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# Molecular Epidemiology Issues of BVDV Infection In the Eastern Ukraine

**B. T. Stegniy[1], A. P. Gerilovych[1], S. Vilcek[2], E. Peterhans[3], R. O. Kucheriavenko[1], M. Yu. Stegniy[1], I. V. Goraichuk[1], V. I. Bolotin[1], O. S. Solodiankin[1]**

*[1]National Scientific Center – Institute of Experimental and Clinical Veterinary Medicine*
*83, Pushkinska Str., Kharkiv, Ukraine, 61023*
*[2]University of Veterinary Medicine and Pharmacy in Kosice*
*73, Komenskeho Str., Kosice, Slovakia, 04001*
*[3]Institute of Veterinary Virology, University of Bern*
*Laenggass-Str. 122, CH-3001 Bern, Switzerland*
*e-mail: antger@rambler.ru*

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**Aim.** This study was focused on (i) detection of specific BVDV-antibodies within selected cattle farms, (ii) identification of persistently infected (PI) animals and (iii) genetic typing of selected BVDV isolates. **Methods.** RNA extraction, real-time polymerase chain reaction, ELISA technique, sequencing. **Results.** Specific BVDV-antibodies were detected in 713 of 1,059 analyzed samples (67.3 per cent). This number is in agreement with findings in many cattle herds around the world. However, the number of positive samples differed in the herds. While 57 samples out of 283 (20.1 per cent) were identified in the first herd, 400 out of 475 (84.2 per cent) and 256 out of 301 (85 per cent) animals were positive in the second and third herd. High number of animals with BVDV RNA was detected in all herds. The real-time PCR assay detected BVDV RNA in 5 of 1068 samples analyzed (0.5 per cent). 4 positive samples out of 490 (0.8 per cent) and 1 out of 301 (0.33 per cent) were found in the second and third herd. The genetic materials of BVDV were not found in the first herd. Data on the number of PI animals were in accord with serological findings in the cattle herds involved in our study. The genetic typing of viral isolates revealed that only BVDV, Type 1 viruses were present. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f and revealed that all 4 viruses from the second farm were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from the third farm was typed as BVDV-1f. **Conclusion.** Our results indicated that the BVDV infection is widespread in cattle herds in the eastern Ukraine, that requires further research and development of new approaches to improve the current situation.

**Key words:** bovine viral diarrhea, real-time PCR, ELISA, genotyping, phylogenetic analysis, cattle.

## INTRODUCTION

Pestiviruses display marked genetic and antigenic diversity [1]. There are four main species, namely BVDV-1 and -2, Border disease virus and CSFV. A marked diversity is observed also within the BVD viruses. In particular, BVDV-1 viruses are very heterogeneous, with at least 13 subgroups, whereas two subgroups are differentiated in the more homogeneous BVDV-2 viruses [2].

BVDV is present in the cattle population worldwide [3]. The success of BVDV rests on its capacity to establish persistent infection. Viral persistence is established during a "window of opportunity" early in gestation and is associated with immunotolerance to the infecting viral strain. Different from persistent infections by herpes viruses and lentiviruses, PI animals remain free of antibodies to BVDV [4], which calls for detection of viral antigen or viral RNA as the sole methods for diagnosing persistent infection. Although transiently infected animals may be capable of transmitting virus to susceptible cattle to a limited extent, only PI animals are responsible for viral persistence in the host population. Typically, about one percent of the cattle population is PI and some 60 per cent are seropositive when the infection has reached an equilibrium [5, 6]. Calves born to seropositive cows receive colostral antibodies against BVDV [7]. These antibodies decrease in titer over time and the calves become susceptible for infection. The time span of colostral protection depends on the antibody titer and the level of infectious pressure, which the animals are exposed to. Older animals are more likely to be seropositive, due to a longer time, during which the animals are at risk of being exposed

to PI animals. In contrast, many heifers may still be seronegative during their first pregnancy. When exposed to PI animals during the critical period of development, fetuses may be infected to become PI, thereby assuring viral persistence in the next generation.

The economic losses due to BVDV infection are considerable [8]. These losses, and the epidemiological insight that removal of PI animals efficiently truncates chains of infection, have encouraged control programs at the national and regional levels. Life vaccines may cause fetal infection or even trigger mucosal disease [9], whereas inactivated vaccines are safe, but confer insufficient protection, especially against fetal infection [10]. Consequently, the cornerstone of all current eradication programs is detection and elimination of PI animals. The approaches used to achieve this goal, however, differ. In countries where seroprevalence is low, PI animals may be detected by determining herd immunity to BVDV. High seroprevalence signals the presence of PI animal(s) in the herd, which may then be identified by virus detection. This approach is blunted by a high overall seroprevalence in the cattle population.

