Part 2. Biotechnology

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DEVELOPMENT OF RECOMBINANT POSITIVE CONTROL FOR DETECTION OF PORCINE CIRCOVIRUS TYPE 3 BY POLYMERASE CHAIN REACTION

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Summary. This work aimed to obtain positive control using recombinant DNA technology for detection by PCR of a new poorly studied pathogen — porcine circovirus type 3. Recombinant positive control was designed using Clone Manager Basic. As a vector in the creation of recombinant control we used plasmid pTZ57R/T, as an insert — a fragment of the gene *rep* PCV-3 with the length of 418 nucleotide pairs, obtained by classical PCR. Transformation of competent cells of *E. coli* strain DH5a was carried out by chemical poration, followed by plating on LB-medium with the addition of ampicillin at a final concentration of 100 μg/ml. The selection of *E. coli* cell colonies was performed by the marker of antibiotic resistance to ampicillin. The presence of a specific insert was checked by PCR with electrophoretic visualization of the results. The developed recombinant positive control can be used for the monitoring of biological samples from pigs for the presence of genetic material PCV-3 using molecular technologies

Keywords: PCV-3, PCR, plasmid pTZ57R/T, gene *rep*, *E. coli* strain DH5a

Introduction. One of the branches of animal husbandry that is actively developing all over the world, including Ukraine, is pig breeding. The level of development of this industry and the quality of the products obtained depend on the welfare of pig breeding concerning infectious animal diseases. One of the most common diseases among pigs today is circovirus infection, which causes significant economic damage to the industry and is characterized by severe immunosuppression and multiorgan pathology. Losses from PCV infection are estimated by the death of piglets at the stages of rearing and fattening; failure to gain weight of piglets; losses due to pathology of reproduction; ineffectiveness of antibiotic therapy in the presence of bacterial coinfections.

The causative agent of PCV disease is mainly porcine circovirus type 2, which belongs to the family Circoviridae, is characterized by pronounced pathogenicity and is the cause of many syndromes and diseases of pigs. In 2016, it was reported about a new type of porcine circovirus — PCV-3 in sows with clinical signs of dermatitis and nephropathy, as well as with reproductive problems (Palinski et al., 2017).

Currently known data indicate the prevalence of PCV-3 among pigs and wild boar populations in many countries in different geographical regions of the world (Bera et al., 2020; Hayashi et al., 2018; Serena et al., 2021; Saraiva et al.., 2019; Saporiti et al., 2020b; Yuzhakov et al., 2018; Franzo et al., 2018; Souza et al., 2021).

PCV-3 is associated with cardiac and multisystem inflammations, dermatitis and nephropathy syndrome, abortion and reproductive disorders in pigs, respiratory diseases; it is able to cause intrauterine infections in the absence of obvious reproductive disorders (Jiang et al., 2019; Ouyang et al., 2019; Phan et al., 2016; Saporiti et al., 2020c).

At present, for the detection of PCV-3 and rapid assessment of the epizootic situation, methods based on amplification technologies, including the use of different formats of polymerase chain reaction (PCR), is mainly used (Ji et al., 2019; Liu et al., 2019; Wang et al., 2019; Kim et al., 2020; Yuan et al., 2020; Zheng et al., 2020).

An important component that ensures the high quality of molecular genetic tests based on PCR and the level of reliability of the results obtained regarding the detection of PCV-3 is a positive control, which often uses material containing the virus. The use of such control is caused by the need to periodically obtain the appropriate material with a limited shelf life.

Therefore, the **aim of this work** was to construct a recombinant positive control containing an insert of gene *rep* PCV-3 with a length of 418 bp.

Materials and methods. Virtual design of recombinant positive control was performed using Clone Manager Basic v. 9 (Sci Ed Software, USA).

Plasmid vector pTZ57R/T, which is part of the commercial kit 'InsTAclone PCR Cloning Kit' (Fermentas, Latvia), was used to create *in vitro* plasmid control.

A fragment of the *rep* PCV-3 gene with a length of 418 bp was used as an insert. It was obtained by classical PCR using the commercial 'Maxima Hot Start Green PCR Master Mix' (Thermo Scientific, Lithuania) and the PCV-3 primer system F (5'-TTGTGGTGCTACGAGTG TCC-3'); PCV-3 R (5'-CGTCTCCGTCAGAATCCGAG-3') (Saporiti et al., 2020a).

