

MOL-PCR and xMAP technology – a novel approach to the detection of African swine fever virus DNA

Magdalena Krásná¹, Jakub Hrdý¹, Jana Prodělalová², Petra Vašíčková¹

Veterinary Research Institute, ¹Department of Microbiology and Antimicrobial Resistance, Research Group of Food and Environmental Virology, ²Department of Infectious Diseases and Preventive Medicine, Research Group of Molecular Epidemiology of Viral Infections, Brno, Czech Republic

Received January 19, 2022

Accepted February 1, 2022

Abstract

African swine fever (ASF) is highly contagious haemorrhagic viral disease of domestic pigs and wild boars. The causative agent can be transmitted by direct contact with infected animals or via a contaminated environment, fomites, feed, meat and products thereof. Soft ticks (genus *Ornithodoros*) are known reservoirs and transmission vectors of the virus. As the disease causes serious problems in many countries, rapid detection of the agent and early diagnosis could help in prevention of its spread. Therefore, a multiple-analyte profile (xMAP) technology based on multiple oligonucleotide ligation followed by polymerase chain reaction (MOL-PCR) was introduced and verified. A system targeting two independent loci of the virus genome was designed to increase the likelihood of different strains detection and an internal control was employed to verify the correct course of the analysis. The sensitivity was experimentally determined as 10 genomic copies of the virus in one µl of isolated DNA. The system was verified on samples originating from a recent ASF outbreak in the Czech Republic (six spleen) and the Czech market (eight liver and heart tissues) with real-time polymerase chain reaction used as a reference method. The results of both methods were in agreement, even in samples with a low concentration of the virus genome (9.45×10^1 genomic copies/µl of DNA). The system introduced represents an open method allowing the detection and semi-quantification of up to 50 targets/agents in one reaction. It can, therefore, be used for rapid one-step screening and as an effective tool for risk management.

Domestic pig, wild boar; diagnostic, molecular methods, multiplex analysis

African swine fever (ASF) is a highly contagious haemorrhagic disease affecting members of the family *Suidae*, including both domestic pigs and wild boars (*Sus scrofa* f. *domestica*, *Sus scrofa*). The causative agent of the disease, *African swine fever virus* (ASFV), is the only member of the family *Asfarviridae*, genus *Asfivirus* (<https://talk.ictvonline.org/taxonomy/>). It is an enveloped virus whose genome consists of double-stranded DNA. The size of the genome (170 to 193 kbp) depends on the individual isolates and reflects the number of open reading frames (150 to 167) that encode proteins involved in virus replication or the virus interaction with host cells. Twenty-four genotypes have been described so far on the basis of nucleotide sequence analysis of the p72 genome region. Despite this variability, there are only two genotypes found in Europe: genotype I exclusively in Sardinia and genotype II especially in the countries of Central and Eastern Europe (Gaudreault et al. 2020).

The ASFV shows extreme stability in the environment. The virus is highly resistant especially to higher temperatures and an acid pH; inactivation has been demonstrated at 56 °C after 70 min, at 60 °C after 20 min (Bellini et al. 2016) or after 22 h at pH 3.1 (Plowright and Parker 1967). The virus can survive for up to seven months at 8 °C, and the presence of a protein-rich environment such as blood, serum, and uncooked meat or products thereof increases its stability: from weeks to months in chilled meat, and even up to several years in frozen meat. This stability together with irresponsible human behaviour has a significant effect on the spread of the ASFV (Dee et al. 2018).

Address for correspondence:

Petra Vasicckova
Veterinary Research Institute
Hudecova 296/70, 621 00 Brno, Czech Republic

Phone: +420 777 786 756
E-mail: vasickova@vri.cz
<http://actavet.vfu.cz/>

In European countries, the ASFV epidemiology shows differences from that observed in the sub-Saharan Africa where the virus is endemic. In particular, the spread occurs by the natural migration of wild boars. The virus can be transmitted through direct contact between infected and healthy animals. Indirect transmission occurs via a contaminated environment, with the main source of contamination being ASFV-positive wild boar carcasses. Additionally, the human factor plays a crucial role in indirect transmission of the virus caused by contaminated meat products, fomites, transport of caught game from the affected zone and the feeding of domestic pigs with contaminated leftovers or feed (Bosch et al. 2017). This human factor played an important role in the introduction of the ASFV into the Czech Republic, as the most probable source of the infection of wild boar was contaminated meat or animal products (OIE 2019).

