PhD THESIS

African Swine Fever: epidemiology, diagnostic and molecular characterisation

PhD student **Paul Andrei Ungur**

Scientific coordinator **Prof.univ. dr. Cornel Cătoi**

INTRODUCTION

African swine fever is a viral hemorrhagic disease of pigs that has spread throughout Europe, wreaking havoc on pig production and the economy, disrupting trade in pigs and pig products, and even affecting social welfare in affected areas (BERGMANN et al., 2021). African swine fever virus, the causative agent of the disease, is a large, complex, enveloped DNA virus that belongs to the family Asfarviridae and genus Asfivirus and is known for its exceptional stability in the environment (MAZUR-PANASIUK et al., 2019; BLOME et al. et al. 2020). Infectious ASF virus can be recovered from pig tissues for months or even years at low temperatures, especially from tissues such as blood, muscle or skin stored at -20 or 4 °C (FISCHER et al., 2020). The disease affects both wild and domestic pigs, most often with an acute course. Infected animals show hyperthermia, anorexia, skin hyperemia, respiratory and vascular disorders. The mortality rate can reach 100% (BELLINI et al., 2021). Montgomery first reported the disease in Kenya in 1921 (MONTGOMERY, 1921), but the disease is now widespread in Africa, Europe and Asia, causing massive economic and livestock losses (SAUTER-LOUIS et al., 2021).
In 2017, the disease was also reported in Romania, the first case being reported in two domestic pigs from Satu Mare county, in the north-west of the country. A year later, it was reported in the Danube Delta, also in domestic pigs, and then it was confirmed in wild boars. The appearance of the virus was attributed to the illegal trade in meat and pork products in Satu Mare and due to the migration of wild boars near the country borders in the Danube Delta (BOKLUND et al., 2018). Since Romania has a high consumption of pork, pig farming is an important branch of agriculture in the country, and economic losses are felt by both households and large industrial farms. In order to provide warnings or advice to regulatory agencies responsible for the protection of public health in general and animal health in particular regarding trends in disease transmission in herds, veterinary epidemiology helps by studying the spread of disease in animals (SALMAN, 2009). External biosecurity risk factors were considered to have a substantial influence on the introduction and spread of ASF in domestic pig herds. Biosecurity measures are considered to be a fundamental tool for preventing the introduction and spread of disease in animal populations, and have been shown to be so. Many biosecurity risk factors have been found to play a crucial role in the spread and maintenance of ASF in domestic pig populations (VILTROP et al., 2021).

THE STRUCTURE OF THE THESIS

In the specialized literature, there is little data on African swine fever in Romania.

The work entitled "African swine fever: epidemiology, diagnosis and molecular characterization" contains 141 pages and is written according to the norms in force, being structured in two parts.

The first part, namely the bibliographic part, is structured in 7 chapters and comprises 34 pages. In this part of the thesis, I synthesized the current general framework of knowledge of the etiology, epidemiology, pathogenesis, clinical and pathological picture, diagnostic methods but also the prevention and control of African swine fever.

In the second part, extended over 88 pages and structured in 5 chapters, I detailed the personal research carried out in the period 2018-2022. Each chapter is divided into sub-chapters that present the purpose and objectives, the materials and methods used, the results obtained with discussions on their novelty compared to other studies carried out and the partial conclusions reached after carrying out each individual study. The research results were illustrated in a number of 33 figures and summarized in 3 tables. The work ends with the cited bibliography (231 titles).

RESULTS OF RESEARCH

In the second part of this paper, we studied the clinical, pathological, molecular and epidemiology aspects of African swine fever, in the period 2018-2022, in holdings from all regions of Romania. The main objectives we proposed:

- Description and characterization of the clinical picture and macroscopic lesions encountered in pigs positive for the African swine fever virus in Romania;
- Microscopic description of the lesions that appear in pigs positive for the African swine fever virus;
- Evaluation and immunohistochemical characterization of the lesions that appear in pigs infected with the African swine fever virus;
- Highlighting the epidemiological aspects found among the population of domestic pigs affected by ASF in Romania and their possible association with new outbreaks of the disease;
- Identification of the African swine fever virus circulating in Romania, through molecular examinations and its molecular characterization through genotyping and phylogenetic analysis.

