

False positive results using PCR detection method for African swine fever virus in wild boars from northern Romanian hunting zones

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Abstract: African swine fever virus (ASFV) is an acute virus with a tropism for the pig macrophage and the ability to persist. In the absence of a vaccine, a good understanding of the ecology and epidemiology of the disease is fundamental to implement effective control measures. The recent occurrence and spread of ASFV in East Europe is perceived as a serious risk for the pig industry in the European Union. The aim of this study was to evaluate the classic detection method using polymerase chain reaction (PCR). A total number of 107 wild boar blood, tissues, and organs samples were collected from hunting areas of Romania's northern counties, from which 24 samples were positive by conventional PCR using the OIE manual for ASFV diagnostics. The positive samples were analyzed by sequencing techniques and the results were negative. Furthermore, we obtained only sequences that corresponded with a predicted uncharacterized mRNA from the *Sus scrofa* genome, leading to false positive results. Due to these results, an improvement in the detection method should be made, diagnostics should be based on multiple molecular tests, and a continuous monitoring plan for Romania should be applied to avoid the appearance of outbreaks.

Key words: Wild boar, surveillance, diagnostics

1. Introduction

African swine fever (ASF) is a devastating disease of the swine industry, which is caused by an icosahedral enveloped DNA virus that replicates in the cytoplasm of infected cells. It is the only recognized DNA arbovirus and also the only member of the family *Asfarviridae* and genus *Asfivirus* (1). The virus infects both domestic pigs and European or African wild boars, and it can easily be transmitted by arthropods. The virus can persist in ticks for years, even in quiescent ticks waiting for host feeding. The ASF virus (ASFV) is a highly contagious virus, resulting in up to 100% morbidity in previously unexposed pig herds and with mortality varying between 0% and 100% depending on the virus, the host, the dose, and the route of exposure to the virus. In domestic pigs, ASF was originally described to cause acute hemorrhagic fever, leading to the death of all animals infected. One of the main features of the infection is the lack of induction of neutralizing antibodies, which has prevented the production of a conventional vaccine (2,3).

The viral genome is a double-stranded DNA molecule of about 180 kbp with a conserved central region of about 125 kb and variable ends. A high level of variability is observed mainly within the 35 kb at the 3' end and the 15

kb at the 5' end of the genome (170–190 kb) (4,5). These variable regions encode five multigene families that are directly involved with the variability of the virus genome (6). The viral particle contains about 50 proteins, including cell proteins like actin and tubulins that are specifically encapsulated. In contrast to most enveloped viruses, there is no glycoprotein in the virus particle. ASFV isolates have been previously characterized by sequencing of different genome regions or by restriction enzyme site mapping. Partial sequencing of the *B646L* gene encoding the major capsid protein (p72) has so far led to the identification of 22 ASFV genotypes (7–9). Polymerase chain reaction (PCR) amplification and sequencing of more variable genome regions have been used to distinguish between closely related isolates and identify virus subgroups within several of the 22 genotypes (10). In addition, genotype I viruses were identified in East African sylvatic hosts for the first time, which is significant as this genotype was previously thought to be restricted only to the West African region where it occurred in domestic pigs. Despite more than 50 years of circulation in West Africa, Europe, and South America, the limited accumulation of genetic changes has made it impossible to discriminate among isolates within genotype I (8).

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In April 2007 the remarkable potential of transboundary spread of ASFV was demonstrated by the appearance of the virus in the Republic of Georgia with subsequent outbreaks in Armenia, Azerbaijan, and southern Russia (11–13). This broad distribution of genotype I viruses in Europe was altered in Georgia when the first nongenotype I virus was responsible for an outbreak outside the endemic area in Africa and was classified within South East African ASFV genotype II (9).

The recent occurrence in East Europe is perceived as a serious risk for the pig industry in the European Union (EU) (14). More recently, a report by the World Organisation for Animal Health (OIE) stated that in 2014 ASF was confirmed in dead wild boars in Lithuania, Poland, and Ukraine (http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home).

Due to the latest concerns a strategic national plan should be applied in order to have permanent surveillance of the epidemiological status of ASFV. This study is based on molecular observations of wild boars in order to evaluate the infection status of boar populations using molecular biology techniques based on the OIE manual for ASFV (15).

