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DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY BASED ON THE *C962R* GENE FOR AFRICAN SWINE FEVER VIRUS DETECTION

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Aim. The aim of this study was to develop a loop-mediated isothermal amplification (LAMP) assay for African swine fever virus (ASFV) detection. **Methods**. Primer design was performed using publicly available full genome sequences of ASFV. A panel of heterologous DNA samples and reference ASFV DNA samples were used for the assay specificity testing. The limit of detection (LOD) was assessed using purified and quantified serial dilution of the amplified target sequence. LAMP product detection was performed via gel-electrophoresis and via ethidium bromide fluorescence under UV after adding the ethidium bromide directly to the tube with the LAMP product. **Results**. Three primer sets amplifying different regions of ASFV gene *C962R* were developed, of which the set № 2 providing the most intense product synthesis with the most vivid and clear pattern was selected for further studies. The optimal concentration of reaction mix components for the most effective primer set was established. In the final protocol the LAMP reaction was carried out at 60 °C for 40 min. The limit of detection (LOD) of the assay was 50 copies of the target sequence per reaction. In a preliminary testing the assay proved specific, using 10 reference and 4 heterologous viral and two bacterial DNA samples. Our LAMP assay detected ASFV genotypes I and II that are currently spread in Europe, Asia, and the Pacific and IX, occurring in Africa. **Conclusion**. A LAMP assay was developed based on the *C962R* gene that proved in preliminary validation to be specific and sensitive and was able to detect down to 50 copies per reaction of purified target gene within 40 minutes. Classical gel electrophoresis and direct staining using ethidium bromide were used for product visualisation in this study. Colorimetric approaches or the use of lateral flow devices in the visualisation step could make the assay less equipment dependent. Further validation of the assay, determining analytical specificity, selectivity and reproducibility performance characteristics also using clinical samples under field conditions and inclusion of an internal control would possibly enable its use as a test of choice at point-of-care and at low resource laboratories.

Key words: African swine fever virus, isothermal amplification, molecular diagnostics, rapid test, DNA.

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INTRODUCTION

African swine fever (ASF) is a contagious viral disease of various species of domestic and wild Suidae (suids). An acute form of the disease results in up to a 100 % mortality rate (Beltrán-Alcrudo D et al, 2017). ASF is a notifiable disease, since it causes economic loses and affects natural populations of wild boars. The causative agent, African swine fever virus (ASFV), a large, enveloped double-stranded DNA virus, is the only member of genus *Asfivirus* from *Asfarviridae* family and one of the most complex livestock viruses (Alonso C et al, 2012; Balasuriya U et al, 2017).

ASF is widely spread in Africa, Eastern Europe, Caucasus region, and Asia. The disease is endemic in most countries of sub-Saharan Africa. In Western Europe, ASF has been eradicated after numerous outbreaks in 1960-1990 in Italy, Spain, France, Malta, Belgium, and the Netherlands. Those outbreaks were caused by ASFV of genotype I. The only European territory where ASF became endemic after the outbreaks in the 20th century is the island of Sardinia (Italy) (Beltrán-Alcrudo D et al, 2017; Cwynar P et al, 2019; Dixon L

et al, 2019). In 2007, ASFV was detected in Georgia for the first time. The virus of genotype II from Africa was probably introduced to this new region via sea transport. Since 2007, ASFV has subsequently rapidly spread into Eastern Europe and W. Asia, including Azerbaijan, Armenia, Russia, Belarus, Ukraine, Poland, Estonia, Lithuania, Latvia, Moldova, Chechia, Romania, Hungary, Bulgaria, Slovakia, Serbia, North Macedonia, Italy, where it caused numerous outbreaks among domestic pigs and wild boars (APHA, 2022; Beltrán-Alcrudo D et al, 2017; Cwynar P et al, 2019; Gaudreault NN et al, 2020; EFSA, 2021; OIE-WAHIS, 2022). In August 2018, ASF was for the first time confirmed in China, which is the world biggest pork producer. Until now, the disease has spread in Asia to Vietnam, Cambodia, Indonesia, Mongolia, the Republic of Korea, the Democratic People's Republic of Korea, Myanmar, the Philippines, Laos, Timor-Leste, Papua New Guinea, India, Malaysia, Bhutan, Thailand (OIE, 2019a, FAO, 2022).

