The role of molecular-genetic techniques in BVDV eradication in Lower Austria

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Summary
A voluntary bovine viral diarrhoea virus (BVDV) control programme, which later became a compulsory eradication programme, based on the Swedish model was introduced in Lower Austria in 1997. The persistently infected animals were detected by Ag-ELISA and all samples were re-tested by the improved single-tube RT-PCR, employing panpestivirus primers targeting the 5'-UTR of the virus genome. In 2010, the BVDV eradication programme, which became compulsory from 2004, reached the final stage with only five remaining infected herds in which BVDV was difficult to eradicate. To resolve the problem in those herds, a molecular epidemiology approach was used. No differences in the spectrum of BVDV-1 subgenotypes at the beginning and at the final stage of eradication programme were found. The genetic study revealed the importance of human risk factor when finishing an eradication programme. Molecular epidemiology was also used to analyse BVDV isolates associated with re-introductions to BVDV-free herds.

Introduction

Bovine viral diarrhoea virus (BVDV) is the causative agent of bovine viral diarrhoea and mucosal disease (BVD/MD) (Baker, 1995; Brownlie *et al*., 1984) which is responsible for significant economic losses in cattle farms (Houe, 2003). Infected animals develop a spectrum of clinical signs and may suffer from infertility, abortion, malformation and immunosuppression (Lanyon *et al*., 2014). The infection of pregnant animals in the first trimester of gestation (30^{th} – 125^{th} day) is most dangerous because immunotolerant persistently infected (PI) cattle may be born (Brownlie *et al*., 1998). They are constant carriers of virus during their lifetime. PI animals have no or very rare low levels of specific antibodies but they are the main transmitters of BVDV within and between the herds.

Two different approaches were applied to prevent the spread of BVDV infection in cattle populations. The first approach uses the application of BVDV specific vaccines (Bolin, 1995). However, vaccination cannot change health status of PI animals because their immune response is poor. In principle, vaccination of cattle can reduce the impact of BVDV but does not result in successful eradication in large geographic regions. Despite that, it is widely applied in America and Asia at least to partially improve health status of cattle herds (Van Campen, 2010).

In contrast, a zoosanitary approach represented by a control program without vaccination has been widely used in many European countries. These control/eradication programmes are based on the identification and elimination of PI animals from the herds (Harkness, 1987). This was documented in Sweden where the first BVDV eradication programme without vaccination was successfully introduced in 1993 (Lindberg and Alenius, 1999). Later, similar BVDV eradication programmes began in other Scandinavian countries (Bitsch *et al*., 2000; Nuotio *et al*., 1999; Valle *et al*., 2005).

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Molecular-genetic techniques have benefitted biological research as well as having many practical applications. Methods such as RT-PCR, and real-time RT-PCR are widely used for the detection of BVDV in clinical samples. The sequencing of the virus genome fragments coupled with computer-assisted phylogenetic analysis are used in molecular epidemiology (Cerutti *et al*., 2016; Giammarioli *et al*., 2008; Toplak *et al*., 2004).

The BVDV eradication programme started in Lower Austria in 1997. After an enormous effort, farmers, veterinarians and other specialists achieved BVDV eradication in this region nearly 10 years ago. As several European countries or regions are introducing BVDV control/eradication programmes, we believe that experience from Lower Austria can be useful for specialists involved in control of BVDV infection on cattle farms.

The aim of this minireview is to show how the molecular-genetic techniques were used in the BVDV eradication programme in Lower Austria and their contribution to achieve the final stage of eradication and to control of unwanted re-introduction of viral infection.

Basic principle of BVD eradication programme in Lower Austria

Although cattle management in Lower Austria is to some extent different from that in Scandinavia, Austrian farmers and veterinarians were inspired by the results of the Scandinavian BVDV eradication program. Therefore, the control scheme for BVDV infections in cattle herds in Lower Austria was introduced according to the Swedish model in 1997, becoming compulsory in 2004. In principle, the control strategy included the same steps as in Scandinavia: (1) dividing the herds into presumed non-infected and infected, (2) protection of non-infected herds and (3) systematic identification of PI animals and virus clearance in the herds by the elimination of infected cattle. More details on the BVD eradication program in Lower Austria can be found in other papers (Rossmanith *et al*., 2005; Rossmanith *et al*., 2010).