Chapter 9, entitled "Clinical and morphological evaluation of pigs infected with African swine fever virus" aimed to describe and characterize the clinical picture and macroscopic lesions found in pigs positive for African swine fever virus in Romania. To achieve this goal, a series of objectives were established: carrying out an evaluative study of the main clinical signs encountered in 9 pig holdings where the ASF virus was confirmed; carrying out the necropsy diagnosis on at least one individual from each unit and the macroscopic evaluation of the identified lesions and the presentation of the anatomopathological picture characteristic of each target organ of the ASF virus. Despite the general knowledge of the ASF virus as being highly infectious, specific clinical signs of ASF were absent (Figure 5A) in the surviving individuals from 3 holdings, and in the commercial farm, about a quarter of the individuals could be declared as clinically healthy. The clinical evaluation performed by inspection revealed clinical signs that could be associated with ASF, such as apathy, prolonged lateral recumbency, respiratory distress, cough, hyperthermia, changes in skin surface color, and abortion. Post mortem examination was performed on 13 individuals sacrificed on site for this purpose, but only 9 of these showed changes detectable to the naked eye. Thoracic effusions were found in 7 individuals and abdominal effusions in 2. Only one individual had color changes in the ears. Visible changes in the respiratory system and heart were identified in all 9 pigs, while lymph node and spleen lesions were observed in all cadavers. Hemorrhages of the renal parenchyma were visible in 7 individuals, while those of the urinary bladder only in 3 pigs. Only two individuals had visible changes in the liver and intestines, and gallbladder edema and hemorrhagic lesions were seen in the gallbladders of 7 individuals.

Chapter 10, entitled "Evaluation and histological characterization of the lesions found in ASF positive pigs" had as its main purpose the microscopic description of the lesions appearing in African swine fever virus positive pigs. To achieve this goal, we proposed the following objectives: the creation of a database with tissue samples from 118 ASF positive pigs, received from the Institute for Animal Diagnosis and Health (IDSA); morphologic characterization of lesions found in lymph nodes, lungs, heart, kidneys, spleen, liver, and gall bladder and evaluation of the distribution and severity of specific lesions found in ASF virus target organs. The microscopic lesions that are part of the specific picture of ASF were identified in the vast majority of the samples. Hemorrhages of various sizes were the most noticeable, the most affected organs being the spleen with a percentage of 93.1%, the kidneys with 87.7% and the lymph nodes with 83.7%. Edema lesions were found in almost all systems, but the gallbladder wall was the most affected (77.8%) followed by lungs with 69.4% and heart with 69.2%. Necrotic lesions, respectively lymphoid depletion was most frequent in the spleen (68.1%) and lymph nodes (61.5%). The inflammatory infiltrate was evident in the kidneys (54.3%) and lungs (52.5%). Microvascular thromboses were observed in almost all systems, but the highest incidence (57.2%)

was in the lymph nodes and kidneys (50.8%). Lesions typical of vasculitis were found in 31.5% of the examined kidneys and in 11.9% of the lymph nodes.

Chapter 11, entitled "Identification and immunohistochemical characterization of African swine fever virus in tissues from positively confirmed pigs" had as its main purpose the evaluation and immunohistochemical characterization of lesions that appear in pigs infected with African swine fever virus. In order to achieve this goal, we proposed the following objectives: the creation of a database with tissue samples from 118 ASF-positive pigs, received from the Institute for Diagnosis and Animal Health (IDSA), the immunohistochemical characterization of the lesions that appear in ASF, by using protein markers p72 in the viral capsid and qualitative and quantitative assessment of the presence of ASF virus in the affected tissues. The major ASF virus capsid protein p72 was detected in almost all tissue samples examined. In some cases, due to autolysis, characterization of infected cell types was not possible. Moderate to large numbers of macrophage-like cells were immunolabeled in most tissue samples examined. In the cytoplasm of infected cells, three types of immunostaining patterns were observed: granular, similar to some inclusions or diffuse, with varying intensities. Diffuse immunolabeling was so strong in some cells that it sometimes masked their nuclei. (Figure 24C). At the level of a few cells, the inclusion-type immunolabeling reactions were so strong, completely covering the cell nucleus that it was very similar to nuclear immunostaining (Figure 24C). The major capsid protein p72 was also detected extracellularly as fine to coarse granular immunostaining both in karyorrhexis tissue areas and in the lumen of the vasculature (Figure 24D, 25A,C, 26B,D). Mild diffuse background staining was observed in many of the samples examined. No immunostaining was detected in the tissue samples from the negative control groups.

Chapter 12, entitled "The influence of climatic and environmental factors in the spread of African Swine Fever" started with the aim of highlighting the epidemiological aspects found among the population of domestic pigs affected by ASF in Romania and their possible association with new outbreaks of the disease. The study describes the correlation between the number of outbreaks and the number of affected pigs with the climatic and environmental characteristics of the outbreak areas. In order to achieve this goal, we proposed the following objectives: carrying out a retrospective study of ASF outbreaks that evolved in Romania between February 6, 2020 and March 2, 2021 and creating a database and conducting statistical studies highlighting the correlation between the factors climatic (average annual humidity, temperature during spring, summer, autumn and winter), environmental factors (altitude, distance between forest, lake and river) and occurrence of ASF outbreaks. A very weak negative correlation was identified between longitude and number of cases (Spearman correlation, $rs = -0.19$, $p = 0.001$) or number of outbreaks (Spearman correlation, $rs = -0.16$, $p = 0.006$), with more many cases of infection in the western part of Romania. A positive linear regression was detected between the number of cases and longitude ($p = 0.02$, $R2= 0.013$),

showing an increase in the number of cases in the west of the country. A very weak negative correlation (Spearman correlation, rs = -0.13, p = 0.01) was identified between the number of affected pigs and longitude. humidity between 50-60% (Figure 29A). After statistical analysis, it was found that both the number of affected pigs and the number of outbreaks were found to be higher in localities with an average spring temperature of 10–14 °C. The number of infected pigs is much lower ($p < 0.001$) in localities with an average spring temperature between 6 and 10 °C (Figure 29B). During spring, a significantly lower number of affected pigs was recorded compared to the other seasons (Chi-square correlation test, $X2 = 9403.8$, $p < 0.001$) (Figure 28B-D).

