Epidemiological reflections on bovine viral diarrhoea virus control in Belgian cattle herds based on experimental infections and observational studies

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Epidemiologische overwegingen in de bestrijding van boviene virale diarree virus op Belgische rundveebedrijven gebaseerd op experimentele infecties en observationele studies

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We learn more by looking for the answer to a question and not finding it

than we do from learning the answer itself.

Lloyd Alexander (1924 - 2007)

Table of contents

List of abbreviations

Ab	Antibody
Ag	Antigen
AI	Artificial insemination
BDV	Border Disease Virus
BT	Bovine turbinate
Btest	Bovine testicle
BVD	Bovine viral diarrhoea
BVDV	Bovine viral diarrhoea virus
CI	Confidence interval
cp	Cytopathic
CSFV	Classical Swine Fever Virus
Ct	Threshold cycle
DIVA	Differentiating infected from vaccinated animals
dpi	Days post inoculation
ELISA	Enzyme Linked Immunosorbent Assay
IPMA	Immunoperoxidase Monolayer Assay
MD	Mucosal disease

MDBK	Madin-Darby Bovine Kidney
MLV	Modified live vaccine
ncp	Non-cytopathic
OR	Odds ratio
PI	Persistently infected
R	Reproduction ratio
R_0	Basic reproduction ratio
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Real-time reverse-transcription polymerase chain reaction
SD	Standard deviation
SE	Standard error
$TCID_{50}$	50% tissue culture infective dose
TI	Transiently infected
VI	Virus isolation
VN	Virus neutralization

An apparently new disease affecting cattle was first described in North America in 1946 (Childs, 1946; Olafson et al., 1946). In some herds one to two animals displayed severe clinical signs such as watery and haemorrhagic diarrhoea, tenesmus, depression, anorexia, ulceration of oral mucosa, nasal discharge and hypersalivation. These animals died within a week (Childs, 1946). The latter report was considered to be the first description of ***mucosal disease*** (MD) in cattle (Fig. 1) (Pritchard, 1963). Attempts to reproduce these clinical signs failed, but resulted in a transmissible disease with additional, but less severe clinical signs: respiratory disease, leukopenia, drop in milk production and increased abortion rates (Olafson et al., 1946). Rinderpest virus and winter dysentery virus were excluded as causal agents of this disease and as no bacteria could be isolated from the inoculum used for a transmission experiment, this reproducible disease with varying severity became known as ***virus diarrhoea*** or ***bovine viral diarrhoea*** (BVD) (Olafson et al., 1946; Pritchard, 1963).

Fig. 1. This 18-month-old bull suffering from mucosal disease was presented at the Faculty of Veterinary Medicine of Ghent University (2010). Typical clinical signs were erosions of the gastrointestinal tract causing hypersalivation and haemorrhagic diarrhoea with tenesmus. The animal succumbed soon after arrival.

Further research demonstrated by using virus neutralization that BVD en MD were different disease manifestations, caused by the same virus, ***Bovine Viral Diarrhoea Virus*** (BVDV) (Gillespie et al., 1961; Kniazeff et al., 1961; Thomson and Savan, 1963). In 1984 McClurkin et al. described the experimental infection with BVDV of cattle in early pregnancy. This resulted in the production of calves persistently infected (PI) with BVDV (i.e. the calves were continuously BVDV viraemic) and immune tolerant (i.e. the calves

remained BVDV seronegative after infection with the same BVDV strain). That same year MD was experimentally reproduced in PI cattle (Brownlie et al., 1984).

It was believed that cases of severe clinical disease associated with BVDV all resulted from MD in PI cattle, while BVD was designated as a mild disease. However, outbreaks of thrombocytopenia resulting in haemorrhages and death associated with BVDV in non-PI cattle were reported in the late 1980s (Perdrizet et al., 1987; Rebhun et al., 1989). Furthermore, acute BVD outbreaks with high mortality and without explicit signs of haemorrhagic disorder were described in Canada and the United States in the 1990s (Pellerin et al., 1994; Carman et al., 1998).

This short historical overview already briefly touched the complex nature of BVDV, whose epidemiology, broad range of clinical manifestations, diagnostic tests and factors associated with an increased risk of BVDV infection will be further introduced. By the aforementioned, predominantly American references the reader could assume BVD-MD is especially a problem in North America, but numerous epidemiological studies have demonstrated that the virus is worldwide spread (Houe, 2005), including Belgium. Taking into account the economic consequences of BVD-MD, control strategies have been established. The present doctoral thesis aimed at gaining insight into the current BVDV prevalence and implementation of control measures in Belgian cattle herds. The obtained results were used to make recommendations for a future mandatory national control programme.

References

Brownlie, J., Clarke, M.C., Howard, C.J., 1984. Experimental production of fatal mucosal disease in cattle. Veterinary Record 114, 535-536.

Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A., Anderson, N., 1998. Severe acute bovine viral diarrhea in Ontario, 1993-1995. Journal of Veterinary Diagnostic Investigation 10, 27-35.

Childs, T., 1946. X disease of cattle - Saskatchewan. The Canadian Journal of Comparative Medicine 10, 316-319.

Gillespie, J., Thompson, J., Baker, J.A., Coggins, L., 1961. Comparison by neutralization tests of strains of virus isolated from virus diarrhea and mucosal disease. The Cornell Veterinarian 51, 155-159.

Houe, H., 2005. Risk Assessment, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 35-64.

Kniazeff, A.J., Huck R.A., Jarrett W.F.H., Pritchard W.R., Ramsey F.K., Schipper I.A., Stober M., Liess B., 1961. Antigenic relationship of some bovine viral diarrhea-mucosal disease viruses from the United States, Great Britain and West Germany. Veterinary Record 73, 768-769.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine-Revue Canadienne de Medecine Comparée 48, 156-161.

Olafson, P., McCallum, A.D., Fox, F.H., 1946. An apparently new transmissible disease of cattle. The Cornell Veterinarian 36, 205-213.

Pellerin, C., Vandenhurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus-strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Perdrizet, J.A., Rebhun, W.C., Dubovi, E.J., Donis, R.O., 1987. Bovine virus diarrhea – clinical syndromes in dairy herds. The Cornell Veterinarian 77, 46-74.

Pritchard, W.R., 1963. The bovine viral diarrhea-mucosal disease complex. Advances in Veterinary Science 8, 1-47.

Rebhun, W.C., French, T.W., Perdrizet, J.A., Dubovi, E.J., Dill, S.G., Karcher, L.F., 1989. Thrombocytopenia associated with acute bovine virus diarrhea infection in cattle. Journal of Veterinary Internal Medicine 3, 42-46.

Thomson, R.G., Savan, M., 1963. Studies on virus diarrhea and mucosal disease of cattle. Canadian Journal of Comparative Medicine and Veterinary Science 27, 207-214.

Chapter 1

General Introduction

The viruses

BVDV are positive sense, single stranded RNA viruses belonging to the *Pestivirus genus*, within the *Flaviviridae* family. Pestiviruses were considered to be host-specific and were therefore divided into groups differentiating between porcine, bovine and ovine viruses (Ridpath, 2013a). However, although the natural host of BVDV is bovine, natural infections of pigs and sheep with BVDV are reported (Vilcek et al., 1997; Tao et al., 2013). Nowadays, genomic sequence and antigenic relatedness are used to distinguish subgroups in the *Pestivirus genus*, which currently comprises four recognized species: Border Disease Virus (BDV), Classical Swine Fever Virus (CSFV), BVDV-1, and BVDV-2. Because of the high rate of genetic mutations observed with these pestiviruses on the one hand and with the improvement of virus detection on the other hand, it is assumed that the *Pestivirus genus* will continue to expand (Ridpath, 2013a). Four additional "atypical" *Pestivirus* species have been proposed: Giraffe, Pronghorn, Bungowannah and HoBi (Bauermann et al., 2013) (Fig. 1). Because of a high degree of genomic sequence and antigenic relatedness to BVDV-1 and BVDV-2, the HoBi species was proposed as BVDV-3 (Liu et al., 2009).

Single stranded RNA viruses, such as BVDV, are subject to genomic modifications due to high rates of point mutations and recombinations, resulting in heterogeneity within recognized species (Bolin and Grooms, 2004; Ridpath, 2013a). Because some BVDV isolates were as different from each other as from CSFV (Ridpath et al., 1994), the previously recognised Bovine Diarrhoea Virus species was split up into the currently recognised species BVDV-1 and BVDV-2 (ICTV 7th Report, 1999). Both ***species (types)*** are divided into several genetic ***subspecies (subtypes)***. The biological significance of these subgroupings is a matter of debate (Ridpath, 2005). Even between ***strains (isolates)*** of a same subspecies antigenic differences were noticed (Vilcek et al., 2001). In conclusion, BVDV is not one virus, but a group of many genetic variants.

Both BVDV species are worldwide spread, although the prevalence of BVDV-2 is clearly higher in North America compared to Europe (Ridpath, 2005). In Belgium most field isolates are identified as BVDV-1b (Couvreur et al., 2002), while also BVDV-2 has been described (Couvreur et al., 2002; Letellier et al., 2010). Isolates belonging to the HoBi-like (BVDV-3) species have not yet been reported in Belgium, but natural infections have been described in Italy (Decaro et al., 2011).

Regardless of the species, BVDV may exist as one of two biotypes. Based on their ability to cause cytopathic effects in cell cultures, strains are classified as cytopathic (or cytopathogenic, cp) and non-cytopathic (or non-cytopathogenic, ncp) biotypes (Peterhans et al., 2010). Although this distinction is made *in vitro*, the difference between the two biotypes plays a role in the pathogenesis of BVD. Through genetic recombination cp viruses arise from ncp viruses, with the combination of cp and ncp viruses in PI animals leading to MD (Goens, 2002; Peterhans et al., 2010).

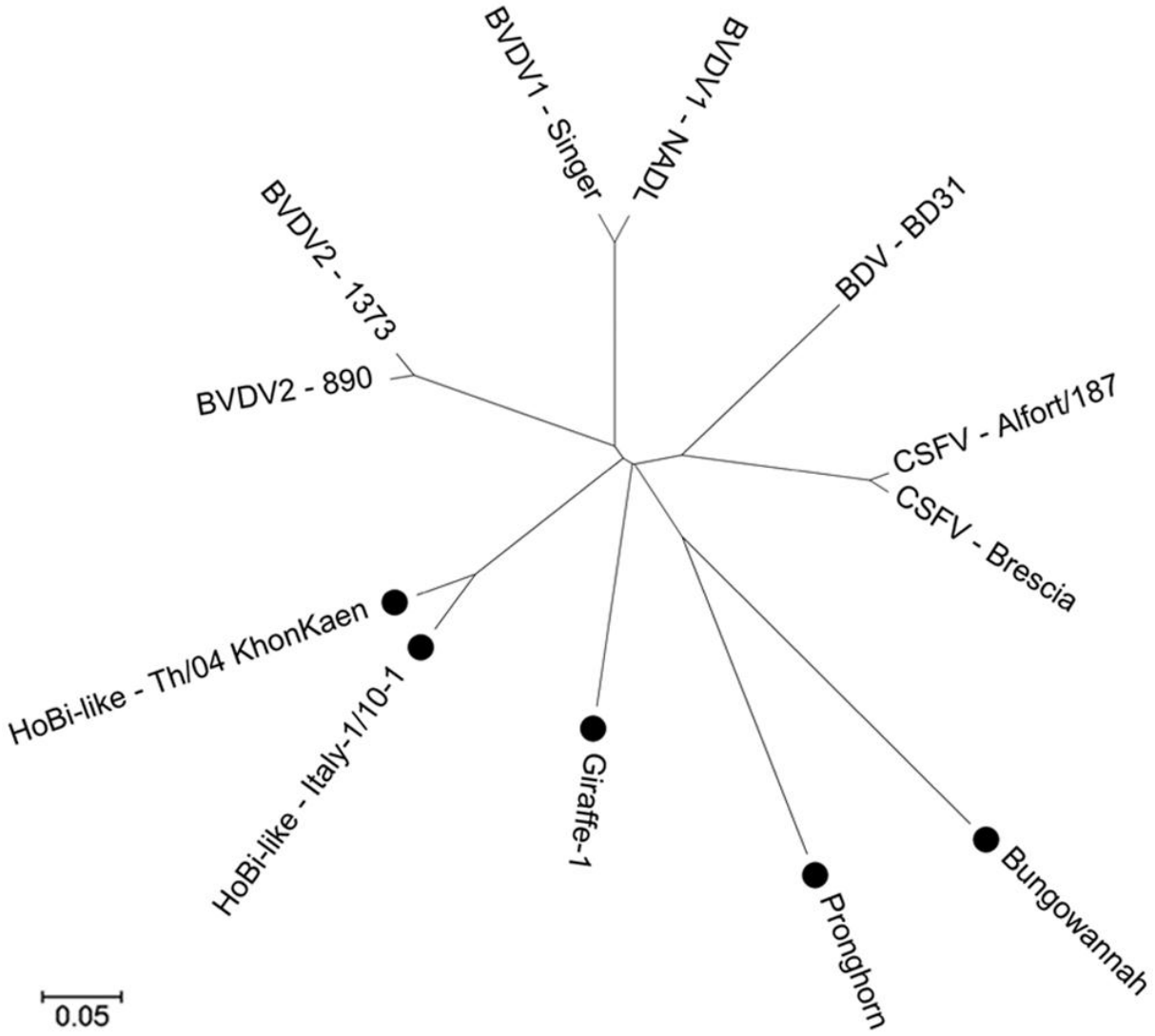

Fig. 1. Phylogenetic tree of *Pestivirus* genus. The currently recognized species (BVDV-1, BVDV-2, CSFV and BDV) and four "atypical" species (Giraffe, Pronghorn, Bungowannah and HoBi), marked with '●', are shown. The genetic distances between species and strains is proportional to the branch lengths. Analysis was performed on partial sequences, which consisted of the amino acids from the first five polypeptides Npro; C; Erns; E1 and E2. Source: Bauermann et al., 2013.

Transmission

BVDV can be maintained within a susceptible cattle population through horizontal and vertical transmission, depending on when the animal became infected. Following an acute infection acquired postnatally, infected animals can shed the virus transiently. Following a congenital infection between the second and fourth month of gestation, animals can become persistently infected (PI) and continuously shed the virus (Fig. 2). BVDV can also be spread indirectly. Both active immunity (following a transient BVDV infection) and passive immunity (colostral antibodies or vaccination) have a considerable impact on the transmission rate of BVDV, which can be expressed by the reproduction ratio.

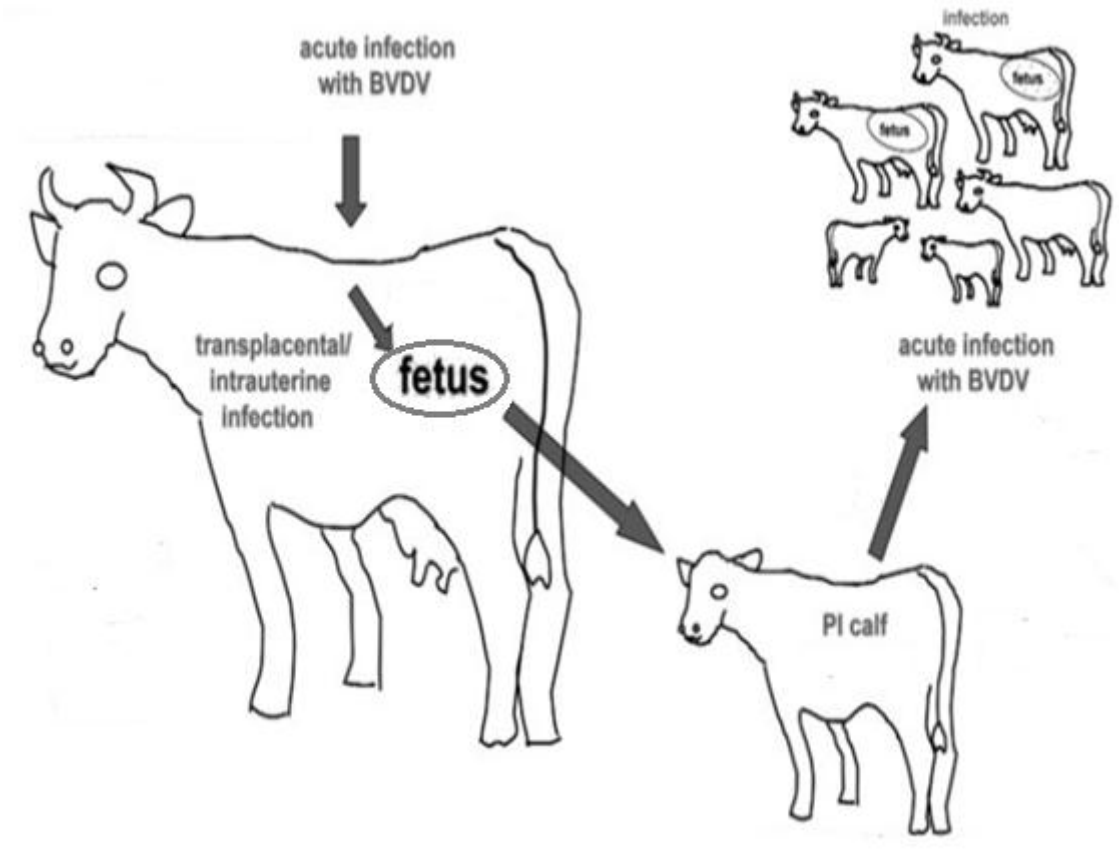

Fig. 2. BVDV transmission in a susceptible cattle population is principally maintained by persistently infected (PI) calves, but also transiently infected (TI) animals can spread the virus. PI calves are congenitally infected (vertical transmission) with BVDV and continuously shed the virus. TI animals result from acute, postnatal BVDV infections (horizontal transmission). TI animals in early gestation can give birth to new PI animals. Source: Liebler-Tenorio, 2005.

Transient infections

The most frequent route of BVDV infections is by oro-nasal uptake of BVDV. Acute natural BVDV infections of BVDV seronegative cattle result in a transient viraemia, starting 3 days post-infection (Lanyon et al., 2014). The duration of virus shedding by transiently infected (TI) animals likely depends on the virulence of BVDV strains and their efficiency to replicate (Bolin and Ridpath, 1992; Houe, 1999) and varies from less than 1 day to 2 weeks (Thurmond, 2005; Lanyon et al., 2014).

TI animals shed the virus through all excretions and secretions, such as tears, milk, saliva, urine, nasal discharge, semen, foetal fluids and faeces (Meyling et al., 1990), although faeces are a poor source of virus (Brownlie et al., 1987). Following the clearance of the virus from the blood and a systemic immune response, a prolonged spread of BVDV may still be possible, due to sequestration of the virus (Baule et al., 2001; Givens and Marley, 2013). In a study performed by Baule et al. (2001), nasal swabs were intermittently positive in virus isolation up to 31 days following experimental infection of calves. Similarly, protected by the blood testicle barrier the semen of TI bulls may remain BVDV positive for more than 2 years (Kirkland et al., 1991; Voges et al., 1998; Niskanen et al., 2002a; Givens et al., 2009).

Because of the short duration of the infection and the intermittent shedding of relatively low amounts of virus, TI animals are believed to be of minor importance in the epidemiology of BVDV (Lindberg and Houe, 2005). Some studies demonstrated no spread of BVDV by experimentally infected animals (Niskanen et al., 2000; Niskanen et al., 2002b). Nevertheless, it has been suggested during severe outbreaks with BVDV-2 TI animals significantly contribute to virus spread (Ridpath et al., 2006). A recent outbreak of severe disease associated with BVDV-2 in Germany and the Netherlands is believed to have spread solely through TI animals, since no PI animals were detected (Doll and Holsteg, 2013; Moen, 2013). Also other reports suggest that BVDV can be maintained in the population only by transient infections (Moerman et al., 1993; Moen et al., 2005). However, sufficient evidence to prove that transmission was not due to PI animals that have escaped identification should be provided in these cases (Lindberg and Houe, 2005).

Persistent infections

During infection with BVDV the pregnant uterus, placenta and foetus easily become infected (Fredriksen et al., 1999a). Depending on the biotype of the BVDV strain and stage of gestation, infection of the foetus results in early embryonic death, abortion, congenital malformations, the birth of PI calves or the birth of healthy calves (Grooms, 2004). When the infection of the foetus occurs with ncp virus between 30 and 125 days of gestation (McClurkin et al., 1984; Blanchard et al., 2010), viral proteins are recognized as self-antigens by the foetus, since it is not immunocompetent at that stage (Peterhans and Schweizer, 2013). These PI calves may be born apparently healthy, but remain lifelong infected and will persistently shed the virus (Brownlie, 1990).

Pregnant PI dams invariably transmit the virus to the foetus, resulting in new generations of PI calves (Moennig and Liess, 1995). PI calves thus always result from congenital BVDV transmission, either by transmission from a PI dam to her foetus or via acute infection of the dam with ncp virus between day 30 and 125 of gestation. Semen from PI bulls contains large amounts of virus (Voges et al., 1998) and breeding with such semen can result in acute infection of BVDV seronegative dams and possibly a PI calf (Meyling and Jensen, 1988).

During their life PI animals, and only PI animals, can suffer from MD, which is a highly fatal form of BVD and develops when cp BVDV appears in PI animals. The cp biotype arises from the persisting ncp biotype by mutations or recombinations in the PI animal or by superinfection with a cp strain from another PI animal suffering from MD and which is antigenically homologous to the persisting ncp strain (Brownlie et al., 1984; Peterhans et al., 2010). While MD is in general fatal within 2-3 weeks (Brownlie et al., 1984), some PI animals develop chronic MD and may survive when superinfection occurs with a more heterologous cp strain (Baker, 1995). PI animals usually die young, but they can also reach the normal life span of commercial cattle and be used for reproduction since it was demonstrated that more than 10% of PI animals were older than 2 years (Presi et al., 2011). Taken into account this high mortality among PI animals, the prevalence of PI animals in a population infected with BVDV will usually be no more than 2% (Houe, 2005).

Notwithstanding the low prevalence and their short life expectancy PI cattle play the principal role in BVDV transmission. Once colostrum-derived BVDV antibody titres have declined they continuously shed BVDV in large quantities through all their secretions and

excretions (Lindberg and Houe, 2005). In herds where cattle are housed in close contact a PI animal can infect up to 97% of susceptible cattle during a period of 6 months (Houe and Meyling, 1991; Houe et al., 1993).

Indirect transmission

Like other enveloped viruses, BVDV is easily inactivated by organic solvents. However, considering the massive amounts of virus shed by PI animals, several studies have demonstrated that susceptible animals can become BVDV infected through indirect transmission. BVDV are shed by the ***foetal fluids*** at the birth of PI animals and can infect animals exposed to the uterine lochia or housed in pens where PI calves were born (Lindberg et al., 2004). ***Pens*** where PI animals have been housed can be a source of BVDV for a limited period of time and infect susceptible cattle (Niskanen and Lindberg, 2003). ***Material*** such as nose tongs or rectal gloves previously used on PI animals can transmit BVDV (Gunn, 1993; Lang-Ree et al., 1994). ***Flies*** can serve as mechanical vectors in BVDV transmission (Gunn, 1993; Tarry et al., 1991). Cattle housed near a PI calf without direct contact can get infected through ***ambient air*** transmission (up to 10 m) (Niskanen and Lindberg, 2003). ***Iatrogenic*** transmission can occur by repeated use of the same needle (Gunn, 1993), contaminated vaccines (Barkema et al., 2001; Niskanen and Lindberg, 2003) or clothing (Ståhl et al., 2005). Embryos originating from BVDV infected animals (Brock et al., 1997) or washed with contaminated foetal calf serum (Bolin et al., 1991; Bolin and Ridpath, 1998) can transmit the virus to susceptible recipients. Although indirect BVDV transmission is of minor importance compared to direct animal contacts, attention should be paid to this transmission route as a control programme proceeds and the probability of direct contacts decreases (Hult and Lindberg, 2005).

Immunity

Colostral antibodies have a considerable impact on BVDV transmission. First, they decrease the amount of virus spread by infected animals. Secondly, the number of animals susceptible to infection are reduced and clinical signs are less pronounced. Low neutralizing antibody titres of 1:16-1:32 (i.e. two-fold dilution series that neutralize BVDV) are believed to be sufficient to offer protection against clinical signs associated with BVDV infections (Howard et al., 1989; Bolin and Ridpath, 1992). Depending on the quality and quantity of

colostrum consumed, calves are protected from BVDV infection during the first 2 to 4 months of life. In a study of Munoz-Zanzi et al. (2002) neutralizing colostral antibody titres in half of the calves declined to < 1:16 after about 110 days and 80 days of age for BVDV-1 and BVDV-2, respectively. The half-life of colostral antibodies in PI calves was much shorter compared to non-PI calves (5 to 11 days versus 3 weeks), resulting in a clearance of these antibodies in PI animals by 8 weeks of age (Palfi et al., 1993). The same study revealed that PI calves under protection of colostral antibodies shed only low amounts of BVDV. Colostral antibodies also interfere with diagnostic tests (Palfi et al., 1993; Zimmer et al., 2004; Fux and Wolf, 2012) and vaccination (Endsley et al., 2003) by blocking the virus or its antigens. For example, due to the neutralizing capacity of antibodies, BVDV is no longer able to propagate in cell culture and young PI calves can therefore be considered as false negative since they could not be detected by virus isolation (Palfi et al., 1993).

A transient BVDV infection evokes an immunosuppression by an impairment of the innate immune response and a lymphopenia (Chase et al., 2004). This immunosuppression is followed by the development of both a cell mediated and humoral immune response (Chase, 2013). The humoral immune response is characterized by the presence of serum antibodies from 2-3 weeks post-infection onwards (Howard et al., 1992). Animals remain BVDV seropositive during their entire life (normal life span of commercial cattle) (Brownlie, 1990; Fredriksen et al., 1999b). The fact that a TI animal remains seropositive for BVDV during its entire life may result from repeated stimulations of the immune response when BVDV is maintained within a population following the birth of one or more PI animals and subsequent infection of susceptible cattle in early gestation (Lindberg and Alenius, 1999). However, this could also indicate that the virus is still replicating and being presented to the lymphoid tissue in BVDV infected, recovered and immune cattle (Thurmond, 2005). This is supported by a study that was capable of isolating wild-type BVDV from peripheral blood mononuclear cells in BVDV seropositive cattle (Gogorza et al., 2005). Another study demonstrated that BVDV seropositive cattle can continue to carry virus in peripheral blood mononuclear cells for at least 98 days and transfer of infection was possible through blood transfer (Collins et al., 2009). Although this experimental transfer of infection was possible, natural transfer of infection is believed to be unlikely (Lanyon et al., 2014). Protection against BVDV infection is dependent on the virus strain and the level and isotype of antibodies produced (cross-protection). Neutralizing antibodies prevent disease development following homologous

challenge (Potgieter, 1995), but animals with neutralizing antibodies can develop viraemia (Nobiron et al., 2003).

Immunization following ***vaccination*** will be introduced below.

The reproduction ratio

The transmission rate of an infectious disease, i.e. the potential to spread, in a population can be expressed by its reproduction ratio, R. The basic reproduction ratio, R_0, is defined as the mean number of secondary infections arising from one typical infectious case introduced in a fully susceptible population (Kroese and de Jong, 2001; Lindberg and Houe, 2005; Velthuis et al., 2007). The use of R_0 is restricted to the situation without intervention (fully susceptible population) while the transmission rate is usually described by R in situations with control measures (e.g. vaccination) (Velthuis et al., 2007).

A value for R_0 of 4 means that one infectious animal can infect on average four new susceptible animals. R_0 has thus a threshold value equal to 1. This means that an infection may spread when $R_0 > 1$, possibly resulting in a major outbreak, and will fade out when $R_0 < 1$, always resulting in a minor outbreak (Lindberg and Houe, 2005; Velthuis et al., 2007). R_0 is determined by the following parameters (Lindberg and Houe, 2005; Thurmond, 2005): the probability of transmission during a contact between an infectious and susceptible animal (β), the number of contacts per time period (k) and the duration of the infectious period (d).

As stated above, PI animals continuously shed massive amounts of virus, resulting in high values for the parameters β en d. The amount of virus spread by TI animals is much lower because of a much shorter duration of the infectious period and the relatively low amounts of virus shed, resulting in low values for the parameters β en d. PI animals are therefore considered as very successful virus transmitters (R_0 assumed > 1), whereas TI animals are believed to be less important in spreading BVDV (R_0 assumed < 1).