Importance of diagnostic methods selection

As in all BVDV control/eradication programmes based on biosecurity without application of vaccination, the identification of PI animals is the most important step. The selection of good diagnostic methods to identify BVDV infected animals in the herds was also a challenge for specialists working on the eradication programme in Lower Austria.

At the beginning of the BVDV eradication programme, antibody tests were used to divide cattle farms into infected and noninfected premises. The approaches included the detection of BVDV antibodies in tank milk, spot tests of milk samples from young cows and spot tests of blood samples of young animals aged from 6 to 12 months. Specific BVDV antibodies in blood and milk samples were detected by the use of an indirect ELISA (Svanovir™, Boehringer Ingelheim Svanova, Uppsala, Sweden). The non-infected farms were carefully protected with strong biosecurity measures.

While cell culture virus isolation was mainly used in Sweden to identify PI animals in the group of infected herds, in Austria more modern, simple, Ag-ELISA and RT-PCR (single-tube reverse transcriptase-polymerase chain reaction) methods were used for this purpose. In infected herds where spot tests of 6-12 months old cattle were BVDV antibody positive or a PI animal had been introduced, a virus clearance was performed. Young cattle with maternal derived BVDV antibodies, BVDV antibody negative cattle or cattle with low levels of antibody were tested for BVDV-specific antigen using an Ag-ELISA assay (HerdCheck BVDV antigen leucocytes, HerdChek BVDV antigen/serum, IDEXX Scandinavia, Österbybruck, Sweden). All samples were retested by RT-PCR employing the panpestivirus 324/326 primer pair, which targeted the 5'-untranslated region (5'-UTR) of the pestivirus genome (Vilcek *et al*., 1994). To facilitate high throughput analysis, virus was detected by RT-PCR in pooled serum samples.

In the first two years of the control programme, the results suggested an inadequate sensitivity for the detection of virus antigen by Ag-ELISA. Some samples were negative or doubtful by Ag-ELISA but positive in RT-PCR assays. To exclude the possibility that this phenomenon was due to an unusual BVDV variant, the genetic typing of incriminated virus isolates was carried out. Phylogenetic analysis of BVDV isolates did not confirm this idea because although most of these isolates were found in the well-established BVDV-1f subgenotype, some of them were typed as BVDV-1b and BVDV-1h as well (Rossmanith *et al*., 2001). To improve the results of the commercial Ag-ELISA kit, the preparation of leukocytes was modified (Rossmanith *et al*., 2001). Instead of mixing an equal volume of blood sample and 0.17 M ammonium chloride solution, as proposed in the instruction manual, one part of the blood sample was diluted with four parts of 0.17 M ammonium chloride solution. This was done to increase the efficiency of haemolysis of erythrocytes and to minimise the contamination of the final leucocyte pellet with non-haemolysed erythrocytes. The modified procedure led to a cleaner pellet of leukocytes and increased the Ag-ELISA test sensitivity.

The detection of one-tube RT-PCR products was also modified.

To avoid the use of carcinogenic ethidium bromide for visualisation of PCR products, a more sensitive and less dangerous silver staining procedure was used (Gottlieb and Chavko, 1986). For convenience, commercially available polyester films were used to prepare thin layer agarose gels. Such gels could be dried and stored at ambient room temperature for future experiments (Rossmanith *et al*., 2001).

In 2015, the single tube RT-PCR was replaced by a Real-Time PCR (ViroReal® Kit BVD Virus, Ingenetix Ltd, Vienna, Austria). In addition, sample identification was controlled by a bar code procedure allowing transfer of metadata and diagnostic results directly to the computer.

Progress with BVDV eradication programme

At the beginning of the voluntary BVDV control program (1997), 5 024 breeding herds took part. From the year 2005 onwards, in the compulsory BVDV eradication programme, nearly all 13 382 herds with animals for breeding have been included.

