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PERFORMANCES OF TWO EXTRACTION KITS OF AFRICAN SWINE VIRUS GENOME

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Abstract

Currently, the ASF (African Swine Fever) diagnosis is carried out by the detection of viral DNA. The successful amplification of the targeted DNA fragment needs a proper quantity of the genetic material. The aim of this study was to compare the yield and quality of DNA extracted using two dedicated commercial kits. It was compared the product obtained using the Pure Link Genomic DNA Mini Kit (Invitrogen) and QIAamp Cador Pathogen Mini Kit (Qiagen). The DNA has been quantified using Qubit DNA HS Assay Kit (Qubit 3.0 Fluorometer, ThermoFisher Scientific), a highly selective over RNA and accurate tool for DNA at levels between 10 pg/µL and 100 ng/µL.
The standard DNA of the ASF virus has been diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} as both extracts obtained with Pure Link Genomic DNA Mini Kit (Invitrogen) and the QIAamp Cador Pathogen Mini Kit (Qiagen). According to the dilutions (10^{-2} to 10^{-6}), the DNA yield with the QIAamp Cador Pathogen Mini Kit was 132 ng/µl, 71 ng/µl, 45 ng/µl, 21.2 ng/µl and 10.2 ng/µl, respectively.
The yield obtained with the Pure Link Genomic DNA Mini Kit was 126 ng/µl, 65 ng/µl, 31 ng/µl, 15.2 ng/µl and 4.2 ng/µl, respectively.
The highest concentration was obtained using the QIAamp Cador Pathogen Mini Kit (Qiagen). In conclusion, the sensitivity of the Qiagen Kit is more suitable to be used for further investigation on the ASF genome.

Key words*: ASFV, DNA extraction, fluorometer.*

INTRODUCTION

ASF (Africane Swine Fever) is caused by African swine fever virus (ASFV) (DNA genome), belonging to *Asfivirus* genus, *Asfarviridae* family (Dixon et al., 2005). The transmission is made by direct contact with the infected animals (Sánchez-Vizcaíno J.M., 2016) or indirect contact by secondary source of infection like: feed, water, other materials, means of transport contaminated with secretions and excretions from contaminated animals or through contaminated clothing of pig farmers and veterinarians (Răpuntean & Răpuntean, 2014).
African swine fever (ASF) is a significant and intricate notifiable disease affecting both domestic and wild pigs. It is caused by the African swine fever virus, classified under the genus *Asfivirus* within the Asfarviridae family Uniquely, ASFV is the only known DNA virus transmitted by an arthropod vector (Pietschmann et al., 2016).
ASFV and Classical Swine Fever virus (CSFV) are unrelated pathogens. Pigs immunized against CSFV do not gain protection against ASFV, and the same is true the other way around. This lack of antigenic relationship necessitates separate and specific laboratory diagnostic methods for detecting ASF (Mânzat, 2005).
There is a continuing risk of the spread of ASF from these areas due to the uncontrolled movement of infected individuals, pork products, fomites and wild boar. Pig industry of each country is at risk. The low biosecurity of small and medium farms is particularly vulnerable. In the absence of a vaccine or effective treatment, the best strategy against ASF is to put in place an early detection strategy, together with an early response mechanism in case of outbreaks. In this context, awareness

raising and training of veterinary professionals and other front-line staff will be essential (Costard et al., 2009).

The pig food industry and their relationship with wild pigs has an important role preserving the virus in local and regional systems. By looking the pig market in the European countries, it can be noted the necessity to see this structure in the surveillance systems of pig healthy and in the outbreaks diseases (Guinat et al., 2016).

The potential for live pig trade networks between EU member countries to face and spread ASF, is increasing particularly when there is a long period between infection appearance and reporting of disease, has also been noticed.

The first outbreak of ASF in Romania has been confirmed by the Institute of Diagnosis and Animal Health in a farm located on the outskirts of Satu Mare in 2017. This outbreak was related to the large scale spread of the virus in the neighboring countries: Ukraine and Republic of Moldova (Ladoși et al., 2023). The losses suffered by Romanian's economy was made by a massive depopulation strategy.