2. Materials and methods

A total of 107 samples of boar blood (in anticoagulant, EDTA, tissues, and organs (tonsils, lungs, mediastinal lymph nodes, liver, spleen, and kidneys) were collected from wild boars that were hunted/captured for regular monitoring from different hunting areas in northern Romania (Iasi, Neamt, Suceava, and Botosani counties) in 2013 and 2014 (Figure 1). After the samples were collected, they were kept on ice at 4 °C during transport; after arriving at the laboratory they were stored at –80 °C.

DNA was extracted using the commercial High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) and 200 µL of tissue homogenate/blood supernatant was processed in accordance with the manufacturer's instructions. One positive and one negative control were included in each nucleic acid extraction run. Positive control samples were represented by 200 µL of 1/10 tissue spleen homogenate obtained from the EURL-ASF Laboratory in Spain. The negative control was represented by 200 µL of tissue homogenate from a naive boar, ASFV-free. Controls were processed with the test samples. The purity and the integrity of extracted DNA were evaluated using a BioSpec-nano spectrophotometer (Shimadzu Biotech, Japan).

PCR detection of ASFV was performed using primers PPA-1 and PPA-2 that partially amplify the *B646L* gene, encoding the major capsid protein p72. The expected size of the PCR product using primers PPA1 and PPA2 primers was 257 bp.

The PCR reaction was optimized on the positive control (EURL-ASF tissue homogenate) regarding the annealing temperature of PPA-1 and PPA-2 primers. Gradient PCR was done to evaluate the optimal annealing temperature, ranging between 55 and 63.9 °C. The gradient PCR annealing temperatures of 6 steps were respectively 55.0 °C, 55.8 °C, 57.1 °C, 58.9 °C, 62.3 °C, and 63.9 °C (Figure 2). The optimal annealing temperature was fixed at 62.3 °C.

The reaction was carried out in a final volume of 23 µL using Hot Start Taq Gold DNA polymerase 5 U/µL (0.125 µL) (Promega, USA), 10X PCR Buffer II (2.5 µL) (Promega), magnesium chloride 25 mM (2 µL) (QIAGEN, USA), dNTP mix 10 mM (0.5 µL) (QIAGEN), PPA-1 forward 10 pmol/µL (0.25 µL) (5'-AGT-TAT-GGG-AAA-CCC-GACCC-3'), PPA-2 reverse 10 pmol/µL (0.25 µL) (5'-CCC-TGA-ATC-GGA-GCA-TCC-T-3'), and nuclease-free water (17.375 µL). We added 2 µL of extracted sample template to each PCR tube, including a positive reaction control (2 µL of ASFV DNA), a negative reaction control (2 µL of nuclease free water), and a negative extraction control (from an ASFV-free boar). The optimal primer concentration was established by performing a PCR on the positive ASFV control and on a supposedly positive sample from a wild boar from the Botosani hunting area (Figure 3).

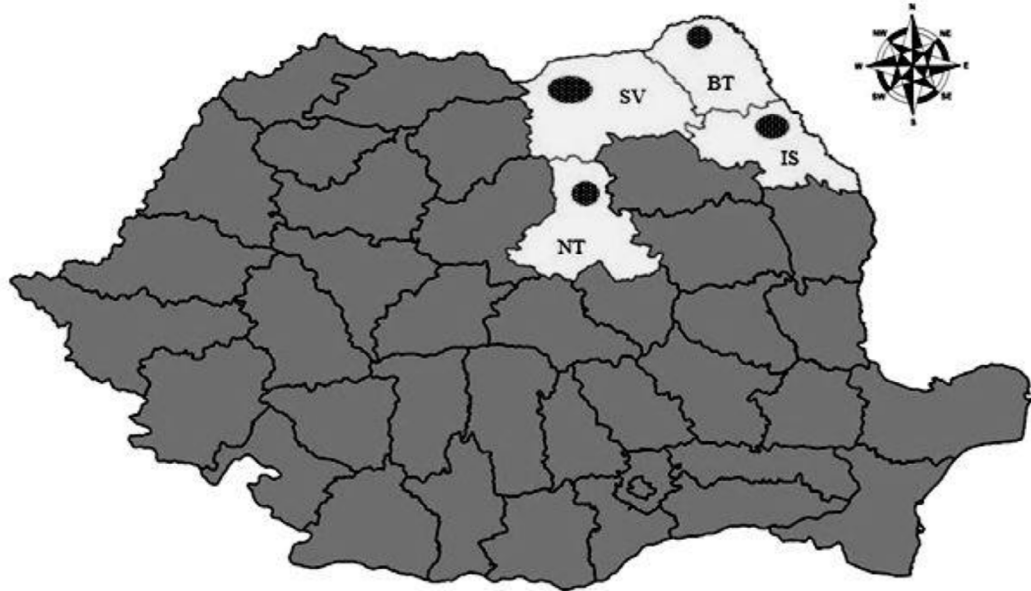
PCR was performed using an iCycler Bio-Rad thermocycler with the following program: 10 min at 95 °C - 1 cycle; 15 s at 95 °C, 30 s at 62.3 °C, and 30 s at 72 °C - 40 cycles; 7 min at 72 °C - 1 cycle; and 4 °C ∞. The PCR products were visualized on 2% agarose gels stained with ethidium bromide.