The good progress of BVDV eradication in Lower Austria is documented in Table I. From the introduction of the compulsory programme, the percentage of farms with detected PI animals progressively decreased and the number of BVDV-free herds significantly increased. The BVDV eradication programme finished in 2012 (Table I, bolt numbers), when nearly all cattle herds were BVDV-free.

Table I. *Elimination of PI animals from BVDV infected herds.*

Year	Herds sub-jected to BVD-law in L. Austria	Number of herds with PI animals detected	% of herds with PI animals	Total number of PI animals detected	Number of BVDV free herds	% of BVDV free herds
2005	13 382	248	1,85	511	7 931	59,26
2006	12 857	124	0,96	269	9 982	77,63
2007	12 273	46	0,37	115	11 166	90,98
2008	12 031	22	0,18	45	11 017	91,57
2009	11 733	10	0,09	12	10 951	93,33
2010	10 713	5	0,05	7	10 073	94,02
2011	10 703	5	0,05	14	10 357	96,76
2012	**10 369**	**0**	**0**	**0**	**10 144**	**97,83**
2013	10 105	0	0	0	9 857	97,54
2014	9 530	0	0	0	9 347	98,07
2015	9 262	1	0,01	2	9 048	97,68
2016	8 959	1	0,01	1	8 772	97,91
2017	8 699	1	0,01	1	8 468	97,34
2018	8 478	0	0	0	8 256	97,38
2019	8 076	0	0	0	7 893	97,73
2020	7 766	0	0	0	7 676	98,84

Application of molecular epidemiology to resolve problems in last infected herds

Experience in the field has shown that the most difficult part of the eradication programme was the final stage. In Lower Austria, 5 farms were the most resistant to finish the eradication programme in 2010. Despite enormous effort, the virus was still not eradicated from those farms. At the beginning, it was hypothesized that an unusual BVDV subgenotype might be responsible for continued circulation of virus in the herds. To resolve this problem, the methods of molecular epidemiology, namely the nucleotide sequencing of PCR products obtained from 5'-UTR coupled with the computer-assisted phylogenetic analysis were used. To see the relationship between infected herds, the broader collection of 23 BVDV isolates identified in PI animals in the years 2010, 2009, 2008 and 2006 were sequenced and analysed.

The genetic typing of BVDV isolates did not reveal the occurrence of any new subgenotype which would be prevalent or specific to the final stage of

the eradication programme. While BVDV-1 subgenotypes a, b, d, e, f, g, h and k had been identified in thebeginning of the eradication program (Vilček *et al*., 2001) or in other parts of Austria (Hornberg *et al*., 2009; Kolesárová *et al*., 2004; Vilček *et al*., 2003), the subgenotypes b, e, f, g and h were found on the problematic farms at the final stage of the eradication programme. However, the phylogenetic analysis revealed that there were three phylogenetic clusters with the same isolates despite originating from different farms (Rossmanith *et al*., 2014). Data in the first cluster of the phylogenetic tree indicated that two farms had the same isolates. Their close proximity (around 100 m apart) and employment of common workers explained this observation. The second cluster of farms with the same isolate was served with the same animal carrier and veterinarian. A poor practice on farms falling to this cluster was parking the transport vehicle loaded with animals from herds with unknown BVDV status very close to the animal stable (less than 10 m). Such practices may have contributed to the spread of BVDV between animals. The same milk collector or veterinarian regularly visited other farms (the 3rd phylogenetic cluster) with an identical circulating BVDV isolate. Their visits could also contribute to the spread of virus. Although the exact mode of transmission between herds with identical isolates has not been definitely clarified by genetic study, the results of this molecular analysis significantly contributed to focus on the risk factors for transmission of virus at the final stage of the eradication programme in Lower Austria. All concerned groups of farmers, animal owners, animal carriers, veterinarians and farm visitors were informed on their critical role to prevent the spread of viral infections in cattle farms. Subsequently, the epidemiological situation on the farms investigated has been improved resulting in the final elimination of BVDV from cattle farms and the completion of the BVDV eradication programme in Lower Austria in 2012 (Rossmanith *et al*., 2014).At the start of the eradication program in Lower Austria, participants learned that livestock trade, shared grasslands and animal contacts over fences were the greatest risks recognized for the transmission of BVD viruses (Rossmanith et al., 2005). At the final stage of the eradication programme the new risk represented by human factor emerged, which was revealed by complex analysis of the cattle management and the application of molecular epidemiology approach (Rossmanith *et al*., 2014).

