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DEVELOPMENT OF RECOMBINANT POSITIVE CONTROL FOR AFRICAN SWINE FEVER VIRUS PCR DETECTION

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Recombinant plasmids containing target sequences are widely used as positive controls for PCR laboratory diagnostics. The aim of the study was development of recombinant positive control containing a fragment of *B646L* gene of African swine fever virus. The sequence of interest encodes targets of all the PCR assays for African swine fever laboratory diagnostics recommended by World Organisation for Animal Health. A plasmid containing 1763 bp insertion was cloned in *E. coli* DH5α strain. After purification, the plasmid ten-fold serial dulutions were used as a positive control while PRC testing. A minimal detectable copy number was 20 copies per reaction for both conventional and real-time PCR assays. The developed plasmid could be used as a safe and effective positive control while ASF laboratory diagnostics by PCR.

Key words: African swine fever virus, molecular cloning, PCR, positive recombinant control.

African swine fever (ASF) is a viral disease, affecting domestic pigs and wild boars. Acute ASF form is characterized by hemorrhagic fever and results in high mortality. The causative agent is the ASF virus (ASFV), a giant enveloped DNA virus, the only member of the *Asfarviridae* family. African wild boars and soft ticks of the *Ornithodoros* genus are the natural virus hosts. In the wild boar reservoir the infection is usually asymptomatic [1, 2]. Since a vaccine and treatment are unavailable, the disease causes significant economic losses and poses a threat to pig industry in all European countries. For these reasons, ASF is a notifiable disease according to the World Organization for Animal Health [3].

ASF is endemic in sub-Saharan countries and Sardinia. In 2007 ASFV was detected in Georgia for the first time. Probably, the virus of the genotype II from Africa was introduced to the new territory via sea. Since 2007 ASFV has rapidly spread to Azerbaijan, Armenia, Russia, Belarus, Ukraine, Poland, Estonia, Lithuania, Latvia, Moldova, the Czech Republic, Romania, Hungary, Bulgaria, Serbia, the Slovak Republic and Germany, where it caused numerous outbreaks among domestic pigs and wild boars. In September 2018 ASFV has been detected in wild boars in Belgium for the first time since eradication in 1985. Belgium is the first country of Western Europe, where the disease reoccurred after its introduction to Georgia [4, 5]. In August 2018 ASF for the first time was confirmed in China, which is the world biggest pork producer. Until now the disease has spread to Vietnam, Cambodia, Indonesia, Mongolia, the Republic of Korea, the Democratic People`s Republic of Korea, Myanmar, Philippines, Laos, Papua New Guinea, India and other Asian countries [5]. Thus, rapid virus detection and strict quarantine measures implementation is crucial for the disease control.

Different laboratory tests are used for ASF diagnostics. Assays, directed towards virus

isolation, antigen or antibodies detection are time-consuming and may generate false-negative results. Molecular methods, such as conventional and real-time PCR, are highly sensitive and rapid technique for virus detection. They are appropriate for routine diagnostics and surveillance of the ASF and could be used for a wide isolates variety of different virus genotypes testing. According to the OIE guidelines, conventional and real-time PRC assays are recommended for ASFV detection [6]. These assays are aimed at identifying the sequences in positions 115–371 [7], 648–714 [8] and 1627–1877 [9] of gene *B646L* that encodes viral protein p72.

PCR assays require using positive controls for valid results obtaining. It`s preferable not to use virus culture as a positive control for biosafety and biosecurity providing. Plasmid positive controls use is reliable for such purposes.

The aim of the study was to develope a universal positive recombinant control containing all the mentioned sequences, wich can be used for both conventional and real-time PCR assays recommended by OIE.

Materials and Methods

The plasmid map was designed using the Clone Manager Professional 9 (Scientific and Educational Software, USA). The insertion of 1763 bp *B646L* gene fragment in positions 115–1877 was synthesized using conventional PCR. The following primers were used for this assay:

Primer PPA-1: AGTTATGGGAAACCCGACCC [7];

King 2: GATACCACAAGATCAGGCCGT [9].