Few studies were performed to determine R_0 for horizontal BVDV transmission by TI animals. In pigs a limited horizontal transmission of BVDV-1b by experimentally infected pigs was demonstrated (Wieringa-Jelsma et al., 2006). Moerman et al. (1993) estimated that the horizontal BVDV transmission rate by TI cattle had an R_0 of 3.3 (95% CI 2.6; 4.1). However, although separated from the study group, PI animals were present in the herd during this study. As these animals shed massive doses of virus, they may have attributed to

virus spread through indirect transmission (Niskanen and Lindberg, 2003; Lindberg et al., 2004), resulting in an overestimation of R_0.

Clinical features

Virulence of BVDV

The clinical outcome of infection depends on host factors (e.g. immune status and pregnancy status), environmental factors (e.g. concurrent infections) and viral factors. It is generally assumed that variation in virulence exists between different BVDV isolates. However, the basis for clinical variation at the virus level is not understood (Bolin and Grooms, 2004). Disease severity is likely to be linked to the degree of viraemia (Walz et al., 2001) and thus the propensity to replicate of the infecting strain (Bolin and Ridpath, 1992).

Initially BVDV-2 was only associated with severe BVD outbreaks while BVDV-1 was linked to mild and subclinical infections. However, also BVDV-1 infections can result in severe clinical disease (Vilcek et al., 2001) and BVDV-2 isolates of low virulence are likely to predominate over virulent BVDV-2 isolates (Ridpath et al., 2000). Severe clinical signs linked with BVDV-1 and BVDV-2 infections have been described in Belgium (Letellier et al., 2010; Laureyns et al., 2011a; Laureyns et al., 2011b; Laureyns et al., 2013b).

Ncp BVDV predominate in nature while cp viruses are rare and usually found in association with MD, which achieve a high mortality rate (Ridpath, 2010). Cp BVDV amplifies viral RNA at a much higher level (Kummerer et al., 2000). However, cytopathogenicity of a pestivirus does not correlate with its virulence, since most virulent strains are of the ncp biotype (Peterhans and Schweizer, 2010).

Effects of BVDV infections on disease and production

In a study of Loneragan et al. (2005) ***PI animals*** were on average treated twice as much compared to other calves that received treatment. Eventually, the PI animals were removed from the herd before the age of slaughter because of chronic illness (147 days after arrival) or death (58 days after arrival). In the same study PI animals were more likely to die

before the age of slaughter as their prevalence among dead cattle was 2.5% while at arrival this was only 0.3% (Loneragan et al., 2005). When evaluating the national BVDV eradication campaign in Switzerland, Presi et al. (2011) noticed that only 10% of PI animals were older than 2 years.

Occasionally ***severe outbreaks*** with high mortality rates and whether or not associated with haemorrhagic disorders are reported (Corapi et al., 1990; David et al., 1994; Pellerin et al., 1994; Carman et al., 1998; Doll and Holsteg, 2013; Moen, 2013). During an outbreak in a feedlot 76% of the calves became clinically ill and 31% eventually died (Hessman et al., 2012). During the recent BVDV-2c outbreak in Germany mortality rates up to 20% and 80% were reported in a dairy herd and veal calf herd, respectively (Doll and Holsteg, 2013).

It has been estimated that 70% to 90% of acute BVDV infections occur with only mild fever and leukopenia (Ames, 1986; Baker, 1995). Other mild clinical signs in TI cattle are for instance depression, anorexia, diarrhoea and nasal discharge. However, also subclinical infections impair animal production. Moerman et al. (1994) reported a 10% ***drop in milk production*** for several days in subclinically infected cows. After recovery milk production increased again, but remained 5% lower than before the drop in half of the cows. Another study estimated that milk production was 5.8% lower in herds with a high BVDV antibody titre in bulk tank milk compared to herds with a lower titre (Heuer et al., 2007). ***Calf mortality*** within one year of birth was found to be 1.35% and 3.05% higher in BVDV seropositive herds compared to negative control herds for beef and dairy herds, respectively (Gates et al., 2013a).

BVDV infects cells of the immune system, which evokes an ***immunosuppression*** leading to a decreased response to other infectious agents (Chase et al., 2004) and can aggravate the clinical signs of other infectious diseases (Ridpath, 2010). A 7% increase was noted in the incidence rate of clinical ***mastitis*** in herds exposed to BVDV as compared with non-exposed herds (Waage, 2000). A significant lower bulk milk somatic cell count (211,390 cells/ml versus 242,790 cells/ml) was observed in BVDV naïve herds compared to BVDV seropositive herds (Laureyns et al., 2013c). The number of cases of mastitis, retained placenta and miscellaneous diseases increased in herds with an increased BVDV antibody milk titre (Niskanen et al., 1995).

In a study on morbidity and mortality in white veal calves in Belgium 26% of the necropsied and virologically tested calves was BVDV RT-PCR-positive and a positive test

was associated with chronic pneumonia (OR= 21.6; 95% CI 5.7; 81.9) and pleuritis (OR= 4.9; 95% CI 1.5; 16.3) (Pardon et al., 2012). Among calves entering a feedlot, 45% of calves treated for ***respiratory disease*** had seroconverted for BVDV while this was only 22% of untreated calves (Martin and Bohac, 1986). Eradication of BVDV in a dairy herd resulted in a decrease of ***diarrhoea*** within one month of birth from 71% to 19% (Klingenberg et al., 1999).

Reproductive consequences of BVDV infections such as reduced fertility, congenital defects, abortion and of course persistent infections are well described (Baker, 1995; Grooms, 2004). In an experimental study reduced ***conception rates*** were demonstrated in heifers intranasally infected with BVDV before insemination: 44% compared to 79% for the control group (McGowan et al., 1993). Calves congenitally infected with BVDV were 2.3 times more likely to have a severe illness (requiring at least three days of treatment or resulting in death) during the first 10 months after birth compared to calves without ***congenital infection*** (Munoz-Zanzi et al., 2003).

Zootechnical impact of BVDV infections

Economic consequences of BVDV infections

One cannot expect to find all of the different clinical signs in one herd or during one outbreak (Laureyns, 2014). The costs following BVDV infection will therefore vary according to the type of clinical signs, the severity of the outbreak (virulence of the infecting strain) and the initial herd immunity (Houe, 1999).

For an affected ***dairy herd*** Heuer et al. (2007) estimated the losses per cow per year to be €53 (NZ$83), especially due to the reduced milk production, increased abortion rates, increased time to conception, loss among PI animals and calf losses as a consequence of abortion or induction. For a similar outbreak another study estimated the costs to vary between €48 and €67 (Fourichon et al., 2005). These estimated costs lie within the range of the costs usually reported for an average outbreak in dairy herds: between €21 ($25) and €135 ($160) per cow per year (Houe, 2003; Lindberg et al., 2006). The mean financial loss per cow per year in ***beef herds*** where the disease is not diagnosed quickly and without intervention or

re-infection was estimated to be €46 (£37) (Gunn et al., 2004). Depending on the initial BVDV status of the herd and the management practices undertaken to prevent virus transmission, the costs of re-infection with BVDV were estimated to vary between almost €0 and €51 (£40) per cow per year (Stott et al., 2010). Losses from more severe BVD outbreaks are estimated to be more than €340 per cow (Houe, 2003; Lindberg et al., 2006).

When deciding to eradicate or control at the herd level or at regional/national level, it is clear that the expenses of the control measures should not exceed the costs associated with BVDV infections. Several cost-benefit analyses, usually at the national level, have been published. A cost-benefit study on the control and eradication programme in Norway showed that the programme was already cost-effective from the second year onwards (Valle et al., 2005). A study in New Zealand showed that all considered control options – vaccination, test and cull, increased biosecurity or combinations – would be economically favourable (Reichel et al., 2008). Stott et al. (2012) estimated that the payback periods of a potential eradication programme in Ireland for the beef and dairy sector were respectively 1.2 and 0.5 years, which corresponded to a cost-benefit ratio of 1:5 and 1:14, respectively. An eradication programme in France was estimated to be cost-effective only after 15 years (Dufour et al., 1999). For this estimation extensive blood sampling on individual animals was taken into account, which raised the costs of the programme considerably. Currently more efficient diagnostic tests such as analysis of ear notch samples (see below) are available, which can reduce the costs of a control programme, as shown by Stott et al. (2012). These studies show that the control of BVDV is beneficial, but that the period for a programme to become cost-effective varies and depends on the design (Saatkamp et al., 2006).

Other beneficial effects of BVDV control

As described above, BVDV infections involve a higher need for disease treatment and BVDV control could therefore result in a ***reduction in antimicrobial usage*** (Pardon, 2012). Furthermore, the impact of BVDV infections on ***animal welfare*** is often overlooked, e.g. PI animals suffering from MD (Lindberg et al., 2006). As the performance of a herd is related to the ***job satisfaction*** of a farmer (Hanna et al., 2009), a successful BVDV control programme could enhance the job satisfaction. Finally, if neighbouring countries also would implement national BVDV control and Belgium remains endemic, this could possibly hamper ***international trade***.

Risk factors for occurrence of BVDV infections

Risk factors for BVDV infection usually are identified at the herd level. A herd can be considered as BVDV infected by the presence of BVDV seropositive animals following serologic examination (e.g. Valle et al., 1999) or by the presence of virus-positive animals (e.g. Graham et al., 2013). Some risk factors such as the purchase of cattle are obvious risk factors, while others may be confounded by other factors. Whenever the association between the risk factor and the occurrence of BVDV infections was not so obvious, a possible explanation is given by the authors of the corresponding study.

Valle et al. (1999) identified the following statistically significant risk factors for Norwegian herds being BVDV seropositive: over-the-fence pasture contact, purchasing animals, not asking information about BVDV status when purchasing animals, exchange of calves and sharing of cattle housing with other farmers during summer, not consulting dairy-association advisors, being a younger farmer and use of common pasture. Younger farmers purchased more cattle and tended not to ask for health certificates when purchasing animals more often than older farmers. The use of common pasture was also identified as a risk factor in Croatia by Bedekovic et al. (2013).

In New Zealand, Cuttance and Cuttance (2014) identified the purchase of animals (introduction of replacement heifers) as a risk factor for being BVDV seropositive at the herd level, together with the farmer considering BVD was an issue on the farm and an increasing number of heifers on the farm. A larger number of heifers could suggest a higher probability of infecting at least one susceptible dam in early gestation, resulting in the birth of a PI animal. Vaccination of introduced breeding bulls resulted in a decreased odds of being BVDV seropositive.

Similar to the previous study, a larger herd size was found to be a risk factor for Brazilian dairy herds being BVDV seropositive when considering dairy herds BVDV seropositive based on bulk tank milk analysis (Almeida et al., 2013). Furthermore, the use of artificial insemination (AI) was significantly associated with herds being BVDV seropositive. Interestingly, the semen used for AI was used from selected bulls from well-known AI centres and should therefore be BVDV negative. This result suggested that other factors, such as the visits of AI technicians, were likely to be associated with BVDV infection and illustrates that

risk factors only demonstrate an association and not necessarily a causal relation between risk factor and disease.

Other studies using bulk tank milk samples demonstrated that vaccination for BVD, suspicion of BVD, housing of cows in gestation with calves, the number of cows, the proportion of cows that were dry, the purchase of cattle and the number of breeding farms within a 10 km radius were associated with an increased level of BVDV antibodies in bulk tank milk in Scottish cattle herds (Humphry et al., 2012; Gates et al., 2013b). Regarding the risk factor 'the proportion of the herd that were dry', a possible explanation could be that BVDV infected herds had lower conception rates and animals were therefore dry for a longer period of time.

A study using data of more than 8,000 Danish dairy herds demonstrated the following significant associations with the presence of PI animals: herd size, distance to neighbouring farms and the presence of BVDV infected neighbours (Ersbøll and Stryhn, 2000).

In Ireland, Graham and co-workers (Graham et al., 2013) used the presence of at least one virus-positive animal based on analysis of ear notches as outcome of interest and identified the following risk factors: province, herd size (log number of cows), introduction of cattle during 2009-2011, the number of tested calves whose dams had been purchased within 9 months of their calving date and calf mortality. When the vaccination status of the herds was included, a significant interaction between the vaccination status and log number of cows was observed. Vaccination reduced the probability of positive test results and the impact of vaccination was bigger in larger compared to smaller herds.

Diagnosis

Laboratory diagnosis

As most BVDV infections occur subclinically and because of the broad range of clinical disease manifestations, the diagnosis of BVDV based upon clinical signs is not obvious and should therefore be supported by laboratory tests (Laureyns, 2014). Fortunately, these tests are generally considered to be very reliable, given their high sensitivity and

specificity (Saliki and Dubovi, 2004; Sandvik, 2005; Dubovi, 2013). Two diagnostic approaches to detect BVDV infections are used: direct tests (detection of the virus or viral components) and indirect tests (detection of the immune response to BVDV).

Detection of the virus or viral components

Virus isolation (**VI**) of BVDV out of samples in cell cultures, is the gold standard to which other virus detection tests are compared (Dubovi, 2013). The following cell lines are most widely used for VI: bovine turbinate (BT), bovine testicle (Btest) and Madin-Darby Bovine Kidney (MDBK) (Saliki and Dubovi, 2004). Studies have shown that MDBK cells are less sensitive to BVDV compared to BT and Btest cells (Onyekaba et al., 1987; Dubovi, 2013). Regarding the sample, the highest sensitivity is obtained with a blood sample from which viable mononuclear cells can be harvested. In this way virus can even be isolated from samples containing antibodies. Viable cells come in close contact with the indicator cells permitting infection without the virus coming in contact with neutralizing antibodies. Freezing and thawing of the mononuclear cells to release the virus is unnecessary and permits the neutralization of the virus by antibodies, leading to false negative results (Dubovi, 2013). Since most BVDV field isolates in samples are ncp virus, they cause no visual cytopathic effects and the inoculated cells are therefore examined for the presence of BVDV either by immunofluorescence or immunoperoxidase staining with a specific BVDV antiserum. A reference method for VI can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2014). The main advantage of VI is the detection of viable virus, but it is time-consuming and expensive (Lanyon et al., 2014). VI should therefore be used as back-up and reference test for other diagnostic tools (Sandvik, 2005).

Antigen Enzyme Linked ImmunoSorbent Assays (**Ag-ELISA**) are used to detect the presence of BVDV antigen in various sample matrices, such as non-coagulated blood, serum, skin biopsies (ear notches), tissue samples (spleen, lung, liver, kidney) and milk (Sandvik, 2005; Lanyon et al., 2014). The samples should contain sufficient material, as there is no amplification of the target (Sandvik, 2005). Suitable viral proteins for detection are the envelope glycoprotein Erns and the nonstructural protein NS3 (Dubovi, 2013). Several commercial Ag-ELISA kits are available. The kits used by the regional centres for animal disease control “Dierengezondheidszorg Vlaanderen (DGZ)” in Flanders (northern part of Belgium) and “Association Régionale de Santé et d’Identification Animales (ARSIA)” in

Wallonia (southern part of Belgium) detect the Erns Ag. The Ag-ELISA is robust, simple and cost-efficient (Lanyon et al., 2014), but false negative test results may occur due to the presence of colostral antibodies that capture the antigens and make them unavailable for the test (Zimmer et al., 2004; Fux and Wolf, 2012). However, even in the presence of colostral antibodies Erns Ag remains detectable in ear notch samples and Ag-ELISA that target Erns in combination with ear notch sampling is therefore preferred to test young animals (Fux and Wolf, 2012).

Reverse-Transcription Polymerase Chain Reaction (**RT-PCR**) detects the presence of viral RNA and is widely accepted as the standard for BVDV diagnosis as it is faster and less expensive compared to VI, but also highly sensitive and suitable for a large variety of samples, including blood, milk, saliva, follicular fluid and tissue samples (Lanyon et al., 2014). RT-PCR consists of four steps: extraction of RNA from the sample, reverse transcription to cDNA, amplification of cDNA and finally detection of the amplified products (Sandvik, 2005). Combining the last three steps in a single tube reduces the risk of false positive results due to cross-contamination (Sandvik, 2005; Dubovi, 2013). The main advantages of RT-PCR are a high sensitivity, no interference of antibodies with the detection of the virus genome and the possibility to pool the samples as a consequence of the high sensitivity. By using specific primers (and probes) it is possible to distinguish between BVDV-1 and BVDV-2 (Letellier et al., 1999). Quantitative or real-time RT-PCR (RT-qPCR) can give semi-quantitative results in terms of a threshold cycle (Ct), with lower values indicating a higher amount of viral RNA, or quantitative results, expressed in the number of BVDV copies present in the sample (Letellier and Kerkhofs, 2003).

Detection of BVDV infection based on the immune response

Virus neutralisation (**VN**) is the gold standard and quantifies the inhibitory effect of specific neutralizing antibodies on virus replication in cell cultures. The result is expressed as the highest serum dilution able to inhibit replication of a quantified amount of the challenge virus (Sandvik, 2005). The sensitivity of the test depends on the cells used (e.g. MDBK versus BT) and the antigenic relatedness between the antibodies in the sample and the virus strains used in the test (Lanyon et al., 2014). For example, when an animal was infected with BVDV-2, a higher sensitivity is obtained with a BVDV-2 strain than with a BVDV-1 strain as challenge virus, since BVDV-1 and BVDV-2 are antigenically distinct (Ridpath et al., 2000).

If cp reference strains are used for VN tests, then viral inhibition is directly visible, otherwise immunoperoxidase staining is needed. A reference method for VN can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2014). VN is very specific and sensitive, but time-consuming and expensive.

Antibody ELISA (Ab-ELISA) to detect the presence of BVDV-antibodies are less expensive and less time-consuming compared to VN and can be used for a large amount of samples (Sandvik, 2005). Several commercial Ab-ELISA kits are available. Serum and milk samples are suitable matrices for Ab-ELISA. Ab-ELISA also return quantitative results in terms of optical densities (Lanyon et al., 2014) and a positive association between Ab-ELISA results and VN titres was demonstrated (Lanyon et al., 2013). A reference method for Ab-ELISA can be found in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE, 2014). Interference of vaccination with Ab-ELISA tests is an issue as no marker vaccine to differentiate between infected and vaccinated animals (DIVA) is available up to date and no Ab-ELISA tests are able to differentiate vaccine-induced antibodies from antibodies following natural infections (Makoschey et al., 2007; Raue et al., 2011).

Diagnostic approaches in the field

Diagnostic tests are indispensable to BVDV control and several test strategies have been described (Brock, 2004; Larson, 2005; Houe et al., 2006). The strategy recommended by the regional centres for animal disease control and faculties of veterinary medicine in Belgium has been described (Laureyns et al., 2010; Laureyns, 2014). Whatever strategy is used, the following diagnostic objectives should be achieved (Lindberg et al., 2006):

1) to detect new cases of BVDV infection,
2) to identify free herds and confirm their status and
3) to evaluate the progress by estimating the prevalence.

Each of these objectives can be performed both at the herd level and animal level.

Determination of the BVDV status at the herd level

The BVDV status of a herd is verified without testing every animal separately, but by testing samples from multiple representative individuals in a herd (Houe et al., 2006). The

spot test or testing bulk tank milk for the presence of BVDV antibodies are convenient test strategies for this purpose. The spot test is an indirect screening for the presence of PI animals in the herd by testing a limited number of young stock (3 to 10 animals) for BVDV specific antibodies (Houe, 1992; Houe, 1994). The advantage of testing young stock is that it detects a recent or on-going infection given the young age of the animals (Brülisauer et al., 2010). Bulk tank milk samples can be used to detect antibodies amongst lactating cows by Ab-ELISA. Antibody concentrations are associated with the prevalence of immune cows and thus the likelihood of a herd being infected (Beaudeau et al., 2001). However, vaccine-induced antibodies might hamper the interpretation of bulk milk testing (Houe et al., 2006).

Identification of individual infected animals

When the presence of PI animals is suspected, every individual animal has to be tested for the presence of BVDV. Bulk tank milk can be tested for animals contributing to the bulk tank milk at the moment of sampling. One should be aware that only information is obtained about the animals contributing to the bulk tank milk and additional animals should be tested separately (Laureyns et al., 2010; Lanyon et al., 2014). The BVDV status of a group of animals can be verified with one single RT-PCR test by pooling samples such as blood, serum and ear notches (Munoz-Zanzi et al., 2000; Kennedy et al., 2006). An optimal pool size of 20 is advised (Munoz-Zanzi et al., 2000). A positive test result involves testing of the individual samples for which the cheaper Ag-ELISA can be used (Mars and van Maanen, 2005; Hanon et al., 2014).

The detection of PI calves has to be continued for one year after the removal of the last identified PI animal (Laureyns et al., 2010). Ear notch samples from new-born calves, which are obtained when placing the official ear tag, are very suitable for this purpose. Furthermore, all newly acquired cattle or animals at suspicion of a BVDV infection based on clinical signs should be tested for the presence of BVDV (Smith and Grotelueschen, 2004; Houe et al., 2006), with special attention for the Trojan cow, i.e. a TI dam carrying a PI foetus (Laureyns et al., 2010; Lanyon et al., 2014).

A single positive test result in VI, Ag-ELISA or RT-PCR can originate from a PI or a TI animal. To distinguish between PI and TI animals a second sampling is needed. The requested time between sample collection is usually set to 3 weeks as BVDV can be cleared from the blood of TI animals within 14 to 21 days post-infection (Howard, 1990). A TI

animal positive by Ag-ELISA or VI during the first sampling should have a negative test result 3 weeks later using the same tests. However, viral RNA has been shown to be longer detectable than viable virus (Givens et al., 2002) and RT-PCR should therefore not be used for resampling (Laureyns, 2014). Studies also suggest that differentiation between PI and TI is possible with one single RT-qPCR test (Bhudevi and Weinstock, 2001; Hanon et al., 2014). The interpretation of a single quantitative result may be complicated by the variation in levels of viraemia such as intermittent viraemia in PI cattle (Laureyns et al., 2010).

Elements for BVDV control

When considering BVDV control, two main strategies used to be proposed: vaccination versus application of biosecurity measures (Lindberg and Houe, 2005; Houe et al., 2006). Now it is generally accepted that every BVDV control programme should involve a combination of different measures (Ridpath, 2013b). A general model for successful BVDV control was proposed by Lindberg and Houe (2005) and consists of three essential elements: biosecurity, virus elimination and monitoring. An optional element is immunization (Fig. 3).

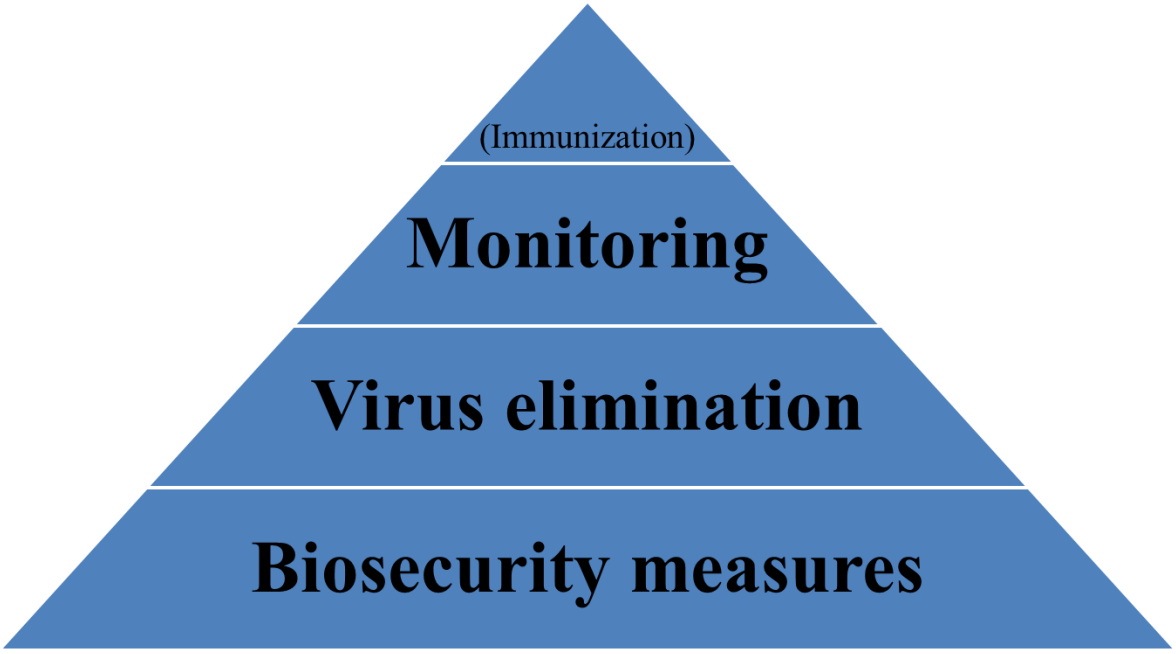

Fig. 3. The general model for BVDV control as proposed by Lindberg and Houe (2005) consists of three essential measures: preventive measures (biosecurity), removal of PI animals (virus elimination) and follow-up of the BVDV status (monitoring). Immunization through vaccination is as an optional fourth element.

Biosecurity

Farm biosecurity embraces all aspects of the prevention of pathogens entering and spreading within a group of animals (Laanen et al., 2013). It can be divided into external biosecurity, which includes all measures preventing pathogens from entering a herd, and internal biosecurity (or biocontainment), which embraces measures reducing the spread of pathogens within a herd (Villarroel et al., 2007; Laanen et al., 2013).

Biosecurity is not a new concept in animal agriculture, but rather a scientifically underpinned redefinition of earlier ideas and practices historically considered to be good animal husbandry (Barrington et al., 2002). Biosecurity became somewhat of a 'buzzword' in the United Kingdom livestock industry following the foot and mouth disease outbreak of February 2001 (Gunn et al., 2008). Indeed, biosecurity is usually associated with collective action for disease control in case of large epidemic outbreaks (Heffernan et al., 2008). However, it is also a crucial element in the control of endemic diseases.

Expected consequences of the reduced disease spread and thus indirectly from applying biosecurity measures are improved production characteristics and thus greater profits, better animal welfare, improved immune responses to vaccines and enhanced job satisfaction for farmers (Brennan and Christley, 2012). Recently it was shown in pig production that a higher biosecurity status is associated with a reduction in antimicrobial usage and an improved production (Laanen et al., 2013).

Although biosecurity not necessarily focuses on one particular infectious disease and aims at upgrading herd health in general, measures are based on the knowledge of the epidemiology of specific pathogens (Barrington et al., 2002). Despite the fact that between pathogens there are considerable epidemiologic differences such as the reservoir, modes of transmission and incubation period (Barrington et al., 2002; Villarroel et al., 2007), the basic principle is to reduce contact between disease agents and susceptible animals (Callan and Garry, 2002). As a result, most biosecurity measures are not specific to a single infectious agent (Barrington et al., 2002).

The most important route of disease transmission is generally considered to be direct contact between live animals, but also indirect transmission should not be ignored (Barrington et al., 2002; Callan and Garry, 2002; Brennan et al., 2008; Nöremark et al., 2013) (Fig. 4).

Considering external biosecurity, all cattle (both purchased and returning) must be put in quarantine and preferably tested for endemic diseases such as BVD, neosporosis and paratuberculosis (Villarroel et al., 2007). Furthermore, contact on pastures with cattle from neighbouring farms should be avoided (Wells et al., 2002). The access for vehicles and professional visitors entering the farm should be restricted and farm-specific protective clothing and boots should be provided.

Considering internal biosecurity, new-born calves and cows in the periparturition period are very susceptible to disease (Villarroel et al., 2007) and a clean and disinfected calving box where no diseased animals are housed should be available (Wells et al., 2002). Since older animals are more resistant to disease and as they often are reservoirs of disease agents, compartmentalization into age groups and working from younger towards older animals may reduce this type of disease spread (Barrington et al., 2002).

Animate vectors such as pets, insects, rodents and wild birds can spread disease both between and within farms and specific measures can be implemented. For example, the access of pets into sheds should be restricted (Wells et al., 2002) and manure should be handled in such a way to reduce the breeding sites of flies (McCluskey, 2002).

