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EXPRESSION OF RECOMBINANT P24 PROTEIN IN ESCHERICHIA COLI FOR SEROLOGICAL DIAGNOSIS OF BOVINE LEUKOSIS

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Abstract

Bovine leukemia is an infectious lymphoproliferative disease in cattle caused by the bovine leukemia virus (BLV). Production losses associated with BLV include increased cull rates, decreased milk production, or reduced fertility. Serological tests are used for diagnosis of BLV, of which the most sensitive is enzyme-linked immunosorbent assay (ELISA). Capsid p24 protein is a diagnostically important antigen of the BLV. The gene of p24 protein was cloned into pET-28 plasmid vector. The recombinant p24 antigen was obtained by using Escherichia coli ArcticExpressRP(DE3)_pET-28/p24 strain. The total yield of the purified p24 protein was 76 mg. Testing of the recombinant p24 protein on control sera showed that the p24 binds to positive control sera and has high antigenic activity. The pilot testing of the recombinant p24 protein on 48 animal samples resulted in 83% positive and 17% negative. The results indicate that the recombinant p24 protein is promising in enzyme-linked immunosorbent assay for the diagnosis of bovine leukemia.

Keywords: bovine leukemia virus, antigen, ELISA, retrovirus, DNA, recombinant protein, expression

Introduction

Bovine leukemia is an infectious lymphoproliferative disease in cattle caused by the bovine leukemia virus (BLV) [1,2]. A feature of this disease is that the majority of animals remain constantly infected without any external signs of infection. Approximately 29% of BLV-infected cattle develop persistent lymphocytosis, while less than 5% of BLV-infected cattle develop lymphosarcoma [3]. Production losses associated with BLV include increased cull rates, decreased milk production, or reduced fertility [4-6]. The economic impact of BLV infection is also related to restrictions on commercialization between countries in the field of animal husbandry.

Bovine leukemia virus belongs to the Retroviridae family, Oncovirinae subfamily, Oncovirus genus. Viruses are spherical, 40-90 nm in size and consist of a nucleicapsid with a cubic type of symmetry and a lipoprotein shell that forms protrusions on the surface. The nucleotide contains a diploid genome, represented by two single-stranded linear RNA molecules. The virus is unstable to environmental factors and is inactivated at high temperatures (56 °C and above), UV radiation, under the influence of ultrasound. Virus contain external glycoprotein and internal polypeptide antigens. Glycoprotein antigens have hem-agglutinating activity and induce the formation of virus neutralizing antibodies that protect animals from infection. Polypeptide antigens induce the formation of precipitating and complement-binding antibodies. BLV strains are related to each other, but differ from other retroviruses and viruses of other genera in antigenic properties, morphogenesis, formation in monolayer cell cultures, and the property of reverse transcriptase.

The virus is localized in the lymphoid cells of various organs, although it can be detected only in a colostrum and a milk. Structural proteins of BLV: gp51 (Env) and p24 (Gag) have diagnostic value and are present in cow's milk exosomes from BLV-infected cattle [7]. In cells of infected animals, the virus persists for life. In infected lymphocytes (in provirus stage), the virus is protected from the action of neutralizing antibodies and can be transmitted to offspring during cell division. A provirus can change the expression of a cell's

genes, which leads to its transformation and the development of a tumor stage of leukemia.

The diagnosis of leukemia is made based on epizootological, clinical, pathological data and laboratory results, which play the main role. Serological tests are used for diagnosis, of which the most sensitive are agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA). They allow the detection of specific antibodies in infected animals from 6 months of age and older. ELISA is used to detect specific antibodies in blood serum, milk and colostrum. Diagnostic kits for indirect and competitive ELISA variants are used. This method allows large-scale studies of epizootic leukemia in cattle herds. Seropositivity can serve as a reliable indicator of infection, since animals infected with the BLV develop a stable immune response characterized by a high titer of antibodies directed both to the gp51 envelope glycoprotein and to the capsid protein p24 [8]. AGID and ELISA are diagnostic tests approved by the World Organization for Animal Health, and both are accepted as recommended diagnostic methods for sanitary services. Both methods are a good diagnostic tool in BLV serological diagnosis [9-11].

