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Genetic diversity of pestivirus isolates in cattle from Western Austria

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Abstract

The genetic diversity of bovine viral diarrhoea virus (BVDV) isolates in infected cattle from Tyrol and Vorarlberg (Austria) was investigated. Blood samples were collected within the compulsory Austrian BVDV control programme during 2005 and 2006. The 5´-untranslated region (5´-UTR) and partially the N-terminal autoprotease (Npro) were amplified by one-step reverse transcriptase-polymerase chain reaction (RT-PCR) and the PCR products were subsequently sequenced. Phylogenetic analysis based on 5´-UTR and Npro sequences demonstrated that almost all isolates (307/310) were of the BVDV-1 genotype. They were clustered into eight different subtypes, here listed by their frequency of occurrence: BVDV-1h (143), BVDV-1f (79), BVDV-1b (41), BVDV-1d (28), BVDV-1e (6), BVDV-1a (4), BVDV-1g (3) and BVDV1-k (3). Two pestivirus isolates were typed as BVDV-2 and one isolate as BDV closely related to Gifhorn strain (BDV-3). Correlation among isolates could only be observed at the farm level, i.e., within a herd. However, no correlation between the genetic and geographical distances could be observed above the farm level. Because of the wide distribution of certain BVDV-1 subtypes and the low prevalence of herd-specific strains, a determination of tracing routes of infection was not possible. Furthermore, recombination events were not detected.

Keywords: pestivirus, BVDV, sequencing, genetic typing


Introduction

The bovine viral diarrhoea virus (BVDV) is an important cattle pathogen and generates significant economic losses (Houe, 1999). Although BVDV infections usually cause no or only mild clinical symptoms such as inappetence, mild diarrhoea, oculo-nasal discharge, oral lesions, transient fever or reduced milk yield, severe forms elicit diarrhoea, pyrexia and decreased milk production (Baker, 1995). Several outbreaks of hemorrhagic syndrome were also described (Baker, 1995). The highest financial damage is caused by transplacental infections (Houe, 1999). Clinical signs are abortion, mummification, congenital defects, stillbirth and birth of weak or persistently infected (PI) calves. The extent depends on the timing of the infection relative to the period of gestation. PI cattle are immunotolerant to BVDV and shed the virus throughout their lifetime. Therefore, they are the main viral reservoir and source for viral transmission. Superinfections of PI animals induce the fatal mucosal disease, which is characterised by profuse and watery diarrhoea, anorexia, pyrexia, weakness and oral lesions (Baker, 1995).

The genus Pestivirus belongs to the family Flaviviridae and includes the following species: bovine viral diarrhoea virus 1 (BVDV-1), bovine viral diarrhoea virus 2 (BVDV-2), border disease virus (BDV) and classical swine fever virus (CSFV). As a tentative species in the genus, a pestivirus isolated from a giraffe was also added (Fauquet et al., 2005). Pestiviruses were primarily classified according to their host of origin, but many investigations demonstrated interspecies transmission (Paton, 1995). Therefore, the classification of pestiviruses based on genetic and antigenic characteristics is rather appropriate (Becher et al., 1999).

Pestiviruses are enveloed viruses of spherical shape. Their genome is a single-stranded, positive-sense RNA of a size of approximately 12.3 kb. The large open reading frame (ORF)
encodes a polyprotein of about 3900 amino acids and is flanked by the 5´- and the 3´-
untranslated region (UTR) (Fauquet et al., 2005).

Most of the phylogenetic analyses of the bovine viral diarrhoea virus have been based on the
5´-UTR of the viral genome (Vilcek et al., 1999; Falcone et al., 2003; Hurtado et al., 2003;
Vilcek et al., 2003; Kolesarova et al., 2004; Stahl et al., 2005; Uttenthal et al., 2005; Barros et
al., 2006; Pizarro-Lucero et al., 2006), but also on the N-terminal autoprotease (N\text{pro}) (Becher
et al., 1997; Vilcek et al., 2001; Toplak et al., 2004) and on the structural envelope protein E2
(Hamers et al., 1998; Tajima et al., 2001). BVDV can be subdivided into two species, the
BVDV-1 and the BVDV-2, based on the sequence variations within the highly conserved 5´-
UTR (Ridpath et al., 1994; Wolfmeyer et al., 1997). Genetic typing of BVDV-1 isolates from
different countries revealed at least eleven subtypes (1a-1k) (Vilcek et al., 2001; Vilcek et al.,
2004), whereas recently a new subtype (subtype 1l) was found (Jackova et al., 2008). BVDV-
2 is divided into two to four different subtypes (2a-2d) (Giangaspero et al., 2001; Tajima et
al., 2001; Flores et al., 2002).