The amplicons were purified using the Wizard PCR Preps DNA Purification System (Promega). DNA sequencing reactions were performed using the BigDye Terminator Kit v3.1 (Applied Biosystems, USA) on a 3130 Genetic Analyzer (Applied Biosystems). The sequences were aligned using the CLUSTAL W application from the MEGA5 program (16). The sequenced fragments were edited using BioEdit version 7.1.3.0 (17).

3. Results

A total of 107 wild boar blood, tissue, and organ samples (Figure 1) were collected from northern Romanian hunting areas from hunted specimens or specimens found dead from natural causes (different diseases, car accidents, age, etc.). The samples were harvested and sent to the laboratory for molecular detection and analysis.

The conventional PCR results, following the OIE manual assay for ASF diagnostics, supposedly showed multiple positive samples (Figures 2 and 3). The negative controls showed that the PCR reactions were correct. In the first stage of the study we conducted the PCR reaction



● - hunting areas from where the samples were collected

SV, NT, BT, IS - counties where the hunting areas are located

Counties	Number of samples	Blood in EDTA	Tissue homogenates
Suceava (SV)	47	19	28
Botosani (BT)	61	25	36
Iasi (IS)	44	28	15
Neamt (NT)	15	-	15

Figure 1. Geographical map of Romania, number, and locations where the samples were collected.

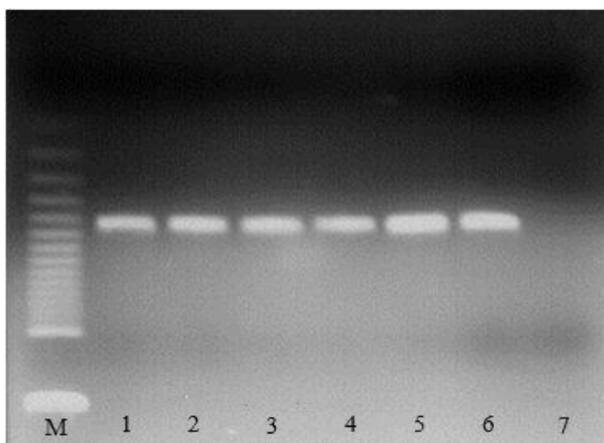


Figure 2. Gradient PCR on the ASFV positive control. First lane is represented by a 50-bp molecular marker. Lane 1 - 55.0 °C, lane 2 - 55.8 °C, lane 3 - 57.1 °C, lane 4 - 58.9 °C, lane 5 - 62.3 °C, and lane 6 - 63.9 °C are the different annealing temperatures. Lane 7 is represented by a negative PCR control. As observed, the best annealing temperatures are 62.3 °C and 63.9 °C.

