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RAPID AND RELIABLE REAL-TIME PCR WORKFLOW FOR DETECTION OF AFRICAN SWINE FEVER VIRUS

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Abstract

ASF is a fatal haemorrhagic disease affecting pigs, posing a significant threat to global agriculture as it rapidly spreads across the UE, Asia, and Oceania. Due to the lack of a vaccine or treatment, each outbreak requires pursuit of stringent biosecurity measures and trade restrictions. The disease's emergence is causing significant economic losses due to the stamping out of pig holdings in the affected areas and the pursuit of disease control measures, including trade restrictions to avoid disease spread. Since the onset of the ASF, Romania has been severely affected economically and socially, suffering losses in commercial and backyard farms, unemployment among workers in the pig breeding and meat processing industries, and significant expenditure on disease control and surveillance. ASF remains a major threat with no available vaccine and challenging eradication. Rapid, sensitive diagnostics are crucial. The proposed workflow offers multiple advantages, including the ability to verify extraction, amplification efficiency for each sample through amplification of two internal control systems, ensuring accurate pathogen detection regardless of nucleic acid type presence, and confirming sample cellularity to validate result accuracy and prevent false negatives due to inadequate collection, transport, or storage.

***Key words**: ASF, ASFV, cellularity, diagnosis, Real-Time PCR.*

INTRODUCTION

African swine fever (ASF) is a viral disease exclusively affecting domestic and wild pigs. Clinically, it involves a haemorrhagic syndrome; it is quite contagious and has a lethal course in most cases (Sauter-Louis et al., 2021).

The emergence of the disease is causing significant economic losses due to the stamping-out procedures in the affected areas, the implementation of disease control measures and the trade restrictions to prevent the spread of the disease (Saatkamp et al., 2000).

African Swine Fever (ASF) through various transmission cycles involving different hosts, such as domestic pigs, wild boar, wild African swine, and possibly other species. The epidemiology of ASF varies significantly between Africa and Europe due to differing environmental factors, with ticks playing a key role in the virus's transmission (Sánchez-Vizcaíno et al., 2015). In regions where these ticks are found, increased surveillance and comprehension of the interactions among different hosts are essential for creating effective control and eradication strategies (Costard et al., 2013).

While the African Swine Fever virus (ASFV) represents a significant threat to livestock, it does not pose a risk to human health (Sánchez-Vizcaíno et al., 2009).

This distinction is important for shaping public health policies. Nevertheless, ASFV is contagious among pigs, requiring strict biosecurity protocols in veterinary environments. A comprehensive understanding of ASF's epidemiology, including its routes of transmission and the roles of various hosts and vectors, is crucial for effectively managing outbreaks and safeguarding pig populations worldwide through targeted research and surveillance initiatives.

The only way to stop the spread of the virus is to slaughter and destroy all pigs on infected holdings, followed by rigorous disinfection and restrictions on animal movements and trade in the affected area (Busch et al., 2021).

The African swine fever virus was originally grouped within the *Iridoviridae* family but has since been reclassified into the *Asfivirus* genus of the *Asfarviridae* family. While unclassified viruses such as faustoviruses, kaumoebavirus, and Pacmanvirus possess about 30 genes similar to those found in the African swine fever virus, their genomes are substantially larger - approximately 400 kilobase pairs compared to the 170-194 kilobase pairs of the African swine fever virus (Alonso et al., 2018). It is much more resistant and environmentally stable than the classical swine fever virus. This virus can cause extremely high mortality and affects pigs of all ages. Due to the lack of a safe effective vaccine and the common presence of infected wild boars in particular areas, the only method to control the disease is strict biosecurity measures allied to international cooperation on this matter. Knowledge and epidemiological understanding of how the virus may be introduced into susceptible populations of pigs is crucial to provide awareness to prevent the outbreaks and detect and control them immediately and appropriately when they do occur (Mazur-Panasiuk et al., 2019).

Since the onset of the ASF outbreak, Romania has been severely affected economically and socially, suffering losses in commercial and backyard farms, unemployment among workers in the pig and meat processing industries, and significant expenditure on disease control and surveillance.

