Hungary-Slovakia-Romania-Ukraine

ENI CBC Programme 2014-2020

„BIOSECURITY" - JOINT ACTION IN EMERGENCY SITUATIONS IN CASE OF THE IDENTIFICATION OF DANGEROUS AND WIDESPREAD INFECTIONS IN CARPATHIAN REGION”

HUSKROUA/1901/8.1/0010

Summary

Report on the Hungarian molecular biological studies based on samples taken from wild boars and food

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Hungary Slovakia Romania Ukraine

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Report on the Hungarian molecular biological studies based on samples taken from wild boars and food

African swine fever is an acute disease of domestic pigs and European wild boars, characterized by fever, haemorrhages, and in many cases, results in high mortality. It was first described in Kenya in 1921 and from there, it spread throughout Africa. From Africa, it was imported multiple times to Europe, Central and South America, and then to Georgia in 2007, from where it started and has caused the biggest pandemic in recent times. The African swine fever virus belongs to the Asfarviridae family, within the Asfivirus genus. It has a complex structure, large, multi-layered, with icosahedral symmetry, and is enveloped. Its natural reservoirs are the Ornithodoros ticks and wild pigs living in Africa.

The virus is resistant to environmental effects. From the perspective of spreading the infection, particularly important are raw meat, frozen meat, raw smoked, salted, marinated meat products, undercooked or poorly cooked foods, swill, food leftovers, and kitchen waste. The virus strains can be classified based on their virulence, genetic properties, and serological behaviour.

In Hungary, the first case was identified in 2018 in a dead wild boar in Heves County. The isolate showed 99-100% similarity to the Georgia 2007 strain. The virus gradually spread among wild boars, reaching as far as Fejér County. Since the case in Fejér County, the infection has not spread to other counties, so the risk classification was downgraded, and other areas were exempted from restrictions. African swine fever has not been identified in domestic pigs in Hungary.

The hosts of the virus are the warthog (Phacochoerus africanus), the giant forest hog (Hylochoerus meinertzhageni), the bushpig (Potamochoerus sp), the Ornithodoros ticks, the European wild boar, and the domestic pig. Infection among animals and herds can occur through direct contact with an infected, sick animal, or indirectly. The organs contain the virus at high titers; 1 gram of spleen tissue can contain up to 10^{12} viruses, and 1 ml of blood can contain 10^{8} viruses. The epidemiology of the infection varies by region, and different infection cycles (African cycle, tick-domestic pig cycle, domestic pig cycle) are distinguished. Since 2014, in Eastern Europe, a cycle associated with European wild boar habitats that is different from the previous

ones has been observed. In a "normal case", the epidemic spreads slowly among wild boars (1-2 km/month). Among domestic pigs, or from wild boars to domestic pigs, it spreads with human intervention, by violating epidemic protection rules. The antibodies produced as a result of the infection do not have a sufficient virus-neutralizing effect. Despite intensive vaccine development experiments, an effective and safe commercial vaccine is not yet available.

High virulence strains cause the hyper acute and acute forms of the disease, medium virulence strains lead to the acute and sub acute forms, while low virulence strains result in the chronic form of the disease. In the acute form, animals lose their appetite, become lethargic, and their body temperature reaches 40-42°C. The skin becomes flushed, turns cyanotic (bluish), and small necrotic areas and subcutaneous hemorrhages can be seen. Animals suffering from the acute form have a 90-100% mortality rate within a week of the first symptoms appearing. A hyperemic enlargement of the spleen is very characteristic of the disease. Hemorrhages can occur in the lymph nodes, the renal cortex, and the mucous membrane of the urinary bladder. The low virulence strains cause a chronic form of the disease with nonspecific symptoms.

African swine fever cannot be definitively diagnosed based solely on clinical and/or pathological examination. Therefore, laboratory tests are essential for accurate disease diagnosis and for making successful control measures. Direct methods are available for the detection of the causative agent of the disease, while indirect methods are used to detect the antibodies produced. From the perspective of controlling the disease, detecting the virus is of greater significance a few days after infection at the onset of viremia, while detecting antibodies becomes more important in the later stages of the infection. Virus positivity with antibody negativity indicates a current, fresh infection; virus positivity with antibody positivity indicates an ongoing infection, whereas antibody positivity with virus negativity indicates a past infection.

For the determination/exclusion of African swine fever, samples recommended by the European Union's African Swine Fever Reference Laboratory and the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021 Chapter 3.9.1 should be examined. For diagnosis, the laboratory examination of the tonsils, submandibular, retropharyngeal, preauricular lymph nodes, mesenteric lymph node, spleen, kidney, lung, ileum, tubular bone, sternum, native blood sample, and EDTA-anticoagulated blood sample is required. For domestic pigs, it is recommended

to remove the spleen or a part of it through a cut made along the left rib arc, while for wild boars, sampling using a swab from the gunshot channel is also suggested. Superficial inguinal lymph nodes, dried blood samples on Whatmann 903 filter paper or "dried blood spot sampling", FTA cards (Flinders Technology Associates), and dried swab samples can also be suitable for examinations. African swine fever has no public health implications; therefore, the examination of food samples is primarily of scientific importance.

Methods for virus detection

The causative Asfivirus can be isolated. The virus can be detected from the organs of the infected animal using immunofluorescence, its antigens can be identified using antigen-detection ELISA rapid tests (pen-side test, lateral flow devices), and its nucleic acid can be detected using real-time polymerase chain reaction (real-time PCR) and gel-based PCR reaction. Hemadsorption or immunofluorescence staining supplemented virus isolation is a sensitive confirmatory method for detecting infectious virus, but it is unsuitable for a large number of routine examinations. In everyday laboratory diagnostics, due to its high sensitivity and specificity, the potential for automation, and mass testing capabilities, real-time PCR has become widespread.

Detection of the ASF virus using the real-time PCR method

The PCR test can be completed within a few hours (a maximum of one working day), allowing for immediate implementation of epidemic control measures. Virus cultivation is not necessary, but the procedure requires separate rooms, a laboratory equipped with special instruments, and trained personnel. PCR can detect strains belonging to all 24 genotypes. PCR can still yield positive results even when infectious virus can no longer be detected through virus isolation. The costs of the tests can be reduced by pooling samples to some extent without significantly decreasing the sensitivity of the reaction.

Due to the high sensitivity of PCR, special attention must be paid to both false positive reactions and false negative results. False positivity can arise from an ASF virus-containing sample, cross-contamination from nucleic acid extraction control or the positive control, and contamination from reagents and equipment in the laboratory. To avoid false positives during PCR, different stages of the test should be conducted in well-separated rooms, and workflows should be organized in one direction in accordance with the increasing level of DNA exposure. Disposable

gloves, sterile single-use filtered pipette tips, and generally sterile single-use instruments should be used. In the room where the master mix is prepared, clean protective clothing (not used for other workflows) must be worn. Instruments, equipment, and surfaces must be continuously disinfected and decontaminated. Mistakes during sample collection, especially when done in large numbers on-site, can lead to false positive results. False results (but not false PCR reactions!) can be caused by contamination of the collector's clothing or gloves during sample collection, failure to decontaminate the used tools (knife, scissors, scalpel), and incorrect sample collection techniques.

Detection of the infection using serological methods

In animals that have been infected, antibodies appear which can be detected for a long time, even for years. In Europe, due to the absence of a vaccine and vaccination, seropositivity clearly indicates past exposure. Antibodies can be detected using ELISA, immunoblotting techniques, indirect fluorescent antibody testing, indirect immunoperoxidase tests, and rapid antibody tests. Infections caused by the virulent genotype II virus result in acute disease, so during the current outbreak, animals typically die before the appearance of antibodies. In practice, due to their simplicity, speed, and automation capabilities, ELISA methods have become the most widespread.

The study details the most important steps of the examinations and disinfection procedures used in 5 annexes. (Annex 1: Sample preparation, homogenization, inactivation, heated-off board lysis nucleic acid extraction (IndiMag Pathogen kit for KingFisher heated OFF-BOARD lysis nucleic acid extraction), Annex 2: Detection of the ASF virus using real-time PCR method (Virotype ASFV 2.0 PCR Kit), Annex 3: Detection of antibodies against the African swine fever virus using ELISA (Ingezim PPA Compac K3 ELISA test), Annex 4: Detection of antibodies against the African swine fever virus using ELISA (ID Screen® African Swine Fever Indirect Confirmation Test), Annex 5: Disinfection at Debrecen Immunological, Virological, and TSE Laboratory).

The report contains a detailed presentation of work organization and biological safety rules pertaining to laboratories dealing with the examination of African swine fever (including the laboratory layout and equipment, disinfection, access to the laboratories, and special rules concerning the operation of the ASF laboratory). For the reliable inactivation of the pathogen,

the sample is heat-treated in a thermo-block at 72°C for 30 minutes, and then it is submerged in a disinfectant solution for 15 minutes in the transfer window before being removed.

The study outlines the EU and Hungarian regulations, instructions, and guidelines related to African swine fever, its diagnosis, and laboratory examinations.

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- A brief overview of the African swine fever (world) epidemics and a description of the pathogen's structure. Institutions authorized and licensed to conduct the tests
- The epidemic caused by the genotype II virus from 2007 to the present day in the world, Europe, and in Hungary. The spread of the pathogen, epidemiology, and infection cycles.
- An overview of laboratory diagnostic options: virus isolation, antigen detection, PCR, sequencing, ELISA
- Diagnostic manual and emergency plan guidelines
- Sampling options from wild boars, advantages and disadvantages of specific samples. Accompanying documents, precise identification, traceability
- Packaging, sampling, packaging errors
- Theory and practice of serological diagnosis
- Steps of virological diagnosis
- Reaction mixture calibration, master mix compilation, reaction evaluation, and communication of results
- Key organizational and biosecurity aspects of operating a laboratory focused on African swine fever research
- Participation and suggestions at roundtable discussion
- Education, training, presentation of Hungarian laboratory tests (online or face-to-face)
- Sharing experiences on the installation of new equipment (Uzhhorod, Baia Mare)
- Delivering lectures upon request (online or face-to-face)
- Compiling summary materials on studies, with a maximum volume of 2-3 pages for website publication
- List of laws, regulations, and instructions

The Hungarian diagnostic laboratories for African swine fever

The **National Reference Laboratory for African Swine Fever (NRL)** is the Laboratory of the NÉBIH Animal Health Diagnostics Directorate in Budapest (NÉBIH ÁDI, 1143 Budapest, Tábornok u. 2). In case of suspicion of African swine fever, the NÉBIH ÁDI Lab in Kaposvár sends the test material to the NÉBIH ÁDI Lab in Budapest. The laboratories at the NÉBIH ÁDI in Debrecen (Debrecen Pathological and Bacteriological Laboratory and Debrecen Immunological, Virological, and TSE Laboratory) also conduct pathological and PCR tests. The service laboratory operated at the site of the PROPHYL Animal Health, Diagnostic, Research, and Service Limited Liability Company conducts only PCR tests of blood samples before transportation. In the case of ASF suspicion, it forwards the test material to ÁDI.

The regional ÁDI laboratories are involved in conducting serological tests aimed at detecting African swine fever.

Should there be unfavourable results from the tests, the National Reference Laboratory notifies immediately:

- the Chief Veterinary Officer of the country;
- the National Epidemic Prevention Centre;
- the NÉBIH ÁÁI director;
- the chief veterinary officer of the relevant county and the local epidemic prevention centre; and,
- the submitting veterinarian.

The official diagnosing the decease and evaluation of laboratory tests results

Based on the laboratory tests conducted, the official ASF diagnosing is the responsibility of the district chief veterinary officer. A confirmatory examination by the NRL (National Reference Laboratory) is required for the first diagnosis in a given area. The evaluation of laboratory tests and the official diagnosis of the disease must be carried out in accordance with the details specified in the contingency plan (86).

