OVIDIUS UNIVERSITY OF CONSTANTA DOCTORAL SCHOOL OF APPLIED SCIENCES

BIOLOGY / BIOCHEMISTRY FIELD

DOCTORAL THESIS

AFRICAN SWINE FEVER IN DOMESTIC PIGS AND WILD BOARS THROUGH THE INVESTIGATIONS USED IN THE MOLECULAR BIOLOGY LABORATORY THAT ALLOW THE VIRAL GENOME TO BE HIGHLIGHTED BY THE REAL TIME PCR AND ELISA TECHNIQUE

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CONTENT

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OBJECTIVES AND PURPOSE OF THE WORK

In recent years, due to the high number of reported outbreaks of African swine fever (ASF) and the economic consequences, this virus is considered the most important of all diseases that manifest themselves in pigs both in our country and globally. African swine fever (ASF) is a viral disease of domestic pigs and wild boars.

We chose to study this disease due to the special medical and socio-economic impact it has on international trade and which requires that the reporting of new cases of the disease be compulsorily notifiable to the WOAH (World Organization for Animal Health). The disease is caused by a distinct virus, the only one in the *Asfaviridae family.* Infected animals present a wide variety of clinical forms that vary depending on the virulence of the virus but also on the immunological characteristics of the host. The current absence of effective prophylaxis or countermeasures makes it an economic danger for both the affected and neighboring regions. That is why we consider it imperative to follow up on aspects regarding epidemiology, such as the mode of transmission of the disease, the reaction of the host organism, in order to be able to prevent transmission and also to implement strict biosecurity measures and control and eradication strategies.

Starting from these aspects stated above, the present paper aims to identify the ASF genome through laboratory techniques carried out within DSVSA Constanta by identifying positive cases of ASF and aims at the following objectives:

Establishing the epidemiological curve and ASF incidence in Constanta County over a six-year period (2018-2023) and also a comparison of the transmission dynamics of ASF outbreaks between different countries.

A second epidemiological objective is to highlight the existence of a possible seasonal dynamics that allows the close analysis of possible transmissible vectors.

Sequencing data analysis and phylogenetic tree generation were performed using GenBank sequencing data to highlight similarities between our isolates versus isolates from other countries.

Comparison of two types of samples examined by the Polymerase Chain Reaction (PCR) method that showed based on the analysis of Cycle Treshold (CT) values the detection of the PPA viral genome in predominant quantity in ethylenediaminetetraacetic acid (EDTA) blood samples compared to tissue and organ samples, indicating that the blood on EDTA has a higher specificity for the detection of the PPA viral genome, both in the early stages of the disease and in the late stages of evolution.

Detection and monitoring of ASF virus infection in domestic pigs and wild boars, through a bilateral approach, using viral genome detection methods as well as detection of anti-virus antibodies. By examining a wide range of samples, it was highlighted that the PCR method allows the detection in the pre-viremic stages of ASF infection as well as in the stage of systemic dissemination of the virus leading to the onset of viremia, while the enzyme-linked immunosorbent test (ELISA) method allows the detection of the presence of ASF-specific antibodies starting from the 14th day after contact with the virus. Therefore, it is not a reliable method of analysis for confirming the disease, but it can be used to monitor the post-ASF infection immunological status of domestic pig and wild boar populations.

We considered another method of analysis to detect positive cases of ASF as quickly as possible, based on highlighting changes in blood parameters in the different stages of viremia by comparing positive cases with negative cases.

CHAPTER 1

AFRICAN SWINE FEVER VIRUS

1.1 Structure of the genome

This chapter delves into the characteristics and significant diversity between isolates of the PPA virus genome that is part of the ASF family. *Asfarviridae*, being the only member of this family, the species African swine fever virus (ASF), genus *Asphivirus* classified as a *DNA Arboviruses* (Alejo et al., 2018). The ASF virus has been classified into 24 genotypes based on the sequencing of the B626L gene, which encodes the p72 protein. There was a distribution of these genotypes in different geographical regions with varied distribution depending on the area (Chapman et al., 2008).

The structure of the viral genome has been studied since the onset of the disease and the results showed that this large double-stranded DNA molecule 170-193 kilobase pairs (kbp) encodes between 151-175 open read frames (ORFs) (L. Wang et al., 2020). The PPA virus has a multilayer structure, the viral nucleus is enveloped by a lipid double layer, by a capsid that gives the icosahedral shape of the virion, by an inner envelope derived from

the endoplasmic reticulum and a base envelope that surrounds the viral nucleus, this envelope is composed of two polyproteins, pp220 and pp62. Research has shown that in the absence of pp62 the infection produces viral particles without a nucleus (Suarez et al., 2010).

The genome consists of a central region that is very well conserved and the regions at the ends that are variable and play a role in its adaptability and diversity (Bao et al., 2022) being rich in multigene families (FGM), they contribute to the virulence and adaptation of the virus (Domelevo Entfellner et al., 2024). The variation between the genomes of the different ASF isolates is most commonly due to the gain or loss of members of multigene families, MGF 100,110,300,360,505/530 impact that the loss of MGF 110 has on how the virus increases its pathogenicity by avoiding the immune response (Vega et al., 1990).

68 structural proteins and another 100 nonstructural proteins were identified (Y. Wang et al., 2021). Among the structural proteins p72, p15 and p35 are involved in virion formation and non-structural proteins such as deoxyurinine triphosphate (dUTP) are involved in replication and transcription (G. Wang et al., 2021), but also flexible genes with histamine-like proteins and transcription factors that encode proteins involved in avoiding the host immune response such as the ASFV 5-HL gene, which encodes the Bcl-2 gene that prolongs the survival of apoptotic cells, thus helping the virus avoid the effects of the host's immune response. While the 4CL gene, although it has a similar structure, does not show apoptotic activity (Neilan et al., 1997).

PART II

PERSONAL CONTRIBUTION

INTRODUCTION

Given that there are no scientific data on the evolution of ASF disease in Constanta County, Romania, we focused the present research on the diagnosis and confirmation of ASF outbreaks and implicitly on the epidemiological aspects encountered among wild boars and the domestic pig population. The human risk factor was considered to have an important role in the spread and maintenance of ASF in the population of domestic pigs raised in the private household system. ELISA testing was a useful tool used to show the spread of ASF over the course of six years after the first ASF case was confirmed. The present work aims to identify the ASF genome through laboratory techniques carried out at DSVSA Constanta for the confirmation of positive ASF cases and viral phylogenetic analyses carried out in the Genomics laboratory of the University of Agricultural Sciences and Veterinary Medicine in Cluj-Napoca.

CHAPTER 5

MATERIALS AND METHODS, TECHNIQUES, EQUIPMENT

5.1 Method of analysis for the epidemiological study of the evolution of African swine fever in the area of Constanta County

5.1.1 Epidemiological investigation

In order to identify the ASF virus, the host and the environmental factors that cause this disease, an entire team, represented by veterinarians and engineers from the Animal Health Office within DSVSA Constanta, which has a specific structure and responsibilities regarding the eradication of ASF at the local level based on the requirements of the Operational Manual for ASF (Terrestrial Code Online Access, n.d.), develops the contingency plan and associated responsibilities. These steps are established by the Local Center for Disease Control (CLCB), organized in three distinct divisions: the Local Decision Unit (ULD), the Local Operational Unit (UOL), which also supervises the Center for Field Investigations (CIT) and the Local Support Unit (ULS).

Epidemiological methods are used to establish the origins of ASF disease, the mechanisms of its transmission and the necessary prevention and control strategies. The initial phase involves the collection of data through the surveillance programme, including information on the onset of symptoms, disease incidence and mortality among the domestic pig population on a specific farm or the wild boar population in the vicinity of the outbreak, as well as the clinical status of the affected pigs. Based on an in-depth evaluation of the data collected, the epidemiology team classifies and synthesizes this information, subsequently identifying discrepancies and drawing conclusions on potential causal factors for disease transmission or associated risk elements. Finally, it proposes and executes strategies through intervention measures aimed at reducing the further spread of the disease and informing farmers, hunters and the wider public about the significance of their behaviour in relation to the spread of the disease. The eradication protocols are instituted by the CIT team following the official confirmation of ASF disease in the laboratory, requiring the immediate euthanasia of all pigs.

The CLCB has the obligation to establish a 3 km protection perimeter around the ASF outbreak and will order the slaughter of all pigs in the affected premises, which will be placed under official veterinary supervision to

mitigate any potential for viral transmission. Sampling procedures are performed on all pigs at the time of euthanasia, in accordance with the Terrestrial Code Online Access to determine how the virus is introduced into the farm and to determine the duration of its presence before the disease is notified. The disposal of the carcasses and all hazardous materials resulting from the outbreak is carried out by an alternative method of neutralization, in particular by burial in a location selected by the Environmental Protection Agency of Constanta (APM), the Administration of the Dobrogea Coastal Water Basin (ABADL) and the Autonomous County Water Administration (RAJA). Complete measures of mechanical cleaning and disinfection are instituted, along with the incineration of all sources of contamination. Appropriate disinfection measures shall be established at the entrances to the farm and in the stables. A comprehensive mapping of all areas in the protection area shall be carried out. The entry or exit of animals of any species in and/or in the protection area is strictly prohibited. Samples are taken from both sick and deceased animals and sent to the laboratory for confirmation. These samples are to be collected, recorded, statistically processed and the data subsequently used in ASF control and eradication efforts. Gatherings of animals of all species, including fairs, exhibitions, circuses, etc., are expressly prohibited. The application of measures in the protection zone will persist until the completion of the cleaning and disinfection protocols in the infected premises. All pigs located in all farms in the protection zone will be subject to clinical and laboratory evaluations for a period of 45 days. After completing these procedures and confirming the absence of the disease, the restrictions will be lifted.

The ASF surveillance area shall be established at a distance of 10 km around the ASF outbreak and a mapping of all holdings in the surveillance area shall be carried out. The provision of information and education to breeders must be ensured.

Animal owners are mandated to carry out a passive clinical assessment of their pigs and are obliged to report any change in the health status of these animals to the officially appointed veterinarian. The movement of domestic animals outside the surveillance area is strictly prohibited, except for pigs designated for slaughter and those that have received authorization from local veterinary authorities. Gatherings of animals of all species in the context of animal fairs and exhibitions is expressly prohibited. Any deceased or sick pig on a particular farm must be promptly reported to the official veterinarian, who will initiate the necessary investigations in accordance with the protocols outlined in the African Swine Fever Operational Manual (Terrestrial Code Online Access, n.d.). Communication with the county forestry directorates and branches of the County Association of Sport Hunters and Fishermen (AJVPS) regarding the provisions of the CNCB is essential. The quantification and delimitation of areas inhabited by wild boars shall be evaluated in order to avoid any interaction with the surveillance area.

In the context of small-scale operations, such as private households, the restocking process is prefaced by the introduction of sentinel pigs that have had a negative test for ASF antibodies or come from farms that are not affected by ASF restrictions. Sentinel pigs shall be strategically distributed throughout the holding in accordance with the provisions laid down by the veterinary authority. After a duration of 45 days, these pigs will be tested to establish the presence of antibodies against the ASF virus, according to the Terrestrial Manual Online Access (ASF).