ASFV is a large, enveloped virus with icosahedral morphology and a double-stranded DNA genome of 170 to 190 kbp (Fauquet & Fargette, 2005), containing heads with terminal inverted repeats and closed by hairpin loops (Salas & Andrés, 1999). The ASFV replication cycle is mainly cytoplasmic, but an early stage of replication in the nucleus has been described (Garcia-Beato et al., 1992; Rojo et al., 1999).

Accurate diagnosis is essential for the rapid control of ASF, particularly due to the rising prevalence and the ongoing emergence of variant strains. Commonly used methods for detecting ASFV include the hemadsorption test (HAD), conventional PCR, real-time PCR, antigen detection via the fluorescent antibody test, and enzyme-linked immunosorbent assay (ELISA) (Muzykina et al., 2024).

The disease confirmation is done in accordance with the procedures, sampling methods and criteria for the evaluation of laboratory test results described in the Operational Manual for Intervention in ASF Outbreaks emitted by Romania's National Sanitary and Food Veterinary and Food Safety Authority (NSVFSA) World Organization for Animal Health (2019). The diagnosis was based on epizootiology, clinical, pathological data, laboratory tests and bioassays. Currently, the confirmation of ASF is carried out by PCR. The viral DNA purification is done by lysing cells and solubilizing DNA, followed by removal of contaminated substances: proteins, RNA and other macromolecules (Agüero et al., 2004).

The field of molecular biology has developed very rapidly, since `80, when the polymerase chain reaction (PCR) was discovered in 1987 by biochemist Karry Mullis (Agüero et al., 2004). The PCR method can detect any pathogen that has nucleic acid in its structure: bacteria, viruses, protozoa, fungi, parasites because through this method a fragment of a DNA molecule is multiplied/amplified in numerous copies. The main advantages of this technique are the speed, the sensitivity and the undoubtful specificity (O'Donnell et al., 2017).

Using the PCR method, molecular biology changed the course once the enzymatic synthesis of target DNA was achieved *in vitro*. The PCR method is based on two fundamental properties of nucleic acids:

a) reversible thermal denaturation at 90°C of the two chains of the DNA molecule;

b) renaturation based on the complementarity of the nitrogenous bases: adenine - thymine in the case of DNA and adenine - uracil in the case of RNA, and guanine - cytosine.

The primary structure of nucleic acids is always oriented from the 5′end of the molecule towards the 3′ end so that a DNA double helix has two complementary anti-parallel mono-strands, one "sense" (5′→3′) and the other "antisense" (3′→5′) (Minchin & Lodge, 2019).

Nucleic acid extraction is a non-specific step, the whole nucleic acids being extracted from the biological sample. Extraction is done by cell lysis, inactivation of nucleases and removal of cell debris. The technique includes mechanical destruction (shredding, homogenization), chemical treatment or enzymatic digestion with proteinases.

Purification of nucleic acids differs depending on the nucleic acid extracted, DNA or RNA.

DNA extraction from the biological sample is carried out according to the type of the biological sample (serum, blood, animal tissue, virus, bacteria, etc.) and according to the desired DNA quality (genomic DNA, highly pure

DNA), without PCR - inhibitors (hemoglobin, plant, or synthetic phenols).
DNA can be collected from a range of biological samples by isolating it from the cellular mixture. This purified DNA can then be used in a variety of molecular biology analyses. DNA isolation can be performed by manual and automated methods using standardized commercial kits or the classical phenol-chloroform-isoamyl alcohol method can be used.
The successful amplification of the targeted DNA fragment needs a proper quantity of the genetic material. The aim of this study was to compare the yield and quality of DNA extracted using two dedicated commercial kits. It was compared the product obtained using the Pure LinkGenomicDNA Mini Kit (ThermoScientific) and QIAamp Cador Pathogen Mini Kit (Qiagen). The DNA has been quantified using Qubit DNA HS Assay Kit (Qubit 3.0 Fluorometer, Thermo Fisher Scientific), a highly selective over RNA and accurate tool for DNA at levels between 10 pg/µL and 100 ng/µL.