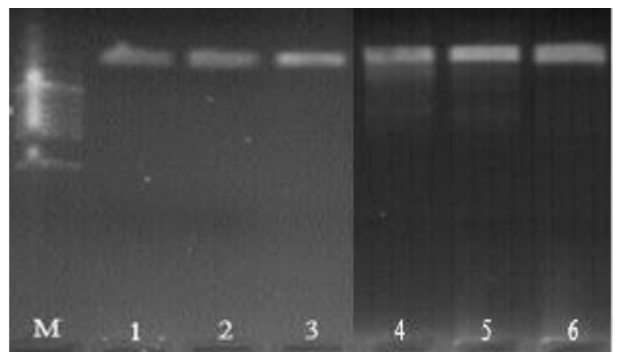


Figure 3. Two electrophoresis showing PCR results for PPA-1 and PPA-2 primers using different concentrations between 10 and 20 pmol/μL on ASFV positive control and on a possible positive sample. Lanes 1–3 are positive ASFV control samples. First lane is represented by a 100-bp molecular marker. Lanes 4–6 are possible positive tissue homogenate samples from Botosani. Lane 1 has a primer concentration of 10 pmol/μL, lane 2 of 15 pmol/μL, lane 3 of 20 pmol/μL, lane 4 of 10 pmol/μL, lane 5 of 15 pmol/μL, and lane 6 of 20 pmol/μL.

on tissues samples from the Botosani, Suceava, and Neamt hunting areas and EDTA-treated blood samples from Iasi. Noticing the specific bands of interest at 257 bp, we redid the experiment using blood samples from Botosani and tissue samples from Suceava and Neamt.

In the second experiment, blood samples from Botosani originated from the same hunting area and tissue homogenate samples from Suceava and Neamt originated from other hunting areas than in the first experiment.

In both experiments we obtained specific bands at 257 bp for the *B646L* gene, encoding the capsid protein p72. We noticed that a concentration of 10 pmol/ μ L was optimal for our PCRs (Figure 3).

Twenty-four samples from a total of 107 were positive by conventional PCR. The amplicons were purified and sequenced using the Sanger dideoxy sequencing method, and the obtained sequences were aligned and compared with the positive control and reference sequences from Ukraine and Italy from the GenBank database (JX857521 and FJ174371).

In Figures 4–6, we present the sequences of one sample of blood from the Iasi hunting area corresponding to sample 8 (Figure 4), one blood sample from the Botosani hunting area corresponding to sample 7 (Figure 6), one sample from tissue homogenate from the Suceava hunting zone corresponding with sample 10 (Figure 6), and one sample of tissue homogenate from the Neamt hunting

zone corresponding with sample 10 (Figure 5).

Interesting, the reference sequences were aligned with each other, while less so with our sequences (Figure 7). All 24 sequences from northern Romania presented multiple mismatches with reference sequences JX857521 and FJ174371 and the positive control used in this study.

Using the NCBI's Basic Local Alignment Search Tool (BLAST) for the Romanian sequences, it was shown that our sequences corresponded with a predicted uncharacterized mRNA from *Sus scrofa* (Figure 8), leading us to the conclusion that the primers from the OIE manual can attach in different parts of the boar genome.

4. Discussion

Countries like Finland, Romania, Germany, Poland, and France obtained high-degree risk scores for transmission and spreading ASF through several pathways. These countries must benefit from further national research to elucidate which pathways are of higher risk for them and which actions can be implemented to prevent the risk of transmission (<http://faostat.fao.org/site/339/default.aspx>). The recent reappearance of ASF in the Russian Federation has increased the risk of emergence of the disease in the EU.

The northern part of Romania is known to be an important region for animal hunting, especially wild boars. Due to the recent outbreak of ASF in East Europe, mainly