Integration of the plasmid molecule into the culture of competent cells of *E. coli* strain DH5a was performed

by chemical poration followed by plating on LB medium (Sigma-Aldrich, USA) with the addition of ampicillin at a final concentration of 100 μg/ml.

A commercial kit 'Plasmid Miniprep Kit' (GeneJET, Lithuania) was used to extract the plasmid DNA.

Measurement of DNA concentration and evaluation of DNA quality was performed using a NanoDrop spectrophotometer (DeNovix, USA).

Electrophoretic analysis of amplification products was performed by horizontal electrophoresis (horizontal electrophoresis chamber Biorad (USA) in 1.5% agarose gel.

For electrophoretic analysis we used agarose produced by Biozym (Germany), ethidium bromide manufactured by Sigma-Aldrich (USA), molecular weight markers manufactured by Invitrogen (USA), Promega (USA) and Fermentas Gene ruler (Latvia) with a discreteness of 100 bp.

Results. In order to obtain a recombinant positive control sample for the detection of PCV-3 genetic material by PCR, we constructed a virtual model of a vector molecule based on plasmid pTZ57R/T with an embedded fragment of the *rep* PCV-3 gene with a length of 418 bp The total length of the theoretically modeled plasmid molecule was 3,304 bp (Fig. 1).

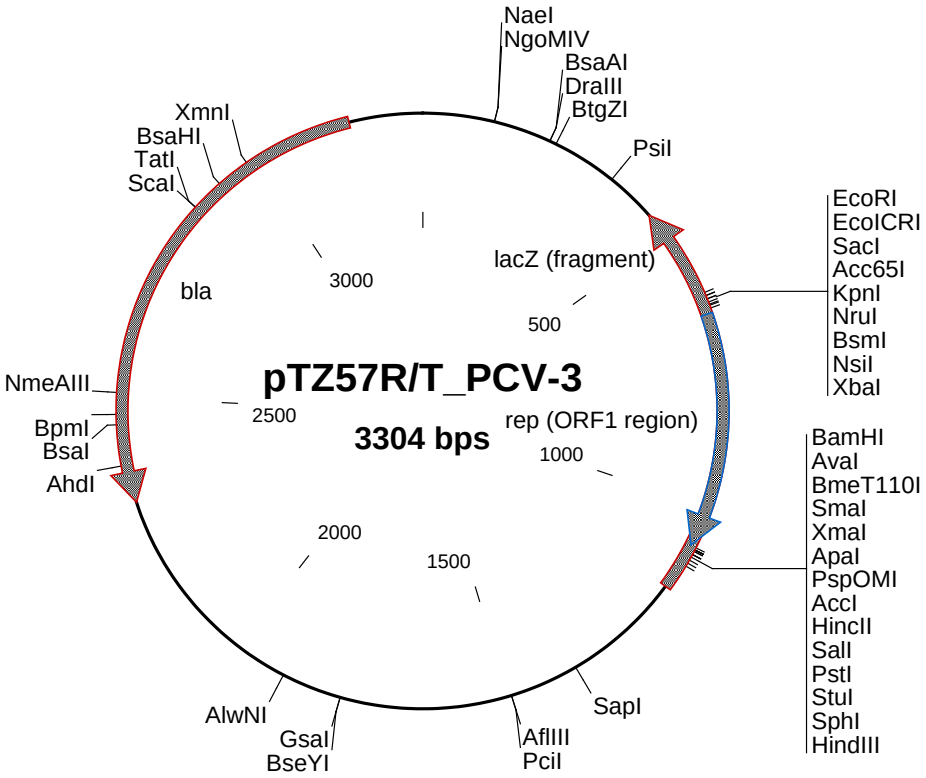

Figure 1. Scheme of recombinant plasmid pTZ57R/T_PCV-3

To create a plasmid control *in vitro* in the first stage of our work, a fragment of the *rep* PCV-3 gene with a length of 418 bp was developed. To do this, we used a DNA sample obtained from pig liver homogenate, which we had previously described as positive for the presence of PCV-3 genetic material. The analysis by horizontal gel electrophoresis confirmed the presence of an amplicon of the required length — 418 bp.

The resulting amplicon was purified and ligated to the plasmid vector pTZ57R/T, which transformed competent *E. coli* DN5a cells.

Because this vector contained an ampicillin resistance gene, it was a marker of selective traits during subsequent cloning of strain DN5a in *E. coli* culture.