Constant danger – re-introduction of BVDV infection

It should be mentioned that after finishing the BVDV eradication programme in Lower Austria in 2012, three cases of unwanted re-introduction of BVDV infections into BVDV-free herds were observed in the period from 2015-2017. The re-introductions were due to purchase of untested animals from infected herds. Apart from a young bull for fattening which originated from an infected herd, the import of lambs from Hungary and which were housed with pregnant cattle resulted in the birth of a PI calf in 2016. Genetic analysis of the viral isolate revealed that the calf was infected with a border disease virus. Another PI calf infected with BVDV, born from a purchased untested pregnant heifer, which originated from the neighbouring Czech Republic, was detected in 2017. Since then, no more infections or PI animals have been detected in the BVDV-free cattle livestock of Lower Austria. To maintain this BVDV-free status, bulk tank milk samples of all herds are tested twice a year for BVDV antibodies with nearly 100 % negative results. In herds without milk production serology is carried out on blood samples from young home-bred cattle.

There is a constant challenge to maintain BVDV-free status after eradication has been declared and continuing education remains very important for maintaining a favourable situation (Lindberg & Alenius, 1999). When unwanted re-introduction will appear, the molecular-genetic methods can significantly contribute to the identification of a pestivirus isolate.

BVDV eradication programmes are evolving

Meanwhile, BVDV eradication programmes in Europe have evolved. The Austrian BVDV eradication program, similar to that in Scandinavia, was based on the application of serological methods to separate infected and non-infected herds and then on the identification of PI animals in infected herds by Ag-ELISA or RT-PCR (in Sweden mostly by virus isolation technique). However, the present control and eradication programmes applied in European countries have changed strategy. PI animals were directly identified by screening the entire cattle population in Switzerland (Pressi and Heim, 2010; Pressi *et al*., 2011) or in newborn calves and their mothers in Germany, without application of serological investigation. The serum or blood samples for the identification of PI animals were mostly replaced by tissue samples from newborn calves punched out with special ear tags. Virus continues to be mostly detected with sensitive Ag-ELISA or one tube real-time RT-PCR methods. This approach with modifications has been applied in many Western European countries, such as Belgium, UK, Ireland (Graham *et al*., 2021; Russell *et al*., 2017) and in other parts of the world (Lindberg *et al*.,

2006; Moenning *et al*., 2005). Whereas methods of molecular epidemiology were used in the eradication programme in Sweden only sporadically (Stahl *et al*., 2005), then more so in Lower Austria (this paper), and now this approach is widely used for typing of BVDV isolates during control programs (Guelbenzu-Gonzalo *et al*., 2016; Russell *et al*., 2017; Wenike et al., 2017). A good example is the phylogenetic typing of most BVDV isolates detected in PI animals in Scotland (Guelbenzu-Gonzalo *et al*., 2016). In Switzerland, more than 10 000 isolates were typed so far which revealed epidemiological links between cattle farms related to the history of the Swiss Federation (Stalder *et al*., 2016; Stalder *et al*., 2018).

Conclusion

The BVDV eradication programme based on the elimination of PI animals without vaccination is a powerful tool to eliminate BVDV from cattle farms and it should be introduced in more, if not all European countries to improve the health status of cattle herds and the welfare of animals. In principle, there are many possibilities to modify programmes to take into account epidemiological specificities of each country. However, in our opinion the omission of serological methods in eradication programmes with direct detection of PI animals by Ag-ELISA or RT-PCR is not an optimal approach. The serological investigation provides a unique opportunity to obtain epidemiological insights in investigated herds and can significantly contribute to the identification of PI animals with minimal additional economic cost. No doubt, the application of molecular-genetic techniques has become an important part of BVDV control/eradication programmes. The methods are mainly used for the identification of PI animals within herds, in molecular epidemiology studies to find the risk factors for transmission of virus between herds and to identify re-introduced BVDV infection into BVDV-free herds.

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