Although the disease is not transmissible to humans or other animal species, it has a large socio-economic impact as it fundamentally reduces the breeding of domestic pigs and the overall production of pork in affected localities and endangers food safety. Economic losses are caused mainly by the introduction of necessary measures (immediate depopulation of the entire farm) and restrictions in the national and international trade in animals, meat, and products thereof. The absence of treatment or a vaccine against the ASFV significantly limits the possibilities of controlling the spread of the infection and encourages the development of rapid and reliable diagnosis of the disease (EFSA 2015).

A number of protocols have been developed to detect the ASFV and the European Union Reference Laboratory for African Swine Fever (EURL-ASF) has defined several standard operating procedures (<https://asf-referencelab.info/asf/en/procedures-diagnosis/sops>). Methods based on nucleic acid detection represent fast, sensitive, reliable and affordable techniques. Real-time PCR (qPCR) is the most commonly used protocol due to its high sensitivity and specificity. Based on this knowledge, the aim of the present study was to optimise and validate an alternative rapid method, whose characteristics (sensitivity, specificity) are comparable to those of qPCR. A multiple-analyte profile (xMAP) technology based on multiple oligonucleotide ligation (MOL) followed by polymerase chain reaction (MOL-PCR) was introduced (Luminex Corporation, Austin, TX; Plate III, Fig. 1). This method enables the detection and semi-quantification of up to 50 targets/agents in one reaction and represents an open system for rapid one-step screening of different pathogen (e.g. multiplex detection of important porcine viruses; Hrdy et al. 2021). The system was designed to be used in routine diagnostics, for which reason its basic parameters were determined.

Materials and Methods

The viral strain and the origin of the samples used for system verification

The ASFV strain Ba71V obtained from the EURL-ASF was used for optimisation and diagnostic characterisation of the system. Subsequently, the newly introduced system was verified on DNA isolated from six spleen samples originating from wild boar cadavers found in a recent ASF outbreak in the Czech Republic (2017) and four pork liver and four heart tissue samples obtained from the Czech market. The DNA from strain Ba71V was purified using the NucliSENS miniMAG system (bioMérieux, Marcy-l'Etoile, France), while DNA from 50 mg of spleen, liver and heart tissue was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Both nucleic acid isolation procedures were performed according to the manufacturers' instructions. Aliquots of isolated DNA were stored at -70°C until analysis. Since ASFV should be handled with an appropriate level of bio-containment, manipulation with the potentially infectious virus and ASFV-positive samples has been performed under biosafety level-3 laboratory conditions.

Quantification of ASFV DNA by q-PCR

qPCR was introduced and optimised as a reference method to detect and quantify the ASFV genome according to SOP EURL-ASF standard operating procedures (https://asf-referencelab.info/asf/images/ficherosasf/PROTOCOLOS-EN/SOP-ASF-PCR-2_REV2021.pdf) with slight modifications, due to introduction of an internal amplification control (IAC; Vojkowska et al. 2015). The reaction mix contained 10 μl of LightCycler 480 Probes Master (Roche Molecular Diagnostics, Mannheim, Germany), 10 pmol of King-F, King-R, IAC F and

Table 1. List of MOLigos and universal primers.

Name	Target	Sequence (orientation from 5' to 3' end)	Length
p72_01_M1	ASFV p72	PHO-TTAAACAATTTCCGTAACCTGCTTCTCACTTCTTACTACCGCG	43 nt
p72_01_M2	ASFV p72	ACTCGTAGGGAATAAACCGTatgagtgttgattgaattgaacCGTATCCGATCACATTACCTATTA	68 nt
pCVR_04_M1	ASFV CVR	PHO - ATGTGTGCAGATACCAATGTAGATTCTACATTTCTTACTACCGCG	45 nt
pCVR_04_M2	ASFV CVR	ACTCGTAGGGAATAAACCGtttggtgttgattgaattgagatTGTAGACACCTGTGCAAGC	63 nt
IC_2_M1	internal control	PHO- ATTAGCACAAATGAATAATCATCTGCTCTCACTTCTTACTACCGCG	43 nt
IC_2_M2	internal control	ACTCGTAGGGAATAAACCGTatttggaagaagaagaattTATACACACGCAATCACCCAC	64 nt
uni FW	universal forward primer	CGCGGTAGTAAAGAGTGAGA	20 nt
uni REV	universal reverse primer	BODIPY-TMRX - ACTCGTAGGGAATAAACCGT	20 nt