ASF virus strain Georgia 2007 was used as a positive DNA template. The reference DNA of the ASF virus was obtained from the European Union Reference Laboratory for African Swine Fever (Spain). Reaction mix was prepared according to the following protocol (per 1 sample): AmpliTaq Gold polymerase (Applied Biosystems, USA) 1.25 U/50 mcl — 0.13 mcl, PE-buffer 1X — 2.5 mcl, dNTP mix 0.2 mM — 0.5 mcl, $MgCl_2$ 1.5 mcM — 1.5 mcl, primer PPA-1 10 mcM — 0.5 mcl, Primer-2 10 μM — 0.5 mcl, DNA matrix — 5 mcl, adjusted with water up to the final volume of 20 mcl. Thermal cycling was performed using the following program: one cycle at 95 °C for 5 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 10 min. Amplification was verified by amplicons visualising in 1% agarose gel. The size of the PCR product was 1763 bp. The amplicons were isolated from the gel using GeneJET Gel Extraction Kit (Thermo Scientific, USA). The DNA concentration was determined using a DS-11 spectrophotometer (DeNovix, USA).

PCR product was inserted into the vector using InsTAClone PCR Cloning Kit (Thermo Scientific, USA) with the vector to insertion ratio 1:5. Ligation was performed overnight at +4 °C. *E. coli* DH5α (Thermo Scientific, USA) Calcium Chloride competent cells were transformed via heat-shock with the ligated plasmid. Transformed cells were verified using blue-white screening and confirmed by the mentioned above PCR. After the transformed bacteria cultivation, the plasmid was isolated using GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA).

Isolated plasmids were used as a positive template for PCR assays with PPA1/2 [7] and King 1/2 [9] sets. The reaction mixture for conventional PCR per 1 reaction contained 12.5 mcl of DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, USA), 1 mcl of 10 pM PPA-1 primer, 1 mcl of 10 pM PPA-2 primer, 5.5 mcl of water and 5 mcl of a template. The cycling was performed using the following protocol: one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, final elongation at 72 °C for 5 min and 257 bp amplicons were visualized by electrophoresis in 1.5% agarose gel. Real-time PCR reaction mixture was prepared using 10 mcl of TaqMan Fast Advanced Master Mix (2X) (Applied Biosystems, USA), 0,5 mcl of 10 pM probe, 0,4 mcl of each 20 pM primer, 6,7 mcl of water, 2 mcl of a template. Real-time PCR was carried out according to the following protocol: one cycle at 95 °C for 5 min, 40 cycles at 95 °C for 15 s, 58 °C for 30 s, 60 °C for 30 s, the fluorescent signal was recorded at FAM channel at every 60 °C step.

Results and Discussion

The plasmid with a 1763 bp *B646L* gene fragment ligated into the pTZ57R/T vector was constructed. The total length of the plasmid is 4649 bp (Fig. 1).

The vector encodes an ampicillin resistance and a *lacZ* genes, which were used as selective markers for transformed *E. coli* DH5α clones. Ten white single colonies were screened with conventional PCR and confirmed as containing a 1763 bp *B646L* gene fragment.

The series of ten-fold dilutions, containing 10^5, 10^4, 10^3, 100 and 10 copies of

