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## IDENTIFICATION OF THE BOVINE ATYPICAL PESTIVIRUS IN BIOLOGICAL SAMPLES

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### Abstract

Atypical cattle pestivirus (BVDV3; HoBi-like) is an unclassified candidate for the genus *Pestivirus* of the *Flaviviridae* family. The agent was isolated for the first time in 2004 from fetal bovine serum from Brazil, and shows a high degree of similarity with the BVDV1 and BVDV2. Its presence in the cattle populations can potentially reduce the effectiveness of control programs of bovine viral diarrhea. The article presents the results of developing a method for identifying of BVDV3 in the biological samples based on polymerase chain reaction. Synthetic oligonucleotide primers complementary to positions 9202-9218 and 9501-9521 of the reference strain D32/00 "HoBi" genome were selected. The basic parameters of the reaction have been worked out. The sensitivity of PCR was  $7.4 \times 10^{-1}$  copies/ $\mu$ l. It has high specificity and does not reveal RNA of the BVDV1, BVDV2 and classical swine fever virus. With the help of the developed PCR 18 samples of fetal bovine serum (FBS) from various sources, 11 types of continuous cell culture lines used for virus cultivation in several Russian research institutes, 10 attenuated live vaccines, 189 internal organs, 1383 blood sera from cattle, 168 blood sera from reindeer, and 63 blood samples from red deer were investigated. The virus was revealed only in seven lots of FBS obtained from two manufacturers and produced in South America. Phylogenetic analysis of amplicons showed all positive lots grouped with BVDV-3 strain D32/00 "HoBi" (Brazilian group). Given the potential risk of using contaminated fetal serum, further research of the spread of BVDV3 in Russia is needed. The virus was not found in continuous cell culture lines, vaccines used for human, cattle and small domestic animals. Additionally, no evidence has been found out of virus circulation among cattle of various breeds, including those imported from another countries, reindeers and red deer in the Krasnodar territory, Siberia and the Republic of Kazakhstan. The presence of the virus in the FBS used in the production of vaccines does not exclude its spread in Russia. The findings confirm the need for continuous updating and improvement of methods for diagnosing pestiviruses and tightening the rules for the international FBS trading.

Keywords: atypical pestivirus, BVDV3, HoBi-like virus, primers, polymerase chain reaction, cell cultures, fetal bovine serum, vaccines, phylogenetic analysis, cattle, reindeers, red deer

Pestivirus infections cause significant economic losses in dairy and beef cattle farming worldwide. Nowadays, the greatest concern is Bovine viral diarrhea virus (BVDV) which affects mucous membrane, especially two prototype BVDV species of the genus, BVDV types 1 and 2 (BVDV1 and BVDV2). Infection of non-immune animals leads to subclinical pathologies, immunosuppression, diarrhea, respiratory diseases, reproductive pathology and mucosal disease of persistently infected calves [1-4].

Atypical pestiviruses are new, officially not classified group of *Pestivirus*, *Flaviviridae*, tentatively called Bovine viral diarrhea virus type 3 (BDVV3) or atypical pestiviruses (HoBi-like) [5]. The agent was first isolated in Europe from fetal bovine serum imported from Brazil [6]. Later, the pathogen was identified in fetal serums, which were isolated in Australia, Mexico, the USA and pack-

aged in Europe [7-10]. Cattle and buffalo natural infection caused by BVDV3 was reported in Southeast Asia [11-12], in Italy [13-15], and in India [16]. BVDV3 infection is closely related to bovine viral diarrhea — mucosal disease (BVD-MD) and may discredit its control causing false-positives results in diagnostics and impaired prophylactic efficiency of vaccination [5, 17]. Wide use of fetal serum and intensive international trade in purebred breeding animals may lead to transmission of infection worldwide [5, 18], and therefore it is necessary to develop modern highly sensitive and specific diagnostics to detect the viruses of this group, and studying their circulation in different areas, including Russia.

In accessible domestic literature, we could not find any report about PCR detection of atypical bovine pestivirus. Here, this is the first such report on rapid and sensitive method we suggested and used to reveal and sequence atypical bovine pestivirus genome in seven imported batches of fetal bovine serum.

The aim of the research was development of polymerase chain reaction protocol for detection of atypical pestivirus in biological products and studying its circulation among domestic and wild ruminants.

