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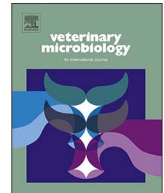

Eradication of bovine viral diarrhea virus in Germany—Diversity of subtypes and detection of live-vaccine viruses

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A B S T R A C T

Bovine viral diarrhea (BVD) causes high economic losses in the cattle population worldwide. In Germany, an obligatory control program with detection and removal of persistently infected animals is in force since 2011. For molecular tracing of virus transmission, a comprehensive sequence data base of the currently circulating BVD viruses was established. Partial sequences of 1007 samples collected between 2008 and 2016 were generated. As dominant viruses, subtypes 1b (47.0%) and 1d (26.5%) could be identified with no marked geographic or sampling year effect, a much higher amount of BVDV-2c was detected in 2013 compared to other years, predominantly in Western Germany. In addition, subtypes 1a, 1e, 1f, 1h, 1g, 1k, and 2a were found. Interestingly, besides field-viruses, two different live-vaccine viruses were detected in tissue samples of newborn calves (n = 37) whose mothers were immunized during pregnancy.

1. Introduction

Bovine viral diarrhea virus (BVDV), which is endemic in cattle populations worldwide, is a pestivirus within the family *Flaviviridae* and is closely related to the ovine Border disease virus (BDV) and classical swine fever virus (Fauquet et al., 2005). BVDV exists in two species (BVDV-1 and BVDV-2) which are further subdivided into subtypes based on genetic analysis (Vilcek et al., 2004, 2001). During the last years, several additional pestiviruses were described which represent further putative species of this genus (Firth et al., 2014; Kirkland et al., 2007; Schirrmeier et al., 2004). According to their growth in cell culture, BVD viruses are classified into two distinct biotypes: cytopathic (cp) and non-cytopathic (ncp) (Ridpath, 2008). Clinical signs of bovine viral diarrhea (BVD) range from inapparent infections to non-specific symptoms such as diarrhea, fever, hemorrhagic lesions, pneumonia or to the inevitably fatal mucosal disease (MD). Dependent of the phase of gestation, fetal infections with ncp BVDV may result in abortion, stillbirth, teratogenic effects or the birth of immunotolerant, persistently infected (PI), viremic calves (Baker, 1995). MD is associated with the appearance of a cp biotype arising from mutations of the ncp BVDV which is already circulating in the PI animal or with the superinfection of the animal with a cp strain that is homologous to the persisting ncp strain (Lanyon et al., 2014).

BVD is one of the most prevalent infectious diseases of cattle causing major economic losses (Houe, 1999, 2003). Therefore, BVD eradication programs have been implemented in several European countries including the Scandinavian countries, Switzerland, Lower Austria or Ireland (Moennig and Becher, 2015; Moennig et al., 2005b; Stahl and Alenius, 2012). Despite the common goal of BVDV eradication from the respective cattle populations different approaches were selected. The Scandinavian model, which was also implemented in Lower Austria, is based on the preselection of farms with a higher risk for the presence of PI animals within the herd by using e.g. large scale bulk milk serology combined with a strict non-vaccination policy. Subsequently, all animals from higher PI risk prevalence herds were tested, the detected PI animals were removed, and an ongoing serological monitoring was established. Under the Scandinavian conditions, the freedom of BVDV might be achieved within a timeline of about 10 years (Lindberg and Alenius, 1999; Stahl and Alenius, 2012). An alternative approach to BVD control, based on the direct testing (genome or antigen) of all animals of the entire cattle population for BVDV within the first year, and the following testing of tissue (ear notch) or blood samples of each newborn calf in the subsequent years, proved beneficial especially for countries with a high initial BVDV prevalence, a high level of cattle trading and transport partly combined with ongoing vaccination campaigns. Switzerland started a program following this approach without vaccination in 2008 and the prevalence of PI animals decreased to 0.02% till the end of 2012 (Bachofen et al., 2013). In Ireland the same approach was initiated, and an obligatory program focusing on the testing of ear notch samples of all newborn calves has started in 2013 (Clegg et al., 2016; Graham et al., 2014).

In Germany, BVD is one of the notifiable diseases of cattle since

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2004. In December 2008, a federal regulation for an obligatory nationwide control program was decreed by the Federal Ministry of Food and Agriculture which came into force on the 1st of January 2011. Due to high and varying BVD prevalences, diverse sizes and structures of cattle holdings and traditional differences in agricultural, commercial and trading structures between the federal states, the German eradication program has in the first phase the major goal of the fast and efficient reduction of PI animals, and the establishment of so-called "unsuspicious (non-PI)" animals and farms with a certified status. The defined basis rules are the obligatory investigation of every newborn calf for BVDV antigen/genome in the first 6 months of life, since June 2016 in the first 4 weeks of life, the immediate elimination of all detected PI animals, and trade only with certified unsuspicious animals. Reinfections of cattle holdings are predominantly prevented by biosecurity measures and in contrast to most European countries by voluntary vaccination. For the prevention from BVDV infections several inactivated vaccines, either as monovalent preparations or in combination with immunogens against further pathogens, as well as two live vaccines (Bovela® [Boehringer Ingelheim Vetmedica GmbH, Ingelheim/Rhein, Germany] and Vacoviron® FS [Merial GmbH, Hallbergmoos, Germany]), are licensed in Germany (Paul-Ehrlich-Institut, 2016). Two-step immunization based on inactivated vaccines for priming and following booster vaccination by a live attenuated vaccine is recommended to induce a long lasting immune response and to prevent fetal infection (Moennig et al., 2005a).