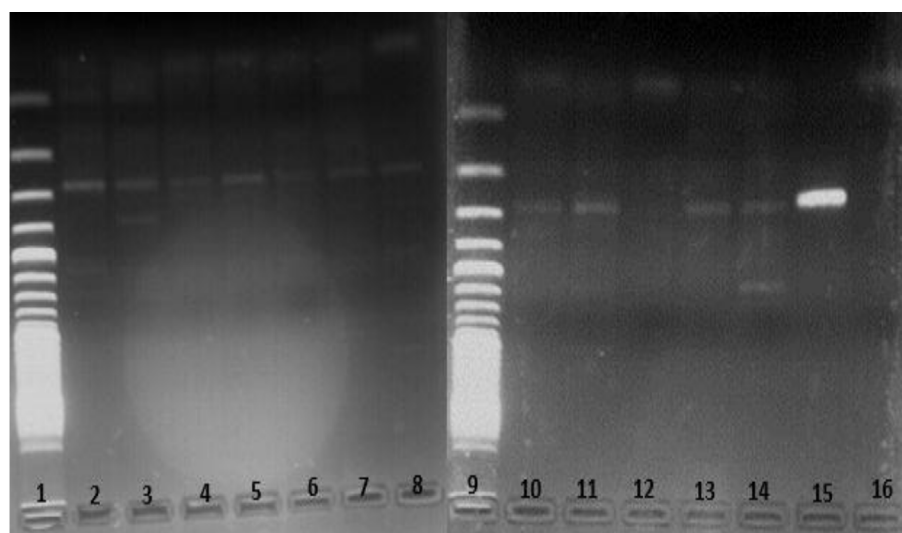


Figure 4. Results from conventional PCR using PPA-1 and PPA-2 primers. First lane is represented by 100-bp molecular ruler; lanes 2–8 are samples (lanes 2 and 3 are samples from tissue homogenates from Botosani, lanes 4 and 5 are tissue homogenate samples from Suceava and Neamt, and lanes 6, 7, and 8 are blood samples from Iasi). Lane 9 is represented by a 100-bp molecular ruler, and lanes 10–14 are samples (lane 10, sample of blood from Suceava; lane 11, sample of blood from Botosani; lanes 12 and 13, samples of blood from Iasi; lane 14, tissue homogenate sample from Iasi); 15 is the positive ASF control; and lane 16 is the negative extraction control represented by tissue homogenate from an ASF-free boar.

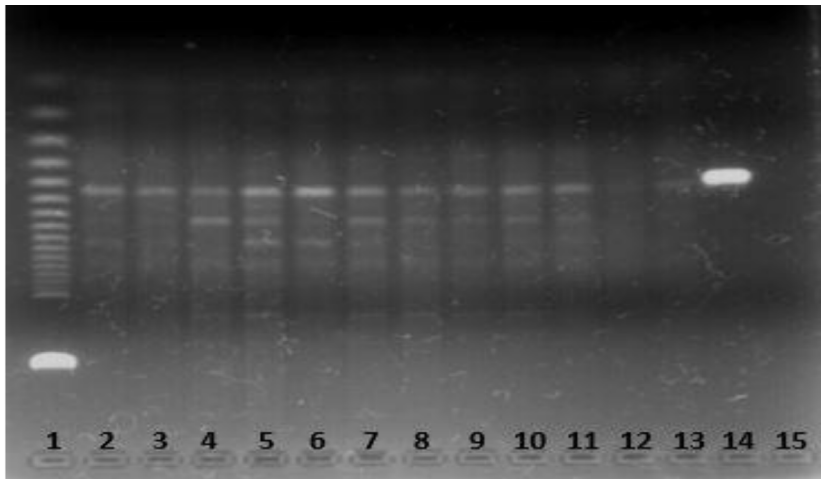


Figure 5. Results from conventional PCR using PPA-1 and PPA-2 primers. First lane is represented by 50-bp molecular ruler; lanes 2–13 are samples (lanes 2, 3, 4, and 5 are blood samples from Botosani county; lanes 6, 7, 8, and 9 are samples from tissue homogenates from Suceava county; lanes 10, 11, 12, and 13 are samples from tissue homogenates from Neamt county); lane 14 is the positive ASF control; and lane 15 is the negative control represented by nuclease-free water.