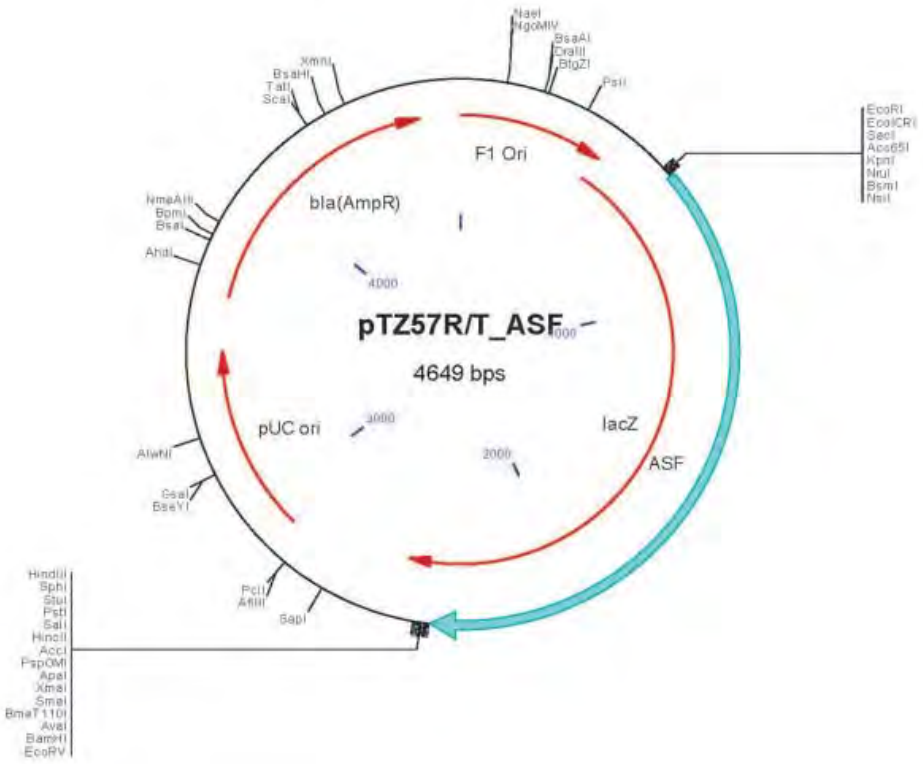

Fig. 1. **The plasmid pTZ57R/T_ASF map**

the plasmid DNA in 1 mcl, were prepared for a minimum copy number detection testing. The pTZ57R/T_ASF plasmid is detected by both real-time PCR (Fig. 2) and conventional PCR (Fig. 3) at dilution up to 10 copies per µl, corresponding to 20 copies per reaction. The successful result for both target regions proved that the whole sequence of interest was ligated correctly into the vector and can be used as a positive control sample.

PCR is considered to be a "gold standard" test for an early ASFV detection. However, its results can be regarded as true only in case of positive and negative controls use, which ensure performance of the reaction and help to avoid false-posive and false negative results obtaining [10].

Substantions of different types containing the gene of interest can be used as positive control. DNA extracted from reference culture of microorganism is an optimal kind of positive control sample. However, this approach has limitations while dangerous diseases diagnostics, as laboratory must meet special biosafety requirements to be permitted to cultivate that kind of agents. Even though a laboratory has the permittion, a cultivation of hazardous agents, biomass producing and concentration is associated with additional risks. In case of inactivated microorganisms use, resiaduals of inactivating substances (formaldehyde, ethanol, etc.) may affect PCR acting as inhibitors [10]. DNA extracted from clinical material can also be used as positive control. Advantage of this type of material is a close simulation of tested samples, so the influence of sample matrix on result eliminates. However, variation of Ct values obtained during real-time PCR testing of samples from different patients/animals as positive control should be taken into account [11].

Recombinant plasmids are widely used as both inner and outer positive controls while PCR testing. Many kits available commercially include recombinant plasmid containing gene of interest as a positive control sample. OIE suggests using recombinant plasmid DNA as positive control while diagnostics testing by PCR [12]. They can be easily obtained in big amount from transformed bacteria during short time. This process does not require special conditions and can be performed even in BSL-1 laboratory as bacteria strains used for cloning are safe for a researcher and environment. Plasmid DNA is also an excellent material for the absolute quantification and qPCR assay validation [13]. Plasmid purification kits ensure that the final product contains

