DOI: 10.29326/2304-196X-2023-12-4-315-321

Testing and identification of bovine viral diarrhea virus isolates recovered in Russia between 2019 and 2022

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ABSTRACT

Studying the agents of bovine viral diarrhea (BVD) is an important task given the high probability of new isolate introduction into the Russian Federation, as well as the need to take into account the genotype and subgenotype of the virus circulating in a herd when developing vaccines and diagnostic kits for the infection. During the work performed, 6 BVD virus isolates were recovered and identified. The recovery of these isolates in the lamb testicle cell subculture revealed that Bashkiria/2019, Kirov/2020 and Samara/2020 isolates belong to non-cytopathic bovine viral diarrhea virus biotypes, Chelyabinsk/2021 isolate demonstrated the characteristic cytopathic effect in the monolayer and was classified as a cytopathic variant of the virus, the adaptation of Belgorod/2021 and Udmurtiya/2020 isolates to this cell system was not possible. The study also identified the species of the recovered isolates. Based on the analysis of the nucleotide sequence of genome 5′-untranslated region (5′-UTR) fragment, these isolates were classified as belonging to three genotypes of the virus. The phylogenetic analysis showed that Chelyabinsk/2021 and Udmurtiya/2020 isolates belong to genotype 2 and demonstrate, respectively, 98% and 99% homology with reference 890 strain of BVD virus. The recovered Bashkiria/2019, Samara/2020, Kirov/2020 isolates were classified as belonging to subtypes 1i, 1f and 1b of genotype 1, and Belgorod/2021 isolate represents genotype 3 of the virus. The findings from the study confirm the presence of all three genotypes of bovine viral diarrhea virus in the Russian Federation and reiterate the need for the development of specific prevention and diagnosis tools for the disease.

Keywords: bovine viral diarrhea, pestiviruses, genotype 2, isolate, polymerase chain reaction, sequencing

Acknowledgements: The study was conducted under the governmental programme "Detection of transboundary animal disease agents, studies of their biological properties, introduction and spread patterns of diseases caused by these agents, potential transmission factors". The authors are grateful to N. G. Zinyakov, A. A. Kozlov, A. V. Sprygin for advisory and technical support during sequencing and phylogenetic tree construction.

For citation: Bubyakin R. I., Kononova S. V., Shumilova I. N., Byadovskaya O. P., Krotova A. O., Kononov A. V. Testing and identification of bovine viral diarrhea virus isolates recovered in Russia between 2019 and 2022. *Veterinary Science Today*. 2023; 12 (4): 315–321. DOI: 10.29326/2304-196X-2023-12-4-315-321.

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

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УДК 619:616.98:578.833.3:578.5.001

Изучение и идентификация изолятов вируса вирусной диареи крупного рогатого скота, выделенных на территории России с 2019 по 2022 г.

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РЕЗЮМЕ

Изучение возбудителей вирусной диареи крупного рогатого скота является важной задачей в связи с высокой вероятностью заноса новых изолятов на территорию Российской Федерации, а также с необходимостью учитывать генотиповую и субгенотиповую принадлежность циркулирующего в стаде вируса при разработке вакцин и средств диагностики инфекции. В ходе проделанной работы было получено и идентифицировано 6 изолятов возбудителя вирусной диареи крупного рогатого скота. При выделении данных изолятов в субкультуре клеток тестикул ягненка установили, что изоляты Bashkiriya/2019, Kirov/2020 и Samara/2020 относятся к нецитопатогенным биотипам вируса вирусной диареи крупного рогатого скота, изолят Chelyabinsk/2021 проявлял характерное цитопатическое действие в монослое и был отнесен к цитопатогенному варианту вируса, а изоляты Belgorod/2021 и Udmurtiya/2020 не удалось адаптировать к данной клеточной системе. Также при проведении исследования была определена видовая принадлежность полученных изолятов. При анализе нуклеотидной последовательности фрагмента 5′-нетранслируемой области (5′-UTR) генома данные изоляты отнесены к трем генотипам вируса. Филогенетический анализ показал, что изоляты Chelyabinsk/2021 и Udmurtiya/2020 принадлежат к генотипу 2 и имеют соответственно

98%-ю и 99%-ю гомологию с референтным штаммом 890 возбудителя вирусной диареи крупного рогатого скота. Выделенные изоляты Bashkiria/2019, Samara/2020, Kirov/2020 были отнесены к субтипам 1i, 1f и 1b генотипа 1, а изолят Belgorod/2021 является представителем генотипа 3 вируса. Данные исследования подтверждают присутствие всех трех генотипов вируса вирусной диареи крупного рогатого скота на территории Российской Федерации и необходимость разработки средств специфической профилактики и диагностики против данного заболевания.