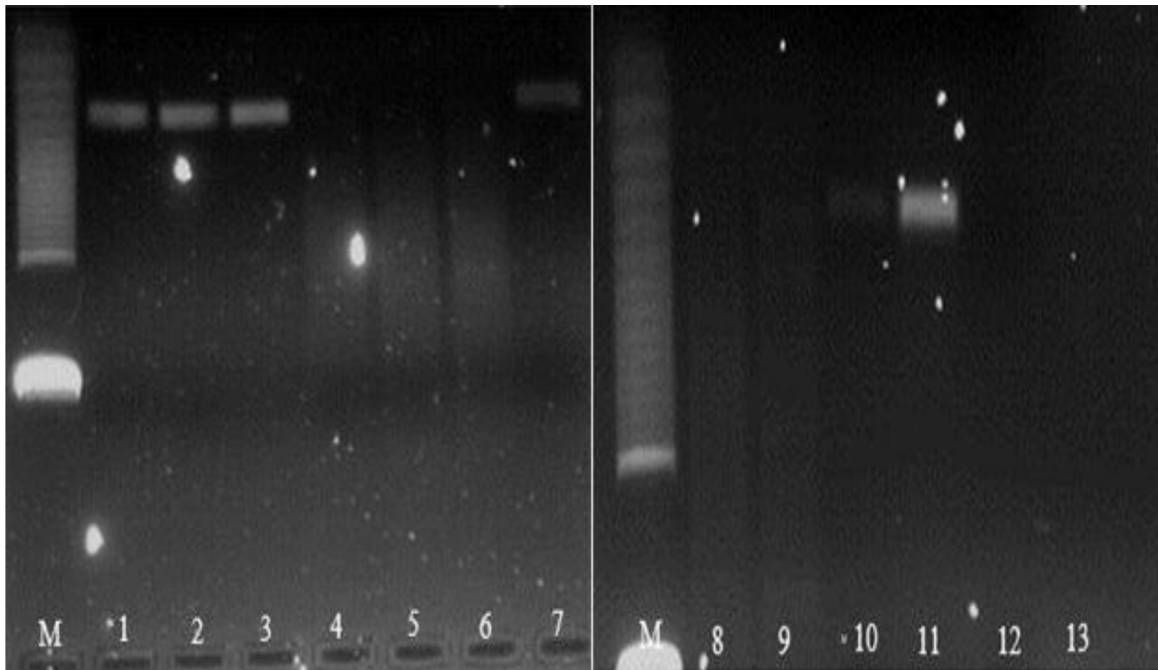


Figure 6. Results from conventional PCR using primers PPA-1 and PPA-2 at a concentration of 10 pmol/μL. M lane is represented by molecular ruler of 50 bp. Lanes 1–3 are dilutions of the ASF positive control (lane 1 - 15X, lane 2 - 10X, and lane 3 - 5X). Lanes 4, 5, and 6 are pig tissue homogenate samples from Botosani, Neamt, and Iasi intensive farms. Lane 7 is a blood sample from a wild boar from the Botosani hunting area. Lanes 8 and 9 are blood samples from a swine farm in Suceava county. Lane 10 is a tissue homogenate sample from a wild boar from the Suceava hunting area. Lane 11 is the ASF positive control and lanes 12 and 13 are negative extraction control and negative PCR control.

in Ukraine, the risk of transboundary spread of the virus to the northern counties of Romania is very high. This is why this region has been chosen for investigations on

the detection of ASFV. That implies a series of periodical checks, control of animal imports, a national surveillance program, and wild boar monitoring. The present study

