, the feed truck from the farm group enters the farm perimeter and goes around the area where the barns are.

- This feed truck comes from a feed mill located in another area, so, the truck gets the roads to arrive at the farms and enters the farm.

- At the finishing barn, there is not a footbath at the entrance, nor a station to clean and disinfect boots.

- The cleaning of pens is not good in most barns. Only three barns, which are under the supervision of one specific employee, have a good cleaning of urine and feces.

- There are some pens built with a water blade on one of the ends of the pens (the idea is to cool off the animals), however, this part of the pen is too deep and accumulates feces, urine, and water, making it a “pool of waste”, and it is not clean as it should be, so, pigs are in a complete swamp of feces, this is a strong source for bacteria and virus contamination and spread.

- There is not a system of “all-in all-out” for the finishing barn. Some pens are crowded beyond the capacity (welfare??)

- Pigs located in pens close to the ends of barns are receiving direct sun light, it can be a distress, adding the beyond pen-capacity (welfare??)

- Only 2 days, on average, of downtime between leaving ready animals and arriving the new batch from the maternity/nursery site.

- The farm is having a problem with effluent management; the volume of liquid waste seems to be higher than the farm capacity of waste lagoons. Odor and flies can be a consequence of this issue.

- There is not a specified area to bury dead pigs, or rests of slaughtered pigs (sometimes they sell slaughter animals, but it is in a small scale, and also they slaughter for food consumption of employees). They burn the carcasses in an open pit, where vultures are there eating rest.

- There are families leaving on the perimeter of the farm, and they are instructed not to buy salami, ham, etc. However, there is no control regarding compliance when the veterinarian is not on the farm.

- The area where the evisceration occurs is an opened space with a cover, a water box, and an adapted tub that is used to clean the carcasses with hot water. It attracts flies.

**Animal health-related aspects:**

- Pigs arrive at 11 to 12 weeks of age and stay until 23 to 24 weeks when they are sold

- Pigs haven't shown signs of diarrhea, fever, reddish areas, or hemorrhages, only one with depression, reddish areas in the years, and nostril (this animal had blood collected during the visit). But coughs and some with the difficulty of breading. The mortality rate is still high, so the farm cannot say if they experience a peak in mortality.

- This farm vaccinates pigs against CSF with a modified live vaccine ("Pestiffa" from Merial) in the nursery site, before moving the pigs to the finishing site (Farm A).

- There is not a division or separate pen for sick pigs, like a "pen-hospital".

- The purchases for replacement are few, and they import pigs from the USA.

**Short-term suggestions for improvement of biosecurity and herd health:**

- Have a specific small truck for receiving the feed that comes in bags from the outside, that is, doesn't allow the feed truck to come into the farm perimeter. This small vehicle would be exclusive for feed and never would go outside the farm.

- Exchange the format of the pen with a deep-water blade (pool of waste) for a slatted floor (I'm adding some material to support best practices for pig barns from Embrapa poultry & pigs from Brazil, which have similar climate characteristics to the Dominican Republic and can help for improvement in the stalls).

- Decrease the water to wet pigs in pens because it is necessary to make the pens drier, allowing to remove feces (solid waste) with brooms or shovels, and then, use water to clean the pens. The

current situation is not correct, and there is a waste of water, besides the combination of high humidity, and organic material is a great hazard for bacteria and virus infections in the pigs.

- Improve the capacity for effluent drains and lagoons, avoiding overflowing wastewater (sewer) increasing environmental contamination, and also attracting flies and rodents.

- Manage the number of pigs per pen. (I understand that because of the ASF outbreak there was a decrease in demand for pork, so, maybe an economic evaluation for starting its own slaughterhouse could be a solution for crowding pens in finishing or decreasing the number of sows in the maternity/ nursery site.)

- The carcasses and remains should be buried, to avoid scavengers.

- Have a specific tool for holding pigs, being a way of not hurting either the animal or the person holding the animal.

- Increase flies' control.

**Long-term suggestions for improvement of biosecurity and herd health:**

- Build a footbath for people entering the barns, since the outdoor pavement is not with a surface that allows cleaning and disinfection. (This is an easy measure, however, with the current condition of feces and urine at the barns, there is no sense to create a footbath now, later, after the improvement of cleaning and removing wastes, a footbath can be useful to keep pathogens away from inside barns.

- Have a specific station for cleaning and sanitation of boots and clothes for the employees.

- Build doors in the slaughter/ eviscerated area, to avoid flies, with ceramic floor allowing cleaning and disinfection of the area.

