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DEVELOPMENT OF THE RECOMBINANT POSITIVE CONTROLS OF BOVINE VIRAL DIARRHEA VIRUS 1 AND 2 FOR PCR ASSAY

Aim. *The method of amplifying and cloning the E^{rns} gene of Bovine Viral Diarrhea Virus was developed to obtain the positive controls for polymerase chain reaction.*
Methods. *The strains used in this study were BVDV-1b (Ossloss) and BVDV-2 (Kosice). Viral RNA was extracted by the silica-based extraction method. Using the specific primers, a part of E^{rns} gene was amplificated. The PCR product was inserted into the cloning vector pTZ57R/T. Furthermore, E. coli DH10B bacteria were transformed to amplify the recombinant plasmid. Recombinant clones were identified by antibiotic selection on agar plate and confirmed by PCR. Moreover, insert of E^{rns} gene was verified by restriction enzyme digestion assay using EcoRI and HindIII.*
Results. *It was shown that we had constructed the recombinant plasmids with insertion E^{rns} gene fragment (826 base pair) of BVDV-1 and BVDV-2.*
Conclusion. *The obtaibed recombinant plasmids can be used as a positive control for PCR.*

K e y w o r d s: Bovine Viral Diarrhea Virus, PCR, cloning, pTZ57R/T, restriction enzyme digestion analysis.

Bovine Viral Diarrhea Virus (BVDV) is a member of the genus *Pestivirus* within the family *Flaviviridae* [8]. BVDV comprises two species, type 1 (BVDV-1) and type 2 (BVDV-2) [12]. Other representives of this genus are classical swine fever virus and border disease virus of sheep. All four *Pestivirus* species are closely genetically and antigenically related. Whereas cattle are naturally infected with BVDV-1 and BVDV-2, sheep can be infected by both bovine viruses in addition to BDV, and swine can be infected with all four Pestivirus species. [2].

The pestiviral genome consists of a single-stranded positive-sense RNA of about 12.3 kb that contains one open reading frame (ORF) coding for a

polyprotein of about 4000 amino acids [3]. The ORF is at both ends flanked by untranslated regions (UTR) [10]. The nucleocapsid protein C and the glycoproteins E^{rns}, E1, and E2 are structural components of the virion [9]. Both E^{rns} and E2 regions are found on virions [14], induce neutralizing antibodies [7], and elicit protective immunity in their natural host [13]. The considerable amount of E^{rns} is also secreted from the infected culture cells.

BVDV has a significant economic impact on the cattle industry due to abortions, production of weak calves, congenital deformities, respiratory infections, thrombocytopenia, persistent infections, and mucosal disease [5]. The strains of BVDV-1 usually cause only mild diarrhea in cattle that were immunized, whereas some strains of BVDV-2 are highly virulent and cause severe thrombocytopenia and hemorrhage or mucosal inflamation illness [6].

Due to the increase of using cellular biotechnology, the risk of viral contamination of their products rises [1]. For today the control methods for sterility, in particular, eliminate viral contamination of animal origin raw materials and ready preparations are important.

In the study of animal origin raw material for the screening of pestiviruses contamination preferable to use the methods in which there are not used the culture of the viruses as a positive control. The aim of this work was to construct a recombinant plasmid vector including insertion of region E^{rns} gene of BVDV type 1 and 2 for further work on the production of recombinant positive control used in the polymerase chain reaction (PCR) diagnostic of BVDV.

Materials and methods

The samples of dried blood on filter papers contained BVDV-1b (strain Ossloss) and BVDV-2 (strain Kosice) was kindly provided by Prof. Stefan Vilcek (The University of veterinary medicine and pharmacy in Kosice, Slovak republic).

Extraction of RNA from dried blood on filter papers was performed using silica-based extraction method [4]. The second strand cDNA synthesis and amplification was applied using «GenePak RT-PCR Core» and «GenePak PCR Core» (Ltd. Lab. Isogene, Russian Federation) accordingly.

