Open Access

Berl Münch Tierärztl Wochenschr 129, 196–201 (2016)
DOI 10.2376/0005-9366-129-15102

ISSN 0005-9366

Korrespondenzadresse:
karl.schoepf@ages.at

Eingegangen: 28.08.2015
Angenommen: 19.12.2015

Online first: 23.03.2016
http://vetline.de/open-access/158/3216/

Institute for Veterinary Disease Control Innsbruck, Austrian Agency for Health and Food Safety (AGES), Innsbruck, Austria[1]
Institute for Veterinary Disease Control Mödling, Austrian Agency for Health and Food Safety (AGES), Mödling, Austria[2]
Division for Data, Statistics and Risk Assessment, Austrian Agency for Health and Food Safety (AGES), Graz, Austria[3]

Retrospective epidemiological evaluation of molecular and animal husbandry data within the bovine viral diarrhoea virus (BVDV) control programme in Western Austria during 2009–2014

Retrospektive epidemiologische Untersuchung anhand molekularbiologischer Daten und Einzeltierdaten im Rahmen des Bovinen Virus Diarrhoe Virus (BVDV) Bekämpfungsprogrammes in Westösterreich im Zeitraum 2009–2014

Karl Schoepf[1], Sandra Revilla-Fernández[2], Adolf Steinrigl[2], Reinhard Fuchs[3], Andreas Sailer[1], Joachim Weikel[1], Friedrich Schmoll[2]

Summary

A retrospective epidemiological investigation of molecular and animal husbandry data collected over an observation period of five years (2009–2014) within the compulsory bovine viral diarrhoea virus (BVDV) control programme in Western Austria, covering the federal provinces of Tyrol and Vorarlberg is presented in this study. Samples collected from 232 infected calves were phylogenetically classified based on the 5' untranslated region (5'UTR). All but 13 samples, which were typed as border disease virus subtype 3 (BDV-3), belonged to the bovine viral diarrhoea virus genotype 1 (BVDV-1) and clustered within six different subtypes (1b, 1e, 1f, 1h, 1d and 1k). Movement data and survival times from infected individual animals were analysed because of their potential of passing on infection to naive herds. From the moment of submission of the laboratory results, 180 animals were culled within the first month, 13 lived longer than two but not longer than six months and seven infected animals lived longer than one year. 13 of the infected animals were born on alpine pastures and eleven infected animals were grazed on mountain pastures during summer. The movement of infected animals and the role of trade in alpine areas are a possible source for spreading the infection, thus hampering the progress of eradication.

Keywords: bovine viral diarrhoea virus, epidemiology, diagnosis, phylogenetic analysis

Zusammenfassung

Es wird über eine retrospektive epidemiologische Erhebung mit Auswertung von molekularbiologischen Daten und Einzeltierdaten über einen Zeitraum von fünf Jahren (2009–2014) im Rahmen des Bovine Virus Diarrhoe/Mucosal Disease Bekämpfungsprogrammes in Österreich berichtet. Proben von 232 infizierten Kälbern aus den Bundesländern Tirol und Vorarlberg wurden einer phylogenetischen Analyse basierend auf der 5' nicht-translatierten Region (5'UTR) unterzogen. Mit Ausnahme von 13 Proben, die als Border Disease Virus (BDV 3) identifiziert wurden, konnten alle der Bovinen Virus Diarrhoe Virus Spezies 1 (BVDV-1) zugeordnet werden. Innerhalb dieser Spezies konnten sechs Subtypen identifiziert werden (1b, 1e, 1f, 1h, 1d und 1k). Bewegungsdaten und Überlebenszeiten der infizierten Tiere wurden auf ihre Risikorelevanz analysiert, neue Infektionen in freien Beständen hervorzurufen. 180 Tiere wurden innerhalb des ersten Lebensmonates nach der Übermittlung des positiven Laborbefundes geschlachtet bzw. getötet. Sieben infizierte Kälber lebten länger als ein Jahr, die Lebensspanne von 13 Tieren war länger als zwei, aber kürzer als sechs Monate.

13 der untersuchten Tiere waren auf Almen geboren und elf Tiere waren während der Sommermonate auf Almen aufgetrieben. Handel und Alpung von infizierten Kälbern sind eine mögliche Quelle der weiteren Virusverbreitung und können den Fortschritt des Bekämpfungsprogrammes negativ beeinflussen.