Capsid protein of BLV – p24 is a diagnostically important antigen of the virus [12, 13]. This antigen with gp51, is actively used as a marker for diagnosing diseases in cattle and in experimental studies to study the resistance of BLV to antibodies and the action of H2O2. [14,15]. The activity of human immunoglobulins against the p24 capsid protein was used to detect antibodies in human serum [16]. Obtaining natural viral p24 antigen seems to be a laborious and costly work, however, the use of recombinant DNA technology makes it possible to effectively solve this problem.

To obtain antigens in retroviral serology, recombinant technology is used: the expression of gp51 and p24 proteins in heterologous expression systems, such as *Escherichia coli, Saccharomyces cerevisiae,* baculoviruses [17-21].

The aim of the work is the expression of the recombinant capsid p24 protein of the bovine leukemia virus in Escherichia coli cells and its testing as an antigen in the serum of bovine leukemia.

Materials and methods of research

Strain and vector

To express the p24 gene of the bovine leukemia virus, the Escherichia coli ArcticExpress(DE3)RP strain with genotype ompT hsdS(rB – mB–) dcm+ Tetr gal λ(DE3) endA Hte [cpn10 cpn60 Gentr] [argU proL Strr] was used. Plasmid pET-28/p24 from Biobank NCB was used as an expression vector.

Mediums

The Lennox Broth (LB) (1% tryptone, 0.5% yeast extract, 0.5 % NaCl, pH 7.5) and Super Optimal Broth (SOC) (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM MgSO4, 20 mM glucose, pH 7.5) were used. Preparation of media was carried out in accordance with the Maniatis protocol [22].

SDS-PAGE and determination of protein concentration

SDS-PAGE was performed as described by Laemmli [23] in 12% polyacrylamide. The gel was stained with 10% acetic acid in 50% ethanol containing 2% Coomassie Brilliant Blue R-250 (AppliChem, Darmstadt, Germany) for 2 h. The gel was destained in 7.5% acetic acid with 25% ethanol. The results were analyzed using the molecular weight marker - Pierce Unstained Protein MW Marker (Cat.#26610, Thermo Scientific). Protein concentration was determined by Bradford method [24] using Bio-Rad assay reagent (Bio-Rad Protein Assay Day, Munich, Germany) and bovine serum albumin as the standard. The measurements were carried out in three repetitions, with the average of the three repetitions being reported as the specified results.

Expression of p24 in E.coli cells

Competents cells of E.coli ArcticExpressRP(DE3) were transformed with pET-28/p24 plasmid vector by electroporation (MicroPulser, Bio-Rad). The selection of clones was carried out on agar LB medium with 50 μg/mL kanamycin. The selected clone was cultured in 1000 mL of LB broth supplemented with kanamycin (50 μg / mL) in the incubator shaker (Climo-Shaker ISF1-X, Kuhner, Switzerland) at 37 °C and 150 rpm. In the middle of the logarithmic growth phase (OD600 = 0.6) 0.2 mM isopropyl β-d-1-thiogalactopyranoside (IPTG) was added and the culture was incubated for 16 hours at 30 °C and 150 rpm. The cells were collected by centrifugation at 6,000 × g for 7 min at 4 °C.

Purification of p24 protein

Inducted cells of ArcticExpressRP(DE3)_ pET-28/p24 strain were suspended in lysis buffer

(20 mM NaCl, 20 mM Hepes-NaOH pH 7.6) and lysed with lysozyme (2 mg/mL) at room temperature (RT) for 30 minutes followed by ultrasound sonication. The lysate was clarified by centrifugation (40000 × g, 1 hour, 4 °C) on Beckman Coulter Avanti J-26S XP. The prepacked HiTrap Chelating HP 1 mL (General Electric) column was washed with 100 mM NiCl2 for activation with Ni2+ ions and equilibrated by buffer (500 мМ NaCl, 20 mM Imidazole, 20 mM Hepes-NaOH pH 7.6). The supernatant was loaded into the column. The column washed with the same buffer and the recombinant protein was eluted by linear gradient with imidazole (20-500 mM) using FPLC (AKTA Purifier 10, General Electric). The purity of fractions was checked by SDS-PAGE, the positive fractions were polled and dialyzed against phosphate-buffered saline (PBS) in 1000 mL volume for 18 hours at 4 °C.The membrane with 14 kDa cut-off (Sigma-Aldrich, St. Louis, USA) was used for dialysis and the buffer was changed every 6 hours.