Different methods can be used for ASFV laboratory detection, depending on the clinical sample type and condition, the purpose of the test and laboratory equipment available. Among methods such as virus isolation, hemadsorption test, immunofluorescent test, and ELISA for antigen detection, PCR remains the most widely used gold standard for the first-line laboratory diagnosis (Lvov DK et al, 2015; Beltrán-Alcrudo D et al, 2017; OIE, 2019b). Isothermal amplification assays can be a rapid alternative to PCR. Loop-mediated isothermal amplification (LAMP) is an assay developed by Notomi T et al. in 2000. The LAMP reaction can be carried out under isothermal conditions (60–65 °), as opposed to PCR, due to the use of the strand-displacing *Bst* DNA polymerase which obviates the need for the DNA denaturation step at 95 °C. The recognition of six target regions by three different primer pairs makes LAMP highly specific, whereas the use of *Bst* polymerase ensures the rapidness of the method. Since the reaction is carried out at constant temperature, LAMP requires very simple equipment.

Several LAMP assays for ASFV detection have been developed to date. Genes coding structural proteins p72 (Dokphut A et al, 2021, Tran DH et al, 2020, Zhu YS et al., 2020), p22 (Elsukova AA et al, 2019), p10 (Wang D et al, 2020), uncharacterised protein K205R (Wu X et al, 2016), C717R (Zhu YS et al, 2020), enzymes topoisomerase II (James HE et al, 2010), helicase (Zhu YS et al., 2020) have been used as target sequences. However, there is a need for other molecular markers for molecular diagnostics. The use of the *KP177R* gene coding protein p22 as a molecular marker for ASFV detection (Elsukova AA et al, 2019) is an example, proving this necessity. Gene *KP177R* is located closely to the 5′-end of the ASFV genome and its homologous sequence *L10L* is located at the 3′-end of the viral genome. However, both sequences are located in highly variable regions, where insertions and deletions occur quite often (Dixon LK et al, 2012). Thus, a wild ASFV strain with 5′-end deletion, detected in Estonia, carries mutations affecting both sequences. *KP177R* is a part of deleted 14 kbp fragment and has been completely lost (Zani L et al, 2018). More to the point, p22 appears to be not essential for virus virulence and replication (Vuono EA, 2021) and in case of the gene loss, the virus could possibly spread and cause the disease, being able to avoid detection by *KP177R* LAMP assay. Since Ukraine is the territory where ASFV is well-established, the assays used for the disease diagnostics in the country should be aimed at highly conservative genes located in stable genome parts. Although the rest of the target sequences are located in the central conservative region of ASFV genome, plentiful mutations are described in this genome part. The genes, coding proteins p10 and K205R, are located next to each other (Dixon LK et al, 2012) and the mutations in this region might mean a loss or change of target sequences for two assays (Wu X et al, 2016; Wang D et al, 2020). Recombination events are a part of ASFV evolutionary strategy and in case of indel occurrence a target sequence of an assay can be changed (Zhu Zh et al, 2019). Therefore, the development of assays targeted at other molecular markers of ASFV remains important.

Thus, the aim of our work was to develop a LAMP assay for ASFV detection using the *C962R* gene, which is involved in DNA replication and located in central conservative region of ASFV genome leading possibly to a stability of the target sequence in different virus isolates to be possible used for express diagnostics in laboratories with limited resources.

MATERIALS AND METHODS

Primer design. Twenty-six publicly available in GenBank full genome sequences of ASFV were used for the primer design (https://www.ncbi.nlm.nih.gov/genbank). Multiple sequence alignment and conservative region search were performed using BioEdit, v. 7.0.5.3 software. LAMP primer sets were designed using the online tool PrimerExplorer V5 (http://primerexplorer.jp). Three primer sets amplifying different regions of

the *C962R* gene, which encodes putative DNA primase of ASFV, were designed, for two of them the loop primers were generated (Table 1)

Reference samples and DNA templates. 170 bp long amplicons generated with F3/B3 primers were used as a positive template during the LAMP protocol optimization. PCR for the amplicon generation was performed as follows: 2X Hot Start Green PCR MasterMix – 12.5 µl, F3 primer (5 pmol/µl) – 1 µl, B3 primer (5 pmol/µl) – 1 µl, PCR grade water – 5.5 µl, DNA template (ASFV DNA) – 5 µl. The amplification protocol: 5 min denaturation at 95 °C, 40 cycles of amplification at 95 °C – 15 s, 60 °C– 30 s, 72 °C– 30 s, 5 min elongation at 72 °C. The amplicons were cleaned using Monarch® PCR & DNA Cleanup Kit prior to using them as a template. The amplicons were quantified using micro-volume spectrophotometer and used for the preparation of serial 10-fold dilutions containing from 10^{10} to 1 copies of the gene of interest per 1 µl. The panel of serial dilution was used for the LAMP limit of detection (LOD)determination.

