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EPIDEMIOLOGICAL ANALYSIS OF BVDV INFECTION IN CATTLE FARMS OF KHARKOV REGION, UKRAINE*

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SUMMARY: Bovine viral diarrhea is a widespread infection of cattle caused by bovine viral diarrhea virus (BVDV),a member of the Pestivirus genus of the Flaviviridae family. The virus persists in the cattle population by a unique combination of transient and persistent infections. Persistently infected (PI)animals may succumb to mucosal disease, which is characterized by lesions in the gastrointestinal tract and its invariably lethal outcome. This study was focused on identification PI animals in cattle farms of Kharkov region, Ukraine. For this reason 1080 blood samples from three different farms were tested for presence BVDV specific antibody by ELISA and viral genetic materials by real-time RT-PCR. In this study 5 PI animals were detected in two farms. Following phylogenetic analysis in 5'-UTR (245 bp fragment) was used for the genetic typing of revealed BVDV isolates into subgenotypes. The genetic typing indicated that all 4 viruses from second farm were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR. The virus from third farm typed as BVDV-1f.

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

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INTRODUCTION

*Pestiviruses*family displays marked genetic and antigenic diversity (Becheret al., 2003). There are four main species, namely, BVDV-1 and -2, Border disease virus and CSFV in the family. A marked diversity is observed also within the BVD viruses. In particular BVDV-1 viruses are very heterogenous, with at least 13 subgroups, whereas two subgroups are differentiated in the more homogenous BVDV-2 viruses (Jackovaet al., 2008).

BVDV is present in the cattle population worldwide (Nettleton and Entrican,1995). The success of BVDV rests on its capacity to establish persistent infection. Viral persistence is established during a "window of opportunity" early in gestation andis associated with immunotolerance to the infecting viral strain. Different from persistent infectionsby herpesviruses and lentiviruses, persistent infected(PI) animals remain free of antibodies to BVDV(Chase et al., 2004), which calls for detection of viral antigen or viral RNA as thesole methods for diagnosing persistent infection. Although transiently infected animals maybe capable of transmitting virus to susceptible cattle to a limited extent, only PI animals areresponsible for viral persistence in the host population. Typically, about one percent of thecattle population is PI and some 60 percent are seropositive when the infection has reachedan equilibrium (Houe, 1999; Hessmanet al., 2009). Calves born to seropositivecows receive colostral antibodies against BVDV (Peterhans et al., 2010). These antibodies decrease in titer overtime and the calves become susceptible for infection. The time span of colostral protectiondepends on the antibody titer and the level of infectious pressure to which the animals areexposed. Older animals are more likely to be seropositive, due to a longer time during whichthe animals are at risk of being exposed to PI animals. In contrast, many heifers may still beseronegative during their first pregnancy. When exposed to PI animals during the critical periodof development, fetuses may be infected to become PI, thereby assuring viral persistencein the next generation.

The economic losses due to BVDV infection are considerable (Weldegebrieletal., 2009). These losses, and the epidemiological insight that removalof PI animals efficiently truncates chains of infection, have encouraged control programs atthe national and regional levels. Life vaccinesmay cause fetal infection or even trigger mucosal disease (Becheret al., 2001), whereasinactivated vaccines are safe but confer insufficient protection, especially against fetalinfection (Van Oirschotet al., 1999). Consequently, the cornerstone of allcurrent eradication programs is detection and elimination of PI animals. The approachesused to achieve this goal however differ. PI animalsmay be detected by determining herd immunity to BVDVin countries where seroprevalence is low. High seroprevalence signalsthe presence of PI animal(s) in the herd, which may then be identified by virus detection. Thisapproach is blunted by a high overall seroprevalence in the cattle population.

Ukraine currently has 4.5 million cattle (http://minagro.gov.ua, 2013). These animals are kept in 4350 herds, of which 10 to 20% have 900 and more animals, with the biggest ones approaching 7000 animals. In addition to the very large herds, there are an unknown number of small privately owned herds in Ukraine. About 60% of the herds produce milk and about 30% 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 is there a systematic approach to controlling BVD. 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 animalsin selected cattle farmsand with followed genetic typing of selected BVDV isolates.

MATERIAL AND METHODS

*Cattle and sample collection.*1080 blood samples of cattle from 3 different farms in North-East territory of Ukraine were used for this study. The samples were collected from November 2011 to June 2012. Animals were selected of different ages from newborns.

A total number of animals are 815 cattle in the first farm, 900 and 5431 cattle in the second and the third farm. 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.

Antibodies capture ELISA. The ELISA test was performed by the commercially available ELISA Kit HerdChek BVDV Ab Test (IDEXX Laboratories, Switzerland), in which microtitre 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, Inc. Winooski, VT, USA). The result could be read visually where the optical density (OD) value was measured at450 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) for each sample was considered as follow:

$$\frac{S}{P} = \frac{Sample\ A_{450} - NC\bar{x}A_{450}}{PC\bar{x}A_{450} - NC\bar{x}A_{450}}$$

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

Samples with S/P values 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 RT-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 real-time RT-PCR was applied, using *cador*BVDV RT-PCR Kit (QIAGEN, Germany) 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 sec. 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 Neighbor Joiningand Maximum parsimony algorithms. Pair distance was determined by Murakami algorithm. All phylogeny trees buildings and analyses were done with modules of MEGA 5 software.

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

RESULTS AND DISCUSSION

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

The real-time PCR assay detected BVDV RNA in 5 of 1068 samples analyzed (0.5%). 4 positive samples out of 490 (0.8%) and 1 out of 301 (0.33%) were found in the second and the third herd. The genetic materials of BVDV were not found in the first herd.