Specific preventive measures concerning BVDV spread have been described (Smith and Grotelueschen, 2004; Villarroel et al., 2007). Moreover, the implementation of biosecurity is even considered as the most essential pillar for BVDV control (Lindberg and Houe, 2005) (Fig. 3).

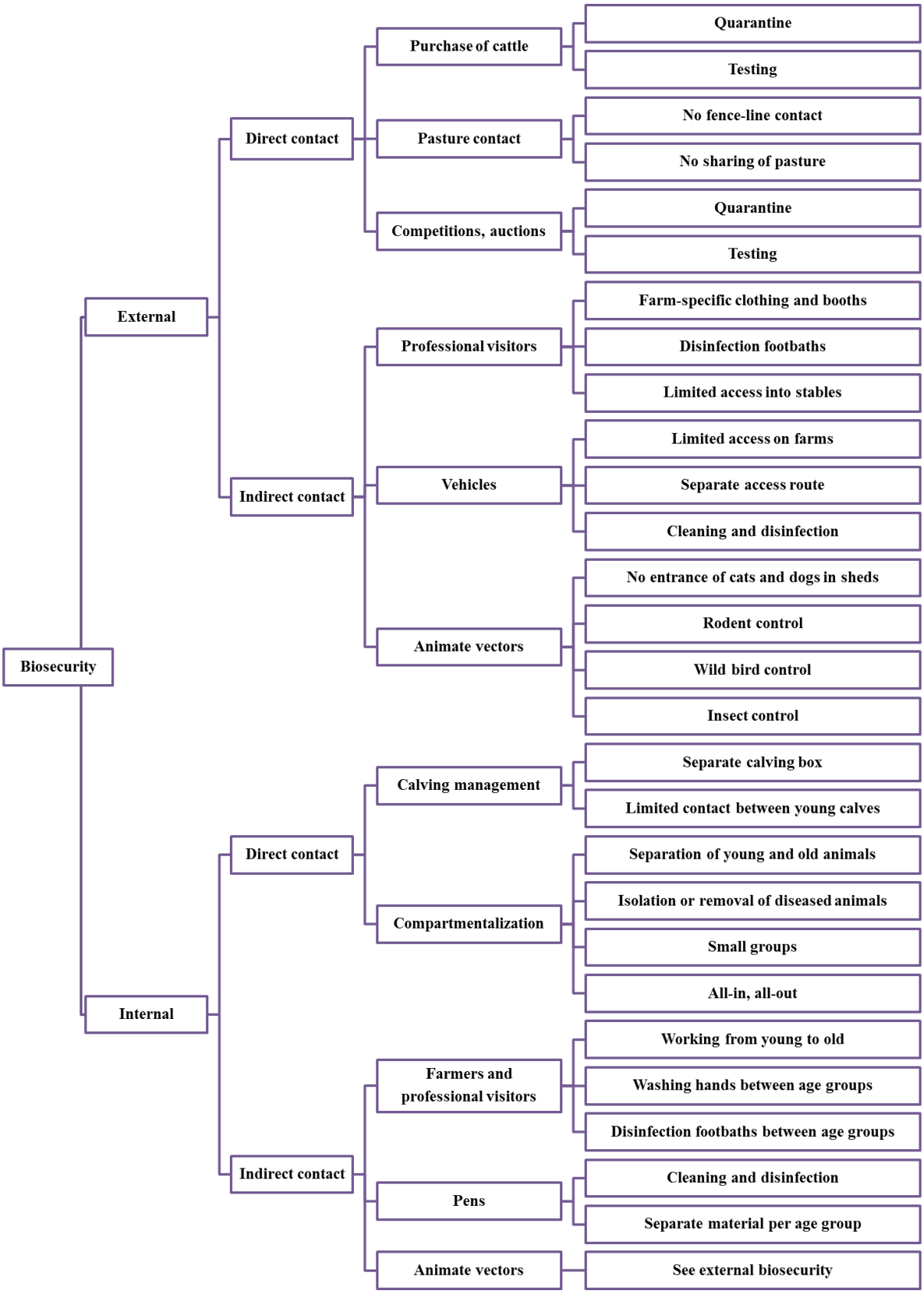

Fig. 4. The basic principle in biosecurity is to reduce contact between disease agents and susceptible animals. For both external and internal biosecurity this can be divided into avoiding direct and indirect transmission, further translated into biosecurity measures applicable for upgrading herd health in general.

Virus elimination

When herds are BVDV infected it is essential to eliminate the virus from the herd. Since PI animals are generally considered to be the source of infection as they continuously shed massive amounts of virus (Lindberg and Houe, 2005), this measure can actually be reduced to the removal of PI animals. So far it was assumed that TI animals did not significantly contribute to BVDV spread. However, the recent BVDV-2c outbreak in Germany and the Netherlands has challenged this assumption. Since no PI animals were detected during this outbreak (Doll and Holsteg, 2013), it is believed that BVDV has spread solely through TI animals and therefore the importance of these TI animals in the epidemiology of BVDV is questioned. However, as mentioned above, sufficient evidence to prove that transmission was not due to PI animals that have escaped identification should be provided (Lindberg and Houe, 2005). Furthermore, other transmission routes such as iatrogenic transmission due to contaminated vaccines should be taken into account (Barkema et al., 2001).

Monitoring and surveillance

Monitoring, or follow-up of the BVDV status, is a third essential measure for successful BVDV control and evaluates the effectiveness of elimination of BVDV in infected herds. Monitoring also serves for detection of new infections and therefore also herds with no history of BVDV infections should be strongly encouraged to monitor the BVDV status, as (re-)infections with BVDV often occur. In the Netherlands, where BVDV control is voluntary at the herd level, every year antibodies are detected in the monitoring system in 5 to 10% of these herds, indicating virus circulation in the herd. New-born PI animals were found in half of these herds (Mars and Van Maanen, 2005). Denmark started a national BVDV eradication programme in 1994. In 1998, 333 herds were found to be re-infected with BVDV after previously having been found free (Bitsch et al., 2000).

Whenever monitoring involves specific actions in case of a possible (re-)infection, this is defined as surveillance (Lindberg et al., 2006). Specific actions include for instance testing of new-born calves to early detect new PI animals or testing diseased animals for BVDV viraemia.

Immunization

Immunization is a fourth control measure and does not cancel out the need for the other measures proposed in the general model for BVDV control (Lindberg and Houe, 2005). The need for immunization, which is usually obtained by vaccination, as an additional control measure depends on the risk of re-infection of herds that cleared BVDV (Lindberg et al., 2006). Vaccination as a single measure is often assumed, by farmers and veterinarians, to be sufficient in the control of BVDV, thereby neglecting the three essential measures (Laureyns et al., 2013a). However, incorrect use of vaccination can lead to incomplete protection (Meadows, 2010) and should therefore be implemented in combination with the previously mentioned essential control measures. The birth of PI animals in vaccinated herds has been reported (Van Campen et al., 2000).

Undoubtedly, some drawbacks are associated with vaccination. First, as mentioned above, it can interfere with diagnostic tests. Secondly, BVDV vaccines currently registered in Belgium only contain BVDV-1 strains. This may be of concern as studies have demonstrated incomplete foetal protection against BVDV-2 strains in animals vaccinated with BVDV-1 vaccines (Ridpath et al., 1994; Brock and Cortese, 2001). Furthermore, concerns related to BVDV vaccination with modified live vaccines (MLV) are discussed by Ridpath (2013b): BVDV contamination of MLV, immunosuppression and post vaccinal mucosal disease, i.e. MD following vaccination of PI animals with MLV containing cp virus. A commercial BVDV-1 MLV was used as emergency vaccination during a recent BVDV-2c outbreak in Germany (Doll and Holsteg, 2013). Currently no MLV vaccines are registered in Belgium. Recently, the European Medicines Agency (EMA) has approved the marketing authorisation of a new MLV BVDV vaccine (EMA, 2014). The vaccine contains both a BVDV-1 and a BVDV-2 strain and its launch is planned for spring 2015 (Boehringer-Ingelheim, 2014).

Immunization following ‘controlled natural exposure to a PI animal’ was also suggested by Lindberg and Houe (2005). However, several disadvantages are involved with this immunization procedure: welfare implications concerning the PI animal, possible clinical manifestations in TI cattle, immunosuppression associated with BVDV infections and no guarantee that every animal is immunized. Moreover, biosecurity measures may fail to avoid infection of pregnant cattle.

BVDV control at the regional/national level

Given the variability in animal density, movement of animals, production structures, BVDV prevalence and the diagnostic tests available, there is no "one-size-fits-all" approach to the design of BVDV control and eradication programmes (Ridpath and Fulton, 2009; Brülisauer et al., 2010; Presi and Heim, 2010). Several European countries have initiated diverse national and regional campaigns with Denmark, Finland, Norway and Sweden already in 1993-1994 (Sandvik, 2004; Lindberg et al., 2006). Also the Shetland Islands started in the early 1990s and it became mandatory in entire Scotland in 2011 (Voas, 2012). A regional voluntary programme was launched in Austria in 1997 and was made compulsory for the entire country in 2004 (Rossmanith et al., 2010). Eradication and control programmes using ear notch testing were launched in Switzerland (2008) and Germany (2011) (Presi and Heim, 2010; Ståhl and Alenius, 2012). A programme was made compulsory in Ireland in 2013 (Graham, 2013). Regional programmes are operative in France and Italy.

Until now BVDV control in Belgium was on a voluntary basis at the herd level. The only legislation was the annulment of sale when purchasing a PI animal. Recently, the outline of the first phase of a Belgian mandatory BVDV control programme has been published (Royal Decree on the control of BVD, 18 June 2014) and the programme has been launched in January 2015.

References

Almeida, L.L., Miranda, I.C.S., Hein, H.E., Neto, W.S., Costa, E.F., Marks, F.S., Rodenbusch, C.R., Canal, C.W., Corbellini, L.G., 2013. Herd-level risk factors for bovine viral diarrhea virus infection in dairy herds from Southern Brazil. Research in Veterinary Science 95, 901-907.

Ames, T.R., 1986. The causative agent of BVD - its epidemiology and pathogenesis. Veterinary Medicine 81, 848-869.

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Veterinary Clinics of North America-Food Animal Practice 11, 425-445.

Barkema, H.W., Bartels, C.J.M., van Wuijckhuise, L., Hesselink, J.W., Holzhauer, M., Weber, M.F., Franken, P., Kock, P.A., Bruschke, C.J.M., Zimmer, G.M., 2001. Outbreak of bovine viral diarrhoe on Dutch dairy farms induced by a BHV1 marker vaccine contaminated with BVDV type 2. Tijdschrift Voor Diergeneeskunde 126, 158-165.

Barrington, G.M., Gay, J.M., Evermann, J.F., 2002. Biosecurity for neonatal gastrointestinal diseases. Veterinary Clinics of North America-Food Animal Practice 18, 7-34.

Bauermann, F.V., Ridpath, J.F., Weiblen, R., Flores, E.F., 2013. HoBi-like viruses: an emerging group of pestiviruses. Journal of Veterinary Diagnostic Investigation 25, 6-15.

Baule, C., Kulcsar, G., Belak, K., Albert, M., Mittelholzer, C., Soos, T., Kucsera, L., Belak, S., 2001. Pathogenesis of primary respiratory disease induced by isolates from a new genetic cluster of bovine viral diarrhea virus type I. Journal of Clinical Microbiology 39, 146-153.

Beaudeau, F., Assie, S., Seegers, H., Belloc, C., Sellal, E., Joly, A., 2001. Assessing the within-herd prevalence of cows antibody-positive to bovine viral diarrhoea virus with a blocking ELISA on bulk tank milk. Veterinary Record 149, 236-240.

Bedekovic, T., Lemo, N., Barbic, L., Cvetnic, Z., Lojkic, I., Benic, M., Cac, Z., Lojkic, M., Madic, J., 2013. Influence of category, herd size, grazing and management on epidemiology of bovine viral diarrhoea in dairy herds. Acta Veterinaria Brno 82, 125-130.

Bhudevi, B., Weinstock, D., 2001. Fluorogenic RT-PCR assay (TaqMan) for detection and classification of bovine viral diarrhea virus. Veterinary Microbiology 83, 1-10.

Bitsch, V., Hansen, K.E.L., Ronsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994-1998 with special reference to legislation and causes of infection. Veterinary Microbiology 77, 137-143.

Blanchard, P.C., Ridpath, J.F., Walker, J.B., Hietala, S.K., 2010. An outbreak of late-term abortions, premature births, and congenital deformities associated with a Bovine viral diarrhea virus 1 subtype b that induces thrombocytopenia. Journal of Veterinary Diagnostic Investigation 22, 128-131.

Boehringer-Ingelheim, 2014. New vaccine against Bovine Viral Diarrhea: Boehringer Ingelheim receives European marketing authorization for Bovela®. www.boehringer-ingelheim.com (Accessed 30 December 2014)

Bolin, S.R., Grooms, D.L., 2004. Origination and consequences of bovine viral diarrhea virus diversity. Veterinary Clinics of North America-Food Animal Practice 20, 51-68.

Bolin, S.R., Littledike, E.T., Ridpath, J.F., 1991. Serologic detection and practical consequences of antigenic diversity among bovine viral diarrhea viruses in a vaccinated herd. American Journal of Veterinary Research 52, 1033-1037.

Bolin, S.R., Ridpath, J.F., 1992. Differences in virulence between 2 noncytopathic bovine viral diarrhea viruses in calves. American Journal of Veterinary Research 53, 2157-2163.

Bolin, S.R., Ridpath, J.F., 1998. Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum. Journal of Veterinary Diagnostic Investigation 10, 135-139.

Brennan, M.L., Christley, R.M., 2012. Biosecurity on Cattle Farms: A Study in North-West England. Plos One 7 (1), e28139.

Brennan, M.L., Kemp, R., Christley, R.M., 2008. Direct and indirect contacts between cattle farms in north-west England. Preventive Veterinary Medicine 84, 242-260.

Brock, K.V., 2004. Strategies for the control and prevention of bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 20, 171-180.

Brock, K.V., Cortese, V.S., 2001. Experimental fetal challenge using type II bovine viral diarrhea virus in cattle vaccinated with modified-live virus vaccine. Veterinary Therapeutics: Research in Applied Veterinary Medicine 2, 354-360.

Brock, K.V., Lapin, D.R., Skrade, D.R., 1997. Embryo transfer from donor cattle persistently infected with bovine viral diarrhea virus. Theriogenology 47, 837-844.

Brownlie, J., 1990. Pathogenesis of mucosal disease and molecular aspects of bovine virus diarrhea virus. Veterinary Microbiology 23, 371-382.

Brownlie, J., Clarke, M.C., Howard, C.J., 1984. Experimental production of fatal mucosal disease in cattle. Veterinary Record 114, 535-536.

Brownlie, J., Clarke, M.C., Howard, C.J., Pocock, D.H., 1987. Pathogenesis and epidemiology of bovine virus diarrhea virus-infection of cattle. Annales de Recherches Veterinaires 18, 157-166.

Brülisauer, F., Lewis, F.I., Ganser, A.G., McKendrick, I.J., Gunn, G.J., 2010. The prevalence of bovine viral diarrhoea virus infection in beef suckler herds in Scotland. The Veterinary Journal 186, 226-231.

Callan, R.J., Garry, F.B., 2002. Biosecurity and bovine respiratory disease. Veterinary Clinics of North America-Food Animal Practice 18, 57-77.

Carman, S., van Dreumel, T., Ridpath, J., Hazlett, M., Alves, D., Dubovi, E., Tremblay, R., Bolin, S., Godkin, A., Anderson, N., 1998. Severe acute bovine viral diarrhea in Ontario, 1993-1995. Journal of Veterinary Diagnostic Investigation 10, 27-35.

Chase, C.C.L., 2013. The impact of BVDV infection on adaptive immunity. Biologicals 41, 52-60.

Chase, C.C.L., Elmowalid, G., Yousif, A.A.A., 2004. The immune response to bovine viral diarrhea virus: a constantly changing picture. Veterinary Clinics of North America-Food Animal Practice 20, 95-114.

Collins, M.E., Heaney, J., Thomas, C.J., Brownlie, J., 2009. Infectivity of pestivirus following persistence of acute infection. Veterinary Microbiology 138, 289-296.

Corapi, W.V., Elliott, R.D., French, T.W., Arthur, D.G., Bezek, D.M., Dubovi, E.J., 1990. Thrombocytopenia and hemorrhages in veal calves infected with bovine viral diarrhea virus. Journal of the American Veterinary Medical Association 196, 590-596.

Couvreur, B., Letellier, C., Collard, A., Quenon, P., Dehan, P., Hamers, C., Pastoret, P.P., Kerkhofs, P., 2002. Genetic and antigenic variability in bovine viral diarrhea virus (BVDV) isolates from Belgium. Virus Research 85, 17-28.

Cuttance, W., Cuttance, E., 2014. Analysis of individual farm investigations into bovine viral diarrhoea in beef herds in the North Island of New Zealand. New Zealand Veterinary Journal 62, 338-342.

David, G.P., Crawshaw, T.R., Gunning, R.F., Hibberd, R.C., Lloyd, G.M., Marsh, P.R., 1994. Severe disease in adult dairy-cattle in 3 UK dairy herds associated with BVD virus-infection. Veterinary Record 134, 468-472.

Decaro, N., Lucente, M.S., Mari, V., Cirone, F., Cordioli, P., Camero, M., Sciarretta, R., Losurdo, M., Lorusso, E., Buonavoglia, C., 2011. Atypical pestivirus and severe respiratory disease in calves, Europe. Emerging Infectious Diseases 17, 1549-1552.

Doll, K., Holsteg, M., 2013. BVD-virus type 2 – An outbreak in Germany. Cattle Practice 21, 216.

Dubovi, E.J., 2013. Laboratory diagnosis of bovine viral diarrhea virus. Biologicals 41, 8-13.

Dufour, B., Repiquet, D., Touratier, A., 1999. Economic studies in animal health decision-making: the cost-benefit ratio of eradicating bovine virus diarrhoea in France. Revue Scientifique et Technique - Office International des Epizooties 18, 520-532.

Endsley, J.J., Roth, J.A., Ridpath, J., Neill, J., 2003. Maternal antibody blocks humoral but not T cell responses to BVDV. Biologicals 31, 123-125.

Ersbøll, A.K., Stryhn, H., 2000. Epidemiological modelling of infectious disease in animals: Bovine virus diarrhoea in Danish dairy herds. In: Proceedings of the 9th International Symposium on Veterinary Epidemiology and Economics. Breckenridge, Colorado, USA, 520-522.

European Medicines Agency (EMA), 2014. CVMP summary of positive opinion for Bovela. http://www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/veterinary/003703/WC500175224.pdf (Accessed 30 December 2014)

Fourichon, C., Beaudeau, F., Bareille, N., Seegers, H., 2005. Quantification of economic losses consecutive to infection of a dairy herd with bovine viral diarrhoea virus. Preventive Veterinary Medicine 72, 177-181.

Fredriksen, B., Press, C.M., Loken, T., Odegaard, S.A., 1999a. Distribution of viral antigen in uterus, placenta and foetus of cattle persistently infected with bovine virus diarrhoea virus. Veterinary Microbiology 64, 109-122.

Fredriksen, B., Sandvik, T., Loken, T., Odegaard, S.A., 1999b. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Veterinary Record 144, 111-114.

Fux, R., Wolf, G., 2012. Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? Veterinary Microbiology 161, 13-19.

Gates, M.C., Humphry, R.W., Gunn, G.J., 2013a. Associations between bovine viral diarrhoea virus (BVDV) seropositivity and performance indicators in beef suckler and dairy herds. The Veterinary Journal 198, 631-637.

Gates, M.C., Woolhouse, M.E.J., Gunn, G.J., Humphry, R.W., 2013b. Relative associations of cattle movements, local spread, and biosecurity with bovine viral diarrhoea virus (BVDV) seropositivity in beef and dairy herds. Preventive Veterinary Medicine 112, 285-295.

Givens, M.D., Marley M.S., 2013. Immunology of chronic BVDV infections. Biologicals 41, 26-30.

Givens, M.D., Riddell, K.P., Edmondson, M.A., Walz, P.H., Gard, J.A., Zhang, Y.J., Galik, P.K., Brodersen, B.W., Carson, R.L., Stringfellow, D.A., 2009. Epidemiology of prolonged testicular infections with bovine viral diarrhea virus. Veterinary Microbiology 139, 42-51.

Givens, M.D., Riddell, K.P., Galik, P.K., Stringfellow, D.A., Brock, K.V., Loskutoff, N.M., 2002. Diagnostic dilemma encountered when detecting bovine viral diarrhea virus in IVF embryo production. Theriogenology 58, 1399-1407.

Goens, S.D., 2002. The evolution of bovine viral diarrhea: a review. Canadian Veterinary Journal - Revue Veterinaire Canadienne 43, 946-954.

Gogorza, L.M., Moran, P.E., Larghi, J.L., Segui, R., Lissarrague, C., Saracco, M., Braun, M., Esteban, E.N., 2005. Detection of bovine viral diarrhea virus (BVDV) in seropositive cattle. Preventive Veterinary Medicine 72, 49-54.

Graham, D., 2013. The Irish BVD eradication programme. BVD Day 2013. http://bvd-day2013.eu/conferences2/ (accessed 18 Aug. 2014)

Graham, D.A., Clegg, T.A., Lynch, M., More, S.J., 2013. Herd-level factors associated with the presence of bovine viral diarrhoea virus in herds participating in the voluntary phase of the Irish national eradication programme. Preventive Veterinary Medicine 112, 99-108.

Grooms, D.L., 2004. Reproductive consequences of infection with bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 20, 5-19.

Gunn, H.M., 1993. Role of fomites and flies in the transmission of bovine viral diarrhea virus. Veterinary Record 132, 584-585.

Gunn, G.J., Heffernan, C., Hall, M., McLeod, A., Hovi, M., 2008. Measuring and comparing constraints to improved biosecurity amongst GB farmers, veterinarians and the auxiliary industries. Preventive Veterinary Medicine 84, 310-323.

Gunn, G.J., Stott, A.W., Humphry, R.W., 2004. Modelling and costing BVD outbreaks in beef herds. The Veterinary Journal 167, 143-149.

Hanna, D., Sneddon, I.A., Beattie, V.E., 2009. The relationship between the stockperson's personality and attitudes and the productivity of dairy cows. Animal 3, 737-743.

Hanon, J.B., Van Der Stede, Y., Antonissen, A., Mullender, C., Tignon, M., Van den Berg, T., Cay, A.B., 2014. Distinction between persistent and transient infection in a bovine viral diarrhoea (BVD) control programme: appropriate interpretation of real-time RT-PCR and antigen-ELISA test results. Transboundary and Emerging Diseases 61, 156-162.

Heffernan, C., Nielsen, L., Thomson, K., Gunn, G., 2008. An exploration of the drivers to bio-security collective action among a sample of UK cattle and sheep farmers. Preventive Veterinary Medicine 87, 358-372.

Hessman, B.E., Sjeklocha, D.B., Fulton, R.W., Ridpath, J.F., Johnson, B.J., McElroy, D.R., 2012. Acute bovine viral diarrhea associated with extensive mucosal lesions, high morbidity, and mortality in a commercial feedlot. Journal of Veterinary Diagnostic Investigation 24, 397-404.

Heuer, C., Healy, A., Zerbini, C., 2007. Economic effects of exposure to bovine viral diarrhea virus on dairy herds in New Zealand. Journal of Dairy Science 90, 5428-5438.

Houe, H., 1992. Serological analysis of a small herd sample to predict presence or absence of animals persistently infected with bovine viral diarrhea virus (BVDV) in dairy herds. Research in Veterinary Science 53, 320-323.

Houe, H., 1994. Bovine Virus Diarrhea Virus - Detection of Danish dairy herds with persistently infected animals by means of a screening-test of 10 young stock. Preventive Veterinary Medicine 19, 241-248.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterinary Microbiology 64, 89-107.

Houe, H., 2003. Economic impact of BVDV infection in dairies. Biologicals 31, 137-143.

Houe, H., 2005. Risk Assessment, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 35-64.

Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. Journal of Veterinary Diagnostic Investigation 18, 427-436.

Houe, H., Meyling, A., 1991. Prevalence of bovine virus diarrhea (BVD) in 19 Danish dairy herds and estimation of incidence of infection in early-pregnancy. Preventive Veterinary Medicine 11, 9-16.

Houe, H., Pedersen, K.M., Meyling, A., 1993. The effect of bovine virus diarrhea virus-infection on conception rate. Preventive Veterinary Medicine 15, 117-123.

Howard, C.J., 1990. Immunological responses to bovine virus diarrhoea virus infections. Revue Scientifique et Technique - Office International des Epizooties 9, 95-103.

Howard, C.J., Clarke, M.C., Brownlie, J., 1989. Protection against respiratory-infection with bovine virus diarrhea virus by passively acquired antibody. Veterinary Microbiology 19, 195-203.

Howard, C.J., Clarke, M.C., Sopp, P., Brownlie, J., 1992. Immunity to bovine virus diarrhoea virus in calves: the role of different T-cell subpopulations analysed by specific depletion in vivo with monoclonal antibodies. Veterinary Immunology and Immunopathology 32, 303-314.

Hult, L., Lindberg, A., 2005. Experiences from BVDV control in Sweden. Preventive Veterinary Medicine 72, 143-148.

Humphry, R.W., Brülisauer, F., McKendrick, I.J., Nettleton, P.F., Gunn, G.J., 2012. Prevalence of antibodies to bovine viral diarrhoea virus in bulk tank milk and associated risk factors in Scottish dairy herds. Veterinary Record 171, 445.

ICTV 7th Report, 1999. International Committee on Taxonomy of Viruses (ICTV). http://www.ictvonline.org/taxonomyHistory.asp?taxnode_id=20020934&taxa_name=Bovine viral diarrhea virus 2 (accessed 17 Aug. 14).

Kennedy, J.A., Mortimer, R.G., Powers, B., 2006. Reverse transcription-polymerase chain reaction on pooled samples to detect bovine viral diarrhea virus by using fresh ear-notch-sample supernatants. Journal of Veterinary Diagnostic Investigation 18, 89-93.

Kirkland, P.D., Richards, S.G., Rothwell, J.T., Stanley, D.F., 1991. Replication of bovine viral diarrhea virus in the bovine reproductive-tract and excretion of virus in semen during acute and chronic infections. Veterinary Record 128, 587-590.

Klingenberg, K.D., Vagsholm, I., Alenius, S., 1999. Incidence of diarrhea among calves after strict closure and eradication of bovine viral diarrhea virus infection in a dairy herd. Journal of the American Veterinary Medical Association 214, 1824-1828.

Kroese, A.H., de Jong, M.C.M., 2001. Design and analysis of transmission experiments. Society for Veterinary Epidemiology and Preventive Medicine, Proceedings, xxi-xxxvii.

Kummerer, B.M., Tautz, N., Becher, P., Thiel, H.J., Meyers, G., 2000. The genetic basis for cytopathogenicity of pestiviruses. Veterinary Microbiology 77, 117-128.

Laanen, M., Persoons, D., Ribbens, S., De Jong, E., Callens, B., Strubbe, M., Maes, D., Dewulf, J., 2013. Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. The Veterinary Journal 198, 508-512.

Lang-Ree, J.R., Vatn, T., Kommisrud, E., Loken, T., 1994. Transmission of bovine viral diarrhea virus by rectal examination. Veterinary Record 135, 412-413.

Lanyon, S.R., Anderson, M.L., Bergman, E., Reichel, M.P., 2013. Validation and evaluation of a commercially available ELISA for the detection of antibodies specific to bovine viral diarrhoea virus (bovine pestivirus). Australian Veterinary Journal 91, 52-56.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. The Veterinary Journal 199, 201-209.

Larson, R.L. 2005. Management Systems and Control Programs, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 223-238.

Laureyns, J., 2014. Challenges in the Control of Bovine Viral Diarrhoea Virus: Implications for a Belgian Eradication Programme. Merelbeke, Belgium: Ghent University. Faculty of Veterinary Medicine. http://hdl.handle.net/1854/LU-4231850

Laureyns, J., Booth, R., Sarrazin, S., Deprez, P., Pfeiffer, D., Dewulf, J., De Vliegher, S., 2013a. Assessment of two essential elements of BVDV control on selected Flemish dairy and beef farms. Vlaams Diergeneeskundig Tijdschrift 82, 356-362.