Target-hybridising complementary sequences are underlined, xTAG sequences (specific binding to microspheres) are differentiated by lowercase letters
 ASFV - African swine fever virus; p72 - conserved part of gene p72 coding the major capsid protein; CVR - central variable region of gene B602L;
 PHO - phosphorylation; nt - nucleotide; BODIPY-TMRX - fluorescence label

IAC R primers, 4 pmol of King-probe, 2 pmol of IAC probe, 10^3 copies of IAC DNA, 0.2 U of Uracil DNA Glycosylase (Roche) and 5 μ l of template DNA. The optimised duplex qPCR was run in a total volume of 20 μ l. Amplification and fluorescence detection was performed on a LightCycler 480 Instrument (Roche) under the following conditions: initial denaturation at 95 °C for 4 min and 45 cycles of 95 °C for 10 s, 60 °C for 30 s and 72 °C for 10 s. The DNA standards (plasmid; concentration 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 1×10^2 and 1×10^1 copies/ μ l) were used to construct a gradient that served for quantitation of ASFV (genomic equivalents; GE) and as a positive control. Analysis of the results was carried out using the “Fit point analysis” option of the LightCycler 480 Software release 1.5.0 (version 1.5.0.39).

Internal control of the analysis

An internal control is used in the detection system to verify the correct course of the analysis; to distinguish between true and false negative results caused by the inhibition of MOL-PCR. Therefore, 10^3 copies of plasmid DNA containing unique mitochondrial sequences of two extinct organisms, the thylacine (*Thylacinus cynocephalus*, Acc. No. FJ515781.1) and the giant moa (*Dinornis struthoides*, Acc. No. AY326187.1), were used for artificial contamination of each sample of isolated DNA (Jelinkova et al. 2021).

MOLigos and primer design

MOLigos targeting two independent loci of ASFV genome were designed to increase the likelihood of detection of different ASFV strains (Table 1). The first set of MOLigos targets the highly conserved part of gene p72 coding the major capsid protein of ASFV, while the second set is specific for the central variable region (CVR) located in gene B602L.

In order to meet the optimal condition of the designed MOLigos in accordance with Reslova et al. (2019), parameters (melting temperature, GC content, hairpin and dimer formation) were calculated using the OligoAnalyser Tool (Integrated DNA Technologies, Coralville, IA). Subsequently, the designed MOLigos were tested *in silico* against available records in BLAST program of the National Centre for Biotechnology Information to approve their homology and specificity for all ASFV genotypes with special attention paid to genotype II.

Both universal primers used for subsequent singleplex PCR were adopted from the study by Thierry et al. (2013) (Table 1).

MOLigos and the universal forward primer were provided by Generi-Biotech (Hradec Kralove, Czech Republic). The BODIPY-TMRX labelled universal reverse primer was obtained from Eurofins Genomics (Ebersberg, Germany).

MOL-PCR

The ligation reaction (MOL) was physically separated from the following singleplex PCR to avoid interference between reaction mixtures. Only the pair for one target was tested independently and subsequently, all functional pairs were gradually mixed together into one ligation reaction to reveal possible interactions between MOLigos targeting the ASFV genome. The optimisation or redesign of individual targets was conducted if an undesirable interaction was observed between MOLigos. Optimised ligation reaction conditions were described by Hrdy et al. (2021). Briefly, the ligation mix contained 125 pmol of each MOLigo, 2.5 μ l of $10 \times$ Hifi *Taq* DNA ligase reaction buffer, 0.5 μ l of Hifi DNA ligase (both New England Biolabs, Ipswich, Massachusetts, USA) and 2.5 μ l of template DNA. The reaction was run in a total volume

of 25 µl under the following conditions: initial denaturation at 95 °C for 10 min, 20 cycles of 95 °C for 30 s and 59 °C for 1 min. Samples were stored at 4 °C until further processing.