DNA sample extracted from swine lymph nodes was used as a negative template control during the LAMP protocol optimization.

A panel of reference DNA samples (n = 10) isolated from different sources was used for the LAMP performance assessment (Table 2). The reference samples were kindly provided by PD Dr. Sandra Blome (Friedrich Loeffler Institute, Institute of Diagnostic Virology, Germany).

Additionally, nucleic acid samples of viruses and bacteria, causing symptoms near or similar to ASF, (n = 6) were used for the LAMP specificity assessment. The panel consisted of purified DNA (cDNA in case of RNA viruses) samples of classical swine fever virus (CSFV), Aujeszky's disease virus (ADV) (kindly provided by PD Dr. S. Blome), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV 2), *Erysipelothrix rhusiopathiae* and *Pasteurella multocida* (kindly provided by PhD in veterinary sciences, A. I. Buzun, National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine", Ukraine). RT-

Table 1. Primers developed for the detection of ASFV using *C962R* gene-based LAMP assay

Primer name	Primer type	Length (bp)	Genome position (according to isolate ASFV Georgia 2007/1*	Sequence (5′–3′)
1ASFV_F3	Forward outer	18	90501–90518	CTGAAGTGGTCCAGGGCA
1ASFV_B3	Reverse outer	19	90710–90728	GTTGGTAGGGCTTGTGGTT
1ASFV_FIP	Forward inner (F1c+F2)	41	90584–90604, 90529–90548	GGCATCGTGCTGTAGGGATCC-CCATGTGCTCATTCCTGGTC
1ASFV_BIP	Reverse inner (B1c+B2)	38	90627–90646, 90687–0704	AGCAGGGCGTTGCAAATCCT-TGGAGGAGCCGTAGAGGA
1ASFV_LF	Forward loop	20	90549–90568	GGTAGAAGCCGCCAGCTTGA
1ASFV_LB	Reverse loop	20	90647–90666	GAGTCCTGTCTGGACCCCCA
2ASFV_F3	Forward outer	20	92116–92135	GCGGGGATATGGGTACTTTG
2ASFV_B3	Reverse outer	19	92303–92321	GCAGTCTTCTCCATGTGCC
2ASFV_FIP	Forward inner (F1c+F2)	42	92178–92199, 92137–92156	TCGCCCGGATTTACCATTTCCT-GGAAACCAACAAAAGCGAGG
2ASFV_BIP	Reverse inner (B1c+B2)	41	92238–92258, 92280–92299	TTCAGATGACGGCCACCATGG-GTCCGTCGTGTCAATGATGA
2ASFV_LF	Forward loop	20	92158–92157	TCAGCCGCGACGTATTTAGA
2ASFV_LB	Reverse loop	20	92260–92279	CGCCGCGTCCAACTATAACT
3ASFV_F3	Forward outer	18	91339–91355	GGGCAACAAGTTTGTGGT
3ASFV_B3	Reverse outer	19	91515–91533	TGGTATTTGATGTGCTCCG
3ASFV_FIP	Forward inner (F1c+F2)	38	91407–91426, 91367–91382	TCCCTGATTCATTGGCTGGC-ACTCAAACGGGAAGTACG
3ASFV_BIP	Reverse inner (B1c+B2)	38	91435–91454, 91495–91512	GAAGTGGCGCAAGGAGGTAA-ATTCGGTCCATCACCCTT

* Isolate ASFV Georgia 2007/1 genome assembly, complete genome: monopartite GenBank: FR682468.2)

PCR for RNA samples was performed using REVERTA-L RT reagents kit (AmpliSens). The sensitivity and specificity assessment tests were performed in 3 repeats with the selected LAMP primer set № 2.

Primary LAMP assessment and protocol optimization. Bst 2.0 DNA polymerase ("New England Biolabs") was used for the primary assessment of performance of all three assays developed. The reaction mixture was prepared according to the standard manufacturer's protocol apart from the modifications mentioned below.