BVDV-1 was first isolated in the 1940s (Olafson and Rickard, 1947) and it occurs worldwide.
In contrast, BVDV-2 was discovered in the 1990s and was initially identified only in North
America (Ridpath et al., 1994). In recent years, it has been also found in Europe (Tajima et
al., 2001; Vilcek et al., 2001; Couvreur et al., 2002; Vilcek et al., 2003), Japan (Nagai et al.,
2004), Korea (Park et al., 2004) and South America (Jones et al., 2001; Flores et al., 2002;
Pizarro-Lucero et al., 2006).

In the 1990s, non-obligatory BVDV control schemes were adopted in several provinces of
Austria, e.g., Lower Austria, Styria, Tyrol and Vorarlberg. In 2004, a compulsory national
BVDV control programme was implemented. The aim of this programme was the annual herd
screening to identify PI animals and their elimination without using vaccination (Lindberg
and Alenius, 1999; Schöpf et al., 2005).
The aim of this study was to type BVDV isolates from infected cattle in Tyrol and Vorarlberg, two provinces of Western Austria. All viruses were typed in 5′-UTR, selected isolates were also analysed in the Npro region. Within the ongoing national BVDV control programme we collected blood samples from the years 2005 and 2006. The highly conserved 5′-UTR sequences were tested for recombination using the likelihood permutation test (McVean et al., 2002), since recombination may cause changes in the genetic composition, such that phylogenetic distances become irrelevant. Furthermore, we analysed the correlation between genetic and geographical distances. Therefore, we performed a test based on permutation, comparing the phylogenetic to the geographical distance (Mantel, 1967).

Materials and methods

Serum samples and isolation of RNA

Blood samples of 353 cows from 163 different farms in the western Austrian provinces, Tyrol and Vorarlberg, were used for this study. The samples were collected from January 2005 to December 2006 within the national BVDV control programme. The geographical origin and herd information of all specimens were recorded and all samples were tested ELISA positive. The ELISA test was performed at the Austrian Agency for Health and Food Safety (AGES) in Innsbruck by the commercially available ELISA Kit HerdChek BVDV Ag/Serum Plus (IDEXX Scandinavia, Oesterbybruk, Sweden). After serological testing, the samples were sent to the Austrian Agency for Health and Food Safety (AGES) in Moedling for further molecular analysis.

Viral RNA was isolated from the 353 sera by using the QIAamp® Viral RNA Mini Kit (QIAGEN, Vienna, Austria) following the manufacturer's instructions.
For initial pestivirus RNA detection and in order to estimate the amount of RNA, a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems, Vienna, Austria) and the QuantiTect Probe RT-PCR Kit (QIAGEN) following the protocol described by Gaede et al. (2005) with the modifications that 0.2 µM TQ-Pesti Probe and 0.5 µM of each primer (Pesti 3 and Pesti 4) (Hyndman et al., 1998) were used. Three µl total RNA was added to the reaction mix. Reverse transcription was performed at 50° C for 30 min followed by 45 cycles of PCR amplification persisting of denaturation at 95° C for 30 s, primer annealing at 60° C for 30 s and elongation at 72° C for 30 s.

Both reverse transcription and PCR amplification of the viral RNA prior to sequencing were done in one-step using the One-step® RT-PCR Kit (QIAGEN). Template RNA (2.5 µl) was added to the reaction mix (22.5 µl) containing RNase Inhibitor (Invitrogen, Lofer, Austria), 1x one-step Buffer, 1 µl RT-PCR Enzym Mix, dNTP Mix (0.4 mM each) and 0.5 µM of each primer. For the 5´-UTR amplification the primer pair 324 and 326 (Vilcek et al., 1994) was used whereas amplification of the N-terminal autoprotease (N\textsuperscript{pro}) was done with the primer pair BD1 and BD3 (Vilcek et al., 2001). Reverse transcription was performed at 50° C for 30 min. PCR conditions were as follows: initial denaturation at 95° C for 15 min followed by 40 PCR cycles of denaturation at 94° C for 30 s, primer annealing at 50° C for 30 s (primers 324/326) or 62° C for 50 s (primers BD1/BD3) and elongation at 72° C for 1 min. The final elongation was extended to 5 min at 72° C. The PCR products of 288 bp (5´-UTR) and 428 bp (N\textsuperscript{pro}) were separated by gel electrophoresis in 1.5 % agarose gel stained with ethidium
bromide. The fragments were visualized using a UV transilluminator. DNA bands of the expected sizes 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.