Schlüsselwörter: Bovines Virus Diarrhoe Virus (BVDV), Epidemiologie, Diagnose, phylogenetische Analyse

Introduction

Bovine viral diarrhoea virus (BVDV) was first described by Olafson et al. (1946). Together with classical swine fever and border disease viruses (BDV), it belongs to the genus Pestivirus within the family Flaviviridae. BDV causes primarily disease in sheep and goats. In domestic animals, the interspecies infection appears to occur particulary in the direction of cattle to sheep, but also vice versa (Krametter-Frötscher et al., 2008). BVDV is responsible for one of the economically most significant and interesting infectious diseases of cattle and other ruminants worldwide (Houe, 1999). In the majority of cases, infection in cattle leads to a transient or acute course and is clinically associated with gastrointestinal symptoms, abortions and drop in milk production. The majority of transiently infected (TI) animals show a mild clinical course and develop viraemia for up to 2–3 weeks (Hanon et al., 2012). After acute infection, animals develop immunity that is assumed to be life-long (Viet et al., 2007). Infections of the reproductive tract can cause abortion, stillbirth, embryonic death, congenital defects, or the birth of weak, undersized and persistently infected (PI) calves. The birth of immunotolerant PI calves results from exposure of an immunologically naive heifer or cow to non-cytopathogenic BVD virus between day 42 and 125 of gestation (McClurkin et al., 1984). Those PI animals remain life-long infected, are sources of constant virus shedding and are most likely developing the fatal Mucosal Disease (MD). The ability to cause two types of infection, which can lead to a long lasting viraemia, contributes to the worldwide distribution of BVDV infection in farm ruminants. Distinction between TI and PI animals is difficult, unless longitudinal frequent resampling of the same individual is possible (Hanon et al., 2012). PI animals are responsible for maintaining the infection in the herds (Baker, 1995), although both PI and TI animals excrete the virus and can induce horizontal transmission (Hanon et al., 2012).

The high genetic diversity is a hallmark of BVDV. BVDV are currently grouped into three species or genotypes, BVDV-1, BVDV-2 and BVDV-3. At least 13 subtypes can be differentiated within BVDV-1 (Bachofen et al., 2013). In a previous two years study performed during the time period 2005 to 2006 in Western Austria pestivirus positive samples were collected and typed by molecular methods. Except two samples clustering with the genotype BVDV-2 and one unique BDV-3 strain, all other samples belonged to the BVDV-1 genotype and clustered within eight different subtypes (Hornberg et al., 2009).

The BVDV control programme in Austria based on the national legislation of 2007 is focused on the detection and culling of infected animals (test-and-cull) in order to disrupt infection routes without using vaccination. Monitoring of the disease is accomplished by serological surveillance via antibody detection in bulk tank milk and serum from young stocks. Since 2009, every new-born calf in Tyrol is tested by skin biopsies (ear notch samples) for the presence of virus antigen. Samples obtained during ear tagging were demonstrated to be a useful alternative to blood samples for very early in vivo diagnosis of PI animals (Cornish et al., 2005; Fulton et al., 2006; Goyal, 2005; Houe et al., 2006) immunohistochemistry (IHC. Due to national legislation implemented in 2007 (Bundeskanzleramt RIS), ear notch samples are collected by the farmers in Tyrol, whereas in Vorarlberg only the offspring of infected farms are tested using this method. Sampling in Vorarlberg is performed by official veterinarians.

Herd management and movement of infected animals are likely to influence viral spread within and between herds. The risk of spreading the infection within a herd by a PI animal is likely depending on the length of its presence on the farm; therefore, the remaining lifespan of each pestivirus-positive animal starting from submission of positive laboratory results was recorded. Furthermore the transmission of BDV from clinically healthy PI sheep to cattle has recently been detected as a source of infection of pestivirus-free cattle herds. Calves kept together with sheep can seroconvert and pose a diagnostic problem in BVD eradication (Braun et al., 2014; Krametter-Frötscher et al., 2008; Preyler-Theiner et al., 2009). Thus, co-housing of cattle and sheep on the same premises was also recorded.

The aim of this study was to provide current data on molecular epidemiology over an extended time period in order to allow a comparison to previous published data and to conclude regarding a possible impact on the ongoing control programme. Thus, molecular typing of pestivirus positive field samples collected from cattle in Tyrol and Vorarlberg over a time period of five years (2009–2014) was performed. Recent results were compared to previous data obtained from the same geographical area studied by Hornberg et al. (2009).