The final protocol of the singleplex PCR was performed using 12 µl of EliZyme HS Robust MIX (Elisabeth Pharmacon, Brno, Czech Republic) with 1.5 pmol of universal forward primer, 6 pmol of BODIPY-TMRX labelled universal reverse primer and 6 µl of ligation product in a total volume of 24 µl (Hrdy et al. 2021). The thermal cycling protocol for singleplex PCR was started with incubation at 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 15 s. The samples were stored at 4 °C until further processing.

Magnetic microspheres coating and hybridisation

Commercially available magnetic microspheres (Luminex Corporation) were used for detection by xMAP technology (MAGPIX instrument; Luminex Corporation). To achieve the best signal-to-noise ratio the microspheres were coupled with anti-TAG sequences by an in-house carbodiimide method according to an adapted Bio-Plex BEAD Coupling protocol (Reslova et al. 2019).

Ten µl of the singleplex PCR product was mixed with 5 µl of the microspheres solution (2,500 microspheres per reaction) and subjected to a hybridisation protocol: 96 °C for 90 s followed by 37 °C for 30 min. Thereafter, 45 µl of analysis buffer containing 10 nmol/µl of Tris-Cl (pH 8.0; Sigma Aldrich, St. Louis, PA), 0.1 nmol/µl of EDTA (VWR International, Randor, Germany), 90 nmol/µl of NaCl (Carl Roth, Karlsruhe, Germany) and 0.02% Tween 20 (Alpha Diagnostics, San Antonio, TX) were added to the hybridisation mix to increase the reaction volume to 60 µl.

MAGPIX analysis

A MAGPIX instrument utilising xPONENT software version 4.2 (both Luminex Corporation) was used for the analysis of the fluorescence signal produced by singleplex PCR products hybridised to microspheres. Median fluorescence intensity (MFI) was calculated from the analysis of at least 50 microspheres of each target per sample and the results were evaluated. The samples were considered positive, when the MFI of the samples was higher than 100 after subtracting the value of the negative control fluorescence signal. Semi-quantitative detection can be performed since the MFI decreases with a smaller number of targets detected.

The sensitivity and specificity of the system

The sensitivity (i.e. limit of detection; LOD) of the system was determined according to the general principles of qPCR evaluation. The above mentioned qPCR was used to quantify the GE of ASFV DNA isolated from strain Ba71V. Subsequently, the LOD of the system was tested on 10-fold serially diluted DNA ranging from 1×10^4 to 1×10^1 GE/µl. Dilution was performed in Ultra-PCR water (Top-Bio, Vestec, Czech Republic) and concentration series were tested in eight repetitions. The LOD was specified as the lowest concentration of ASFV GE that can be detected with 100% probability.

To approve the specificity of the system, the designed MOLigos were tested primarily *in silico* against available records BLAST program of the National Centre for Biotechnology Information. All MOLigos were controlled for possible interaction with each other, i.e. monoplex reactions were run in comparison with multiplex reactions. Further verification of the specificity was performed using genetic material of agents that may be present in tested matrices and may interfere with the system: Porcine reproductive and respiratory syndrome virus (PRRSV) CAPM V-502 (strain Strachotice) and CAPM V-536 (strain Mn 1b), Suid herpesvirus 1 (SuHV1) CAPM V-166 (strain Aujeszky), Swine influenza virus (SIV) CAPM V-1 (strain Merotin 1957), *Staphylococcus aureus* CAPM 5736 (strain M 85/67), *Yersinia enterocolitica* CAPM 6154 (strain 49) and *Toxoplasma gondii*. Viral and bacterial strains were provided by the Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute (CAPM, Czech Republic). Oocysts of *Toxoplasma gondii* were obtained from the University of Veterinary Sciences (Brno, Czech Republic). Genetic material of these agents was extracted using the NucliSENS miniMAG system (bioMérieux) according to the manufacturer's instructions.