Ukraine currently has 4.5 million heads of livestock. These animals are kept in 4,350 herds, 10 to 20 per cent of which have 900 and more animals, with the biggest ones approaching 7,000 animals. In addition to the very large herds, there are an unknown number of small privately owned herds in Ukraine. About 60 per cent of the herds produce milk and about 30 per cent are in mixed milk and beef production. The rest are beef herds. The extent of animal traffic between the herds is not known in detail, but most likely a large fraction of male calves end up in beef herds, because only a relatively small number of bulls are required for natural breeding and artificial insemination. The intensity of import and export is not known at this time. There is no systematic testing for BVD, nor a systematic approach to BVD controlling exists. Inactivated and attenuated BVDV vaccines are in use, but the extent of use and the efficacy of these vaccines are unknown.

This study was focused on detection of BVDV specific antibodies by ELISA and viral RNA by real-time PCR, identification of persistently infected animals in selected cattle farms and with followed genetic typing of selected BVDV isolates.

## MATERIAL AND METHODS

*Cattle and sample collection.* 1,080 blood samples of cattle from 3 various farms in the North-East territory of Ukraine were used for this study. The samples were collected from November 2011 to June 2012. Animals were selected of various age, starting from newborns.

A total number of animals are 815 cattle in the first farm, 900 and 5,431 cattle in the second and third farms correspondingly. A detailed questionnaire was completed for each herd with the owner's support. The variables of interest related to individual animals as well as to the herd and comprised the type of farm, animal movements, general management, feeding, prophylactic health measures, disease incidence, and BVDV disease awareness.

*Antibody capture ELISA.* The ELISA test was performed by the commercially available ELISA Kit HerdChek BVDV Ab Test (IDEXX Laboratories, Switzerland), in which micro titre plates were coated with immobilizing BVDV antigen. BVDV antibodies of the sample were bounded to the antigen on the plates. After incubation of the test sample in the well, captured BVDV antibodies are detected by anti-bovine horseradish peroxidase conjugate. Unbound conjugate is washed away and a substrate/chromogen solution is added. In the presence of enzyme, substrate is converted into a product, which reacts with the chromogen to generate a blue color. The reaction was terminated by the addition of stop solution to each well and finally the absorbance at 450 nm was monitored in ELISA reader (BIO-TEK Instruments, USA). The result could be read visually where the optical density (OD) value was measured at 450 nm. Positive and negative controls were used as indicated in the kit. The presence or absence of BVDV antibody in the sample is determined by the corrected OD value (S/P) considered for each sample as follows:

$$SIP = \frac{Sample\ A_{450} - NC\bar{x}A_{450}}{PC\bar{x}A_{450} - NC\bar{x}A_{450}},$$

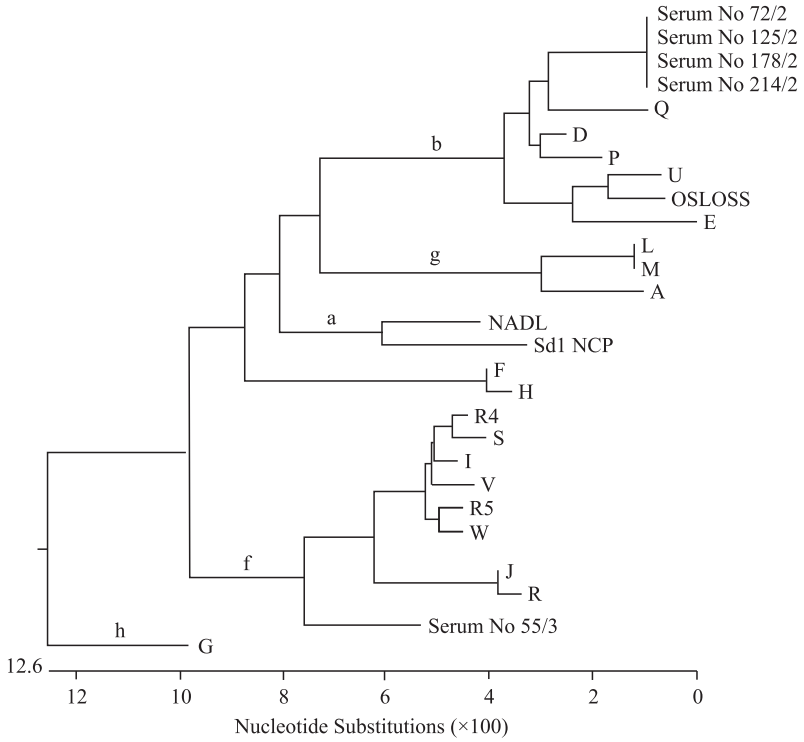
where $NC\bar{x}$ is negative control; $PC\bar{x}$ is positive control.