Material and Methods

The cattle population of the province Tyrol is estimated to be 179 067 animals, kept on 9627 farms. In Vorarlberg, 64 539 cattle are owned by 2543 farms (Statistik Austria, 2013). In contrast to eastern Austrian provinces , the cattle herds in Tyrol and Vorarlberg consist of 19.1 and 29.5 animals on an average, respectively. The main purposes for cattle breeding are milk production and the export of pedigree animals, which plays an important economical role. During the period between May and September, about 60% of the entire cattle population of Western Austria graze on 2627 pastures (Tyrol: 2100, Vorarlberg: 527) (BMLFUW, 2013). On these alpine pastures cattle are kept in close proximity with sheep and goats. Moreover, pasturing takes place with cattle from neighbouring countries as Germany, Switzerland and Italy.

TABLE 1: *Pestivirus typing results based on the 5´-UTR region sequences from 232 calves over the period 2009–2014*

	2009	2010	2011	2012	2013	2014	Total	Proportion
Positive blood	42	45	39	29	15	16	186	80.2%
Positive ear notch	0	14	13	5	9	5	46	19.8%
BVDV-1b	26	10	23	7	3	1	70	30.2%
BVDV-1d	1	0	0	0	0	0	1	0.4%
BVDV-1e	5	0	0	0	4	0	9	3.9%
BVDV-1f	1	37	18	14	1	0	71	30.6%
BVDV-1h	9	9	10	11	11	17	67	28.9%
BVDV-1k	0	1	0	0	0	0	1	0.4%
BDV-3	0	2	1	2	5	3	13	5.6%
Total	42	59	52	34	24	21	232	100%

Blood and tissue samples

In general, all blood and ear tissue samples from new born calves in Tyrol were initially tested with a commercial ELISA (Herdcheck® BVDV Ag/serum plus, IDEXX, Hoofddorp, Netherlands). Until October 2009, tissue samples were obtained by using the ear tagging system TypiFix™ (Agrobiogen GmbH, Germany), afterwards ear tags from Caisley International (Bocholt, Germany) were used. About 80 000 tissue samples are processed yearly in the Province of Tyrol according to the ELISA manual. Selected positive ear tissues were suspended in ELISA buffer and selected positive blood samples were frozen at –20°C and sent for further analyses to the BVDV National Reference Laboratory. The samples were collected within the routine control programme during the period from January 2009 to December 2014. Excluding Ag ELISA-positive but PCR-negative or weak positive samples, 186 positive blood and 46 positive ear notch samples were included in this study and successfully typed. The 232 animals originated from 133 farms, 77 located in Tyrol and 56 in Vorarlberg.

Viral RNA detection

RNA extraction from 100 µl of serum was performed by an automated procedure, using the Freedom EVO® 150 platform (Tecan, Grödig, Austria) and the Nucleospin® 96 Virus kit (Macherey-Nagel, VWR, Vienna, Austria). Elution of bound nucleic acid was performed with 100 µl of buffer. Ear tag tissue solved in ELISA buffer, was manually recovered from the original ELISA tubes and put into a 2 ml tube with a stainless steel bead (Qiagen), ß-mercaptoethanol and RA1 lysis buffer. After homogenisation with the TissueLyser II (Qiagen), the supernatant of lysates were subjected to automated RNA extraction with the Nucleospin 96® RNA-Kit (Macherey-Nagel, VWR, Vienna, Austria). Finally, the RNA was recovered with 75 µl of RE elution buffer.

RT-PCR of RNA templates was performed following the protocol described in a previous publication (Hornberg et al., 2009), which is based on amplification of the 5´UTR using the primer pair 324 and 326 (Vilcek et al., 1994). The PCR products of 288 bp were separated by gel electrophoresis in 1.5% agarose gel stained with ethidium bromide. The fragments were visualized using an UV transilluminator. DNA bands of the expected size were excised from the agarose gel and recovered using the QIAquick® Gel Extraction Kit (Qiagen) according to the manufacturer´s protocol and finally the purified DNA was stored at –20°C.