Between the onset of the mandatory testing in 2011 and the end of 2016, the proportion of animals classified as PI among all new-born calves in Germany was considerably reduced from 0.5% (23,792 PI among 4929,160 new-born calves) to less than 0.03% (1005 PI among 4915,421 calves) (Wernike, 2017). The number of cattle holdings with PI animals decreased likewise from 7808 (3.44%) in the year 2011 to 324 (0.16%) in 2016. The challenge of the coming years will be e.g. the detection and removal of the remaining PI animals as soon as possible.

As demonstrated in the final phase of the BVDV programs in Switzerland or Lower Austria, the typing of viral isolates and subsequent comparison of the identified subtypes with previously present strains is a valuable tool for the identification of relationships between BVD outbreaks, and helps to find viruses responsible for a longer circulation in individual cattle holdings, or to identify potential risk factors for virus transmission (Rossmanith et al., 2014; Stalder et al., 2016). However, in order to use the molecular tracing more efficiently, a comprehensive sequence data base of the currently circulating viruses is required for the comparison with new samples. Hence, the 5′ untranslated region (UTR) sequences of a considerable number of samples collected throughout Germany during the last years were generated to be used for molecular tracing in the final phase of the German eradication program.

2. Materials and methods

From 2008 to 2016, a total of 1007 BVDV-positive viral RNA or blood samples or ear notch samples in ELISA or PCR buffer were submitted from veterinary diagnostic laboratories to the national reference laboratory. The diagnostic submissions included samples from 12 of the 16 German federal states (Table 1).

Ear notch samples soaked in ELISA or PCR buffer were manually recovered from the original tubes, homogenized in RLT buffer (Qiagen, Hilden, Germany), and viral RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendation. From blood samples, viral RNA was extracted using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany). The presence of pestiviral RNA was confirmed by a previously published panpesti real-time PCR (Hoffmann et al., 2006) with minor modifications of the FAM-labelled probe (5′-TGC YAY GTG GAC GAG GGY ATR C-BHQ1-3′). From PCR-positive samples, sequence information of the 5′ untranslated genome region (5′ UTR) was generated as described previously (Schaarschmidt et al., 2000). The sequences were assembled and aligned, and the pairwise similarity was calculated using Geneious version 9.1.6 (Biomatters, Auckland, New Zealand). To identify the BVDV subtypes, sequences of representative reference strains available at NCBI GenBank were used.

3. Results

Of the 1007 samples analyzed in the present study, 934 (92.8%) belonged to BVDV-1 and 73 (7.2%) to BVDV-2. The dominant subtypes were 1b (47.0%) and 1d (26.5%), independent of the federal state (Fig. 1) and year of sampling. However, a remarkably amount of BVDV-2c was detected during 2013 (Fig. 2), predominantly in North Rhine-Westphalia (NW) and Lower Saxony (NI) (Fig. 1). Additionally, BVDV of subtypes 1a (4.1% of the analyzed samples), 1e (3.5%), 1f (7.8%), 1h (3.5%), 1g (0.3%), 1k (0.1%), and 2a (3.1%) were also present in Germany (Figs. 1 and 2). BVDV-1g and 1k were only found in Southern Germany (Bavaria [BY]), while the other subtypes were distributed all over the country (Fig. 1).

A total of 41 BVDV-1a sequences were identified with 34 of them being identical to the vaccine virus Oregon C24V which is used since the 1960s in Europe (Balint et al., 2005; Coggins et al., 1961) and is part of the BVDV vaccine Vacoviron® FS. The mean quantification cycle value (Cq) of the corresponding ear notch samples was 32.4 (± 1.8) when tested with a panpesti real-time PCR system (Hoffmann et al., 2006).

Three out of the 473 BVDV-1b sequences were identical to the vaccine strain KE-9 (Bovela®). In all these cases the double individual genomic deletions (N^{pro} protease codons 5–168, and E^{rns} RNase codon 349 (European-Medicines-Agency, 2014)), which were introduced into KE-9 to attenuate the virus, were confirmed by sequencing of the ear notch samples as described above using the primers Bovela1-241F (5′-GCA CAT CTT AAC CTG AGC GG-3′) and BVD-1-1671R (5′-CAC ATC ACT TGC GGC TAT TTC-3′).

In one of those cases of the detection of the vaccine strain KE-9 (Bovela®), the pregnant dam was vaccinated in the 3rd month of gestation and BVDV-genome was detected by the panpesti real-time PCR in the ear notch sample taken from the newborn calf with a Cq value of 26. When the calf was culled 5 days after the first sampling, a second ear tissue sample was analyzed. Even though the BVDV genome was confirmed (Cq 24), no virus could be isolated. In an additional blood sample taken at the second sampling, viral RNA was not detectable. However, the blood sample was also tested for BVDV-specific antibodies by a standard microneutralization test against the BVDV-1b strain "Paplitz" as described in the German official collection of test methods for bovine viral diarrhea (Friedrich-Loeffler-Institut, 2016). An antibody titer of 1/640 was calculated.

In the second and third case of vaccine strain KE-9 (Bovela®) detection in ear notch samples (Cq 24 and 25), the calves succumbed after birth or were slaughtered immediately after the first positive BVDV result. Therefore, no further samples were available.

4. Discussion

As shown in other European countries with ongoing BVDV eradication programs, the molecular tracing of BVDV is a very valuable tool e.g. for the identification of relationships between BVD outbreaks, to identify potential risk factors and to interrupt chains of infections (Rossmanith et al., 2014; Stalder et al., 2016). For the efficient use of this molecular approach also in Germany, a comprehensive data set of the circulating viruses was established by generating 5′ UTR sequences of the viruses present in the cattle population since 2008. Although more suitable genomic regions such as the Npro or E2 coding regions might exist, the 5′ UTR is the established standard, and there is the highest number of sequences in the databases available. The 5′ UTR was therefore also selected in the present study and it provides sufficient

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