Nucleotide sequencing

Sequencing reactions were performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Five µl of purified DNA was added to five µl of the sequencing reaction. Standard sequencing reaction was carried out. The RT-PCR primers were also used for sequencing of the 5´-UTR and Npro products. Afterwards, unincorporated dye terminators were removed using the DyeEx™ 2.0 Spin Kit (QIAGEN) following the protocol of the manufacturers. Each PCR products was sequenced from the 5´ and from the 3´ direction utilizing capillar electrophoresis on the 3130xl Genetic Analyzer (Applied Biosystems).

Computer-assisted analysis

In our analysis, additional sequences of representative strains of BVDV-1, BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV) from the NCBI (National Center for Biotechnology Information) GenBank were included. The nucleotide sequences were aligned using the SeqScape® Software Version 2.5 (Applied Biosystems). Phylogenetic trees were constructed using the Dnadist, Neighbor, and Consense programmes of the Phylogeny Inference Package (PHYLIP, Seattle, USA) Version 3.6 (Felsenstein, 2005). For BVDV-1, the strain Suwa (BVDV-1k) served as an outgroup sequence for the 5´-UTR and Npro phylogenetic trees. The 5´-UTR and Npro phylogenetic trees were constructed using 245 nucleotides long sequences flanked by 324/326 primers and 348 nucleotides flanked by
BD1/BD3 primers, respectively. Bootstrap analysis was performed on 1000 replicates with the Seqboot programme. Representative sequences of the 5´-UTR (n = 26), including two BVDV-2 and one BDV isolate, and of the N\textsuperscript{pro} (n = 14) obtained in this work were deposited in the NCBI GenBank with the following accession numbers: EU224221-EU224260 (Table1).

To test for the presence of recombination, a composite likelihood permutation test was carried out (McVean et al., 2002). Briefly, first the population recombination rate per site using the composite-likelihood estimation of 4N\textsubscript{e}r (N\textsubscript{e} = effective population size, r = genetic map distance across the region analysed) was calculated, using an extended version of Hudson’s composite likelihood estimator and a finite-sites model. Then, the recombination rate of the unpermuted data was compared with a data set where the genomic location of segregating sites was permuted. If the observed recombination rate had a higher value than the permuted data set, recombination would be estimated. This test can also be applied to RNA viruses with high mutation rates.

Furthermore, the geographical distance was compared with the genetic distance among several BVDV-1 subtypes using the permutation test (Mantel, 1967). For this purpose, either the samples from the same herd and samples from different herds were differentiated (the compared farm was encoded with 0, all other farms with 1) or the geographical distances between the farms were considered.

Results

For the identification of pestivirus RNA, we performed both, real-time and conventional RT-PCR. Real-time RT-PCR amplification with the pestivirus specific TaqMan\textsuperscript{®} probe and the primer-pair Pesti 3 and Pesti 4 (Hyndman et al., 1998) detected 301 of the 353 ELISA-positive samples. However, by the conventional one-step RT-PCR targeting the 5´-
untranslated region (5´-UTR) with the pestivirus specific primers 324 and 326 (Vilcek et al., 1994) we amplified these 301 and nine additional samples. These amplification primers were used for direct DNA sequencing of the PCR products in both directions. For phylogenetic analysis, a 5´-UTR sequence of about 245 bp was used. Sample B293/06, B384/06 and B385/06 could not be sequenced in this region and were therefore excluded from the 5´-UTR comparison.

To verify the BVDV-1 classification obtained from the 5´-UTR sequences, 33 samples representing all BVDV-1 subtypes and the three sera B293/06, B384/06 and B385/06 were examined in the N-terminal autoprotease (Npro) with another conventional one-step RT-PCR using the primer pair BD1 and BD3 (Vilcek et al., 2001). All samples were successfully analysed and a phylogenetic tree could be constructed with 348 bp long sequences.