MATERIALS AND METHODS

We used in the present study molecular biology methods for the detection of ASF nucleic acid, standard ASF (synthetic standard) from the diagnostic test ASFV Monodose dtec-qPCR (GPS genetic PCR solution, Orihuela, Spain) to optimize the molecular biology methods. We made serial dilution, quantified. For 2 µl synthetic standard we add 8 µl ultrapure water for 10^{-2} and so on.
Most of the time extraction is done by using dedicated extraction kits from different manufacturers. The quality of the extracted nucleic acids is important in order to avoid PCR inhibitors and to have the best-preserved sequences.
DNA purification is done by lysing cells and solubilizing DNA, followed by the removal of contaminated substances: proteins, RNA and other macromolecules (Agüero et al., 2004).
The reference materials were used and protocols were followed. One kit extracts DNA and RNA and the other extracts only DNA, this comparison aimed to evaluate the quantity of extracted material from each kit.
The QIAamp Cador Pathogen Mini Kit is designed for the isolation of all types of nucleic acids out of many biological samples like blood, swabs, and organ tissues. The principle used is a rapid spin-column technique, where the contaminants and inhibitors are eliminated in order to extract the nucleic acids. The nucleic acid extract can be used in classical PCR or real-time PCR. The benefits of the QIAamp Cador Pathogen Mini Kit are: unique protocol for all types of nucleic acids (RNA, DNA), the isolation out of many biological samples - blood, swabs etc., the extract of isolated nucleic acids ready for analysis by real-time PCR or classical PCR.
QIAamp Cador Pathogen Mini Kit uses silica column principle in order to extract RNA and DNA from different type of samples. There are used optimized buffers and different enzymes in order to lyse the samples. The nucleic acid is bound to the silica membrane while the contaminants will pass through the membrane into the column. Then are used washing buffers in order to eliminate all kinds of PCR inhibitors like bivalent cations and proteins. Nucleic acids are then eluted with AVE buffer (according the manual of the kit: Samples are lysed under highly denaturing conditions at room temperature (15-25°C) with proteinase K and Buffer VXL to inactivate nucleases. Buffer ACB is then added to adjust the conditions for binding DNA and RNA. The lysate is transferred to a QIAamp Mini column, where nucleic acids attach to the silica membranes during centrifugation, while contaminants pass through. Two thorough wash steps remove any remaining impurities and enzyme inhibitors, and the nucleic acids are eluted in Buffer AVE). These nucleic acids can be used in all kinds of molecular biology methods. Compared to other kits that are using the silica membrane, in this kit can be used up to 200 µl of blood sample, without producing the clogging of the column filter.

Table 1. Nucleic acid extraction protocol (QIAamp Cador Pathogen Mini Kit)

Reagent	µl/sample	No. samples	Total
Proteinase K	20 µl		
Sample	200 µl		
Buffer VXL	100 µl		
Pipetting/vortex mixing			
Incubate for 15 minutes at room temperature			
Spin centrifuge for liquid collection			
Buffer ACB	350 µl		
Pipetting / vortex mixing			
Spin centrifuge for liquid collection			
Transfer of samples to purification column			
Centrifuge at 8000-10.000 rpm for 1 minute. Replacement manifold tube.			
Buffer AW 1	600 µl		
Centrifuge at 8.000 – 10.000 rpm for 1 minute.			
Eluted remove			
Buffer AW2	600 µl		
Centrifuge at 8.000 – 10.000 rpm for 1 minute. Replacement manifold tube.			
Eluted remove			
Centrifuge - maximum speed - 2 minutes. Introduction of columns into the collection tube.			
Buffer AVE	50 µl		
Incubation for 1 minute at room temperature.			
Centrifuge at maximum speed for 1 minute.			
Storage the elute at 1°C - 2°C until the amplification step.			