During the LAMP protocol optimization, the amplification temperature (57–64 °C, step 1 °C) and time (10–60 min, step 10 min), concentration of $MgSO_4$ (4–10 mM, step 1 mM), betaine (0.8–1.2 mM, step 0.1 mM) and *Bst* polymerase (2–8 U, step 2 U) were tested. The amplification was carried out using a Biometra TAdvanced Twin 48 Gradient Thermal Cycler (Analytik Jena, Germany). The amplicons were visualized by electrophoresis in 1.5 % agarose gel. The alternative rapid visualization was performed by adding 1 µl of ethidium bromide (0.5 µg/ml solution) with further illumination with UV-light.

RESULTS

Initially, the primary assessment of the three LAMP assays developed was performed. The analysis of the reaction products via gel-electrophoresis showed that two primer sets generated amplicons (Fig. 1). However, in the reaction with primer set No. 2, the ladder pattern of the amplicon was more legible and clearer. The reaction with a primer set including only inner and outer primers (set No. 3) was not successful and no product was generated.

The primer set 2 was selected for further studies, since the amplification result obtained using it had been the most robust. During the LAMP protocol optimization, the difference between LAMP products synthesised under different conditions was hardly observed in the use of gel-electrophoresis for product visualisation. For this reason, the product was additionally visualized by adding ethidium bromide and subsequent UV illumination, which helped to visualise larger amount of the generated LAMP product and the tube with the most intensive LAMP product formation (Fig. 2).

A little difference in LAMP product synthesis was observed for the LAMP reactions carried out at different temperatures, however, the brightest fluorescence was observed in case of the amplification at 59 °C and 60 °C. An increase of the amplification time for more than 40 min did not affect the LAMP result. In view of this, the amplification for 40 min at 60 °C was selected as the optimal condition for the assay. The optimized protocol for 25 µl LAMP mixture preparation was 0.2 µM of 2F3/2B3, 0.4 µM 2LF/2LB, 1.6 µM 2FIP/2BIP, 6 mM DNTPs mixture, 1× Isothermal Amplification Buffer, 6 mM $MgSO_4$, 1 mM betaine, 10U of *Bst* DNA polymerase. Five (5) µl of template should be added to 20 µl of reaction mixture.

A 10-fold serial dilution of 170 bp target sequence for primer set № 2 generated by PCR, cleaned up and quantified, was used for LOD assessment. Testing the DNA templates, including those from 10^{10} to 1 copy of the sequence of interest, resulted in the production of a clear ladder-patterned LAMP product for 10 samples out of 11 (Fig. 3). Hence, the LOD for the LAMP was 10 copies of target sequence per 1 µl, which equalled 50 copies per reaction.

The initial LAMP analytical specificity assessment was performed by testing DNA and RNA samples of ASFV and other swine viruses and bacteria, causing similar symptoms. ASFV reference DNA panel includ-

Table 2. Panel of reference samples used for the LAMP assay specificity testing

Matrix	Inoculum	Genotype	Quantification
Spleen	ASFV "Sardinia"	I	$9.41*10^3$
Lung	ASFV "Sardinia"	I	$1.87*10^3$
Spleen	ASFV "Estonia"	II	$5.57*10^2$
Lung	ASFV "Estonia"	II	$1.44*10^2$
Spleen	ASFV "Kenia05"	IX	$3.86*10^3$
Lung	ASFV "Kenia05"	IX	$3.68*10^3$
Macrophage culture	ASFV "Netherlands86"	I	$2.66*10^3$
Macrophage culture	ASFV "Estonia"	II	$1.64*10^2$
Macrophage culture	ASFV "Kenia05"	IX	$1.93*10^3$
Macrophage culture	ASFV "Sardinia"	I	$1.54*10^3$

ed samples extracted from three different matrixes and three of the 24 described genotypes of ASFV. In our preliminary experiments using DNA of a limited number of sources/samples the LAMP assay, using primer set № 2 showed high specificity, generating neither false positive, nor false negative results (Fig 4). No LAMP products were observed in the DNA samples, extracted from swine lymph nodes, used as a negative control during the protocol optimization.

DISCUSSION

Despite strict control measures, ASF has been constantly spreading in European and Asian countries among both wild boars and domestic pigs, since its introduction to the Caucasus region in 2007 (Beltrán-Alcrudo D et al, 2008) In the absence of commercially available vaccines and treatment, the diagnostics of ASF became the crucial factor for the prevention of wide spreading of this disease. Many (molecular) methods have been developed for ASF diagnostics, including conventional techniques such as ELISA, IFAT, IBT, and PCR. However, the use of PCR tests requires well-equipped laboratories with sophisticated thermocyclers, which are not always available in remote regions. The transportation of the material to the sophisticated laboratories may take (too) much time and makes fast decision-taking impossible. The LAMP assay fits this gap, since the reaction can be carried out under field conditions using such simple devices as a water bath or thermoblock. Different simple product visualisation techniques are also compatible with LAMP, which makes it a good tool for rapid molecular diagnostics (Wong Y-P et al, 2017; Mee PT et al., 2020).