In the event that the primary ASF outbreak (African Swine Fever) has not yet been determined in the given county, or if the animal suspected of ASF cannot be linked to an outbreak confirmed in another county during the epidemiological investigation, then the PCR positive result should be considered as an officially confirmed suspicion of ASF until the sequencing results become known or until a positive virus isolation result is obtained. Appropriate action must be taken accordingly.

African swine fever

(A brief overview of African Swine Fever (ASF) epidemics worldwide, an introduction to the structure of the causative agent, its resistance, disinfectants, classification of virus strains, the early history of the disease, the disease in present times, the epidemic caused by the genotype II virus from 2007 to the present worldwide, in Europe, and in Hungary, the spread of the pathogen, epidemiology, the role of arthropods, infection cycles, virus carriage, clinical symptoms, pathological changes, and differential diagnosis).

African swine fever is an acute disease of domestic pigs and European wild boars, characterized by fever, haemorrhages, and in many cases, results in high mortality within 1-2 weeks. The decrease was first described in Kenya in 1921 and from there, it spread throughout Africa, where there are over 35 countries infected nowadays. From Africa, it was imported multiple times to Europe, Central and South America, and then to Georgia in 2007, from where it started and has caused the biggest global pandemic in recent times (75, 48).

General virological overview

The African swine fever virus belongs to the Asfarviridae family, Asfivirus genus (7). It has a complex structure, being a large, multi-layered virus with icosahedral symmetry and an envelope. Its genome, made of double-stranded DNA, is aprx. 170-193 in size and contains 150-160 ORFs (9, 24, 67, 106). In addition to the structural proteins (aprx. 54), a large number of non-structural proteins are also produced during the virus's replication. These proteins are essential for nucleotide metabolism, DNA transcription, and replication. The virus genome also encodes various repair enzymes, other metabolic enzymes, proteins that influence the host cell-host organism function, and proteins that modify interferon production and the immune response (the CD2v lectin-like protein).

The virus replicates in the cytoplasm of monocyte/macrophage cells. Its natural reservoirs are Ornithodoros ticks and African wild swine. ASFV is the only DNA virus that can be transmitted by arthropods (soft ticks, which are not native to Central and Eastern Europe). In Africa, it is transmitted by the Ornithodoros moubata, and in Europe/the Iberian Peninsula by the Ornithodoros erraticus. Genes belonging to the multigene family, often found in multiple copies and located in the variable regions at both ends of the genome (such as MG 100, MG 110, MG 300, MG 360, MG 506/530), play a decisive role in pathogenicity and virulence (24).

Resistance

The virus's detectability in various materials (live animals and arthropod vectors, animal tissues and meats, meat products, secretions, infected environments) and its survival time has been summarized in a review by the EFSA Panel on Animal Health and Welfare and Wales in 2021 (32, 104). The virus is resistant to environmental influences: it can be isolated from feces for up to 11 days, from an infected barn environment for up to 30 days, from meat on bone at 4°C for 150 days, from decaying blood for 15 weeks, from blood at 4°C for 18 months, from decaying animal carcasses for 3-5 weeks, from Serrano ham for 140 days, from Parma ham for 399 days, and can be detected in frozen meats for up to 1000 days (29, 31, 35, 104).

Due to the above, in terms of spreading the infection, raw meat, frozen meat, raw smoked, salted, marinated meat products popular in international tourism, undercooked or raw foods, swill, leftover food, and kitchen waste are of particular importance. The virus can be inactivated at 56°C in 70 minutes; at 60°C in 20 minutes, and it dies within a few minutes at 70°C. It is stable between pH <3.9 and >11.5. It is sensitive to lipid solvents (ether and chloroform) due to its lipoprotein envelope.

Disinfectants

For disinfection against ASF, one can use organic and inorganic acids, alkalis (NaOH), aldehydes (glutaraldehyde), chlorine and chlorine-based agents (NaClO), iodine-containing agents, oxidizing agents (hydrogen peroxide vapour, potassium peroxide, Virkon), phenol-containing agents, and quaternary ammonium salts, or combinations thereof (Virocid, Perfect kombicid). The effectiveness of chemical agents can be significantly influenced by the organic matter content of the environment, the quality of the surface to be disinfected, pH, temperature, applied concentration, and exposure time (13, 104). The WOAH recommendation against ASF includes: sodium hydroxide 0.8% for 30 minutes, sodium hypochlorite with a free chlorine content between 0.03 and 0.5% for 30 minutes, ortho-phenylphenol 3.0% for 30 minutes, and formalin, 0.3% for 30 minutes (83). A list of disinfectants effective against ASF and authorized in Hungary can be found at

https://portal.nebih.gov.hu/documents/10182/902001/ASP+ellen+ajanlott+fertotlenitoszerek.xl

sxAt the ÁDI Immunology, Virology, and TSE Laboratory in Debrecen, we use a 1:100 solution of Virocid for disinfection (26).

Virus Strains Classification

The virulence of the strains varies widely. Distinctions are made between strains of high virulence, such as Lisboa60 (L60), Benin 97/1, Lithuania LT14/1490; those of medium virulence, including Malta 78, Netherlands 1986, Portugal 1960, Dominican Republic; and those of low virulence, like NH/P68, OURT88/3 (Portugal 1968, 1988, China 2021), BA71V (Spain 1971 VERO), and Brazil 1978. There are naturally attenuated strains, such as Lv17/WB/Rie1 (non-HAD) from Latvia in 2017, and artificially attenuated strains like ASFV-G-DI177L (virulence deletion) (88). Previously, strains of low virulence were only detected in areas with endemic infections (Africa, Iberian Peninsula). However, recent data suggests that an increasing number of surviving individuals are appearing in the Baltic states, especially Estonia, indicating the emergence of low-virulence viruses (111).

Based on genetics, 24 genotypes are distinguished based on the C-terminal end of the B646L gene that encodes the major capsid protein (p72) (74, 93). Within these genotypes, further typing can be done by examining different genetic elements. Based on repeating sequences (TRS) in the central variable region (CVR) of the B602L gene, the strains can be classified into 31 types (80). The insertion of the GGAATATATA sequences in the intergenic region between the I73R and I329L genes allows for classification into four IGR variants (45). Variations found in the O174L (DNA polymerase PolX gene) and the K145R gene region, including TRS and SNP variants, as well as variants between the MGF 505-9R/MGF505-10R genes and between the I329L and I215L genes, can also be differentiated. Further differences among the strains can be made by examining the EP402R (encoding the CD2v protein) and the E183L (p54) genes (46, 95).

Based on hemadsorption inhibition, strains can be classified into eight serogroups (1-8). The current Eurasian epidemic is caused by viruses belonging to the eighth serogroup (71). The genetic basis for serological specificity is linked to the CD2v and C-type lectin genes (70, 71, and 100).

The Early History of the Disease

The virus was first described in East Africa, particularly within the pig populations of settlers in Kenya. It subsequently emerged in South Africa and gradually spread throughout the continent. However, until 1957, it remained confined within Africa (89).

Currently, in regions in south to the Sahara in Africa, some genotypes (1-24) are endemically present. In some areas, up to 54% of domestic pigs transported to slaughterhouses can be seropositive, and 12% might test positive for the virus via PCR (11). In 1957, the virus was introduced to Portugal through food waste from a ship arriving from Angola. The outbreak, which had a nearly 100% mortality rate, was successfully eradicated. However, after three years, it was reintroduced, spreading not only in Portugal but also in Spain and France. Between 1971 and 1983, because of three separate introductions, the virus appeared in Cuba, the Dominican Republic, Haiti, and Brazil. In 1978, through the use of contaminated food waste as pig feed, the disease reached the island of Sardinia, where despite the continuous control measures, the disease remains endemic to this day (76).

Outside Africa, only two genotypes have been observed. Historically, genotype I sporadically appeared outside Africa in places such as Portugal, Spain, Central and South America, and Sardinia. However, in more recent times, genotype II, which originated in East Africa (specifically countries like Mozambique, Malawi, Zambia, Tanzania, and Madagascar) and spread via Georgia, has become widespread throughout Eurasia (81). In 2021, reports emerged from China detailing the appearance of low-virulence strains belonging to genotype I. Furthermore, recombinant strains between these genotype I strains and the genotype II strains have also been detected (33, 81).

Current State of the Disease Due to the Emergence of the ASFV Genotype II

In 1998, new outbreaks of African swine fever were observed in East Africa (Madagascar) (69). It is believed that the virus likely spread there from Mozambique. In April 2007, symptoms indicative of African swine fever among domestic pigs were observed in the vicinity of the Poti port in Georgia. These symptoms were laboratory-confirmed by June 5th. The suspected origin of the virus in this case was also contaminated food waste from ships (95). Subsequently, the virus spread to the Caucasus region (including Armenia and Azerbaijan) and reached Russia, where it was also detected in wild boars (49). From Russia, the virus spread westward, passing through Ukraine and Belarus and reaching the eastern parts of the European Union in 2014, before continuing to its

central and western regions. To date, 15 EU member states (Bulgaria, Belgium, Czech Republic, Germany, Italy, Latvia, Lithuania, Estonia, Poland, Romania, Slovakia, Croatia, Hungary, Greece, and Sweden) and 6 non-EU countries (Belarus, Moldova, North Macedonia, Serbia, Ukraine, and Bosnia-Herzegovina) have been infected. Despite increasingly intensive control measures, the virus continues to spread throughout significant parts of Europe.

In August 2018, the disease reached China, the world's largest pork producer (110), and spread at an exceptionally rapid pace to several other Asian countries (4). Nearly forty years later, in 2021, it returned to Central America, appearing in the Dominican Republic and Haiti. As a result, African swine fever has achieved unprecedented prevalence and significance over the past 25 years (15). In the meantime, the genotype II virus has been introduced to previously uninfected southern African countries (Tanzania) and spread to western African regions (Nigeria) (55).

Through the combined analysis of six genomic regions, the genotype II viruses found in Europe have been classified into 24 groups. The largest group contained 50.3% of the isolates, with the highest variability (9 variants) observed among the Polish viruses (46).

Epidemiology of African swine fever

The hosts of the virus include the warthog (Phacochoerus africanus), the giant forest hog (Hylochoerus meinertzhageni), the bushpig (Potamochoerus sp), Ornithodoros ticks, the European wild boar, and domestic pigs.

Infection can spread among animals or herds through direct contact with an infected, diseased animal (or with an infected wild boar or its carcass and its blood), or indirectly. Due to the virus's high resistance, it can be spread by virtually any means or fomite: shoes, clothing, animal transport vehicles, other vehicles, animal loading equipment, livestock handling tools, meat, ham, sausage, salami, bacon, leftover food, sandwiches, kitchen waste, dishwater, swill, infected grass, green forages, straw, grain seeds, pig-derived feed additives, infected liquid manure, needles and knives contaminated with blood, and trophies. Aerosol (within-stable) transmission (85) is known but of subordinate significance. In Africa and southern Europe, certain ticks (Ornithodoros sp) play a particularly important role. The virus shedding through semen and its spread via artificial insemination have also been confirmed (41).

The organs contain the virus at high titers. 1 gram of spleen tissue can contain up to 10^{12} viruses, while 1 ml of blood can contain 10^8 viruses. The 50% infectious dose (ID_{50}) is 10^4 with dry feed, only 10^2 with liquids, and even lower at 10^1 $TCID_{50}$ when administered intramuscularly (79). Thus, 1 ml of blood from a viremic animal could be sufficient to infect up to 50 million animals. The virus appears in the bloodstream 2-5 days after infection, is excreted in high titers with secretions and blood, and is present in the "meat" of the infected animal, maintaining its infectivity for an indeterminably long time when frozen. The "meat" is of paramount importance in both local and distant transmission, and frozen meat can cause new outbreaks even years later. The virus can be detected in nasal secretions and saliva even before its appearance in the bloodstream and the onset of clinical symptoms. Although its quantity is relatively low, it's sufficient for transmission.

