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GENETIC POLYMORPHISM OF THE BOVINE VIRAL DIARRHEA VIRUSES IN BIG DAIRY FARMS IN SIBERIA

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Abstract

Bovine viral diarrhoea is a widespread disease in the Russian Federation and causes significant economic damage to dairy cattle, especially under intensive commercial farming. The seropositivity of livestock in various Russian regions reaches 65-100 %. The disease is caused by two different virus species, BVDV-1 and BVDV-2, of which the latter is considered more virulent. The persistently infected (PI) animals which are a constant endogenous source of the virus in the herd play a major role in maintaining the stationary trouble of the farms. Short-term sources of the pathogen are transitively infected (TI) animals in which disease proceeds in acute form. Genetic structure of viruses circulating in local livestock populations gives necessary information about the evolution, geography and pathways of the pathogen. However, studies on the genetic polymorphism of viruses in Russia are not enough. This paper is the first to report on a phylogenetic analysis of two types of viral diarrhoea virus, isolated from animals of foreign and domestic origin with different clinical manifestations of the disease. These data show the prevalence of BVDV-1b in PI and TI animals, as well as BVDV-2b and BVDV-2c circulation among Siberian dairy complexes (note, BVDV-2b and BVDV-2c in animals of foreign and domestic origin are detected in Russia for the first time). Phylogenetic analysis of viruses circulating among PI and TI of highly productive dairy cattle was based on a comparison of conserved region of viral genome (5'-UTR) using reverse transcriptase PCR (RT-PCR) method. Studies were conducted in five regions of Western and Eastern Siberia: Tyumen, Novosibirsk, Irkutsk regions, the Krasnoyarsk Territory and Northern Kazakhstan on big dairy farms with a population of 800 or more milking cows with an average annual productivity of 7,000-10,000 liters, where at the time of the research vaccination was not carried out or inactivated vaccines were used. The imported livestock was kept mainly in the Tyumen region and the Republic of Kazakhstan. Biomaterial (blood, serum, nasal discharge, lymphoid organs, lungs, vaginal discharges) was collected from clinically healthy persistently infected animals, transitively infected animals with reproductive disorders and respiratory syndrome, as well as aborted fetuses. A total of 479 samples were examined. According to our findings, two BVDV species circulate among the PI and TI animals on the big dairy farms. The phylogenetic analysis reveals seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), 1p (5 %) and two subtypes of BVDV-2, i.e. 2b (10 %) and 2c (5 %). Taking into account the fact that the strategy of livestock breeding is changing in Russia, the number of dairy mega farms" is increasing, which receive animals with different infectious status from many sources. Therefore, the study of the genetic polymorphism of the virus is topical. Comparison of data on the origin of animals with the results of phylogenetic analyses can help in determination of the sources and ways of bringing pathogens into a particular region and in identifying and tracking new and highly virulent strains of viruses. This is especially important during the implementation of vaccination programs for animals when the genetic profiles of vaccine strains do not coincide with the profiles of viruses circulating among animals in a particular area.

Keywords: cattle, bovine viral diarrhoea, subtypes, BVDV-1, BVDV-2, genome, polymerase chain reaction, 5'-nontranslated region (5'-UTR), nucleotide sequence, molecular epidemiology, phylogenetic analysis

Bovine viral diarrhea (mucosal disease) is widespread throughout the world [1], including Russia where the infection of cattle in different regions reaches 65-100 % [2-6]. The importance of this infection in intensifying dairy and beef farming is increasing. The most economically significant consequences of the diseases are the reproductive disorders and pathologies of the respiratory tract; therefore, seronegative heifers of the mating age and calves up to 6 months of age are more susceptible to infection [7-9]. Permanent adverse epidemiological situation on farms is maintained through the presence of persistently infected (PI) animals, which become a permanent endogenous source of the pathogen. Transiently infected (TI) animals in which the disease proceeds in an acute form can be short-term sources [1].

The disease is caused in cattle by two genetically different types of virus, BVDV-1 and BVDV-2 [10] of cytopathogenic (CP) and non-cytopathogenic (NCP) biotypes [1, 11]. The distribution of species and subtypes of the virus is region-specific and depends on the type of livestock breeding, herd density, productivity, the frequency of introduction of new animals and other factors [12]. The first type of virus is spread all over the world [1], but is found more often in the European countries. The largest number of subtypes (up to 21) was found in cattle in Italy [13] and China [14]. BVDV-2 in cattle was found in the USA [15], Canada [16], Brazil [17], Argentina [18], Uruguay [19], Germany [20], Slovakia [21], Italy [22], South Korea [23], Japan [24] and Mongolia [25]. This type, which is considered more virulent, is divided into six subtypes (2a-2f) [26] and prevails in the USA and Canada (up to 50 % of all isolated strains) [1]. For virus species differentiation, the nucleotide sequencing of genomic RNA is used with the study of a 5'-nontranslated region (5'-UTR) suitable for amplification [28, 29]. In Russia, the works on the phylogenetic analysis of isolates are fragmented, i.e. BVDV-1 was found to be widely distributed among the cattle in the Central region [30] and two antigenically different strains of the virus (1m and 1a) were detected in the population of wood bison and livestock [31].

