https://doi.org/10.29326/2304-196X-2024-13-1-57-63

Construction of prokaryotic system for expression of porcine circovirus type 2 *ORF-2* gene fragment

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ABSTRACT

Porcine circovirus-associated diseases (PCVDs) are among the most significant challenges for pig farming in developed countries. Porcine circovirus type 2 (PCV-2) is considered the main etiological agent of postweaning multisystemic wasting syndrome in piglets. Mass PCVD occurrence has been reported in most regions of the world, that results in serious economic consequences. Optimal PCVD prevention is known to be achieved through a set of veterinary and sanitary measures in combination with vaccination. High evolutionary virus variability facilitating new genotype and strain emergence requires development of new candidate recombinant vaccines against PCV-2 infection. The study was aimed at construction of prokaryotic system for PCV-2 *ORF-2* gene fragment expression and its functionality assessment. A genetic insert constructed from the most immunogenic type-specific PCV-2 epitopes based on genotype 2a, 2b, 2d strain and isolate consensus sequence was cloned into the expression vector pET-22b(+) that was incorporated into the *Escherichia coli* strain Rosetta 2(DE3). The transformants were selected based on the marker gene of ampicillin resistance on a selective medium. Target gene expression was induced by adding of isopropyl-*β*-D-1-thiogalactopyranoside at different concentrations. As a result, *Escherichia coli* Rosetta 2(DE3)/pET-22b-ORF-2 strain, a producer of capsid protein fragment (92–233 amino acid residues), was constructed. It was found that in the presence of 1 mM isopropyl-*β*-D-1-thiogalactopyranoside, the expression level of soluble truncated rCap was 35–40 mg/L 6 hours after induction. The expression product was tested for its specificity with indirect ELISA using whole-virion PCV-2-hyperimmunized porcine serum. It was shown that the positivity coefficient of producer strain cell lysates averaged to 4.34 ($p < 0.005$). The recombinant rCap protein is suitable for serological diagnosis and is also of interest as a vaccine component, which is the goal of our further studies.

Keywords: porcine circovirus type 2, postweaning multisystemic wasting sindrome, *ORF-2*, prokaryotic expression system

Acknowledgements: The authors thank senior researcher of the Laboratory "OpenLab Gene and cell technologies", Kazan (Volga Region) Federal University, Cand. Sci. (Biology) R. F. Khairullin for providing of *Escherichia coli* expressing strain, as well as employees of the Shchelkovo Biocombinat, Dr. Sci. (Biology), Professor I. N. Matveeva and Cand. Sci. (Biology) N. V. Khaustova for providing PCV-2 hyperimmune serum and fruitful discussion.

For citation: Galeeva A. G., Akhunova A. R., Usoltsev K. V., Khammadov N. I., Efimova M. A. Construction of prokaryotic system for expression of porcine circovirus type 2 *ORF-2* gene fragment. *Veterinary Science Today*. 2024; 13 (1): 57–63. https://doi.org/10.29326/2304-196X-2024-13-1-57-63

Conflict of interests: The authors declare no conflict of interests.

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УДК 619:616.98.578:636.4:575.113:616-085.37

Конструирование прокариотической системы экспрессии фрагмента гена *ORF-2* цирковируса свиней 2-го типа

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РЕЗЮМЕ

Цирковирусные болезни свиней на сегодняшний день являются одной из наиболее значимых проблем свиноводства в развитых странах. Цирковирус свиней 2-го типа (ЦВС-2) считается основным этиологическим агентом синдрома мультисистемного послеотъемного истощения поросят. Случаи массовой заболеваемости свиней цирковирусными болезнями зарегистрированы в большинстве регионов мира, что влечет за собой серьезные экономические последствия. Известно, что оптимальный профилактический эффект в отношении данных инфекций достигается за счет проведения комплекса ветеринарно-санитарных мероприятий в сочетании с вакцинацией. Учитывая высокую эволюционную изменчивость вируса, способствующую появлению новых генотипов и штаммов, вопрос разработки новых кандидатных рекомбинантных вакцин против цирковирусной инфекции, вызванной ЦВС-2, остается