Depending on the temperature, the virus can survive for a long time, remaining viable in a frozen carcass for months. Even in the absence of live wild boars, a new cycle of infection can begin in the spring. This is why the collection and disposal of carcasses, internal organs, and offal, as well as the reduction of viral load in habitats, are especially important. Both the virus DNA and the virus itself can be detected from the soil underneath the decomposed, infected wild boar carcass. The half-life depends on the environmental temperature (and on proteases and lipases), but it is longer in urine than in feces.

The role of arthropods in the spread of the virus

Blood-feeding arthropods that act as true biological vectors consume infected blood during their feeding. The virus multiplies within them (intestine, salivary gland, and ovary) and is then transmitted during a subsequent blood meal. In the case of mechanical vectors, the virus does not multiply. Instead, their mouthparts and the initial part of the digestive tract become contaminated with the virus, which they can transmit during a later blood meal. During passive virus transmission, it can also occur that the body of the arthropod becomes contaminated with the virus, which can then infect the pigs (18).

Flies (Green bottle fly, Lucilla sericata, and Blue bottle fly, Calliphora vicina) were not found to carry the live virus in their maggots, but the virus's DNA was detected using PCR. Since wild boars often forage for these maggots, the maggots increase the chances of contact with infected carcasses. The stable fly (Stomoxys calcitrans), which can be found in animal barns and surrounding

areas, can maintain the viable virus in its mouthparts and digestive tract for up to 48 hours (87). Thus, the stable fly can contribute to the spread of the virus within a herd, either through the fly's bite or by animals ingesting the flies. Horseflies (Tabanidae) can also spread the virus, even though they have a lower chance of sustaining the virus (10).

Mosquitoes (Culex pipiens) did not show detectable nucleic acids when tested using PCR, although their role as mechanical vectors cannot be ruled out. In the context of industrial animal farming, the role of mosquitoes can be considered negligible (107). No signs of virus replication were found in ticks such as Ixodes and Dermacentor (56). The role of common houseflies, lice, mites, and other arthropods in the spread of the virus remains unclear and not sufficiently elucidated (18).

Epidemiological Cycles of the Infection

The epidemiology of the infection varies by region, distinguishing different infection cycles.

In the African cycle (sylvaticus, original, natural) Ornithodoros ticks, transmit the virus to wild African swine, primarily to the warthog (*Phacochoerus africanus*). After the bite of an infected tick, transient viremia (lasting up to 11 days, with a maximum of $10^{3.7\text{-}4.0}$ $HAD_{50/ml}$) develops in animals younger than 3 months. However, in many cases, the infection remains asymptomatic, and these viremic animals subsequently transmit the virus to the next generation of ticks (101). A tick can remain infectious for up to 3 years, while a warthog can stay infected for 25 weeks (or potentially for its entire life). The virus can reach titers of 10^5-10^6 $HAD_{50/g}$ in lymph nodes, but it is not get expelled from the body. Thus, adult warthogs spread the virus neither horizontally nor vertically. Direct contact with warthogs has not been proven to cause infection in domestic pigs. However, infection has been established after feeding domestic pigs with tissues from infected warthogs (108).

In the tick-domestic pig cycle, infected ticks can occasionally transmit the virus to domestic pigs (or wild swine) in areas like Africa and the Iberian Peninsula. When these ticks feed on viremic domestic pigs, they can get re-infected. Given that Ornithodoros ticks are not present in Europe, with the exception of the Iberian Peninsula, this mode of transmission is not a concern during the ongoing outbreak.

In the domestic pig cycle, the virus spreads through contact among domestic pigs, without the presence of ticks as natural reservoirs, and occasionally with the involvement of wild boars. Transmission can occur through direct contact, but also indirectly through fomites, swine products, and other contaminated objects. Anthropogenic factors play a decisive role in maintaining this cycle and in the long-distance spread of the virus.

Since 2014 in Eastern Europe, a cycle different from the previous ones, associated with European wild boar habitats, was observed (34). The wild boar habitat cycle is characterized by the virus persisting in the wild boar population independently of domestic pigs and ticks. The spread among wild boars can occur through direct contact or indirectly (contaminated habitat, removal of remains from their peers). Contact with carcasses found in the habitat (scavenging/cannibalism) is

a more significant epidemic spreader than direct contact between living animals. The blood and organs contain the virus at a high titer, so it is crucial to remove the carcasses as soon as possible. Wild boars infect their environment, from where the virus can spread to both small and large pig populations (82). A clear correlation was demonstrated between the occurrence of infection and the number of wild boars and the density of the population. The epidemic does not spread below a population density of 0.5 animals/km^2. Cold, wet weather significantly increases the virus's survival, remaining infectious for years when frozen. In carcasses (around freezing temperatures, in winter), the virus can be preserved (overwinter). Where the wild boar is the virus reservoir, almost all-domestic pig cases can be linked to the wild boar.

The infection cycles are independent of each other, transmission between cycles is possible, but only anthropogenic factors are involved in the domestic pig cycle. During the ongoing epidemic in Eastern and Western Europe, the wild boar is the true reservoir, while in Southeast Europe (Romania, Bulgaria, and Croatia), backyard pigs are the main reservoirs of the virus. However, the two main epidemiological cycles often intersect.

In a "normal case", the epidemic spreads relatively slowly among wild boars (1-2 km/month, 30-60 km/year) (30). The virus spreads among domestic pigs or from wild boars to domestic pigs with human intervention, violating epidemic protection rules (anthropogenic spread). If the virus appears in wild boars, it soon enters the domestic pig population as well (Baltic States, Poland, Romania, Slovakia, Germany) (52, 82). The virus can only cover long distances with human mediation (through illegal activities): Belgium, Czech Republic, Pacific Islands, East Timor, Indonesia, Papua New Guinea, Honduras, Italy, and Sweden.

Following the most common oronasal infection, virus replication begins in the tonsils and the mucosa of the pharynx. Through the lymphatic circulation, the virus reaches the submandibular and retropharyngeal lymph nodes. 15-20 hours after the infection, it reaches the bone marrow, spleen, liver, lymph nodes, and lungs through the bloodstream. General virus replication begins 30-72 hours after the infection, and the maximum virus titer in the tissues is reached after 72 hours (42).

Serology and Immunity

In East African regions endemically infected with genotype I, it has been observed that the produced antibodies do not have an adequate virus-neutralizing effect. However, these antibodies play a role in the disease's pathogenesis. Pigs that survive a natural or artificial infection are resistant to reinfection with the virulent virus. However, cross-protection between strains is weak. Antibodies appear in 7-10 days after infection and can be detected for 2-3 years (and potentially for a lifetime). Sows transfer antibodies to piglets through colostrum (98), and these antibodies persist for up to 3 months. Both the virus and the specific antibodies can simultaneously be present in the bloodstream. Despite intensive vaccine development efforts, an effective and safe commercial vaccine is not currently available (8, 112).

Virus Carriers

While the disease generally results in a near 100% mortality rate across all age groups, in an infected herd, a few animals might survive acute, sub-acute, or chronic forms and recover. These surviving animals, though no longer exhibiting clinical symptoms, can remain persistently infected. The role of such animals in spreading the disease remains a controversial topic in analytical works (64).

In experimental infections using a medium virulence virus (DR'79), the virus could be detected in lymph nodes and tonsils for up to 12 weeks (62). Animals that have recovered from the disease, under experimental conditions, transmitted the medium virulence virus (Malta/78) through direct contact for up to 30 days; this extended up to 56 days when there was contact with blood (e.g., due to fighting). The virus could be isolated from the lymph node 6 months after infection (Wilkinson, 1984). Although DNA was detectable by PCR in peripheral blood mononuclear cells even 500 days post-infection, the virus couldn't be isolated by then (20). Generally, pigs that have survived the infection do not shed the virus for more than 30-40 days.

In studies involving I genotype virus (Netherlands'86), the virus could not be isolated from organs or tissues after the 60th day, but viral DNA remained detectable in peripheral blood mononuclear cells up to day 91 (90). Pigs infected with the ASPV II genotype virus (POL/2015/Podlaskie/Lindholm) had stables that remained infectious for only 1 day. Animals introduced on days 3, 5, and 7 post-infection did not display symptoms, and the viral nucleic acid was undetectable (84). Observations during the East European ASP epidemic indicate that

survivors do not seem to spread the virus, and no chronically infected animals have been identified so far.

The ASPV II genotype is present in the blood at titers of $10^{6.4}$ -$10^{8.6}$, in feces at titers of $10^{1.0}$-$10^{2.0}$, in nasal discharge at titers of $10^{1.0}$ -$10^{4.0}$, and in organs at a titer of $10^{6.0}$ (22, 53).

The clinical symptoms and pathological changes of African swine fever

The clinical symptoms and pathological changes of the disease can vary depending on the virulence of the virus strains, the mode of infection, the amount of infectious virus, the species of the animals, and their immune status. Strains with high virulence cause the hyperacute and acute forms of the disease, strains with medium virulence cause the acute and sub-acute forms, while strains with low virulence lead to the chronic form of the disease (38, 61).

In the case of the **hyper-acute form** caused by strains with high virulence, symptoms include a fever of 41-42°C, loss of appetite, lethargy, rapid breathing, and reddening of the skin. Animals suddenly die within 1-4 days following the onset of the first clinical symptoms.

The disease most commonly presents in the **acute form** caused by strains with high or medium virulence. The animals huddle together, show a loss of appetite, become lethargic, and have a body temperature of 40-42°C, with signs of laboured breathing and pulmonary edema observed (88, 96). After a week of fever, the affected animals die with symptoms of shock; during this time, frothy content may appear around the mouth and nostrils. The tips of the ears, the tail area, the limbs, the sides of the chest, the abdomen, and the perianal region show skin reddening and become cyanotic, with small areas of skin necrosis and subcutaneous haemorrhages visible. Rarely, there may be mucous nasal discharge, nosebleeds, vomiting, abdominal pain, intestinal stasis, and diarrhea (initially mucous, later bloody). Miscarriages in pregnant sows are rare. 90-100% of animals suffering from the acute form of the disease die within a week of the onset of the first symptoms.

Concurrent with the appearance of skin redness and cyanosis, a very characteristic hyperaemic splenic enlargement can develop in the animals that have died; in such cases, the spleen can enlarge up to six times its size, with rounded edges. Its texture becomes fragile and its colour turns to a deep red-black, potentially filling almost the entire abdominal cavity. Haemorrhages appear

in the medulla of the gastrohepatic and peri-renal lymph nodes, making the lymph nodes appear marbled. There may be pinpoint haemorrhages in the renal cortex and pelvis. Haemorrhages can also occur in the mucosa of the urinary bladder, under the outer and inner linings of the pericardium, as well as on the pleura (40, 61, and 96).

Strains with medium virulence cause the **sub-acute form** of the disease. During this phase, symptoms and changes similar to the acute form are observed, but they are milder. However, the consequences of vascular changes, such as haemorrhages and edemas, are more pronounced (51). Miscarriage is often the first symptom of the sub-acute form. Affected pigs die between days 7 to 20, with a mortality rate of 30-70%. The survivors recover within 3-4 weeks, but they can continue to shed the virus for up to 6 weeks (61). The animals exhibit moderate fever, ascites, pericardial effusion, and edematous infiltration of the gallbladder wall, which is considered characteristic. Significant edemas can also develop around the kidneys. The spleen is moderately to partially hyperemic. The lymph nodes are haemorrhagic, edematous, fragile, and often appear as blackish-red hematomas (50). Kidney haemorrhages can be more pronounced than in the acute form of the disease.