Previously, the main gender and age groups of animals, most at risk of infection, were identified, which can be used as an indicator and be a source of the pathogen in the herd [4].

In this paper, we conducted for the first time the phylogenetic analysis of two types of viral diarrhea virus (mucosal disease) isolated from animals of foreign and domestic origin with various clinical signs, and revealed the prevalence of BVDV-1b in PI and TI animals and the circulation of BVDV-2b and BVDV-2c at the dairy complexes of Siberia.

The aim of the work is to study the genetic polymorphism of pathogens of viral diarrhea of cattle, circulating among persistently and transiently infected animals, including the imported ones, at large dairy complexes.

Techniques. Seven dairy complexes were surveyed in 2006-2017 in Western and Eastern Siberia (Tyumen, Novosibirsk, Irkutsk regions, the Krasnoyarsk Territory, and Northern Kazakhstan). In each livestock complex the population of dairy black-and-white cows, with average annual milk productivity of 7,000-10,000 liters and above, was 800 heads and more; neither specific prophylaxis of the disease nor vaccination using inactivated strains were performed [4]. Feeding and housing conditions complied with physiological and zootechnical standards.

Biomaterial (blood, serum, nasal discharge, lymphoid organs, lungs, vaginal discharges) was collected from clinically healthy persistently infected animals, transiently infected animals with reproductive disorders and respiratory syndrome, as well as aborted fetuses. Persistent infection was diagnosed only when viral RNA was detected in paired serum samples taken at 30-day intervals. A total of 479 biomaterial samples were analyzed.

For the isolation of viral RNA and reverse transcription, the commercial kits RIBO-sorb and Reverta-L (Central Research Institute of Epidemiology of Rospotrebnadzor, Russia) were used.

Viral RNA in samples of the biomaterial was detected in reverse transcription polymerase chain reaction (RT-PCR) using universal primer pairs: 324 (sense) — 5'-ATGCCC(T/A)TAGTAGGACTAGCA-3', 326 (antisense) — 5'-TCAACTCCATGTGCCATGTAC-3' [33], flanking the 5'-UTR region, with subsequent phylogenetic analysis. In 30 µl reaction mixture there were 5 µl cDNA; 1× Taq buffer without Mg²⁺ (Medigen Laboratory LLC, Russia), 0.4 mM dNTP, 3.3 mM MgCl₂, 0.15 µM of each primer, 1.5 IU SmartTaq DNA polymerase, water (up to 30 µl). Amplification mode: 5 min at 95 °C (1 cycle); 20 s at 95 °C, 30 s at 54 °C, 40 s at 72 °C (45 cycles); 7 min at 72 °C (1 cycle). PCR products were analyzed electrophoretically in 2 % agarose gel with Tris-borate buffer (pH 8.0) according to the standard methodology.

The nucleotide sequence of the viral genome was determined with the use of BigDye 3.1 kit (Applied Biosystems, USA) by sequencing both DNA strands. The resulting nucleotide sequences were analyzed with BioEdit 7.0.0 (<https://bioedit.software.informer.com>) and Lasergene 7.1.0 software packages (<https://lasergene.software.informer.com>). The phylogenetic dendrogram was made using the nearest neighbor method with MEGA v7.0 software (<https://www.megasoftware.net/>). The topology of the dendrogram was confirmed by the bootstrap analysis method (1000 steps of replication) [13, 34]. The nucleotide sequences of the synthesized fragments were analyzed by alignment with the sequences of other strains BVDV-1 and BVDV-2 from GenBank (NCBI) using the ClustalW software [35].

Results. RNA of the virus was found in 20 (4.17 % of the examined number) samples, including 10 samples from the Tyumen Region, 6 from the Novosibirsk Region, 1 from the Irkutsk Region, 2 from the Krasnoyarsk Territory and 1 from the North Kazakhstan (Table). It was established that two types of the virus circulate among PI and TI animals. The phylogenetic analysis revealed seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), and 1p (5 %). The second type of the virus was found in 15 % of samples, out of them BVDV-2b in 10 %, BVDV-2c in 5 %. The predominant subtype was BVDV-1b (Fig., posted on the website <http://www.agrobiology.ru>).