The primer sets P1: 5'-AACAAACATGGTTGGTGCAACTGGT-3' (forward primer) and P2: 5'-CTTACACAGACATATTTGCCTAGGTTCCA-3' (reverse primer) designed by D. Sullivan and R. Akkina et al. [11] were used for PCR amplification of E^{rns} gene. The PCR-amplification was performed with temperature program consisting of initial denaturation (94 °C, 2 min) and 35 cycles of denaturation (94 °C, 1 min), primer annealing (55 °C, 1 min) and primer extension (72 °C, 1 min) in an automatic thermalcycler Biometra T3000. A final step of extension at 72 °C for 10 min. 25 μL of the PCR products were evaluated by gel electrophoresis in 1% agarose gel with ethidium bromide (0.1%).

DNA products were purified from gel using «Silica Bead DNA Gel Extraction Kit» (Fermentas, Lithuania). Eluted DNA products were ligated into the plasmid vector pTZ57R/T as recommended by the supplier («InsTAclone PCR Cloning Kit», Fermentas, Lithuania). 10 μL of DNA eluted from one PCR reaction mixture was ligated to 3 μL of TA vector at 4 °C overnight with 1μL T4 ligase.

DH10B E. coli cells were used as the host for cloning experiments with pTZ57R/T vector. Stock culture was maintained at -70 °C in 25% glycerol. All culture was grown in LB broth at 37 °C in shaker incubator. In order to insert recombinant vector into the component cells, «InsTAclone PCR Cloning Kit» (Fermentas, Lithuania) was used. Transformed *E. coli* of DH10B strain were grown in LB-Ampicillin agar plates at 37 °C.

Transformed bacterial colonies were screened directly for presence of recombinant plasmids by PCR using primers P1/P2 and M13/pUC_F/R. Plasmids DNA were purification from recombinant *E. coli* culture using «Plasmid Miniprep Kit» (GeneJET, Lithuania). cDNA inserts in plasmids were further characterized by restriction endonuclease digestion with restriction endonucleases *Eco*RI and *Hind*III in reaction buffer B2 (Jena Bioscience, Germany). For confirming 2 μL 10x buffer B2, 14 μL dH_20, 2 μL product and 2 μL of restriction enzymes were mixed and incubated at 37 °C for 16 h. Further the inactivation was conducted by incubating at 65 °C for 20 min. The results were checked by electrophoresis in a 1.5% agarose gel.

Results and discussion

As the first step of our study the viral RNA from 2 samples of dried blood on filter papers was used as a template for cDNA synthesis using reverse transcription followed by specific amplification by the polymerase chain reaction. The PCR product was checked by agarose gel electrophoresis followed extraction from gel (fig. 1). In gel electrophoresis the specific bands were observed at the position corresponding of Erns gene 826 bp in length.

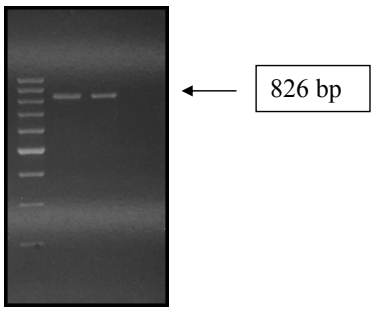

Fig. 1. Electrophoregram of PCR amplified product using the primers P1/P2:
M — the DNA marker (100 bp DNA Ladder, Ltd. Lab. Isogene, Russian Federation);
1 — BVDV 1; 2 — BVDV 2; 3 — negative control.

The resulting PCR product was inserted into the corresponding site of cloning vector pTZ57R/T. The constructed plasmids pTZ57R/T-VD1 and pTZ57R/T-VD2 (fig. 2) had fragment of insert E^{rns} gene and providing resistance to ampicillin beta-lactamase gene (bla(Ap^R)), nucleotide complementary to primer M13/pUC sequence provided the selection of bacterial cells containing recombinant plasmids.

Two recombinant plasmids were transformed into the cells of *E. coli* DH10B. Screening of plasmids containing *E. coli* clones was performed in selective medium with adding of ampicillin. For eliminating the artifact DNA structures there were conducted PCR screening of all colonies with acquired resistance to ampicillin.