Ключевые слова: вирусная диарея крупного рогатого скота, пестивирусы, генотип 2, изолят, полимеразная цепная реакция, секвенирование

Благодарности: Работа выполнена в рамках государственного задания по теме «Выявление возбудителей трансграничных заболеваний животных, изучение их биологических свойств, особенностей заноса и распространения болезней, вызываемых данными возбудителями, возможных факторов передачи». Авторы выражают благодарность Н. Г. Зинякову, А. А. Козлову, А. В. Спрыгину за консультативную и техническую поддержку при проведении секвенирования и составлении филогенетического древа.

Для цитирования: Бубякин Р. И., Кононова С. В., Шумилова И. Н., Бьядовская О. П., Кротова А. О., Кононов А. В. Изучение и идентификация изолятов вируса вирусной диареи крупного рогатого скота, выделенных на территории России с 2019 по 2022 г. *Ветеринария сегодня*. 2023; 12 (4): 315–321. DOI: 10.29326/2304-196X-2023-12-4-315-321.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus*, family *Flaviviridae,* and is represented by different genotypes: genotype 1 (pestivirus A, *Pestivirus bovis*, BVDV-1), genotype 2 (pestivirus B, *Pestivirus tauri*, BVDV-2), genotype 3 (pestivirus H, HoBi-like pestivirus, *Pestivirus brazilense*, BVDV-3), as well as by two phenotypes – cytopathic (CP) and non-cytopathic (NCP). At the same time, the genotypes are divided into subtypes: 21 subtypes of genotype 1 (1a–1u), 6 subtypes of genotype 2 (2a–2f) and 4 subtypes of genotype 3 are known [1, 2].

BVDV infection has a wide variety of clinical manifestations and leads to significant economic losses in meat and dairy farming all over the world. The infection is accompanied by reproductive disorders, respiratory diseases, immune system malfunction, erosive-ulcerous inflammation of gastrointestinal mucosa, chronic diseases with predisposition to the development of secondary bacterial and other viral infection. A distinctive feature of the virus is its ability to cross the placental barrier and, depending on gestation period in a cow, to infect a fetus and thus to cause persistent infection [3]. As a result, immunotolerant calves are born that act as a continual source of the pathogen for non-immune animals.

The key feature of BVDV is its genotypic and phenotypic diversity that underlies the polymorphism of the clinical presentations of the disease, as well as virulence and antigenicity variation of the virus. BVDV genome is a single-stranded, positive-sense RNA with a length of around 12.3 thousand nucleotides. It has one open reading frame (ORF) of about 4,000 codons in length that encodes 12 structural and non-structural proteins (Npro-C-Erns-E1-E2-p7-NS2/NS3-NS4A-NS4B-NS5A-NS5B), flanked at 5′- and 3′-ends by untranslated regions (5′-UTR and 3′-UTR), p80-125, and basic protein gp53 responsible for virus neutralizing antibody (VNA) induction [4].

The specific feature of the virus, which is its antigenic variability and differences in virulence and reproductive properties, may be associated with genomic reorganizations, mutations or recombinations.

The distribution of the virus types and subtypes has regional specificities and depends on animal husbandry practices, stocking density, livestock performance, new animal introduction frequency and other factors. Genotype 1 BVDV is spread globally, with its outbreaks being most commonly reported in the countries of Europe. The largest number of subtypes (as many as 21) was detected in cattle in Italy and China. The isolation of genotype 2 pestivirus from cattle was reported in the USA, Canada, Brazil, Uruguay, Germany, Slovakia, Italy, South Korea, Japan, Mongolia and Russia [5].