If the test results are negative, the complete restocking process can begin. In the case of commercial holdings, the restocking of pigs is carried out in accordance with the established legislative directives and is conditional on the total restocking of all pigs from holdings that are not subject to ASF restrictions. Pigs from the newly populated herd are subjected to serological evaluation in accordance with the Terrestrial Manual Online Access (ASF). Sampling for this investigation shall be carried out no earlier than 45 days after the arrival of the final group of pigs. The research team, composed of veterinarians, uses a variety of sources and methodologies to collect data in the field, with the aim of investigating and evaluating the epidemiological background of the disease. A standardized questionnaire (Figure 1.) is used to gather accurate information regarding the type of production, owner details, the number of additional farms owned by the same owner, animal identification and the total number of each animal species owned, as well as geographic coordinates and environmental characteristics such as neighboring farms or agricultural areas, as well as primary and secondary routes and arteries according to the Operational Manual for Intervention in ASF outbreaks (Operational Manual for Intervention in African Swine Fever Outbreaks – 4th Edition – 2019 – A.N.S.V.S.A., 2019)

VETERINARY SANITARY AND FOOD SAFETY DIRECTORATE
CONSTANTA,
CSVSAO ...

epidemiological investigations

for African swine fever in non-professional Holdings,
type a commercial holdings and "peasant farm"holdings according
to law no. 6/2022

Performed in the unit...
Unit type non-professional sexploitation, a commercial holding Type A a holding type "peasant household" according to law no. 6/2022
Name and surname of owner / owner of animals ...
Address: County... commune ... locality ...
No. phone ... Holding code ...
Geographical coordinates holding: Longitude ... Latitude...
CSVSAO... Narrie of authorized veterinarian
Official veterinarian conducting the epidemiological investigation ...

1. Farm information:
1.1 schtta exploittet (hand drawing)

1.2 location of the holding in the locality / area with the description of the oblective principalities:
(Holdings with or without livestock, agricultural land, forest, mairl and secondary roads, etc.)

1.3 Holding and/or managing one or more pig Holdings. Yes No If
Yes: Location (address)...
Geographical coordinates: Longitude ... Latitude ...

1.4 status of livestock of species other than pigs

Speoie	Cattle	Equines	sheep	goats	PMPs	Other, speoify
Number of heads						

2. Biosafety infrastructure:
2.1 minimum biosecurity requirements
- Location holding la level locality:
- egg fencing continuous fence so that the OU porcine shelter does not have access to other porcines, other domestic animals of other species, wild porcines Yes c No c;

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- At the entrance to the perimeter of the swine shelter, the change of shoes is ensured Yes Walnut:
- At the entrance to the perimeter of the pig shelter there are facilities to ensure hand washing Yes c
- Theentrance to the pig shelter there is a disinfectant for disinfection shoes Yes No
If so, the substance used (e.g. oloros lime, other virucid substance)

Observations

2.2 artificial/natural seeding is practiced yes a no a,
if so, detail (name and address of the Seeder, source of the semen / source of the boar, date seeding)

2.3 in the case of artificial installation/seeding, the traceability should be established boar / Seeder in the last 30 days before suspicion / appearance of the first signs of disease ...

2.4 in the case of the purchase/sale of pigs, indicate which means of transport were used, vehicle registration number ...

2.5 other biosafety issues:...

2.6 in the last 30 days before the suspicion / appearance of the first signs of the disease there were:
- source of entry of meat or pork products Yes No c,
If so, mention the provenance
- family events Yes No
If so, expand the epidemiological survey on participating individuals and the source supply of meat / pora meat products
- repair/construction activities were carried out Yes No
c, If so, expand the epidemiological survey on the participating individuals and source of supply of used materials ...
- visits made by foreign persons in the perimeter of the OU porcine shelter Yes No d,
If so, extend the epidemiological investigation to these people

2. 7. Checking the activity of family members or employees in:
- silvatio environment possibly affected by PPA (V), material management activities Woody, mushroom picking etc)
- ogricole activities
activities units food industry, SNCU, feed, farm etc

2.8 Edisto: Wild Boars on the hunting grounds by the way? Yes s no c;
If so, when was the last time a positive case was detected? The last case will be mentioned virologically positive or last case serologio positive and virologically negative

2.9 feed
- from own production Yes No ;

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If so, tarla location / cultivated area (traces of hogs have been observed)

.....

- purchased Yes No c,

Yes, mention: date ac acquisition origin and registration number of the means of transport used and expand the epidemiological investigation with respect to other EF transport performed of those animals means d transport to ra ltee xploitatiile pigs, 30 days prior to suspicion / appearance of the first signs of disease

.....

- Cereals used in pig feed were stored year it's been at least 30 years days, prior to the suspicion / appearance of the first signs of illness Yes No s, not known

2.10 administration of fresh green mass from infected PPA-if observed traces of Wild Boars (e.g. in alfalfa field, hay, forest edge, garden without fencing etc.)

YES NO c,

If so detail: .....

.....

2.11 administration of food waste (waste) from your own kitchen or from third parties (waste) from washing fresh/frozen meat as a result of shaping, etc.)

Yes c No c,

2.12 bedding-use straw bedding Yes Walnut,

If so, the straw has been stored prior to use for at least 90 days, yes c No s, no known =

when using straw as bedding, determine their origin

.....

2.13 other aspects of forage/soohygiene / maintenance

.....

.....

2.14 findings medio official veterinarian (dsvsa/CSVSAO veterinarian) conducting the investigation at suspicion / confirmation:

2.14.1. No. ... date ... . the latest PPA biosafety advisory sheet developed by the MVLPI ... .;

2.14. 2. Establishing an epidemiological link with a confirmed case / outbreak of PPA, Yes No c, if so, detail

.....

.....

3. PPA Suspictunea

3.1 date of announcement of the veterinarian regarding the illness / death of animals ... .

3.2 doctor's Name announced ... .

3.3 suspicion was made on the date of ... . based on:

The olinic exams Yes No c,

- Figet surveillance PPA no. ... date ... and/or

- necropsy Act No. .... date ... .

issued / issued by dr. ...

practice empowered/veterinarian dsvs / 5vsothe quality of medio veterinarian of free

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A number of ... ... samples .....for laboratory examination that were sent to lsvsa arrondissement/IDSA .....

3.4 number of sick/dead pigs at the date of notification by the animal owner/keeper the veterinarian... .....

.....

.....

3.5 date / time of occurrence of the first olinic signs declared by the owner/holder of animals... .....

3.6 what olinic signs were observed by the owner / keeper of animals

.....

.....

3.7 date / period of occurrence of the first deaths according to the declaration of the owner / holder of animals ... .

3.8 number and category of dead poroes ... .

3.9 evidence taken on suspicion in order to confirm or disprove suspicion; and test result:

Date sampling	Tip Probă*	Trimitere date LSVSA/IDSA	LSVSA/IDSA	Testefec .ut	B.A. nr./data	Rezultat

*Set organs, abortions, blood on EDTA, whole blood

3.10 inventory in case of PPA suspictune

3.10.1 updated inventory on categories, number of Animals held in the establishment, number of animals that died or showed clinical signs at the time of suspicion / investigation:

Categoria deporci	Nr. porci, conform SNB	Nr. TOTAL porci în exploatație	Nr. de porci clinic sănătoși				Nr. de porci cu semne clinice				Nr. de porci moarte/eliminați de neatate			
			Concordanță cu SNB	Neconcordanță cu SNB	Născute în exploatație și nedentificate	Provenite din alte exploatații și nedentificate	Concordanță cu SNB	Neconcordanță cu SNB	Născute în exploatație și nedentificate	Provenite din alte exploatații și nedentificate	Concordanță cu SNB	Neconcordanță cu SNB	Născute în exploatație și nedentificate	Provenite din alte exploatații și nedentificate
Scroafe														
Vieri														
Purcei nelnțărcați														
Tineret														
Porci la îngrășat														
Total														

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3.10.2 identification data of pigs in accordance with Delegated Regulation (EU) 2019/2035 (there will be attached a report of the exploitation of the BND extracted at the date of suspicion of the disease / investigation for the period at least a year and a half ago).

3.11 checking farm records

3.11.1 verification of the connoordance of the inventory mentioned in item 3.10 with the co-States of holding;

(please note if and what inconsistencies are found) .....

.....

.....

.....

3.11.2 checking the concordance between ear tags from the BND and those existing in the field

.....

.....

.....

3.11.3 verification of entries in the BND of pigs, with minimum 30 days retroactive from the date suspicion / appearance of the first signs of the disease, mention shall be made of the origin

their... .....

.....

3.11.4 verification of pig BND outputs, with minimum 30 days retroactive from the date suspicion / the appearance of the first signs of illness, mention should be made of the destination.

.....

.....

3.11.5 verification of su events regarding the slaughter of pigs own consumption, with minimum 30 days retroactive from the date of suspicion / appearance of the first olinic signs, the reason will be mentioned slaughter

.....

.....

.....

3.11.6 in the case of events referred to in pot. 3.1.5. the destination of the meat shall be mentioned

.....

.....

3.11.7 verification of events found at the holding level and not recorded in BND, their detailing

.....

.....

.....

3.11.8 in the case of pigs present on the holding and not registered to determine their origin and period their introduction to esploitation

.....

.....

3.11.9 the date of the last visit and its purpose by MVLPI / health personnel (conf. the health staff statement, veterinary anaitar, registry of consultations and treatments, BND on identification animal ilioer ex in ..... question, ve sanitary actionsderinary mandatory, eto)

.....

.....

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3.11.10 what other swine holdings have been visited by veterinary staff who have visited controlled holding in the last 30 days prior to suspected illness?

.....

.....

4. Confirmation of PPA

4.1 confirmation was made on ... ...based on bulletin no. ... date ... ., teste performed ... .

4.2 management of cremated bodies, unit ... . s burial.

4.3 uois/dead pigs from the first clinical signs Yes No ,

4.4 number and category of poroes killed / dead .....

.....

.....

4.5Official retention / neutralization meat, offal, gastrointestinal meal

Yes No a, if yes what quantity and what is their destination?

.....

4.8 treatments performed in the last 30 days (who performed the treatment, medication administered name of products used, date/period of treatment)

.....

4.9 number of pigs undergoing treatment ... .

4.10 symptomatology and diagnostio olinio cnsigned in the Register of oconsultations and treatments

.....

.....

.....

5. Increased risk period-PRC established:

Probable date of introduction of the PPA virus into the holding

Date	Date	Date of Investgation	Oel moment	The moment
suspicion:	confirmation:	epidemiological;	further afield:	closer:

....