Strains with low virulence cause the **chronic form of the disease**, which is characterized by non-specific symptoms. In Spain, Portugal, and the Dominican Republic, clinical pictures have been observed with skin necrotic changes, arthritis, growth retardation, lameness, and mild respiratory symptoms. What characterizes the chronic form is the absence of vascular changes and the appearance of secondary bacterial infections (fibrinous pleuritis, fibrinous pericarditis, necrotic pneumonia, fibrinous arthritis, skin necroses, and necrosis in the tonsils and tongue) (59). The low virulence, non-hemadsorbing strains cause subclinical infection, often with the only sign being serological conversion.

The clinical symptoms and pathological changes during an outbreak caused by ASPV genotype II

ASPV genotype II is highly virulent, and the acute form of the disease results in nearly 100% mortality in both wild boars and domestic pigs, regardless of age, gender, or mode of infection. The incubation period is 3-5 days, and animals typically die between 7-13 days post-infection, before the onset of antibody production. Exceptionally, the disease might have a more prolonged course, with death occurring within 21 days, or recovery is even possible (the chronic form is rarely

observed, and to date, there have been no reports of persistently virus-shedding individuals). The incubation periods observed in infected suids and the maximum detectable duration of the infectious virus have been summarized by the European Food Safety Authority (EFSA) (29).

During the outbreak caused by ASPV genotype II between 2007-2020, the incubation period was 3-4 days. Virus shedding was observed between 2-9 days, and the animals typically died within 7-9 days post-infection (16, 43).

The clinical symptoms observed in wild boars are not typical. Their behaviour changes and the animal becomes easily susceptible to "accidents". Rarely, symptoms such as loss of appetite, lethargy, uncoordinated movement, staggering, laboured breathing, and skin redness, cyanosis at the tips of the ears, and possibly pinpoint bleedings on the limbs can be noticed. Moribund animals, due to the fever, seek watery, cool, and damp habitats. As a result, the carcasses of these animals are often found near water sources. Post-mortem examinations reveal pulmonary edema, sero-hemorrhagic free fluid in the abdominal cavity, widespread hemorrhages throughout the body, a spleen enlarged several times its normal size (indicative of the disease), a protruding red splenic pulp on the cutting surface, edematous, swollen, and hemorrhagic lymph nodes, and hemorrhages in the kidneys. The gastrohepatic and peri-renal lymph nodes are most severely affected, and edematous infiltration of the gallbladder wall can also be observed (21, 23, 68).

Of the infected animals, 75-90% die within 7-14 days. Antibody positivity is rare, and death occurs before the antibody response develops. Among the animals found dead, 70-95% are virus-positive. Of the animals shot during hunting, 0.5-3% are virus-positive, and 0.0-2% are antibody-positive.

Differential Diagnosis

When diagnosing the disease, especially in initial cases involving a small number of sick and dead animals, assessing the less characteristic symptoms/lesions requires great care. The disease picture in wild boars and domestic pigs must be differentiated from classical swine fever, circovirus infections (PCAD), high pathogenicity PRRS, erysipelas, salmonellosis, pasteurellosis, streptococcosis, eperythrozoonosis, actinobacillosis, Glasser's disease (infection from Glaesserella parasuis), Aujeszky's disease, leptospirosis, edema disease, swine dysentery, thrombocytopenic hemorrhages, warfarin poisoning, heavy metal poisonings, and other septicemic or hemorrhagic

conditions. Due to the above, laboratory examinations are indispensable for the confirmation of African swine fever and for ruling out similar diseases.

The dynamics of the African swine fever virus infection and the progression of the disease

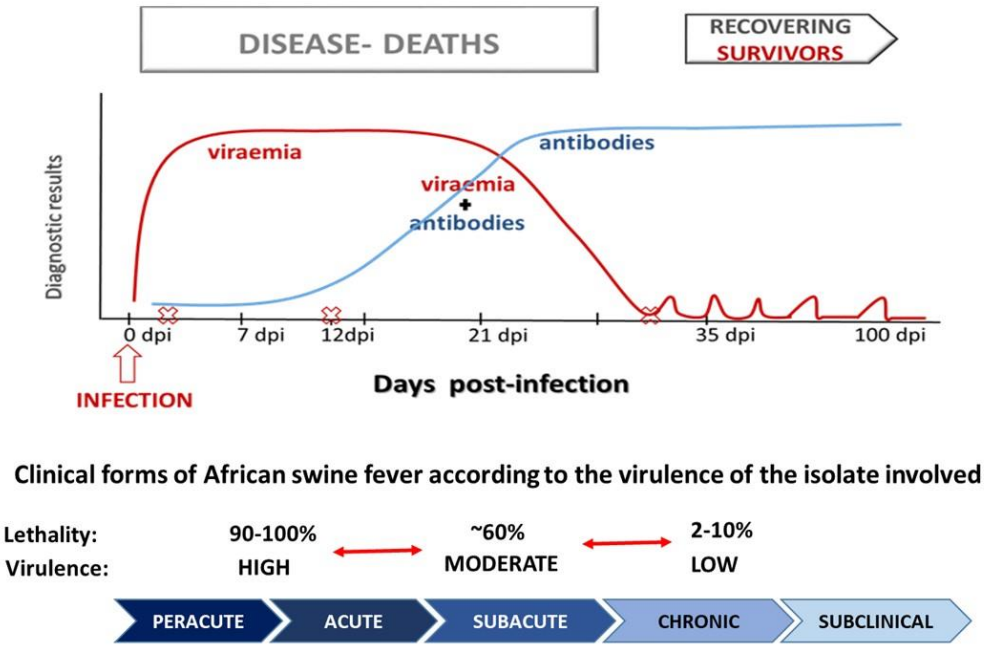


Diagramme 1. After 1-2 days following the infection, viremia begins and lasts for 3-4 weeks. Antibodies appear on days 9-14 and can persist for years.

source: https://asf-referencelab.info/asf/en/procedures-diagnosis/diagnostic-procedures

Laboratorium tests

The African swine fever cannot be unequivocally diagnosed by clinical and/or pathological examination, hence laboratory tests are indispensable for accurate diagnosis and the implementation of successful control measures. For detecting the causative agent of the disease, we have direct methods, and for identifying the produced antibodies, indirect methods are available (44). Ideally, both pathogen detection methods and antibody detection procedures can be employed for diagnosing the disease. However, the high costs of (molecular) investigations, the lack of conditions necessary for virus isolation, difficulties in sampling, various epidemiological situations, and the specificity and sensitivity required to handle these may alter the utilized procedures. From the standpoint of disease control, a few days after infection (even before the onset of clinical symptoms), the detection of the virus is essential at the onset of viremia, while in later stages of the infection (chronic or subclinical infections), the detection of antibodies becomes

more significant (Figure 1). Virus positivity with antibody negativity indicates the current, fresh infection (within 7-14 days), virus positivity and antibody positivity indicate an ongoing infection (beyond 7-14 days), while antibody positivity with virus negativity represents past infection (the animal survived the infection or was infected with an attenuated strain) (37).

Sampling, sample dispatch, accompanying documents, packing

Reliable laboratory tests can only be achieved with sample collection appropriate for the purpose of the tests (disease determination, disease exclusion, epidemiological investigation, and proof of immunity). For the diagnosis/exclusion of African swine fever, samples should be examined with recommended by the European Union's African Swine Fever Reference Laboratory (https://asf-referencelab.info/asf/en/) and the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021 Chapter 3.9.1 African swine fever (109). Previously, the types of samples to be included in the investigation were regulated by the annex to the Commission Regulation 2003/422/EC on the approval of the African swine fever diagnostic manual (ASPV diagnostic manual) (3).

In the event of suspicion of the disease, laboratory examination of the tonsils, submandibular, retropharyngeal, sub-auricular lymph nodes, mesenteric lymph node, spleen, kidney, lung, ileum, long bone, sternum, native blood sample, and anticoagulated blood sample (EDTA) is required from pigs (wild boars) that have died or have been culled for diagnostic purposes. Whole long bone samples containing spongy bone tissue from deceased animals (such as femur, humerus, possibly sternum) can still be successfully examined even months after the animal's death. From the organs, a piece about the size of a sugar cube should be taken individually and sent to the laboratory. From a hunted healthy wild boar, a blood clot sample taken from the heart or major vessels and a tonsil sample are sufficient. In our laboratory, the bone ends are drilled with a power drill used exclusively for this purpose (decontaminated after every use), and the resulting bone shavings are placed in an eppendorf tube containing a steel ball, which is then examined in the same manner as the organs.

The samples must be sent to the laboratory accompanied by an accompanying document containing the necessary information for diagnosis and any further measures. The accompanying document must indicate the location, where the animals are kept, the owner, the main clinical

symptoms observed, pathological changes, in the case of wild boars, the location and time of the shooting and/or finding of the carcass, the wildlife identification number, the name of the wildlife management unit, the name of the hunter who shot/found the animal, the exact geographical coordinates of the shooting location, as well as the intended destination of the animal body and the potential purpose of its use. In Hungary, every shot or deceased wild boar receives a six-digit identification number, known as a wildlife identification number, either at the time of shooting or when the carcass is found (12, 78). Among other things, during the laboratory examination, we identify the sample using this number.

For the purpose of professional sampling from wild boars, a unified package is available, which the authority provides free of charge. The samples must be sent to the territorial NÉBIH ÁDI. NÉBIH organizes regular sample collection circuits, through which samples can be directly delivered to the examining laboratories. The velcro-fastened bag, self-adhesive flap, containing an accompanying document, with the plastic blood collection tube inside and the approx. 50-100 ml sample collection container, with its striking red colour, already draws attention to ASF from outside. Direct contact between the accompanying document and the sample containers should be avoided, and care should be taken to ensure that the most important data required for sample identification are visible from outside. Proper identification of the samples is essential, as is sealable, leak-proof packaging, and the earliest possible (immediate in case of suspicion, within 48 hours in other cases) refrigerated transport. Freezing of the samples should be avoided. In case of ASF suspicion, the examining laboratory must be notified about the dispatch of such a sample. The packaging of the samples must indicate the address of the receiving laboratory and also state, "Animal pathological material; Perishable; Fragile; Do not open outside the ASF laboratory." (12, 86).

Alternative sampling options

During the defence against ASF, it is of paramount importance to reduce the virus load in the environment (at the sampling location) and to simplify the sampling process. For domestic pigs, instead of full dissection, it is recommended to perform a cut along the left rib arch of a pig laid on its right side to extract the spleen or part of it. Some believe that for wild boars, taking samples from the bullet channel using a swab can be suitable for detecting both the virus and any possible

antibodies (60). The examination of superficial inguinal lymph nodes has also been found to be a sensitive method (63). Dried blood samples on Whatman 903 filter paper, known as "dried blood spot sampling", or FTA cards (Flinders Technology Associates), and dried swab samples may be suitable for the detection of antibodies and/or the viral nucleic acid due to their specificity and sensitivity. Their main advantages are that after air drying the sample, they do not require refrigeration, are not very expensive, the sampling does not require great expertise (can be carried out by hunters) and can be stored for a longer period, even up to 2 months (17, 19, 60, 66, 91, 94).

Evaluation of Individual Samples

Anticoagulated Blood Sample (Whole Blood), Serum (Native Blood Sample)

For each individual, a minimum of 2-5 ml blood should be taken from the anterior vena cava. Coagulation should be prevented using EDTA (ethylenediaminetetraacetic acid). The use of other anticoagulants (like heparin) is not recommended as they can inhibit the PCR reaction (leading to false-negative results) or cause false positivity in the HAD test. Blood samples that do not contain anticoagulants should be kept at room temperature for 8-10 hours for the serum to separate, after which it can be decanted post-centrifugation. High-quality serum is clear, transparent, non-opalescent, and does not contain formed elements. Reddish discoloration of the serum indicates haemolysis, and a more severe degree of haemolysis can inhibit the ELISA reaction. Special care should be taken when handling blood samples collected from shot wild boars.