Mucosal disease, one of the forms of diverse BVD clinical presentation, develops following persistent infection and is accompanied by animal death at the age between 6 months and 2 years. The disease occurs when an animal persistently infected with an NCP virus is superinfected with a homologous CP variant of the virus [6]. Outbreaks may also be caused by an NCP virus of genotype 2 not accompanied by a CP virus [6, 7].

The isolates of genotype 2 BVDV are less common than genotype 1 BVDV isolates. They cause acute and hyperacute disease characterized by high mortality, thrombocytopenia and hemorrhages [8, 9, 10, 11]. Studying the agents of BVD is very important given the high probability of new isolate introduction into the Russian Federation. Besides, the genotype and subgenotype of the virus circulating in a herd should be taken into account when developing specific prevention and diagnosis tools for the infection [6, 7].

Specific immunity plays an important role in combating BVD. Due to its wide spread, a long latency and virus shedding period, the high number and density of animals on farms, the infection eradication is difficult and even impossible to achieve without vaccine prevention. Immunization is intended to shield animals from viremia and spread of the virus, to prevent the infection of target reproductive and lymphatic system cells in order to protect a fetus against the infection and immune suppression development. A few decades ago, most vaccines contained genotype 1 BVDV strains. But, taking into account

the antigenic variability of BVDV, the development and production of both attenuated and inactivated vaccines based on the strains of two virus genotypes were initiated. There are more than 180 licensed vaccines against BVD in the USA [9]. In Russia, there are no registered immunological products containing genotype 2 BVDV.

The aim of the study is to test the material isolated from cattle with respiratory and reproductive disorders, collected on farms of the Russian Federation in 2019–2022, as well as to perform the phylogenetic characterization of the recovered isolates.

MATERIALS AND METHODS

Biological material samples were submitted to the Reference Laboratory for Bovine Diseases for testing for the presence of BVDV RNA. Before isolation, sample preparation was carried out under laboratory conditions. Stabilized blood, nasal swabs and/or a 5–10% suspension of pathological material were used for the tests. To prepare the suspension, a sample of the material was homogenized into a paste using a sterile porcelain mortar and a pestle. Then a 10% suspension was prepared by adding nuclease-free water to the mortar and mixing it with the homogenate. The total RNA was extracted from 0.1 mL of the tested biological material using a "RIBO-sorb" test kit (Central Research Institute for Epidemiology of the Federal Service for Surveillance on Consumer Rights Protection and Human Wellbeing (Rospotrebnadzor), Russia), or a "Viral RNA kit" (QIAGEN, Germany), or equivalent in accordance with the manufacturer's instructions.

The detection of genotype 2 BVDV genome was carried out using the following oligonucleotide primers: BD1 5′-GTAGTCGTCAGTGGTTCG-3′ (positions 188–205 nt, NADL strain), BD4 5′-GCCATGTACAGCAGAGAT-3′ (positions 383–366 nt, NADL strain), BD2 5′-CGACACTCCATTAGTTGAGG-3′ (positions 204–223 nt, 890 strain), BD3 5′-GTCCATAACGCCACGAATAG-3′ (positions 320–301 nt, 890 strain), flanking the conservative virus genome region with a length of 261 base pairs, and a method for the virus detection and strain differentiation based on genome 5′-UTR region [12] in a programmable Rotor-Gene™ 6000 thermal cycler (Corbett Research Pty Ltd., Australia).

The sequencing of the samples was carried out using an automated ABI Prism® 3100 sequencer (Applied Biosystems, the USA) in accordance with the manufacturer's instructions.

The nucleotide sequences of the synthesized fragments were analyzed using sequence alignment methods with BVDV-1, BVDV-2 and BVDV-3 sequences received from the GenBank international database and the genetic database of the Federal Centre for Animal Health.

The resulting nucleotide sequences were analyzed using BioEdit 7.0 software package. The phylogenetic tree was constructed by maximum likelihood method using Mega 11 software. Branch topology was confirmed by bootstrap analysis.