Probable period of time elapsed since the appearance of PPA

6. Hypotheses on the probable origin of the disease and the means of response:

6.1 link to pigs held, their epidemiological units and units, structures in the food or feed sector, establishments producing by-products of origin animal or any other place where animals of the species listed for the listed disease suspected may have been infected:

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Hypothesis	Findings	Probability* Excluded/Low/Medium / High
Epidemiological link with another unit (holding pigs, etc.), by: Movement of pigs (purchase / sale of pigs, temporary movement of pigs in view of reproduction)		
-Movement of people		
- Veterinary sanitary activities (treatments, surgeries)		
- Identification activities and animal registration		
- Flax activities of artificial insemination		
-Products of animal origin		
- Animal by-products		
- Means of transport		
- Possibly contaminated feed		
- Machinery / Equipment		
- Bedding		
-Other		

6.2.Link to wild pigs (wild environment)

Hypothesis	Findings	Probability* Exelus / Small/Medium / Large
Epidemiological link through - Contact of pigs held with Wild Boars		
- Products from Wild Boars positives		
- Vs. vs. vs. vs.		
- People performing activities the management a materialului lemnos		
- Persons performing activities in the forest environment (mushroom picking, fruit of pidure, ete)		
- Agricultural activities		
-Mismanaged boar corpses		
- Other		

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7. Concluded

Probable origins of boil: .....
Means of delivery: .....

Probable time of occurrence of PPA vaults / probable date of virus introduced in holding: .....

Epidemiological units and units within them, any other sites where pigs for suspected disease may have been infected: .....

Means by which the pathogen could have access during the relevant period of prior to notification of suspected or confirmed PPA: .....

Official Veterinarians who have conducted an epidemiological survey Name and surname,signature	Responsible species Name and surname,signature
.....	.....
Headteenic SCVSA Name and surname/signature	Director / adj director. Name and surname/signature
.....	.....

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Figure 1. Model Epidemiological Survey for ASF in non-professional farms. Type A commercial holdings and in compliant household type holdings (contains 8 pages).

The epidemiological investigation focuses on 30 days before the first signs of the disease or suspicion of disease. Data is also collected when the owner uses artificial insemination, the date on which the inoculation took place, the name and address of the semen supplier and the traceability of the donor.

Data on the entry of contaminated meat products and by-products into the farm, if confirmed, the owner must provide details of origin and thus the epidemiological investigation must be extended and thus all participants and the main source of supply of contaminated meat and meat products are recorded. The checks of the activities carried out on the farm are carried out on all employees or family members, such as stable maintenance activities, hunting activities, wood cutting or mushroom picking, agricultural activities that provide animal feed, interaction with other farms.

If the farm is located near a hunting ground confirmed positive for ASF, it is recorded when the last positive case was confirmed by the Real Time PCR or ELISA method and when the last negative test was using the same analysis methods. As regards feed, the owner must specify whether he uses cereals from his own production in this case and whether he has noticed wild boar tracks on his agricultural land. If the owner purchases the feed, the date of purchase, the source, the license plate of the vehicle used for transport and all deliveries to other pig farms are recorded 30 days before the first clinical signs from which the onset of ASF appears. It is also recorded if the pigs have been fed with kitchen scraps and if they have not been heat treated. As for the bedding used (straw), it is recorded if it was stored at least 90 days before the outbreak of the epidemic.

5.1.2 Statistical analysis

For the statistical processing of the study data, IBM SPSS Software Descriptive Statistics for Windows, version 29.0, was used. (30-day trial) Armonk, NY: IBM Corp. Nominal data were presented as absolute frequency and percentage, and continuous variables were expressed as mean and standard deviation. A value of the coefficient of statistical significance $p<0.05$ was considered significant.

5.1.3 Geographic analysis

The tracing of ASF outbreaks on the map was done by using the images of the Geographic Information System on Google Earth Pro, which allows the integration of longitude and latitude data collected by the epidemiology team with spatial information. The map content includes the physical layout of land, cities, roads, customs borders, and the exact location of ASF outbreaks obtained by longitude and latitude to predict the occurrence of ASF outbreaks and to generate hypotheses on the possible association between risk factors such as wild boars and human intervention in disease transmission.

The laboratory methods used to confirm ASF outbreaks were Real Time PCR for the detection of the ASF virus genome and passive serological surveillance by using a test antigen for the detection of ASF antibodies in the blood serum sample.

5.2 Method of analysis for genetic examinations by virus test: sequencing and phylogenetic analysis

In order to identify the circulating ASF strain in the investigated area, the sequencing of amplicons (PCR products) obtained after DNA extraction and purification (step detailed in chapter 5.3) of ten positive samples of domestic pigs and wild boars was performed.

5.3 Method of analysis for virological examination by the method of identification of the PPA virus genome using the method of polymerase chain reaction in time

QPCR real

The polymerase chain reaction is based on the use in a mixture of eluate reaction (template DNA), thermostable DNA polymerase, primers and the four excess deoxyribonucleoside triphosphates (dNTPs) in a buffer solution. The tubes containing the reaction mixture are subjected to repetitive cycles at different temperatures, 40 times inside the heating block of a thermo-block system such as the Applied Biosystems 7900HT Fast Real-Time PCR (Applied Biosystems, USA) or CFX96 Touch System Real – Time PCR (Bio-Rad, USA) The device allows the duration and sequence of temperature step cycles to be established.

a complete PCR reading.

Obtaining CT values. It represents that cycle in which the fluorescent signal of the reaction crosses the threshold line (treshold). The CT value is inversely proportional to the starting amount of the DNA target, in conclusion, the amount of DNA decreases and the CT value will increase. The Real Time PCR treshold

represents the level of a fluorescent signal that reflects a significant increase above the calculated baseline signal. The thresold is meant to distinguish the relevant amplification signals from the background (noise area), it is automatically set by the Real Time PCR instrument software to a value ten times multiplied by the standard deviation of the baseline fluorescence value. The treshold is represented by a horizontal line in the amplification graph that is set where the amplification curves are straight and parallel to each other and where the precision of the replicates is higher. In Figure 2. CT values obtained during the analysis of positive samples are observed.

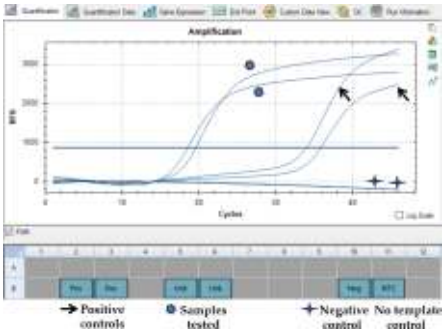


Figure 2. Real-time PCR amplification graph highlighting two samples tested positive for ASF infection. Unk: tested samples; Pos: positive controls (strain reference BA71VR); Wart: negative witness (water sample); NTC: negative warning light (contains only Master Mix).

5.3.4 Equipment

Amplification phase analysis

Amplification was performed on two devices, namely by using the Applied Biosystems 7900HT Fast Real-Time PCR using 2.4 software (Figure 3) and the Bio Rad CFX96 Touch System Real-Time PCR using CFX Maestro 2.3 software (Bio-Rad, USA) (Figure 4) both devices use fluorescent-based polymerase chain reaction to provide quantitative detection of nucleic acid sequences using real-time analysis. The following thermal profile was used: initial denaturation for 2 min at 95 °C(1 cycle) followed by 45 cycles for 10 s at 95°C, alignment of primers for 15 s at 58°C and extension for 45 s at 60°C. The plates were read with the FAM filter activated at the end of the 45 cycle extension. The result was considered negative for CT values ≥ 39 (Angel et al., 2022).

Figure 3. 7900HT Fast Real-Time PCR Amplification Equipment Applied Biosystems.

Figure 4. CFX96 Touch System Real-Time PCR Bio Rad Amplification Equipment.

5.4 Method of analysis for serological examination using the method of detection of anti-virus PPA antibodies by the immuno-enzymatic technique ELISA

5.4.4 Equipment

The ELISA method was used for the detection of anti-virus ASF antibodies by the indirect immunological technique. Plasma and serum samples were analyzed using the ID VET kit and the Ledetect 96 Led Based ELISA reader; Channel Microplate Reader Austria (Figure 5). Multi-antigen indirect ELISA Kit- ID Screen African porcine swine fever The indirect screening test (Louis Pasteur- Grabels, France) was used for the detection of

antibodies against P32, P62 and P72 of ASF (sensitivity, 99% correspondence with OIE ELISA and specificity – 100% OIE ELISA correspondence).

Figure 5. Reading equipment Ledetect 96 Led Based Channel Microplate Reader Austria.

5.5 Method of analysis for haematological examination by analysis of blood constituents on EDTA in animals tested positive for ASF virus

The study was carried out in a household, where the day before an outbreak of ASF was confirmed by the Real Time PCR method, on a group of 15 domestic pigs (*Sus Scrofa Domesticius*) composed of 14 young pigs aged 6 months and one sow aged 18 months. The pigs were housed in four pens arranged linearly and separated by a metal mesh fence. The first group composed of 3 pigs (a sow and two piglets) that showed critical clinical signs fever (41ºC) and prolonged decubitus moving only assisted, the second group composed of 5 pigs showed disorientation, lack of appetite and intermittent ataxia, the third group was composed of another 4 pigs with reduced mobility and lack of appetite and the fourth group consisted of 3 pigs that did not show clinical signs of disease. These pigs were finally disposed of in accordance with European and national legislation on animal welfare and care. The negative control group was 5 pigs, all 9 months old, from another farm that showed no clinical signs associated with any known disease.

The blood samples on EDTA were analyzed at the Quick Vet clinic, Constanta within 6 hours of collection with the help of an automatic veterinary hematology analyzer (URIT – 3000Plus) manufactured by Urit Medical Electronics for the determination of total white blood cells (WBC), mean platelet volume (MPV), red blood cells (RBC), hemoglobin concentration (HGB), hematocrit (relative volume of HCT erythrocytes), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution (RDW_CV). All results were compared with the calibrated references of the URIT-3000Plus analyzer for *the domestic sow top.*

The plasma and serum samples were analyzed at the serology laboratory of DSVSA Constanta for the detection of antibodies to African swine fever virus by the enzyme-linked immunoswine technique (ELISA) using the ID Screen African Swine Fever Indirect Screening Test Kit and for the reading of the plates the ELISA reader - Ledetect 96 Led Based, Channel Microplate Reader was also used.

5.5.4 Equipment

To analyze the samples, we used a URIT-3000Plus URIT Medical Eectronic hematology analyzer (Figure 14) and an ELISA- Ledetect 96 Led Based, Channel Microplate Reader for the detection of anti-virus PPA antibodies by enzyme immunosorbent assay (ELISA) technique (Figure 13).

Figure 6. URIT-3000Plus URIT Medical Eectronic automatic hematology analyzer equipment.

CHAPTER 6

OWN EXPERIMENTS

6.1 EPIDEMIOLOGICAL STUDY OF THE EVOLUTION OF ASF IN THE AREA OF CONSTANTA COUNTY

(Anghel et al., 2025)

The prevention and control of the ASF virus is difficult due to the ease with which it spreads, the resistance to environmental conditions but especially the lack of effective immunoprophylaxis, this affects the country's economy due to losses in trade and pig production by implementing drastic and costly control strategies to eradicate the disease. Worldwide, at present, according to the ASF Self-Declaration Report to the World Organisation for Animal Health (WOAH), the only ASF-free country is Belgium and according to the same report, two countries in Europe have reported new ASF events, while nine countries in Europe have updated their ASF situation as evolving (Self-Declared Disease Status – World Organisation for Animal Health, n.d). No new outbreaks have been reported in 2025 by countries in the Americas, Asia, Africa or Oceania.