Results

The sensitivity of the system

The sensitivity (LOD) was determined to be 25 and 2,500 ASFV GE of p72 and CVR targets per MOL-PCR reaction (i.e. 10 and 1,000 GE/µl of isolated DNA), respectively. These values were achieved in all eight repetitions of 10-fold serially diluted DNA originating from ASFV strain Ba71V (Fig. 2). Subsequently, the sensitivity of the system was confirmed by analysis of biological samples when qPCR was used as a reference method (Table 2).

Decreases in MFI values were observed in accordance with a falling number of both targets detected (Fig. 2).

Table 2. Comparison of detection system introduced and real-time polymerase chain reaction (qPCR) results of biological samples originating from recent African swine fever outbreak in the Czech Republic (spleen) and from the Czech market (liver and heart tissues).

Sample	MOL-PCR combined with xMAP technology		qPCR (GE/ μ l)*
	Target p72	Target CVR	
spleen 1	positive	positive	9.45×10^1
spleen 2	positive	positive	4.48×10^4
spleen 3	positive	positive	2.27×10^3
spleen 4	positive	positive	2.43×10^3
spleen 5	positive	positive	1.89×10^3
spleen 6	positive	positive	9.49×10^4
heart 1	negative	negative	0
heart 2	negative	negative	0
heart 3	negative	negative	0
heart 4	negative	negative	0
liver 1	negative	negative	0
liver 2	negative	negative	0
liver 3	negative	negative	0
liver 4	negative	negative	0

* African swine fever virus genomic equivalents (GE) in 1 μ l of isolated DNA

MOL-PCR - multiple oligonucleotide ligation followed by polymerase chain reaction; xMAP - a multiple-analyte profile; p72 - gene p72 coding the major capsid protein of the African swine fever virus; CVR - central variable region located on gene B602L of the African swine fever virus genome

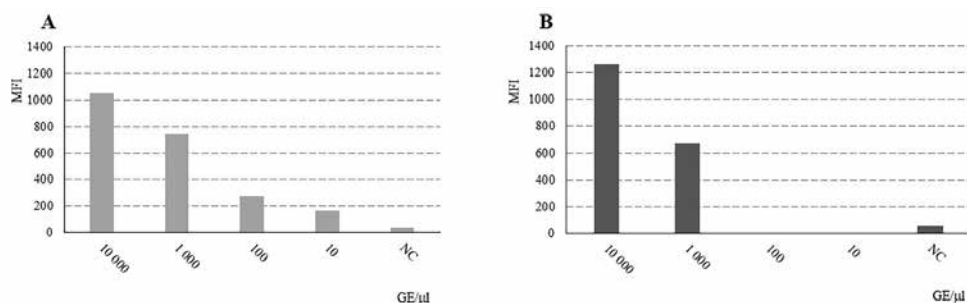


Fig. 2. Evaluation of the sensitivity (limit of detection; LOD) of the system by eight independent reactions. A) MOLigos targeting conserved part of gene p72 coding the major capsid protein, B) MOLigos targeting central variable region located on gene B602L (CVR) of the African swine fever virus genome.

GE/ μ l - genomic equivalents per μ l of isolated DNA; MFI - median florescence intensity; NC - negative control

The specificity of the system

In silico analysis of designed MOLigos using the BLAST program revealed homology to the reference sequences of ASFV available in the National Centre for Biotechnology Information database and no identity to other presented sequences within the database was observed here. Subsequently, it was determined that 10^3 GE of internal control plasmid DNA did not interfere with the MOL-PCR reactions even at low concentrations such as 25 ASFV GE per reaction. No interaction between MOLigos targeting the ASFV genome was found (Fig. 3). No false positive identification was observed when testing the genetic material originating from the viruses, bacteria, and parasites mentioned above or from real biological samples.