ELISA

The NUNC immuno plates were coated overnight at 4 °C with 100 µL per well of recombinant p24 antigen at a concentration of 1 µg/mL in 50 mM carbonate/bicarbonate buffer (pH 9.6). Plates were washed 100 mL PBST 3 fold and blocked with 100 µL of PBS with 10% of equine serum and 0.2% Tween 20 (PBST) for 1 hours at 37°C. Serum samples were incubated in a 1/10 dilution in PBST for 60 minutes at RT. After serum incubation plates were washed with PBST and 100 µL of diluted 10000 anti-Bovine IgG peroxidase labelled conjugate horseradish peroxidase (HRP) was added and incubated for 60 minutes at RT. Color development was carried out with 3,3',5,5' tetramethylbenzidine (TBM, Abcam, UK) according to the manufacture's recommendations. After 10 minutes the reaction was stopped with 1M sulphuric acid and absorbance was read immediately in a microplate reader at 450 nm.

Results pET-28/p24 vector is a plasmid with an integrated gene for the p24 capsid protein of the BLV. The p24 protein gene has a length of 657 bp and is inserted under the control of the bacteriophage T7 promoter. Figure 1 shows the plasmid map of pET-28/p24 vector. The open reading frame of p24 polypeptide carries a hexahistidine tag from the N-terminus (6His-tag) fused to p24 with the serine-glycine joint. The calculated mass of the recombinant p24 protein with additional amino acid residues is 26.1 kDa. pET-28/p24 vector contains the resistance marker (KanR) to the antibiotic kanamycin.

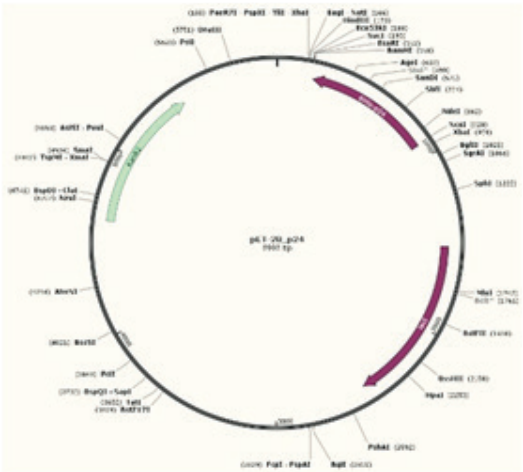


Figure 1 – Plasmid map of pET-28/p24 vector

Escherichia coli ArcticExpress(DE3)RP was used as a host strain. The proteome of the strain contains two chaperons from psychrophile Olespira antarctica – Cpn10 and Cpn60. Due to the action of these chaperones in the ArcticExpress(DE3) RP strain, the process of folding of heterologous proteins proceeds more efficiently, including at low temperatures: 18°C-30°C. This strain also contains eukaryotic codons for proline and arginine. After transformation of competent ArcticExpress(DE3) RP cells with the pET-28/p24 and selection of clones on LB-agar with kanamycin, the producing strain of the recombinant p24 protein was obtained. The presence of 6His-tag in the structure of the recombinant p24 protein made it possible to quickly and efficiently purify it by metal affinity chromatography (Figure 2).