Despite the fact that ASF has been one of the viral diseases studied for more than 100 years (Penrith and Kivaria, 2022), it continues to cause significant economic losses that can only be prevented by understanding as thoroughly as possible how the ASF virus is distributed, in order to target disease control methods as effectively as possible.

6.1.1 Purpose of the study

In the current study, we investigated the evolution of a total number of 93 ASF outbreaks confirmed on the territory of Constanta County by testing a total number of 3085 samples collected, from animals susceptible to the disease, during the period 2018-2023. We have analyzed the geographical area in the western area of the county where we found a number of 35 outbreaks in wild boars and thus we can predict that ASF outbreaks have spread to other areas due to the route of wild boars through their migration from the Danube Delta to the (western) forest area of Constanta County (Update of the situation regarding the evolution of African swine fever (ASF) - ANVSA., 2023).

We conducted an analysis of the epidemiological curve to deduce where and when the distribution of the disease occurred in the county and we concluded that in some regions the most likely way of spreading the epidemic is represented by wild boars, which are considered the main reservoir of the disease, but by following the geographical map of outbreaks we can also observe areas where the virus could have been transmitted by human animal interaction.

Starting with July 5, 2018, it was confirmed for the first time in Constanta County in a private household in the province of Istria. The index case described during the investigation was a pregnant sow found dead in the stable. Following the notification of the official veterinarian in the affected area, the samples were collected and sent to the DSVSA Constanta molecular biology laboratory for Real Time PCR analysis, which resulted in positive results for ASF. The affected area has been placed under official surveillance, in accordance with the control measures established by the Operational Manual of the PPA (Terrestrial Code Online Access, n.d.).

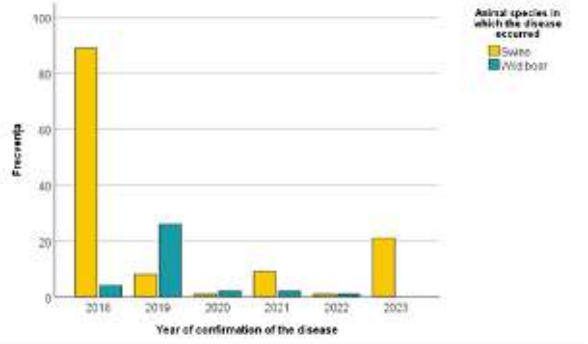
Subsequently, on July 11, 2018, a second ASF outbreak was confirmed when another seventeen pigs died in a private household farm in Cheia, about 32 km from Istria. Restriction and surveillance action was taken in both outbreaks to comply with At. 5 of EU 429/2016 with subsequent additions and amendments (Regulation-2016/429-RO-EUR-Lex, n.d.) and in accordance with EU 1099/2009 with subsequent additions and amendments (Council Regulation (EC) No. 1099/2009 of 24 September 2009 on the protection of animals at the time of killing, 2009) on the method of killing and protection of animals. Thus, animals, products and waste can be destroyed by burial and burning methods in an approved location. During the killing, strict biosecurity procedures were followed by spraying the carcasses, tissues, blood, shelter and yard with disinfectant. Measures have been implemented to prevent the spread of ASF virus by restricting animals, vehicles and equipment to and from the outbreak and establishing the protection zone (3 km) and surveillance zone (10 km) (Figure 7). Despite efforts to contain each outbreak, 164 ASF outbreaks had been reported by the end of 2023.

Figure 7. The protection and surveillance areas of the first ASF case in Istria measure at least 3 km and 10 km respectively.

An epidemiological curve was used to identify how the ASF virus was transmitted. The curve indicates 93 new ASF outbreaks declared in 2018 (254 pigs and 4 wild boars), followed by a decrease, reaching 34 ASF outbreaks in 2019 (19 pigs and 50 wild boars) and 3 ASF outbreaks in 2020 (4 pigs and 2 wild boars). In 2021, 11 ASF outbreaks were confirmed (62 pigs and 2 wild boars); in 2022, only 2 ASF outbreaks (4 pigs and 1 wild boar) were confirmed, followed by 21 ASF outbreaks confirmed in 2023 (76 pigs) (Table 1; Graph 1). This graph shows a propagated epidemic trend, as there is no common source of infection. The progress of ASF is represented by the large number of confirmed cases of domestic pigs in 2018. A large number of notifications in wild boars and domestic pigs were confirmed in 2019, 2020 and 2021. However, in 2023, no cases of ASF in wild boars were reported.

Year	Swine		Wild boar		Total number
	Number	Percentage (%)	Number	Percentage (%)	
2018	89	95.7	4	4.3	93
2019	8	23.5	26	76.5	34
2020	1	33.3	2	66.7	3
2021	9	81.8	2	18.2	11
2022	1	50	1	50	2
2023	21	100	0	0	21

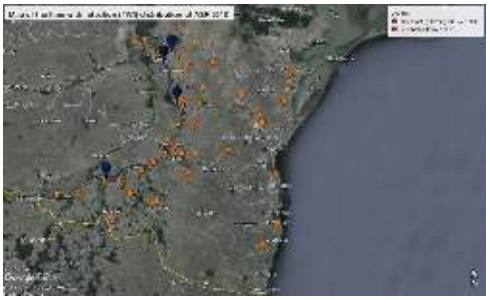
Table 1. The year of confirmation and the species in which ASF appeared in Constanta County.



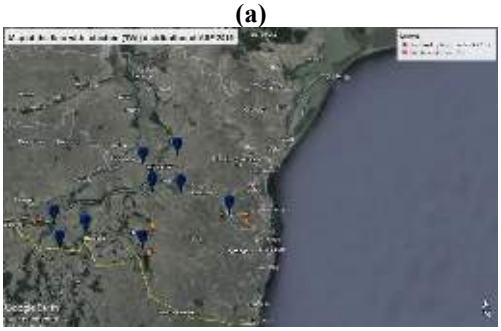
Graph 1. Graphical representation of the species of animals in which ASF appeared each year.

The annual distribution of ASF confirmed outbreaks by species in which the disease occurred.

The maps below indicate the location of confirmed ASF outbreaks in Constanta County each year. A large number of outbreaks were identified in 2018, both in domestic pigs in private households and in wild boars (Figure 8 a), following a decrease in the number of outbreaks recorded in 2019 (Figure 8 b).



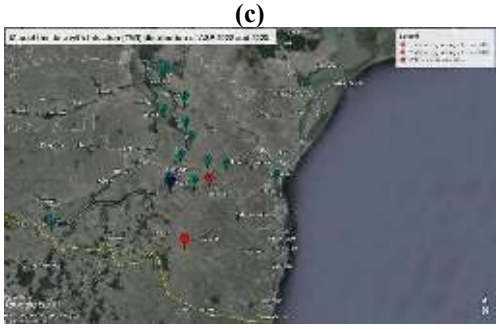
(a)



(b)

Figure 7. Map of the temporal distribution of ASF virus in Constanta County: (a) The dots labeled with the letter H in blue color indicate the location of wild boar ASF outbreaks and the dots labeled with 1 in orange color indicate the location of traditional pig breeding with confirmed ASF outbreaks in 2018; (b) Dots labelled with the letter H in blue colour indicate the location of wild boar ASF outbreaks and dots labelled with orange 2 indicate the location of traditional pig farming with confirmed ASF outbreaks in 2019.

(c)



(d)

Figure 7. Map of the temporal distribution of ASF virus in Constanta County: (c) The dots labeled with the letter H in blue color indicate the location of the 2020 wild boar outbreaks and the dots labeled with the letter C in purple indicate the location of the 2021 wild boar outbreaks. Dots labeled 3 in dark green color indicate the location of traditional pig breeding with confirmed ASF outbreaks 2020 and dots labeled 4 in light green color indicate the location of traditional pig breeding with confirmed ASF outbreaks 2021; (d) Dots labelled with the letter H in blue colour indicate the location of wild boar outbreaks in 2022 and dots labelled 5 in red colour indicate the location of traditional pig farming with outbreaks confirmed by ASF 2022, and dots labelled with 6 in green colour indicate the location of traditional pig farming with outbreaks confirmed ASF 2023.

All confirmed cases of ASF occurred in domestic pigs raised in a private household system. The statistical analysis confirmed that several ASF outbreaks were identified in domestic pigs in August (54 outbreaks), July (26 outbreaks), so we can conclude that ASF outbreaks occur during the summer due to wild boars that become dependent on areas with water (rivers, ponds) but also on shade, conditions provided by forests, in an attempt to cope with high temperatures and low food resources. But also in September (22 outbreaks), once the food becomes more abundant, the wild boars are attracted to the agricultural fields, where they can feed on cereals. Another aspect taken into account was the mating period of the wild boars November – December in which our statistical data show in November (4 ASF positive boars), in December (5 ASF positive boars) and January – April in February were the most wild boars confirmed with ASF (7 ASF positive boars) (Table 2).

Month	Swine outbreaks		Wild boar outbreaks	
	Number	Percentage (%)	Number	Percentage (%)
January	3	2.32	3	8.57
February	2	1.55	7	20
March	4	3.1	5	14.2
April	3	2.32	2	5.7
May	0	0	4	11.4
June	1	0.77	0	0
July	26	20.1	0	0
August	54	41.8	0	0
September	22	17	3	8.57
October	7	5.42	2	5.7
November	6	4.6	4	11.4
December	1	0.77	5	14.2
Total	129	100	35	100

Table 2. The months in which ASF outbreaks were confirmed in Constanta County.

Animals killed and destroyed.

A total of 1985 animals were killed and destroyed: 1406 in 2018, 134 in 2019, 70 in 2020, 107 in 2021, 16 in 2022 and 252 in 2023 (Table 3)

Animal species in which the disease occurred	Year	Number of outbreaks	Total animals killed	Media	Standard deviation	Minimum	Maximum
Swine	2018	89	1403	15.76	24.088	0	123
	2019	8	100	12.50	20.771	0	63
	2020	1	68	68.00	0	68	68
	2021	9	105	11.67	13.029	0	37
	2022	1	15	15.00	0	15	15
	2023	21	252	12.00	15.238	0	57
Wild boar	2018	4	3	0.75	0	0	2
	2019	26	34	1.31	1.408	0	5
	2020	2	2	1.00	0	1	1
	2021	2	2	1.00	0	1	1
	2022	1	1	1.00	0	1	1

Table 3. Animals killed and destroyed.

An epidemiological curve was used to identify how the ASF virus was transmitted. The curve indicates 93 new ASF outbreaks declared in 2018 (254 pigs and 4 wild boars), followed by a decrease, reaching 34 ASF outbreaks in 2019 (19 pigs and 50 wild boars) and 3 ASF outbreaks in 2020 (4 pigs and 2 wild boars). In 2021, 11 ASF outbreaks were confirmed (62 pigs and 2 wild boars); in 2022, only 2 ASF outbreaks (4 pigs and 1 wild boar) were confirmed, followed by 21 ASF outbreaks confirmed in 2023 (76 pigs) (Table 1; Graph 1). A maintenance of the epidemiological curve is observed, the difference between years in terms of the number of outbreaks, the number of susceptible animals and the number of sick animals at the date of declaration is not statistically significant, as shown by the results of the Kruskal-Wallis test below (Table 7), for both pigs and wild boars.