Note: Hemolysis is the breakdown of red blood cells. When haemolysis occurs, it can release the haemoglobin inside red blood cells into the serum, which can interfere with certain laboratory tests. The presence of free haemoglobin in the serum due to haemolysis can give it a reddish tinge.

Blood Clot

In the case of wild boars, blood clots should be collected directly from the heart or the major veins immediately after the animal is shot or the carcass is discovered. The blood clot can be used for PCR tests similarly to various other tissues, and the serum separated from it can be used for ELISA (serological) examinations.

Organs

The Chief State Veterinary Officer's regulation mentioned above specifies that from swine (wild boars) that have died or have been culled for diagnostic purposes, the examination of samples from the tonsils, submandibular, behind the throat, below the ear, intestinal lymph nodes, spleen, kidney, lung, ileum, tubular bone, and sternum is required. Spleen, lymph nodes, and tonsils are the most crucial among theses, as they contain the virus in significant amounts and, except for the spleen, are relatively resistant to autolysis. The examination of bone marrow becomes indispensable for older, already autolyzed carcasses, often it is the only available option. The joints, or the synovial fluid, can be useful in the investigation of viruses with reduced virulence.

Examination of faecal matter, oral fluid, and meat juice samples can provide important information about the disease's progression and the spread of the virus. However, they cannot be used to determine infection or to confirm immunity in the current European phase of defence against ASF.

Foods

African swine fever has no public health implications, so the examination of food samples has primarily scientific significance. The examination of animal-derived (pig, wild boar) food products provides indispensable data related to the pathogen's survival and epidemiological investigation. The detection of the disease is based almost exclusively on animal illnesses, fatalities, or post-diagnostic slaughtering, and not on the examination of food samples. During border control checks, it may be justified to subject pork products illegally brought in through tourist traffic from infected areas to ASF testing. When evaluating the results, it is important to consider that the virus (or its nucleic acid) detected using various techniques might indicate an active epidemic spread or, in the case of heat-treated products, just indicate the origin's infection status (105).

Virus detection methods

The causative Asfivirus can be isolated from porcine bone marrow cells, porcine white blood cells in cell culture, or various cell lines. The virus can be detected from the organs of the infected animal (tonsils, spleen) using the immunofluorescence method, its antigens can be identified by antigen-detection ELISA, rapid tests (pen-side test, lateral flow devices) such as *(Eurofins INgezim ASFV CROM Ag rapid antigen test, Bionote Anigen ASFV Ag Rapid Test, PenCheck Rapid Screening Test for ASFV, and Shenzhen Lvshiyuan Biotechnology Co. ASF Antigen Detection rapid test)* (97). The

virus's nucleic acid can also be detected with real-time polymerase chain reaction (real-time PCR) and gel-based PCR reactions (47). Virus isolation supplemented with hemadsorption or immunofluorescent staining is a sensitive confirmatory method for detecting infectious virus. However, it's unsuitable for a large number of routine tests. The antigen-detection ELISA (Eurofins INgezim PPA DAS 2.0 double antibody ELISA) allows for the virus to be detected from anticoagulated blood, cell culture supernatant, or pig spleen using two monoclonal antibodies. Rapid antigen detection tests, which can be conducted in 15-30 minutes from a few drops (about 20 µl) of fresh or chilled, anticoagulated blood, are highly valuable in practice, when the laboratory and trained personnel are unavailable or hard to reach. These rapid tests are specific, but their sensitivity can be low due to poor sample quality, autolysis, and the appearance of antibodies in subacute or chronic cases. Such rapid tests are useful for supplementing clinical and pathological examinations, but they cannot be used for official disease diagnosis or to confirm immunity.

In everyday laboratory diagnostics, real-time PCR has become widespread due to its high sensitivity and specificity, automation capabilities, and the possibility of mass testing. For the initial confirmation of the disease in a given area, a confirmatory examination by the NRL (National Reference Laboratory) is required.

Detection of the ASFV virus using real-time PCR method

In recent years, the number of PCR tests suitable for detecting the ASFV genome has rapidly increased, e.g. *Virella ASFV seqc real-time PCR kit (Gerbion, Kornwestheim, Germany), VetMaxTM ASFV Detection kit (Thermo Fisher Scientific, Lissieu, France), ViroReal® Kit ASF Virus (Ingenetix, Vienna, Austria), Kylt ASF (AniCon Labor GmbH, Höltinghausen, Germany), Virotype ASFV PCR kit (Indical, Leipzig, Germany), Virotype ASFV 2.0 PCR kit (Indical, Leipzig, Germany), ID GeneTM African Swine Fever Duplex (Innovative Diagnostics, Grabels, France), Real PCR ASFV DNA Test (IDEXX, Hoofddorp, Netherlands), VetAlert ASF PCR Test Kit (Tetracore, Rockville, USA), INgene q PPA (Ingenasa, Madrid, Spain), Adiavet ASFV Fast Time (Bio-X Diagnostics, Rochefort, Belgium), GeneReach POCKIT African Swine Fever Virus iiPCR, GeneWorks LAMP African Swine Fever Virus test kit (GeneReach, Taichung, Taiwan), ID GeneTM African Swine Fever Triplex (Innovative Diagnostics, Grabels, France)* (5, 39, 73, 92, 102). Multiplex PCR procedures have also been developed for simultaneous detection of ASF and classical swine fever, which are significant when

considering the circulation of both viruses in endemic areas or the introduction of the viruses in free areas (6, 54).

The PCR test can be completed in a few hours (up to one working day), allowing epidemic control measures to be taken immediately. There is no need for virus propagation, but it does require separate rooms, a laboratory equipped with specialized instruments, and trained personnel. Using PCR, strains belonging to all 24 genotypes can be detected, regardless of their virulence or hem adsorption capability. With PCR, we attempt to detect a much-conserved segment of the virus, a part of the gene encoding the VP72 protein (25, 39). Traditional PCR requires more time, effort, and resources. PCR can still yield a positive result, even when no infectious virus can be detected through virus isolation (e.g., autolyzed tissues, decomposing animal tissues, or samples taken from convalescent pigs). The cost of the tests can be reduced by pooling samples to a certain extent without significantly decreasing the sensitivity of the reaction.

Due to the high sensitivity of PCR, special attention must be paid to false positive reactions (contamination) and to false negative results due to poor quality samples (degraded nucleic acid, presence of inhibitory substances) (99). False positivity can arise from samples containing the ASF virus, from nucleic acid extraction controls, or from positive controls via cross-contamination (transfer of nucleic acid from one sample/control to another: endogenous contamination), or from contaminants in the laboratory reagents and equipment (exogenous contamination). To avoid false positives during PCR, different stages of the examination should be carried out in separate, easily distinguishable areas (sample preparation, nucleic acid extraction, primers-probes-master mix measurement, reaction setup, gel documentation-agarose gel analysis). The workflow should be organized in one direction according to increasing DNA load (clean→dirty). Workers must wear disposable gloves, change them frequently, use disposable sterile filtered (aerosol-resistant) pipette tips for pipetting, and generally use sterile disposable equipment during the examination. Clean protective clothing (not used in other work processes) must be worn in the room, where the master mix is prepared. Tubes containing PCR product should only be opened in the designated room. Reagents used should be aliquoted in multiple steps, measurements should be performed in a safety cabinet or PCR workstation. Doors must be kept continuously closed, and excessive air movement should be avoided. Equipment, devices, and surfaces must be continuously disinfected and decontaminated. Using an NTC (Non Template Control; contains all reagents, but water is

measured instead of sample nucleic acid) helps to detect contamination of reagents or samples. False positive results can be caused by sampling errors made during on-site, sometimes mass sampling. False results (but not false PCR reactions!) can be caused by contamination of the sampler's clothing and gloves, failure to decontaminate the tools used (knife, scissors, scalpel), or incorrect sampling technique.

In our laboratory, we use the Virotype RT-PCR Kit 2.0 for the detection of the African swine fever virus based on the real-time PCR method. For the tests, we process pig (wild boar) organs and coagulation-inhibited blood samples according to the Handbook for DNA detection from African swine fever virus (ASFV) (25, 103).

Infection diagnosing by serological methods

Antibodies appear in animals that have undergone infection on days 6-8, which can be detected for a long time, even for years. In Europe, due to the absence of a vaccine and vaccination, seropositivity clearly indicates prior exposure. Serological tests cannot differentiate between maternal antibodies and those produced due to infection. Antibodies can be detected with ELISA, immunoblot technique (IBt), indirect fluorescent antibody test (IFAT), indirect immunoperoxidase test (IPT), and rapid antibody tests (LFT) (47). The ELISA tests sensitivity is low especially in the initial phase of infection (7-12 days); however, they are very useful in later stages of infection. Infections caused by the virulent genotype II virus result in acute illness; therefore, during the current epidemic, animals usually die before the appearance of antibodies. Experiments indicate that only 10% (3/30) of definitely infected and still living animals are seropositive by days 16/21. In endemic areas, serological procedures are indispensable for the detection of asymptomatic infections caused by low virulence viruses. In practice, due to their simplicity, speed, and potential for automation, ELISA procedures have become the most widespread (e.*g., Eurofins Ingenasa INgezim ASF ASFV-R indirect ELISA (cp312 and p30), Eurofins Ingenasa INgezim PPA COMPAC blocking ELISA (P72), Svanova Svanovir ASFV-Ab Indirect ELISA (p30), ID Screen® African Swine Fever Indirect - Screening test, ID Screen® African Swine Fever Indirect – Confirmation test)*. Rapid tests operating on the immunochromatography principle *(e.g., Eurofins INgezim ASFV CROM Ab rapid antibody test (p72), Eurofins INgezim ASFV/CSFV CROM Ab rapid antibody test (p72), Global Dx GDX70-2 Herdscreen® ASF Antibody rapid tests)* have a sensitivity and specificity similar to

laboratory-conducted ELISA tests, detecting antibodies in 10-20 µl of fresh, cooled, or frozen serum and anticoagulated blood samples within 10-30 minutes. There are also rapid tests suitable for the simultaneous detection of ASF/CSF antibodies. During ELISA examination the expected negative samples are acceptable; however, positive results must be confirmed with reinforcing serological procedures, such as immunoblot technique (IBt), indirect fluorescent antibody test (IFAT), and indirect immunoperoxidase test (IPT). In our laboratory, we use the Ingezim PPA Compac K3 ELISA test (Ingenasa) and/or the ID Screen® African Swine Fever Indirect Confirmation test (57, 58).

Communicating the test results

After conducting the reactions, the results are recorded in our special registry system (LABOR system). These results can be downloaded by the commissioned laboratory and are immediately accessible for the county government offices. Applying their unique secured passwords, officials of the authority can obtain information about the tests related to their jurisdiction and their status. The results of the tests are made available to those prescribed by law both through the platform provided by the LABOR system and in the form of an electronic letter.

Work organization and biosafety regulations for laboratories dealing with African swine fever (14, 65, and 72)

Entry to the laboratory

Entrance doors should display the international symbol for biological hazard and a corresponding warning inscription. Laboratory doors must remain closed. Children are not allowed to visit the laboratory. Only those with appropriate authorization can access the laboratory workspace. Visitors may only enter the laboratory with permission from the laboratory manager and must always be accompanied while inside.

Entry to the high-containment laboratory is highly restricted, primarily for essential personnel (like maintenance). Those intending to enter must be informed in advance about the behavioural protocols in the laboratory. Before entering the high-containment area, they must be made aware of their obligations and the specific requirements of the high-containment facility: complete disrobing before entry, wearing specialized work attire and footwear, washing hands with a disinfectant solution, disrobing before exiting, and washing three times with soap and shampoo in the designated shower area. They should be made aware that they couldn't bring anything into the laboratory area. If equipment entry is unavoidable, such items can only be removed after disinfection approved by the laboratory manager. Only individuals who acknowledge and accept these conditions are allowed to entry.