Laureyns, J., Letellier, C., Meganck, V., Pardon, B., Deprez, P., de Kruif, A., 2011a. Severe disease in neonatal calves with detection of cytopathic BVDV. Veterinary Record 169, 101.

Laureyns, J., Pardon, B., Cay, A.B., Sarrazin, S., Deprez, P., 2013b. Spontaneous bleeding in a neonatal calf persistently infected with BVDV1b. Vlaams Diergeneeskundig Tijdschrift 82, 87-89.

Laureyns, J., Pardon, B., Letellier, C., Deprez, P., 2011b. Periparturient infection with bovine viral diarrhea virus type 1 causes hemorrhagic proctocolitis in a cow. Canadian Veterinary Journal - Revue Veterinaire Canadienne 52, 1135-1139.

Laureyns, J., Piepers, S., Ribbens, S., Sarrazin, S., De Vliegher, S., Van Crombrugge, J.M., Dewulf, J., 2013c. Association between herd exposure to BVDV-infection and bulk milk somatic cell count of Flemish dairy farms. Preventive Veterinary Medicine 109, 148-151.

Laureyns, J., Ribbens, S., de Kruif, A., 2010. Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. The Veterinary Journal 184, 21-26.

Letellier, C., Kerkhofs, P., 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. Journal of Virological Methods 114, 21-27.

Letellier, C., Kerkhofs, P., Wellemans, G., Vanopdenbosch, E., 1999. Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5 ' untranslated region. Veterinary Microbiology 64, 155-167.

Letellier, C., Pardon, B., Van der Heyden, S., Deprez, P., 2010. Circulation in Belgium of a bovine viral diarrhoea virus type 2 closely related to North American hypervirulent viruses. Veterinary Record 166, 625-627.

Liebler-Tenorio, E.M., 2005. Pathogenesis, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 121-143.

Lindberg, A., Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Veterinary Microbiology 64, 197-222.

Lindberg, A., Brownlie, J., Gunn, G.J., Houe, H., Moennig, V., Saatkamp, H.W., Sandvik, T., Valle, P.S., 2006. The control of bovine viral diarrhoea virus in Europe: today and in the future. Revue Scientifique et Technique - Office International des Epizooties 25, 961-979.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Veterinary Record 154, 463-467.

Liu, L.H., Xia, H.Y., Wahlberg, N., Belak, S., Baule, C., 2009. Phylogeny, classification and evolutionary insights into pestiviruses. Virology 385, 351-357.

Loneragan, G.H., Thomson, D.U., Montgomery, D.L., Mason, G.L., Larson, R.L., 2005. Prevalence, outcome, and health consequences associated with persistent infection with bovine viral diarrhea virus in feedlot cattle. Journal of the American Veterinary Medical Association 226, 595-601.

Makoschey, B., Sonnemans, D., Bielsa, J.M., Franken, P., Mars, M., Santos, L., Alvarez, M., 2007. Evaluation of the induction of NS3 specific BVDV antibodies using a

commercial inactivated BVDV vaccine in immunization and challenge trials. Vaccine 25, 6140-6145.

Mars, M.H., Van Maanen, C., 2005. Diagnostic assays applied in BVDV control in The Netherlands. Preventive Veterinary Medicine 72, 43-48.

Martin, S.W., Bohac, J.G., 1986. The association between serological titers in infectious bovine-rhinotracheitis virus, bovine virus diarrhea virus, parainfluenza-3 virus, respiratory syncytial virus and treatment for respiratory-disease in ontario feedlot calves. Canadian Journal of Veterinary Research - Revue Canadienne de Recherche Veterinaire 50, 351-358.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine-Revue Canadienne de Medecine Comparee 48, 156-161.

McCluskey, B.J., 2002. Biosecurity for arthropod-borne diseases. Veterinary Clinics of North America-Food Animal Practice 18, 99-114.

McGowan, M.R., Kirkland, P.D., Richards, S.G., Littlejohns, I.R., 1993. Increased reproductive losses in cattle infected with bovine pestivirus around the time of insemination. Veterinary Record 133, 39-43.

Meadows, D., 2010. A study to investigate the use and application of BVDV vaccine in UK cattle. Cattle Practice 18, 202-215.

Meyling, A., Houe, H., Jensen, A.M., 1990. Epidemiology of bovine virus diarrhoea virus. Revue Scientifique et Technique - Office International des Epizooties. 9, 75-93.

Meyling, A., Jensen, A.M., 1988. Transmission of bovine virus diarrhea Virus (BVDV) by artificial-insemination (AI) with semen from a persistently-infected bull. Veterinary Microbiology 17, 97-105.

Moen, A., 2013. Boviene virus diarree type 2. GD Veterinair 19, 3.

Moen, A., Sol, J., Sampimon, O., 2005. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Preventive Veterinary Medicine 72, 93-98.

Moennig, V., Liess, B., 1995. Pathogenesis of intrauterine infections with bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 11, 477-487.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long-term epidemiologic-study of bovine viral diarrhea infections in a large herd of dairy-cattle. Veterinary Record 132, 622-626.

Moerman, A., Straver, P.L., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1994. Clinical consequences of a bovine virus diarrhea virus-infection in a dairy-herd - a longitudinal-study. Veterinary Quarterly 16, 115-119.

Munoz-Zanzi, C.A., Hietala, S.K., Thurmond, M.C., Johnson, W.O., 2003. Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves. American Journal of Veterinary Research 64, 358-365.

Munoz-Zanzi, C.A., Johnson, W.O., Thurmond, M.C., Hietala, S.K., 2000. Pooled-sample testing as a herd-screening tool for detection of bovine viral diarrhea virus persistently infected cattle. Journal of Veterinary Diagnostic Investigation 12, 195-203.

Munoz-Zanzi, C.A., Thurmond, M.C., Johnson, W.O., Hietala, S.K., 2002. Predicted ages of dairy calves when colostrum-derived bovine viral diarrhea virus antibodies would no longer offer protection against disease or interfere with vaccination. Journal of the American Veterinary Medical Association 221, 678-685.

Niskanen, R., Alenius, S., Belak, K., Baule, C., Belak, S., Voges, H., Gustafsson, H., 2002a. Insemination of susceptible heifers with semen from a non-viraemic bull with persistent bovine virus diarrhoea virus infection localized in the testes. Reproduction in Domestic Animals 37, 171-175.

Niskanen, R., Emanuelson, U., Sundberg, J., Larsson, B., Alenius, S., 1995. Effects of infection with bovine virus diarrhea virus on health and reproductive-performance in 213 dairy herds in one county in sweden. Preventive Veterinary Medicine 23, 229-237.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. The Veterinary Journal 165, 125-130.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Veterinaria Scandinavica 41, 93-99.

Niskanen, R., Lindberg, A., Traven, M., 2002b. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. The Veterinary Journal 163, 251-259.

Nobiron, I., Thompson, I., Brownlie, J., Collins, M.E., 2003. DNA vaccination against bovine viral diarrhoea virus induces humoral and cellular responses in cattle with evidence for protection against viral challenge. Vaccine 21, 2082-2092.

Nöremark, M., Frossling, J., Lewerin, S.S., 2013. A survey of visitors on Swedish livestock farms with reference to the spread of animal diseases. BMC Veterinary Research 9, 184.

OIE, 2014. World Organisation for Animal Health. Manual of diagnostic tests and vaccines for terrestrial animals (mammals, birds and bees). Sixth Edition. http://www.oie.int/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/ (accessed 17 Aug. 14).

Onyekaba, C., Fahrmann, J., Bueon, L., King, P., Goyal, S.M., 1987. Comparison of 5 cell-lines for the propagation of bovine viral diarrhea and infectious bovine-rhinotracheitis viruses. Microbiologica 10, 311-315.

Palfi, V., Houe, H., Philipsen, J., 1993. Studies on the decline of bovine virus diarrhea virus (BVDV) maternal antibodies and detectability of BVDV in persistently infected calves. Acta Veterinaria Scandinavica 34, 105-107.

Pardon, B., 2012. Morbidity, mortality and drug use in white veal calves with emphasis on respiratory disease. Ghent University. Faculty of Veterinary Medicine, Merelbeke, Belgium. http://hdl.handle.net/1854/LU-3007344

Pardon, B., De Bleecker, K., Hostens, M., Callens, J., Dewulf, J., Deprez, P., 2012. Longitudinal study on morbidity and mortality in white veal calves in Belgium. BMC Veterinary Research 8, 26.

Pellerin, C., Vandenhurk, J., Lecomte, J., Tijssen, P., 1994. Identification of a new group of bovine viral diarrhea virus-strains associated with severe outbreaks and high mortalities. Virology 203, 260-268.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterinary Research 41.

Peterhans, E., Schweizer, M., 2010. Pestiviruses: How to outmaneuver your hosts. Veterinary Microbiology 142, 18-25.

Peterhans, E., Schweizer, M., 2013. BVDV: A pestivirus inducing tolerance of the innate immune response. Biologicals 41, 39-51.

Potgieter, L.N.D., 1995. Immunology of Bovine Viral Diarrhea Virus. Veterinary Clinics of North America-Food Animal Practice 11, 501-520.

Presi, P., Heim, D., 2010. BVD eradication in Switzerland – A new approach. Veterinary Microbiology 142, 137-142.

Presi, P., Struchen, R., Knight-Jones, T., Scholl, S., Heim, D., 2011. Bovine viral diarrhea (BVD) eradication in Switzerland-Experiences of the first two years. Preventive Veterinary Medicine 99, 112-121.

Raue, R., Harmeyer, S.S., Nanjiani, I.A., 2011. Antibody responses to inactivated vaccines and natural infection in cattle using bovine viral diarrhoea virus ELISA kits: Assessment of potential to differentiate infected and vaccinated animals. Veterinary Journal 187, 330-334.

Reichel, M.P., Hill, F.I., Voges, H., 2008. Does control of bovine viral diarrhoea infection make economic sense? New Zealand Veterinary Journal 56, 60-66.

Ridpath, J.F., 2005. Classification and Molecular Biologiy, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 65-80.

Ridpath, J.F., 2010. Bovine Viral Diarrhea Virus: Global Status. Veterinary Clinics of North America-Food Animal Practice 26, 105-121.

Ridpath, J.F., 2013a. A need to define characteristics to be used in the taxonomy of the expanding pestivirus genus. Berliner und Munchener Tierarztliche Wochenschrift 126, 462-467.

Ridpath, J.F., 2013b. Immunology of BVDV vaccines. Biologicals 41, 14-19.

Ridpath, J.F., Bolin, S.R., Dubovi, E.J., 1994. Segregation of bovine viral diarrhea virus into genotypes. Virology 205, 66-74.

Ridpath, J.F., Fulton, R.W., 2009. Knowledge gaps impacting the development of bovine viral diarrhea virus control programs in the United States. Journal of the American Veterinary Medical Association 235, 1171-1179.

Ridpath, J.F., Neill, J.D., Frey, M., Landgraf, J.G., 2000. Phylogenetic, antigenic and clinical characterization of type 2 BVDV from North America. Veterinary Microbiology 77, 145-155.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Veterinary Microbiology 114, 196-204.

Rossmanith, W., Deinhofer, M., Janacek, R., Trampler, R., Wilhelm, E., 2010. Voluntary and compulsory eradication of bovine viral diarrhoea virus in Lower Austria. Veterinary Microbiology 142, 143-149.

Saatkamp, H.W., Gunn, G.J., Stott, A.W., Humphry, R.W., 2006. Position paper: socio-economic aspects on BVDV control. EU Thematic Network on BVDV control.

http://www.afbini.gov.uk/chs-thematic-network-position-paper-on-bvd-control.pdf (Accessed 11 September 14)

Saliki, J.T., Dubovi, E.J., 2004. Laboratory diagnosis of bovine viral diarrhea virus infections. Veterinary Clinics of North America-Food Animal Practice 20, 69-83.

Sandvik, T., 2004. Progress of control and prevention programs for bovine viral diarrhea virus in Europe. Veterinary Clinics of North America-Food Animal Practice 20, 151-169.

Sandvik, T., 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. Preventive Veterinary Medicine 72, 3-16.

Smith, D.R., Grotelueschen, D.M., 2004. Biosecurity and biocontainment of bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 20, 131-149.

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe-an update. Japanese Journal of Veterinary Research 60 Suppl, S31-S39.

Ståhl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-Lopez, J., Belak, S., Alenius, S., Lindberg, A., 2005. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. Preventive Veterinary Medicine 72, 103-108.

Stott, A.W., Humphry, R.W., Gunn, G.J., 2010. Modelling the effects of previous infection and re-infection on the costs of bovine viral diarrhoea outbreaks in beef herds. The Veterinary Journal 185, 138-143.

Stott, A.W., Humphry, R.W., Gunn, G.J., Higgins, I., Hennessy, T., O'Flaherty, J., Graham, D.A., 2012. Predicted costs and benefits of eradicating BVDV from Ireland. Irish Veterinary Journal 65, 12-21.

Tao, J., Liao, J.H., Wang, Y., Zhang, X.J., Wang, J.Y., Zhu, G.Q., 2013. Bovine viral diarrhea virus (BVDV) infections in pigs. Veterinary Microbiology 165, 185-189.

Tarry, D.W., Bernal, L., Edwards, S., 1991. Transmission of bovine virus diarrhea virus by blood feeding flies. Veterinary Record 128, 82-84.

Thurmond, M.C., 2005. Virus Transmission, in: Goyal, S.M., Ridpath, J.F. (Eds.), Bovine viral diarrhea virus – Diagnosis, management, and control, first edition. Blackwell Publishing, Ames, pp. 91-104.

Valle, P.S., Martin, S.W., Tremblay, R., Bateman, K., 1999. Factors associated with being a bovine-virus diarrhoea (BVD) seropositive dairy herd in the More and Romsdal County of Norway. Preventive Veterinary Medicine 40, 165-177.

Valle, P.S., Skjerve, E., Martin, S.W., Larssen, R.B., Osteras, O., Nyberg, O., 2005. Ten years of bovine virus diarrhoea virus (BVDV) control in Norway: a cost-benefit analysis. Preventive Veterinary Medicine 72, 189-207.

Van Campen, H., Vorpahl, P., Huzurbazar, S., Edwards, J., Cavender, J., 2000. A case report: evidence for type 2 bovine viral diarrhea virus (BVDV)-associated disease in beef herds vaccinated with a modified-live type 1 BVDV vaccine. Journal of Veterinary Diagnostic Investigation 12, 263-265.

Velthuis, A.G.J., Bouma, A., Katsma, W.E.A., Nodelijk, G., de Jong, M.C.M., 2007. Design and analysis of small-scale transmission experiments with animals. Epidemiology and Infection 135, 202-217.

Vilcek, S., Nettleton, P.F., Paton, D.J., Belak, S., 1997. Molecular characterization of ovine pestiviruses. Journal of General Virology 78, 725-735.

Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Archives of Virology 146, 99-115.

Villarroel, A., Dargatz, D.A., Lane, V.M., McCluskey, B.J., Salman, M.D., 2007. Suggested outline of potential critical control points for biosecurity and biocontainment on large dairy farms. Journal of the American Veterinary Medical Association 230, 808-819.

Voas, S., 2012. Working together to eradicate BVD in Scotland. Veterinary Record 170, 278-279.

Voges, H., Horner, G.W., Rowe, S., Wellenberg, G.J., 1998. Persistent bovine pestivirus infection localized in the testes of an immuno-competent, non-viraemic bull. Veterinary Microbiology 61, 165-175.

Waage, S., 2000. Influence of new infection with bovine virus diarrhoea virus on udder health in Norwegian dairy cows. Preventive Veterinary Medicine 43, 123-135.

Walz, P.W., Bell, T.G., Wells, J.L., Grooms, D.L., Kaiser, L., Maes, R.K., Baker, J.C., 2001. Relationship between degree of viremia and disease manifestations in calves with experimentally induced bovine viral diarrhea virus infection. American Journal of Veterinary Research 62, 1095-1103.

Wells, S.J., Dee, S., Godden, S., 2002. Biosecurity for gastrointestinal diseases of adult dairy cattle. Veterinary Clinics of North America-Food Animal Practice 18, 35-55.

Wieringa-Jelsma, T., Quak, S., Loeffen, W.L.A., 2006. Limited BVDV transmission and full protection against CSFV transmission in pigs experimentally infected with BVDV type 1b. Veterinary Microbiology 118, 26-36.

Zimmer, G.M., Van Maanen, C., De Goey, I., Brinkhof, J., Wentink, G.H., 2004. The effect of maternal antibodies on the detection of bovine virus diarrhoea virus in peripheral blood samples. Veterinary Microbiology 100, 145-149.

Chapter 2

Scientific Aims

To evaluate the effectiveness of interventions in the framework of BVDV control, it is important to start with a good insight in the BVDV prevalence. Furthermore, when considering a control plan at the national level this information is of crucial importance for the design of a such a plan. At the start of this research, only limited and outdated data on the BVDV prevalence in Belgium were available. Therefore the first aim of this doctoral thesis was to determine the BVDV prevalence in Belgium (**Chapter 3**).

It has been stated before that a successful BVDV control scheme should be based on three central elements. First, the elimination of virus sources from infected herds is crucial. Since PI animals are considered to be the main source of infection, this measure generally focuses on the elimination of PI animals. However, recent outbreaks suggest that TI animals may have an impact on BVDV spread as well. Since the role of these TI animals in the epidemiology of BVDV remains highly controversial, the second aim of this research was to determine the role of cattle transiently infected with virulent BVDV strains in the transmission of BVDV (**Chapter 4**).

Furthermore, the implementation of biosecurity measures is crucial to prevent re-infection in free herds. Yet, information on the implementation of biosecurity in Belgian cattle herds, a very densely populated livestock area, is currently lacking. Therefore the third aim of this thesis was to gain insight into the current implementation of biosecurity measures in the daily management of Belgian cattle herds (**Chapter 5**).

Finally, continuous monitoring and surveillance is needed to rapidly detect new infections. To optimize the prevention and early detection of new BVDV infections, knowledge of the risk of BVDV (re-)infection and associated risk factors may be very useful. Therefore the final aims of this thesis were to assess the risk of BVDV re-infection in non-vaccinating herds, given the current implementation of BVDV control measures (**Chapter 6**) and to identify risk factors for infection and re-infection with BVDV (**Chapters 3 and 6**).

Recently a mandatory Belgian BVDV control programme was launched. In the general discussion of this thesis (**Chapter 7**) the obtained results are used to assess the current outline of this control programme and to make some recommendations for further improvements of the programme.

Chapter 3

BVDV prevalence and risk factors

Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds

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Abstract

Bovine viral diarrhoea virus (BVDV) is a worldwide spread virus that most commonly infects cattle and can cause considerable economic losses. To determine the prevalence of BVDV in Belgium, a cross-sectional study was performed between November 2009 and March 2010. Young stock aged between 6 and 12 months from 773 randomly selected Belgian cattle herds were tested for BVDV specific antibodies and antigen. With a target and maximum of 10 animals per sampled herd, a total of 5246 animals were selected. Additionally a questionnaire including different herd management topics and questions about participation in animal health programmes, including BVDV, was sent to 1100 Belgian cattle herds, including the 773 herds for BVDV testing. This paper focuses on results regarding these 773 herds.

The true prevalence of BVDV-specific antibodies and antigen at the herd level was respectively 47.4% and 4.4%, while at the animal level this was 32.9% and 0.3%, respectively. In 44.4% of the herds where BVDV-specific antibodies were detected at least 60% of the sampled young stock was BVDV seropositive. Interestingly, 83.4% of these farmers stated not to have suffered from problems related to BVDV. Moreover, only 8.4% of all farmers who completed the questionnaire (n = 895) reported problems possibly related to BVDV the past 3 years. This result demonstrates that farmers are often unaware of the presence of BVDV in their herd.

Risk factors for a herd to be BVDV seropositive were identified by a multivariable logistic regression model. Large herds were significantly more likely to be BVDV seropositive (OR = 1.004, $p < 0.01$). The interaction between "Antigen positive animal detected in this study" and "BVDV vaccination in 2009" was significant ($p < 0.01$). In non-vaccinating herds, the detection of antigen positive animals was significantly associated with BVDV seropositive herds (OR = 13.8, $p < 0.01$). In herds with no antigen positive animals detected, vaccination resulted in a significant risk factor to be BVDV seropositive compared to non-vaccinating herds (OR = 3.4, $p < 0.01$). Herds reporting BVDV-related problems the past 3 years were more likely to be BVDV seropositive (OR = 1.9, $p < 0.05$). This relation became non-significant (OR = 1.8, $p = 0.08$) when only a subset of herds with no vaccination of animals < 12 months was taken into account. The results of the current study suggest an active circulation of BVDV in a considerable number of Belgian cattle herds.

Introduction

In 1946 bovine viral diarrhoea virus (BVDV) was described in the United States as the cause of a new transmissible cattle disease (Olafson et al., 1946). Up to date many studies have been published and are still being performed in order to understand the complex pathogenesis and epidemiology of this pestivirus.

The complex epidemiology of BVDV partially lies in its ability to infect the foetus. If the infection occurs between the second and fourth month of gestation, the virus is able to cause a persistent infection of the foetus which may result in the birth of a persistently infected (PI) calf (Peterhans et al., 2010). These PI animals are important sources of infection because they continuously shed BVDV in large quantities (Lindberg and Houe, 2005). Clinical symptoms caused by BVDV are highly variable due to the ability of the virus to infect multiple organ systems. Symptoms can range from subclinical to acute infections with high mortality. BVDV infections also affect animal productivity and make animals more susceptible to other diseases (Houe, 1999). For these reasons, the economic impact of BVDV infections is likely to be underestimated. Several studies have demonstrated a negative financial impact of BVDV infections (Houe, 2003; Gunn et al., 2004; Fourichon et al., 2005).

Convinced by the economic importance of BVDV, several European countries launched an eradication campaign. By selective culling of PI animals Scandinavian countries have successfully eradicated BVDV without the use of vaccination. At this moment BVDV is being eradicated in Austria and Switzerland (Houe et al., 2006; Presi et al., 2011) and an eradication programme has been launched in Germany in 2011 (Meier et al., 2010). Given the variability in important influencing factors such as density of animal populations, movement of animals and the incidence of BVDV infections in different countries, there is no "one-size-fits-all" approach to the design of BVDV eradication programmes (Ridpath and Fulton, 2009; Brülisauer et al., 2010). Therefore, it is of crucial importance, before even considering the design of an eradication plan, to have a good insight into the current BVDV prevalence for a specific country (Brülisauer et al., 2010).

In Belgium, an important beef cattle and milk producing country in Europe, only limited and outdated data on the prevalence and the current situation of BVDV were available. Therefore the aim of this study was to estimate both the virological and the serological BVDV prevalence in Belgium using a large representative sample. Additionally, potential risk factors for herds to be BVDV seropositive were studied.

Material and methods

Selection of the herds and animals

In Belgium 32,000 active cattle herds and 2,637,519 domestic bovine animals were registered in the animal identification and registration system (SANITEL) in October 2009. This resulted in an average population density at municipality level of respectively 1.1 herds/km² and 86.4 animals/km².

The BVDV data were collected as part of a larger cross-sectional survey, the cattle winter screening campaign 2009-2010 commissioned by the Federal Agency for the Safety of the Food Chain. The main objective of this survey was to prove the absence of bluetongue virus serotype 8 (BTV-8) circulation in Belgium. Statistical sample size calculations were performed in relation to this main objective. Out of the 32,000 registered Belgian cattle herds (SANITEL, October 9th 2009) 1100 herds were randomly selected with the SURVEYSELECT procedure in SAS® version 9.2 (SAS Institute Inc., Cary, NC, USA). Veal holdings and farms with no cattle present at the time of selection were not included in the study.

Samples for examination of BVDV antigen and antibodies were collected from animals aged between 6 and 12 months. This age category was selected to avoid as much as possible the detection of maternal antibodies and antibodies induced by vaccination. In some herds, no animals between 6 and 12 months of age were present at the time of sampling. Therefore, out of the 1100 selected herds, 773 herds were examined for BVDV antigen and/or antibodies. Per herd a maximum number of 10 randomly selected animals was sampled. If in the age category the required number of animals was not present, all animals in this age category were sampled. A total of 5246 animals were sampled.

Sample collection

All blood samples were collected by the herd veterinarians between November 2009 and March 2010 during the winter screening campaign 2009-2010. Samples for antibody detection were collected in tubes allowing coagulation. Samples for antigen detection were collected in tubes inhibiting coagulation in order to maintain whole blood (EDTA).

Laboratory analysis

For the detection of BVDV-specific antibodies a commercial enzyme-linked immunosorbent assay (ELISA) was used (Herd Chek BVDV Antibody test kit, IDEXX, changed to BVDV Total Ab Test). This ELISA provides a specificity and sensitivity of respectively 99.5% and 96.3% (BVDV Total Ab Test Brochure, IDEXX). Analyses were done on serum samples according to the instructions provided by the manufacturer.

For the detection of BVDV antigen a commercial ELISA was used (Herd Chek Antigen/Serum Plus test kit, IDEXX, changed to BVDV Ag/Serum Plus Test). This test provides a specificity of more than 99.7% and a sensitivity of nearly 100% (BVDV Ag/Serum Plus TestBrochure, IDEXX). Analyses were done on whole blood according to the instructions provided by the manufacturer.

Analyses were performed by the regional centres for animal disease control "Dierengezondheidszorg Vlaanderen (DGZ)" in Flanders (the northern part of Belgium) and "Association Régionale de Santé et d'Identification Animales (ARSIA)" in Wallonia (the southern part of Belgium).

Questionnaire

A questionnaire (in Dutch or French) was distributed during the winter screening 2009-2010 to all 1100 selected herds. The questionnaire contained two questions with regard to farm infrastructure, two questions concerning purchasing policy and general animal health programmes, 18 questions about more specific animal health programmes (BVDV, BHV-1 and BTV) and 9 questions about herd management. With regard to BVDV, closed questions were asked about the occurrence of BVDV problems in the past (detection of PI animals), current BVDV problems and BVDV vaccination. To fill in the questionnaire the farmer could

collaborate with the herd veterinarian. A copy of the questionnaire (in Dutch or French) can be obtained upon request.

Analysis of the test results

A map showing the sample distribution and the cattle density at municipality level was produced using Arc Map® version 3.2.1 (ESRI, Redlands, CA, USA). The animal density data were extracted from SANITEL at the time of selection of the herds.

The estimation of the animal prevalence with 95% confidence interval (95% CI) of BVDV antibodis and antigen was based on a Generalized Estimating Equation (GEE) using the GENMOD procedure in SAS® version 9.2 taking into account the possible correlation among the animals within the same herd. A logit link function and binomial distribution were assumed. An exchangeable working correlation structure was used assuming a constant correlation between any two animals within the same herd. Sampling weights were taken into account by weighting the observation of each animal by the inverse of the probability of this animal being sampled within the age group of 6- to 12-month-old animals present in the herd at the time of herd selection. This probability was calculated based on the sampling fraction of Belgian herds and the number of 6- to 12-month-old animals present in the herd at time of herd selection. Estimations of the animal prevalence of BVDV antibodies and antigen were also obtained by production type. For the latter, herds were classified as dairy, beef or mixed (herds containing beef and dairy cattle) based on the answers to this question in the questionnaire. All obtained apparent animal prevalence estimates and their 95% CI were converted into true prevalence estimates by means of the Rogan and Gladen estimator (1978) taking into account the sensitivity and specificity estimates of the used tests. For the antigen ELISA, a sensitivity of 100% and a specificity of 99.7% were assumed.

The estimation of the herd prevalence (and 95% CI) of BVDV antibodies and antigen was obtained using a logistic regression (GENMOD procedure, SAS® version 9.2). A herd was considered seropositive/antigen positive if at least one of the sampled animals had a positive test result. Estimations were obtained by production type according to the classification described above.

Descriptive statistical analysis was performed to define questionnaire results based on the questionnaire dataset.