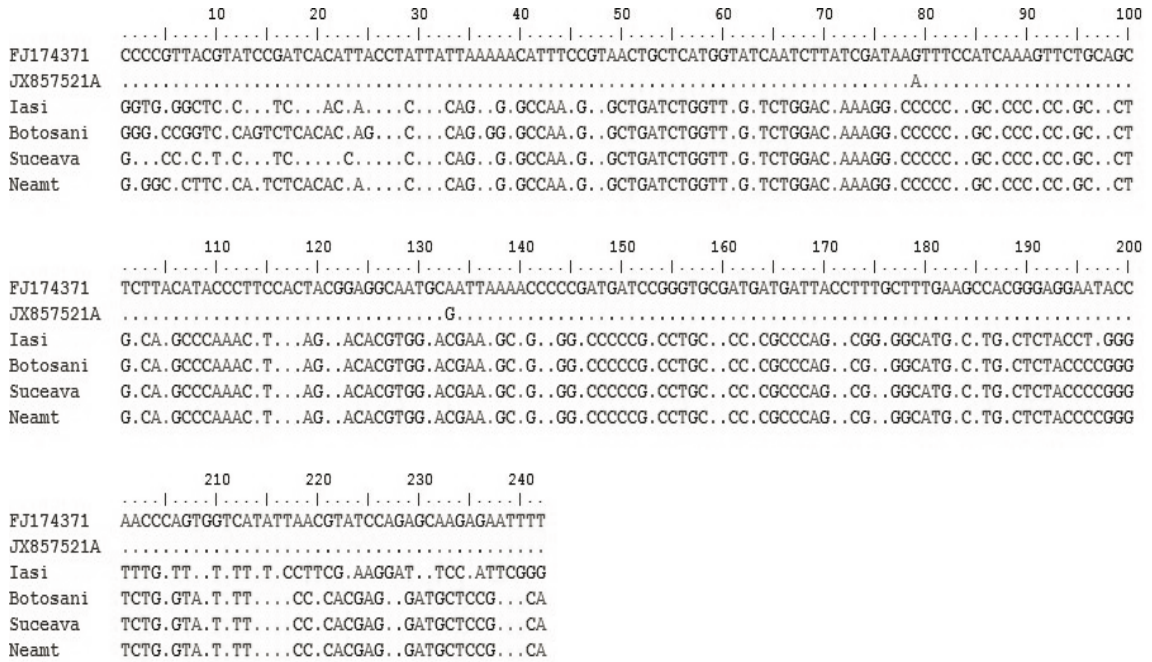


Figure 7. Sequence alignment of Romanian sequences of supposedly PCR-positive samples of ASFV from Iasi, Botosani, Suceava, and Neamt counties with reference sequence from GenBank. Dots represent similarities between the sequences. As observed, the results from the conventional PCR are not consistent with the obtained sequences.

PREDICTED: *Sus scrofa* uncharacterized LOC100736873 (LOC100736873), mRNA
 Sequence ID: [reflXM_005668591.1](#) Length: 1294 Number of Matches: 1

Range 1: 137 to 282 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
222 bits(120)	1e-54	138/146(95%)	3/146(2%)	Plus/Plus
Query 38	CAGAGGAGCCAACGGTGCTGATCTGGTITGTTCTGGACAAAAGGA	ccccccc-gccc-cc	95	
Sbjct 137	CAGAAAGAGCCAACGGTGCTGATCTGGTITGTTCTGGACAAAAGG	ccccccccccccacc	196	
Query 96	g-cccGCCACTGCCAAGCCCAAACCTCACAGCGACACGTGGGACGAAAGCAGCCGGGCC		154	
Sbjct 197	GCCCCGCCACCGCAAAGCCCAAACCTCACAGCGACGCGTGGGACGAAAGCAGCCGGGCC		256	
Query 155	CCGCCCTGCCGCGCGCCAGCCCGT	180		
Sbjct 257	CCGCCCTGCCGCGCGCCAGCCCGT	282		

Figure 8. BLAST alignment of a Romanian sequence that was a false positive on conventional PCR. Results show that the sequence belonged to a predicted uncharacterized mRNA of *Sus scrofa*.

wishes to offer a better perspective of the diagnostic measures used to detect ASFV and to add novel data regarding the current situation in Romania.

In Romania, most laboratories for veterinary diagnosis do not use the sequencing technique due to lack of funds, so diagnostics are based on conventional PCR, which,

as shown, can be misleading, leading to false positive results. Moreover, similar findings in eastern Africa have documented a low incidence of detectable serological responses to ASFV infection using the OIE-approved assays in ASFV-positive swine (18,19).

The real-time PCR assay currently recommended and rigorously validated by four National Reference Laboratories of the European Union for ASF, including the European Union Reference Laboratory, with an internal control will provide a rapid, sensitive, and reliable molecular tool for ASFV detection in pigs in newly infected areas, control in endemic areas, and surveillance in ASF-free areas (20).

Although PCR is a reliable technique for detecting ASFV, it has been shown in previous studies that it can induce false positive results (21).

Although multiple conventional PCR assays for ASFV detection have been described previously (1,22), nowadays these have now mostly been superseded by the development of rPCR assays (20).

Moreover, the use of capture ELISA and immunofluorescence assays is not possible in many countries in which ASFV is currently circulating or that

are at high alert, due to a lack of molecular diagnostic tools and the difficulty in carrying out virus isolation. Cheap assays are available for the detection of the ASF antigen, such as the antigen ELISA and FAT; however, these assays have reduced sensitivity compared to PCR and a preliminary result is only obtained after several hours.

The severe disease manifestations of ASFV combined with the lack of treatment and vaccination options means that early detection is crucial to prevent serious consequences for hunting areas, farms, and some at-risk regions. The high incidence of false positive results using conventional PCR with primers PPA-1 and PPA-2 is worrying for Romania, due to the recent outbreaks in East Europe and due to the lack of sequencing equipment in diagnostic veterinary laboratories that could lead to false positive results. It is advisable that results be confirmed with other diagnostic techniques and a continuous monitoring plan in Romania should be applied.

In conclusion, since the classic PCR assay should be more reliable for obtaining good results, this technique is being left behind, and assays more stable and rapid are beginning to be used.

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