The use of LAMP for infectious diseases field diagnostics has been rather limited since the method development, although it was mentioned as one of the alternative tests to PCR in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2019b). However, the situation has changed after the beginning of COVID-19 pandemic, when cheap mass express testing has become particularly important. Different LAMP assays have been developed for COVID-19 diagnostics and are used as one of the tests of choice (Baek YH et al, 2020; Kashir J et al, 2020; Park G-S et al, 2020)

Numerous LAMP assays for the diagnostics of OIE notifiable viral swine diseases, including classical swine fever, African swine fever, swine vesicular disease and porcine reproductive and respiratory syndrome, have been developed (Mansour SM et al, 2015). The genes of ASFV topoisomerase II (James HE et al, 2010), ma-

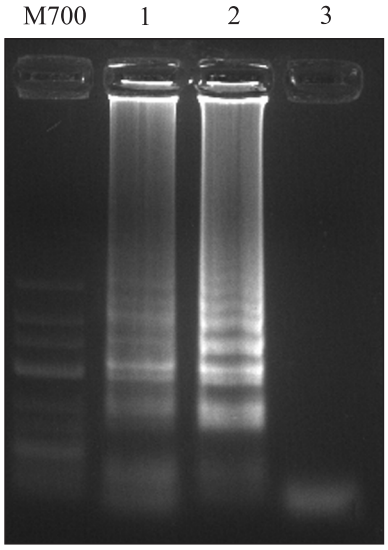

Fig. 1. The results of electrophoresis in 1.5 % agarose gel of the LAMP products (ASF of genotype II, strain ASFV "Estonia"), generated using the LAMP primer sets developed – set1 (1), set2 (2), set3 (3), M700 – Thermo Scientific O'GeneRuler Low Range DNA Ladder. Purified PCR products of each target sequence were used as templates

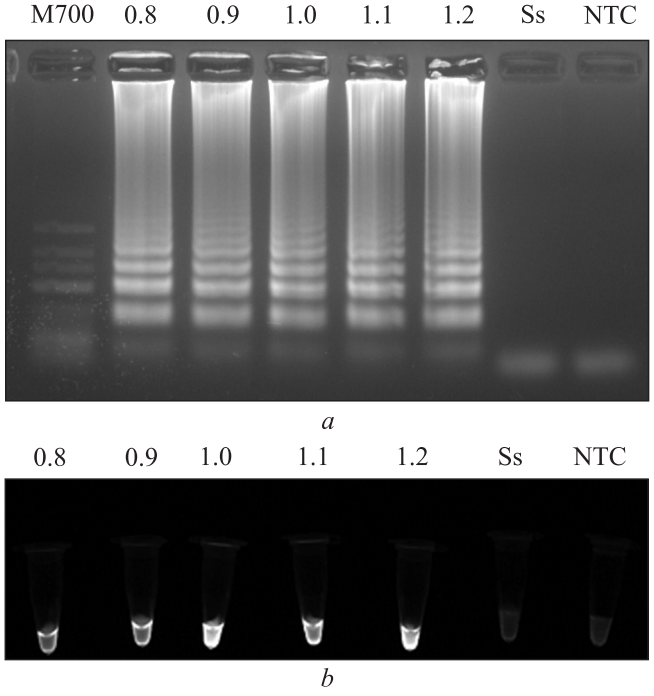

Fig. 2. The visualization of the LAMP product (ASF of genotype II, strain ASFV "Estonia") using gel-electrophoresis (*a*) and UV-light after EtBr addition (*b*). 0.8–1.1 betaine concentration (mM), Ss – *Sus scrofa* DNA, NTC – negative control (deionized water), M700 – Thermo Scientific O'GeneRuler Low Range DNA Ladder. Purified PCR product of target sequence for LAMP primer set № 2 was used as a template