DNA sequencing

Sequencing was performed as published previously (Hornberg et al., 2009) on the 3130xl Genetic Analyzer (Applied Biosystems by Thermo Fisher Scientific, Vienna, Austria) without modifications. The PCR primers were used for direct bi-directional sequencing of the PCR products. After assembling the 232 field sequences with SeqScape v2.5 software (Applied Biosystems by Thermo Fisher, Vienna, Austria) the pestivirus sequences were clustered using the Bionumerics software v6.6 (Applied Maths, Sint-Martnes-Latem, Belgium) and compared to other BVDV strains from western Austria (Hornberg et al., 2009) and pestiviruses reference strains published in GenBank. To investigate the genetic relationship of the recent detected field strains to previous strains, we constructed a phylogenetic tree using the neighbour joining method as implemented in MEGA5 (Tamura et al., 2011). All BVDV-1, BVDV-2 and BDV-3 strains studied by Hornberg et al. (2009) were also included in the cluster analysis. Bootstrap analysis was performed using 500 replicates.

Animal husbandry and herd management data

The data were extracted from the National Veterinary Information System (VIS) and covered the time period 01.01.2009–31.03.2015. The first quarter in 2015 was included in order to check animals movements which were positive detected by the end of 2014. The following parameters were determined: lifespan of virus positive animals, movement of infected animals between farms and parent animal during pregnancy, herd size, contact to small ruminants and herding on alpine pastures. The data was not validated concerning completeness and correctness. The statistical programming language R was used for data analysis (R Core Team, 2015).

Results

Molecular typing of pestivirus strains

For the amplification and sequencing of pestivirus viral RNA from the ELISA antigen positive samples, the 5´UTR was partially amplified. In total, 232 samples were successfully sequenced. BVDV-1 was detected in 94.1% of the 232 typed samples. The phylogenetic analysis revealed 219 sequences belonging to six different BVDV-1 subtypes (1b, 1e, 1f, 1h, 1d and 1k) and 13 BDV-3 sequences. The occurrence of BVDV-1 subtypes, BVDV-1f (71), BVDV-1b (70), and BVDV-1h (67), followed by BVDV-1e (9), BVDV-1d (1) and BVDV-1k (1) are summarised in Table 1. A phylogenetic tree was built using all 232 newly sequenced samples plus reference strains obtained from NCBI GenBank and following the classification used previously by Hornberg et al. (2009) (Fig. 1). In nine farms, three in Tyrol and six in Vorarlberg, animals within the herd were infected with two different subtypes. In one farm animals were infected with genotype 1h and BDV-3 and in a second farm with 1h and 1f. The remaining farms showed a combination of genotype 1f and 1b. The farm where three circulating subtypes (1b, 1f, 1h) were found was located in Vorarlberg.

Movement and husbandry data on individual and farm level

From the 232 infected animals, 138 were female and 91 male; the sex of the remaining three animals was unknown. In 47 out of 133 farms included in this study, cattle and small ruminants were kept on the same premises: eight kept cattle, sheep and goat, 18 cattle and sheep and 21 farms kept cattle and goat. Animal movement data revealed that 180 animals were culled within the first month after submission of the positive laboratory results; 14 animals lived longer than one month but not longer than two months; 13 lived longer than two but not longer than six months from submission of the laboratory results and seven animals lived longer than one year. From 18 animals no lifespan data was available in the database. Regarding husbandry data, eleven animals were grazed on mountain pastures after testing pestivirus-positive and 13 infected calves were born on mountain pastures. Six positive tested animals were moved afterwards out of the farm or were sold to other farms during their remaining lifespan. Seven animals were moved abroad; no further information is available for these animals. Movement data of the female parent animals from the 232 infected calves were also considered. This data showed that 28 cows had been sold and moved to another farm and 122 were on mountain pastures during pregnancy. The 13 BDV-3 positive cattle came from 13 different farms which also kept small ruminants (six farms kept cattle and sheep, three farms cattle and goats and four farms cattle, sheep and goats). Although a total of 47 cattle farms were keeping small ruminants on their premises, 34 of them had no history of BDV infection. The analysis of cattle movements to/from pastures and cattle movements between farms are shown in Figure 2.