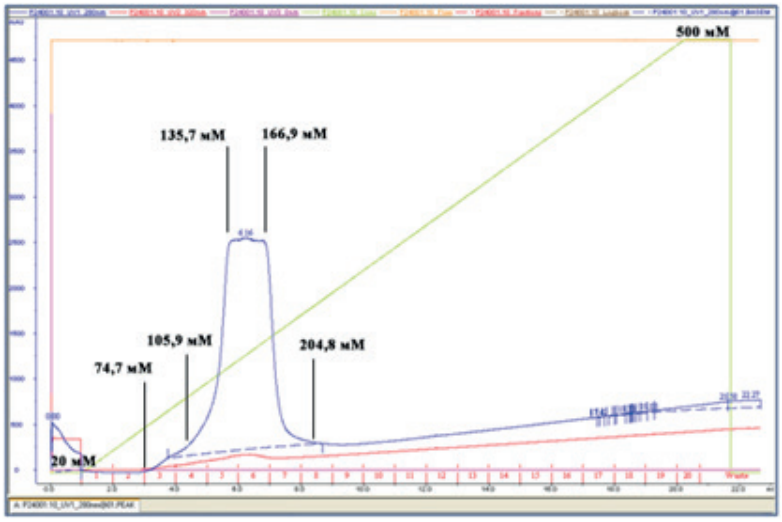
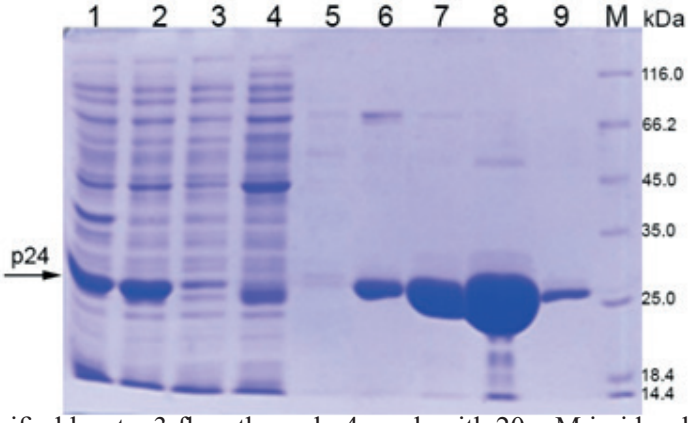


Figure 2 – Chromatogram for purification of the recombinant p24 protein



1-total lysate; 2-clarified lysate; 3-flow through; 4-wash with 20 mM imidasole; 5-elution with 74.7 mM imidasole; 6-elution with 100 mM imidasole; 7-elution with 105.9 mM imidasole; 8-elution with 135.7-166.9 mM imidasole; 9-elution with 204.8 mM imidasole; M-protein marker

Figure 3 – Purification of recombinant p24 protein from induction ArcticExpress(DE3)RP_ pET-28/p24 culture

Table. Results of ELISA with recombinant p24

No	OD_{450}	Result	No	OD_{450}	Result
1	0.270	NC1	26	0.836	POS
2	1.510	PC1	27	0.261	NEG
3	0.742	POS	28	1.181	POS
4	0.632	POS	29	0.923	POS
5	0.461	POS	30	0.577	POS
6	0.613	POS	31	1.136	POS
7	0.647	POS	32	0.689	POS
8	0.238	NEG	33	0.993	POS
9	0.551	POS	34	1.385	POS
10	0.444	POS	35	0.722	POS
11	0.839	POS	36	1.267	POS
12	0.352	NEG	37	1.333	POS

13	1.288	POS	38	1.268	POS
14	0.244	NEG	39	1.204	POS
15	0.300	NEG	40	0.287	NEG
16	0.510	POS	41	0.854	POS
17	0.672	POS	42	1.136	POS
18	0.549	POS	43	0.533	POS
19	0.285	NEG	44	0.616	POS
20	0.723	POS	45	0.711	POS
21	1.161	POS	46	0.805	POS
22	1.183	POS	47	1.241	POS
23	0.641	POS	48	0.261	NEG
24	1.156	POS	49	0.986	POS
25	0.753	POS	50	0.653	POS

Discussion of the results and conclusion

The recombinant p24 protein was overexpressed in the ArcticExpress (DE3)RP_ pET-28/p24 strain. The p24 protein collected in cytosol fraction without transition in including bodies. This process can be explained by the nature of the p24 protein, the folding activity of the Cpn10 and Cpn60 chaperones, and the presence of eukaryotic codons for Pro and Arg. Prolines in the p24 are 17 and Arginine are 10. The concentration of purified p24 in polled eluates was 15.2 mg/mL. The total yield of the purified p24 protein was 76 mg from 1000 mL, which is a very high result. For comparison, Gutierres et al carried out a similar work on obtaining p24 from E. coli and the yield was 10 mg from 200 mL induced bacterial culture [13]. Yield of p24 recombinant protein in [17] was 55 mg from 1 liter of E.coli culture.

Testing of the recombinant p24 protein on control sera showed that the recombinant p24 antigen binds to positive control sera and has high antigenic activity. The results of the pilot testing of the recombinant p24 protein on animal samples showed that the samples showed 83% positive and 17% negative results.