The phylogenetic analysis based on the 5´-UTR sequences revealed 304 isolates belonging to eight different BVDV-1 subtypes (1a, 1b, 1d, 1e, 1f, 1g, 1h and 1k). Genetic typing was performed following the nomenclature by Vilcek (2001; 2004). Clustering of the BVDV-1 isolates showed a high diversity of the virus. Most isolates belonged to the subtypes BVDV-1h (141) and BVDV-1f (78). Forty-one viruses were classified as BVDV-1b, 28 clustered within the BVDV-1d subtype and three within the subtype BVDV-1g. We identified three BVDV-1 subtypes, never before described in Austria: these are four isolates of the subtype 1a, six of the subtype 1e and three of the subtype 1k.

Only two isolates (B91/05 and B253/06) were classified as BVDV-2, both showing a 99 % nucleotide similarity to the 5´-UTR region of the German BVDV-2 strain 104-98, found in Lower Saxony (Acc.No. AJ304381, Tajima et al., 2001).

One cattle (B300/06) from Innsbruck-Land was infected with a border disease virus (BDV). This isolate showed a 96.7 % similarity at the nucleotide level with the BDV strain Gifhorn and therefore phylogenetically clustered as a BDV-3. This was the first time that a BDV was shown in Austrian cattle.
The three samples (B293/06, B384/06 and B385/06), that could not be sequenced at the 5´-UTR, were successfully classified as BVDV-1f (one sample) and BVDV-1h (two samples) by analysing the N\textsuperscript{pro} region. In addition, the BVDV-1 grouping of the other 33 samples could be confirmed.

Due to the large number of samples, a graph of a complete phylogenetic tree based on all 5´-UTR isolates would have been too big. Therefore, a tree was built with only selected isolates (Fig. 1). Reference strains were obtained from the NCBI GenBank and were included in the alignment. These strains comprised different BVDV-1 subtypes, representative BVDV-2, BDV, classical swine fever virus (CSFV) and the Giraffe isolates. Bootstrap analysis was performed on 1000 replicates with bootstrap values 100 %, except for the the BDV genotypes (84 %). This phylogenetic tree clearly indicated the identification of two BVDV-2 isolates (B91/05, B253/06) and one BDV isolate (B300/06) in our collection of pestivirus isolates.

Furthermore, two other trees were constructed, showing the distinct subtypes of BVDV-1, one based on the 5´-UTR nucleotide sequences (Fig. 2) and the other one on the N\textsuperscript{pro} region sequences (Fig. 3).

Following the method of McVean et al. (2002), the recombination rate (\(\rho\)) of the 5´-UTR sequences showed a value of \(\rho = 14.00\). The recombination rate of the permuted samples was inferred to be higher (\(\rho = 16.23\)). Hence, we could not find evidence for recombination.

Originally, our 310 successfully analysed samples derived from 163 different farms. In 66 farms, more than one animal was infected with a pestivirus. In 40 of these cases (60.6 %) the animals were infected with the same BVDV subtype. In 37.9 % of the cases, which represent 25 farms, animals from the same herd were infected with two different genotypes or subtypes.

In one farm (1.5 %), even three different BVDV-1 subtypes occurred. Fig. 4 shows the distribution of the distinct subtypes in Tyrol and Vorarlberg. To quantitatively investigate a possible geographical correlation of the variation of BVDV, a permutation test was performed (Mantel, 1967). Using this test, a correlation of 0.088 between the geographical and the
genetic distance matrices was inferred. When, instead of geographical distances, only individuals from the same or different herds were differentiated, the correlation was 0.109. As the correlation at the farm level was higher than the geographical correlation, we concluded that correlation above farm level was absent.

Discussion

In this study, the genetic diversity of bovine viral diarrhoea virus (BVDV) isolates from two western provinces of Austria was analysed. By molecular analysis, 353 antigen-capture ELISA positive samples were examined. A total of 301 isolates were determined positive (85.3 %) by real-time RT-PCR. With the conventional one-step RT-PCR nine additional BVDV samples could be detected. The sequences of the nine real-time RT-PCR negative samples were examined, but no relevant mismatches in the primer and probe binding regions were observed (data not shown) (Klein et al., 1999). Therefore, we have no explanation for these real-time RT-PCR negatives. One reason for detecting only 310 out of 353 ELISA positives could be due to a poor RNA quality, but in addition, also ELISA false positives were observed in similarly designed investigations (Schöpf et al., 2005).