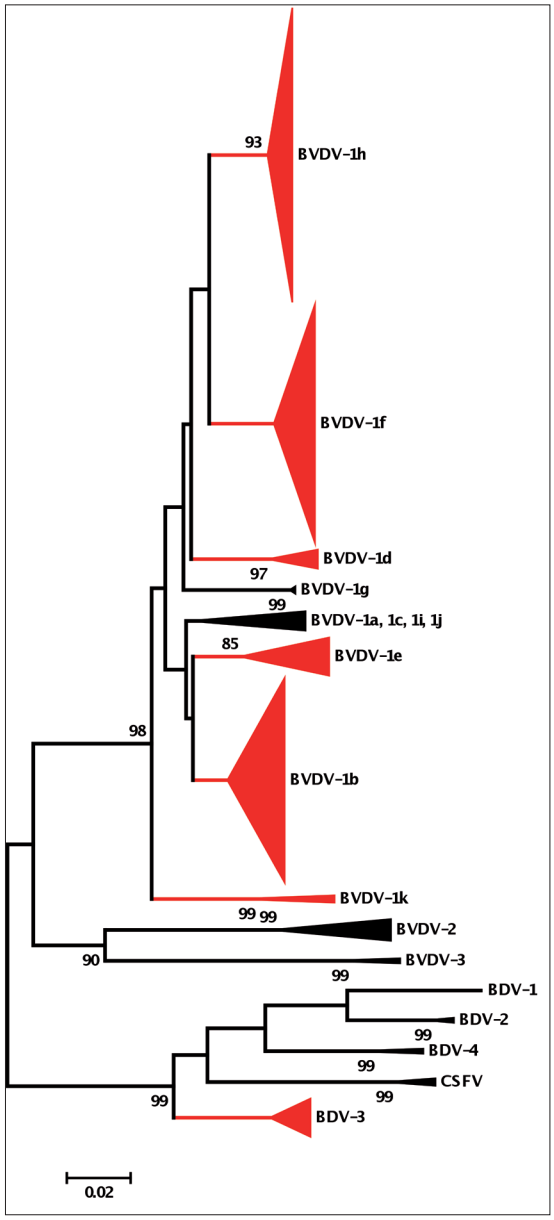

FIGURE 1: *Neighbour-joining tree based on 383 pestivirus sequences from the 5′-UTR region. The Maximum Composite Likelihood substitution model as implemented in MEGA5 was chosen, using the pairwise deletion option for the treatment of gaps and/or missing data. Clusters including pestivirus sequences newly determined in this study (n = 232) are indicated in red. Vertical extension of clusters is proportional to the number of sequences within the cluster. Bootstrap support values from 500 bootstrap iterations are indicated along the branches. Only bootstrap support values ≥ 80% are shown.*

Discussion

A follow-up study on the pestivirus genetic variability in two western Austrian regions was carried out to determine its influence on the eradication programme. In

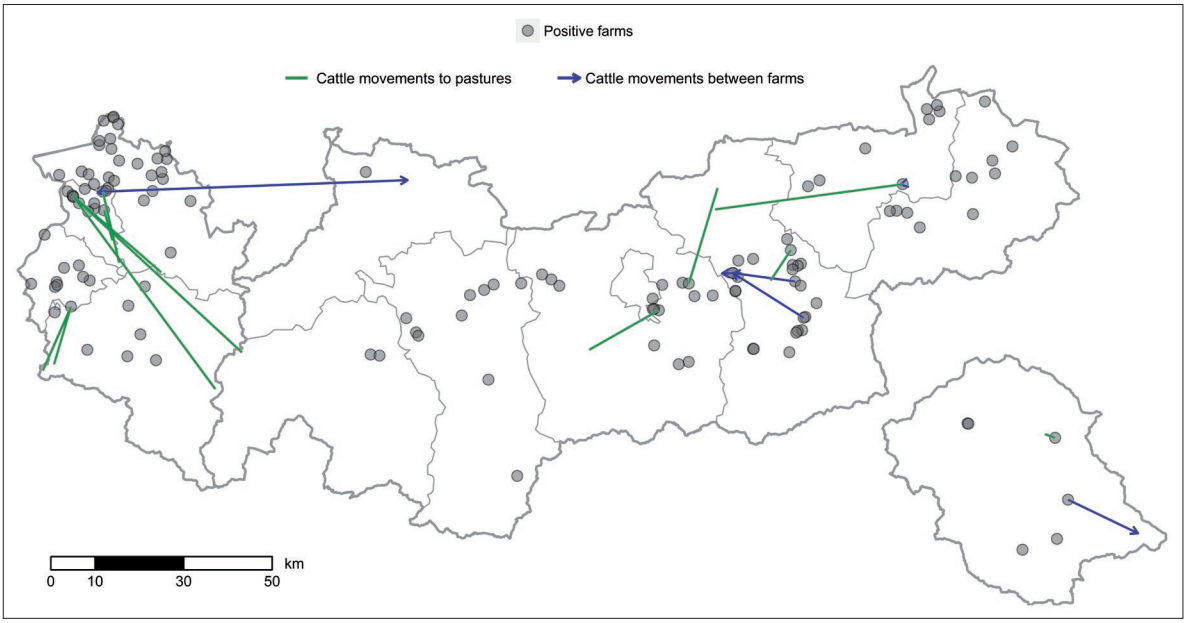

FIGURE 2: *Spatial distribution of farms with positive tested cattle (dots) and movements of positive tested cattle. Green lines represent movements to pastures and blue lines between farms.*