открытым. Целями настоящего исследования явились конструирование прокариотической системы экспрессии фрагмента гена *ORF-2* ЦВС-2 и оценка ее функциональности. Генетическая вставка, сконструированная из наиболее иммуногенных типоспецифических эпитопов ЦВС-2 на основании консенсусной последовательности штаммов и изолятов генотипов 2a, 2b, 2d, клонирована в экспрессионный вектор pET-22b(+), который был реципиирован в штамм *Escherichia coli* Rosetta 2(DE3). Отбор трансформантов осуществляли на селективной среде по маркерному гену устойчивости к ампициллину. Индукцию экспрессии таргетного гена проводили внесением различных концентраций изопропил-*β*-D-1-тиогалактопиранозида. В результате исследований был сконструирован штамм *Escherichia coli* Rosetta 2(DE3)/pET-22b-ORF-2 – продуцент фрагмента капсидного белка (92–233 а. о.). Установлено, что в присутствии 1 мМ изопропил-*β*-D-1-тиогалактопиранозида уровень экспрессии растворимого укороченного rCap достигает 35–40 мг/л через 6 ч постиндукции. Специфичность продукта экспрессии оценивали в непрямом иммуноферментном анализе с сывороткой крови свиньи, гипериммунизированной цельновирионным ЦВС-2. Было показано, что коэффициент позитивности лизатов клеток штамма-продуцента составлял в среднем 4,34 ($p < 0,005$). Рекомбинантный белок rCap пригоден для целей серологической диагностики, а также представляет интерес в качестве компонента вакцины, что является целью наших дальнейших изысканий.

Ключевые слова: цирковирус свиней 2-го типа, синдром мультисистемного послеотъемного истощения, *ORF-2*, прокариотическая система экспрессии

Благодарности: Авторы благодарят старшего научного сотрудника НИЛ OpenLab «Генные и клеточные технологии» ФГАОУ ВО «Казанский (Приволжский) федеральный университет» канд. биол. наук Р. Ф. Хайруллина за предоставление экспрессирующего штамма *Escherichia coli*, а также сотрудников ФКП «Щелковский биокомбинат»: д-ра биол. наук, проф. И. Н. Матвееву и канд. биол. наук Н. В. Хаустову – за предоставление гипериммунной сыворотки к цирковирусу свиней 2-го типа и плодотворную дискуссию.

Для цитирования: Галеева А. Г., Ахунова А. Р., Усольцев К. В., Хаммадов Н. И., Ефимова М. А. Конструирование прокариотической системы экспрессии фрагмента гена *ORF-2* цирковируса свиней 2-го типа. *Ветеринария сегодня*. 2024; 13 (1): 57–63. https://doi.org/10.29326/2304-196X-2024-13-1-57-63

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

Для корреспонденции: Галеева Антонина Глебовна, канд. вет. наук, заведующий лабораторией вирусных антропозоонозов ФГБНУ «ФЦТРБ-ВНИВИ», Научный городок-2, г. Казань, 420075, Республика Татарстан, Россия, *e-mail: antonina-95@yandex.ru*

INTRODUCTION

Today, porcine circovirus-associated diseases (PCVDs) are among the most significant challenges for pig farming in developed countries. Porcine circovirus type 2 (PCV2) is considered the main etiological agent of post-weaning multisystemic wasting syndrome in piglets and also involved in porcine dermatitis and nephropathy syndrome, porcine respiratory disease complex and infectious congenital tremor of piglets [1]. Porcine circovirus-associated diseases can manifest as subclinical and clinical infections, while subclinical infection is the most common form of the infectious process caused by PCV2, and manifested by reduced average daily weight gain without specific clinical signs [2]. The systemic disease mainly affects piglets aged 6–15 weeks and is characterized by anorexia, dyspeptic disorders and lymphadenopathy [3]. Events of high morbidity of pigs caused by circovirus infections have been reported in most regions of the world that results in serious economic consequences for the pig industry [4, 5, 6, 7].