Table 1
PCR tests of pathological material samples, 2019–2022

Region	Number of tested farms	Number of samples	PCR test results	
			positive	negative
Kursk Oblast	1	6	0	6
Ivanovo Oblast	1	10	0	10
Republic of Bashkortostan	**1**	**11**	**5**	**6**
Krasnodar Krai	2	10	0	10
Tambov Oblast	1	33	0	33
Belgorod Oblast	**2**	**24**	**3**	**21**
Stavropol Krai	1	9	0	9
Republic of Mordovia	1	17	0	17
Mari El Republic	1	2	0	2
Novosibirsk Oblast	1	10	0	10
Kirov Oblast	**3**	**40**	**5**	**35**
Udmurt Republic	**1**	**12**	**3**	**9**
Kamchatka Krai	1	4	0	4
Vologda Oblast	1	7	0	7
Kostroma Oblast	1	10	0	10
Chelyabinsk Oblast	**1**	**6**	**1**	**5**
Krasnoyarsk Krai	1	10	0	10
Samara Oblast	**1**	**40**	**1**	**39**
Rostov Oblast	1	2	0	2
Total	23	263	18	245

Table 2
Results of RT-PCR tests of culture fluid samples collected during cultivation of BVDV isolates in lamb testicle cell culture

BVDV isolate	Ct value		Presence of CPE
	passage 2	passage 5	
Bashkiria/2019	25.75	21.93	–
Kirov/2020	26.56	19.63	–
Samara/2020	25.64	18.25	–
Belgorod/2021	30.86	33.22	–
Udmurtiya/2020	29.59	34.51	–
Chelyabinsk/2021	25.26	17.02	+

The samples that tested BVDV RNA positive with polymerase chain reaction (PCR) were used for the virus isolation in cell culture. To exclude contamination with a NCP BVDV, the virus isolation was carried out in the subcultured lamb testicle (LT) cell lines. The infected monolayer was cultivated at a temperature of (37 ± 1) °C. Serum-free semi-synthetic nutrient medium prepared in accordance with the Federal Centre for Animal Health formula, supplemented with a 10% Baytril solution was used as a maintenance medium. The identification of the isolates was carried out after 5 serial passages in LT cell culture by testing the culture fluid with reverse transcription polymerase chain reaction (RT-PCR).

RESULTS AND DISCUSSION

Between 2019 and 2022, 263 samples (nasal swabs, lip erosion scrapings, pathological material samples) submitted to the laboratory from 23 farms located in 19 Subjects of the Russian Federation were subjected to PCR testing.

All the samples were tested for BVDV genome with RT-PCR. Eighteen samples, i.e. 6.8% of the total number of the tested samples, tested positive.

Nasal discharge samples collected from a 1-month-old calf with respiratory dysfunction on a farm located in the Chelyabinsk Oblast tested positive. BVDV genome was also detected in fetal bovine serum samples (the Belgorod Oblast), intestinal mucosa samples from calves with clinical manifestations characteristic of BVD with mucosal inflammation (the Republic of Bashkortostan, the Samara and Kirov Oblasts), samples from the aborted fetus of a first-calf cow recently imported to Russia (the Udmurt Republic). The diversity of the clinical picture of the disease observed in the animals confirms the data on the polymorphism of its clinical presentations.

To recover BVDV isolates, serial passages in LT cell subculture were performed. The virus reproduction was assessed based on characteristic cytopathic effect (CPE), as well as by RT-PCR tests of the culture fluid, on the basis of the fact that the highest cycle threshold (Ct) values correspond to the minimum BVDV accumulation level. In total, 6 BVDV isolates were recovered and subsequently used in the work (Table 2).

Bashkiria/2019, Kirov/2020 and Samara/2020 isolates demonstrated no CPE after 5 serial passages in LT cell culture; however, a significant decrease in Ct values was indicative of the positive dynamics of the virus activity.

The analysis of Ct values for Belgorod/2021 and Udmurtiya/2020 isolates allowed to conclude that the use of LT cell culture for the virus accumulation is ineffective, that is why a search for more sensitive cell systems is required.