Samples with S/P values lower than 0.3 were classified as negative and samples with S/P values higher than 0.3 were classified as positive for BVDV antigen.

After serological testing, molecular analyses were conducted.

*Real-time PCR.* RNA extraction from the serum was performed using QIAamp Viral RNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instruction (vacuum protocol), with the following modifications. Serum samples were combined for five into one pool of 140 µl. The RNA was eluted in 60 µl RNA storage solution. The RT-PCR was applied, using *cador* BVDV RT-PCR Kit (QIAGEN) and carried out in a total volume of 50 µl, containing 20 µl of the eluate from the RNA isolation and 30 µl of the Master Mix and applied the following program in the ABI Prism 7700 Sequence Detection System: 1 × 30 min 50 °C (RT-step), 1 × 10 min 95 °C, and 45 × 30 s 95 °C; 1 min 55 °C (cycling).

*Phylogenetic study.* Samples determined as positive in PCR were studied with sequencing. Phylogenetic analysis in 5'-UTR (245 bp fragment) was used for the genetic typing of BVDV isolates into subgenotypes. Phylogenetic trees were constructed by multiple algorithms. All phylogeny trees buildings and analyses were done with modules of MEGA 3.1. software.




Genetic typing of BVDV isolates in the 5'-UTR region

*Statistical analysis*. Data were transferred to Microsoft Excel spreadsheet (Microsoft Corp. One Microsoft Way, USA) for analysis using NCSS 07.1.21 statistical software (NCSS, LLC, USA).

## RESULTS AND DISCUSSION

As the first step of our study, specific BVDV-antibodies were detected by ELISA in 713 of 1059 samples analyzed (67.3 per cent). This number is in agreement with findings in many cattle herds around the world. However, the number of positive samples differed in the herds. While 57 samples out of 283 (20.1 per cent) were identified in the first herd, 400 out of 475 (84.2 per cent) and 256 out of 301 (85 per cent) animals were positive in the second and third herds.

The real-time PCR assay detected BVDV RNA in 5 of 1,068 samples analyzed (0.5 per cent). 4 positive samples out of 490 (0.8 per cent) and 1 out of 301 (0.33 per cent) were found in the second and third herds. The genetic materials of BVDV were not found in the first herd.

Animals that were virus-positive in the RT-PCR but antibody-negative in ELISA were considered to be persistently infected. Based on these criteria, the results obtained with the antibody detection method and the RT-PCR were concordant in 1,047 of the 1,080 animals. All 5 virus-positive samples were serological negative. Consequently, 5 of these 1,047 (0.48 per cent) animals were persistently infected. The 5 virus-positive animals were 2, 4, 5 and 8 month old.

The genetic typing of viral isolates revealed that only BVDV type 1 were present. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f (Figure) and revealed that all 4 viruses from the second farm were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from the third farm was typed as BVDV-1f.

## CONCLUSION

The genetic typing of viral isolates revealed that only BVDV type 1 were present. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f and revealed that all 4 viruses from the second farm were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from the third farm was typed as BVDV-1f.

**Аспекти молекулярної епізоотології вірусної діареї великої рогатої худоби у Східному регіоні України**

Б. Т. Стегній[1], А. П. Герілович[1], С. Вільчек[2], Е. Петерханс[3], Р. О Кучерявенко[1], М. Ю. Стегній[1], І. В. Горайчук[1], В. І. Болотін[1], О. С. Солодянкін[1]

e-mail: antger@rambler.ru

[1]Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини»
Вул. Пушкінська, 83, Харків, Україна, 61023
[2]Університет ветеринарної медицини і фармацевтики в Кошицях
Вул. Коменського, 73, Кошице, 04001, Словакія
Інститут ветеринарної вірусології,
Бернський університет
Ленггасс-штрасе 122, CH-3001, Берн, Швейцарія