Chapter 13, entitled "Genotyping of the strains of the African swine fever virus circulating in Romania" followed the identification of the African swine fever virus circulating in Romania, through molecular examinations and its molecular characterization through genotyping and phylogenetic analysis. To carry out this study, we proposed the following objectives: the creation of a database with the purified DNA samples received from the Institute for Animal Diagnosis and Health; detection of ASF virus in samples using polymerase chain reaction (PCR) and purification of amplicons for genetic sequencing, sequence editing and phylogenetic analysis. The presence of PPA viral DNA was confirmed in 105 samples tested from 31 counties in Romania (Figure 32). The presence of PPA viral DNA was confirmed in a total of 117 samples tested from 31 counties in Romania (Figure 32), 105 samples were positive for the B646L gene of the p72 protein and 106 samples were positive for the E183L gene of the p54 protein. Some samples were positive for both genes, others only for one. The Romanian samples with the PPA virus were grouped in genotype II with p72 showing 100% identity, and with p54, an identity between 97-100% with the compared vPPA isolates from Georgia (JX857509), Armenia (JX857508), Azerbaijan (JX857515), Belarus (KJ627215), Russia (JX857509), Lithuania (JX857508), Liv. Poland (KJ627218) and Ukraine (JX857521) (Figure 33). This is the first report of genotype II PPA in all of Romania from the present study. The current study is the first to report the presence of vPPA genotype II in Romania.

SELECTED REFERENCES

1. Bellini, S.; Casadei, G.; De Lorenzi, G.; Tamba, M. A Review of Risk Factors of African Swine Fever Incursion in Pig Farming within the European Union Scenario. Pathogens 2021, 10, 84.
2. Bergmann, H.; Schulz, K.; Conraths, F.J.; Sauter-Louis, C. A Review of Environmental Risk Factors for African Swine Fever in European Wild Boar. Animals **2021**, 11, 2692.
3. Blome, S., Franzke, K., & Beer, M. (2020). African swine fever – A review of current knowledge. Virus Research, 287, 198099. doi:10.1016/j.virusres.2020.198099, 7
4. Boklund, A.; Cay, B.; Depner, K.; Földi, Z.; Guberti, V.; Masiulis, M.; Miteva, A.; More, S.; Olsevskis, E.; Šatrán, P.; et al. Epidemiological analyses of African swine fever in the European Union (November 2017 until November 2018). EFSA J. 2018, 16, e05494.
5. Fischer, M.; Hühr, J.; Blome, S.; Conraths, F.J.; Probst, C. Stability of African Swine Fever Virus in Carcasses of Domestic Pigs and Wild Boar Experimentally Infected with the ASFV "Estonia 2014" Isolate. Viruses 2020, 12, 1118.
6. Fischer, M.; Mohnke, M.; Probst, C.; Pikalo, J.; Conraths, F.J.; Beer, M.; Blome, S. Stability of African swine fever virus on heat-treated field crops. Transbound. Emerg. Dis. 2020, 67, 2318–2323.
7. Mazur-Panasiuk, N., Woźniakowski, G. & Niemczuk, K. The first complete genomic sequences of African swine fever virus isolated in Poland. Sci. Rep. 9, 1–9 (2019).
8. Mazur-Panasiuk, N., Żmudzki, J., & Woźniakowski, G. (2019). African swine fever virus – persistence in different environmental conditions and the possibility of its indirect transmission. Journal of Veterinary Research, 63(3), 303-310. doi:10.2478/jvetres-2019-0058
9. Montgomery, R.E. On A Form of Swine Fever Occurring in British East Africa (Kenya Colony). J. Comp. Pathol. Ther. 1921, 34, 159–191
10. Salman, M. The role of veterinary epidemiology in combating infectious animal diseases on a global scale: The impact of training and outreach programs. Prev. Vet. Med. 2009, 92, 284–287.
11. Sauter-Louis, C.; Conraths, F.J.; Probst, C.; Blohm, U.; Schulz, K.; Sehl, J.; Fischer, M.; Forth, J.H.; Zani, L.; Depner, K.; et al. African Swine Fever in Wild Boar in Europe—A Review. Viruses 2021, 13, 1717.
12. Viltrop, A.; Reimus, K.; Niine, T.; Mõtus, K. Biosecurity Levels and Farm Characteristics of African Swine Fever Outbreak and Unaffected Farms in Estonia—What Can Be Learned from Them? Animals 2021, 12, 68