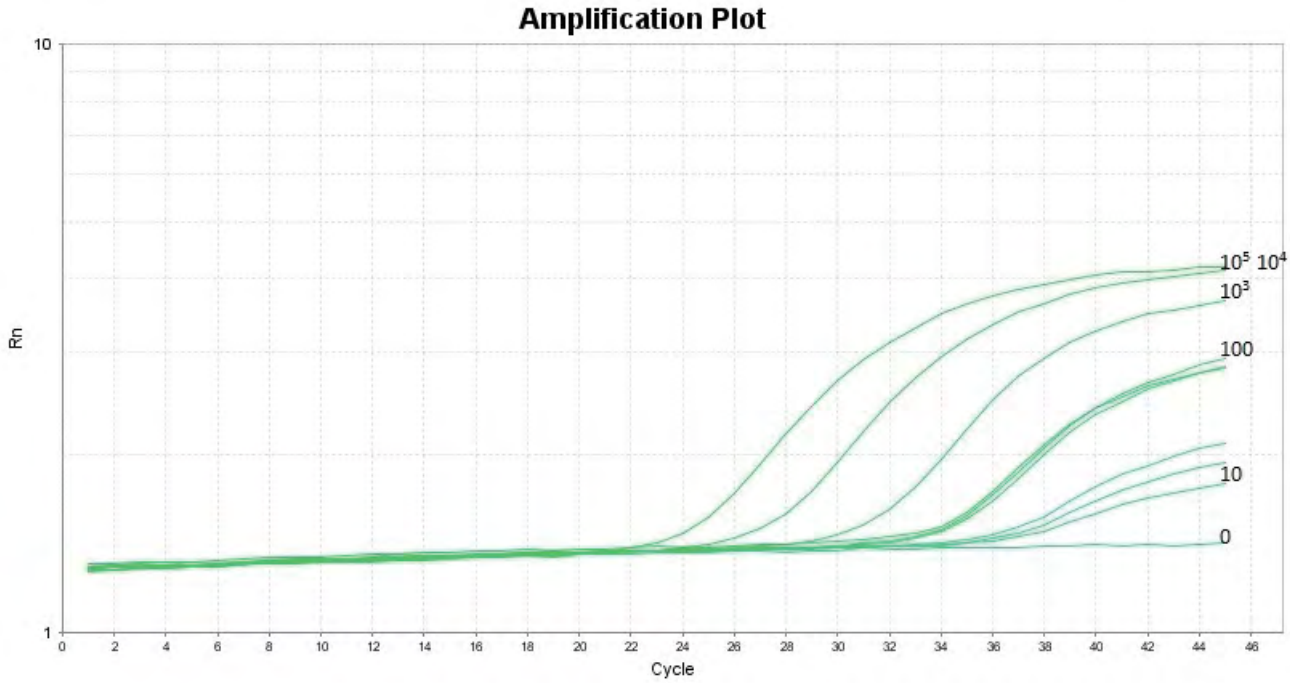

Fig. 2. **The results of pTZ57R/T_ASF minimal copy number detection using real-time PCR**

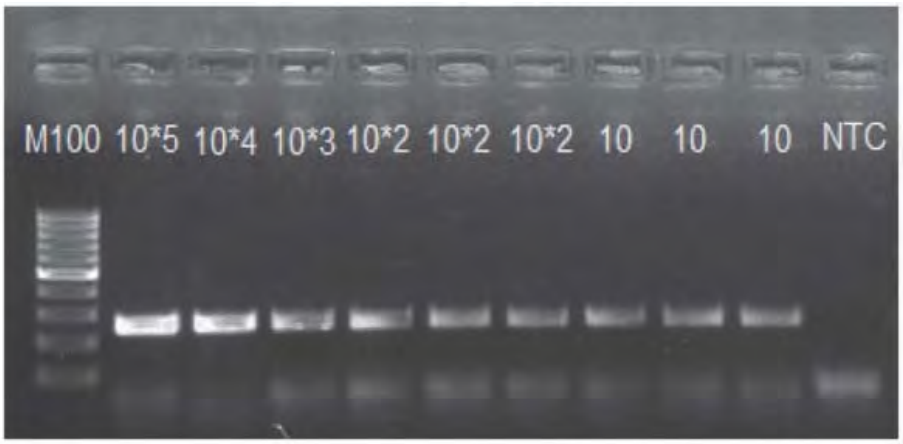

Fig. 3. **The results of pTZ57R/T_ASF minimal copy number detection using conventional PCR**

pure plasmid DNA. Thus, gene copy number calculation in plasmid DNA sample is more accurate than in one containing DNA of both host organism and pathogen.