Potential risk factors for a herd to be BVDV seropositive and/or Ag-positive were identified by means of a multivariable logistic regression model using SPSS 19.0 for Windows (SPSS Inc., Chicago, IL, USA). This was performed both on the full dataset (all BVDV tested herds) and on a subset of the dataset where herds with BVDV vaccination of animals younger than 12 months were excluded to eliminate the possible influence of vaccine-induced antibodies. First, univariable analysis was performed of each potential risk factor. Subsequently, all variables with a *p*-value < 0.2 were combined in a multivariable model. Pearson and Spearman correlation coefficients were estimated to explore the relationship between all selected independent variables. If the correlation between two variables was > 0.6, only the variable with the smallest *p*-value was included in the multivariable model. This model was built in a stepwise backward manner resulting in a model in which only significant risk factors ($p < 0.05$) were retained. Odds ratios, including 95% CI, are reported for all significant variables. Confounding was checked for by monitoring the change in regression parameters and was considered to be present if parameters changed by > 0.25. In the final model all two-way interactions were tested and significant interactions ($p < 0.05$) were taken up in the model. The goodness-of-fit of the final multivariable model was tested using the Hosmer-Lemeshow test (Hosmer and Lemeshow, 2000) with a significance level set at 5%.

The added value of including herd as random effect in the model and thus analyzing the data at the animal level rather than at herd level was verified by fitting a two-level binomial model in MLwiN 2.02 (Centre for Multilevel Modelling, Bristol, UK) with 'herd' as random effect. A dispersion parameter was calculated to quantify the amount of clustering of animals within a herd by dividing the Pearson chi-square by its degrees of freedom. A value lower than one was considered to indicate underdispersion and thus no need for taking into account clustering of animals within a herd (Dohoo et al., 2009).

Spot test

As the sampling was performed in young stock, the testing can be considered as a spot test for each individual herd. This is an indirect screening for the presence of PI animals in the herd by testing a limited number of young stock (3-10 animals) for BVDV-specific antibodies (Houe, 1992; 1994). To evaluate the sensitivity of the spot test to detect the presence of Ag-positive animals, serological results of herds with a minimum of five animals tested and in which at least one Ag-positive animal was detected were studied in more detail.

Results

Characterization of selected cattle herds

Selected and sampled herds were scattered over the entire country and cattle-dense regions were well represented (Fig. 1). The BVDV sampled herds had an average herd size of 97 animals.

A total of 5212 animals, originating from 770 cattle herds, and 5205 animals, originating from 769 cattle herds, were tested for the presence of respectively BVDV-specific antibodies and antigen and had a valid test result. Serum samples from 34 animals and whole blood samples from 41 animals could not be analysed for the presence of respectively BVDV antibodies and antigen due to loss of samples, empty tubes or an insufficient sample quantity.

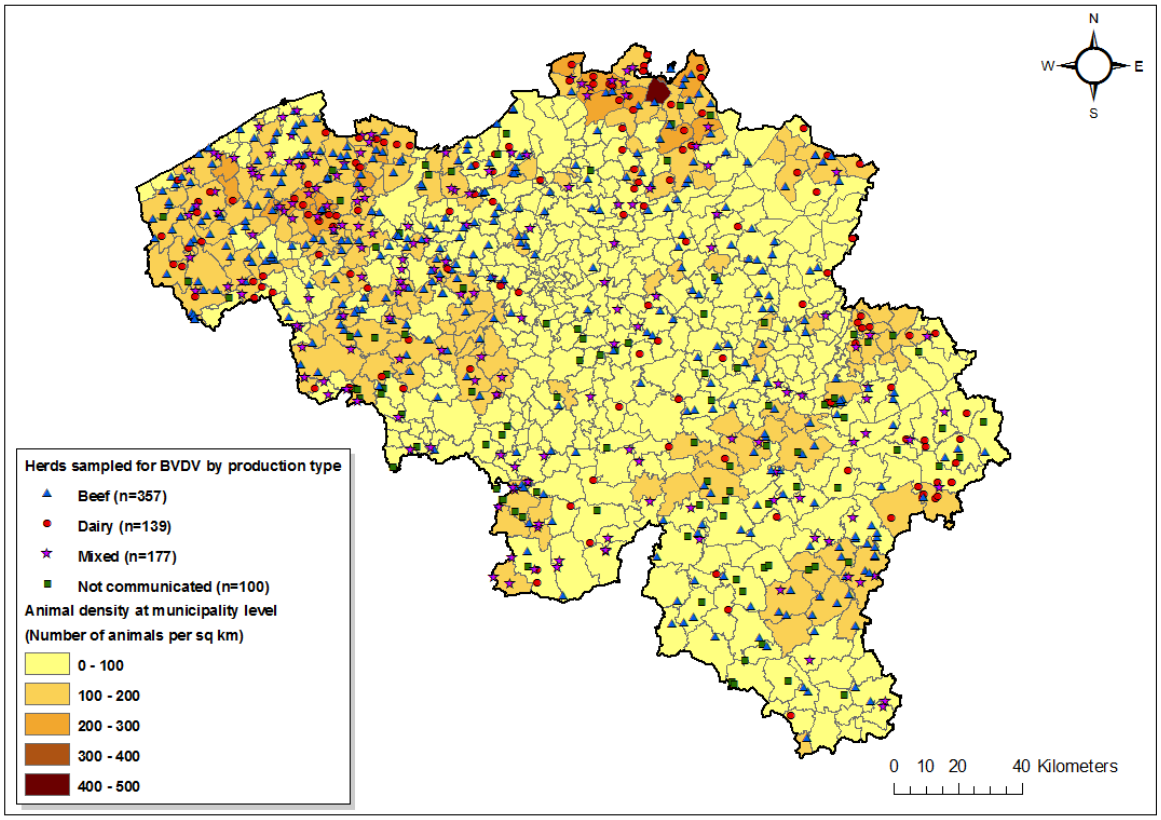

Fig. 1. Production types and municipality level animal densities of the BVDV sampled cattle herds. A total of 773 cattle herds were sampled for BVDV, of which 357 beef herds, 139 dairy herds and 177 mixed herds (herds containing beef and dairy cattle). For 100 herds the production type characteristics were missing in the questionnaire and those herds could therefore not be classified. Cattle densities at municipality level are displayed in the background.

BVDV prevalence in Belgium

Serological and virological BVDV true prevalence estimates at the herd and animal level were stratified by production type based on herds for which the production type was known as a result of completed questionnaires (Table 1).

The seroprevalence at the herd level was significantly lower for beef herds than for mixed herds ($p < 0.05$). This difference was not noticed at the animal level. No other significant differences between production types were present for antibody prevalence. No significant differences were detected between the production types with regard to the virological prevalence at the herd and animal level.

Table 1. Serological en virological BVDV true prevalence in Belgium in 2009-2010. Apparent prevalence estimates were based on a Generalized Estimating Equation and converted into true prevalence estimates by means of the Rogan and Gladen estimator.

	Total	Dairy	Beef	Mixed
Seropositive herds*	351/770	63/139	149/356	93/176
Herd seroprevalence	47.4% (46.0-48.8)	47.1% (45.7-48.5)	43.4% (42.0-44.8)	55.3% (53.9-56.7)
Seropositive animals	1527/5212	298/989	589/2104	425/1422
Animal seroprevalence	32.9% (31.6-34.2)	29.5% (28.2-30.8)	30.6% (29.3-31.9)	36.4% (35.0-37.7)
Antigen positive herds*	36/769	10/139	13/355	6/176
Herd virological prevalence	4.4% (3.8-5.0)	6.9% (6.2-7.6)	3.4% (2.9-3.9)	3.1% (2.6-3.6)
Antigen positive animals	44/5205	13/989	15/2099	9/1413
Animal virological prevalence	0.3% (0.1-0.6)	0.8% (0.5-1.1)	0.3% (0.1-0.5)	0.1% (0.0-0.3)

*= Herd with at least one positive sample; prevalence estimates are reported with 95% CI

A frequency distribution of the within-herd BVDV seroprevalence demonstrated that in 44.4% of the seropositive herds at least 60% of the tested animals was BVDV seropositive (Fig. 2).

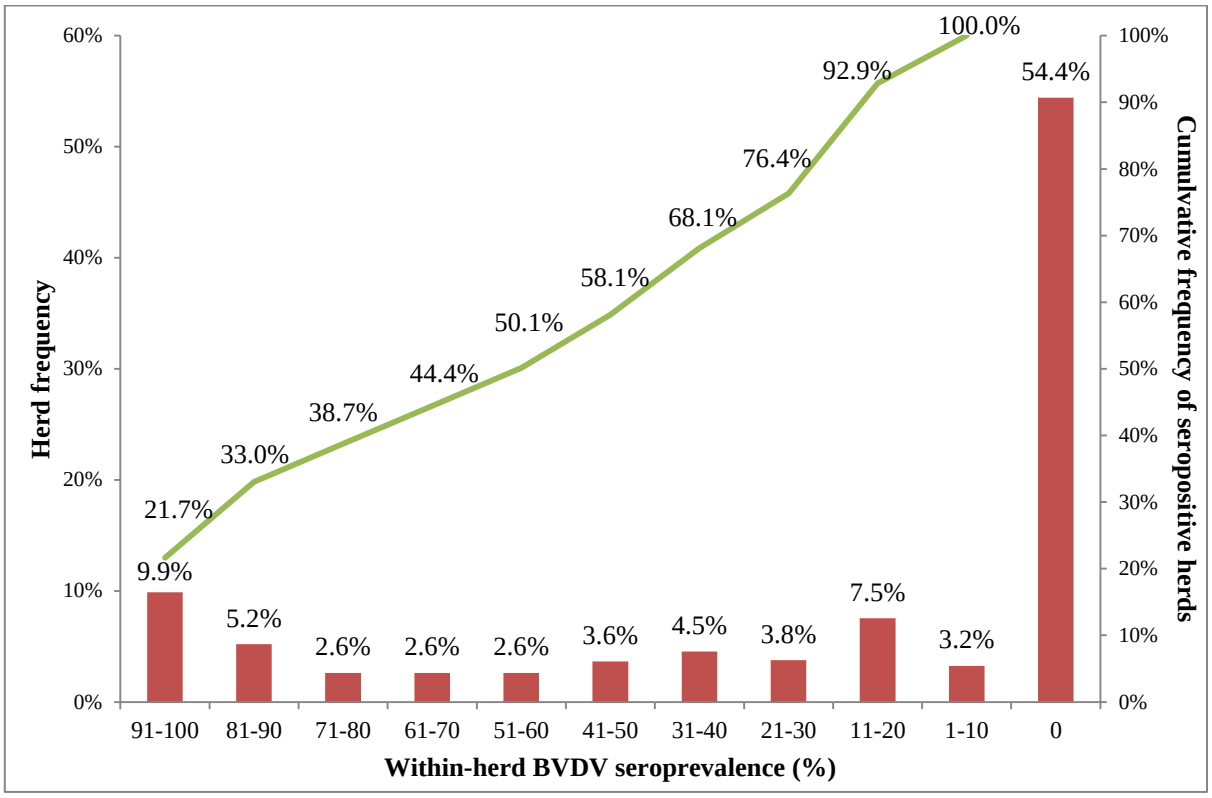

Fig. 2. Frequency distribution of the apparent within-herd BVDV seroprevalence. Percentages above the bars indicate the frequency of the percentage of seropositive animals in the BVDV antibody sampled herds (left vertical axis). The graph line demonstrates the cumulative frequency of seropositive herds (with cumulative percentage for each category) starting with herds with the highest percentage of seropositive animals (right vertical axis). This graph clearly shows that in 44.4% of the seropositive herds at least 60% of the tested animals were BVDV seropositive.

Spot test

Out of the sampled herds, 31 herds in which at least one Ag-positive animal was detected and a minimum of five animals was tested for antigen and antibodies were studied in more detail (Fig. 3). When more than 60% of sampled animals in a herd is BVDV seropositive, this is a strong indication for the presence of a PI animal (Houe et al., 1995a). As such, the sensitivity of the spot test to detect the presence of a PI animal was estimated to be 64.5% (20/31).

In one herd (herd 31) all tested animals were seropositive, including the antigen positive animal.

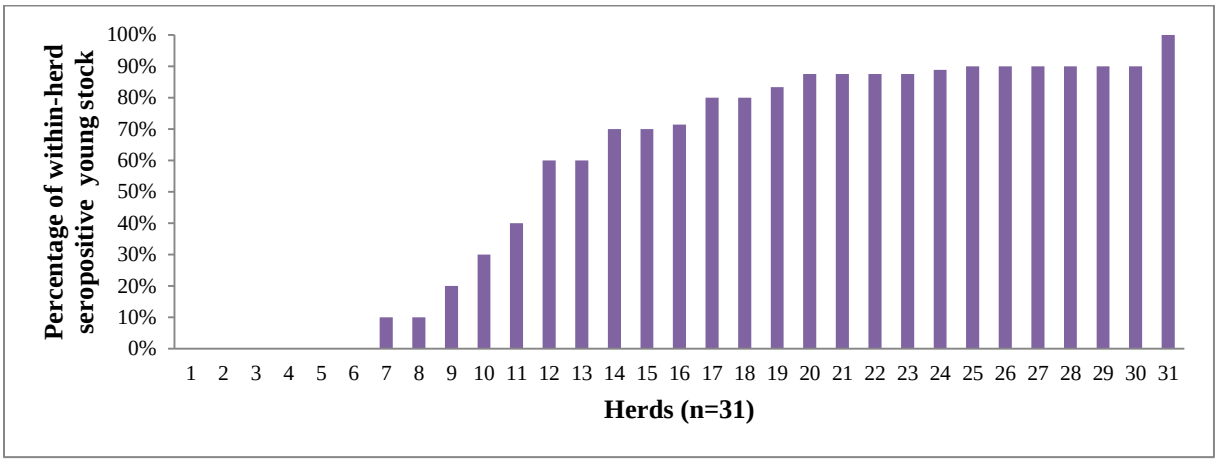

Fig. 3. Percentage of seropositive young stock in herds with a minimum of five animals tested and at least one BVDV antigen positive animal detected in this study. In six herds no BVDV-specific antibodies were detected despite the presence of a BVDV antigen-positive animal. In another five herds less than 60% of the tested animals were BVDV seropositive.

Questionnaire

As 895 of the 1100 herds selected for the winter screening 2009–2010 answered the questionnaire, the response rate was 81.4%. Results of the answers to the BVDV-related questions are listed for all herds and per production type (Table 2).

Table 2. Questionnaire results for BVDV-related questions

Question	Yes	Production type			
		All herds	Dairy	Beef	Mixed
Did the herd suffer from problems possibly related to BVDV the past 3 years?	*n*	75/890	19/165	25/514	31/202
	%*	8.4	11.5	4.9	15.3
Have PI animals ever been detected during a full screening?	*n*	92/868	28/160	36/504	28/196
	%*	10.6	17.5	7.1	14.3
Were animals BVDV vaccinated in 2009?	*n*	158/874	33/159	72/506	53/198
	%*	18.1	20.8	14.2	26.8

*valid percentage (not-applicable and missing values excluded from denominator)

It has to be noted that for the question "Have PI animals ever been detected during a full screening?" the 10.6% herds that did detect a PI animal had done a full screening and detected a PI animal during this screening. No information was available about the proportion of herds that performed a full screening but did not detect a PI animal.

As production types are concerned, differences between beef herds and mixed herds were significant ($p < 0.05$) for all three questions. The difference between dairy and beef herds was only significant for the question "Have PI animals ever been detected during a full screening?" ($p < 0.05$). No significant differences were detected between dairy and mixed herds for any of the three questions.

Risk factor analysis

Herd BVDV seropositivity was significantly associated with seven factors following univariable analysis: "Number of animals" (herd size) ($p < 0.01$), "Antigen positive animal detected in this study" ($p < 0.01$), "BVDV vaccination in 2009" ($p < 0.01$), "Reporting BVDV-related problems the past 3 years" ($p < 0.01$), "Detection of PI animals in the past during a full screening" ($p < 0.01$), "Herd production type" ($p = 0.06$) and "The age of starting BVDV vaccination" ($p < 0.01$). Checking the correlations showed that a relevant correlation was present between "BVDV vaccination in 2009" and "The age of starting BVDV vaccination" ($r = 0.785$). Consequently "The age of starting BVDV vaccination" was not included in the multivariable model.

In the multivariable model four factors remained significantly associated with herd BVDV seropositivity: "Number of animals", "Antigen positive animal detected in this study", "BVDV vaccination in 2009" and "Reporting BVDV-related problems the past 3 years" (Table 3 and Table 4). No confounding was detected. The two-way interaction between "Antigen positive animal detected in this study" and "BVDV vaccination in 2009" was statistically significant ($p < 0.01$) in all herds and in the subset of herds with no BVDV vaccination of animals < 12 months. The model fitted the data sufficiently well, as no significant results were obtained with the Hosmer and Lemeshow test for all herds and herds with no vaccination of animals < 12 months. Similar results were obtained with herd as random effect in a two-level binomial model. The dispersion parameter was 0.451 and 0.421 respectively for all herds and herds with no vaccination of animals < 12 months, indicating overclustering when a two-level binomial model was used.

Due to a lack of power (because of the limited number of antigen positive herds), potential risk factors for BVDV antigen presence could not be identified.

Finally, the relation between BVDV vaccination and the reporting of BVDV-related problems was analysed. The odds for BVDV vaccination was 4.6-fold higher in herds reporting BVDV-related problems compared to herds which did not report such problems (95% CI 2.8; 7.6).

Table 3. Multivariable odds ratios (OR) and 95% confidence intervals (CI) for risk factors associated with a herd to be tested BVDV seropositive in all sampled herds (*n* = 664).

Factor	Category	*n*	Prevalence (%)	OR	95% CI	*p*-value
Number of animals (herd size) (average: 96; range: 1 - 642)	-	-	-	1.004	1.002; 1.006	< 0.01
BVDV vaccination in 2009: No						
Antigen positive animal detected	No	488	35.7	1.0	Reference	
	Yes	25	88.0	13.8	4.0; 46.9	< 0.01
BVDV vaccination in 2009: Yes						
Antigen positive animal detected	No	147	72.1	1.0	Reference	
	Yes	4	25.0	0.1	0.0; 1.5	0.10
Antigen positive animal detected: No						
BVDV vaccination in 2009	No	488	35.7	1.0	Reference	
	Yes	147	72.1	3.4	2.2; 5.2	< 0.01
Antigen positive animal detected: Yes						
BVDV vaccination in 2009	No	25	88.0	1.0	Reference	
	Yes	4	25.0	0.04	0.00; 0.47	< 0.05
BVDV-related problems	No	591	42.8	1.0	Reference	
the past 3 years	Yes	73	68.5	1.9	1.1; 3.3	< 0.05

Table 4. Multivariable odds ratios (OR) and 95% confidence intervals (CI) for risk factors associated with a herd to be tested BVDV seropositive in herds where animals younger than 12 months were not vaccinated (*n* = 579).

Factor	Category	*n*	Prevalence (%)	OR	95% CI	*p*-value
Number of animals (herd size) (average: 88; range: 1 - 642)	-	-	-	1.004	1.002; 1.006	< 0.01
BVDV vaccination in 2009: No						
Antigen positive animal detected	No	484	35.5	1.0	Reference	
	Yes	25	88.0	13.8	4.1; 47.1	< 0.01
BVDV vaccination in 2009: Yes						
Antigen positive animal detected	No	66	63.6	1.0	Reference	
	Yes	4	25.0	0.2	0.0; 2.4	< 0.20
Antigen positive animal detected: No						
BVDV vaccination in 2009	No	484	35.5	1.0	Reference	
	Yes	66	63.6	2.2	1.3; 4.0	< 0.01
Antigen positive animal detected: Yes						
BVDV vaccination in 2009	No	25	88.0	1.00	Reference	
	Yes	4	25.0	0.03	0.00; 0.46	< 0.05
BVDV-related problems	No	530	39.2	1.0	Reference	
the past 3 years	Yes	49	59.2	1.8	0.9; 3.3	0.08

Discussion

This paper describes the results of a BVDV virological and serological prevalence study in Belgium. This is the first complete study for Belgium as in the past studies either described the BVDV prevalence for only certain regions of Belgium (Onclin et al., 1995; Schreiber et al., 1999) or focused on the detection of PI animals (Letellier et al., 2005). The random selection of cattle herds and the large sample of young stock results in an accurate estimation of the virological and serological BVDV prevalence in 6- to 12-month-old animals at both the herd and animal level (Table 1). The within-herd virological and serological prevalence in the entire herd may be biased as only animals aged between 6 and 12 months were sampled. A high between-herd (Table 1) and within-herd seroprevalence (Fig. 2) of young stock suggests an active circulation of BVDV in a considerable number of Belgian cattle herds. Recently, similar results were obtained in beef suckler herds in Scotland (Brülisauer et al., 2010).

When comparing with former BVDV prevalence studies, caution has to be applied to the target population and the methodology used (Brülisauer et al., 2010). None of the previous Belgian studies were performed with a random selection of herds. The selection of the tested herds was based on a history of BVDV in the herd or suspicion of BVDV infection of animals. This can explain why these studies report a higher prevalence of BVDV Ag-positive animals, namely 1.8% (Letellier et al., 2005); or PI animals, namely 2.3% (Wellemans and Vanopdenbosch, 1990), 1.4% (Onclin et al., 1995) and 0.8% (Schreiber et al., 1999). A Danish study selecting dairy herds with an unknown BVDV status obtained a PI animal prevalence of 0.1% (Houe et al., 1995b) which is lower than the 0.3% found in our study. This can be explained by a possible overestimation of PI animal prevalence in our study due to the inclusion of transient infections since the Ag-positive animals have not been retested to differentiate between a transient and persistent infection. Though, a positive test result for BVDV antigen is likely to originate from PI animals (Sandvik, 2005). We also possibly overestimated the PI animal prevalence by testing only young stock compared to testing all animals in a herd since detection of PI animals at adult age occurs less frequently. PI animals usually die from mucosal disease before the age of 2 years (Peterhans et al., 2010) or leave the herd because their production is insufficient (Houe, 2003). Yet, PI animals can sometimes live beyond the age of 2 years (Houe, 1992).

As previous BVDV prevalence studies performed in Belgium focused on PI animals, little information about BVDV seroprevalence is available. Schreiber et al. (1999) estimated a seroprevalence of 65.5% at the animal level after testing all animals in 61 herds. When sampling all animals in a herd, also older animals are included. Obviously, older animals are more likely to have been exposed to the virus and as animals stay seropositive life-long, the herd seroprevalence is likely to be higher when older animals are also included in the sample. Moreover, vaccination is more frequently applied in adult animals than in young stock. The non-random selection of the herds and the different age of the sampled animals make it difficult to compare the seroprevalence results.

The serological results of the young stock can be considered as a spot test for each individual herd and as such can provide useful information about the BVDV infection status of each herd (Houe, 1992). The advantage of testing young stock for the presence of BVDV-specific antibodies is that it detects a recent or on-going infection given the young age of the animals (Brülisauer et al., 2010). When only the herds with a minimum of five animals tested are taken into account, 54.1% (286/529) of the herds were BVDV seropositive (data not shown). Almost half (48.3%) of these seropositive herds had a within-herd seroprevalence ≥ 60%, suggesting the presence of a PI animal in these herds when considering these results as a spot test (Brülisauer et al., 2010).

Pillars and Grooms (2002) obtained a sensitivity of 66.7% to detect the presence of PI animals when at least 3 out of 5 unvaccinated heifers aged 6-12 months were BVDV seropositive. A similar sensitivity of 64.5% was calculated in the present study. This might be an underestimation of the true sensitivity of the spot test due to possible false positive antigen detection results. Given the specificity of 99.7% of the used antigen test and the number of animals tested (n = 5205), about 15 false positive results are expected. It is impossible to determine which of the 44 Ag-positive animals were false positive, but it is likely that at least some of these herds with an apparent antigen positive animal and no seropositive animals had false positive antigen test results. Moreover, also a number of biological factors may impair the spot test sensitivity. A first factor is a limited contact of the PI animal with the sampled animals, for instance when animals are housed separately. If animals are housed in different groups, spot testing should be performed on each cohort (Pillars and Grooms, 2002; Laureyns et al., 2010). When a PI animal was recently added to the group, it is possible that cohort animals have not seroconverted yet (Houe et al., 2006). Therefore the value of the spot test increases by repeating it at regular intervals (Houe, 1999). In herds with spot tests results

ranging between 0% and 60% of the sampled animals testing seropositive, it is favourable to repeat the spot test 3 months later to evaluate the previously obtained results (Pillars and Grooms, 2002 and Laureyns et al., 2010). The same holds for the antigen results since BVDV Ag-positive animals in this study have not been retested to confirm PI status and therefore it is possible that some animals were transiently infected (TI) or even false positive. TI animals are a much smaller source of infection compared to PI animals (Lindberg and Houe, 2005), which may explain why some sampled animals did not seroconvert. In one herd (herd 31, Fig. 3) all tested animals were seropositive including the Ag-positive animal. This may be an indication of a transient infection in this animal.

The influence of the herd production type on BVDV infections is not well known. Few differences between production types were detected in this study. Only between beef herds and mixed herds statistically significant differences could be detected: beef herds had a significantly lower seroprevalence, reported less BVDV-related problems and vaccinated less compared to mixed herds. Fewer beef herds detected PI animals in the past compared to dairy and mixed herds. All these differences were not significant between dairy herds and mixed herds. It is suggested that beef herds purchase less BVDV susceptible animals in early pregnancy, which could possibly explain these findings. Though, one can argue if these statistically significant differences are of any biological importance.

The number of animals present in the herd was identified as a risk factor for a herd to be BVDV seropositive. Large herds were significantly more often seropositive than small herds ($p < 0.01$). This was also found in other studies (Houe, 1999; Talafha et al., 2009). Small herds are more likely to eliminate BVDV infections spontaneously. The probability of this self-clearance depends on the prevalence of susceptible animals in early pregnancy, which is lower in small herds (Lindberg and Houe, 2005).

Despite a high BVDV prevalence in Belgian cattle herds, only 8.4% of the farmers stated to have had problems possibly related to BVDV. Moreover, 83.4% of the farmers with a spot test ≥ 60% seropositive stated not to have had any BVDV-related problems (data not shown). This can be explained by the ability of BVDV to cause a broad range of symptoms and by the fact that 70-90% of BVDV infections occur subclinically. Nevertheless, these subclinical infections also affect animal productivity, for instance by decreasing milk yield (Fourichon et al., 2005).

The fact that farmers mostly are not aware of the presence of BVDV in their herds could explain why "Reporting BVDV-related problems the past 3 years" could not be identified as a significant risk factor for a herd to be BVDV seropositive (Table 4). Such herds had an odds of 1.8 of being seropositive, but this was not statistically significant ($p = 0.08$). One would have expected this effect to be more explicit but the fact that BVDV infections mostly remain undetected has probably attenuated the association between herd seropositivity and this factor.

The influence of BVDV vaccination on BVDV herd seropositivity depended on the detection of Ag-positive animals in the present study. Non-vaccinating herds with Ag-positive animals detected, were significantly more often BVDV seropositive compared to herds with no Ag-positive animals detected ($p < 0.01$). This was to be expected as viraemic animals (TI and especially PI animals) are the most important source of infection (Lindberg and Houe, 2005). In vaccinating herds no significant relation between the detection of Ag-positive animals and seropositive herds could be demonstrated ($p = 0.10$). With only four vaccinating herds where Ag-positive animals were detected and this limited number relative to the vaccinating herds with no Ag-positive animal detected ($n = 147$), no firm conclusions should be drawn from this result. In the subset of herds with no vaccination of animals < 12 months the discrepancy between the number of vaccinating herds with Ag-positive animals ($n = 4$) and without Ag-positive animals detected ($n = 66$) was smaller (Table 4). Nevertheless, the relation between the detection of Ag-positive animals and seropositive herds remained non-significant ($p = 0.20$). When no Ag-positive animals were detected, vaccination was a significant risk factor for a herd to be seropositive ($p < 0.01$). This can partially be explained by the detection of vaccine-induced antibodies (Raue et al., 2011). However, in herds with no vaccination of the sampled animals (but with possible vaccination of older animals), vaccination also appeared to be a risk factor (Table 4). As the sampled animals were not BVDV vaccinated, this means they have BVDV antibodies which must be originating from a BVDV infection. It can be assumed that herds with BVDV-related problems apply vaccination as a measure to control the infection: herds reporting BVDV-related problems applied significantly more often BVDV vaccination ($p < 0.01$) compared to herds non-reporting such problems. In herds where Ag-positive animals were detected, vaccination protected herds of becoming seropositive ($p < 0.05$). Vaccination reduces the number of susceptible animals and is therefore able to decrease the virus spread (Lindberg and Houe, 2005).