However, in the case of pigs, there is a significant difference in the number of confirmed sick animals since the beginning of the epizootic in the period under review 2018-2023.

Since the Kruskal Wallis test does not tell us in which years the differences are statistically significant, we compared the years two by two by applying the Mann-Whitney U test and adjusting the significance threshold according to the number of comparisons (15 in our case), so that $p = 0.05/15 = 0.003$.

Animal species in which the disease occurred	Analysis Method	Outbreaks number	Susceptible animals	Sick animals at the time of declaration of disease	Ill animals confirmed since the beginning of the epizootic disease
Swine	Kruskal-Wallis H	,000	4,154	4,952	14,982
	df	5	5	5	5
	Asymp. Sig.	1,000	,527	,422	,010
Wild boar	Kruskal-Wallis H	,000	4,673	7,430	7,790
	df	4	4	4	4
	Asymp. Sig.	1,000	,323	,115	,100

Table 3. Statistical analysis of data collected from 2018 to 2023.

There have been significant differences in the number of confirmed sick animals since the beginning of the outbreak between 2018 and other years (2019, 2020, 2021, 2022, 2023).

However, in the period 2019-2023, no statistically significant differences are observed in the number of confirmed sick animals since the beginning of the epizootic.

Based on a well-functioning veterinary health service, we should be able to eradicate ASF in domestic pigs at farm level. Considering that all measures are taken on a farm with ASF and follow all the steps to eradicate this disease before it is transmitted to a neighboring farm. As mentioned, it is a slow disease compared to other diseases (foot-and-mouth disease), and in the case of ASF it is time to apply eradication measures. Based on what we have learned about ASF and our knowledge of epidemiology, we have concluded that it is a cross-border disease maintained by wild boar migration and maintained by humans. It depends on how people behave and how they carry the virus. In the past, because there was no compensation scheme for farmers, as they lost many pigs either they died or started to get sick, farmers were tempted to save money by slaughtering sick pigs. The contaminated meat was transported over very long distances. So, individual behavior is very important to control the spread of ASF, only if people are responsible and actively involved can we succeed through education in terms of good farm management, workers, veterinarians and all kinds of people, so as to prevent the spread of the disease.

It is difficult to prevent ASF in the forest. Based on good cooperation with hunters, the wild boar population is under surveillance regarding the evolution of the disease in the forest. Hunters are the ones who ensure the search for carcasses and it is the best approach for identifying clinical signs of disease, in the forest along with testing hunted animals for ASF. As a prospect for the future, high hopes are focused on an approved vaccine. This will likely mitigate the effect of the disease.

The study analyzes the statistical processing of data collected from a study, using IBM SPSS Statistics for Windows, version 29.0. The analysis focuses on presenting nominal data as absolute frequencies and percentages, while continuous variables are expressed as mean values and standard deviations. A significance level of $p<0.05$ is considered statistically significant, indicating that the results are probably not due to chance.

6.2 GENETIC EXAMINATIONS BY VIRUS TEST, SEQUENCING AND PHYLOGENETIC ANALYSIS

(Anghel et al., 2025) In the press

In recent years, an exponential increase in the number of published research reports on ASF disease has been observed, and the latest research studies involve molecular analysis methods that allow the genetic characterization of the ASF virus. There are 24 genotypes identified in Africa. In Europe, genotype I was first identified in Portugal in 1957 and spread rapidly to Spain, France, Malta, Belgium, Italy, and the Netherlands (Gaudreault et al., 2020). In 1978, a new ASF outbreak occurred in Sardinia, Italy (Mur et al., 2016). In Russia, the presence of ASF was first reported in 1977 (Kolbasov et al., 2018). In the late 1970s, the virus was identified in Brazil (Tokarnia et al., 2004), Cuba (Negrin and Frías Lepoureau, 2002), and the Caribbean Islands (Ruiz Saenz et al., 2022).

The study aimed at sequencing the 251 bp amplicons of the B646L gene of the ASF virus (positions 105,320-105,570; GenBank MN194591.1), which encodes the capsid protein p72, was amplified using a TaqMan assay obtained from ten positive ASF samples from Constanta County (5 from pigs and 5 from wild boars) collected from 10 outbreak areas and subjected to phylogenetic analysis by comparing the references with the available ASF sequences available from Europe (Romania: MK850402, OK623917; Hungary: MN715134; Czech Republic: LR722600; Poland: MH681419; Ukraine: JX857521; Georgia: MH910496; Russia: JX857510; Lithuania: MK628478), Asian (China, MT332151; Vietnam, MN393476; South Korea, MN817977) and Africa (Tanzania: MK276921), have shown 100% similarities with ASF strain II. The Neighbor-Join phylogenetic tree, built using the Tajima Nei model with 1000 bootstrap replications, confirmed these findings (Figure 8).

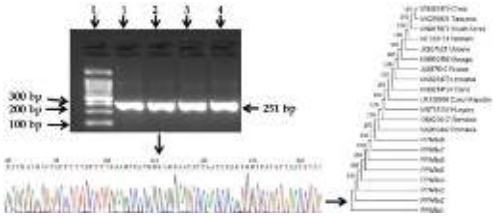


Figure 8. Identification of the circulating strain of ASF virus in Constanta County.

By molecular diagnostic methods: agarose gel electrophoresis revealing 251 bp PCR amplicons obtained from B646L in positive samples for APPV (1-4), L-100 bp scale; Chromatogram and phylogenetic tree sequencing of ASF isolates from Constanta County versus ASF strain II isolates from other countries.

6.3 VIROLOGICAL EXAMINATIONS BY THE METHOD OF IDENTIFICATION OF THE PPA VIRUS GENOME USING THE REAL-TIME POLYMERASE CHAIN REACTION (QPCR) METHOD

(Anghel et al., 2022)

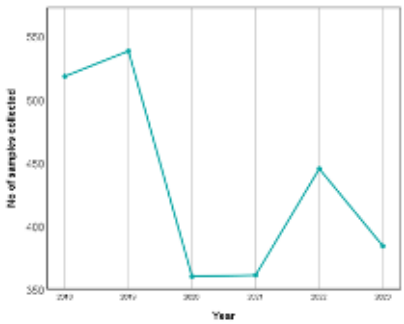
The purpose of the study is to highlight how essential routine ASF surveillance is for the rapid detection of outbreaks in Constanta County, demonstrating how continuous monitoring allows for timely interventions to prevent further spread. Understanding the dynamics of ASF infection can help stakeholders implement better biosecurity measures and reduce economic losses.

6.3.2.1 Detection and monitoring of ASF virus infection in domestic pigs and wild boars over a period of six years using the PCR test

In the period 2018-2023, a number of 2606 samples were taken for the PCR test, of which 518 (19.9%) in 2018, 538 (20.6%) in 2019, 360 (13.8%) in 2021, 445 (17.1%) in 2022, respectively 384 (14.7%) in 2023 (Table 4; Graph 2).

Year	2018	2019	2020	2021	2022	2023	Total
Total frequency	518	538	360	361	445	384	2606
%	19,9	20,6	13,8	13,9	17,1	14,7	100,0

Table 4. The number of samples taken each year.



Graph 2. Graphical representation of the number of samples taken per year.

Place of sampling

Most of the samples were taken from commercial farms (1556). 844 samples were taken from the hunting complex, and 206 (7.9%) from non-professional farms (Table 5; Graph 4).

			Place of sampling			
			Hunting complex	Commercial industrial farming	Tradidional pig farming	Total
Year	2018	Frequency	270	155	93	518
		%	52,1%	29,9%	18,0%	100,0%
	2019	Frequency	153	364	21	538
		%	28,4%	67,7%	3,9%	100,0%
	2020	Frequency	90	262	8	360
		%	25,0%	72,8%	2,2%	100,0%
	2021	Frequency	81	266	14	361
		%	22,4%	73,7%	3,9%	100,0%
	2022	Frequency	147	262	36	445
		%	33,0%	58,9%	8,1%	100,0%
	2023	Frequency	103	247	34	384
		%	26,8%	64,3%	8,9%	100,0%
Total		Frequency	844	1556	206	2606
		%	32,4%	59,7%	7,9%	100,0%

Table 5. Year and place of sampling

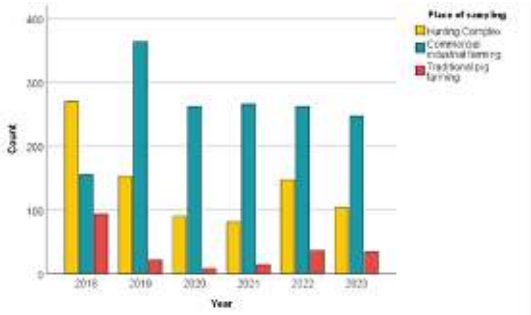


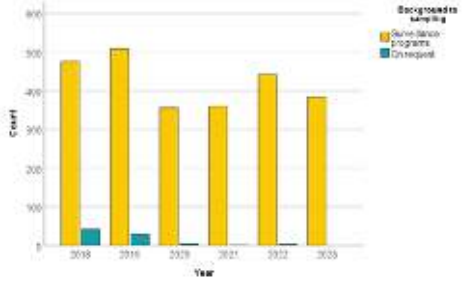
Figure 4. Graphic representation of the place where the samples were taken each year

Context of sampling

The overwhelming majority of samples (97%) were collected in the context of surveillance of the spread of ASF and only 3% were collected on request (Table 6; Graph 5).

			Context of sampling		Total
			Supervision	On request	
Year	2018	Frequency	476	42	518
		%	91,9%	8,1%	100,0%
	2019	Frequency	509	29	538
		%	94,6%	5,4%	100,0%
	2020	Frequency	356	4	360
		%	98,9%	1,1%	100,0%
	2021	Frequency	360	1	361
		%	99,7%	0,3%	100,0%
	2022	Frequency	442	3	445
		%	99,3%	0,7%	100,0%
	2023	Frequency	384	0	384
		%	100,0%	0,0%	100,0%
Total		Frequency	2527	79	2606
		%	97,0%	3,0%	100,0%

Table 6. The context of sampling each year.



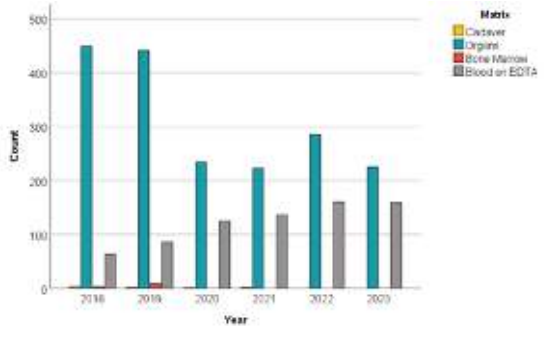
Graph 5. Graphic representation of the sampling context for each year.

Type of samples

The types of samples were predominantly organs (71.3%). Other types of samples collected were: blood on EDTA (28%), bone tissue (0.5%) and animal carcasses (0.3%) (Table 7; Graph 6).