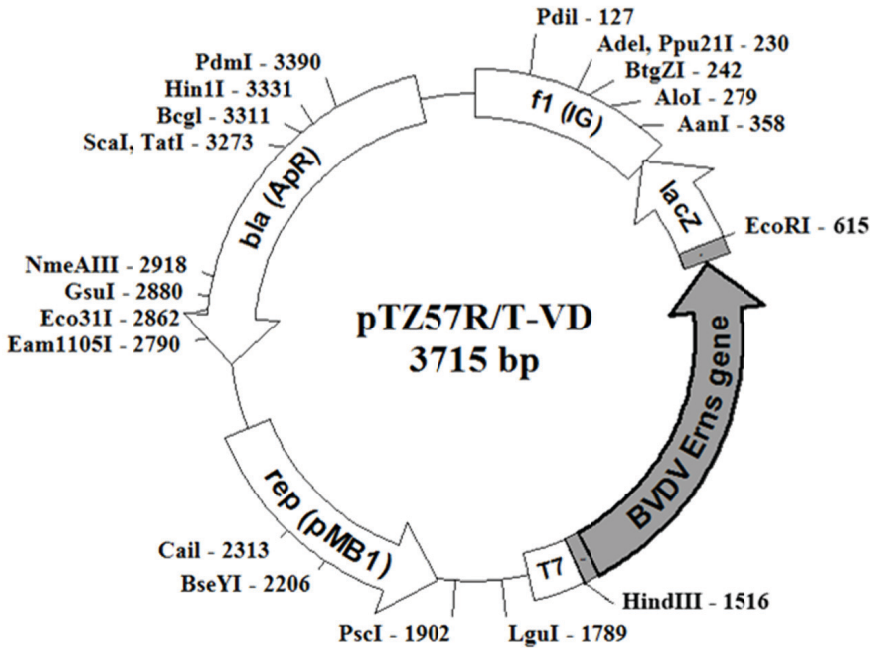

Fig. 2. Restriction site map of the pTZ57R/T cloning vector [15] with insertion of E^{rns}

Therefore during amplification of a target gene using the primers P1/P2 and M13/pUC_F/R there were obtained the clones of *E. coli* containing plasmids with predetermined insert (fig. 3).

The presence of target gene in four recombinant clones were further confirmed by restriction enzyme digestion assay using restriction endonuclease enzymes EcoRI and HindIII cut plasmids in relative restriction sites. According to the results of electrophoregram there were observed the presence of two fragments of expected length — 2814 and 901 bp (fig. 4).

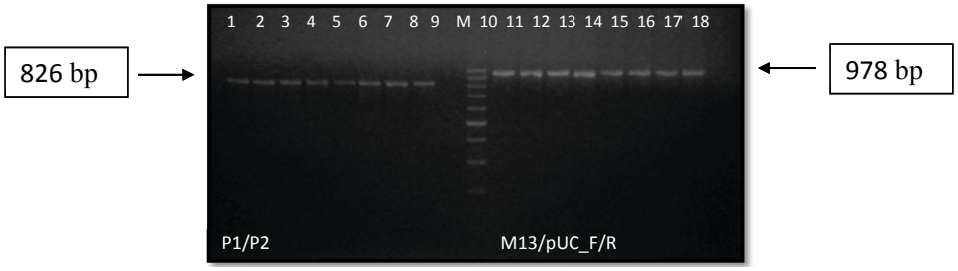

Fig. 3. Electrophoregram of PCR amplified product of target gen using the primers P1/P2 and M13/pUC_F/R:
1–4, 10–13 — recombinant colonies with insertion of Erns gene from BVDV-1;
5–8, 14–17 — recombinant colonies with insertion of Erns gene from BVDV-2;
9, 18 — negative control; M — the DNA marker
(100 bp DNA Ladder, Ltd. Lab, Isogene, Russian Federation).

The culture of *E. coli* cells including the recombinant plasmids with insertion fragment of the E^{rns} gene was obtained. The plasmids can be used as the positive controls for BVDV detection by PCR method.

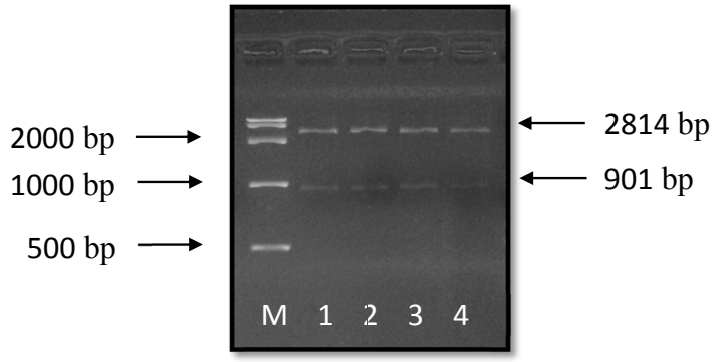

Fig. 4. Electrophoregram of the plasmids pTZ57R/T-VD after restriction enzyme digests:
1–2 — recombinant colonies with insertion of Erns gene from BVDV-1;
3–4 — recombinant colonies with insertion of Erns gene from BVDV-2;
M — the DNA marker (FastRuler High Range DNA Ladder, Fermentas, Lithuania).