Initially, we could successfully sequence the 5´-untranslated region (5´-UTR) of 307 pestivirus isolates using the primers 324 and 326 (Vilcek et al., 1994). However, we could not sequence the 5´-UTR of three isolates (B293/06, B384/06 and B385/06). In order to exclude mispriming (Klein et al., 1999), another RT-PCR based on the 5´-UTR was repeated with the nested primers A11 and A14 (McGoldrick et al., 1998), but results remained unchanged (data not shown).

The most conserved region of the pestivirus genome is the 5´-UTR and therefore Becher et al. (1997) recommended its use for the classification of pestivirus isolates of several genotypes. However, for more detailed subdivision less conserved regions, e.g., the coding N-terminal
autoprotease \( (N^{\text{pro}}) \) region, are better suited. Therefore, for verification of the genetic variability of BVDV-1 observed in the 5´-UTR, a phylogentic analysis based on the \( N^{\text{pro}} \) region was carried out with 33 samples, representing different BVDV-1 subtypes. The results of the 5´-UTR typing were confirmed in all samples and additionally, the three samples that could not be sequenced at the 5´-UTR, could be successfully typed as BVDV-1f (B293/06) and BVDV-1h (B384/06 and B385/06).

Most pestivirus isolates (307/310) from Tyrol and Vorarlberg were classified as BVDV-1. They represented eight distinct subtypes within this genotype. This high BVDV-1 genetic diversity has never been described before in any country, although former publications confirmed a wide genetic range of BVDV-1 in Austria. In Styria five different subtypes (1b, 1d, 1f, 1g and 1h) were identified (Vilcek et al., 2003). In another study, BVDV field samples collected from certain parts of Austria revealed only four distinct subtypes, 1b, 1f, 1g and 1h (Kolesarova et al., 2004). In the present study, the subtype 1h accounted for the largest number of sequenced BVDV isolates (143), but also the subtype 1f (79 isolates) was widespread in Tyrol and Vorarlberg. These results are in accordance with Kolesarova et al. (2004), where three of five samples collected in Tyrol were also typed as BVDV-1h and two as BVDV-1f. However, the subtype 1f appeared to be the most abundant when samples from five different Austrian provinces were considered (Vilcek et al., 2003; Kolesarova et al., 2004). Only three of 310 isolates analysed in this study belong to subtype BVDV-1g. This low prevalence is in agreement with other Austrian investigations (Vilcek et al., 2003; Kolesarova et al., 2004), where only one BVDV-1g isolate was found. Interestingly, all BVDV-1g isolates of this study originated from Tyrol, but from three different districts (Innsbruck Land, Kitzbuehel and Kufstein). The three BVDV-1 subtypes 1a, 1e and 1k represented with four, six and three isolates, respectively, were isolated for the first time in Austria, both in Tyrol and in Vorarlberg. Three isolates of the subtype 1a were found in Tyrol (Schwaz) and one in Vorarlberg (Dornbirn) and four isolates of the subtype 1e were found in
Tyrol (Innsbruck Land, Reutte and Schwaz) and two in Vorarlberg (Bregenz). One cow infected with the subtype 1k was born in Tyrol and shared alpine communal pasturing during at least two summers in this province, so transmission of this subtype on the alp was possible. The other two animals, which were persistently infected, were born in the late summer on a farm in Vorarlberg and died with the age of two and three months. Both mother cows were infected in winter and therefore BVDV transmission occurred within animals in this farm. All 1k subtypes showed a similarity of 100% at the nucleotide level.