Therefore, after the transformation, six white single colonies of *E. coli* with signs of acquired resistance to ampicillin were selected (Fig. 2).

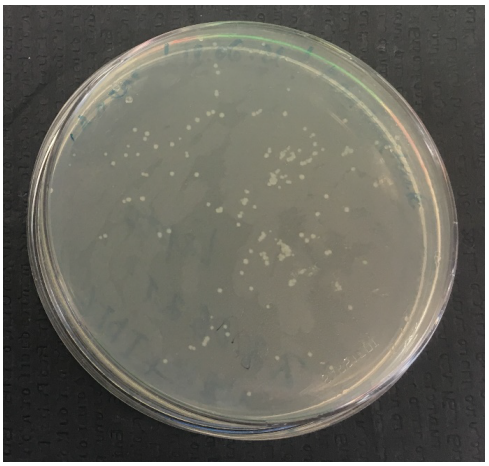

Figure 2. Selection of colonies of *E. coli* cells by the marker of antibiotic resistance to ampicillin

PCR screening of selected colonies showed the presence of a specific insert with the length of 418 bp in each of them (Fig. 3).

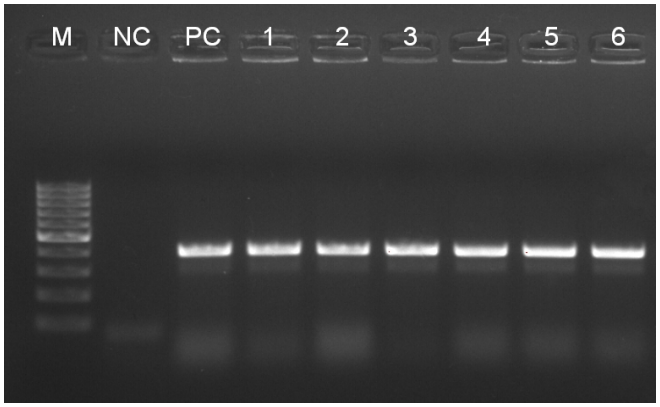

Figure 3. Visualization of the result of PCR screening of selected colonies by electrophoretic analysis in 1.5% agarose gel (M — molecular weight marker, NC — negative control, PC — positive control, 1–6 — samples positive for the presence of PCV-3 DNA)

Three colonies of *E. coli* were selected for cultivation in liquid nutrient medium, after which the resulting bacterial biomass was used for extraction of plasmid DNA. The presence of fragments about 3 thousand bp in length when performing electrophoretic analysis in 1.5% agarose gel of the obtained samples testified to the successful transformation of *E. coli* cells by the recombinant plasmid constructed by us (Fig. 4).

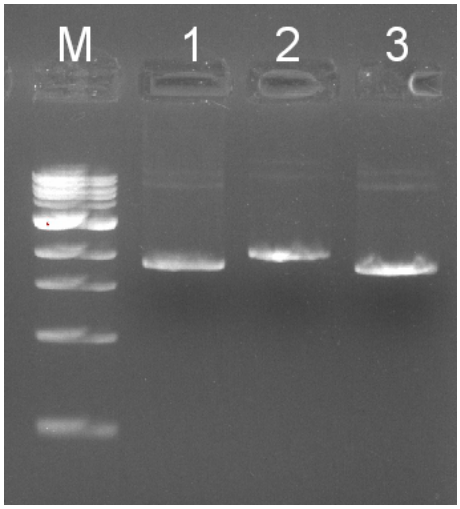

Figure 4. Visualization of the result of electrophoretic analysis of purified plasmid DNA (M — molecular weight marker; 1–3 — positive for the presence of plasmid DNA samples)

The concentration of DNA in the first sample was 106.15 ng/μl, in the second — 132.39 ng/μl, in the third — 83.08 ng/μl (Fig. 5).