Besides the significant relation between reporting BVDV-related problems and applying BVDV vaccination, we noticed that some farmers which did not report BVDV-related problems also applied vaccination. Some hypotheses can be proposed why they vaccinated anyway. Farmers who experienced BVDV-related problems in the past often continue vaccination without considering the necessity of it. Moreover, some herd veterinarians advise all their clients to apply BVDV vaccination, irrespective of the risk of infection. Some farmers will vaccinate as a preventive measure because they fear to get BVDV infected.

Conclusions

This paper describes the first Belgian virological and serological BVDV prevalence study based on a random selection of a young cattle. The high between-herd and within-herd seroprevalence of young stock suggests an active circulation of BVDV in a considerable number of Belgian cattle herds. Despite a high BVDV prevalence, few farmers are aware of the presence of BVDV in their herds. Nevertheless, useful information about the BVDV infection status of a herd can easily be obtained by performing a spot test at regularly intervals. The identified risk factors illustrate that the detection and removal of PI animals is of crucial importance to eradicate BVDV at the herd level, which may be more difficult in larger herds.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

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References

Brülisauer, F., Lewis, F.I., Ganser, A.G., Mckendrick, I.J., Gunn, G.J., 2010. The prevalence of bovine viral diarrhoea virus infection in beef suckler herds in Scotland. The Veterinry Journal 186, 226-231.

BVDV Ag/Serum Plus Test Brochure, IDEXX.

www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/bvdv-ag-serum-plus-test-sheet.pdf (visited on August 13th 2011)

BVDV Total Ab Test Brochure, IDEXX.

www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/bvdv-total-ab-test-brochure.pdf (visited on August 13th 2011)

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary Epidemiological Research, 2nd Edition. VER Inc, Charlottetown.

Fourichon, C., Beaudeau, F., Bareille, N., Seegers, H., 2005. Quantification of economic losses consecutive to infection of a dairy herd with bovine viral diarrhoea virus. Preventive Veterinary Medicine 72, 177-181.

Gunn G.J., Stott A.W., Humphry R.W., 2004. Modelling and costing BVD outbreaks in beef herds. The Veterinary Journal 167, 143-149.

Hosmer, D.W., Lemeshow, S., 2000. Applied Logistic Regression (second ed.). John Wiley and Sons, New York, USA, 143–186.

Houe, H., 1992. Serological analysis of a small herd sample to predict presence or absence of animals persistently infected with bovine viral diarrhea virus (BVDV) in dairy herds. Research in Veterinary Science 53, 320-323.

Houe, H., 1994. Bovine virus diarrhoea virus: detection of Danish dairy herds with persistently infected animals by means of screening test of ten young stock. Preventive Veterinary Medicine 19, 241-248.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterinary Microbiology 64, 89-107.

Houe, H., 2003. Economic impact of BVDV infection in dairies. Biologicals 31, 137-143.

Houe, H., Baker, J.C., Maes, R.K., Ruegg, P.L., Lloyd, J.W., 1995a. Application of antibody titers against bovine viral diarrhea virus (BVDV) as a measure to detect herds with cattle persistently infected with BVDV. Journal of Veterinary Diagnostic Investigation 7, 327-332.

Houe, H., Baker, J.C., Maes, R.K., Wuryastuti, H., Wasito, R., Ruegg, P.L., Lloyd, J.W., 1995b. Prevalence of cattle persistently infected with bovine viral diarrhea virus in 20 dairy herds in two counties in central Michigan and comparison of prevalence of antibody-positive cattle among herds with different infection and vaccination status. Journal of Veterinary Diagnostic Investigation 7, 321-326.

Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. Journal of Veterinary Diagnostic Investigation 18, 427-436.

Laureyns, J., Ribbens, S., de Kruif, A., 2010. Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. The Veterinary Journal 184, 21-26.

Letellier, C., De Meulemeester, L., Lomba, M., Mijten, E., Kerkhofs, P., 2005. Detection of BVDV persistently infected animals in Belgium: Evaluation of the strategy implemented. Preventive Veterinary Medicine 72, 121-125.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Meier, N., Wittkowski, G., Alex, M., Gangl, A., 2010. BVDV eradication will become mandatory for all German states as from 2011. 26th World Buiatrics Congress, Santiago, Chile. http://www.originalprocess.it/wbc2010/AbstractCD/pdf/274.pdf (visited on April 28th 2012)

Olafson, P., McCallum, A.D., Fox, F.H., 1946. An apparently new transmissible disease of cattle. The Cornell Veterinarian 36, 205–213.

Onclin, M., Lambot, M., Limbourg, B., Antoine, H., Czaplicki, G., Pastoret, P.-P., 1995. Prévalence des bovins immunotolérants infectés permanents par le pestivirus responsable de la diarrhée virale bovine, dans l'est de la Belgique, au sein de troupeaux infectés. Annales de Médecine Vétérinaire 139, 429-431.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterinary Research 41, article number 44.

Pillars, R., Grooms, D., 2002. Serologic evaluation of five unvaccinated heifers to detect herds that have cattle persistently infected with bovine viral diarrhea virus. American Journal of Veterinary Research 63, 499-505.

Presi, P., Struchen, R., Knight-Jones, T., Scholl, S., Heim, D., 2011. Bovine viral diarrhea (BVD) eradication in Switzerland – Experiences of the first two years. Preventive Veterinary Medicine 99, 112-121.

Raue, R., Harmeyer, S., Nanjiani, I., 2011. Antibody responses to inactivated vaccines and natural infection in cattle using bovine viral diarrhoea virus ELISA kits: Assessment of potential to differentiate infected and vaccinated animals. The Veterinary Journal 187, 330-334.

Ridpath, J.F., Fulton, R.W., 2009. Knowledge gaps impacting the development of bovine viral diarrhea virus control programs in the United States. Journal of the American Veterinary Medical Association 235, 1171-1179.

Rogan, W.J., Gladen, B., 1978. Estimating prevalence from the results of a screening test. American Journal of Epidemiology 107, 71-76.

Sandvik, T., 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. Preventive Veterinary Medicine 72, 3-16.

Schreiber, P., Dubois, F., Drèze, F., Lacroix, N., Limbourg, B., Coppe, P., 1999. Prevalence of bovine virus diarrhoea virus infection in Belgian white blue cattle in Southern Belgium. Veterinary Quarterly 21, 28-32.

Talafha, A.Q., Hirche, S.M., Ababneh, M.M., Al-Majali, A.M., Ababneh, M.M., 2009. Prevalence and risk factors associated with bovine viral diarrhea virus infection in dairy herds in Jordan. Tropical Animal Health and Production 41, 499-506.

Wellemans, G., Vanopdenbosch, E., 1990. Dépistage des bovins BVD-immunotolérants (BVD-IT) à partir des lymphocytes: un aperçu de 22 mois de contrôle de la population bovine belge. Annales de Médecine Vétérinaire 134, 175-178.

Chapter 4

BVDV transmission by TI cattle

Virulence comparison and quantification of horizontal bovine viral diarrhoea virus transmission following experimental infection in calves

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Abstract

Bovine viral diarrhoea virus (BVDV) causes persistent infections by infecting the foetus of susceptible animals during gestation. These persistently infected (PI) animals are important sources of infection. On the contrary, transiently infected (TI) animals are believed to be less important, but transient infections with a severe BVDV-2 strain can spread explosively.

To assess the importance of TI cattle in the epidemiology of BVDV, two experimental infections were performed to determine basic reproduction ratios (R_0). In each experiment three calves were infected via intranasal inoculation and housed together with seven susceptible animals. Two strains isolated in Belgium were used, a virulent BVDV-1b and a virulent BVDV-2a field isolate, resulting in an R_0 of 0.25 (95% CI 0.01; 1.95) and 0.24 (95% CI 0.01; 2.11), respectively. A PI animal was then introduced to the remaining uninfected animals and produced an R of $+\infty$ (95% CI 1.88; $+\infty$). These results support the suggestion that TI animals, compared to PI animals, contribute only a limited amount to BVDV spread. Additionally, the severe clinical symptoms observed in the field with these isolates could not be reproduced during these experiments suggesting that other factors besides strain virulence influence the clinical manifestations evoked by BVDV.

Introduction

Bovine viral diarrhoea virus (BVDV) is characterized by a strong genetic diversity and can be divided into two genotypes, BVDV-1 and BVDV-2, each subdivided into subgenotypes (Neill, 2013). Disease severity is likely to be linked to the degree of viraemia (Walz et al., 2001).

Acute BVDV infections of BVDV seronegative cattle result in a transient viraemia of 10-14 days, starting 3 days post-infection (Lanyon et al., 2014). The duration of virus shed by these transiently infected (TI) animals varies considerably and is likely to depend on the virulence of the strain (Bolin and Ridpath, 1992). After 5-7 days of incubation about 70% to 90% of acute infections are subclinical, associated with only a mild raise in body temperature and leukopenia (Baker, 1995). By infecting cells of the immune system, BVDV evokes an immunosuppression leading to a decreased response to other infectious agents (Chase et al., 2004). BVDV also causes persistent infections by infecting the foetus in early gestation (Peterhans et al., 2010). Once colostrum-derived BVDV antibody titres have declined, these persistently infected (PI) animals continuously shed BVDV in large quantities (Lindberg and Houe, 2005).

The transmission rate of an infectious disease in a population can be expressed by its reproduction ratio, R. A special case is the basic reproduction ratio, R_0, defined as the mean number of secondary infections arising from one typical infectious case introduced in a fully susceptible population. R has a threshold value equal to 1. This means that an infection may spread when $R > 1$ and will fade out when $R < 1$ (Velthuis et al., 2007). PI animals are very successful virus transmitters (R_0 assumed > 1), whereas TI animals are believed to be less important in spreading BVDV (R_0 assumed < 1), due to the shorter duration of infection and the intermittent shedding of relatively low amounts of virus (Lindberg and Houe, 2005). However, it has been suggested that during severe BVDV-2 outbreaks transient infections significantly contribute to BVDV transmission (Ridpath et al., 2006).

Belgium is characterized by a high cattle density and a high BVDV prevalence; about one-third of the young stock is BVDV seropositive (Sarrazin et al., 2013). In addition, severe clinical symptoms linked to BVDV-1 and BVDV-2 have been described (Letellier et al., 2010; Laureyns et al., 2011a; Laureyns et al., 2011b; Laureyns et al., 2013). We hypothesized

that TI animals may contribute substantially more to BVDV spread than is generally assumed. The objective of the present study was to estimate R_0 for BVDV transmission by TI cattle following experimental infections using Belgian BVDV field isolates.

Material and methods

All experiments were approved by the Ethics Committee of the Belgian Veterinary and Agrochemical Research Centre (reference number 120102-01 BOVIDI1, 2 January 2012).

Animals

For each trial 10 Holstein-Friesian calves were selected from BVDV free herds (no BVDV history despite regular monitoring). The calves were checked twice for the absence of BVDV-RNA and antibodies using real-time RT-PCR (RT-qPCR) and virus neutralization (VN), respectively, as described below. At the time of infection, animals in trials 1 and 2 were aged between 65 and 90 days and between 100 and 128 days, respectively.

Virus isolates

For trial 1, a non-cytopathic (ncp) BVDV-1b strain isolated from a 2-day-old PI animal with spontaneous skin bleeding (Laureyns et al., 2013) was cultivated on Madin-Darby Bovine Kidney (MDBK) cells (one passage). The titre obtained was 2.6×10^6 tissue culture infective dose/mL ($TCID_{50}$/mL). For trial 2, the ncp BVDV-2a isolate 07/3913 was used (Letellier et al., 2010) after cultivation (two passages). The titre obtained was 3.7×10^5 $TCID_{50}$/mL.

Experimental design (Table 1)

During trial 1 calves were housed together in a box 3.2 ×10.0 m on a slatted floor (Fig. 1), which was cleaned daily with water. After an acclimatization period of 14 days, three randomly chosen calves were isolated from the other calves and inoculated with BVDV-1b through intranasal inoculation of 5.0×10^6 $TCID_{50}$. After 2 days of separation the inoculated animals were housed with the seven contact animals. When no infectious animals were left, i.e. all blood samples and nasal swabs were negative by RT-qPCR (69 days after study start), all BVDV seropositive animals were removed, and the trial process was repeated with three of the remaining calves being inoculated with the virus. All 10 calves were slaughtered 38 days after the start of this second phase of the trial (trial 1.2).

For trial 2, housing conditions, acclimatization period and inoculation procedures were identical to trial 1, except that the BVDV-2a strain was used. When no infectious animals were left (56 days after study start), all BVDV seropositive animals were removed, and the trial process was repeated (trial 2.2) with three of the remaining calves inoculated with the virus. Forty-four days later, a 6-month-old calf persistently infected with a BVDV-1b strain was commingled with all 10 calves from trial 2 (seven of which had seroconverted to BVDV-2a and three of which had remained susceptible) (trial 2.3). The serum viral titre of the PI calf was approximately 2.4×10^4 $TCID_{50}$/mL. All 11 animals were slaughtered 34 days after the introduction of the PI animal.

Clinical examination and sample collection

Clinical characteristics and rectal temperatures were recorded during daily examination using a scoring system adapted from Cortese et al. (1998) and Pardon (2012) (see Appendix A, Supplementary material).

Whole blood was collected in EDTA-coated tubes and stored at 4 °C. Serum was collected in tubes with a cloth activator, centrifuged for 10 min at 1800 g and stored at -20 °C. Nasal swabs were dipped in 0.5 mL Minimum Essential Medium (MEM, Invitrogen) containing penicillin, gentamicin and amphotericin B and stored at -80 °C.

Fig. 1. The calves were housed together in a box 3.2 ×10.0 m on a slatted floor which was cleaned daily with water. Freely moving and nose-to-nose contact was always possible, except during feeding and sampling (one hour daily).

Table 1. Overview and results of the experimental design. The status of a calf at the start and the end of each trial is presented. The days of sample collection are shown on the right.

Trial 1: BVDV-1b transmission through TI animals

Days after first inoculation		Calf										Sampling (days after each inoculation)
		1	2	3	4	5	6	7	8	9	10	
First inoculation												
Start	0	I	I	I	S	S	S	S	S	S	S	0; 2-10: daily; 12-24, 27- 31, 34-38: every 2 days; 45
End	69	R	R	R	S	S	S	S	S	S	S	
Second inoculation												
Start	69				I	I	I	S	S	S	S	0; 2-11: daily; 14-18, 21-25: every 2 days; 30
End (slaughter)	107				R	R	R	C	S	S	S	

Trial 2: BVDV-2a transmission through TI animals

Days after first inoculation		Calf											Sampling (days after each inoculation)
		1	2	3	4	5	6	7	8	9	10	PI	
First inoculation													
Start	0	I	I	I	S	S	S	S	S	S	S	-	0; 2-12: daily; 14-18, 21-31: every 2 days; 37; 45; 51
End	56	R	R	R	C	S	S	S	S	S	S	-	
Second inoculation													
Start	56					I	I	I	S	S	S	-	0; 2-4, 7-11, 14-18: every 2 days; 22; 25
End	100					R	R	R	S	S	S	-	
Introduction of PI animal: **BVDV-1b transmission through PI animal**													
Start	100	R	R	R	R	R	R	R	S	S	S	PI	0; 2; 5; 8; 12; 14; 19; 26; 28; 33
End (slaughter)	134	R	R	R	R	R	R	R	C	C	C	PI	

TI, transiently infected; PI, persistently infected; I, infectious; S, susceptible; C, contact infected; R, recovered.

Virus isolation and determination of viral titre

An in-house haemolysis buffer (0.31 M NH_4Cl, 0.02 M $NaHCO_3$ and 0.63 mM Na_2EDTA) was added to 1.5 mL blood. A pellet of leucocytes was obtained after centrifugation for 10 min at 200 g, suspended in 1 mL MEM and used to make 10-fold dilutions. Serial dilutions were inoculated in 96-well microtitre plates containing monolayers of MDBK-cells in MEM, 10% fetal calf serum, L-glutamine and antibiotics.

After incubation for 6 days at 37 °C in humidified air containing 5% CO_2, the supernatant was removed and the monolayers were rinsed with phosphate-buffered saline (PBS) solution, dried overnight at 37 °C and stored at -20 °C for a minimum of 12 h to lyse the cells. MDBK-cells were stained for BVDV antigen using an immunoperoxidase monolayer assay (IPMA) adapted from Jensen (1981). The monolayers were fixed with 4% formalin, rinsed, overlaid with 100 µL of BVDV polyclonal serum diluted 1/200 and incubated for 1.5 h at 37 °C. Washing and staining procedures were performed (Jensen, 1981), using a rabbit anti-bovine peroxidase conjugate (Sigma-Aldrich) diluted 1/500. Reading was done using a light microscope and viral titre was calculated (Reed and Muench, 1938).

RT-qPCR

Total RNA was extracted from haemolysed blood (obtained as described above) and nasal swabs using RNeasy Mini kit (QIAGEN) and MagMAX Pathogen RNA/DNA kit (Life Technologies), respectively. Reverse transcription, using 10 µL RNA and hexanucleotides (Roche), and qPCR assay were performed as described by Letellier et al. (1999) and Letellier and Kerkhofs (2003), respectively. RT-qPCR was used in a qualitative way to follow the infection in the inoculated animals and to verify if the susceptible calves were contact infected.

Virus neutralization

Sera from all calves were tested for virus neutralizing antibodies against the homologous strain using a VN protocol adapted from Edwards (1990). Duplicates of two-fold dilutions, starting at 1:5, were incubated with each virus strain for 2 h. MDBK cells were then added and incubated for a further 3 days. Virus-infected cells were detected by IPMA staining

as described above. Neutralizing antibody titre was defined as the inverse of the highest dilution with complete inhibition of virus growth (Walz et al., 2001).

Haematology

Packed cell volume, haemoglobin concentration and the total number of leucocytes and thrombocytes were determined in EDTA-treated blood samples using an ADVIA 2120i haematology system (Siemens).

Data analysis

R_0 was estimated by maximum likelihood (MLE) according to the final size method (Velthuis et al., 2007). Two-sided 95% confidence intervals (CI) and *p*-values for testing the null hypotheses $R_0 \geq 1$ and $R_0 \leq 1$ were obtained. The design of three infectious and seven susceptible animals at the start was based on power calculations of Kroese and de Jong (2001); these numbers were sufficient to test H_0: $R_0 \leq 1$ (5% significance level). An animal was considered as infected if positive test results were obtained for RT-qPCR and/or virus isolation (VI) and VN.

Fever was defined when the rectal temperature was > 39.0 °C, the one-sided upper limit of the 95% CI obtained for the average rectal temperature of all calves during the last 3 days before inoculation.

To evaluate whether blood composition was associated with BVDV infection, a linear mixed model approach was used in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Normal probability tests and plots of scaled residuals were examined to check whether the assumptions of normality and homoscedasticity were fulfilled. A Bonferroni correction was applied for post-hoc comparisons.

Results

Virus transmission

Following the first inoculation with BVDV-1b (trial 1) no virus transmission by the inoculated calves to the contact animals was detected (Table 1). The experimental infection was repeated by inoculating 3/7 remaining susceptible animals. One of four contact animals was infected (blood samples became RT-qPCR-positive 10 days post infection [dpi]; Fig. 2). For trial 1 the MLE of R_0 was 0.25 (95% CI 0.01; 1.95). During trial 1, the original, undiluted samples of the inoculated and contact infected animals were positive in VI (Fig. 2). However, because the dilution series were negative in VI, the viral titre could not be determined.

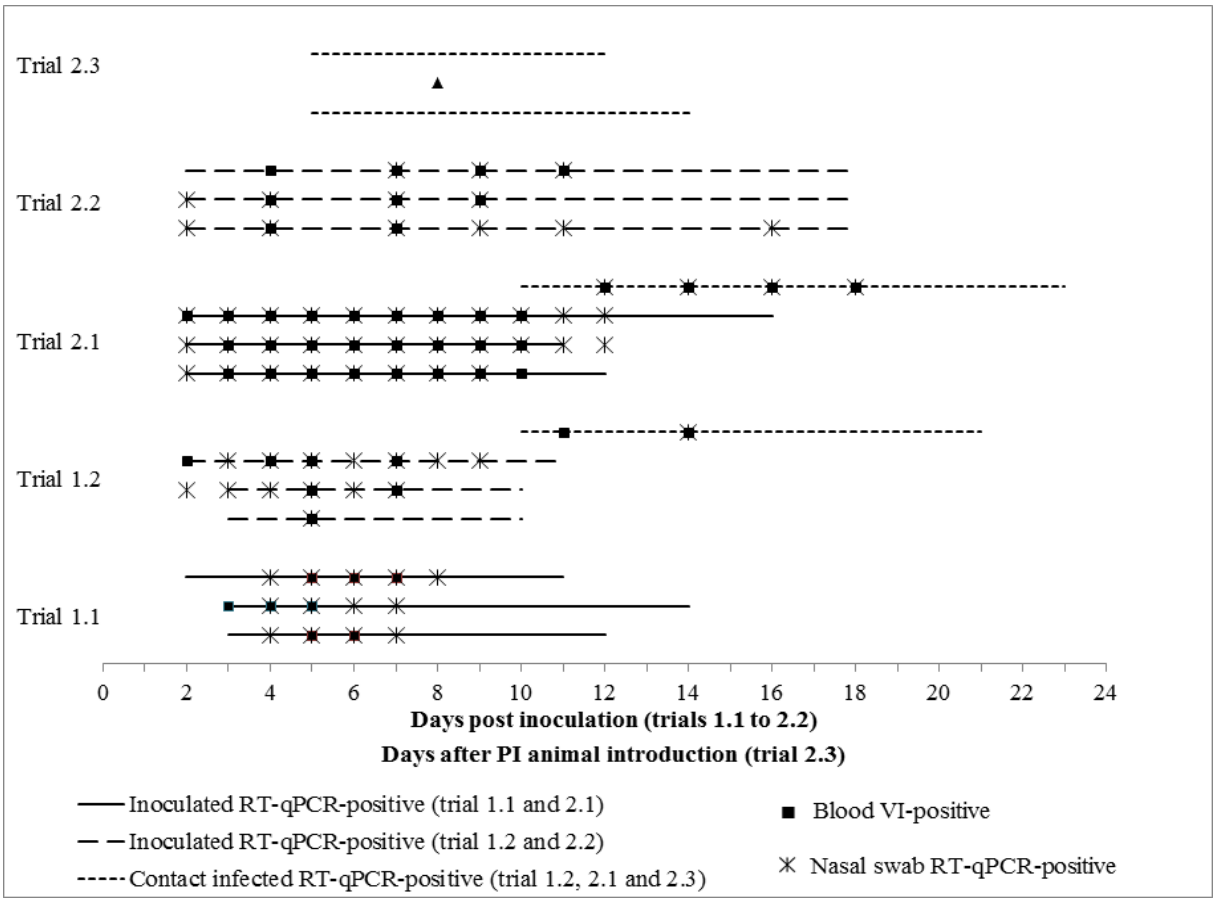

Fig. 2. The period during which inoculated animals were RT-qPCR-positive for the first phase of trials 1 and 2 are represented by a solid line and for the second phase of trials 1 and 2 by a dashed line. A dotted line is used for the animals infected by contact (after the second inoculation in trial 1, and first inoculation and introduction of persistently infected (PI) in trial 2). Days on which whole blood samples were positive for virus isolation (VI) and/or nasal swabs were RT-qPCR-positive are presented. During trial 2, after the introduction of the PI animal, one animal infected by contact was RT-qPCR-positive for one sampling day only (marked with ▲).

During the first part of trial 2, 1/7 contact animals was infected (blood samples became RT-qPCR-positive at 10 dpi; Fig. 2). For the second phase 3/6 remaining susceptible animals were inoculated, but none of the three remaining contact animals became infected. For trial 2, the MLE of R_0 was 0.24 (95% CI 0.01; 2.11). Compared to trial 1 a higher degree of viraemia was obtained and viral titre was able to be determined (Table 2).

After the introduction of the PI animal, all three remaining susceptible calves were infected, with two becoming RT-qPCR-positive 5 days after its introduction (Fig. 2). For this phase of trial 2 the MLE of R was $+\infty$ (95% CI 1.88; $+\infty$). None of the seven animals which seroconverted to BVDV-2a became RT-qPCR-positive after introduction of the PI (which was excreting BVDV-1b).

Table 2. Mean (range) of viral titres (log_{10} $TCID_{50}$/mL) of the inoculated animals (both inoculation phases) and the animal infected by contact (trial 2.1).

dpi	**Trial 2 - first inoculation**		**Trial 2 - second inoculation**
	Inoculated animals	Animal infected by contact	Inoculated animals
2	< 2.30	Not done	Negative
3	< 2.30	Not done	Not done
4	2.66 (< 2.30-2.80)	Not done	< 2.30
5	2.27 (< 2.30-2.63)	Not done	Not done
6	2.30 (< 2.30-2.53)	Not done	Not done
7	2.55 (< 2.30-2.97)	Not done	2.38 (< 2.30-2.63)
8	2.61 (< 2.30-2.97)	Not done	Not done
9	< 2.30	Not done	< 2.30
10	< 2.30	Negative	Not done
11	Not done	Not done	< 2.30
12	Negative	< 2.30	Not done
14	Not done	2.97	Negative
16	Not done	2.30	Not done
18	Not done	< 2.30	Not done
21	Not done	Negative	Not done

dpi, days post inoculation.

Clinical results

A leukopenia and thrombocytopenia was noticed in all RT-qPCR-positive animals during both trials (Table 3). Except for some occasional coughing for two inoculated animals (coughing score = 2) no clinical symptoms were noticed during the first part of trial 1. No clinical symptoms were noticed after the second inoculation. In trial 2 loss of appetite (appetite score = 2) was noticed 2-3 dpi in 2/3 calves inoculated at the beginning of the trial while the third of those calves did not eat at 9 dpi (appetite score = 4). This animal also displayed nasal discharge (score = 2; 8-10 dpi) and apathy (lying down score = 2). Except for some occasional coughing (score = 2; 17 dpi), the calf which became infected showed only subclinical infection. None of the second group of inoculated animals showed any clinical signs, nor did the calves exposed to the PI. All inoculated and contact infected animals showed fever (> 39.0 °C) during infection, with higher and longer periods of pyrexia during trial 2 (see Appendix B, Supplementary Material).

Table 3. Comparison of haematology between RT-qPCR-negative and RT-qPCR-positive animals during both inoculations of trial 1 and after first inoculation of trial 2.

	RT-qPCR-negative		**RT-qPCR-positive**		***p*-value**
	Estimate	95% CI	Estimate	95% CI	
Trial 1 - first inoculation					
Leucocytes (1000/µL)	**10.2**	8.6;11.9	**9.9**	8.1;11.7	0.46
Thrombocytes (1000/µL)	**590**	524;656	**614**	527;701	0.43
Packed cell volume (%)	**31.8**	30.0;33.6	**32.3**	30.2;34.3	0.39
Haemoglobin (g/dL)	**11.0**	10.4;11.7	**11.0**	10.3;11.8	0.95
Trial 1 - second inoculation					
Leucocytes (1000/µL)	**9.7**	8.6;10.9	**7.0**	5.8;8.3	< 0.001
Thrombocytes (1000/µL)	**449**	420;478	**401**	359;442	0.02
Packed cell volume (%)	**31.4**	29.8;32.9	**30.6**	28.8;32.3	0.23
Haemoglobin (g/dL)	**10.9**	10.5;11.4	**10.6**	10.1;11.1	0.03
Trial 2 - first inoculation					
Leucocytes (1000/µL)	**8.5**	7.8;9.2	**5.9**	5.0;6.7	< 0.001
Thrombocytes (1000/µL)	**633**	556;710	**423**	333;513	< 0.001
Packed cell volume (%)	**28.3**	26.3;30.2	**28.8**	26.7;30.9	0.28
Haemoglobin (g/dL)	**10.1**	9.4;11.0	**10.5**	9.7;11.3	0.07

Neutralizing antibody titres

The mean time between infection and rise of neutralizing antibody titres in VN for trial 1 and 2 was 13.0 days (SD 1.7 days) and 11.8 days (SD 2.0 days), respectively (Fig. 3). The contact infected animal in trial 1 seroconverted 18 dpi, while in trial 2 this was 21 dpi. Neutralizing antibody titres peaked at 3840 starting at 36 dpi (trial 1) and 9-12 weeks dpi (trial 2, data not shown). Following the introduction of the PI animal, the contact infected calves seroconverted after 17.3 days (SD 2.9 days).