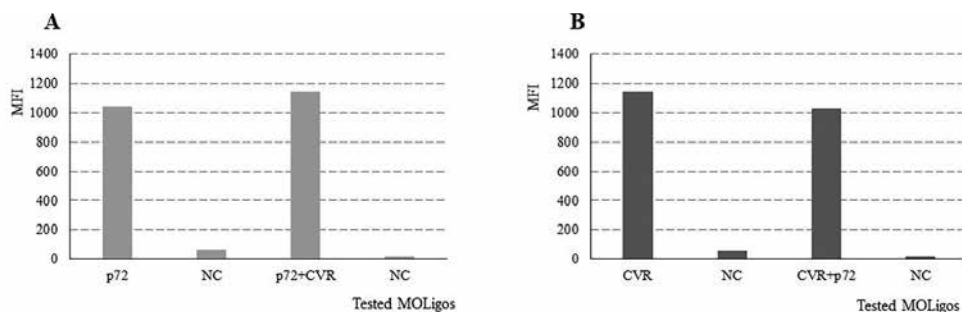


Fig. 3. Evaluation of the specificity (interaction between MOLigos) of the system. A) MOLigos targeting conserved part of gene p72 coding the major capsid protein, B) MOLigos targeting central variable region of gene B602L (CVR) of African swine fever virus genome.

p72 - MOLigos targeting conserved part of gene p72 coding the major capsid protein of the African swine fever virus

CVR - MOLigos targeting central variable region located on gene B602L of the African swine fever virus genome
MFI - median fluorescence intensity; NC - negative control

Verification of the system by qPCR

The system introduced was verified on DNA isolated from real biological samples: six spleen samples originating from a recent ASF outbreak in the Czech Republic and four liver and four heart tissue samples purchased on the Czech market. Each DNA sample was analysed in duplicates by the present system as well as by the qPCR assay. The verification of MOL-PCR combined with xMAP technology using real ASFV-positive samples demonstrated the method's suitability for wider diagnostic use. The optimised system (p72 and CVR targets) confirmed the results determined by qPCR (Table 2), even in the sample where ASFV DNA was present at a low concentration (9.45×10^1 GE/ul of isolated DNA; Fig. 4).

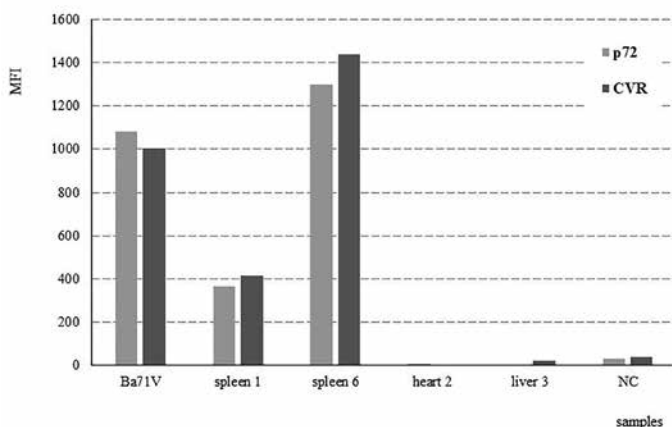


Fig. 4. Results of the verification of the system using spleen samples originating from a recent African swine fever outbreak in the Czech Republic and liver and heart tissue samples purchased on the Czech market. Names of samples correspond to samples shown in Table 2.

Ba71V - African swine fever virus strain Ba71V used as a positive control; p72 - MOLigos targeting conserved part of gene p72 coding the major capsid protein of the African swine fever virus; CVR - MOLigos targeting central variable region located on gene B602L of the African swine fever virus genome; MFI - median fluorescence intensity; NC - negative control

Discussion

The current ASF outbreak (not only in Europe) was preceded by a period of more than 10 years of the virus spreading from Georgia to the west. First European countries were affected in 2014 (Estonia, Lithuania, Latvia and Poland) and the occurrence of the disease was unexpectedly described in wild boars originating from the Zlin District in the Czech Republic in June 2017. Although the situation was successfully managed in this country, the current occurrence of the ASF close to the Polish-Czech and German-Czech borders represents an increased risk of its re-introduction (OIE 2022). The absence of a treatment or vaccine against the causative agents significantly limits the possibilities of controlling the spread of this disease (EFSA 2015). It is, therefore, necessary to address the possibilities of timely and reliable virus detection. The costs of diagnostics are not negligible either.