			Type of samples				Total
			Cadaver	Organ	Bone	Blood on EDTA	
Year	2018	Frequency	3	449	3	63	518
		%	0,6%	86,7%	0,6%	12,2%	100,0%
	2019	Frequency	2	441	9	86	538
		%	0,4%	82,0%	1,7%	16,0%	100,0%
	2020	Frequency	1	234	0	125	360
		%	0,3%	65,0%	0,0%	34,7%	100,0%
	2021	Frequency	2	223	0	136	361
		%	0,6%	61,8%	0,0%	37,7%	100,0%
	2022	Frequency	0	285	0	160	445
		%	0,0%	64,0%	0,0%	36,0%	100,0%
	2023	Frequency	0	225	0	159	384
		%	0,0%	58,6%	0,0%	41,4%	100,0%
Total		Frequency	8	1857	12	729	2606
		%	0,3%	71,3%	0,5%	28,0%	100,0%

Table 7. The type of samples taken each year.



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Graph 6. Graphic representation of the type of samples taken each year.

Animal condition

38.9% of the animals from which samples were taken were dead, 30.9% were shot, 28.9% were with clinical signs of disease. Only 1.2% of the samples were taken from emergency cuts and 0.1% from normal cuts (Table 8; Figure 7).

			Clinical status of animals at the time of harvest					
			With clinical signs of illness	Shot	Death	Normal slaughter	Emergency salughter	Total
Year	2018	Frequency	65	262	191	0	0	518
		%	12,5%	50,6%	36,9%	0,0%	0,0%	100,0%
	2019	Frequency	96	134	308	0	0	538
		%	17,8%	24,9%	57,2%	0,0%	0,0%	100,0%
	2020	Frequency	126	87	146	0	1	360
		%	35,0%	24,2%	40,6%	0,0%	0,3%	100,0%
	2021	Frequency	138	80	143	0	0	361
		%	38,2%	22,2%	39,6%	0,0%	0,0%	100,0%
	2022	Frequency	168	143	106	2	26	445
		%	37,8%	32,1%	23,8%	0,4%	5,8%	100,0%
	2023	Frequency	161	100	120	0	3	384
		%	41,9%	26,0%	31,3%	0,0%	0,8%	100,0%
Total		Frequency	754	806	1014	2	30	2606
		%	28,9%	30,9%	38,9%	0,1%	1,2%	100,0%

Table 8. The clinical status of the animals at the time of sample collection for each year.

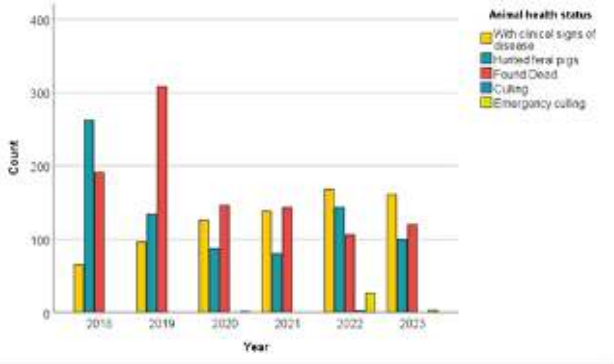


Figure 7. Graphic representation of the clinical status of the animals at the time of sample collection for each year.

No. Positive sample

A total of 218 positive samples were determined from 152 samples (Table 9). Most positive samples were determined in 2018 (112 samples) (Table 9; Table 10; Graph 8).

	No. Positive sample
Number of samples with positive samples	152
Number of positive samples	218

Table 9. The number of samples taken with a positive result and the total number of samples analyzed in which

	No. Positive sample					
	Year					
	2018	2019	2020	2021	2022	2023
Number of samples with positive samples	82	26	2	11	3	28
Number of positive samples	112	44	5	20	3	34

we obtained positive PCR test results during the period 2018-2023.

Table 10. The number of samples taken with a positive result and the total number of samples analysed in which we obtained positive PCR test results per year.

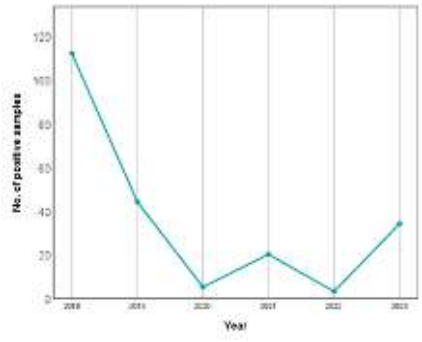


Figure 8. Graphical representation of the number of samples taken per year.

Animal condition	Number of samples with positive samples	Number of positive samples
With clinical signs of illness	63	102
Shot	14	14
Found dead	72	99
Normal slaughter	0	0
Emergency slaughter	3	3

A total of 218 positive samples were determined over the period 2018-2023, of which the majority (102) were taken from animals showing clinical signs of disease (Table 11).

Table 11. The number of samples taken with a positive PCR test result and the condition of the animals at the time of collection during the period 2018-2023.

The study involved surveillance actions carried out by veterinarians and hunters who collected a total of 6820 samples for PCR typing from 2018-2013 the data obtained from the test were statistically analyzed using IBM SPSS Statistic for Windows, version 29.0 emphasizing the advantage of using reliable and advanced statistical tools that can lead to a better understanding and management of ASF disease. This extensive collection of data improves the robustness of the study and allows for a more thorough analysis of health trends over time. The detailed breakdown of samples collected each year on each species in which the disease was confirmed, the number of susceptible animals or showing clinical signs of the disease provides valuable information on temporal changes in ASF disease status data. The methodology and findings presented can serve as a reference for future studies that increase understanding of trends and can lay the foundations for future efforts that can influence decisions and interventions in the field.

Following the statistical analysis, we concluded that the study does indeed present nominal data as absolute frequencies and percentages, which helps to understand the distribution of categorical variables. Continuous variables are expressed by mean values and standard deviations, providing a clear picture of the central trend and variability of the data. This approach improves the interpretability of the results. By establishing a significance level of $p<0.05$, the paper establishes a standard for determining the statistical significance of findings. This criterion is crucial for validating the results and ensuring that they are not due to random chance, thus contributing to the reliability of the research results.

6.3.2.2 Comparison of results obtained on two different types of samples (blood on EDTA and organs/tissues)

The study compares two types of positive PPA samples, blood from EDTA and organs (spleen, kidneys, lymph nodes) collected from both wild boars and domestic pigs. Through this comparison, an evaluation of the viral load in different matrices was allowed, contributing to the understanding of the efficiency of ASF detection by presenting CT (threshold cycle) data, analyzed in the molecular biology laboratory of D.S.V.SA Constanta, focusing on the detection of the ASF virus using the PCR method, a technique that allows the quantification of viral DNA during the amplification process. The use of CT values provides a quantitative measure that can help assess viral load in different samples. The CT value represents the number of the cycle at which the fluorescent signal passes a predetermined threshold. This threshold is essential to determine whether a sample is considered positive for the ASF virus. The study points out that the CT value is inversely proportional to the initial amount of target viral DNA, which means that as the viral load increases, the CT value decreases. CT values serve as an indicator of the viral load present in a sample. A lower CT value suggests a higher amount of PPA viral DNA, indicating that the sample is positive for the ASF virus. In this study, the mean CT score for blood samples was 15.72, significantly lower than the average of 24.33 for organ samples, suggesting that blood contains a higher viral load than organs. The study highlights that CT values obtained from blood samples demonstrate greater specificity for detecting the viral genome using real-time PCR. This means that blood samples are more reliable for early and late detection of ASF, which is essential for timely intervention and the application of control measures.

Out of the total of 1561 samples analyzed in 2019, a positive result was obtained in 20 samples from domestic pigs and 6 wild boars (Table 12).

2019						
Pig	**Total samples analyzed**	**Probe de sânge**	**Positive blood samples**	**Orgă probe**	**Positive organ samples**	**Total positive result**
Domestic	1337	351	12	986	8	20
Wild boar	224	6	0	218	6	6

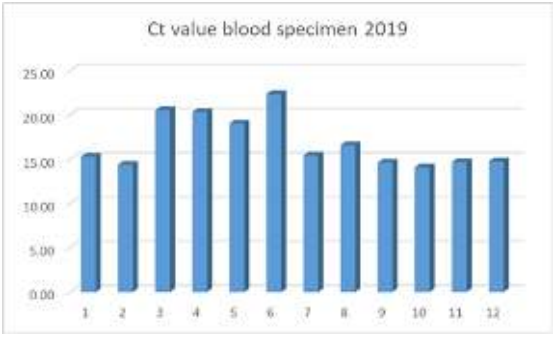
Table 12. Total samples from domestic pigs and wild boars on each matrix and total positive results obtained using the Real Time PCR method in 2019.

Based on the average of 16.82 CT values obtained in 2019, it can be concluded that blood samples on EDTA showed higher viral loads than organ samples, indicating better detection specificity (Table 13; Graph 10, 12).

2019		
The analyzed matrix	**Number of samples**	**CT Values**
Blood	1	15.29
Blood	2	14.38
Blood	3	20.54

Blood	4	20.34
Blood	5	19.03
Blood	6	22.37
Blood	7	15.43
Blood	8	16.59
Blood	9	14.62
Blood	10	14.09
Blood	11	14.67
Blood	12	14.58

Table 13. CT values obtained on the blood matrix in 2019.



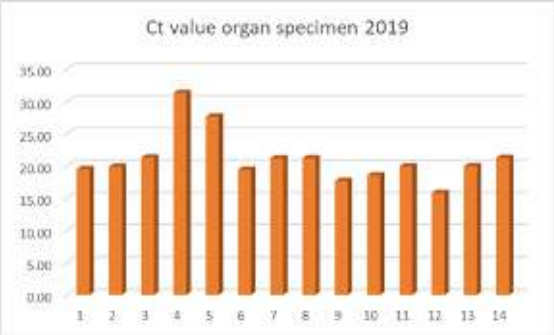
Graph 10. Graphical representation of the CT values obtained on the blood matrix on EDTA in 2019.

Based on the average of 20.89 CT values obtained in 2019, organ samples can be concluded that organ samples had lower viral loads than blood samples on EDTA, indicating better detection specificity (Table 14; Graph 11, 12).

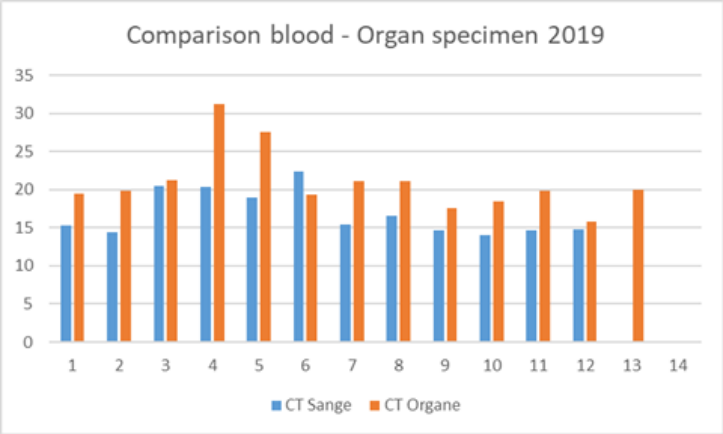
2019		
The analyzed matrix	**Number of samples**	**CT Values**
Organ	1	19. 47
Organ	2	19.85
Organ	3	21.28
Organ	4	19.52
Organ	5	31.26
Organ	6	27.60
Organ	7	19.39
Organ	8	21.16

Organ	9	21.18
Organ	10	17.64
Organ	11	18.51
Organ	12	19.90
Organ	13	15.79
Organ	14	19.93

Table 14. CT values obtained on organ matrices in 2019.