Table 2. Nucleic acid extraction protocol (PureLink® Genomic DNA Kit)

Reagent	µl/sample	No. samples	Total
200µl blood	20 µl		8 ml
Remove the eluate			
Resuspend in Pure Link Digestion Buffer	180 µl		1440 µl
Proteinase K	20 µl		160 µl
Incubation at 55 ºC 1h with vortex during incubation			
RNase A	20 µl		160 µl
Vortex and incubation at room temp.			
PureLink Genomic Lysis/Binding Buffer	200 µl		1600 µl
Ethanol 96º-100º	200 µl		1600 µl
Transfer the lysate 640 µl to the column - centrifugation at 10,000 x g 1 min.			
Remove the supernatant and transfer the column to a new tube			
Wash Buffer 1	500 µl		4000 µl
Centrifuge at 10,000 x g 1 min. Remove supernatant			
Wash Buffer 2	500 µl		4000 µl
Centrifuge at maximum speed 3 min. Remove supernatant			
Transfer the column to a 1.5 ml tube			
PureLink Genomic Elution Buffer 20 µl			
Centrifugation at maximum speed 1 min			
Remove the column and store the DNA at -80ºC			

The PureLink® Genomic DNA Kits (Thermo Scientific, Carlsbad CA USA) are designed for purification of all genomic DNA. The kit can be used for sample like: organ tissues, blood samples, buccal swabs, bacteria, yeast, and FFPE (formalin paraffin-embedded) tissues.
The principle of the kit is similar with other kits using silica membrane where DNA binds in the presence of chaotropic salts (guanidine-HCL).
DNA isolation was done in four steps. In the sample processing step, RNA digestion was performed to prevent contamination of the purified DNA sample with RNA. For a good lysis of tissues and cells it is used proteinase K. DNA is selectively bound to the silica membrane and subsequently washed in two steps to remove all contaminating cellular components. In the last elution step, DNA is released from the silica membrane.
The second extraction of the DNA was made with the PureLink® Genomic DNA Kit according with the manufacturing kit (Table 2).

The advantages of using the PureLink® Genomic DNA kit are: An efficient extraction process for genomic DNA from different sample types, like organ tissues, blood, and swabs, is designed to be quick and yield high-quality DNA, using of the proteinase K, without the mechanical lysis, low quantities of contaminants and performance of purifying DNA for using it in different protocols of molecular biology, not using organic solvents.
The Qubit 3.0 Fluorometer ThermoFisher Scientific system is used for quantifying the DNA concentration of the extracted samples. The Qubit 3.0 Fluorometer, is an equipment that can be used to quantify DNA, RNA, microRNA and proteins using highly sensitive and accurate fluorescence-based quantification assays.
A dedicated DNA quantification kit called Qubit DNA HS Assay Kit has been used to read DNA concentration. The assay is highly selective for DNA and will not determine free RNA, protein or nucleotides.

Common contaminants like salts, free nucleotides, solvents, detergents, or proteins are well tolerated by this kit. The test kit is designed for DNA concentrations in the sample between 250 pg/µl and 100 ng/µl. The kit contains dilution buffer and pre-diluted DNA standards. We dilute the reagent using the buffer provided, add the sample (any volume between 1 µl and 20 µl can be used) and read the concentration. The samples 5 in number, were reference material and read the DNA yield we extract it previously with the dedicated extraction kits.

The photometry reading protocol consists of:

1. We use the 0.5 ml tubes supplied with the kit in which the mixture will be prepared. The tubes will be for both the sample reading and the two standards required to read the DNA concentration.
2. The working solution is prepared in 1/200 dilution with the Qubit DNA HS reagent and the Qubit DNA HS buffer. It is necessary to use a sterile tube each time when it is made the working Qubit solution. It is forbidden to make the working solution in a glass tube. The final volume in each tube will be 200 µl. The standard needs 190 µl of the working solution and samples need any amount between 180- 199 µl. It is necessary to prepare a sufficient volume of working solution in order to read all standards and samples.
3. It is added 10 µl from each standard, mix for 2-3 seconds.
4. It is added each sample to test the concentration, then vortex for 2-3 seconds. The final volume in each tube should be 200 µl.
5. The tubes must be incubated at room temperature for 2 minutes. Then we read the tubes with the solution choosing the appropriate DNA reading program.