**Мета**. Виявити специфічні антитіла до вірусу діареї великої рогатої худоби серед тварин різних господарств

Східного регіону України, ідентифікувати персистентно інфікованих тварин та провести генотипування визначених ізолятів збудника ВД ВРХ. **Методи**. РНК-екстракція, полімеразна ланцюгова реакція у режимі реального часу, ІФА, секвенування. **Результати**. Специфічні до ВД ВРХ антитіла виявлено у 713 з 1059 досліджених зразків (67,3%). Подібні показники зареєстровано в багатьох господарствах по всьому світу. Проте кількість позитивних зразків у господарствах, де проводили дослідження, виявилася різною. Так, у першому з трьох господарств зафіксовано 57 позитивних зразків сироватки крові з 283 проаналізованих (20,1%), у другому – 400 з 475 (84,2%) і в третьому – 256 з 301 (85%). Надалі встановлено присутність РНК вірусу діареї ВРХ у п'ятьох з 1068 зразків (0,5%). Чотири позитивних зразки з 490 (0,8%) і один − з 301 (0,33%) ідентифіковано в другому і третьому господарствах. Генетичний матеріал збудника ВД ВРХ не визначено в зразках з першого господарства. Персистентно інфікованими вважали тварин, у сироватці крові яких знайдено РНК ВД, але не визначено антитіл до цього збудника. В результаті генотипування ізолятів ВД встановлено, що всі вони належать до 1-го генотипу. Виділені ізоляти представлено двома підтипами 1-го генотипу – b та f. Усі чотири зразки збудника ВД ВРХ з другого господарства належать до підтипу 1b та ідентичні в ділянці гена 5′-UTR, проте ВД з третього господарства ідентифікований як представник підтипу 1f. **Висновки**. Встановлено значне поширення збудника ВД ВРХ у господарствах Сходу України, що потребує подальших досліджень і розробки нових методичних підходів для виправлення існуючого стану.

**Ключові слова:** вірусна діарея великої рогатої худоби, ПЛР у режимі реального часу, ІФА, генотипування, філогенетичний аналіз, велика рогата худоба.

**Аспекты молекулярной эпизоотологии вирусной диареи крупного рогатого скота в Восточном регионе Украины**

Б. Т. Стегний[1], А. П. Герилович[1], С. Вильчек[2], Э. Петерханс[3], Р. А. Кучерявенко[1], М. Ю. Стегний[1], И. В. Горайчук[1], В. И. Болотин[1], А. С. Солодянкин[1]

*[1]Национальный научный центр «Институт экспериментальной и клинической ветеринарной медицины»*
*Ул. Пушкинская, 83, Харьков, Украина, 61023*
*[2]Университет ветеринарной медицины и фармацевтики в Кошицах*
*Ул. Коменского, 73, Кошице, 04001, Словакия*
*[3]Институт ветеринарной вирусологии, Бернский университет*
*Лэнггасс-штрасе 122, СН-3001, Берн, Швейцария*

*e-mail: antger@rambler.ru*

**Цель**. Выявление специфических антител к вирусу диареи (ВД) крупного рогатого скота (КРС) среди животных разных хозяйств Восточного региона Украины, идентификация персистентно инфицированных животных и генотипирование обнаруженных изолятов возбудителя ВД КРС. **Методы**. РНК-экстракция, полимеразная цепная реакция в режиме реального времени, ИФА, секвенирование. **Результаты**. Специфические к ВД КРС антитела выявлены в 713 из 1059 исследованных образцов (67,3%). Подобные показатели зарегистрированы во многих хозяйствах по всему миру. Однако количество положительных образцов в хозяйствах, где проводили исследования, оказалось различным. Так, в первом из трех хозяйств зафиксированы 57 положительных образцов сыворотки крови из 283 проанализированных (20,1%), во втором − 400 из 475 (84,2%) и в третьем − 256 из 301 (85%). Далее установлено наличие РНК вируса диареи КРС в пяти из 1068 образцов (0,5%). Четыре положительных образца из 490 (0,8%) и один − из 301 (0,33%) выявлены во втором и третьем хозяйствах. Генетический материал возбудителя ВД КРС не определен в образцах из первого хозяйства. Персистентно инфицированными считали животных, в сыворотке крови которых обнаружена РНК ВД, но не выявлены антитела к этому возбудителю. В результате генотипирования изолятов ВД установлено, что все они относятся к 1-му генотипу. Выделенные изоляты представлены двумя подтипами 1-го генотипа − b и f. Все четыре образца возбудителя ВД КРС со второго хозяйства принадлежат к подтипу 1b и идентичны в области гена 5′-UTR, однако ВД с третьего хозяйства идентифицирован как представитель подтипа 1f. **Выводы**. Установлено значительное распространение возбудителя ВД КРС в хозяйствах Восточного региона Украины, что требует дальнейших исследований и разработки новых подходов для улучшения существующего положения.

**Ключевые слова:** вирусная диарея крупного рогатого скота, ПЦР в реальном времени, ИФА, генотипирование, филогенетический анализ, крупный рогатый скот.

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