In addition to ELISA, an actively used serological method is the AGID [25]. However, with comparatively the same indicators of the specificity of both methods, the enzyme immunoassay, in contrast to the immunodiffusion reaction, revealed 2 times more positively reacting animals, which is an undoubted advantage in the mass examination of animals [13]. Gutierrez G et al investigating the analytical sensitivity and specificity of ELISA based on recombinant p24, determined that ELISA detected antibodies against the p24 antigen in all positive control sera with a high degree of sensitivity [13]. In general, it can be noted that the use of enzyme-linked immunosorbent assay for mass studies of cattle is the best test when carrying out measures to eliminate the disease, since the sensitivity and specificity of the test systems used was 88-100 % [25].

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ЭКСПРЕССИЯ ГЕНА РЕКОМБИНАНТНОГО БЕЛКА P24 В КЛЕТКАХ *ESCHERICHIA COLI* ДЛЯ СЕРОЛОГИЧЕСКОЙ ДИАГНОСТИКИ ЛЕЙКОЗА КРУПНОГО РОГАТОГО СКОТА

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Аннотация

Лейкоз крупного рогатого скота является инфекционным лимфопролиферативным заболеванием животных, вызываемое вирусом лейкоза КРС. Потери производства, связанные с данным заболеванием, включают повышенный процент выбраковки, снижение производства молока и фертильности животных. Для диагностики лейкоза используются серологические тесты, из которых наиболее чувствительным является иммуноферментный анализ. Капсидный белок p24 является важным антигеном для диагностики лейкоза КРС. В рамках данной работы ген белка p24 клонировали в плазмидный вектор pET-28. Рекомбинантный антиген p24 был получен с использованием штамма Escherichia coli ArcticExpressRP (DE3)_pET-28/p24. Общий выход очищенного белка p24 составил 76 мг с 1 литра культуры. Тестирование рекомбинантного белка p24 на контрольной сыворотке показало, что рекомбинантный антиген p24 связывается с сывороткой положительного контроля и обладает высокой антигенной активностью. Результаты пилотного тестирования рекомбинантного белка p24 на 48 образцах животных показали, что образцы показали 83% положительных и 17% отрицательных результатов. Полученный результаты свидетельствуют о перспективности полученного рекомбинантного p24 белка в иммуноферментном анализе для диагностики лейкоза КРС.

Ключевые слова: вирус лейкоза крупного рогатого скота, антиген, ИФА, ретровирус, ДНК, рекомбинантный белок, экспрессия

ІРІ ҚАРА ЛЕЙКОЗЫНЫҢ СЕРОЛОГИЯЛЫҚ ДИАГНОСТИКАСЫ ҮШІН *ESCHERICHIA COLI* ЖАСУШАЛАРЫНДА P24 РЕКОМБИНАНТТЫ АҚУЫЗ ГЕНІНІҢ ЭКСПРЕССИЯСЫ

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Түйін

Ірі қара малдың лейкозы-бұл жануарлардың жұқпалы лимфопролиферативті ауруы. Осы аурумен байланысты өндірістік шығындарға қабылдамаудың жоғары пайызы, сүт өндірісінің төмендеуі және жануарлардың құнарлылығының төмендеуі жатады. Лейкозды диагностикалау үшін серологиялық сынақтар қолданылады, олардың ішіндегі ең сезімтал иммуноферменттік талдау болып табылады. Капсид ақуызы p24 лейкозды диагностикалау үшін маңызды антиген

болып табылады. Осы жұмыс аясында p24 ақуыз гені pET-28 плазмид векторына клондалды. Рекомбинантты p24 антигені ArcticExpressRP (DE3)_pET-28/p24 штаммын қолдану арқылы алынды. Тазартылған p24 ақуызының жалпы өнімі 1 литр дақылдан 76 мг құрады. P24 рекомбинантты ақуызын бақылау сарысуында сынау рекомбинантты p24 антигенінің оң бақылау сарысуымен байланысатынын және жоғары антигендік белсенділікке ие екенін көрсетті. Жануарлардың 48 сынамасындағы рекомбинантты ақуызды p24 пилоттық тестілеу нәтижелері үлгілердің 83% оң және 17% теріс нәтиже бергенін көрсетті. Алынған нәтижелер ІҚМ лейкозын диагностикалау үшін иммуноферменттік талдауда алынған рекомбинантты p24 ақуызының перспективасын көрсетеді.

Кілт сөздер: ірі қара малдың лейкозның вирусы, антиген, ИФТ, ретровирус, ДНҚ, рекомбинантық ақуыз, экспрессия