Porcine circovirus type 2 belonging to the *Circovirus* genus of *Circoviridae* family is a small icosahedral non-enveloped DNA-containing virus with a circular genome that is 1,766–1,768 bp long [8]. There are 4 known types of PCV showing high nucleotide identity (68–76%) and similar genomic organization [9, 10]. The genomic DNA of PCV2 consists of several open reading frames encoding main viral proteins – *ORF-1* (replicative protein), *ORF-2* (capsid protein), *ORF-3* (apoptotic protein), *ORF-4* (apoptosis inhibitor) [11]. *ORF-2* encoding a capsid protein with a molecular weight of ≈30 kDa is of the greatest interest for development of tools for specific prevention and diagnosis of diseases caused by PCV2 [12]. To date, 5 main PCV2 genotypes have been identified: 2a, 2b, 2c, 2d, 2e; over the past 20 years 2a, 2b and 2d genotypes have been predominant in different regions of the world [13].

Optimal PCVD prevention is known to be achieved through a set of veterinary and sanitary measures in combination with vaccination. Currently, two vaccines against circovirus infection (PCV2): "Circostop" whole-virion inactivated vaccine (Shchelkovo Biocombinat, Russia) and "VERRES-CIRCO" recombinant vaccine based on *ORF-2* protein produced in the baculovirus expression system (Vetbiochim-Russia), are available on the Russian market [14]. However, considering high evolutionary PCV2 variability promoting potential emergence of different genotypes and strains [15], it can be stated that development of new candidate recombinant vaccines conferring cross-protection against strains of different virus genotypes requires further discussion. An analysis of commercial vaccines available in the world has shown that vaccines based on the recombinant Cap protein produced in baculovirus expression system are successfully used. However, use of this eukaryotic system is often time-consuming and expensive [16], and therefore it is very important to produce a vaccine protein using other expression tools.

The study was aimed at construction of prokaryotic system for PCV2 *ORF-2* gene fragment expression and its functionality assessment.

MATERIALS AND METHODS

Strains and plasmids. To generate a consensus sequence of *ORF-2* gene fragment, the following nucleotide sequences of PCV2 genotype 2a, 2b and 2d epizootiologically significant strain and isolate genomes deposited in the GenBank database of the National Center for Biotechnological Information (NCBI) were used:

– 2a genotype: IAF2897 (ID AF408635.1), ID AY094619.1, DE99/2014 (ID MW262923.1), AUT-1 (ID AY424401.1), SPA1 (ID AF201308.1), 212 (ID AY256455.1);

– 2b genotype: QZ0401 (ID AY691169.1), NL_Control_1 (ID AY484407.1), 24657 NL (ID AF201897.1), n10eu (ID DQ629116.1), ADDLPP 10069 (ID EU594437.1), DE1054/2014 (ID MW262924.1), AUT5 (ID AY424405.1);

– 2d genotype: 28031_Mantova_32_13/12/2013 (ID KP231140.1), Uy99 (ID KP867050.1), BDH (ID HM038017.1), DE222-13 (ID KP698398.1).

Expression pET-22b(+) vector (Novagen, Germany) was used for the target gene cloning and the following *Escherichia coli* Rosetta 2(DE3) strain was used for the target gene expression: F-*omp*T *hsd* SB (rB- mB-) *gal dcm* (DE3) pRARE2 (CamR) (Novagen, Germany), kindly provided by R. F. Khairullin, Cand. Sci. (Biology).

Antigenic construction design. PCV2 amino acid sequences were analyzed using the Immune Epitope Database (IEDB, USA) resource; density of B-cell epitopes was the main criterion for selection of the fragment for expression. BLAST-analysis was used for searching for homologous amino acid sequences of epizootiologically significant PCV2 strains and isolates. Physical and chemical properties of truncated *ORF-2* gene were predicted using Peptide Property Calculator (Innovagen AB, Sweden), homologous modelling of three-dimensional protein structure was performed with SWISS-MODEL webserver (SIB, Switzerland). Truncated *ORF-2* gene sequence (276–699 bp) was optimized by codons for expression into *E. coli* without changes in its amino acid composition and synthesized by the external institution (Eurogene, Russia) and then cloned in pET-22b vector at BamHI and EcoRI restriction sites. Presence of the whole gene in the vector was confirmed by sequencing. Recipient strain cells were transformed by heat-shock followed by further selection on agar medium supplemented with antibiotics (ampicillin – 200 μg/mL, chloramphenicol – 34 μg/mL).