The correct diagnosis of ASF should include detection of the virus genome or antigen in pig samples and/or the demonstration of a specific antibody response. Those techniques offer high specificity and sensitivity, making it both rapid and suitable for large-scale applications. Among them, PCR is capable of analysing any type of clinical specimen, achieving near-perfect accuracy with almost 100% specificity and sensitivity. PCR is particularly advantageous when working with decomposed tissues, where other methods may falter. It also serves as a valuable tool for analysing *Ornithodoros* soft ticks, which are vectors for several pathogens. The multiplex assay feature enables the simultaneous and differential detection of multiple pathogens, streamlining the diagnostic process. Notably, PCR does not require direct handling of live viruses, though biosecurity precautions are essential until any suspected samples are properly inactivated to ensure safe handling. Additionally, robustness decreases in samples with weak positive results (World Organisation for Animal Health, 2019). Given the similar symptoms of ASF, classical swine fever (CSF), and porcine dermatitis and nephropathy syndrome (PDNS), this method is crucial for swift and reliable diagnosis, enhancing existing molecular diagnostics for identifying ASF in suspected cases (King et al., 2003). A fully validated PCR test with high sensitivity and specificity will detect pigs with low viremia levels, including during early and late infection stages or with attenuated virus strains (Fernández-Pinero et al., 2013). The purpose of this paper is to describe a sensitive and robust workflow for ASF genome detection from various samples, with stringent validation and quality monitoring of the results.

MATERIALS AND METHODS

For this kind of extraction, it is preferred to use 2ml round-bottom tubes (U-shaped bottom) for easy resuspension of magnetic particles and to avoid sedimentation (Potop et al., 2014). Also, for certain sample types, a pretreatment is necessary in order to bring the nucleic acids into solution and make it available for binding to magnetic particles, as described in Table 1.

Table 1. Pre-treatment of dry tissues and pads

Step	Action	Description	Observation
Tissue	Primary lysis	In 2 mL tubes will be pipetting the next reagents in the next order: Approx. 20 mg biological sample 20 µL Proteinase K 300 µL Reagent E0	Incubate at 56°C with occasional shaking or homogenisation by inversion until complete dissolution of the tissue.
Dry swabs	Resuspension biological material	Insert buffer in the tube	Sectioning the buffer rod so that what remains does not go beyond the edge of the tube Energetic vortexing. Spin for liquid collection
		Pipette 500 µL saline into each tube.	

Table 2. Nucleic acid extraction

Step	Action	Description	Observation
Step 1	Lysis of biological samples Nucleic acids binding	In a 2 mL tube pipette the following reagents in the order below: - 20 µL Proteinase K - 200 µL biological sample - 100 µl Reagent E1 - 400 µl Reagent E2 - 20 µl Reagent E3	Homogenisation by pipetting after sample transfer Reagent E1 has a high viscosity, wait 2 seconds after the pipette plunger release
Incubation on shaker at minimum 1200 rpm for 15 minutes, temperature 25°C. Spin centrifugation at the end			
Tubes insertion in the magnetic stand so that they can be opened towards the operator			
Liquid removal by inserting the pipette tip on the opposite side of the pipette, gradually up to the bottom of the tube, with total liquid removal (volume set for the pipette around 900µl)			
Step 2	Nucleic acid wash	1. Pipette 600 µL Reagent E4	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 3	Nucleic acid wash	1. Pipette 600 µl Reagent E5	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 4	Nucleic acid wash	1. Pipetting 600 µL Reagent E6	-
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Spin centrifugation at the end
		3. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator.
		4. Liquid removal	Insert the pipette tip on the opposite side of the pipette, gradually until the bottom of the tube, with total removal of the liquid (volume set for the pipette around 800 µL)
Step 5	Magnetic particle drying	1. Tubes insertion on thermoblock, 5 minutes at 55°C	Tube caps open!
		2. Tubes insertion in the magnetic stand	The tubes are positioned so that they can open towards the operator.
Step 6	Nucleic acid elution	1. Pipetting 90 µL Reagent E7	Tube caps closure!
		2. Incubation on shaker at minimum 1200 rpm for 5 minutes, room temperature	Stir gently before to resuspend the particles in E7 reagent
			Spin centrifugation at the end.
		3. Tubes insertion in the magnetic stand	The tubes are positioned with the opening towards the operator
		4. Transfer 80 µl liquid in a new tube	Keep refrigerated for up to one hour or in the freezer until analysed

Pathogen nucleic acid extraction was performed as described previously (Turcitu, 2024), with a custom protocol based on Qiagen chemistry and using magnetic beads for nucleic acids manipulation. Briefly, 0.2 mL of sample was incubated at 56°C with constant shaking along with 20 µL of Proteinase K and lysis buffer, then washed and eluted in 100 µL nuclease-free buffer. Eluates were stored at -80°C until amplification (Table 2).