During Chelyabinsk/2021 isolate recovery, no apparent changes were observed in the cell monolayer at passages 1 and 2. At passage 3, the detachment of individual cells with a changed morphology from the monolayer surface was observed. CPE was also characterized by the rounding of cells, which gradually formed separate aggregations. Low Ct values based on RT-PCR test results for Chelyabinsk/2021 isolate by passage 5 were indicative of the virus accumulation in LT cell culture [13].

The identification of the species of the recovered isolates was carried out by PCR product sequencing. The nucleotide sequences of 5′-UTR region fragments were identified and compared with the sequences available in the GenBank international database and the genetic database of the Federal Centre for Animal Health. The constructed phylogenetic tree is presented in the figure.

As the dendrogram shows, the recovered virus isolates belong to different BVDV genotypes, the genotypes demonstrate a 70% homology.

Among 6 tested isolates, 2 isolates were classified as belonging to genotype 2. Chelyabinsk/2021 isolate genome sequencing revealed its 98% identity with reference 890 strain of genotype 2 BVDV (sequence U18059.1). This strain was first isolated during the infection outbreak in the USA in the 1990s [7]. Udmurtiya/2020 isolate was 99% homologous to reference 890 strain of genotype 2 BVDV (sequence FJ795044.1) recovered from a fetal bovine serum sample [14].

Genotype 1 BVDV is widely spread in Russia and all over the world. Based on the test results, Kirov/2020 isolate was found to be identical to reference Osloss strain of subtype 1b BVDV; Samara/2020 isolate was found to be 98% homologous to subtype 1f BVDV isolate. Bashkiria/2019 isolate demonstrated a 98% identity with 23-13 strain classified as subtype 1i BVDV (sequence FJ493484.1) [15].

The genetic analysis of Belgorod/2021 isolate revealed its close relationship with 2 isolates of genotype 3 BVDV (sequences JN967748.1 and JN967731.1) and FBS 37 strain (sequence MK017821.1) previously isolated from fetal bovine serum samples [16].

Thus, the findings from the study confirm reports that genotype 1 BVDV prevails in Russia and is spread not only in the central part of the country, but also in the Volga Federal District (subtypes 1b, 1f and 1i).

Over the past 15 years, BVD outbreaks caused by genotype 2 BVDV were reported in some regions of the Russian Federation. Respiratory disorders caused by the virus of the said genotype were detected in calves in the Ural region and Siberia [1, 4]. Koteneva S. V. et al. [17] detected genotype 2 BVDV in internal organ samples from an aborted fetus and a deadborn calf of a local breed (the Novosibirsk and Tyumen Oblasts). Three BVDV-2 subtypes (2a, 2b and 2c) were detected in imported and domestic animals in Siberia. Based on the previously conducted studies, it was found that subtype 2a was recognized as one of the major etiological agents responsible for reproductive disorders in cows [17, 18, 19, 20]. This study revealed the presence of genotype 2 BVDV in the European part of Russia (the Udmurt Republic and the Chelyabinsk Oblast areas located in Europe). According to the international classification (ICTV) of pestiviruses, both isolates (Udmurtiya/2020 and Chelyabinsk/2021) represent subtype 2a BVDV.