The BVDV-2 genotype was detected in samples B91/05 and B253/06 (Acc.No. EU224242 and EU224225). Both isolates showed a nucleotide similarity of 99% with the German virus strain 104-98 from Lower Saxony (Tajima et al., 2001). This was the first time that BVDV-2 was detected in Western Austria. Sample B91/05 was collected in the district Kitzbuehel, whereas sample B253/06 originated from a farm in the district Lienz, where cattle are imported from Bavaria. In 2003, one BVDV-2 isolate was described in Styria, Austria (Vilcek et al., 2003) for the first time (see Fig. 1, isolate 37-Gr), showing a nucleotide similarity of about 93% with the two BVDV-2 isolates of this study. In Europe, the prevalence of BVDV-2 compared to BVDV-1 was always considered to be very low: in Southern Germany two of 61 analysed BVDV isolates were BVDV-2 (Tajima et al., 2001) and in Portugal three of 34 typed viruses were BVDV-2 (Barros et al., 2006). In Slovakia, two BVDV-2 positive samples were detected (Vilcek et al., 2002). BVDV-2 was not present in field studies from Spain (Hurtado et al., 2003), Italy (Falcone et al., 2003), Switzerland (Stalder et al., 2005), Slovenia (Toplak et al., 2004), Denmark (Uttenthal et al., 2005) or England and Wales (Vilcek et al., 1999). A higher prevalence of this genotype has been described in Germany and Belgium, where 15.9% and 24.1% of the isolates, respectively, were classified as BVDV-2 (Wolfmeyer et al., 1997; Couvreur et al., 2002).

Surprisingly, one isolate in this study (B300/06, Acc.No. EU224227) was identified as a border disease virus (BDV), genotype BDV-3. So far, four different genotypes (BDV 1-4)
have been identified and until now, the genotype BDV-3 was only found in Germany (Becher et al., 2003), Switzerland (Stalder et al., 2005) and Austria (Krametter-Froetscher et al., 2007). The BDV isolate B300/06 showed a 96.7% similarity at the nucleotide level in the 5′-UTR to the BDV strain Gifhorn. The infected cow derived from a farm in the district Innsbruck Land (Tyrol), where cattle were kept in the neighbourhood of sheep. This was the first time that BDV was found in Austrian cattle, since BDV was only recently characterised in Austrian sheep (Krametter-Froetscher et al., 2007). We suggest that the transmission from sheep to cattle appears to be the most likely route of infection, as an interspecies transmission of ovine pestiviruses has also been described before by Paton (1995). In contrast, in a British study the transmission of BDV from sheep to cattle could not be proved, whereas BVDV transmission from cattle to sheep was not uncommon (Vilcek et al., 1999).

Because mutation and recombination have been observed in pestiviruses, this work also aimed to test for RNA recombination. Therefore, we analysed the 5′-UTR sequences using the composite likelihood permutation test (McVean et al., 2002). However, no evidence for recombination could be detected. The high diversity of BVDV found in Tyrol and Vorarlberg might evolve from the intense trade practices in this region.

In terms of epidemiological features, the possible geographical correlation to the prevalence of certain BVDV-1 and BVDV-2 subtypes occurring in the Western part of Austria was investigated. Only a low correlation between the genetic and geographical distances at the farm level was found. This is in contrast to other works, where viruses of the same farm were closely related (Hamers et al., 1998; Paton et al., 1995). Principally, this could be due to the communal pasturing and frequent trading with young animals, which are common practices in Western Austria. In contrast to other Austrian regions the average size of 17 animals per herd is relatively low. During the summer period, which lasts from May until September, about 60% and of the Tyrolean cattle population is kept on approximately 2,600 alps. In Vorarlberg about 80% of the cattle population pastures on approximately 500 alps. Additionally, sheep
and goats are kept on alps in close proximity with cattle during summer. During this period of grazing contact with wild ungulates is also possible. All these factors might explain the absence of herd-specific strains. Hamers et al. (1998) suggested that a high variability of BVDV sequences may derive from the horizontal transfection from a BVDV-immunocompetent to a susceptible animal, while herd-specific strains may derive from the transmission from PI animals within the farm. In the present work, evidence for geographical clustering of genotypes or subtypes above the farm level was not determined. These findings are in accordance with other previous investigations (Vilcek et al., 1999; Stalder et al., 2005). In Sweden, where the BVD eradication programme has been running since 1993, the molecular epidemiology is actually used to trace sources and routes of BVDV infections. The comparison of existing BVDV sequences to new cases has been used as an important tool to trace the origin of new outbreaks (Stahl et al., 2005). The samples of this study were collected over two years (2005 and 2006) within the Austrian compulsory BVDV control programme, which was implemented in 2004. So far, it has not been possible to retrace routes of BVDV infections due to the wide geographical distribution of several BVDV subtypes in Tyrol and Vorarlberg and to the low prevalence of herd-specific strains. Tracing the origin of BVDV infections in the future may gain importance during the final phase of the BVD control programme, when the prevalence will be reduced and almost all herds will have a BVDV-free status. The development of a sequence database, as established in Sweden (Stahl et al., 2005), including all characterised isolates from BVDV infected animals from Austria and neighbouring countries, will be a useful tool for eradication and for studying the epidemiology of BVD. Then, genetic typing of BVDV isolates could be used for tracing new infection routes more economically.
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References