As has been reported, the amplification of multiple targets in the viral genome can increase the sensitivity of detection (Vasickova et al. 2012). Therefore, MOL-PCR combined with xMAP technology targeting two independent loci of the ASFV genome was introduced. The LOD of the presented system, which was specified as the lowest concentration of ASFV GE that can be detected with 100% probability, guaranteed detection of at least 25 copies of the p72 gene and 2,500 copies of CVR loci in one reaction, i.e. 10 copies of p72 and 1,000 copies of CVR regions per 1 µl of isolated total DNA. This definition does not exclude detection of lower numbers of ASFV GE, though the probability of successful detection in repeated experiments would be lower than 100%. This was confirmed during the verification of the system on spleen samples originating from the recent outbreak, in which it was shown that MOLigos targeting CVR are able to detect a smaller number (9.45×10^1 GE/µl) of ASFV genomes than the actual LOD determined on laboratory ASFV strain Ba71V (Figs 2 and 4, Table 2). Observed decreases of MFI values associated with a falling number of both detected targets confirm the possibility applying the present system in semi-quantitative analysis of tested samples (Fig. 2).

The introduced system was verified on biological samples originating from the recent ASF outbreak and from the Czech market. This verification was performed on a limited number of samples: six ASFV positive (spleen) and eight negative (liver, heart) samples. However, as long as the general rules of valid detection by molecular methods are maintained, the system can be used to analyse DNA of various origins. The first rule is a critical step for proper DNA isolation. The second lies in the correct course of the analysis, i.e. in distinguishing between true and false negative results caused by inhibition of the reactions. Since the aim of the present study was to introduce a novel approach for the detection of ASFV DNA, the system developed deals only with the control of the correct course of MOL-PCR and detection of fluorescence by xMAP technology. Therefore, plasmid DNA containing unique mitochondrial sequences of two extinct organisms was used for artificial contamination of each sample of isolated DNA (Jelinkova et al. 2021). However, this plasmid DNA can be used in the same way prior to isolation of DNA to verify the extraction procedure for any kind of sample (e.g. animal tissues and products thereof, environmental samples such as water or soil, animal feed).

As MOL-PCR combined with the xMAP technology represents an open system and allows detection and semi-quantification of up to 50 targets/agents in one reaction, it can be used in rapid one-step screening and as an effective tool for risk management (Hrdy et al. 2021). Therefore, further studies can be focused on the optimization and verification of the system for multiplex detection of important porcine viruses such as ASFV, classical swine fever virus (CSFV), PRRSV, SuHVI and SIV in one reaction.

Acknowledgements

The work was supported by grants QK1920113 and MZE-RO0518 of the Ministry of Agriculture of the Czech Republic. The authors thank the National Reference Laboratory for classical swine fever and African swine fever, State Veterinary Institute in Jihlava, the Czech Republic for providing biological samples originating from a recent African swine fever outbreak.