*Techniques.* To select oligonucleotide primers, we aligned available genomic sequences of BVDV1, BVDV2 and BVDV3 strains from the GenBank (<http://www.ncbi.nih.gov>) with the use of ClustalW software [19]. BVDV1 strains were NADL (AJ133738.1), Singer (DQ088995.1), Osloss (M96687.1), and PT-810 (AY078406.1); BVDV2 stains were US890 (Z79772.1) and Giessen-6 AY379547.1; and four atypical pestivirus strains were D32/00\_“HoBi” (AY489116.1), Th/04\_KhonKaen (NC\_012812), SVA/cont-08 (FJ232692.1) and IZSPLV\_To (HM151361.1). Primers were chemically synthesized by amidophosphate method (an automatic synthesizer ASM-102U, Bioset, Russia). Concentration of primers in the stock solutions was determined spectrometrically.

Viral RNA was isolated using commercial RIBO-prep kit reagents (Central Institute of Epidemiology of The Federal Service for Supervision of Consumer Rights Protection and Human Well-Being, Russia) according to the manufacturer’s instruction. The resultant cDNA was diluted 2-fold with 1× TE buffer to 40 µl volume and used for PCR. PCR products were analyzed by horizontal electrophoresis in 2 % agarose gel in Tris-borate buffer with 0.4-0.5 µg/ml ethidium bromide followed by visualization in short-wave UV at  $\lambda = 254$  nm (UVT-1 Transiluminator, Biokom, Russia). PCR outcomes were considered positive when a 320 bp fragment was obtained.

Positive control samples (PCS) were obtained by molecular transformation of *Escherichia coli* with pDrive plasmid which contains specific DNA insertion. Plasmid DNA concentration was determined using Quant-iTdsDNA, HS assay kit (Invitrogen, USA) on a QUBIT fluorometer (Invitrogen, USA); the final concentration was 0.333 µg/µl ( $7.4 \times 10^{10}$  copies/µl). To assess PCR sensitivity, 10-fold dilutions of PCS were used. The analytical sensitivity was the last PCS dilution resulted in positive PCR outcomes. The specificity was assessed with strains Oregon C24VBVDV1, BL BVDV2 and Shimen isolate of classical swine fever virus (collection of Siberian Federal AgroBioTech Center RAS).

RNA fragment specificity was confirmed by nucleotide sequencing and purification on Sephadex G-50 superfine (GE Healthcare, USA). PCR fragment sequencing was performed for both DNA strands. Primary data of sequencing was analyzed by Sequencher v.4.0.5 software (Gene Codes Corporation, USA). Sequencing was performed with BigDye 3.1 kit (Applied Biosystems, USA) according to the manufacturer’s protocol. The reaction mixture (5 µl volume) contained 2 µl solution from the sequencing assay kit, 5 pM oligonucleotide primer and 0.5 µg DNA template. PCR was carried out in a programmed thermostat GeneAmp PCR-system 6700 (Applied Biosystems Inc., USA) and included 30

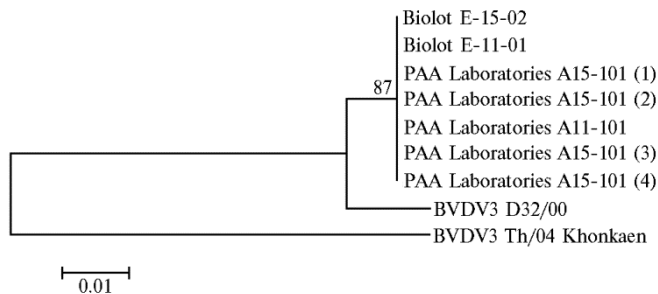
cycles as follows: 10 s at 96 °C, 15 s at 50 °C, and 4 min at 50 °C. After amplification, unbound labeled nucleotides were removed from the reaction mixture on a G-50 Sephadex superfine column. Both DNA strands were sequenced. Primary data of sequencing (chromatograms) was analyzed by Sequencher v.4.0.5 software. A highly conserved 5'-UTR region of pestivirus genomes was used for sequencing. The synthesized fragments were analyzed by a comparison with known sequences of other BVDV3 strains (particularly, BVDV3\_D32/00 and BVDV3\_Th/04\_Khonkaen) using ClustalW multiple sequence alignment program [19].