general, the genetic diversity of the BVDV-1 sequences obtained in recent years was shown to be high as at least six different subtypes of BVDV-1 were still found. When comparing data reported by Hornberg et al. (2009), two BVDV-1 subtypes (BVDV-1a, BVDV-1g) which were identified previously could not be found in this study. The most common subtypes found in virus positive cattle were 1f, followed by 1b and 1h, all three representing about 90% of the samples typed. Pestivirus sequences from the two periods 2005–2006 and those from 2009–2014 sharing ≥ 98.8% sequence identity were clustered into a total of 102 different "strains". 37 strains were only detected during the 2005–2006 period and were considered therefore as eliminated. From those eliminated, 24 belonged to BVDV-1f and 1h subtypes. Three of these 37 eliminated strains clustered either with BVDV-2, BVDV-1a or BVDV-1g. In contrast, 58 new strains were found in the present study, mostly belonging to BVDV-1b (18), 1f (15) and 1h (13), followed by BDV-3 (9) and BVDV-1e, 1d and 1k, each with 1 strain. Only ten strains were consistently found in both periods and six out of them belonged to the subtype BVDV-1h, followed by BVDV-1b, 1e, 1f and BDV-3, with each one strain.

Almost all pestivirus positive samples from Tyrol and Vorarlberg were classified as genotype BVDV-1. Surprisingly, 13 samples could be identified as BDV-3, compared to only one animal found during the period 2005 to 2006. As mentioned by Braun et al. (2014), BDV infection in cattle seems to be a common finding when BVDV eradication is tailing out. Cattle exposed to infected small ruminants will show seroconversion and possible mild clinical signs. This fact clearly poses a diagnostic problem for ongoing national schemes demonstrating freedom of disease by using antibody detection in serum and bulk milk (Braun et al., 2014). Also, the BVDV-1a and 1g subtypes were not further detected during the present study. However, BVDV-1f and BDV-3 became more frequent. The most abundand subtypes found in our study in descending order was 1f, 1b and 1h. Compared to the results from previous observation subtypes in descending order were 1h, 1f and 1b (Hornberg et al., 2009). In some cases health and epidemiological data of farm ruminants pasturing in the Austrian alps from neighbouring countries remains to be unknown. BDV can induce persistent infection and is able to perpetuate among cattle and sheep. It can also infect a wide array of wildlife species including deer and chamois (Schirrmeier et al., 2008).

Transhumance livestock management accounts for seasonal movement of about half of the entire cattle population of both Western Austrian Provinces. According to old traditions, heifers originating from different herds and regions are kept together during summer months on common pastures (Peterhans et al., 2010). This gathering of animals from different farms implies a high risk for re-infection of free herds and for the new development of PI animals (Bachofen, 2009). Other routes of infection such as over-the-fence pasture contact, or via contaminated equipment such as tools, needles, boots or clothes, and trade with young stock must be also taken into account as potential sources of infection.

For the first time, animal husbandry and movement data from selected pestivirus-positive cattle were recorded from a well described alpine area. Our hypothesis is that movement of infected animals together with extended life span seems likely to be a high risk for spreading virus into free farms. As laid down in the national BVDV Regulation, infected animals have to be separated as soon as laboratory results are available and removed from the herd without delay. The results of our study underline that these requirements might not be always efficiently implemented in daily routine. In future, it would be essential to centralise all information regarding a new BVD outbreak within the national Veterinary Information System (VIS).

The role of increased BDV-3 prevalence in cattle in alpine areas should be monitored and the possible role of wildlife as a reservoir should be addressed in further studies.

Acknowledgement

The authors gratefully acknowledge the technical assistance of the laboratory teams in Innsbruck and Mödling. We also would like to thank the Veterinary Department of the regional governments of Tyrol and Vorarlberg for good collaboration.

Conflict of interest

The authors declare no conflicts of interest.

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Address for correspondence:
Dr. Karl Schöpf
Institute for Veterinary Disease Control Innsbruck
Austrian Agency for Health and Food Safety
Technikerstraße 30
6020 Innsbruck
Austria
karl.schoepf@ages.at