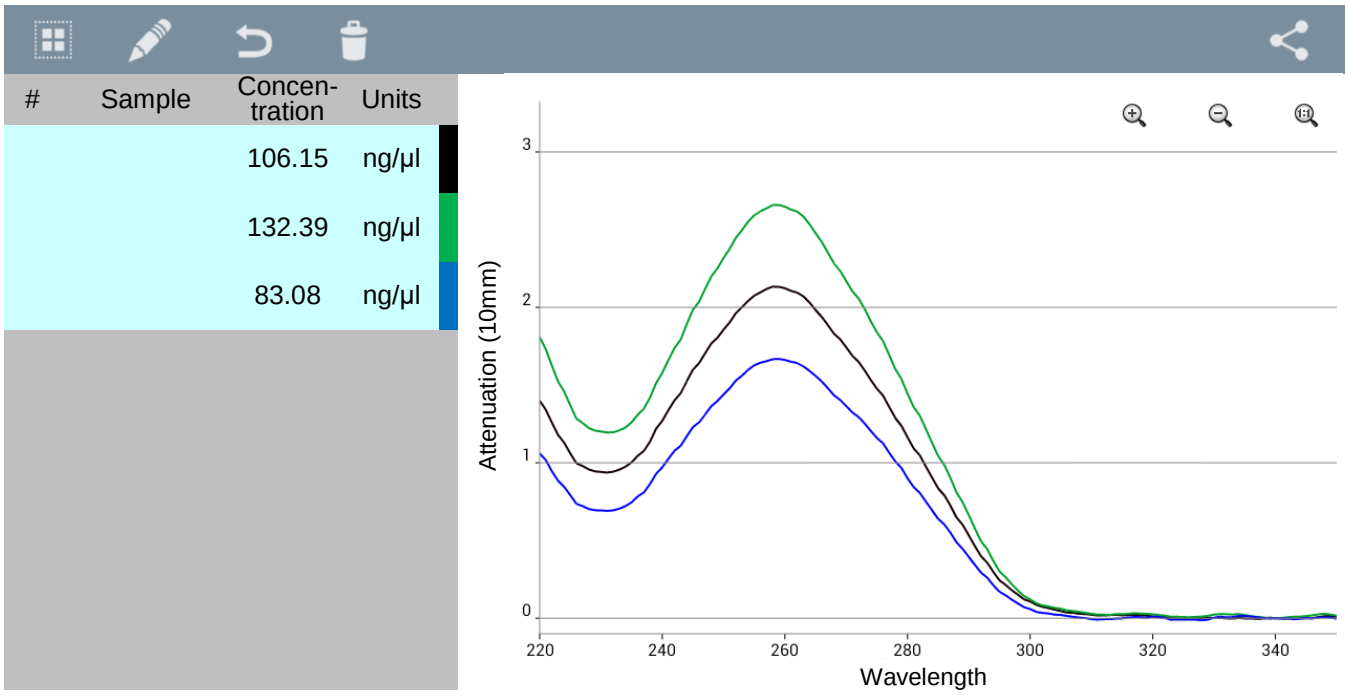

Figure 5. Results of plasmid DNA concentration measurement

The sample with the highest concentration of plasmid DNA was selected for our further studies.

Thus, we have developed a recombinant sample that contains a fragment with a length of 418 bp porcine circovirus type 3 *rep* gene. It can be used as a positive control in the detection of PCV-3 by PCR.

Discussion. Issues related to the selection and design of adequate positive controls for different PCR formats have always received considerable attention, primarily due to the potential risk to biosafety during the study (Chan, Jiang and Tan, 2016; Caasi et al., 2013; Chen et al., 2006; Lion, 2001).

Considerable factors in ensuring the high accuracy and efficiency of molecular genetic analysis based on PCR are, in particular, the conditions and form of storage of biological material used as a positive control.

At present, the use of positive control in the form of a recombinant plasmid containing a fragment of certain genomic DNA has become widespread in the detection of infectious diseases in farm animals, poultry (Yao et al., 2019; Das et al., 2017) and humans (Camacho et al., 2016), food pathogens (Gokduman et al., 2016), food allergens (Miyazaki et al., 2019), determination of GMOs (Taverniers, Van Bockstaele and De Loose, 2004) due to the long shelf life of such a structure, high copy capacity, the possibility of recovery (Matange, Tuck and Keung, 2021).

An equally important advantage of recombinant positive controls is that they do not require the permits provided by the Cartagena Protocol on Biosafety to the Convention on Biological Diversity, which was signed by Ukraine in 1992. Therefore, the recombinant positive control developed by us can be used to monitor biological samples obtained from pigs for the presence of PCV-3 genetic material using molecular technologies.

Conclusions. Thus, we obtained an ampicillin-resistant clone of *E. coli* DN5α, transformed with the constructed plasmid pTZ57R/T_PCV-3 with insertion of the *rep* gene with a length of 418 bp, which can be used as a positive control sample for the detection of the PCV-3 genetic material by PCR method.

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