Eighteen batches of fetal bovine serum from South America, the USA, and New Zealand (twelve, five and one batch, respectively) were tested. In tests, we used continuous cell cultures of bovine coronary artery (BCA), bovine kidneys (MDBK and Taurus), embryonic bovine testis (EBT), rabbit kidneys (RK-13), African green monkey kidney (VERO), mouse fibroblasts L929 and MF, baby Syrian hamster kidney (BHK-21), fetal bovine trachea (FBT), fetal lamb kidney (FLK), feline kidneys (FK-81) (collections of research institutes). Additionally, ten live vaccines for medical and veterinary uses were tested. Also, the biomaterials for PCR assay of viral genome were organs, blood and blood serum of animals of different age and sex groups from Novosibirsk, Tyumen, Omsk, Kemerovo and Kurgan regions, Altai, Krasnoyarsk and Krasnodar territories, the Republic of Kazakhstan, and also of Taimyr and Yamal reindeer and marals from Altai Krai.

*Results.* We found several species-specific regions of BVDV3 genome. Within each, using Oligo Analyser v.6.31 program, we chose oligonucleotide primers which provide specific amplification of BVDV3 RNA. In pre-tests, the most successful primers, in terms of the resulting PCR product quality, were those complementary to positions 9202-9218 and 9501-9521 of a prototype strain D32/00\_“HoBi” (AB871953.1) genome. These primers, i.e. SEQIDNO:1 5'-TTTGCAGCCGAGCGTAG-3' and, SEQIDNO:2 5'-CCTCCTGCATAC-TGTCACCTT-3', were used in PCR tests. The sensitivity of the developed PCR was  $7.4 \times 10^{-1}$  copies/ $\mu$ l, with no amplification of genome fragments of other viruses. Most researchers use the 5'-UTR for sequencing. It provides the most accurate results, especially in attributing isolates to species or types (genotypes). 5'-UTR sequences are most often chosen as primers [8, 10, 11, 18]. We also used this region as a target. The primers we developed had sensitivity and specificity similar to those reported by other researchers, and, when used in sequencing, revealed Brazilian group virus in tested fetal sera.

HoBi-like virus was first isolated and characterized by H.G. Schirrmeyer et al. [6] in Germany in a fetal serum batch derived from Brazil and packed in Europe. The isolate designated as D32/00\_“HoBi” was considered a Brazilian group virus prototype. Then, the genetically varying subtypes having regional distribution, in particular a Thai group, were identified [11, 12]. N. Mishra et al. [16] supposed the existence of a third, Indian group of strains. There may also be a fourth virus group outside the Indian region, particularly in Italy [13-15]. Hence to date, four genetic virus groups have been identified. In available domestic literature, there is a report on detecting atypical pestivirus in a commercial vaccine against plague of small ruminants in the Republic of Tajikistan [22]. HoBi-like virus was identified in seven samples of fetal sera from two producers of South America (Fig.). Phylogenetic analysis revealed clustering of all tested samples with D32/00\_“HoBi” strain of Brazilian group.

It should be noted that we did not find BVDV3 either in tested 10 live vaccines, intended for immunization of human, farm and small domestic animals, or in 11 tested continuous cell lines. Also, the BVDV3 genome was not found in



**Unrooted phylogenetic tree constructed by a Neighbor joining method (NJ) in MEGA v.4 using Kimura model.** Bootstrap support values are shown in the nodes. Samples PAA Laboratories A15-101 (1-4) came in from different sources.

of fetal sera that have been used for several years in four Russian institutes not only in researches but also for vaccine production.

Thus, based on PCR with reverse transcription, we developed a highly sensitive specific method for atypical pestivirus detection and identification in biological material. The use of synthetic oligonucleotide primers, complementary to positions 9202-9218 and 9501-9521 of the reference strain D32/00\_“HoBi” (AB871953.1) genome, allows detection of virus sequences with a sensitivity of  $7.4 \times 10^{-1}$  copies/ $\mu$ l. The BVDV3 contamination of seven fetal bovine serum samples from South America has been determined. Phylogenetic analysis revealed the similarity of the identified viruses to each other and to the strain BVDV3 D32/00\_“HoBi” (Brazilian group). We did not find evidence of the atypical pestivirus circulation among domestic and wild animals on the territory of some Russian regions and the Republic of Kazakhstan. The obtained data confirms the need to continuously update and improve bovine pestivirus diagnostics, and to revise international regulations for fetal serum trade, including strict control of their enforcement.

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189 samples of organs and in 1383 blood serum samples of various cattle breeds, including imported ones, and in 168 blood sera of reindeer and 63 blood samples of marals. Although we have not ascertained atypical pestivirus circulation in the surveyed areas, its presence in Russia is quite possible, because the pestivirus was detected in seven batches

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