**Feedback from Senior vet student and employee of the farm, who is performing the EPS protocol:**

- According to him, the owners of the farm don't allow people to visit the farm very often, and the maternity/nursery site has higher biosecurity than the finishing site.

- At this moment they are struggling with low demand for finishing pigs, and because of that, the number of animals at the farm is beyond the capacity of the barns. I suggested seeing costs for creating their own slaughterhouse, so they would have the entire pork chain, and this vulnerability wouldn't happen.

- I asked about his impression of the EPS protocol being performed by producers if it would be difficult or easy for producers to apply the protocol. And he said that overall, it is easy to follow, but the necropsy parts can be hard for producers. So, the idea of spreading a booklet with images of lesions and clinical signs that are suggestive of ASF or CSF should be included in an EPS protocol.

**Sampling for ASF / CSF detection:**

Because our protocol registered a 58% increase in the score from week 14 to week 15, we suggested another round of sampling, and this happened on week 16 of our study, which coincided with my visit there. So, 25 sick or poor performance pigs were bled and the Official Veterinarian responsible for that municipality passed in the farm and filled out the form for being delivered together with the samples at the Official Laboratory in Santo Domingo. Because of ASF outbreaks happening in backyard farms close to Farm A, the Official Veterinarian is not entering the farm to perform an active search, he comes when producers call them.

When the ASF outbreak started in the Dominican Republic in 2021, the outbreak mitigation actions were restraint to the infected farm, now the OVS is shifting to an active search for cases within a radius of 3 and 5 km, according to information from the Official Veterinarian that spoke with me at the entrance of Farm A.

**Pictures registered in my visit to Farm A:**

Figure 1- Gate at the entrance of the farm.

Figure 2 – Disinfection procedure for cars entering at the farm perimeter.