Animals that were virus-positive in the real-time 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 real-time RT-PCR were concordant in 1047 of the 1080 animals. All 5 virus-positive samples were serological negative. Consequently, 5 of these 1047 (0.48%) 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 viruses were presented. The phylogenetic analysis confirmed two BVDV-1 subtypes, namely b and f (Fig. 1) and revealed that all 4 viruses from second farm were typed as BVDV-1b and all of them were absolutely identical in 5'-UTR, but virus from third farm were typed as BVDV-1f.

The genetic diversity, demonstrated in the study, releases the belonging of characterized viruses to BVDV-1b strains with the distance not more 2-4 %. This is typical in the current genetic studies of worldwide characterized viruses. Allocated viruses of this subtype are truly same inside this clade of Ukrainian viruses.

Another detected subtype was 1f. This group of BVDV-1 was also detected in several countries of the Central and Western Europe, so they are not unique. Characterized isolate had 4.5 % differences among subtype-belonged related viruses of BVDV-1f

genotype.

Current scientific literature explains the significant role of the BVDV-1 in the epidemiology of bovine viral diarrhea all over the World. It demonstrates distribution in all European countries, only several countries have been eradicated this disease by the implementation of the eradication strategies based on PI-animals elimination and/or vaccination of susceptible animals.

Viral genetic divergence studies allows to study the molecular diversity of virus for the creation of effective prevention means, and gives the opportunity to determine viral origin and source for recognition of the epidemiology of bovine viral diarrhea and its eradication strategy development.

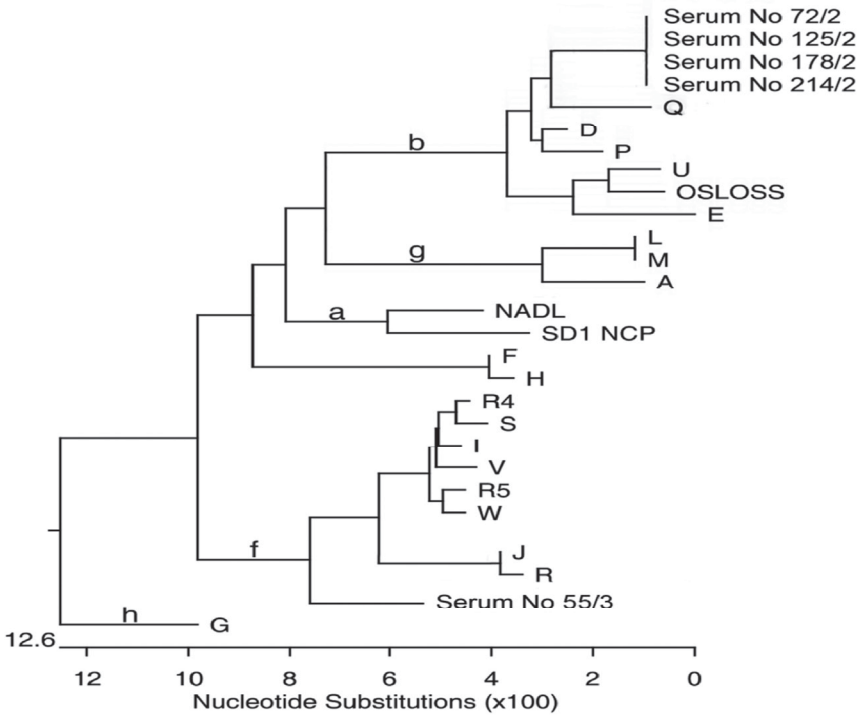

Fig. 1.Genetic typing of BVDV isolates in the 5'-UTR region

CONCLUSION

High seroprevalence levels for BVDV (20.1 - 85%) were demonstrated in the cattle herds.The real-time PCR assay detected BVDV RNA 0.33 - 0.8% of cattle have been tested. Detected viruses belonged to BVDV-1 type, subgenotypes 1b and 1f by 5'-UTR region sequences.

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EPIDEMIOLOŠKA ANALIZA BVDV INFEKCIJE NA FARMAMA GOVEDA U HARKOVSKOJ OBLASTI, UKRAJINA

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Izvod

Bovina virusna dijareja je oboljenje goveda, svetske distribucije, izazvano virusom bovine virusne dijareje (BVDV), koji pripada rodu *Pestivirus* i familiji *Flaviviridae*. Virus se održava u populaciji goveda jedinstvenom kombinacijom tranzitorne i perzistentne infekcije. Perzistentno inficirane (PI) životinje mogu razviti oboljenje sluznica, koje se karakteriše lezijama na gastrointestinalnom traktu sa letalnom ishodom. Ovo istraživanje se odnosi na identifikaciju PI životinja na farmama goveda u Harkovskoj oblasti, Ukraina. Prikupljenih 1080 uzoraka krvi, sa tri različite farme,

ispitano je na prisustvo specifičnih antitela na BVDV pomoću ELISA testa, kao i na prisustvo genoma BVDV primenom real-time RT-PCR testa. U ovoj studiji je registrovano 5 PI životinja na dvema farmama. Za genotipizaciju otkrivenih BVDV izolata u subgenotipove korišćena je filogenetska analiza 5'-UTR (245 bp fragment). Genetska tipizacija ukazala je da su sva 4 virusa sa druge farme klasifikovana kao BVDV-1b i imali su identičan region 5'-UTR. Virus sa treće farme klasifikovan je kao BVDV-1f.

Ključne reči: bovina virusna dijareja, real-time RT-PCR, ELISA, genotipizacija, filogenetska anliza, goveda.

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