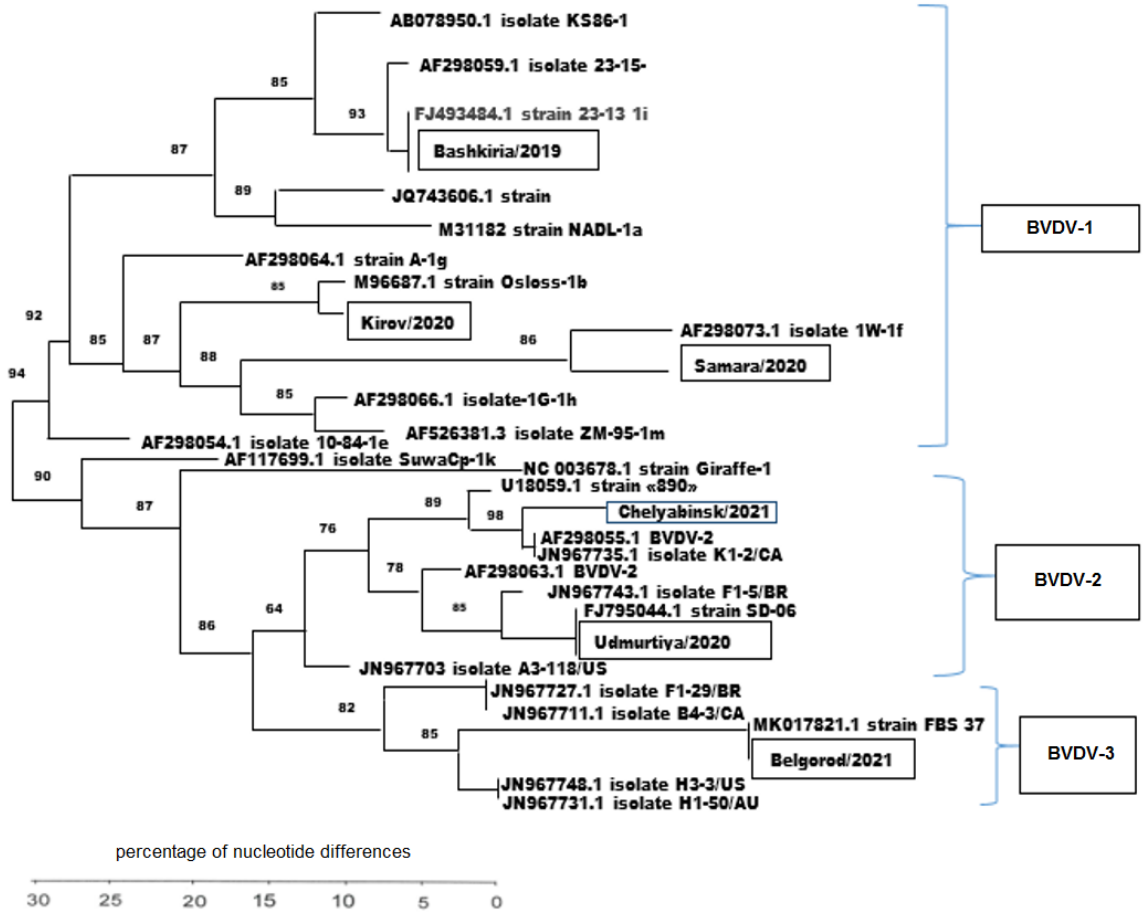

Fig. Dendrogram constructed based on BVDV genome 5'-untranslated (5'-UTR) region fragment sequencing results. The recovered isolates are shown in squares

Genotype 3 BVDV was previously detected on livestock farms in Russia [1, 3, 21, 22]. This study reports the detection of genotype 3 BVDV in the Central Federal District of the country.

CONCLUSION

RT-PCR tests of the biological material samples submitted from livestock farms to the Reference Laboratory for Bovine Diseases detected BVDV genome in 6.1% of the samples. During the work performed, 6 BVDV isolates were recovered in the cell system and identified. The isolates were classified as belonging to different BVDV biotypes: Bashkiria/2019, Kirov/2020 and Samara/2020 isolates were classified as belonging to non-cytopathic phenotypes, Chelyabinsk/2021 isolate demonstrated the characteristic cytopathic effect in LT cell culture monolayer and was classified as a cytopathic variant of the virus, the adaptation of Belgorod/2021 and Udmurtiya/2020 isolates to this cell system was not possible.

Besides, the genetic identification of BVDV was carried out based on conservative 5'-UTR region. The phylogenetic analysis showed that Chelyabinsk/2021 and Udmurtiya/2020 isolates belong to genotype 2 and are, respectively, 98% and 99% homologous to reference 890 strain of subtype 2a BVDV. The recovered Bashkiria/2019, Samara/2020 and Kirov/2020 isolates were classified as belonging to subtypes 1i, 1f and 1b, respectively. Belgorod/2021 isolate represents genotype 3 BVDV.

The findings from the study confirm the presence of three genotypes of BVD virus in Russia.

The recovered Chelyabinsk/2021 isolate will be used for further studies of the cultural properties of genotype 2 BVD virus.

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Received 10.10.2023
Revised 30.10.2023
Accepted 23.11.2023

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