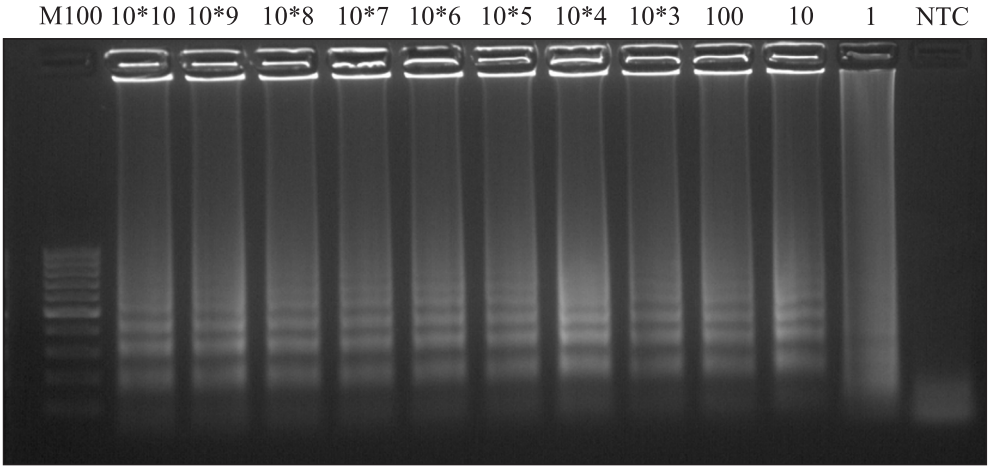

Fig. 3. Analytical sensitivity of the LAMP assay using purified PCR product (ASF of genotype II, strain ASFV "Estonia") of target sequence for LAMP primer set № 2, detecting ASFV gene *C962R*. 10^{10}-1 – analyte concentration (copies/µl), NTC – negative template control (deionized water)

jor capsid protein p72 (Dokphut A et al, 2021, Tran DH et al, 2020), p22 (Elsukova AA et al, 2019), p10 (Wang D et al, 2020), K205R (Wu X et al, 2016) were used as target sequences for LAMP assays developed in the previous studies. In our case we choose, gene *C962R* coding putative DNA primase, which has been used as a molecular marker for ASF diagnostics using LAMP for the first time. The main advantage of *C962R* use as a molecular marker is the fact that the protein coded by this gene is one of the most conservative proteins of ASFV (>97 % identity across 11 isolates of genotype II) (Chapman DAG et al, 2011; Bao J et al, 2019). Although the protein is not essential for virus virulence it is included in viral replication and located in the highly conservative central core region of the ASFV genome (Ramirez-Medina E et al, 2020; Bao J et al, 2019). The development of additional diagnostic assays, based on diverse molecular markers, is useful as backup diagnostic tools in case of emerging virus variants.

The LAMP method is known to be less sensitive to the factors inhibiting amplification, which enables its use for testing samples that include inhibiting contaminants, such as environmental samples, blood with anticoagulants or oral fluids collected using baits. Diluted blood, serum, stool samples were tested with LAMP without prior DNA extraction (Francois P et al, 2011; Mee PT et al, 2020; Phillips DE et al, 2021).

Due to the ability to amplify a target sequence across relatively wide temperature values, LAMP is a robust method. Thus, equally effective amplification was observed at the temperature varying from 57 to 64 °C in our study. The range of 10 °C (57–67 °C) allowed for consistent LAMP results in the study for *Salmonella spp*. detection (Francois P et al, 2011). LAMP amplicon was generated at the temperature values from 51 to 70 in the study of Tran DH et al (2020). Additionally, neither exposition of LAMP reagent to ambient temperature, nor prolonged reaction mixture preparation were shown to yield false results (Francois P et al, 2011).

The LAMP LOD varies greatly depending on the target sequence, and especially the type of sample material used. The LOD of the *C962R* LAMP assay, assessed using serial 10-fold dilutions of purified and quantified PCR product, was 10 copies/µl which corresponds to 50 copies per reaction. This rate is consistent with LOD of p72 LAMP assay (Dokphut A et al, 2021), which was 368 copies of plasmid DNA per µl, the assay detecting topoisomerase II gene – 330 copies of plasmid DNA per reaction (James HE et al, 2010) and 400 copies of synthesized DNA template per reaction (Mee PT et al, 2020), 1 copy of synthesized DNA template per reaction (Tran DH et al, 2020), the assay detecting gene coding p10 – 30 copies of plasmid DNA per µl (Wang D et al, 2020). LOD criteria of OIE recommended assays for ASFV detection are rated as 100 gene copies for TaqMan PCR (King DP et al, 2003), and 18 gene copies for UPL PCR (Fernández-Pinero J et al, 2013). Thus, the analytical sensitivity of the LAMP assay using purified DNA is comparable with this characteristic of the OIE recommended assays for molecular detection of the ASF causative agent (OIE, 2019b)