Graph 11. Graphical representation of the CT values obtained on the organ matrix in 2019.



Graph 12. Graphical representation of the comparison of CT values obtained on the blood matrix on EDTA versus the organ matrix in 2019.

The record pack includes a population consisting of wild boars and domestic pigs, and the CT values obtained for the blood matrix and organs allow us to compare the average CT values for the blood matrix is 15.72 versus the average CT values for the organ matrix is 24.33. We can conclude that following the comparison made between the two matrices we can say that in the blood the amount of virus is higher than in the organs.

6.4 SEROLOGICAL EXAMINATION USING THE METHOD OF DETECTION OF ANTI-VIRUS PPA ANTIBODIES BY THE ELISA ENZYME-LINKED IMMUNOSORBENT TECHNIQUE

(Anghel et al., 2023)

6.4.2.1 Comparative study of the results obtained by two diagnostic methods Real Time PCR and ELISA

According to the European Union Reference Laboratory for ASF, based in Valdeolmos, Spain recommends that both methods be used to establish the dynamics of infection in case of positive results of the detection of the

viral genome, but also the detection of antibodies demonstrates that the animals were infected at the time of sampling. In case of a positive antibody test result in the absence of detection of the viral genome, it indicates an ongoing or previous infection (Guidelines, Community Reference Laboratory for African Swine Fever, n.d.). Investigations show that only 6 cases of wild boars were confirmed by a positive result using both methods of analysis (Table 15, 16). These animals were still in the viremia phase, with clinical signs and in the seroconversion period (> 10 days) (Guidelines, Community Reference Laboratory for African Swine Fever, n.d.). Also, one of the two wild boars had a poor positive result using the PCR method (Table 15), the same animal was tested using the ELISA method and a negative antibody test result was obtained (Table 16) which demonstrates that this animal has recently been infected with ASF and seroconversion has not yet occurred (< 7 days after contact), Clinical signs were not obvious and antibodies were not yet produced. A second wild boar tested weakly positive for PCR (Table 15) and positive for antibody detection (Table 16). In these particular cases, the animal has passed the infection and may not show clinical signs. This wild boar could have been infected with an attenuated strain (Guidelines, Community Reference Laboratory for African Swine Fever, n.d.).

Matrix	Feral pigs	Positive	Weak Positives	Negative
Blood on EDTA	6	0	0	6
Organ	229	5	2	222
Bone Marrow	21	21	0	0

Table 15. The results obtained from samples collected from wild boars analyzed using the Real Time PCR method during 2019.

Matrix	Wild boars	Positive Rezults	Weak Positive Results	Negative Rezults
Blood serum	199	17	1	181

Table 16. Results obtained from wild boar samples using the ELISA method during 2019.

Of the total samples analyzed using the ELISA test from pigs from private households (879), the results obtained showed that 6 tested positive for antibodies and from this group 3 samples were positive for the PCR test. These pigs were still in the viremia phase, with clinical signs and seroconversion occurred (> 10 days) (Table 17).

Matrix	Pigs from private households	Positive	Weak positive	Negative
Blood on EDTA	50	7	0	43
Organ	11	5	0	6
Marrow	0	0	0	0
Cadaver	3	3	0	0

Table 17. Results obtained from samples from domestic pigs from private households analyzed using the Real Time PCR method during 2019.

The ELISA test is more suitable for detecting the presence of specific antibodies to ASF starting from the 14th day after contact with the virus. Therefore, it is not recommended if the animals have recently been infected with the ASF virus. However, it can be used to monitor the post-infection immunological status of domestic pig and wild boar populations in response to the ASF virus. ELISA can statistically help keep track of the percentage of pigs that have become immune to the disease.

6.5 HAEMATOLOGICAL EXAMINATION CARRIED OUT BY ANALYSIS OF BLOOD CONSTITUENTS ON EDTA IN ANIMALS TESTED POSITIVE FOR ASF VIRUS

(Anghel et al., 2022)

Due to the high contagiousness of the disease and most of the time proving to be fatal for both domestic pigs and wild boars, we studied the fastest possible detection of clinical symptoms associated with the ASF viremia stage using the interpretation of the values associated with blood compounds obtained using automatic hematological analyzers. We aimed to emphasize the correlation between blood parameters in the blood count and the different stages of ASF viremia in order to determine whether this method of analysis can be used in the future in clinical practice when natural ASF virus infection is suspected. By diagnosing the disease as quickly and accurately as possible, we can better control its evolution and spread.

In this study we investigate the changes in the blood parameters of the 15 pigs confirmed with ASF by the Real Time PCR test, which were housed in four pens, in order to demonstrate the viral dynamics in the different stages of viremia. In this regard, blood count tests were performed to show the different stages of viremia of ASF by analyzing the number of white blood cells (WBC) that increases rapidly in the range of 0-1dpv (day after the onset of viremia) and drops sharply to 2-6 dpv (day after the onset of viremia) (Oh et al., 2022).

We also performed the ELISA test on all 15 pigs and at the creation we obtained negative results. The reason why we used the ELISA test was to demonstrate that the pigs in the outbreak were in the viremia stage and to exclude the possibility of passing more than 14 days of infection for the detection of specific African swine fever antibodies.

The first group of three pigs in the first pen in which the onset of the disease was the most rapid with clinical signs of fever and prolonged decubitus are considered, based on the data shown in Table 18, to be virus donors through direct contact with other animals (Wilkinson and Donaldson, 1977) (de Carvalho Fereira et al., 2012) from the immediately adjacent pens or through excrement (Davies et al., 2017). Thus, starting from his study (Guinat et al., 2014), which demonstrated that the detection of PPA viral DNA is associated with the appearance of clinical signs, hematological tests of blood on EDTA were performed to show that they are still the viremic period of the disease to ensure the control of outbreaks by diagnosis.

Analyzed parameters [1]	**Reference values**	**Sow**	**Pig 1**	**Pig 2**
Leukocyte count, x10^9/L	11.0 - 22.0	6.7	6.3	7.2
Thrombocyte, x10^9/L	200 – 700	74	33	16
MPV, Fl	6.0 - 12.0	7.6	7.4	7.4
RBC x10^12/L	5.00 - 9.50	1.86	5.24	5.87
HGB, g/dl	9.9 - 16.5	2.8	7.4	10.5
HCT, %	32.0 - 50.0	10.8	30.9	42.0
MCV, FL	51.0 - 68.0	58.5	59.0	71.6
MCH, p	17.0 - 22.0	15.0	14.1	17.8
MCHC, g/ Dl	30.0 - 38.0	25.9	23.9	25.0
RDW_CV, %	14.0 - 19.0	16.1	13.7	13.8

Table 18. Comparison of the blood parameters of the first group of a confirmed ASF outbreak in Constanta County, represented by 3 domestic pigs in critical condition and presenting clinical signs of fever and prolonged decubitus.

Among the pigs in the second pen, changes in blood parameters can be observed, such as leukocytopenia only in pigs number 3, 4 and 5, which was observed according to his studies (Karalyan et al., 2012) in the last days of life, another change that is maintained is the thrombocytopenia observed in pigs 3, 4, 6 and 7 in accordance with low hemoglobin values in pigs 3, 4, 5, 6 and 7, which indicates the onset of anemia proven by the low hematocrit value recorded in pigs 5 and 6, and the below normal MCHC values observed in all pigs in the pen, RDW_CV low values in pig 5.

Parameters analysed [1]	Reference values	Pig 3	Pig 4	Pig 5	Pig 6	Pig 7
Leukocyte count, x10^9/L	11.0 - 22.0	8.0	8.3	10.0	11.2	11.9
Thrombocyte, x10^9/L	200 – 700	111	28	263	36	53
MPV, Fl	6.0 - 12.0	6.7	8.0	8.6	7.0	7.6
RBC x10^12/L	5.00 - 9.50	6.48	5.68	5.29	5.33	5.54
HGB, g/dl	9.9 - 16.5	9.3	7.8	7.8	7.6	8.0
HCT, %	32.0 - 50.0	38.2	32.2	31.7	30.7	33.3
MCV, FL	51.0 - 68.0	59.1	56.8	60.1	57.6	60.2
MCH, p	17.0 - 22.0	14.3	13.7	14.7	14.2	14.4
MCHC, g/ Dl	30.0 - 38.0	24.3	24.2	24.6	24.7	24.0
RDW_CV, %	14.0 - 19.0	14.4	15.0	13.4	14.8	14.1

Table 19. Comparison of the blood parameters of the second group from a confirmed outbreak of ASF in Constanta County, represented by 5 domestic pigs that showed clinical signs such as lack of appetite, ataxia, and the blood parameters are moderately altered.

In infection with the WBC PPA virus, they can exceed normal values immediately after infecting with the PPA virus (0-1dpv) (Oh et al., 2022), which is also found in pores number 12, 13 and 14 in the third table 3. Thrombocytopenia in pigs number 11,12,13 and 14 is a hematological change frequently observed during the period of viremia (Blome et al., 2013) (Ramiro-Ibáñez et al., 1997). Hemoglbin decreased in pigs 11 and 13 MCH, MCHC decreased in pigs 11,12,13 and 14. Low RDW_CV values only in pig number 12.

Parameters analysed [1]	Reference values	Pig 11	Pig 12	Pig 13	Pig 14
Leukocyte count, x10^9/L	11.0 - 22.0	21.9	23.6	23.6	22.8
Thrombocyte, x10^9/L	200 – 700	191	37	57	52
MPV, fL	6.0 - 12.0	7.1	8.8	6.8	7.9
RBC x10^12/L	5.00 - 9.50	5.15	7.53	6.98	5.20
HGB, g/dl	9.9 - 16.5	7.9	12.6	9.7	11.6
HCT, %	32.0 - 50.0	32.0	48.6	39.2	47.8
MCV, fL	51.0 - 68.0	62.3	64.6	56.3	62.9
MCH, p	17.0 - 22.0	15.3	16.7	13.8	15.9
MCHC, g/ Dl	30.0 - 38.0	24.6	25.9	24.7	24.1
RDW_CV, %	14.0 - 19.0	15.1	13.9	15.1	14.1

Table 20. Comparison of the blood parameters of the third group, from a confirmed outbreak of ASF in Constanta County, represented 4 domestic pigs, and which did not present clinical signs, but the WBC number has values above normal limits.

Thrombocytopenia in pig number 9 and MCH, MCHC with low values in pig number 8, 9 and 10.