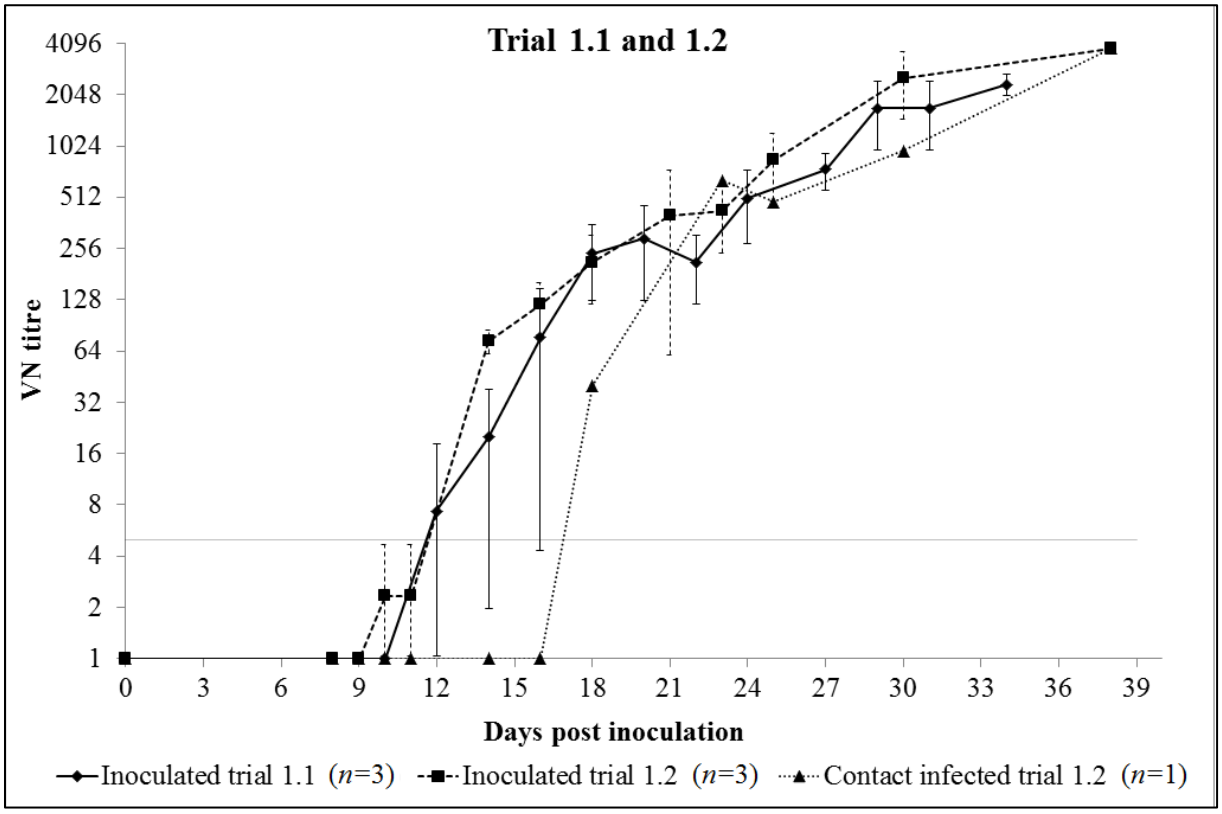

Fig. 3. Neutralizing antibody titres resulting from virus neutralization (VN) using the homologous strain are presented on a logarithmic scale per replication of each trial for the inoculated and animals infected by contact. Mean values with their standard deviation are presented for the inoculated animals and the animals infected by the persistently infected (PI) animal. Animals were considered BVDV-seropositive when the neutralizing antibody titre rose above a threshold of 5 (reference line). The remaining susceptible animals and the PI animal were BVDV seronegative, so their results are not shown.

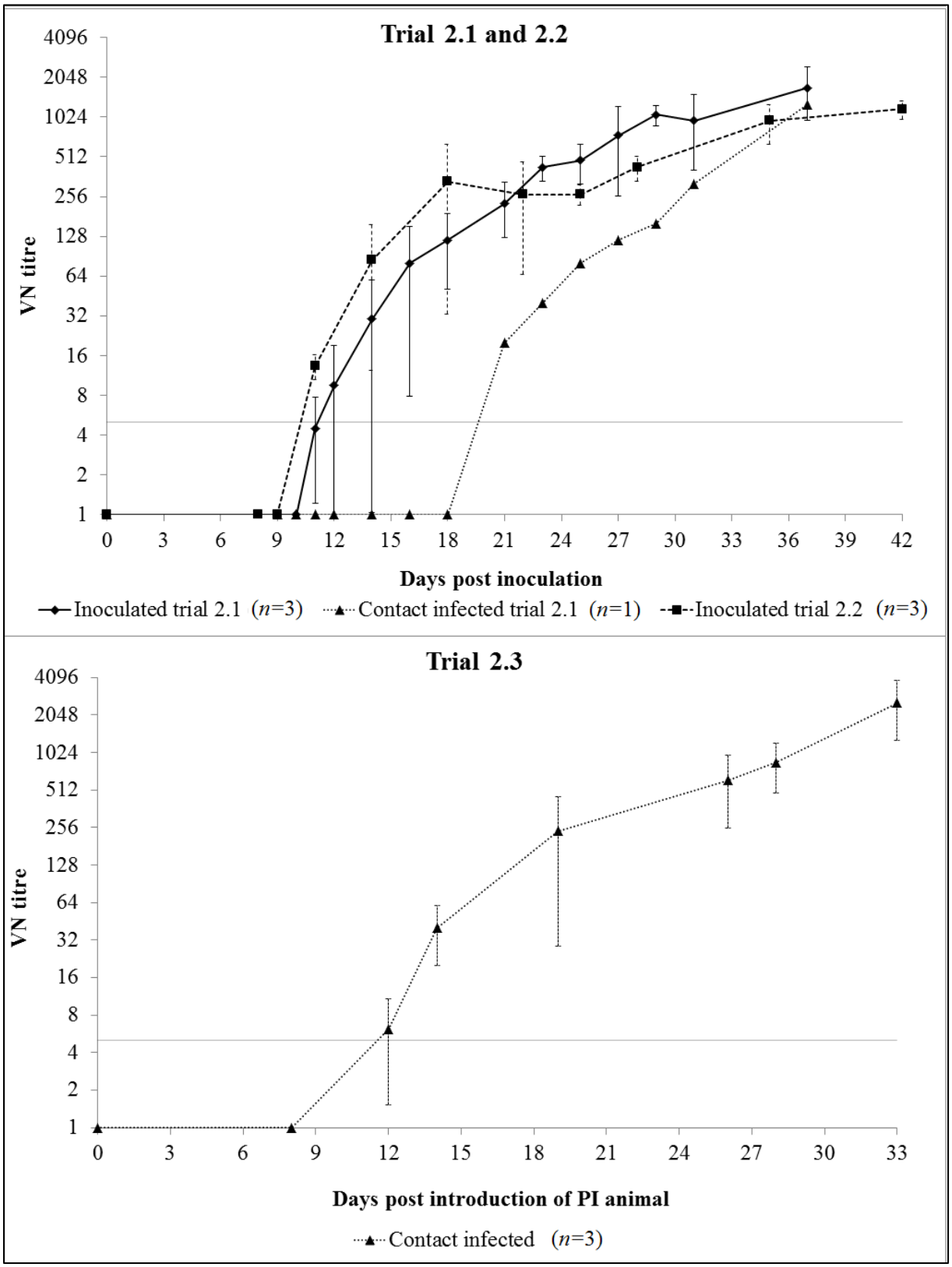

Fig. 3. Neutralizing antibody titres resulting from virus neutralization (VN) using the homologous strain. (*continued*)

Discussion

During this study, limited BVDV transmission by TI animals was observed, in contrast to very rapid transmission after the introduction of a PI animal. These results are consistent with the assumption that TI cattle do not substantially contribute to BVDV spread, but nevertheless the estimation of R_0 provides additional information on BVDV transmission by TI animals.

Moerman et al. (1993) estimated that horizontal BVDV transmission rate by TI cattle had an R_0 of 3.3 (95% CI 2.6; 4.1). However, although separated from the study group, PI animals were present in the herd during this study. As these animals shed massive doses of virus, they may have attributed to virus spread through indirect transmission (Niskanen and Lindberg, 2003; Lindberg et al., 2004), resulting in an overestimation of R_0.

Although the experimental setup in our study was designed to approach field conditions, there were limitations that have to be taken into account. First, under experimental conditions, animals are likely to be less challenged by other pathogens and environmental conditions that may contribute to a depressed immune response, disease development and pathogen spread. Calves were housed on a slatted floor which was cleaned daily with water to remove all faeces. Although direct nose-to-nose contact is considered to be the most effective route for transmission, these housing conditions may have hampered viral spread as contaminated pens may be a source of infection (Niskanen and Lindberg, 2003). Nonetheless, virus transmission was observed during both trials 1 and 2.

Secondly, during both trials non-infected in-contact animals were re-used to increase the power of the experimental design. These non-infected animals had already had been exposed to BVDV, making them possibly more resistant to infection. However, inoculation resulted in active infection in all three calves in both trials and susceptible animals were infected during the second phase of trial 1 and after introduction of the PI animal in trail 2. This suggests that the remaining susceptible calves were not refractory to infection. To our knowledge, natural resistance to BVDV infection in apparently susceptible cattle has not been described.

The dose of virus used for intranasal inoculation dose was similar to those used in other studies to ‘create’ TI animals (Hamers et al., 2000; Walz et al., 2001; Kelling et al.,

2002). In our opinion, it is unlikely that the inoculation dose influenced the transmission rate as the amount and duration of virus shed is believed to depend on the intensity of replication within the host (Bolin and Ridpath, 1992).

During this study a PI animal rapidly infected three susceptible animals in a group with seven BVDV seropositive calves. This resulted in R significantly > 1, which confirms that PI animals can maintain BVDV infection, as already described (Lindberg and Houe, 2005; Fulton et al., 2006; Nickell et al., 2011). The use of R_0 is restricted to fully susceptible populations (Velthuis et al., 2007) and therefore we reported R for trial 2.3, as the BVDV seropositive animals from trial 2.1 and 2.2 were not removed at the start of trial 2.3. Nonetheless, it is unlikely that these animals contributed to the infection of the remaining susceptible animals. This would have been detected by RT-qPCR as it distinguishes between BVDV genotypes: the PI animal transmitted BVDV-1b while BVDV-2a was used in trial 2. None of the contact infected animals during trial 2.3 tested positive for BVDV-2.

As described by Ridpath et al. (2006), acute BVDV infections can spread explosively. By using virulent strains we had expected to observe sufficient virus transmission to reject H_0: $R_0 \leq 1$. However, very limited transmission was observed; each trial was therefore repeated with the remaining susceptible animals in an attempt to reject H_0: $R_0 \geq 1$. However, there was insufficient power to reject H_0. Small point estimates ($R_0 < 1$) were obtained, which means that the infection would theoretically fade out. Although statistically not significant, these results support the assumption that TI cattle, unless in early gestation, are of little importance for BVDV spread (Lindberg and Houe, 2005), as already demonstrated (Niskanen et al., 2000; Niskanen et al, 2002).

BVDV infection was associated with leukopenia (trials 1 and 2) and thrombocytopenia (trial 2 only), but, except for fever, few additional clinical symptoms were noticed, even though the strains were isolated from animals suffering from clinical BVDV infections (Letellier et al., 2010; Laureyns et al., 2013). This is probably not surprising as most BVDV infections are subclinical (Baker, 1995), and the experimental conditions meant that the decreased immunity resulting from BVDV infection was not challenged by concurrent pathogens. In addition, other studies using virulent BVDV strains have also failed to reproduce the severe symptoms observed under field conditions (Hamers et al., 1999, Hamers et al., 2000 and Kelling et al., 2002). Age appears to be a key influence on the clinical manifestations associated with BVDV infection (Hamers et al., 2003), as studies producing

severe clinical symptoms under experimental conditions have used calves that were at most 2 months old (Corapi et al., 1989; Bolin and Ridpath, 1992; Bezek et al., 1994; Cortese et al., 1998; Ellis et al., 1998; Walz et al., 2001).

BVDV is genetically highly variable (Vilcek et al., 2001) and changes in the genome with shifts in strain virulence following virus cultivation may also explain the mild clinical manifestations. To avoid genetic change as much as possible, the first and second passage of the cultivated virus were used for trial 1 and 2, respectively. Changes in the viral genome could be verified by sequence analysis after each passage, but virulence markers on a genetic level have not, so far, been described (Bolin and Grooms, 2004).

Conclusions

The limited viral spread from TI animals in this study indicates that they only make a limited contribution to BVDV transmission, whereas, under the same conditions, a PI animal was demonstrated to be a successful virus transmitter. This supports the conclusion that the removal of PI animals is essential for BVDV control, whereas a focus on TI animals should not be necessary. The severe clinical symptoms observed in the field could not be reproduced, which suggests that other factors besides strain virulence influence the clinical manifestations of BVDV infection.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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References

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Veterinary Clinics of North America-Food Animal Practice 11, 425-445.

Bezek, D.M., Grohn, Y.T., Dubovi, E.J., 1994. Effect of acute infection with noncytopathic or cytopathic bovine viral diarrhea virus isolates on bovine platelets. American Journal of Veterinary Research 55, 1115-1119.

Bolin, S.R., Grooms D.L., 2004. Origination and consequences of bovine viral diarrhea virus diversity. Veterinary Clinics of North America-Food Animal Practice 20, 51-68.

Bolin, S.R., Ridpath, J.F., 1992. Differences in virulence between 2 noncytopathic bovine viral diarrhea viruses in calves. American Journal of Veterinary Research 53, 2157-2163.

Chase, C.C.L., Elmowalid, G., Yousif, A.A.A., 2004. The immune response to bovine viral diarrhea virus: a constantly changing picture. Veterinary Clinics of North America-Food Animal Practice 20, 95-114.

Corapi, W.V., French, T.W., Dubovi, E.J., 1989. Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. Journal of Virology 63, 3934-3943.

Cortese, V.S., West, K.H., Hassard, L.E., Carman, S., Ellis, J.A., 1998. Clinical and immunologic responses of vaccinated and unvaccinated calves to infection with a virulent type-II isolate of bovine viral diarrhea virus. Journal of the American Veterinary Medical Association 213, 1312-1319.

Edwards, S., 1990. The diagnosis of bovine virus diarrhoea-mucosal disease in cattle. Revue Scientifique et Technique de l`Office International des Epizooties 9, 115-130.

Ellis, J.A., West, K.H., Cortese, V.S., Myers, S.L., Carman, S., Martin, K.M., Haines, D.M., 1998. Lesions and distribution of viral antigen following an experimental infection of young seronegative calves with virulent bovine virus diarrhea virus-type II. Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire 62, 161-169.

Fulton, R.W., Johnson, B.J., Briggs, R.E., Ridpath, J.F., Saliki, J.T., Confer, A.W., Burge, L.J., Step, D.L., Walker, D.A., Payton, M.E., 2006. Challenge with bovine viral diarrhea virus by exposure to persistently infected calves: protection by vaccination and negative results of antigen testing in nonvaccinated acutely infected calves.

Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire 70, 121-127.

Hamers, C., Couvreur, B., Dehan, P., Letellier, C., Fischer, L., Brun, A.J., Lewalle, P., Michaux, C., Pastoret, P.P., Kerkhofs, P., 2003. Assessment of the clinical and virological protection provided by a commercial inactivated bovine viral diarrhoea virus genotype 1 vaccine against a BVDV genotype 2 challenge. Veterinary Record 153, 236-240.

Hamers, C., Couvreur, B., Dehan, P., Letellier, C., Lewalle, P., Kerkhofs, P., Pastoret, P.P., 1999. Experimental infection of calves with bovine viral diarrhea virus strains isolated from haemorragic syndromes. Annales de Médecine Vétérinaire 143, 197-200.

Hamers, C., Couvreur, B., Dehan, P., Letellier, C., Lewalle, P., Pastoret, P.P., Kerkhofs, P., 2000. Differences in experimental virulence of bovine viral diarrhoea viral strains isolated from haemorrhagic syndromes. The Veterinary Journal 160, 250-258.

Jensen, M.H., 1981. Detection of antibodies against hog-cholera virus and bovine viral diarrhea virus in porcine serum - a comparative-examination using Cf, Pla and Npla assays. Acta Veterinaria Scandinavica 22, 85-98.

Kelling, C.L., Steffen, D.J., Topliff, C.L., Eskridge, K.M., Donis, R.O., Higuchi, D.S., 2002. Comparative virulence of isolates of bovine viral diarrhea virus type II in experimentally inoculated six- to nine-month-old calves. American Journal of Veterinary Research 63, 1379-1384.

Kroese, A.H., de Jong, M.C.M., 2001. Design and analysis of transmission experiments. Society for Veterinary Epidemiology and Preventive Medicine, Proceedings, xxi-xxxvii.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. The Veterinary Journal 199, 201-209.

Laureyns, J., Letellier, C., Meganck, V., Pardon, B., Deprez, P., de Kruif, A., 2011a. Severe disease in neonatal calves with detection of cytopathic BVDV. Veterinary Record 169, 101-U49.

Laureyns, J., Pardon, B., Cay, A.B., Sarrazin, S., Deprez, P., 2013. Spontaneous bleeding in a neonatal calf persistently infected with BVDV1b. Vlaams Diergeneeskundig Tijdschrift 82, 87-89.

Laureyns, J., Pardon, B., Letellier, C., Deprez, P., 2011b. Periparturient infection with bovine viral diarrhea virus type 1 causes hemorrhagic proctocolitis in a cow. Canadian Veterinary Journal-Revue Veterinaire Canadienne 52, 1135-1139.

Letellier, C., Kerkhofs, P., 2003. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. Journal of Virological Methods 114, 21-27.

Letellier, C., Kerkhofs, P., Wellemans, G., Vanopdenbosch, E., 1999. Detection and genotyping of bovine diarrhea virus by reverse transcription-polymerase chain amplification of the 5 ' untranslated region. Veterinary Microbiology 64, 155-167.

Letellier, C., Pardon, B., Van der Heyden, S., Deprez, P., 2010. Circulation in Belgium of a bovine viral diarrhoea virus type 2 closely related to North American hypervirulent viruses. Veterinary Record 166, 625-627.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Veterinary Record 154, 463-467.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long-term epidemiologic-study of bovine viral diarrhea infections in a large herd of dairy cattle. Veterinary Record 132, 622-626.

Neill, J.D., 2013. Molecular biology of bovine viral diarrhea virus. Biologicals 41, 2-7.

Nickell, J.S., White, B.J., Larson, R.L., Renter, D.G., Roque, J., Hesse, R., Oberst, R., Peddireddi, L., Anderson, G., 2011. Onset and duration of transient infections among antibody- diverse beef calves exposed to a bovine viral diarrhea virus persistently infected calf. International Journal of Applied Research in Veterinary Medicine 9, 29-39.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. The Veterinary Journal 165, 125-130.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Veterinaria Scandinavica 41, 93-99.

Niskanen, R., Lindberg, A., Traven, M., 2002. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. The Veterinary Journal 163, 251-259.

Pardon, B., 2012. Morbidity, mortality and drug use in white veal calves with emphasis on respiratory disease. Thesis, Master of Veterinary Science, Ghent University,

Merelbeke, Belgium, p. 243. http://hdl.handle.net/1854/LU-3007344 (accessed 21 May 2014).

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterinary Research 41, 44.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. American Journal of Epidemiology 27, 493-497.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Veterinary Microbiology 114, 196-204.

Sarrazin, S., Veldhuis, A., Meroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Cay, A.B., Piepers, S., Hooyberghs, J., Ribbens, S., Van Der Stede, Y., 2013. Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Preventive Veterinary Medicine 108, 28-37.

Velthuis, A.G.J., Bouma, A., Katsma, W.E.A., Nodelijk, G., de Jong, M.C.M., 2007. Design and analysis of small-scale transmission experiments with animals. Epidemiology and Infection 135, 202-217.

Vilcek, S., Paton, D.J., Durkovic, B., Strojny, L., Ibata, G., Moussa, A., Loitsch, A., Rossmanith, W., Vega, S., Scicluna, M.T., Palfi, V., 2001. Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Archives of Virology 146, 99-115.

Walz, P.W., Bell, T.G., Wells, J.L., Grooms, D.L., Kaiser, L., Maes, R.K., Baker, J.C., 2001. Relationship between degree of viremia and disease manifestations in calves with experimentally induced bovine viral diarrhea virus infection. American Journal of Veterinary Research 62, 1095-1103.

Supplementary Material

Appendix A: Clinical scoring system

Adapted from Cortese et al. (1998) and Pardon (2012).

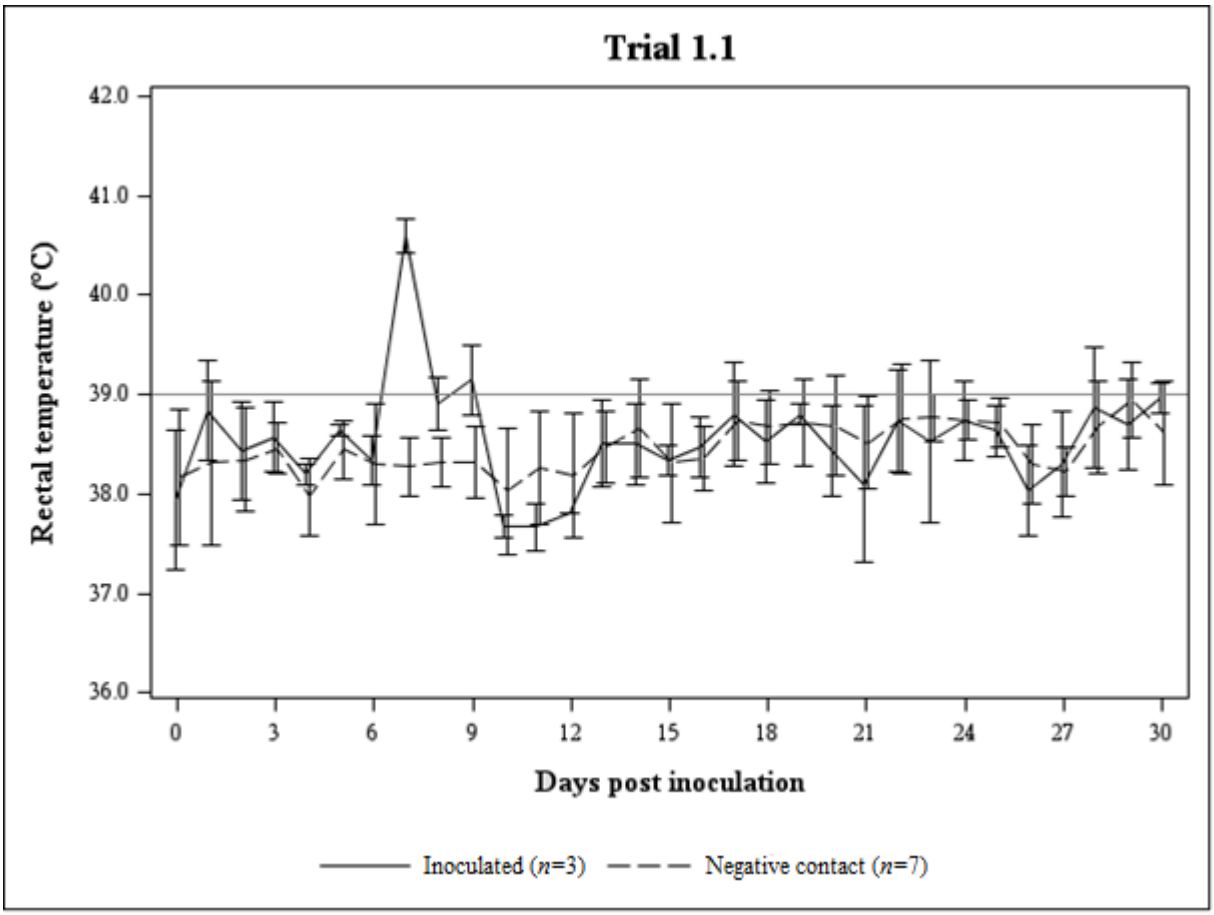
Characteristic	Score	
Posture (apathy)	0 =	The calf is standing or lying down with the head held high.
	1 =	The calf is standing with the head kept down.
	2 =	The calf is lying down with the head on the ground.
	3 =	The calf is in lateral decubitus.
	When lying down,	
	0 =	the calf stands up itself.
	1 =	the calf stands up when approaching.
	2 =	the calf stands up when touching.
	3 =	the calf needs a clear stimulus to stand up itself.
	4 =	the calf does not stand up itself, but does when pulled up.
	5 =	the calf does not stand up itself and does not keep standing when pulled up.
Nasal discharge	0 =	No nasal discharge
	1 =	Clear nasal discharge
	2 =	Mucopurulent nasal discharge
	3 =	Blood
Coughing	0 =	No coughing
	1 =	The calf coughs once
	2 =	The calf coughs occasionally (< 10 times during stable visit)
	3 =	The calf coughs regularly (> 10 times during stable visit)
Respiration rate	0 =	20-24 per minute
	1 =	> 40 per minute
	2 =	> 80 per minute
	3 =	> 80 per minute + difficulty to breath

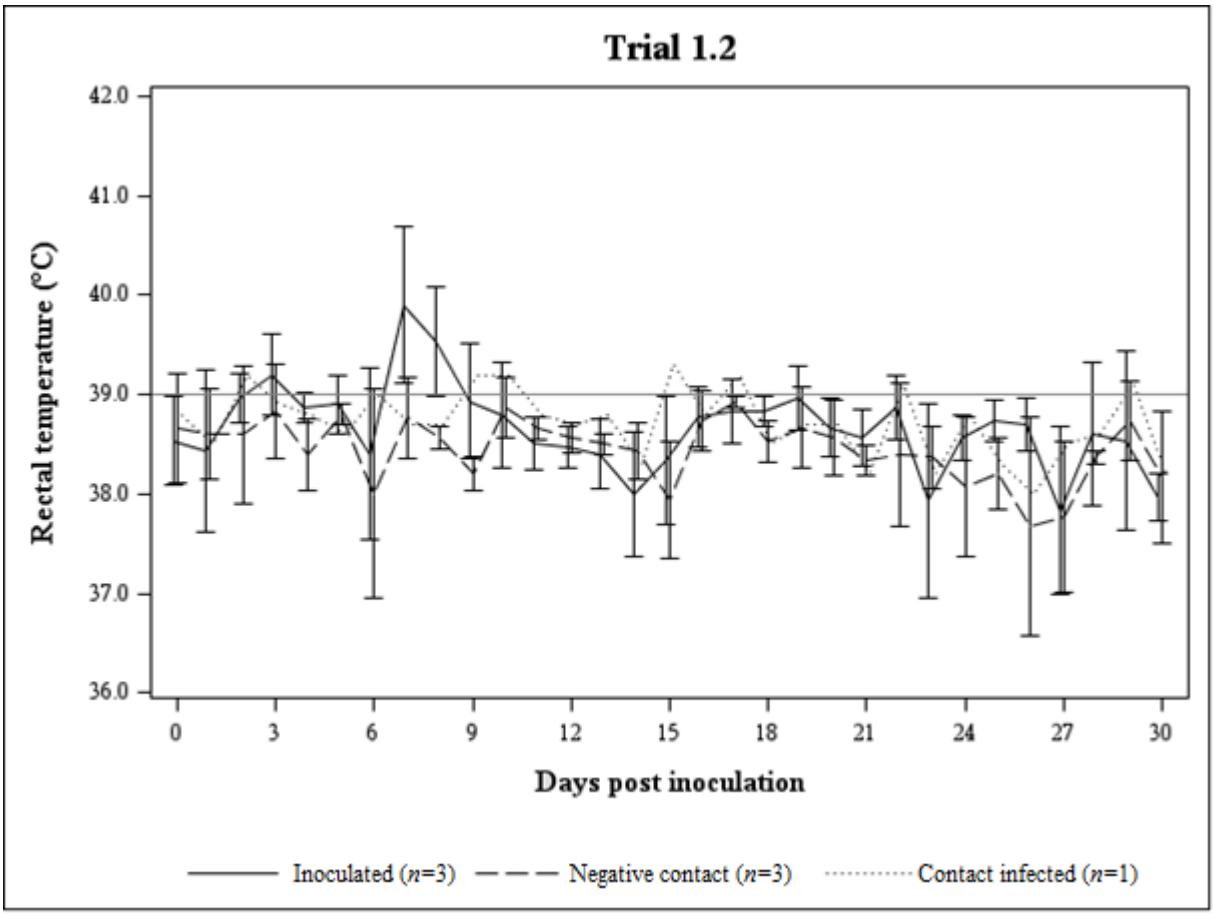
Clinical scoring system (continued)