RESULTS AND DISCUSSIONS

After fluorometer reading of the African Swine Fever standard dilutions 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} of both the extracts obtained with the Pure Link Genomic DNA Mini Kit (Thermo Scientific) and the QIAamp Cador Pathogen Mini Kit (Qiagen), it can be said that the results were in accordance with the dilutions. So, the highest concentrations were registered at dilution 10^{-2} and the lowest at dilution 10^{-6}. For the Pure Link Genomic DNA Mini Kit (Thermo Scientific) the following results were recorded: 125 ng/µl at dilution 10^{-2}, 62 ng/µl at dilution 10^{-3}, 31 ng/µl for dilution 10^{-4}, 15.6 ng/µl at dilution 10^{-5} and 7.8 ng/µl at dilution 10^{-6}.

For the QIAamp Cador Pathogen Mini Kit (Qiagen) the following results were obtained: 134 ng/µl for dilution 10^{-2}, 71 ng/µl for dilution 10^{-3}, 43 ng/µl for dilution 10^{-4}, 21.2 ng/µl for dilution 10^{-5}, 10.3 ng/µl for dilution 10^{-6}.

The concentration of the negative control which is ultrapure water: Ultrapure™ DNase/RNase-Free Distilled Water Invitrogen™, in the extraction was also read for each of the two kits used and the results were negative - "out of range to low" - confirming that the extraction was validated with no contamination. According to the instrument's manual the Qubit will give you an out-range error (it will tell you if too low or too high) so you can dilute your samples to be within range, and will display the message "out of range to low". Preventing and controlling ASF requires the early detection of the disease through rapid field identification and accurate laboratory diagnosis. Essential components of this strategy include robust surveillance programs, adequate facilities and resources, and the preparedness of veterinary services. Handling samples potentially infected with the ASFV necessitates laboratories with appropriate biocontainment levels, which are often limited in ASF-free countries (Kosowska et al., 2021).

The aim of this study was to highlight the effectiveness of some of the most popular extraction kits. The performance of the extraction kit is essential in the case of this virus because depending on the age of the sample and the way it is collected, for example in wild boar carcasses, partially denatured, with a difficult to specify age, which do not provide you with blood or other tissues, the amount of DNA is small. This forces you to have a more efficient extraction that will generate maximum yield.

During the early stages of an outbreak, detecting the virus genome through various PCR assays is the most sensitive and specific method. As the disease progresses, serology becomes more valuable for diagnosis because specific antibodies appear later in the course of the disease (7-10 days).

With no effective vaccine or treatment available, the optimal strategy against ASF is to implement an early detection system paired with a rapid response mechanism for outbreaks. Therefore, it

is crucial to increase awareness and provide training for veterinary professionals and other frontline personnel (Beltran-Alcrudo et al., 2017). The ongoing development of commercial kits with improved sensitivity and specificity is evident. Despite having well-established diagnostic tests for ASF, the global epidemiological situation highlights the need for improved tools to quickly identify new cases and reduce response times. Recent advances in ASF diagnostics, focusing on new sample matrices relevant to Europe's epidemiological context. Rapid and reliable diagnosis depends on selecting appropriate samples and methods (Muzykina et al., 2024).

CONCLUSIONS

DNA concentrations obtained from extraction with the QIAamp Cador Pathogen Mini Kit are higher than those obtained from extraction with the Pure Link Genomic Mini Kit. The sensitivity of the QIAamp Cador Pathogen Mini Kit is higher compared to the Pure Link Mini Kit. This is also because the QIAamp Cador Pathogen Mini Kit extracts both RNA and DNA, and also pathogenic DNA, compared to Pure Link Mini Kit, which extracts total DNA.

Based on the obtained results, we can conclude that the QIAamp Cador Pathogen Mini Kit produced the highest DNA concentration, indicating that it has higher sensitivity compared to the Pure Link Genomic DNA Mini Kit. This suggests that the QIAamp kit may be more effective for applications that require the detection of lower quantities of DNA.

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