The LAMP assay developed is specific and did not show any cross-reaction in our study, neither did other LAMP assays previously developed (Mee PT et al, 2020; Dokphut A et al, 2021; Wang D et al, 2020;

Tran DH et al, 2020; James HE et al, 2010). The assay we developed detected ASFV DNA extracted from three different sources, spleen, liver and macrophage cell cultures. The specificity is not influenced by the ASFV genotype and effectively detects genotypes I and II, which are the two, present in Europe, Asia, and the Oceania (Blome S et al, 2020). Further specificity testing using more and different source material from various origins and under field conditions will be subject of further studies.

Different approaches can be used for LAMP result detection and product visualization. In case of adding fluorescent dyes, the result can be detected in real time using qPCR thermocyclers. Turbidimetry based on monitoring white precipitate of magnesium pyrophosphate, generated during DNA amplification, also allows for real-time LAMP result detection (Wong Y-P et al, 2017). To make the assay less equipment-dependent, the colorimetric approach can be used. Hydroxynaphthol blue, calcein, malachite green, leucocrystal violet are colorimetric indicators changing colour in a positive LAMP reaction (Baek YH et al, 2020; Park G-S et al, 2020). They do not influence the reaction efficiency and enable the detection of LAMP results with the naked eye, although they make interpretation of the test result more subjective (Goto M et al, 2009; Dokphut A et al, 2021, Wong Y-P et al, 2017). Intercalating dyes, such as SYBR Green, Picogreen and ethidium bromide, are used for LAMP result detection under UV light (Wong Y-P et al, 2017). Lateral flow devices (LFD) are also used for LAMP result detection, especially in case of rapid field tests. The principle is based on the interaction between biotin-labelled LAMP product and fluorescein isothiocyanate, allowing for the hybridisation of different ligands and visualisation of the LAMP product band (James HE et al, 2010; Wong Y-P et al, 2017). However, electrophoresis in agarose gel remains an effective end-point method for LAMP product visualization, since it enables visual recognition of ladder-like pattern, which is the characteristic of LAMP amplicon. This approach is more precise and can be used to confirm the results obtained using colorimetric reagents and fluorescent dyes.

Therefore, due to its high specificity and sensitivity, robustness and ability to use different approaches for result detection, the LAMP method is an excellent tool for molecular diagnostics. The generation of consistent results across a wide range of temperatures, allowing for the use of simple equipment and simplicity of visual result detection make LAMP a method of choice for low-resource laboratories and point of need testing, which has been proven by numerous studies (Mee PT et al, 2020; Fillips DE et al, 2021).