Expression of rORF-2. To induce target gene expression, *E. coli* Rosetta 2(DE3)/pET-22b/ORF-2 strain cells were cultivated in Luria – Bertani nutrient medium containing ampicillin and chloramphenicol in ES-20 temperature-controlled shaker (Biosan, Latvia) at temperature of +37 °C and 180 rpm to achieve optical density (OD) of 0.7. Gene expression was induced by adding of isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) (Promega, USA) at concentration of 1 мМ, then cells were cultivated for 5–6 hours. Cell lysates were generated by mechanical disintegration of the biomass in L-buffer (50 мМ tris-HCl, pH 8.0; 0.3% KCl; 2 мМ PMSF); the target products were subjected to initial purification using NEBB-10 bacterial protein extraction kit (Diaem, Russia). The protein concentration was determined with M. Bradford method [17]. The cell lysates were tested for mature recombinant rCap protein with analytical disc electrophoresis in 15% polyacrylamide gel with Coomassie G250 stain.

Indirect enzyme-linked immunosorbent assay (iELISA). rCap was examined for its serological activity with iELISA. For this purpose, immunoglobulins G were purified from the serum prepared by hyperimmunization of pigs with PCV2 (Shchelkovo Biocombinat, Russia) by one-step ion exchange chromatography on DEAE cellulose [18], and then used for coating of medium-binding polystyrene plates (Corning, USA), 50 ng per well, and the plates were incubated at a temperature of +4 °C for 16 hours. Then, the plates were washed thrice with Tween-containing phosphate-buffered solution (T-PBS) and cell lysates diluted at 1:10 with PBS were added to the wells and the plates were incubated at temperature of +37 °C for 1 hour. The cell lysate transformed by pET-22b plasmid without insertion was used as negative control, recombinant E2 antigen of classical swine fever virus produced in similar expression system was used as heterologous control. After washing, specific peroxidase conjugate prepared using modified P. K. Nakane and A. Kawaoi method [19]

Table
The main epitopes of Cap protein included in the antigenic composition

Epitope (sequence, name)	Position in proteome, a. a.	Characteristics
RPWLVHPRHRY (external core epitope)	26–36	Conformational epitope that reacted with PCV-positive porcine sera; it is considered one of the predominant domains based on its PCV2 neutralizing capacity [20]
TRLSRTFGYTVK (P100)	47–58	Peptides demonstrated high binding reactivity with sera from PCV2-immune pigs. It is reported that they can be used for differentiation of vaccinated and convalescent animals [21]
PFEYYRIRKVKVEFWP (P102)	92–107	
CSPITQGDRGVGSSAVILDDNFVT KATALTY (C2)	108–137	
VTMYVQFREFNLKDPPLKP (P106)	215–233	
KATALT (EF-region)	134–139	It was identified as type-specific neutralizing epitope. It was shown to be used for differentiation of pathogenic PCV2 and non-pathogenic PCV1 [22]
YHSRYFT	156–162	Conformational epitope fragment was recognized by PCV1- and PCV2-neutralizing monoclonal antibodies; was detected in transfected PK-15 cells [23]
VLDSTIDYFQPNNKR	166–180	The epitope included in the candidate vaccine based on infectious PCV2b clone prevented virus replication in experimentally infected pigs [24]
VDHVGLGTAFENSIY	193–203	It is a potential heparan sulfate binding site responsible for PCV2 attachment to target cells [25]

was added, 20 ng per well, and the plates were incubated as described for the first step. The plates were washed five times and the substrate, tetramethylenbenzidine, was added. The plates were incubated in the dark place at room temperature for 15–20 minutes. Then, stop solution (0.2 M sulfuric acid solution) was added and test results were read at a wavelength of 450 nm using Model 680 plate reader (Bio-Rad, USA). Optical density (OD) values were interpreted semi-quantitatively with an indication of the positivity index – the ratio of test sample OD value to cut-off value calculated based on minimum absorbance of the samples collected at the post-induction stage. The data were analyzed using Statistica 7.0 software (StatSoft, USA) with Mann – Whitney U test adjusted for multiple comparisons. The p-value < 0.05 was considered the threshold value for statistically significant differences.