Table 1.
Sequences representing Austrian pestiviruses from Tyrol and Vorarlberg and their phylogenetic classification.

Fig. 1.
Typing of selected Austrian pestivirus isolates in the 5’-UTR.
The Austrian isolates B91/05 and B253/06 in the BVDV-2 branch and B300/06 in the BDV branch are written in bolt letters. The selected BVDV-1 Austrian isolates are presented as nonlabelled branches only. They are more precisely analysed on Fig. 2. The origin of the 245 bp nucleotide sequences taken from the NCBI GenBank: Giraffe: H138 (AB040131); BDV: Moredun (U65022); Reindeer (AF144618), Chamois (AY38080), CSFV: Alfort (J04358), Brescia (M31768), C (Z46258); BVDV-2: 890 (U18059), 37-Gr (EU327594), 104-98 (AJ304381); BVDV-1: BVDV-1a – NADL (M31182), BVDV-1b – Osloss (M96687), BVDV-1d – F (AF298065); BVDV-1e – 3-It (AF298062), BVDV-1f – J (AF298067), W (AF298073), BVDV-1g – A (AF298064), BVDV-1h – G (AF298066), BVDV-1i – 23-15 (AF298059), BVDV-1k – Rebe (AF299317). The nucleotide sequence for Gifhorn strain was obtained in this work. The tree was computed by the neighbor-joining method (Kimura 2-parameter; transition/transversion ratio: 2.0) using the PHYLIP Dnadist and Neighbor programmes (Felsenstein, 2005). Bootstrap values are given in percentage for 1000 replicate data sets, performed with the PHYLIP Seqboot programme (Felsenstein, 2005).

Fig. 2.
Genetic typing of selected BVDV-1 isolates in the 5’-UTR.
The phylogenetic tree was constructed from 245 bp 5’-UTR sequences of selected BVDV-1 isolates. The Austrian isolates analysed in this work are labelled in bolt. The tree was generated with the PHYLIP Dnadist, Neighbor and Consense programmes (Felsenstein, 2005). Bootstrap values for 1000 replicate data sets were calculated for all BVDV-1 subgroups. All branches for the BVDV-1 subtypes were supported with 100 percentage support, except BVDV-1b branch supporting with 84.5 %. The nucleotide sequences were taken from the NCBI GenBank as it is shown in the legend of Fig. 1, except Bega (AF049221), Trangie (AF049222), Deer (AB040132), Suwa (AF117699), isolates 71-15 and 71-16 were taken from Jackova et al. (2008).
Fig. 3.
Genetic typing of selected BVDV-1 isolates in the N\textsuperscript{pro} region.
The phylogenetic tree was constructed from 392 bp nucleotide sequences from the N-terminal part of N\textsuperscript{pro}. The Austrian isolates analysed in this work are labelled in bold. Other sequences were taken from the NCBI GenBank with the following accession numbers: BVDV-1a – NADL (M31182); BVDV-1b – Osloss (M96687); BVDV-1c – Bega (AF049221), Trangie (AF049222); BVDV-1d – F (AF287284); BVDV-1e – 3-It (AF287282); BVDV-1f – J (AF287286), W (AF287290); BVDV-1g – A (AF287283); BVDV-1h – G (AF287285); BVDV-1i – 23-15 (AF287279); BVDV-1j – Deer (U80902); BVDV-1k – CH-Suwa (AY894998). The tree was constructed using PHYLIP Dnadist, Neighbor and Consense programmes (Felsenstein, 2005). The bootstrap values presented in percentage supporting particular branch were computed with the Seqboot programme for 1000 replicates.

Fig. 4.
Geographical distribution of all BVDV genotypes and subtypes, respectively, circulating in Tyrol and Vorarlberg.
Every spot represents a farm.
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