Amplification/detection was performed using the QuantiNova Pathogen + IC kit (Qiagen, Hilden, Germany), specially designed for pathogen detection, as the name implies, with the internal exogenous control assay provided for inhibition monitoring and a supplementary assay for cellularity evaluation (mammal housekeeping gene). Primers and probe for ASFV were synthesised according to previous recommendations (King et al., 2003). Pathogen assay was marked with FAM fluorophore, exogenous internal control assay was marked with HEX-like fluorophore, and endogenous internal control assay was marked with Cy5 fluorophore; therefore, a triplex protocol was used (Turcitu, 2024). All components were added according to the manufacturer's recommendations regarding final concentrations. Experiments were conducted on the Q instrument (Quantabio, Beverly, USA).

RESULTS AND DISCUSSIONS

To better evaluate the performance of the designed workflow, serial ten-fold dilution of the original sample was made. Amplification protocol was performed using a singleplex approach (only the pathogen assay was added to the MasterMix) and a triplex approach (all three assays were added to the MasterMix). All reactions were performed simultaneously in replicates, and the results are shown in Figures 1 to 3. Results interpretations were automatically generated using Real Time instrument software features for reaction efficiency and R^2 coefficient calculation (Tables 3 to 5)

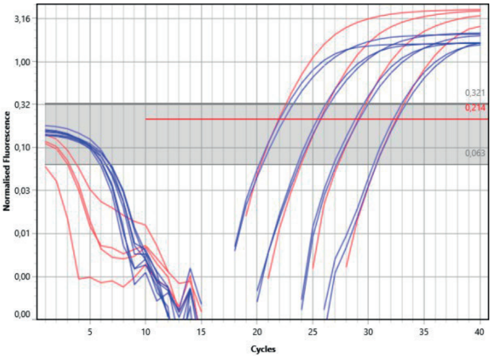


Figure 1. Amplification curves obtained for single-plex assays (red) and multiplex assays (blue) on serial ten-fold dilution of ASF-DNA

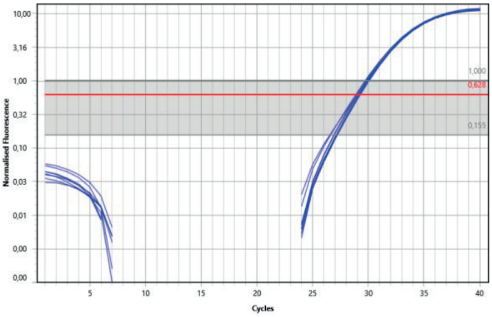


Figure 2. Amplification curves obtained for inhibition (exogenous internal control) assay Signal amplification on the same Ct, showing no inhibitors interference

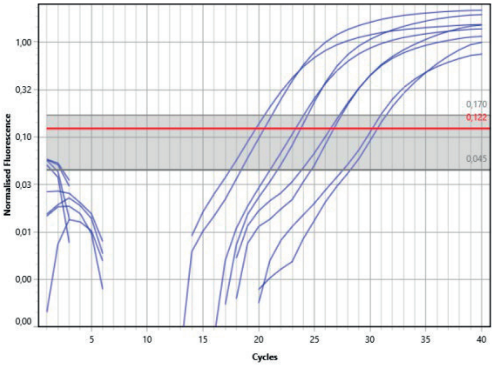


Figure 3. Amplification curves obtained for cellularity evaluation (endogenous internal control) assay on serial ten-fold dilution

Table 3. Results obtained for ASFV target (Green/FAM channel)