Disinfection

The disinfection procedures applied at Immunology, Virology, and TSE Laboratory in Debrecen are detailed in Annex 5.

Special rules for the ASP laboratory operation

The ASP laboratory is an isolated facility, where the presence of pathogens causing severe economically damaging animal diseases must be anticipated. To minimize the risk of these pathogens escaping, specific restrictions and tools are required in addition to the previously mentioned expectations and regulations.

Laboratory equipment and layout

An international biohazard symbol must be displayed at the entrance door. The laboratory windows cannot be opened, and any gaps or openings must be sealed, equipped with a ventilation system under negative pressure. All procedures involving potentially infectious material should be performed in a safety cabinet if possible. Only individuals enlisted by name, trained, and authorized are allowed accessing the closed laboratory workspace. Staff should avoid visiting swine farming locations for 48 hours after leaving the laboratory.

The samples are delivered through a double-door system, where the two doors cannot be opened simultaneously during regular operations, but the electric lock can be opened with a separate switch in the event of an emergency. Even during emergency opening, all measures must be taken to prevent the escape of infection/contamination. In case of an emergency, the assigned laboratory worker can send an audible alert to the gatehouse by directly pressing the emergency (panic) button. It is the responsibility of the person detecting the alert to initiate assistance and alert nearby colleagues

Before entering the sealed laboratory, the work clothing to be used in the infected zone (underwear, coverall) must be prepared. Before entering the laboratory, one must fully change clothes in the anteroom. No tools or devices (e.g. phones) can be brought into the sealed laboratory section. The protective clothing and footwear can only be used within the sealed laboratory. Upon exiting the laboratory, the used protective clothing must be removed and disposed before showering. In the shower, one must wash three times with soap and shampoo, use an electric hairdryer and hygienic paper towels afterward, and then change into the regular laboratory attire.

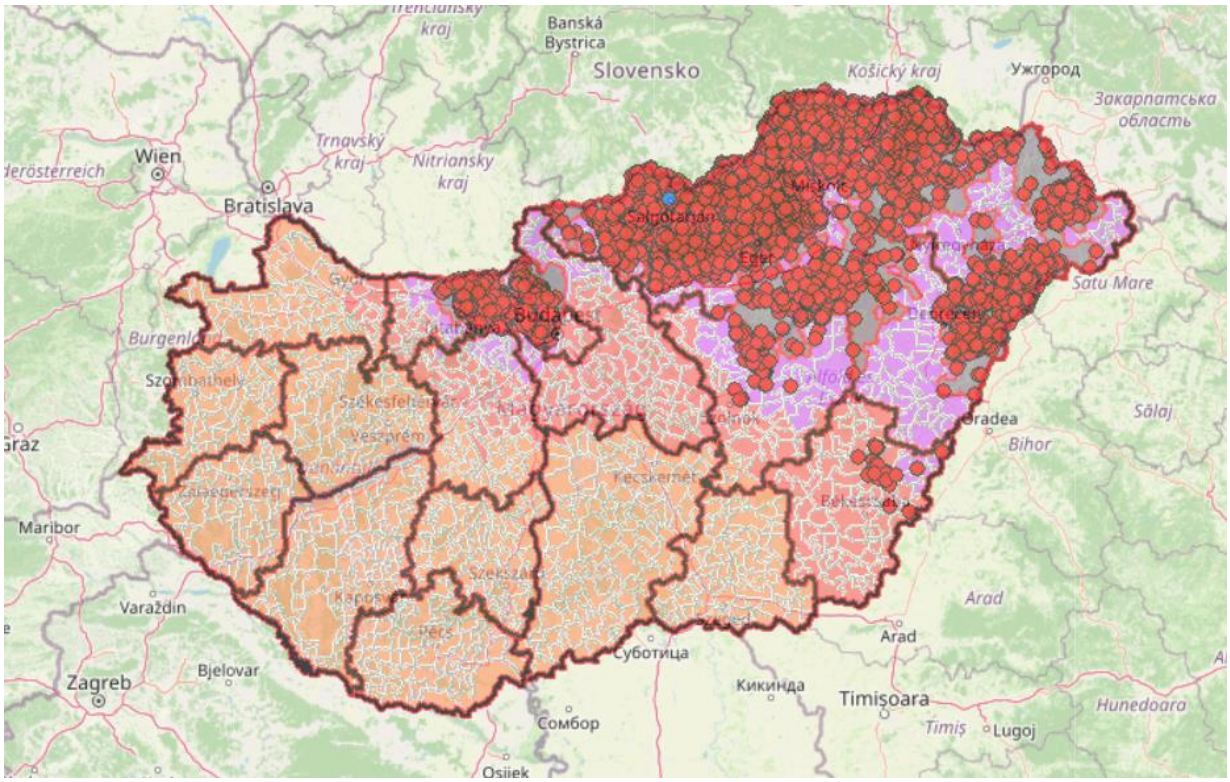
To reliably inactivate the pathogens, the sample is heat-treated at 72°C in a thermo-block for 30 minutes (internal inactivation), and then it is immersed in a 10 x diluted Virocid solution for 15 minutes in the pass-through window to be taken out of the laboratory (external disinfection).

Materials and tools should not be taken out of the laboratory for reuse. Contaminated/infected waste must be collected in strong-walled, approximately 60-liter plastic bags specifically designed for this purpose, ensuring that the bags are not contaminated from the outside. If contamination occurs, a new bag should be used. Sharp objects, including cutting or piercing tools, should be immediately collected in a solid-walled container after use, and then placed in the plastic bag.

The removal of waste from the laboratory must be carried out with special caution, and a record of the removal must be kept, which should also be signed by those involved. Using clean gloves, we seal the uncontaminated bags, remove them from their holder, spray them with a 10 x diluted Virocid solution, then place them in the pass-through chamber between the two doors, ensuring that the bags are touched as little as possible and that they do not become contaminated during the handover in the laboratory. We disinfect them again with another spray in the pass-through chamber. After this, touching as little as possible with disposable gloves from the corridor side, we place them in a previously prepared container, remove the gloves aseptically and place them in the container as well, then seal the container and thoroughly spray-disinfect it from the outside. The international biohazard symbol and the name of the hazardous material should be indicated on the container.

Digital copies should be made of paper-based documents that have been generated in or brought into the laboratory, and then the original document should be destroyed.

Cases of African Swine Fever in Hungary



Wild boar ASF cases in Hungary from 2018-2023. The red dots represent all previous cases, while the blue dots represent the most recent cases from the last week. Infected areas are in purple, high-risk areas are in red, and the orange areas are of medium risk.
Image source: NÉBIH interactive map http://airterkep.nebih.gov.hu/aaijo/asp/asp.htm

In Hungary, the first case was confirmed in a deceased wild boar in Heves County on April 21, 2018. Based on the nucleotide sequence analysis of p72, p54, and B602L genes, the isolate showed 99-100% identity with the Georgia 2007 strain. It was presumed that the virus was brought in by migrant workers active in the region. On May 14, 2018, a dead wild boar was found near Tiszakerecseny in Szabolcs-Szatmár-Bereg County, 1 km from the Ukrainian border. The virus isolated from this case matched 100% with the one in Heves County. It is believed that the virus entered Hungary from Ukrainian territories through the natural movement of the wild population. By the end of September, the infection was confirmed in a healthy wild boar, which was culled for population control purposes, in the area of Tarcal in Borsod-Abaúj-Zemplén County. On October 28, 2018, another deceased wild boar was found with confirmed infection in the vicinity of Pásztó in Nógrád County. The virus gradually spread across the country, and by April 28, 2019, it was detected in a wild boar carcass near the border of Hajdú-Bihar County in Nyírábrány. On August

21, 2019, it was found in a carcass near a town in Heves County (Poroszló) and a few days later in another deceased wild boar in Borsod-Abaúj-Zemplén County (Tiszakeszi). By August 28, 2019, Jász-Nagykun-Szolnok County also became infected. On September 28, 2019, the virus appeared in a carcass from a previously designated low-risk fenced wild boar population in Pest County (Budaörs/Budakeszi), presumably originating from food waste associated with tourist traffic. On December 9, 2019, a dead wild boar found near Biharugra in Békés County bordering Romania became infected likely due to the natural movement of the wild population. On February 15, 2020, the virus was detected in a deceased wild boar in Komárom-Esztergom County, which is believed to have spread from the neighboring infected areas in Pest County through the movement of wild boars. On August 10, 2021, the virus was detected in a dead wild boar in the northern part of Fejér County. The infection in this case was also attributed to the migration of wild boars from nearby areas in Komárom-Esztergom County.

African swine fever has not yet been confirmed in domestic pigs in Hungary. Since the case in Fejér County, there has been no further spread of the infection to new counties in wild boars in Hungary (27, 28, 29, 36).

Due to the effectiveness of the applied epidemiological control and risk reduction measures, in certain districts of Jász-Nagykun-Szolnok, Pest, and Békés counties, there have been no occurrences of African swine fever in both kept and wild swine in the past twelve months. As a result, the risk classification was relaxed in these areas. Meanwhile, certain districts of Jász-Nagykun-Szolnok, Bács-Kiskun, and Csongrád-Csanád counties were removed from the list of the restricted areas (1, 2, 77).

Annex 1: Sample preparing, homogenizing, inactivating, heated-off board lysis nucleic acid extracting (IndiMag Pathogen kit with KingFisher heated OFF-BOARD lysis for nucleic acid extraction).

Organic samples preparing:

Place 0.1-0.5 g of tissue (blood clot, spleen, lymph node, tonsil) in a 2 ml eppendorf tube equipped with a pillow ball inside the safety cabinet. You can mix tissues from the same animal (tissue mix), but examine bone samples separately and do not mix them with other tissues. In the case of serum (from non-coagulated blood), pipette 200-1000 µl of serum into an eppendorf tube, or into a microtube for larger sample numbers. Add 1.0 ml of sterile PBS to each tissue mix (blood clot, spleen, lymph node, tonsil). Place the tubes containing the mix into the TissueLyser homogenizer device. Shake the samples for 3 minutes at a frequency of 25 Hz/min; for more solid samples, repeat the shaking once more. Put the tubes to th centrifuge and centrifuge for 10 minutes at 8,000 rpm.

Diluting and inactivating tissue samples

The measurement of lysis buffer+Proteinase K solution takes place in a separate room, from which it is passed into the sealed laboratory through a transfer door. Measure 520 µl of the mixture per sample into a screw-cap microtube under the safety cabinet. Add 160 µl of PBS to the mixture, and then pipette 40 µl of the supernatant (do not dilute the serum, so measure 200 µl from the serum). Incubate it in a thermal block at 72°C for 30 minutes, then immerse for 15 minutes in a 10 x diluted Virocid solution in the transfer window, ensuring the liquid completely covers the immersion basket and does not touch the inside of the transfer. Continue with the nucleic acid extraction using the KingFisher Flex nucleic acid extraction device on the KF_Flex_cador 96 DW program.

Preparing and homogenizing coagulation-inhibited samples

Homogenize the coagulation-inhibited blood samples by repeatedly inverting them. Measure 200 µl per sample into the appropriate wells of the sample tray. Add 520 µl of the lysis buffer+Proteinase K solution.

Continue with the nucleic acid extraction using the KingFisher Flex nucleic acid extraction device on the KF_Flex_cador 96 DW program."