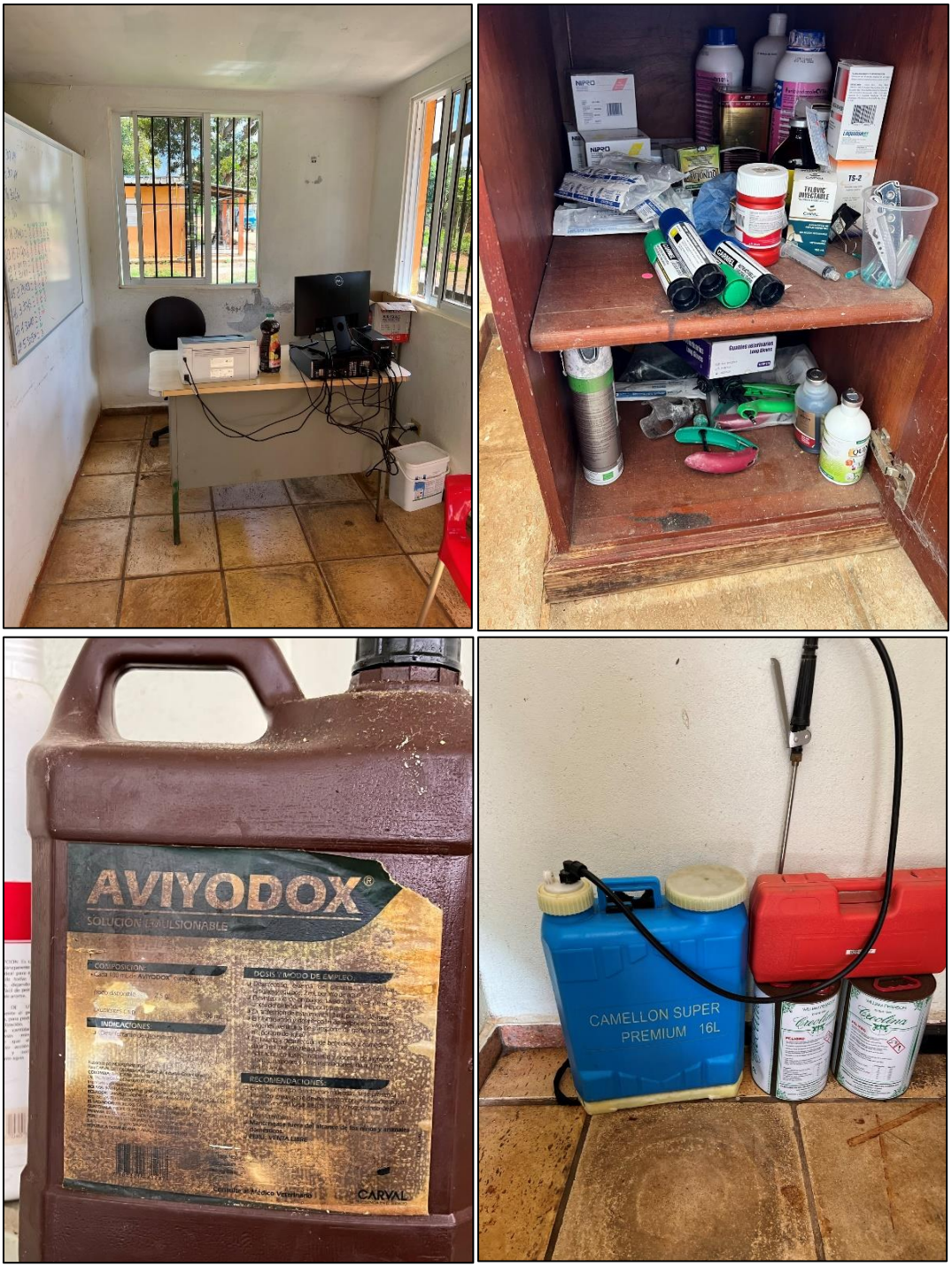


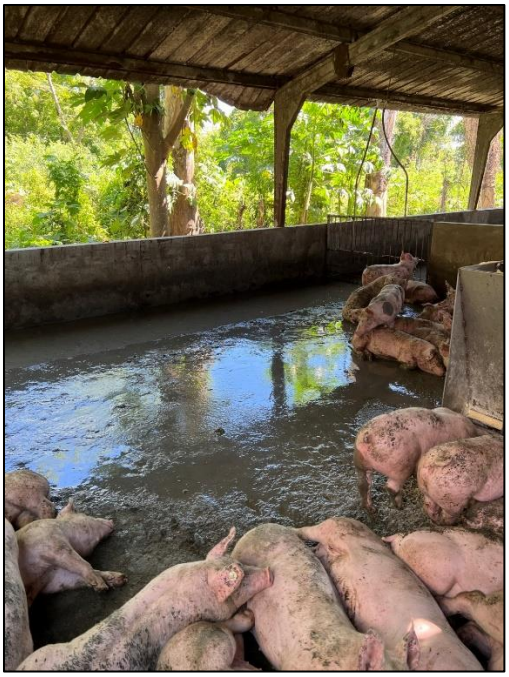

Figure 3 – Office of the farm, where disinfectants are stored.

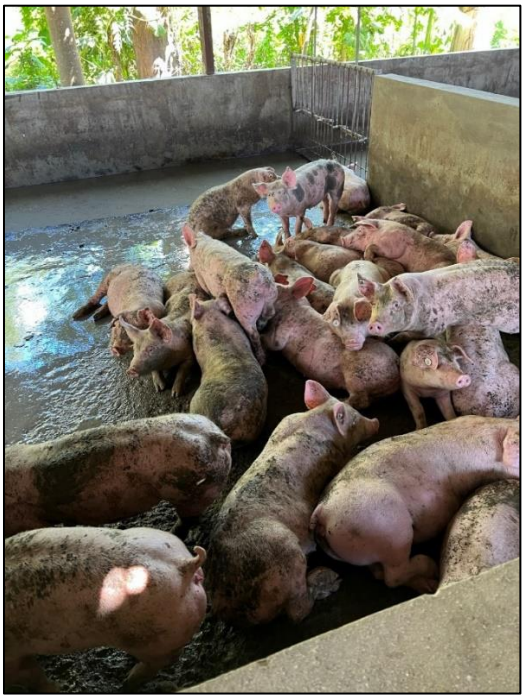
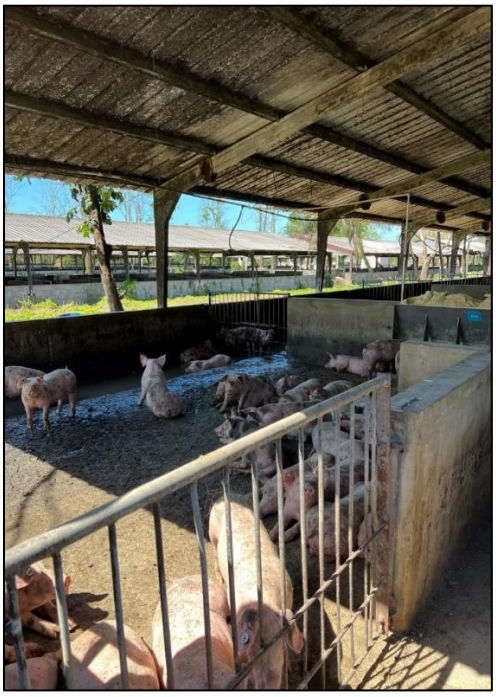
Figure 4: Truck for hauling pigs to loading area. This truck is kept inside the farm perimeter.