RESULTS AND DISCUSSION

Bioinformatics analysis has shown that the 92–233 a. a. fragment of Cap protein is of the greatest interest for designing the antigenic composition structure, since it is characterized by the highest density of type-specific epitopes. Moreover, some researchers previously reported the immunogenic potential of different domains of this fragment (Table).

Thus, modified fragment of truncated Cap amino acid sequence comprises the major significant epitopes being the targets for serological diagnosis. Prediction of the physical and chemical properties of this multi-epitope polypeptide demonstrated that its molecular weight was 17.8 kDa, it was highly soluble and had an optimal half-life (more than 20 hours) *in vivo* for *E. coli*.

At the next stage of the study, pET-22b/ORF-2 expression vector was constructed and used for transformation of *E. coli* Rosetta 2(DE3) cells, then the conditions for producer strain cultivation were optimized. Thus, different modes were used for induced cell cultivation: the different ranges of culture OD values before induction (from 0.5 to 1.0 ODU), different concentrations of added IPTG (from 0.2 to 2.0 mM), different time periods (from 2 to 6 hours) and temperatures (from +25 to +37 °C) were tested. Tests of the polypeptide profiles of cell lysates cor-

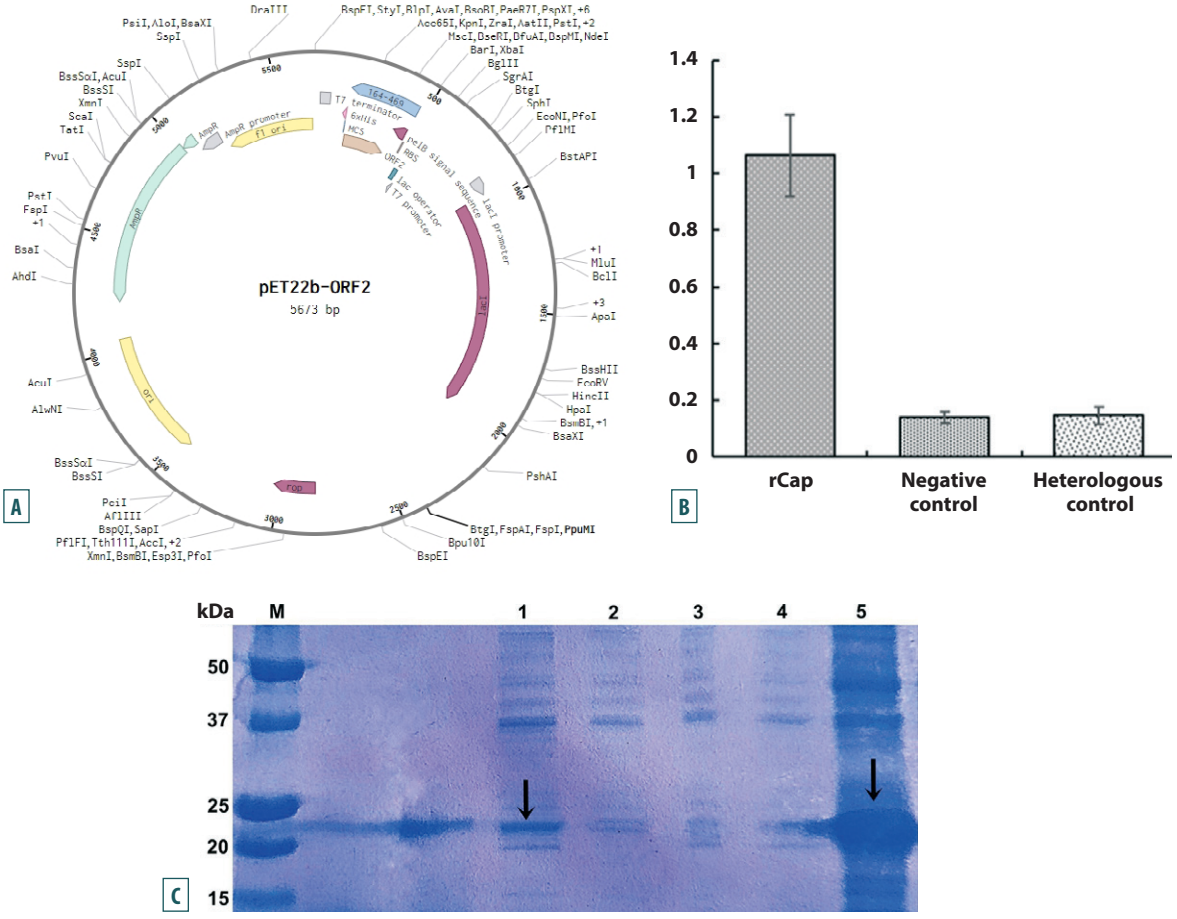

Fig. Characteristics of the prokaryotic PCV-2 rCap expression system:
A – scheme of the pET-22b/ORF-2 expression vector; B – optical density of rCap tested with iELISA using hyperimmune porcine serum, where negative control is lysate of cells transformed with pET-22b plasmid without insertion, and heterologous control is a recombinant E2 protein of classical swine fever virus, produced in the same expression system; C – electrophoregram of expression products, where M band is the molecular weight marker Precision Plus Protein™ Unstained Protein Standards (Bio-Rad, USA), bands: 1 – target protein after primary purification; 2 – negative control (lysate of cells transformed with the pET-22b plasmid without insertion); 3, 4 – lysates of cells transformed with the pET-22b/ORF-2 plasmid before induction; 5 – producer strain cell lysate 6 hours after induction. The target protein fraction is indicated by arrows

responding to each set of cultivation parameters showed that the optimal parameters were as follows: culture OD value before induction – 0.6 ODU, IPTG concentration – 1 mM, post-induction cultivation conditions – 6–7 hours at a temperature of +37 °C. This cultivation mode provides the recombinant product yield at the level of 35–40 mg/L, which is 1.75–2.00% of total biomass of the producer.