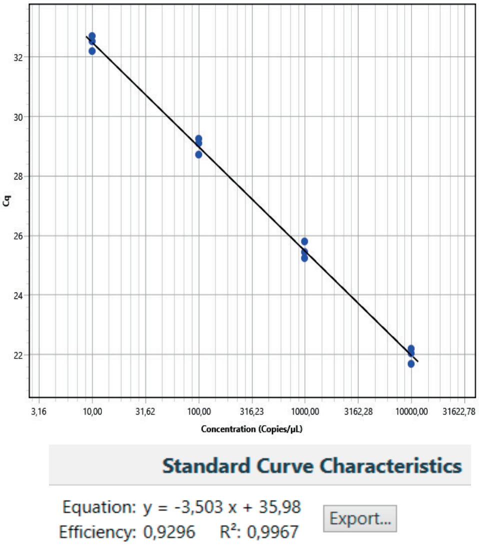
Cq	Efficiency	R^2	Result
PPA -1			x=22.10 σ=0.20
21.91	1.04	0.99714	
22.02	0.87	0.99663	
22.38	0.80	0.99735	
PPA -2			x=25.54 σ =0.13
25.60	0.98	0.99900	
25.36	0.88	0.99727	
25.66	0.83	0.99708	
PPA-3			x=28.98 σ =0.16
29.06	0.93	0.99927	
28.78	0.82	0.99853	
29.13	0.78	0.99794	
PPA-4			x=32.37 σ =0.14
32.41	0.93	0.99875	
32.18	0.90	0.99570	
32.51	0.84	0.99686	

Table 4. Results obtained for inhibition control (Yellow/HEX channel)

Cq	Efficiency	R^2	Result
PPA -1			x=28.91 σ=0.08
28.98	0.76	0.99992	
28.83	0.82	0.99952	
PPA -2			x=29.02 σ =0.04
29.06	0.88	0.99922	
28.97	0.91	0.99914	
PPA-3			x=28.98 σ =0.16
29.15	0.93	0.99917	
29.13	0.93	0.99904	
PPA-4			x=29.20 σ =0.03
29.18	0.93	0.99916	
29.23	0.92	0.99929	

Table 5. Results obtained for cellularity (Red/Cy5 channel)

Cq	Efficiency	R^2	Result
PPA cellularity -1			x=20.05 σ=0.37
20.42	0.62	0.99991	
19.68	0.54	0.99988	
PPA cellularity -2			x=23.47 σ =0.23
23.70	0.64	0.99881	
23.24	0.56	0.99992	
PPA cellularity-3			x=26.53 σ =0.165
26.67	0.64	0.99964	
26.38	0.48	0.99909	
PPA cellularity -4			x=30.36 σ =0.22
30.58	0.54	0.99868	
30.14	0.48	0.99661	



Equation: y = -3,503 x + 35,98
Efficiency: 0,9296 R²: 0,9967

Figure 4. Standard curve for ASFV serial ten-fold dilutions

Results obtained show a good workflow efficiency in terms of removal of sample inhibitors during extraction. as stated by the endogenous internal control amplification curves (Figure 2 - simultaneous amplification of the analysed samples, regardless of ASFV viral load) and Ct results (Figure 4 - comparable values for all samples/dilutions, minimal standard deviation). Moreover, nucleic acid recovery during extraction proved to be adequate for high. medium and low sample viral loads. as stated by the standard curve parameters (slope value -3.5. R^2 coefficient value above 0.99) and replicate values obtained (minimal standard deviation for target as well as cellularity). Besides extraction efficiency. amplification showed a robust pattern. with serial dilution being amplified simultaneously for each dilution and replicate (Figure 1) and standard deviation within accepted range (Figure 4). Moreover, no significant differences were observed when comparing singleplex amplification versus triplex amplification – for each dilution/replicate. the increase of fluorescence starts simultaneously (Figure 1), and the Ct values obtained are comparable (Figure 4); therefore, no loss of sensitivity was observed.

CONCLUSIONS

The workflow described proved to fulfil the requirements for a good, reliable and fast tool for ASF nucleic acid detection. being able to cover all the biological material that can be received for diagnostic and surveillance of the virus. Moreover, by including stringent validation criteria for the obtained results it can give valuable information regarding sample collection, storage and transportation, along with the possibility to detect any inadvertence that might arise during sample processing and nucleic acid detection/amplification.

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