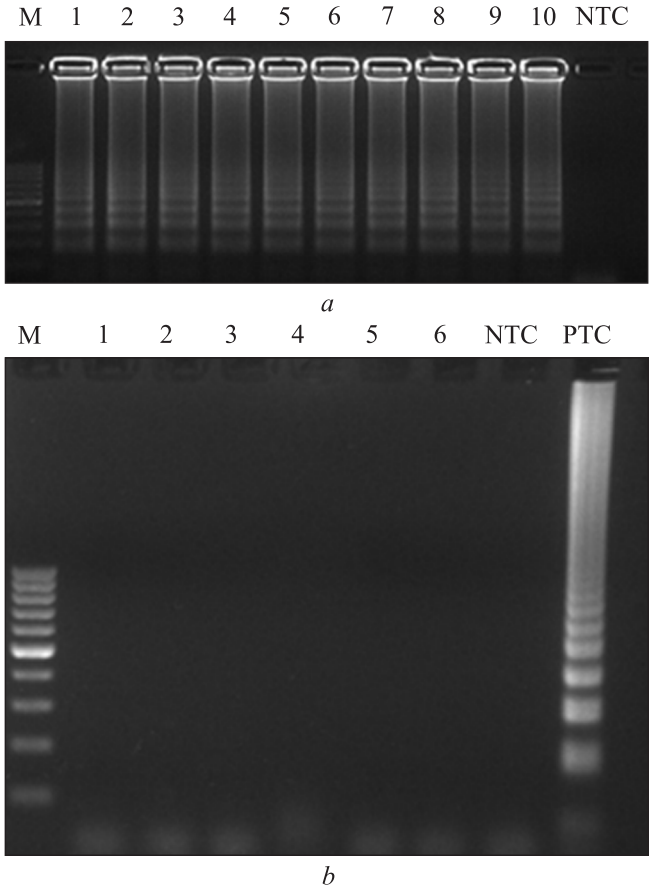

Fig. 4. The LAMP specificity testing, using positive reference (*a*) and heterologous (*b*) DNA samples. A. 1–10 – reference samples (numeration according to Table 2), NTC – negative control (deionized water). B. 1 – PRRSV RNA virus, 2 – PCV 2, 3 – ADV, 4 – CSFV RNA virus, 5 – *P. multocida*, 6 – *Erysipelothrix rhusiopathiae*

CONCLUSION

A LAMP assay, able to detect 10 copies of purified *C962R* ASFV gene per µl DNA within 40 minutes, has been developed and partially validated. Using 3 different sample matrices, purified DNA of 10 known strains of ASFV from 4 different countries and 3 different genotypes (I, II and IX) the primer set 2 appeared to be highly specific. In many studies, the LAMP method has been proven to be an effective tool for rapid testing under both laboratory and field conditions. Further studies of *C962R* gene-based LAMP assay, including the application of colorimetric visualization or LFD, are needed. The assay developed should be further

validated and also tested with clinical samples under field conditions. This will help to implement the LAMP assay in diagnostic practice and enable obtaining fast results and taking decisions to prevent the disease spreading in case of new outbreaks management in less-resource areas.

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Adherence to ethical principles. All experiments described in this paper were non-animal experiments.
Conflict of interest. The authors declare no conflict of interest.
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Розробка способу детекції вірусу африканської чуми свиней на основі петльової ізотермічної ампліфікації гену *C962R*

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Мета. Метою дослідження є розробка методу петльової ізотермічної ампліфікації для детекції вірусу африканської чуми свиней (АЧС). **Методи.** Дизайн праймерів проводили з використанням публічно доступних повногеномних послідовностей вірусу АЧС. Для тестування специфічності методу використовували панель гетерологічних зразків ДНК та референтних позитивних зразків ДНК. Для визначення ліміту детекції (ЛД) використовували очищені серійні розведення ампліфікованої за допомогою ПЛР цільової послідовності з відомою концентрацією аналіту. Продукт реакції петльової ізотермічної ампліфікації детектували за допомогою гель-електрофорезу та флуоресценції броміду етидію в ультрафіолетовому світлі при додаванні його безпосередньо до пробірки з продуктом реакції. **Результати.** Було розроблено три системи праймерів, що детектують різні ділянки гену *C962R* вірусу АЧС, серед яких систему № 2, що забезпечувала найбільш інтенсивний синтез продукту з найбільш чітким патерном, було обрано для подальших досліджень. Було встановлено оптимальну концентрацію компонентів реакційної суміші для обраної системи праймерів. За фінальним протоколом реакцію петльової ізотермічної ампліфікації проводили за температури 60 °С впродовж 40 хв. Ліміт детекції розробленої методики становить 50 копій цільової послідовності на реакцію. В пілотному дослідженні з використанням 10 референтних і 4 гетерологічних вірусних та 2 бактеріальних зразків ДНК було показано, що спосіб детекції є специфічним. За допомогою розробленої методики можна проводити детекцію генотипів I та II вірусу АЧС, що наразі циркулюють в Європі, Азії та Океанії та IX, що виявляють в Африці. **Висновки.** Було розроблено спосіб детекції ДНК вірусу АЧС, що в пілотному дослідженні показав специфічність і чутливість, та виявляє 50 копій очищеного цільового гену впродовж 40 хвилин. У даному дослідженні для візуалізації продукту реакції було використано класичний спосіб гель-електрофорезу та пряме забарвлення за допомогою броміду етидію. Використання колориметрії або тест-смужок на етапі візуалізації продукту зробило б методику менш залежною від обладнання. Подальша валідація розробленого способу детекції, визначення аналітичної специфічності, селективності та відтворюваності з використанням клінічних зразків у польових умовах, потенційно дала б можливість його використання в якості преференційного тесту безпосередньо на місці виявлення спалаху та в лабораторіях із обмеженими ресурсами.

Ключові слова: африканська чума свиней, ізотермальна ампліфікація, молекулярна діагностика, експрес-тест, ДНК.

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