Plasmid DNA containing different fragments of ASFV genome (p72 gene, topoisomerase II gene etc.) are commonly used as a positive control in plentiful diagnostic kits and during scientific studies [14–17]. We have developed the recombinant plasmid containing region of *B646L* gene, which includes target sequences for all the PCR assays recommended by OIE for ASF diagnosis. It makes the developed plasmid the universal positive control for ASFV genome detection by PCR according to OIE guidance [6].

However, there are some limitations for work with plasmids. Since plamid is a circular molecule, this structure is more stable than liner DNA. In case of multicopy plasmid (including pTZ57R/T) use, a huge amount of plasmids are produced during cloning procedure. These issues should be considered while working with plasmids, as they can result in contamination of reagents, equipment or surfaces. Even though plasmid use in the laboratory diagnostics poses a threat of contamination, plasmid contamination of the sample can be easily distinguished from the cross-contamination by

native viral DNA using vector-specific primers (e.g., M13 primers).

Thus, being quantifiable, renewable and stable, recombinant plasmid is a good choice for PCR positive control during laboratory diagnostic testings.

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РОЗРОБЛЕННЯ РЕКОМБІНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЬНОГО ЗРАЗКА ДЛЯ ДЕТЕКЦІЇ ВІРУСУ АФРИКАНСЬКОЇ ЧУМИ СВИНЕЙ ЗА ДОПОМОГОЮ ПЛР

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Рекомбінантні плазміди, що містять цільові послідовності, часто використовують у лабораторній діагностиці як позитивний контроль для ПЛР. Метою дослідження було розроблення рекомбінантного позитивного контролю, що містить фрагмент гена вірусу Африканської чуми свиней. Ця послідовність кодує цільові ділянки усіх ПЛР-методик, що їх рекомендує для лабораторної діагностики АЧС Всесвітня організація охорони здоров'я тварин. Плазміду, що містить вставку розміром 1763 пн, клонували у *Escherichia coli* штаму DH5α. Після очищення десятикратні серійні розведення плазмідної ДНК було використано під час проведення ПЛР. Мінімальна кількість копій плазміди, що детектується класичною ПЛР та ПЛР у режимі реального часу, становила 20 копій на реакцію. Розроблену плазміду можна використовувати як безпечний і ефективний позитивний контрольний зразок у разі лабораторної діагностики АЧС за допомогою ПЛР.

Ключові слова: вірус африканської чуми свиней, молекулярне клонування, ПЛР, рекомбінантний позитивний контроль.

РАЗРАБОТКА РЕКОМБИНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЬНОГО ОБРАЗЦА ДЛЯ ДЕТЕКЦИИ ВИРУСА АФРИКАНСКОЙ ЧУМЫ СВИНЕЙ С ПОМОЩЬЮ ПЦР

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Рекомбинантные плазмиды, содержащие целевые последовательности, часто используются в лабораторной диагностике в качестве положительного контроля для ПЦР. Целью исследования была разработка рекомбинантного положительного контроля, содержащего фрагмент гена вируса африканского чумы свиней. Эта последовательность кодирует целевые участки всех ПЦР-методик, рекомендованных для лабораторной диагностики АЧС Всемирной организацией по охране здоровья животных. Плазмиду, содержащую вставку размером 1763 пн, клонировали в *Escherichia coli* штамма DH5α. После очистки десятикратные серийные разведения плазмидной ДНК были использованы при проведении ПЦР. Минимальное количество копий плазмиды, детектируемой классической ПЦР и ПЦР в режиме реального времени, составляла 20 копий на реакцию. Разработанную плазмиду можно использовать как безопасный и эффективный положительный контрольный образец при лабораторной диагностике АЧС с помощью ПЦР.

Ключевые слова: вирус африканской чумы свиней, молекулярное клонирование, ПЦР, рекомбинантный положительный контроль.