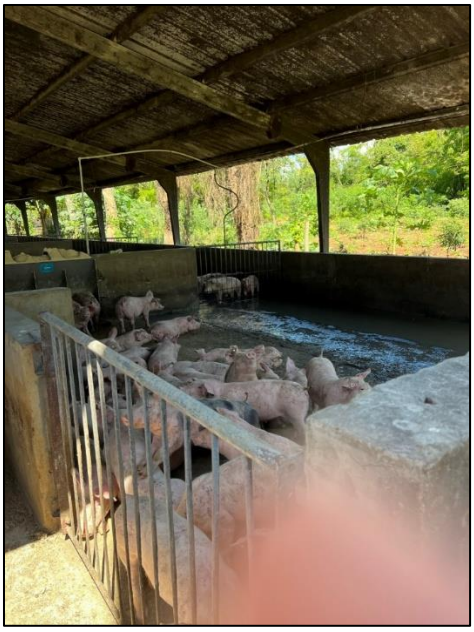
**Sequence of general view of the farm:**

**Sequence of images from inside finishing barn:**

In the sequence it's shown the inefficient cleaning of pens, the pigs are huddled in areas "less dirty". The water blade, which looks like small pool is in a big part of the pen, not allowing pigs have enough space in a dry area. Also, feces and urine are accumulated, so, those animals don't have a dry and clean space to stay. This situation affects the welfare of pigs, and corroborates for diseases, mostly respiratory pathogens.



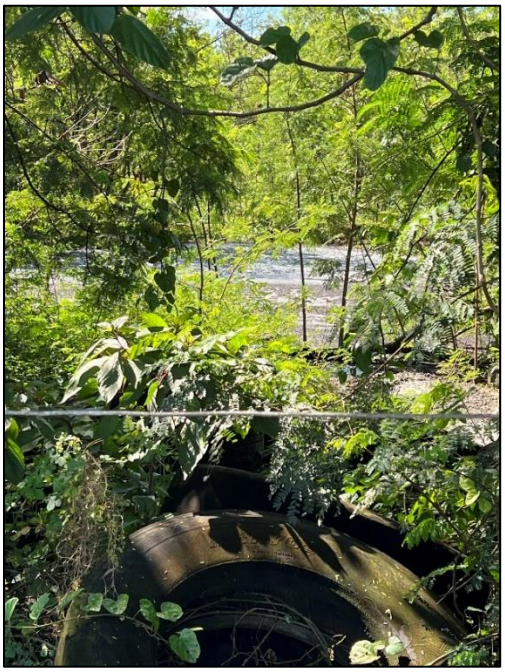



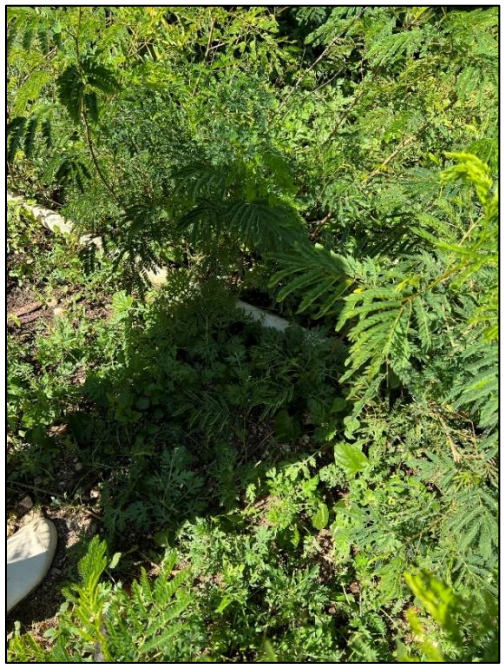


**Sequence of images from slaughter / evisceration area:**

**Sequence of images from carcasses disposal area:**

**Sequence of pictures from the effluent system:**





**Conclusion:**

In terms of biosecurity, this farm is highly vulnerable to entrance of infectious diseases, mostly because of the failure in an efficient cleaning and disinfection of pens.

The drainage of pens should be revised, and the lagoons and effluent management should be increased to support the current capacity of the farm.

**Conclusion for both farms visited:**

Both farms have issues in the biosecurity component, which can impose high risk for introduction of ASF or CSF at those farms. Improvements in short and long term were proposed to them, which can be done gradually but consistently, to achieve better health outcomes.

The other two components, named as syndromic surveillance and necropsy findings, are properly assessed at those farms in a weekly basis.

**Acknowledgments:**

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