The distribution of subtypes had some geographical differences. BVDV-1a was detected only in the Tyumen Region in the internal organs of an aborted fetus. BVDV-1b, additionally to the Tyumen Region, was present in the Novosibirsk Region and the Krasnoyarsk Territory. In the Krasnoyarsk Territory, it was isolated from animals of local breed, in the Novosibirsk Region from heifers imported from Germany and from local cattle, and in the Tyumen Region from animals imported from Holland, USA, Slovenia, and Denmark. PI animals were the source of the virus.

BVDV-1c was found in the Tyumen Region in blood of a calf with respiratory pathology, born by a heifer from Holland, BVDV-1d was present in the Tyumen Region on the farm where the cattle were brought from France, and also in the Novosibirsk Region in calves of local breed with respiratory pathology. BVDV-1f was detected in Northern Kazakhstan in the serum of a calf with respiratory pathology, born by a heifer imported from Germany, in the Irkutsk Region in the serum of a cow of the local breed, in the Krasnoyarsk Region in the serum of a cow of local breed and in the Tyumen region in the serum of a heifer of unknown origin. BVDV-1i was revealed in the Novosibirsk Region in the serum of a calf of the local breed from a troubled farm in respect of respiratory diseases, BVDV1p in the Tyumen Region in the blood of a calf with a simi-

lar pathology, obtained from a heifer imported from Germany. BVDV-2c was found in the Tyumen Region in the internal organs of the aborted fetus from a heifer imported from the USA, BVDV-2b in the Novosibirsk Region in the internal organs of the stillborn calf and aborted fetus of local breed.

Genetic polymorphism of cattle viral diarrhea (mucosal disease) causative agents circulating among persistently infected animals at dairy complexes of Western and Eastern Siberia

Isolate	Type/subtype (5'-UTR)	Region	Source of virus	Origin of animals
N09/16	1b	Novosibirsk Region	Blood serum of a cow	Russia
N08/17	1b		Blood serum of a heifer	Germany
T38/16	1b	Tyumen Region	Blood serum of a PI calf	USA
T11/16	1p		Blood of a calf	Germany
Kz05/15	1f	Northern Kazakhstan	Blood serum of a calf	Germany
N09/15	1i	Novosibirsk Region	Blood serum of a calf	Russia
Ir03/17	1f	Irkutsk Region	Blood serum of a cow	Russia
T13/17	1f	Tyumen Region	Blood serum of a heifer	Unknown
K04/16	1f	Krasnoyarsk Territory	Blood serum of a cow	Russia
K14/16	1b	Krasnoyarsk Territory	Blood serum of a cow	Russia
T11/17	1c	Tyumen Region	Blood serum of a calf	Holland
T15/17	2c		Internal organs of an aborted fetus	USA
T18/17	1a		Internal organs of an aborted fetus	Austria
T01/16	1b		Blood serum of a calf	Slovenia
T30/16	1b		Mesenteric lymph nodes of a calf	Holland
T41/17	1b		Internal organs of a PI heifer	Denmark
T24/16	1d		Lymphoid organs of a calf	France
N19/17	2b	Novosibirsk Region	Internal organs of a stillborn calf	Russia
N12/16	2b		Internal organs of an aborted fetus	
N11/17	1d		Blood serum of a calf	

It is known that pestiviruses, due to the structure of their genome, possess significant mutational activity which is expressed in a constant increase in the genotypic and phenotypic diversity of strains, affecting virulence. Their role in the pathology of animals is not fully studied. In addition, viral polymorphism makes it difficult to diagnose diseases and may reduce the effectiveness of vaccination [1, 12]. BVDV-1a and BVDV-1b are the most common; their role has been described for various forms of the disease, and they, along with BVDV-2a, are the components of commercial vaccines worldwide [1]. BVDV-1f was reported to be found in Slovenia [36] and Austria [33] in PI animals with a high frequency. Sporadically, this subtype of the virus was detected in Italy [37] and Turkey [38], but the data on the caused clinical syndromes are not presented.

In Russia, there are no data on the identification of various subtypes of BVDV-2 and their connection with the clinical forms of the disease. Previously, we established the presence of the virus (second type) in animals with various pathologies; however, it was not possible to conduct subtyping [32]. In this paper, for the first time in Russia, the circulation of the virus of subtypes BVDV-2b and BVDV-2c are established.