Annex 2: Detection of the ASF virus using real-time PCR method (Virotype ASFV 2.0 PCR Kit)

Detection Principle of the ASF Virus using Real-time PCR Method

During the examination, the ASF virus genome's p72 region is targeted with the help of designed primers and probes to detect the virus strains or their genome through polymerase chain reaction (PCR). As the reaction progresses, the quantity of amplification products (amplicons) can be measured in real-time based on the changes in fluorescence intensity. Using different sequence-specific probes, the resulting products can also be qualitatively differentiated. The Virotype ASFV 2.0 PCR Kit contains three different primer/probe combinations. For signaling the ASFV, the FAM probe (470-510 nm) is used, for signaling the internal control (the gene of a protein always present in pig-derived materials, beta-actin) the HEX probe (530-555 nm) is utilized, and for signalling the exogenous internal control (which we add to the lysis buffer during sample processing) the Cy5 probe (650-670 nm) is employed. Both the internal control and the exogenous internal control indicate the occurrence of false-negative reactions. The positive control is used to verify the functionality of the reaction mixture.

Tools needed for the examination

To conduct the examination, the following equipment and tools are required: a refrigerated centrifuge, a microcentrifuge, a homogenizer (TissueLyser), a vortex mixer, a biological safety cabinet, a PCR workstation, pipettes (0.2, 10, 20, 100, 200, 1000 µl), sterile pipette tips with filters (10, 20, 50, 100, 200, 1000 µl), sample holders, PCR tube racks, sterile real-time PCR tubes/strips/plates, and the corresponding caps or seals, various sizes of sterile centrifuge/microcentrifuge tubes, latex gloves, a sealing device, a nucleic acid extraction robot (e.g., KingFisher Flex), a pipetting robot (e.g., Qiagility), and a Q-PCR machine (e.g., Rotor Gen Q real-time PCR, Aria Mx real-time PCR).

Procedure of the Examination

The Virotype ASFV 2.0 PCR Kit is suitable for simultaneous detection of all genotypes. Due to the DNA instability, assemble the reaction mixture continuously and preferably without interruption. Perform the thawing process in a refrigerator, protected from light. Keep the master mix, controls, and samples on ice packs during the measurement. Measure the PCR mix and negative control in a separate, closed area of the PCR laboratory (often referred to as the "clean room"), or inside a PCR box. Before entering the reaction mixture preparation room, one should change their lab coat. Wear disposable gloves (of a noticeable colour that are used exclusively for this purpose) during the master mix measurement. Transfer the master mix to holders on the other side of the transfer window (which should otherwise remain closed). Do not bring samples, controls (positive, negative), or PCR products into the Reaction mixture preparation room. Measure the positive control and samples outside the clean room, under a laminar flow hood. Multiple freeze-thaw cycles reduce the sensitivity of the reaction; therefore, if there is a small number of samples, aliquot both the reaction mixture (master mix) and the controls.

Measure 20 µl of master mix and 5 µl of sample nucleic acid for one reaction. When calculating the master mix, besides the number of samples to be tested take into account also the number of positive and negative controls, as well as the measurement loss (an additional 1-2 reactions).

Fill out the appropriate form or protocol for the PCR test. Dispense 20 µl of the master mix (with an orange cap, which includes enzymes, primers, and probes) into the PCR tubes for each sample, and then measure and add 5 µl of the extracted DNA. The Virotype ASFV 2.0 PCR Kit includes both a positive control (with a red cap) and a negative control (with a blue cap); measure and add 5 µl for each control in place of the sample. The reaction mixture can be assembled manually using pipettes or, in the case of a large number of samples, with automated robots.

PCR programme steps

Enzyme activation: 95°C for 2 minutes

Denaturation: 95°C for 5 seconds

Primer annealing and synthesis: 60°C* for 30 seconds, 40 cycles.

*: Fluorescent signals are detected on green, yellow, and red channels.

The fluorescent dyes found in the mix produce detectable fluorescent signals at different wavelengths. After selecting the appropriate program for the specific PCR devices, launch the reaction in the instrument room.

Evaluation of Test Results:

The test is valid if the positive control gives a signal before the 35th cycle on all three channels, and the negative control does not give a signal on any channel.

The sample is **ASFV positive and the test is valid** if the following criteria are met: the sample gives a signal before the 35th cycle on the FAM channel (regardless of whether it gave a signal on the HEX and/or Cy5 channels; if the sample contains a large amount of ASFV DNA, it can outcompete the controls). The positive control gives a signal on all three channels before the 35th cycle. The negative control does not give a signal on any channel before the 35th cycle.

The sample is **ASFV negative and the test is valid** if the following criteria are met: the sample does not give a signal before the 35th cycle on the FAM channel. The sample gives a signal before the 35th cycle on both the HEX and Cy5 channels. The positive control gives a signal on all three channels before the 35th cycle. The negative control does not give a signal on any channel before the 35th cycle.

The result is **inconclusive and the test is invalid** if the sample does not give a signal on any channel before the 35th cycle.

Considering that the housekeeping gene (beta-actin) is found in every swine cell, the absence of a signal on the HEX channel indicates a failure in nucleic acid extraction or the presence of a minimal amount of extractable nucleic acid in the sample. In the case of PCR inhibitory substances, the signal will also be absent on the Cy5 channel. From our experience, the presence of disinfectant residues, soil contamination, or autolysis can cause failure in nucleic acid extraction and the PCR reaction. In such cases, we can try diluting the sample (1:5, 1:10) and/or extracting the nucleic acid again. If the positive control does not produce a signal on any channel before the 35th cycle, it

could indicate an improper preparation of the reaction mixture, errors in measurement, or incorrect reaction conditions. The reaction should be repeated in such situations.

Annex 3: Detection of antibodies against African swine fever virus applying ELISA (Ingezim PPA Compac K3 ELISA test)

The principle of ELISA method

The ELISA plate is sensitized with inactivated, purified p72 ASFV structural protein antigen. After adding the serum sample to be tested, we measure a peroxidase conjugate labeled with a monoclonal antibody produced against the p72 ASFV protein. Finally, we add the substrate necessary for the colour reaction. If there are ASFV-specific antibodies in the sample, they will inhibit the binding of the peroxidase conjugate labelled with a monoclonal antibody produced against the p72 ASFV protein to the antigen. If the sample does not contain specific antibodies, this inhibition will not occur. With interspersed washing steps, we remove unbound materials. Based on the colour reaction formed after adding the substrate, we deduce the presence or absence of antibodies.

Tool set for the test

For the preparation and testing, you will need pipettes, multi-channel pipettes (50, 100, 300, 1000 µl), sterile plastic pipette tips, a centrifuge, microtubes, a measuring cylinder, a refrigerator, an ELISA washer, an ELISA reader, paper towels, a 37°C thermostat, reagent trays, and a timer.

Procedure of the examination

During preparation, remove the required number of plates and reagents and allow them to reach room temperature (20-25°C). Dilute the wash solution concentrate 25 x distilled or deionized water (1 part wash solution concentrate to 24 parts water). The prepared solution can be stored at 2-8°C. Dilute the control sera in a 1:2 ratio with the specified sample diluent on the plate, similar to the samples. Just before use, dilute the conjugate 100 x with the dilution buffer. Always dilute only the required amount; unused diluted conjugate solution cannot be stored! The dilution buffer, substrate buffer, and stop solution are ready to use. Serum samples should be diluted in a 1:2 ratio with the serum diluent. The dilution can be done directly on the plate (50 µl diluent + 50 µl serum per well).

During the test, add 50 µl of diluent to each well. Measure and add positive and negative controls 50 µl each into two separate wells. For samples, add 50 µl into each well. Cover the plate and

incubate at 36 ± 1°C for 1 hour (± 5 min) or at 20-25°C for 16-20 hours (overnight). After incubation, empty the plate and wash it four times by pouring out the liquid from the wells in a swift motion and filling the wells with the wash solution (300 µl/well), ensuring no air bubbles form. Remove the wash solution from the wells with a swift motion. Repeat this washing step three more times. After the fourth wash, turn the plate (or strip) upside down and tap it vigorously on paper towels to dry. Protect the plate from drying out between washes and before adding the next reagent. Add 100 µl of the diluted conjugate to each well, cover the plate, and incubate for 30 minutes at 36 ± 1°C. Wash the plate five times following the previously described steps. Add 100 µl of substrate solution to each well and incubate at room temperature (20-25°C) for 15 minutes. Stop the reaction by adding 100 µl of pre-warmed stop solution at room temperature. It's essential to pipette the stop solution in the same order and speed as you did with the substrate. Read the results using a photometer at 450 nm wavelength, within 5 minutes after stopping the reaction.

Evaluation of probe results

The test is valid if the optical density (OD) value of the negative control (NC) is at least four times greater than that of the positive control (PC). To evaluate the samples, the cut-off value is calculated using the following formula: Positive cut-off = NC - [(NC-PC) * 0.5)], Negative cut-off = NC - [(NC-PC) * 0.4)].

Sample evaluation

A sample is considered positive if its OD value is lower than the positive cut-off value. A sample is considered negative if its OD value is higher than the negative cut-off value. Results that fall between the two values are considered inconclusive.

ELISA tests can produce false positive reactions in the case of haemolysed samples or those that are highly contaminated.

Annex 4: Detection of antibodies against the African swine fever virus applying ELISA (ID Screen® African Swine Fever Indirect Confirmation Test)

ELISA method principle

The even-numbered columns of the ELISA plate are sensitized with ASPV recombinant proteins p32, p62, and p72, while the odd-numbered columns serving as controls are not sensitized. After the introduction of the test serum into both columns, if there are ASPV-specific antibodies present in the sample, they will bind to the recombinant proteins on the plate. After the addition of the peroxidase-labelled anti-multi-species antibody conjugate and the substrate necessary for the colour reaction, a colour change occurs. Through the incorporated washing steps, unbound materials are removed. Naturally, no colour reaction will occur in the control columns, since they do not contain antigens. The presence or absence of antibodies is inferred from the calculated colour intensity difference between the sensitized and control columns. If antibodies are present, a blue reaction will occur, which turns yellow after the addition of the stopping agent; there is no colour reaction in the absence of antibodies.

Tool set for the test

The following equipment is required for preparation and testing: pipettes, multi-channel pipettes (50, 100, 300, 1000 µl), sterile plastic pipette tips, centrifuge, microtubes, measuring cylinder, refrigerator, ELISA washer, ELISA reader, paper towels, a thermostat set at 37°C, reagent dishes, and a timer.

Procedure of the examination

The conjugate controls, and substrate should be stored at 5 ± 3°C. Other reagents can be stored between 2-26°C. Products with the same name in the IDVET product line can be freely used interchangeably. Before use, wait for the reagents to reach room temperature (21 ± 5°C) and then mix the reagents well. If necessary, allow the washing solution concentrate (Wash Concentrate 20x) to warm to room temperature, mix it well, and then dilute with distilled water at a ratio 20:1.

Measure 190 µl of Dilution Buffer 14 and 10 µl of the negative control into wells A1, A2, B1, and B2. Measure 190 µl of Dilution Buffer 14 and 10 µl of the positive control into wells C1, C2, D1, and D2. Finally, measure 50 µl of Dilution Buffer 14 and 50 µl of each sample serum into both the

antigen-covered even and uncovered odd columns. Incubate the covered plate for 45 (± 4) minutes at room temperature (21 ± 5 °C). After incubation, wash the plates three times with 300 µl of the previously diluted 1:20 Wash Solution. Quickly invert the plate to empty its contents and fill each well with at least 300 µl of wash solution. Thoroughly shake out the washing solution from the plate, and tap it dry on paper towels, but avoid letting the plate completely dry out. The conjugate needs to be diluted 1:10 with Conjugate Dilution Buffer (Dilution Buffer 3). The diluted conjugate should be used immediately. Measure 100 µl of the conjugate previously diluted 1:10 with Conjugate Dilution Buffer (Dilution Buffer 3) into each used well. Incubate the covered plate for 30 (±3) minutes at room temperature (21 ± 5 °C). Repeat the washing step. Measure 100 µl of Substrate Solution into each used well. Incubate the covered plate in a dark place for 15 (± 2) minutes at room temperature (21 ± 5 °C). To stop the reaction, measure 100 µl of Stop Solution into each used well. Measure the color intensity (OD value) of the controls and samples at a wavelength of 450 nm with a photometer.