Parameters analysed [1]	Reference values	Pig 8	Pig 9	Pig 10
WBC number, x10^9/L	11.0 - 22.0	13.4	15.8	18.1
Thrombocyte, x10^9/L	200 – 700	210	86	241
MPV, fL	6.0 - 12.0	7.8	8.1	7.6
RBC x10^12/L	5.00 - 9.50	6.69	6.95	6.87
HGB, g/dl	9.9 - 16.5	9.4	11.2	9.9
HCT, %	32.0 - 50.0	38.9	44.3	40.4
MCV, FL	51.0 - 68.0	58.2	63.8	58.9
MCH, p	17.0 - 22.0	14.0	16.1	14.4
MCHC, g/ Dl	30.0 - 38.0	24.1	25.2	24.5
RDW_CV, %	14.0 - 19.0	15.4	16.2	15.2

Table 21. Comparison of the blood parameters of the fourth group, from an outbreak confirmed by ASF in Constanta County, represented by 3 domestic pigs that did not show signs of disease, and the WBC number is within normal limits.

The mean values were within the reference values of the automatic analyzer, except for MCH, MCHC, RDW_CV that exceed the reference values attributed to a diet with a high concentration of Fe (Deng et al., 2021).

Parameters analysed[1]	Reference values	Pig 1	Pig 2	Pig 3	Pig 4	Pig 5
Leukocyte count, x10^9/L	11.0 - 22.0	11.9	11.8	12.1	11.9	11.8
Thrombocyte, x10^9/L	200 – 700	236	240	235	236	246
MPV, fL	6.0 - 12.0	7.5	7.4	7.5	7.4	7.4
RBC x10^12/L	5.00 - 9.50	5.59	5.64	5.61	5.60	5.58
HGB, g/dl	9.9 - 16.5	15.2	15.3	15.4	15.4	15.4
HCT, %	32.0 - 50.0	36.5	36.9	36.9	36.8	36.8
MCV, FL	51.0 - 68.0	65.4	65.6	65.8	65.8	66.0
MCH, p	17.0 - 22.0	27.1	27.1	27.4	27.5	27.5
MCHC, g/ Dl	30.0 - 38.0	41.6	41.1	41.7	41.8	41.8
RDW_CV, %	14.0 - 19.0	26.8	25.2	25.1	25.8	25.0

[1]WBC = white blood cells; MPV = average platelet volume; RBC = red blood cells; HGB = Hemoglobin concentration; HCT = hematocrit (relative volume of erythrocytes); MCV = average corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; RDW_CV = distribution of red blood cells.

Table 22. Comparison of blood parameters from the negative control group represented 5 domestic pigs, which are not known to have pathologies and in which the blood parameters did not show significant differences from the normal reference values.

The study carried out on blood samples from pigs from the first pen showed a long-lasting viremic period to develop pathological processes that determine a low level of WBC, thrombocytopenia associated with a low level of MPV values. The very low values of the parameters RBC, HGB, HTC, MCH, MCHC found especially in the sow but also in pig number 1 and 2 denote the onset of anemia which also explains the reluctance to move.

Due to the fact that the examined pigs did not have hemorrhagic syndrome (such as cyanosis or bleeding in the skin, ears or bloody diarrhea), no changes in RBC values that are marginally low or within normal limits were observed during this experiment, results also supported by the research of Blome et al. (Blome et al., 2013). For this reason, the RBC parameters are not reliable to diagnose African swine fever in the viremia stage, instead we can rely on the WBC number. The interaction between the host and the viral pathogen as well as the spread of the ASF virus to other hosts can be traced by analyzing this parameter by comparing the parameters obtained at the blood count from the progression of the infection in the body correlated with values exceeding the normal limits of the WBC, followed by the replication and then elimination of the ASF virus by secretion, excretion followed by transmission to other susceptible pigs correlated with the decrease of values below normal the normal limits of the WBC. Depending on how sensitive the host is and the virulence of the viral strain, we concluded that the blood parameters obtained from all pigs in the first pen, but also pigs number 3, 4 and 5 in the second pen, had an advanced stage of infection, probably at 12 -14 dpi, compared to pigs number 11,12,13,14 where the blood parameters showed that they are in the initial stage of the viremia period due to values exceeding the normal limit of WBC.

The results showed a significant correlation between the clinical signs and the values obtained in determining the hematological parameters of the affected pigs compared to the healthy pigs tested and which were not under the incidence of known diseases and in which it can be observed that they did not have significant changes in the parameters analyzed in the blood count.

CHAPTER 7

GENERAL CONCLUSIONS

Analyzing the results obtained through our experimental and original studies par excellence, from this doctoral thesis, entitled **AFRICAN SWINE FEVER IN DOMESTIC PIGS AND WILD BOARS through the prism of the investigations used in the molecular biology laboratory that allow the highlighting of the viral genome by the real time PCR and ELISA technique, we can** formulate the following appreciations, comments and conclusions.

In this study, we evaluated the incidence of ASF in domestic pigs and wild boars over a six-year period (between 2018 and 2023) in Constanta County, a second key area of origin of the outbreak in Romania, where ASF was confirmed in July 2018. This county is of particular importance for understanding the evolution and spread of the ASF outbreak in Romania, due to its close proximity to the wetlands of the Danube Delta and the Danube River, both of which are highly populated with wild boars. These wetlands, together with the largest agricultural land cultivated with crops, play an important role for the ecology of wild boars and pig breeding. An important population of pigs is raised in small private households or outdoors in the Danube Delta area, favoring close contact with wild boars. Moreover, due to its geographical position, the Danube Delta is a key migration route for wild boars through Ukraine from eastern to western countries, where ASF outbreaks were initially reported: Georgia (Rowlands et al., 2008), Russia (Kolbasov et al., 2018), Armenia (Sargsyan et al., 2018).

Since the first cases identified in pigs in Romania in July 2017 in Satu-Mare County (Ardelean et al., 2021) and July 2018 in Constanta County, both affected areas have been placed under official surveillance by the National Veterinary and Food Safety Authority of Romania, according to the control measures established by the diagnostic manual of the Commission of the European Communities (Decision – 2003/422- EN-EUR-Lex, n.d.). Despite initial efforts to control the spread of the disease, by the end of 2023, 164 ASF outbreaks had been reported in Constanta County. The highest proportion of confirmed ASF cases in Constanta County occurred in pigs raised on traditional farms, probably due to low security levels, which favored close contact of pigs with wild boars and/or probably due to feeding pigs with catering waste. At national level, according to the communication of the National Veterinary and Food Safety Authority in the period 2017-2023, a number of 7141 positive ASF cases in wild boars were identified in 5979 areas declared outbreaks, affecting all 41 counties in Romania (Update on the situation regarding the evolution of African Swine Fever (PPA) – A.N.S.V.S.A, March 23, 2023).

We have observed, based on the statistical analysis carried out, a seasonal dynamics of the disease in Constanta County, by confirming the most ASF outbreaks in domestic pigs in August (54 outbreaks), July (26) due to high temperatures and due to reduced feeding conditions, wild boars become dependent on areas with water (rivers, ponds) and shaded areas (forests, prairie). In September, 22 outbreaks of ASF in domestic pigs were confirmed, when wild boars' food becomes more abundant and they are often attracted to agricultural fields where they can feed on cereals.

The number of cases in pigs and wild boars in the country has decreased significantly during this period due to the eradication procedures implemented by the National Veterinary and Food Safety Authority of Romania. These consisted of the implementation of molecular diagnostic procedures and quarantine measures: restricting animal movements, disinfecting stables, vehicles and agricultural equipment, feeding animals exclusively with

cereals and slaughtering all animals in outbreak areas. In addition, the establishment of protection zones at 3 km and a surveillance zone at 10 km around the outbreak site has proven to be a great success in controlling the spread of the disease.

Several studies investigated the molecular epidemiology of ASF to establish the dynamics of ASF transmission between different countries and revealed a similar dynamics of ASF outbreaks with our results. In September 2018, ASF strain II was detected in wild boars from Belgium (Garigliany et al., 2019). Gallardo et al. (C. Gallardo et al., 2023) analyzed the central variable region of 382 isolates collected from 2007 to 2022 and divided the European II-ASFV genotypes into 24 different groups. The authors noted that the strain that caused the 2022 outbreak in North Macedonia is similar to the strain evolving in Romania, Bulgaria, Serbia, and Greece. In Romania, ASF genotype II was first detected in January 2019 in a domestic pig farm in western Romania (C. Gallardo et al., 2023). This strain later caused outbreaks in the rest of the country and in Constanta County. The Romanian strains from 2017, 2018 and 2021 were identified as variant I (C. Gallardo et al., 2023). Our study demonstrates that the positive samples for ASF in Romania analyzed in Constanta County have 100% similarities with ASF strain II. A phylogenetic tree constructed using GenBank reference sequences from Europe, Asia and Africa confirmed these findings.

The paper brings new perspectives regarding laboratory examinations performed for the diagnosis of ASF disease using laboratory technologies that have led to the development of more sensitive and specific testing methods. These improvements facilitate faster and more accurate detection of the disease, which is crucial for confirming ASF outbreaks. The analysis methods used are innovative and improve the way of collecting samples, analyzing and interpreting data that have practical implications, being subsequently applicable in the field by veterinarians and hunters. The Real Time PCR method used in the molecular biology laboratory of D.S.V.S.A Constanta is a molecular testing technique that targets single viral DNA PPA sequences and allows the amplification of small amounts of viral PPA DNA, this finding was made by comparing the CT values obtained from organ samples compared to blood samples on EDTA, from wild boars and domestic pigs, collected over a period of 3 years (2019-2021). Following the comparison made between the CT values obtained between the two matrices, we concluded that the blood matrix on EDTA offers a higher specificity for the detection of the PPA viral genome compared to the organ matrix. Veterinarians may give priority to blood tests in suspected cases of ASF. This can facilitate timely interventions, potentially reducing the spread of the virus among domestic pigs and wild boars.

The paper brings new perspectives by comparing the results obtained in the diagnosis of ASF virus in domestic pigs and wild boars during the period 2019 – 2023, bringing several significant contributions to the understanding and management of ASF disease in Constanta County, through a comprehensive evaluation of two diagnostic methods PCR and ELISA, highlighting how these methods can be effectively used together to monitor ASF infections in both wild boars, and in domestic pigs, providing a pertinent framework for diagnosis.

The study on the results obtained by using the ELISA test, during 2019-2023 as a surveillance measure for ASF disease in Constanta County, showed that ELISA tests are more useful in monitoring the progress of the disease or the effectiveness of the ASF control plan. For example, by regularly measuring anti-ASF antibody levels, veterinarians can check whether the control plan applied has worked or if adjustments are needed. Not only does it help confirm a diagnosis, but it also helps tailor plans to monitor ASF disease progression.

Out of the desire to help veterinarians in the field even more to diagnose ASF disease as quickly and accurately as possible, we correlated the detection as quickly as possible of the clinical signs associated with the ASF viremia stage, based on the interpretation of the values associated with the blood compounds, especially the values obtained for the WBC parameter, in which we found a correlation between the increase in the value of this parameter above the normal limits in the initial stage of the period of viremia and the decrease in the value of this parameter below normal limits during the period of viral replication and elimination of the virus.

The impact of ASF on the global pig industry is significant. An effective and safe vaccine could be a valuable tool for countries where ASF is endemic and could help eradicate the virus in pig and wild boar populations.

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