According to the literature, BVDV-2a and BVDV-2b are recognized as the main etiological agents of the pathology of reproduction and systemic infection and are spread mainly in the countries of North and South America [1, 15, 16].

BVDV-2b was identified in the internal organs of an aborted fetus and a stillborn calf of local breed in the Novosibirsk Region. BVDV-2c is a rare virus subtype detected in Germany in 2013-2014 (North Rhi-ne-Westphalia and Lower Saxony) in seronegative animals during a massive outbreak of cattle viral diarrhea. It caused a decrease in milk productivity in cows, fever, respiratory disease and hemorrhagic enteritis in calves, heifers, and cows [40, 41]. In 2016, its circulation was established among small ruminant animals in Southern Italy [42].

We conducted research in the farms where new animals were imported from various sources, including from abroad. The results showed that the diversi-



Phylogenetic dendrogram of cattle viral diarrhea (mucosal disease) causative agents BVDV-1 and BVDV-2 identified in Siberian farms (2006-2017). Dendrogram is based on sequence analysis of the 5'-nontranslated region (5'-UTR). The ClustalW-based alignment of sequences. The topology of the tree is constructed by the neighbor-joining method. The genetic distance matrix is calculated using the minimal evolution method. The external group is the sequence of the virus BVDV-3. Near each node of the dendrogram, bootstrap support is indicated. Investigated isolates are underlined. For reference strains, the name and number in the database GenBank (NCBI) are indicated.

ty of species and subtypes of the virus is quite large. In previous work, among local and imported cattle during acute outbreaks of viral diarrhea of cattle, we detected the circulation of 6 subtypes of BVDV-1 (a, b, c, g, p, and k) and BVDV-2 without differentiation into subtypes [32]. This study confirms the circulation and the prevalence of BVDV-1b in PI and TI animals. In addition, three new subtypes of the first type of virus (BVDV-1d, BVDV-1f and BVDV-1i) were detected, as well as the circulation of new subtypes BVDV-2b and BVDV-2c not previously identified in Russia. The fact of their detection in the territory of Russia should be treated carefully, taking into account the potential pathogenicity of these agents and their lack in commercial vaccines.

Since the livestock farming strategy is changing in Russia towards dairy mega-farms to which animals with different infectious status come from many sources, the study of the genetic polymorphism of the virus is becoming more and more relevant. The comparison of the data on the origin of animals with the results of phylogenetic studies can help in determining the sources, pathways of pathogens in a particular region, as well as in identifying and tracking new and highly virulent strains of viruses. This is especially important in vaccinating

animals when the genetic profiles of vaccine strains and viruses circulating in a specific area do not match [29]. At present, mainly inactivated and live vaccines based on strains of subtypes 1a, 1b and 2a are used in Russia [1, 12]. In some cases, the genetic profile of vaccine strains may not fully correspond to the genetic spectrum of pestiviruses circulating in a particular country or region. The genetic diversity of viral diarrhea viruses that we detected can reduce the effectiveness of specific prophylaxis of the disease. In addition, for the formation of persistent adverse situation on bovine viral diarrhea of cattle and a continuous epizootic process, the cycle mother-calf is important. This leads to the birth of persistently infected offspring, which becomes a permanent endogenous source of the virus in the herd, reducing the effectiveness of specific prophylaxis. Therefore, vaccination will be more effective if the PI animals are completely removed from the herd [1, 4].

Thus, pathogens of two types of bovine viral diarrhea (mucosal disease) circulate at the dairy complexes of Siberia among persistently and transiently infected animals. The phylogenetic analysis revealed seven subtypes of BVDV-1, i.e. 1a (5 %), 1b (35 %), 1c (5 %), 1d (10 %), 1f (20 %), 1i (5 %), 1p (5 %), and two subtypes of BVDV-2, i.e. 2b (10 %) and 2c (5 %). The predominant subtype is BVDV-1b. For the first time in Russia, the circulation of BVDV-2b and BVDV-2c is established among animals of foreign and domestic origin, and the relationship between the belonging of viruses to the subtype and the clinical syndromes caused by them are revealed. Particularly, BVDV-1a causes reproductive pathology, 1b persists in infected animals, 1c, 1d, 1i, 1p lead to respiratory pathology, 1f is found in PI animals and in case of respiratory pathology, BVDV-2b and 2c are causative agents of reproductive pathologies and systemic infection of animals. Our findings may be useful in studying molecular epizootiology of viruses, in elaborating more accurate diagnostic tests that cover a variety of genetic variants, in creating vaccines, and also in more effective programs for infection control.

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