References

- Bellini S, Rutili D, Guberti V 2016: Preventive measures aimed at minimizing the risk of African swine fever virus spread in pig farming systems. *Acta Vet Scand* **58**: 82
- Bosch J, Rodríguez A, Iglesias I, Muñoz MJ, Jurado C, Sánchez-Vizcaíno JM, de la Torre A 2017: Update on the risk of introduction of African swine fever by wild boar into disease-free European union countries. *Transbound Emerg Dis* **64**: 1424-1432
- Dee SA, Bauermann FV, Niederwerder MC, Singrey A, Clement T, de Lima M, Long C, Patterson G, Sheahan MA, Stoian AMM, Petrovan V, Jones CK, De Jong J, Ji J, Spronk GD, Minion L, Christopher-Hennings J, Zimmerman JJ, Rowland RRR, Nelson E, Sundberg P, Diel DG 2018: Survival of viral pathogens in animal feed ingredients under transboundary shipping models. *Plos One* **13**: e0194509
- EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare) 2015: Scientific opinion on African swine fever. *EFSA Journal* **13**: 4163
- Gaudreault NN, Madden DW, Wilson WC, Trujillo JD, Richt JA 2020: African swine fever virus: an emerging DNA arbovirus. *Front Vet Sci* **7**: 215
- Hrdy J, Vasickova P, Nesvadbova M, Novotny J, Mati T, Kralik P 2021: MOL-PCR and xMAP Technology A multiplex system for fast detection of food- and waterborne viruses. *J Mol Diagn* **23**: 765-776
- Jelinkova P, Hrdy J, Markova J, Dresler J, Pajer P, Pavlis O, Branich P, Borilova G, Reichelova M, Babak V, Reslova N, Kralik P 2021: Development and inter-laboratory validation of diagnostics panel for detection of biothreat bacteria based on MOL-PCR. *Microorganisms* **9**: 38
- OIE 2019: Self-declaration of the recovery of freedom from African swine fever in all suids by the Czech Republic. Available at https://www.oie.int/fileadmin/Home/eng/Animal_Health_in_the_World/docs/pdf/Self-declarations/2019_05_CzechRep_ASF_ANG.pdf
- OIE 2022: African swine fever (ASF) – situation report 3. Available at <https://www.oie.int/app/uploads/2022/01/asf-situation-report-3.pdf>
- Plowright W, Parker J 1967: Stability of African swine fever virus with particular reference to heat and pH inactivation. *Archiv für die gesamte Virusforschung* **21**: 383-402
- Reslova N, Huvarova V, Hrdy J, Kasny M, Kralik P 2019: A novel perspective on MOL-PCR optimization and MAGPIX analysis of in-house multiplex foodborne pathogens detection assay. *Sci Rep* **9**: 2719
- Thierry S, Hamidjaja RA, Girault G, Lofstrom C, Ruuls R, Sylviane D 2013: A multiplex bead-based suspension array assay for interrogation of phylogenetically informative single nucleotide polymorphisms for *Bacillus anthracis*. *J Microbiol Methods* **95**: 357-365
- Vasickova P, Kralik P, Slana I, Pavlik I 2012: Optimisation of a triplex real time RT-PCR for detection of hepatitis E virus RNA and validation on biological samples. *J Virol Methods* **180**: 38-42
- Vojtkovska H, Kubikova I, Kralik P 2015: Evaluation of DNA extraction methods for PCR-based detection of *Listeria monocytogenes* from vegetables. *Lett Appl Microbiol* **60**: 265-272

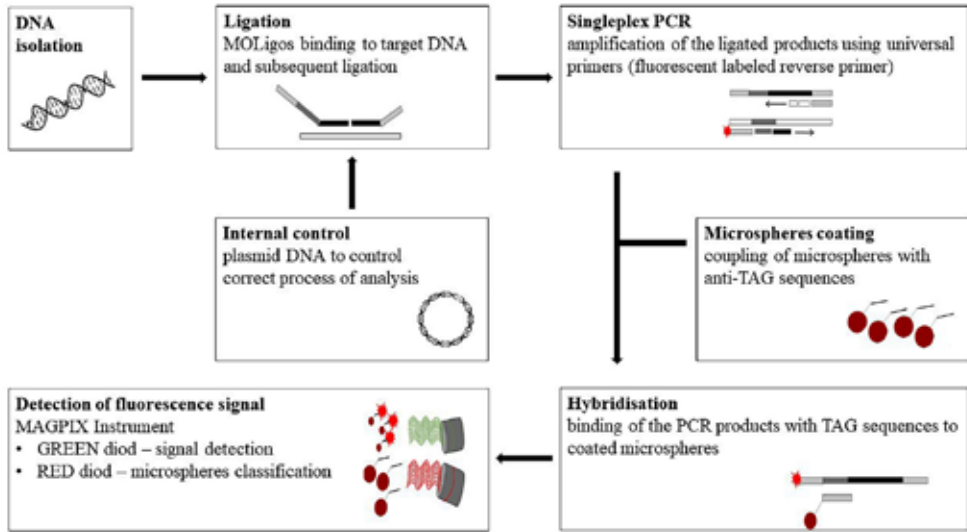


Fig. 1. Flow chart of the system introduced. The system is based on multiple oligonucleotide ligation (MOL) followed by polymerase chain reaction (MOL-PCR). A multiple-analyte profile (xMAP; instrument MAGPIX) technology is used for detection of possible presence of the African swine fever virus DNA.