Evaluation of probe results

To evaluate the results, the net OD value must be determined: net OD = OD of even well - OD of odd well. The test is valid if the average net OD value of the positive control is higher than 0.35 and the ratio of the average net OD value of the positive control to the average net OD value of the negative control is higher than 3.

Sample evaluation

For the evaluation of the samples, we base our analysis on the obtained net OD values, for which we calculate the S/P value using the following formula:

$$\frac{\mathrm{ODsample} - \mathrm{ODnc}}{\mathrm{ODpc} - \mathrm{ODnc}} = \frac{\mathrm{S}}{\mathrm{P}}\ \%$$

The sample is **negative**, if the S/P value lower than 0.3,

positive, if the S/P value is higher, or equal to 0.4,

equivocal, if S/P value is between 0.3 and 0.4.

Annex 5: Disinfection at the Immunology, Virology, and TSE Laboratory in Debrecen

1. Handling Infectious Materials: after the completion of the tests, the following must be treated as hazardous materials and collected in dedicated waste containers: test materials sent from post-mortem examinations and/or directly delivered organs, swab samples, secretions, their remnants, and their containers; blood (serum) samples and their collection vessels; containers holding infected materials and their contents; disposable pipette tips; waste and potentially unused residues from kits and all diagnostic reagents used; materials generated during the tests (e.g. ELISA wash solution, remnants post-centrifugation); and, protective tools and clothing that were used (and potentially contaminated or infected) during the tests.
2. Handling of sample trays, metal or plastic racks, measuring cylinder, stock solution storage bottle: wash with household dish soap and water. If necessary, disinfect with a 6% caustic soda solution, rinse, and dry.
3. Handling of hand instruments (scissors, tweezers): soak for 1-2 hours in a 1% Virocid solution, rinse thoroughly with tap water, then autoclave at 121°C for 20 minutes under 1.2 atmospheres pressure.
4. Air disinfection: for air disinfection after cleaning, disinfecting, and leaving the workspace, the mercury vapour lamps placed in the equipment and on the ceilings of the rooms should be turned on, controlled by a timer, for 1-4 hours daily. Work can only resume in the rooms after turning off the ultraviolet lamp and ventilating.
5. Surface disinfection: at the end of the workday, all work surfaces, tools, instruments, and any other surfaces that could have been contaminated during work must be disinfected. The visibly cleaned surfaces should be treated with Vantropol rapid disinfectant solution using a paper towel. The hard-to-clean, disinfectable places and the inner surfaces of the instruments are sprayed with Vantropol rapid disinfectant spray. Surfaces that cannot be easily decontaminated in other ways should be wiped daily with DNA AWAY and RN-ase AWAY decontamination solution. The laboratory room floors must be mopped with disinfectant water after work every day.

6. Personal hygiene: hand disinfection should be performed after removing gloves, when leaving the laboratory, before and after using the restroom, before and after working in a safety cabinet, when hands become contaminated during work, and at the end of the daily work. Thoroughly wash hands with warm soapy water, dry hands with a disposable paper towel, then take about 5 ml of hand disinfectant solution in one hand, rub it evenly over the backs of the hands, and if necessary, up the arms, and leave it on for the required exposure time. After cleaning hands and drying, apply BradoDerm Soft solution for 2 x 1.5 minutes.
7. Air conditioning units: these must be disinfected annually with the maintenance personnel.
8. For accidental spills: each laboratory room should have a 10 x diluted Virocid solution on hand.
9. For disinfection: the Perfect kombicid solution can be used in place of the Virocid solution, at a similar concentration and exposure time.

Key regulations related to African swine fever and laboratory disease markers

EU regulations

Regulation (EU) 2016/429 of the European Parliament and Council of 9 March 2016 on transmissible animal diseases, amending and repealing certain acts in the area of animal health ("Animal Health Law") *OJ L 84, 31.3.2016, p. 1–208* https://eur-lex.europa.eu/legal-content/HU/TXT/?uri=celex%3A32016R0429.

Commission Delegated Regulation (EU) 2018/1629 of 25 July 2018 amending the list of Annex II to Regulation (EU) 2016/429 of the European Parliament and Council on transmissible animal diseases, amending and repealing certain acts in the area of animal health („Animal Health Law"). http://data.europa.eu/eli/reg_del/2018/1629/oj.

Commission Implementing Regulation (EU) 2018/1882 of 3 December 2018 on the application of certain disease prevention and control rules to categories of listed diseases and establishing a list of species and groups of species posing a significant risk of spreading the listed diseases. http://data.europa.eu/eli/reg_impl/2018/1882/oj.

Commission Delegated Regulation (EU) 2020/687 of 17 December 2019 supplementing Regulation (EU) 2016/429 of the European Parliament and of the Council as regards rules for the prevention of and protection against certain listed diseases. https://eur-lex.europa.eu/legal-content/HU/TXT/HTML/?uri=CELEX:02020R0687-20210714&qid=1673784660292&from=hu.

Commission Implementing Regulation (EU) 2023/594 of 16 March 2023 on the establishment of special epidemic control measures for African swine fever and the repeal of Implementing Regulation (EU) 2021/605. https://eur-lex.europa.eu/legal-content/HU/TXT/?uri=CELEX:02023R0594-20230516&qid=1689368504223.

Commission Implementing Regulation (EU) 2023/1300 of 22 June 2023 amending Annex I to the Implementing Regulation (EU) 2023/594 on the establishment of special epidemic control measures for African swine fever.
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Scientific Opinion on the assessment of the control measures of the category A diseases of Animal Health Law: African Swine Fever,
https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2021.6402.

Regulations of Hungary

Decree No. 98/2003. (VIII. 22.) of the Ministry of Agriculture and Rural Development on measures against African swine fever.
https://net.jogtar.hu/jogszabaly?docid=a0300098.fvm.

Act XLVI of 2008 on the food chain and official supervision
https://net.jogtar.hu/jogszabaly?docid=a0800046.tv

Decision No. 2/2021 of the Chief Veterinary Officer of Hungary
https://portal.nebih.gov.hu/documents/10182/902001/2_2021_OFA_hatarozat.pdf.

National African Swine Fever Contingency Plan.
https://portal.nebih.gov.hu/documents/10182/458753/Afrikai+sert%C3%A9spestis+k%C3%A9szenl%C3%A9ti+terv+20190731.pdf/a7c74fc6-cb91-6465-6ba5-c412c26e9510).

Regulations for laboratories

World Organisation for Animal Health (WOAH) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2021 Chapter 3.9.1 African Swine Fever (https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.08.01_ASF.pdf).
The European Union Reference Laboratory for African Swine Fever (EURL-ASF), https://asf-referencelab.info/asf/en/.
African Swine Fever Detection and Diagnosis, https://www.fao.org/3/i7228e/i7228e.pdf.
Recommendations, operational instructions, guidelines https://asf-referencelab.info/asf/en/procedures-diagnosis/diagnostic-procedures, https://asf-referencelab.info/asf/en/procedures-diagnosis/sops.

The Commission's Decision 2003/444/EC on the approval of the diagnostic manual for African swine fever. Legally it is no longer valid (expiry date: April 20, 2021), but it contains many professional recommendations.

Accreditation documents, internal regulations, instructions, usage instructions for diagnostic reagents, operating manuals for instruments (ELISA, Virotype ASP 2.0 PCR kit, IndiMAG Pathogen kit, etc.).

The **Animal Health Regulation** (Regulation (EU) 2016/429 of the European Parliament and Council) appears as a new, comprehensive, unified regulation (adopted by the European Parliament and the Council of the European Union) in the field of disease control, replacing a large number of previous veterinary laws. The Commission implementing regulations (EU 2018/1629 2018/1882) list diseases based on epidemiological risks, identify affected animal species, and categorize these major infectious diseases in the EU context based on their impact on other animals and public health (zoonosis). The regulation obliges Member States to prepare contingency plans for the management of certain diseases, it sets out the rules on the measures to be taken in the event of a confirmed or suspected outbreak of a disease. It regulates animal transport in terms of both import and export, determining the procedures to follow in emergencies. It establishes the obligations of all actors involved in the preservation of animal

health, clarifying the reporting and monitoring obligations related to animal health (veterinarians, agricultural producers, and authorities).

Structure and Content of the Animal Health Regulation:

Part 1 – General rules (list of diseases, categorization of diseases)

Part 2 – Reporting on diseases and related reporting, supervision, eradication programs, and "disease-free" certification (disease monitoring, disease eradication, disease-free status)

Part 3 – Awareness, preparedness, and response related to diseases (prevention and disease control measures for A, B, and C category diseases: veterinary medicines, use of vaccines: epidemic control measures: contingency plans)

Part 4 – Registration, authorization, traceability, and movement A. - terrestrial animals, breeding materials, and animal-derived products from terrestrial animals B. - aquatic animals and animal-derived products from aquatic animals

Part 5 – Entry and exit to the territory of the European Union

Part 6 – Non-commercial movement of pets

Part 7 – Emergency measures

Parts 8 - 9 – Common, transitional, and concluding measures.

The Commission's (EU) **2020/687 delegated regulation** supplementing rules for the prevention and control of certain listed diseases.

Part I – General provisions; Article 3 deals with the rules for "Clinical examinations, sampling procedures, and diagnostic methods" required for the determination or exclusion of the presence of Category A diseases.

Part II – Terrestrial animals; Chapter I addresses epidemic control measures for terrestrial animals in the case of **Category A diseases**; Chapter II pertains to epidemic control measures for terrestrial animals in restricted zones due to **Category A diseases**; Chapter III covers the repopulation of facilities with terrestrial animals in restricted zones following **Category A diseases**; Chapter IV discusses epidemic control measures for wild animals belonging to the species listed for **Category A diseases**; Chapter V is about epidemic control measures for terrestrial animals in the case of Category B and C diseases.

Part III – Aquatic animals;

Part IV – Final provisions, annexes; Annex I details the clinical examinations, sampling procedures, and diagnostic methods related to Category A diseases as set out in Article 3 of this regulation, as well as matters concerning the transport of samples.2

Based on the above regulations:

Category A disease refers to a listed disease which is generally not present within the territory of the Union and for which eradication measures must be immediately implemented upon detection, as stipulated in Article 9 (1) of Regulation (EU) 2016/429.

Category B disease refers to a listed disease, for which epidemic control measures must be adopted in all Member States in the interest of its eradication throughout the Union, as stipulated in Article 9(1)(b) of Regulation (EU) 2016/429.

Category C disease refers to a listed disease that affects certain Member States and for which measures are needed to prevent its spread to areas of the Union that are officially considered free from the disease or that have eradication programs related to the particular listed disease, as set out in Article 9(1)(c) of Regulation (EU) 2016/429.

Category D disease refers to a listed disease for which measures are needed to prevent its introduction into the territory of the Union or its spread as a result of movement between Member States, as specified in Article 9(1)(d) of Regulation (EU) 2016/429.

Category E disease refers to a listed disease for which surveillance is needed within the Union, as set out in Article 9(1)(e) of Regulation (EU) 2016/429.

According to the above regulations, African swine fever is classified as a Category A, D, and E disease affecting the Suidae family.

Participation in the events, presentations delivered

BioSecurity Conference, Hungary, Vásárosnamény presentation, 16/02/2022

Presentation of ÁDI Virology, and TSE Laboratory in Debrecen, Hungary, 18/02/2022

BioSecurity Conference, Workshop, Hungary, Debrecen, 28/04/2022

BioSecurity Conference, Ukrajna, Uzhhorod online presentation, 26/01/2023

BioSecurity Conference, Hungary, Nyírbátor, 17/03/2023

BioSecurity Conference, Hungary, Miskolc-Tapolca, 13/09/2023

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