rCap serological activity and specificity was confirmed by testing with iELISA. Thus, total protein product OD was 1.065 ± 0.144 ODU, the positivity index was 4.34 ($p < 0.005$). The main characteristics of the prokaryotic rCap expression system are shown in the Figure.

The presented data confirm functionality of the developed expression system that holds great promise for further use of produced rCap for diagnostic and preventive purposes.

Some researchers have been reported successful *coli*-expression for producing anti-PCV2 vaccine components. For example, X. Xi et al. reported that a full-length soluble Cap, expressed in *E. coli,* under neutral pH conditions self-assembles into homogeneous virus-like particle (VLP), corresponding in size to intact PCV2 and providing protection *in vivo* comparable to that one provided by commercial vaccines [26]. Expression of Cap fused with various modified bacterial proteins is of particular interest. In particular, there is evidence that recombinant Cap fused with flagellin has elicited higher virus neutralizing antibody levels in the mouse model thereby promoting humoral and cellular immune responses [27]. However, limitations of prokaryotic expression systems have also been reported. It is known that the full-length Cap can be expressed only in those *E. coli* strains that possess plasmids carrying tRNA genes of rare *E. coli* codons (pRARE). Moreover, the expression product often cannot be purified under native and denaturing conditions so additional optimization of the insertion is required [28].

Despite the acceptable levels of truncated rCap synthesis achieved and the product specificity confirmed during this study, the development of an efficient and effective method of its purification will play a key role in vaccine protein production scaling up.

CONCLUSION

Prokaryotic system for expression of PCV2 *ORF-2* gene fragment containing the most immunogenic capsid protein epitopes was constructed during the study. The expression system functionality was confirmed by presence of mature multi-epitope polypeptide in cell lysates of the producer as well as by serological activity detected by iELISA using anti-PCV2 hyperimmune porcine sera. According to these data, the expressed polypeptide appears to be suitable for serodiagnosis and is also of interest as a component of the recombinant vaccine that will be the goal of our further studies.

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Received 13.12.2023
Revised 17.01.2024
Accepted 14.02.2024

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