The authors express appreciation to Prof. Stefan Vilcek (The University of veterinary medicine and pharmacy in Kosice, Slovak republic) for kindly providing the samples of BVDV-1b (strain Ossloss) and BVDV-2 (strain Kosice).

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РОЗРОБКА РЕКОМБІНАНТНОГО ПОЗИТИВНОГО КОНТРОЛЮ ВІРУСУ ДІАРЕЇ ВЕЛИКОЇ РОГАТОЇ ХУДОБИ І ТА ІІ ТИПУ ДЛЯ ВИКОРИСТАННЯ В ПЛР

Реферат

Мета. Розробка методу ампліфікації та клонування гену E^{rns} вірусу діареї великої рогатої худоби з метою отримання позитивного контролю для полімеразної ланцюгової реакції. ***Методи***. У роботі були використані віруси діареї (ВД) ВРХ І типу штам Ossloss та ІІ типу штам Kosice. Вірусну РНК екстрагували за допомогою методу афінної сорбції. Ампліфікацію ділянки гену E^{rns} здійснювали з використанням специфічних праймерів. ПЛР-продукт був інтегрований до вектору для клонування pTZ57R/T. Для напрацювання рекомбінантних плазмід проводили трансформацію клітин бактерії E. coli DH10B. Плазмідвмісні клони виявляли за допомогою селективного поживного середовища, вставку — методом ПЛР. Наявність вставки гена E^{rns} підтверджували за допомогою рестрикційного аналізу з використанням рестриктаз EcoRI та HindIII. ***Результати***. В ході проведених досліджень були сконструйовані рекомбінантні плазміди, які несуть вставку фрагменту гену E^{rns} (826 пар нуклеотидів) збудників ВД ВРХ І та ІІ типів. ***Висновки.*** Отримані рекомбінантні плазміди можуть використані як позитивний контроль для ПЛР.

Ключові слова: вірусна діарея великої рогатої худоби, ПЛР, клонування, pTZ57R/T, рестрикційний аналіз.

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РАЗРАБОТКА РЕКОМБИНАНТНОГО ПОЛОЖИТЕЛЬНОГО КОНТРОЛЯ ВИРУСА ДИАРЕИ КРУПНОГО РОГАТОГО СКОТА I И II ТИПА ДЛЯ ИСПОЛЬЗОВАНИЯ В ПЦР

Реферат

Цель. Разработка метода амплификации и клонирования гена E^{rns} вируса диареи крупного рогатого скота с целью получения положительного контроля для полимеразной цепной реакции. **Методы**. В работе использованы вирусы диареи (ВД) КРС I типа штамм Ossloss и II типа штамм Kosice. Вирусную РНК экстрагировали с помощью метода аффин-

ной сорбции. Амплификацию участка гена E^{rns} осуществляли с использованием специфических праймеров. ПЦР-продукт был интегрирован в вектор для клонирования pTZ57R/T. Для накопления рекомбинантных плазмид проводили трансформацию клеток бактерии *E. coli* DH10B. Плазмидсодержащие клоны выявляли с помощью селективной питательной среды, наличие вставки — методом ПЦР. Присутствие вставки гена E^{rns} подтверждали с использованием рестрикционного анализа с помощью рестриктаз *EcoR*I и *Hind*III. **Результаты.** В ходе проведенных исследований сконструированы рекомбинантные плазмиды, содержащие вставки фрагмента гена E^{rns} (826 пар нуклеотидов) возбудителей ВД КРС I и II типов. **Выводы.** Полученные рекомбинантные плазмиды могут быть использованы в качестве положительного контроля для ПЦР.

К л ю ч е в ы е с л о в а: вирусная диарея крупного рогатого скота, ПЦР, клонирование, pTZ57R/T, рестрикционный анализ.

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