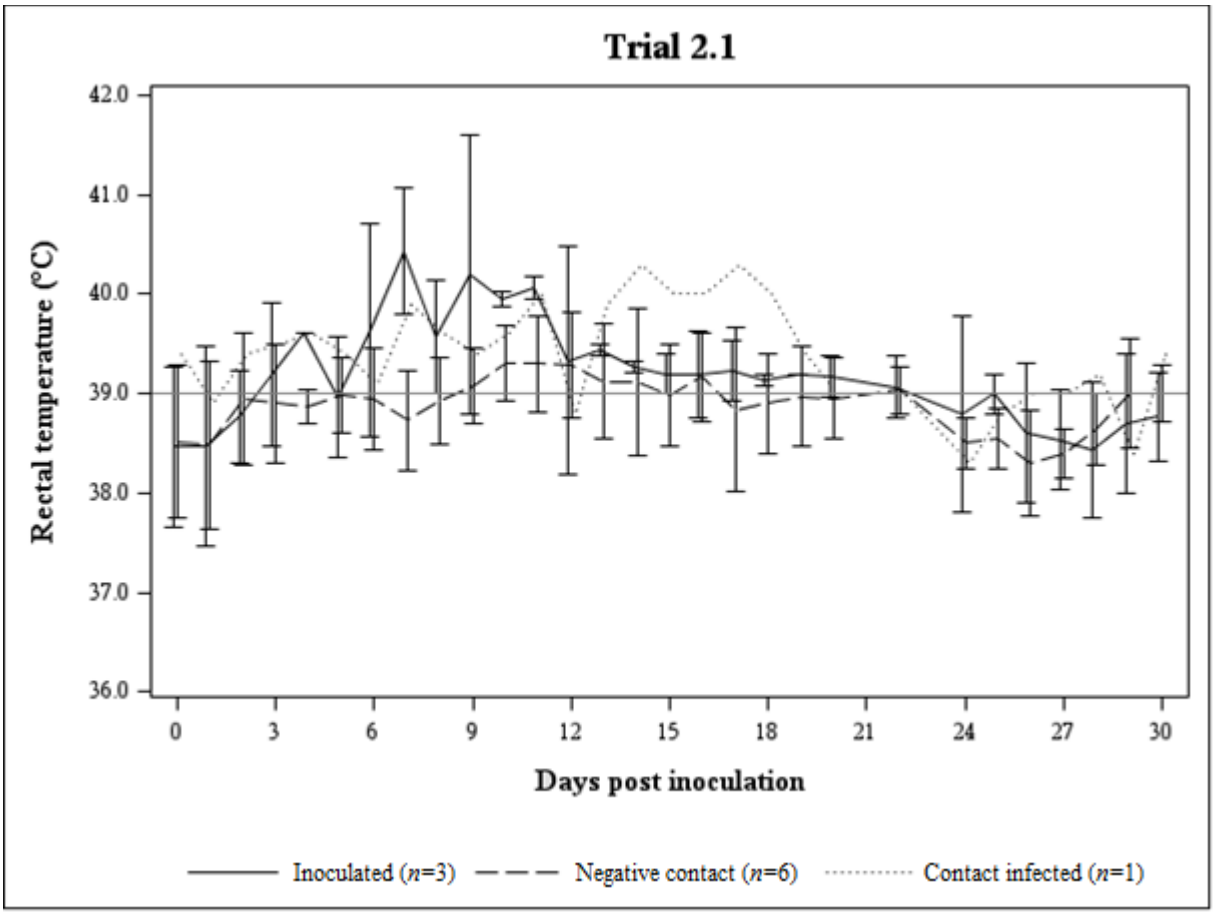
Characteristic	Score	
Behaviour	0 =	The calf comes towards the fence or looking at the person performing the clinical examination.
	1 =	The calf is standing with other calves.
	2 =	The calf is walking alone in the stable.
	3 =	The calf is standing alone and is not interested in the environment.
Appetite	0 =	The calf starts eating immediately.
	1 =	The calf hesitates to eat, but finally eats everything.
	2 =	The calf hesitates to eat and does not eat much.
	3 =	The calf hesitates to come to the fence and does not eat much.
	4 =	The calf hesitates to come to the fence and does not eat.
	5 =	The calf does not come to the fence.
Faeces consistency	0 =	Normal faeces consistency
	1 =	Fluid faeces consistency
	2 =	Watery faeces
	3 =	Blood in the faeces
Rectal temperature = … °C		

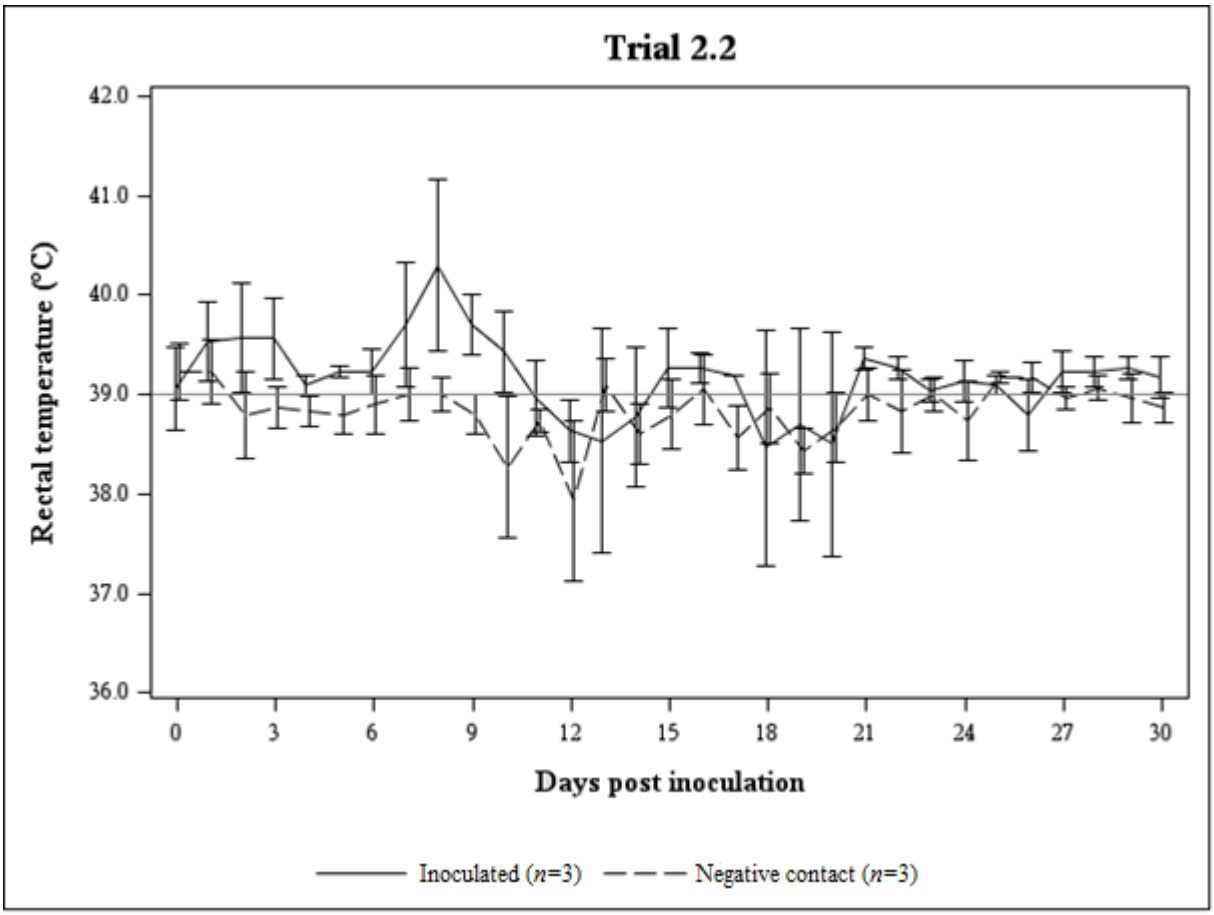
Appendix B: Evolution of rectal temperature per trial

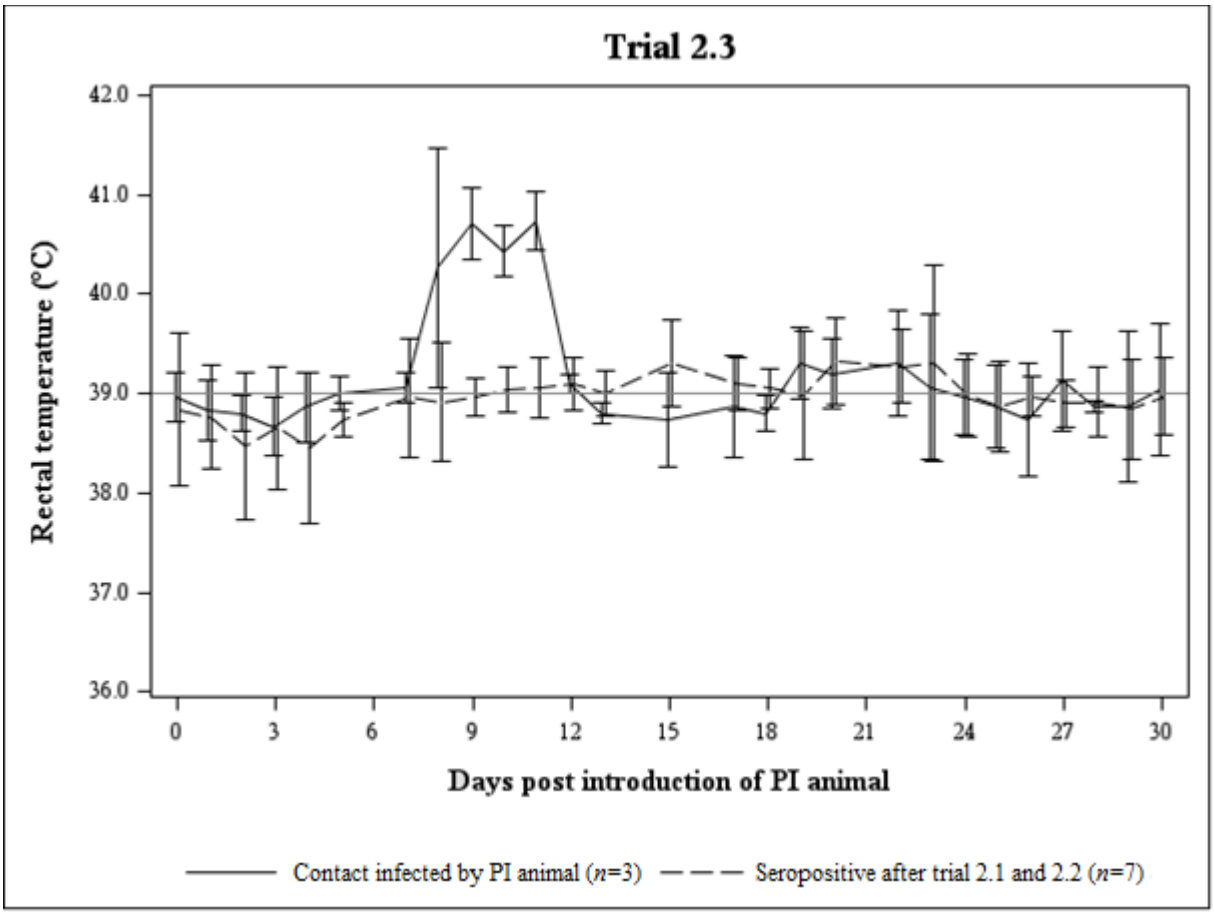
Mean values are associated with their standard deviation. A reference line at 39.0 °C is shown. Fever was defined as a rectal temperature > 39.0 °C, the one-sided upper limit of the 95% CI obtained on the average rectal temperature of all calves during the last 3 days before inoculation.




Trial 1.2
Rectal temperature (°C)
42.0
41.0
40.0
39.0
38.0
37.0
36.0
0
3
6
9
12
15
18
21
24
27
30
Days post inoculation
Inoculated (n=3)
Negative contact (n=3)
Contact infected (n=1)


Trial 2.1
Rectal temperature (°C)
42.0
41.0
40.0
39.0
38.0
37.0
36.0
0
3
6
9
12
15
18
21
24
27
30
Days post inoculation
Inoculated (n=3)
Negative contact (n=6)
Contact infected (n=1)


Trial 2.2
Rectal temperature (°C)
42.0
41.0
40.0
39.0
38.0
37.0
36.0
0
3
6
9
12
15
18
21
24
27
30
Days post inoculation
Inoculated (n=3)
Negative contact (n=3)


Trial 2.3
Rectal temperature (°C)
42.0
41.0
40.0
39.0
38.0
37.0
36.0
0
3
6
9
12
15
18
21
24
27
30
Days post introduction of PI animal
Contact infected by PI animal (n=3)
Seropositive after trial 2.1 and 2.2 (n=7)

A transmission experiment in calves infected with a recently discovered hypervirulent BVDV-2c strain

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Summary

To assess the importance of transiently infected (TI) cattle in the epidemiology of BVDV-2 infections and to describe the clinical signs caused by such an infection, a transmission experiment was performed. Three calves were intranasally infected with a hypervirulent BVDV-2c field strain isolated during a severe BVDV outbreak in Germany and housed together with seven susceptible animals. The clinical signs of the BVDV infected animals varied from very mild disease (fever, loss of appetite) to severe watery and haemorrhagic diarrhoea and death. The clinical signs and the level of BVDV excretion depended on the degree of viraemia. The basic reproduction ratio (R_0) was estimated to be 0.49 (95% CI 0.06; 2.99), suggesting a limited viral spread using the BVDV-2c strain. This suggests that this BVDV-2 infection in TI animals resulted in limited transmission towards other animals.

Introduction

Cattle get infected with bovine viral diarrhoea virus (BVDV) either through a congenital infection (vertical transmission) or through a postnatal, acute infection (horizontal transmission). Vertical BVDV transmission from the second up to the fourth month of gestation can result in the birth of persistently infected (PI) calves (McClurkin et al., 1984; Peterhans et al., 2010). Acute BVDV infections of BVDV seronegative cattle result in transiently infected (TI) animals, which are viraemic for 10-14 days, starting 3 days post-infection (Lanyon et al., 2014).

The transmission rate of an infectious disease can be expressed by its reproduction ratio, R. A special case is the basic reproduction ratio, R_0, defined as the mean number of secondary infections arising from one typical infectious case introduced in a fully susceptible population (Kroese and de Jong, 2001; Lindberg and Houe, 2005; Velthuis et al., 2007). R_0 is determined by the following parameters (Lindberg and Houe, 2005): the probability of transmission during a contact between an infectious and susceptible animal (β), the number of contacts per time period (k) and the duration of the infectious period (d).

Once colostrum-derived BVDV antibody titres have declined, PI animals continuously shed massive amounts of virus, resulting in high values for the parameters β en d (Lindberg and Houe, 2005). The amount of virus spread by TI animals is much lower because of a much shorter duration of the infectious period and the relatively low amounts of virus shed, resulting in low values for the parameters β en d. Therefore, PI animals are considered the main source of infection and can maintain the presence of BVDV in a susceptible population, while TI animals are generally considered to be of minor importance in spreading BVDV (Lindberg and Houe, 2005; Fulton et al., 2006; Nickell et al., 2011; Sarrazin et al., 2014).

However, the virulence of BVDV strains is suggested to influence the amount and duration of virus shed by TI animals (Bolin and Ridpath, 1992). Whereas the spread of low virulent strains is most likely to originate only from PI animals, it is suggested that highly virulent strains can be maintained within a susceptible population with only TI animals as source of infection (Ridpath et al., 2006). Therefore transmission experiments with TI animals were conducted to test this hypothesis, hereby using BVDV strains isolated from individual cattle suffering from severe clinical BVDV-associated signs (Sarrazin et al., 2014). The

results of this study demonstrated a very limited viral spread from TI animals and confirmed the previous believe that TI animals only make a limited contribution to BVDV transmission.

In autumn 2012 and spring 2013 an outbreak of BVDV-2c in Germany and the Netherlands resulted in a high mortality rate, up to 80% in some veal calf herds (Doll and Holsteg, 2013; Moen, 2013). Since no PI animals were detected during this outbreak, it is suggested that BVDV has spread solely through TI animals (Doll, 2013), which could indicate that TI animals may contribute substantially more to BVDV-2c spread than is generally assumed.

The objective of the present study was to perform a transmission experiment according to the experiment performed by Sarrazin et al. (2014) with this BVDV-2c field strain isolated during the severe outbreak in Germany to quantify BVDV transmission by TI cattle.

Material and methods

The experimental design was identical to the protocol of the previously described transmission experiments (Sarrazin et al., 2014). Ten calves were checked for the absence of BVDV-RNA and antibodies using real-time RT-PCR (RT-qPCR) and virus neutralization (VN), respectively. At the time of infection, the animals were aged between 109 and 141 days, respectively. A BVDV-2c field strain (NRW 14-13) isolated during the BVDV outbreak in Germany (Jenckel et al., 2014) was cultivated on Madin-Darby Bovine Kidney cells (two passages) and a titre of 1.3×10^6 tissue culture infective dose/mL ($TCID_{50}$/mL) was obtained. After an acclimatization period of 14 days, three randomly chosen calves were isolated from the other calves and inoculated with BVDV-2c through intranasal inoculation of 5.0×10^6 $TCID_{50}$. After 2 days of separation the inoculated animals were housed together in one box with the seven contact animals and BVDV spread and clinical signs were daily recorded. When no infectious animals were left (i.e. all blood samples and nasal swabs were negative by RT-qPCR), all BVDV seropositive animals were removed and the non-infected contact animals were used in a second infection experiment that started 63 days after the first inoculation. All calves were slaughtered 50 days after the start of this second experiment.

The clinical examination (see Appendix A of Chapter 4.1), sample collection, virus isolation (VI), RT-qPCR, virus neutralization (VN), haematology and estimation of R_0 were performed as previously described (Sarrazin et al., 2014). An animal was considered infected if positive test results were obtained for RT-qPCR, VI and VN. R_0 was estimated by maximum likelihood (MLE) according to the final size method (Velthuis et al., 2007). Fever was defined when the rectal temperature was > 39.0 °C, the one-sided upper limit of the 95% CI obtained for the average rectal temperature of all calves during the last 3 days before inoculation.

Results

Following the first inoculation, blood samples of all three inoculated animals were positive in RT-qPCR (from 2 up to 17 days post inoculation [dpi]) and VI (from 3 up to 11 dpi), but differences in the degree of viraemia were noticed (Fig. 1a-b). One inoculated animal ("Inoculated 1", Fig. 1) died 17 dpi and was still RT-qPCR-positive at that moment. After the first inoculation 2 out of 7 contact animals were infected.. The first contact infected animal became positive in RT-qPCR and VI at 11 dpi and 16 dpi, respectively (Fig. 1a-b), while the second contact infected animal was RT-qPCR-positive between 24 and 38 dpi (Fig. 1b).

The experimental infection was repeated by inoculating 2 out of 5 remaining BVDV negative susceptible animals, but none of the three remaining contact animals became infected. Compared to the first inoculation a lower degree of viraemia was obtained and the viral titre could not be determined. Nevertheless, both inoculated calves were positive in VI (Fig. 2a) and the nasal swabs were RT-qPCR-positive between 2 and 10 dpi (Fig. 2c).

BVDV-infection was confirmed by the development of neutralizing antibodies in all five inoculated animals (from 9 dpi on) and both contact infected animals (from 18 dpi and 30 dpi on). The remaining susceptible animals were BVDV seronegative until the end of the experiment.

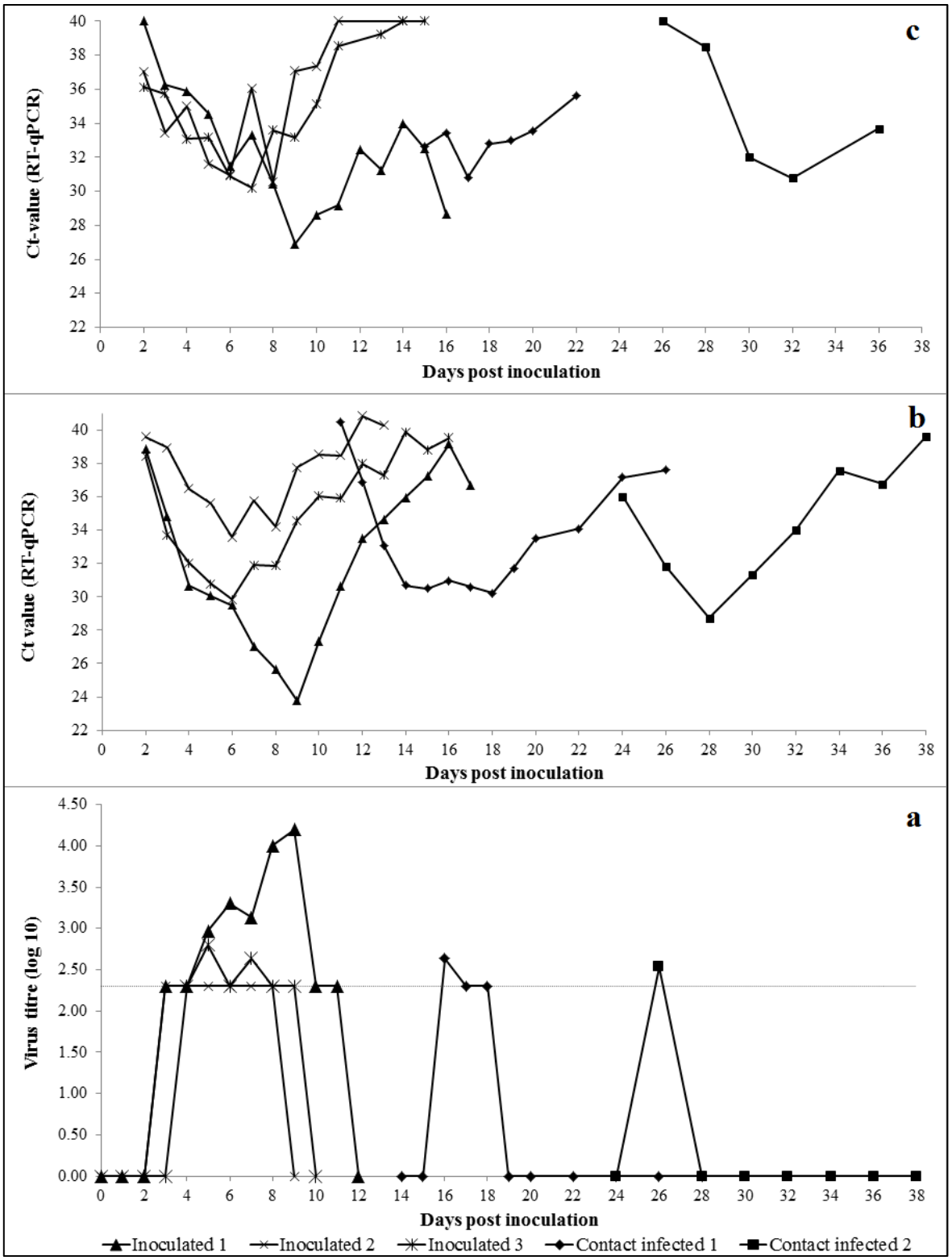

Fig. 1. Virological results from the first inoculation (three inoculated animals and seven susceptible animals). a) Viral titres (log10 $TCID_{50}$/mL) of positive blood samples of the inoculated animals and both animals infected by contact. The dotted line represents the detection limit (2.30). Samples represented on the detection limit are positive in virus isolation, but the viral titre was insufficiently high to determine a viral titre. b) Ct-values of RT-qPCR-positive blood samples. A lower Ct-value indicates a higher amount of viral RNA present in the sample. c) Ct-values of RT-qPCR-positive nasal swabs.

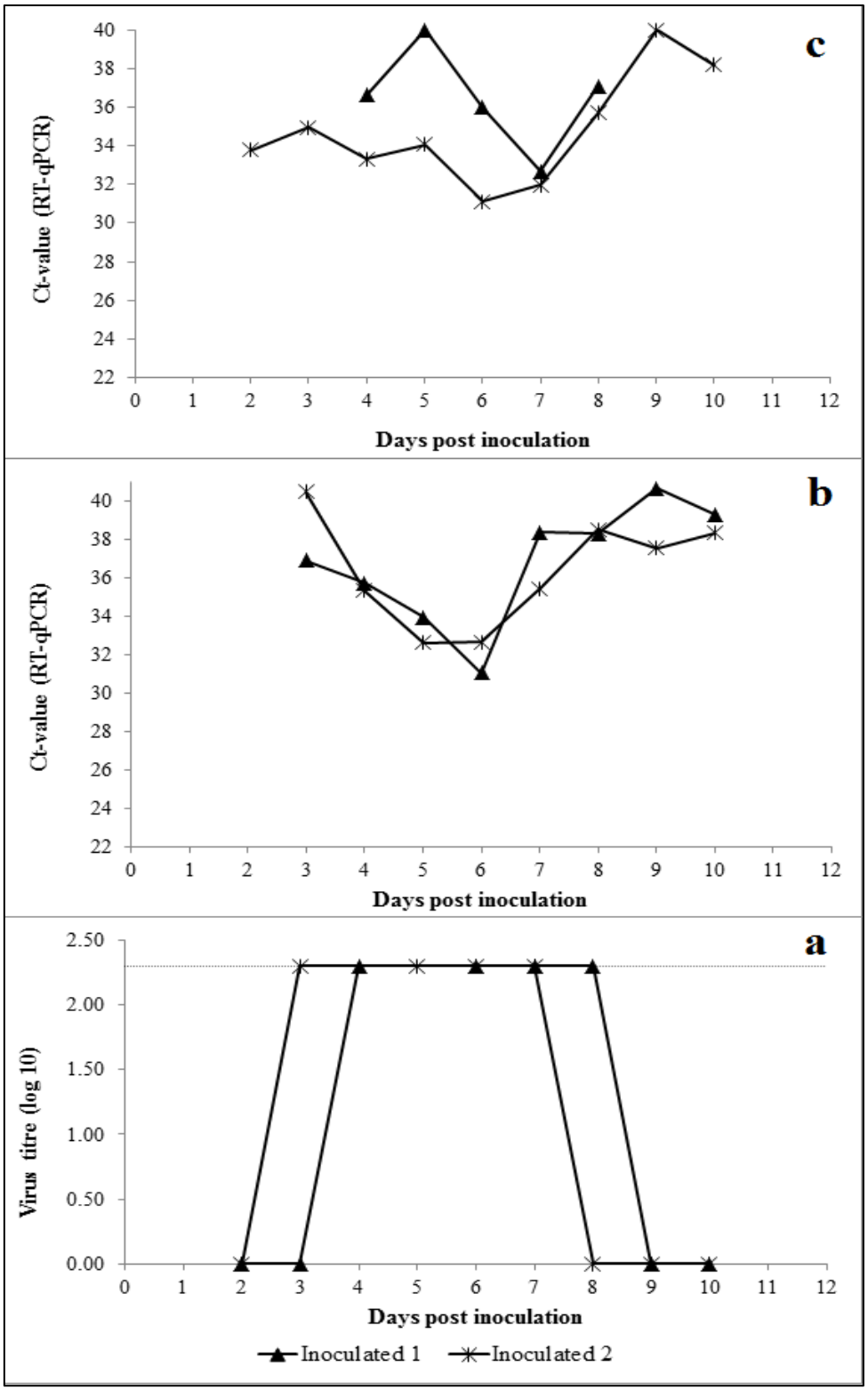

Fig. 2. Virological results from the second inoculation (two inoculated animals and three susceptible animals. a) Viral titres (log10 $TCID_{50}$/mL) of positive blood samples of the inoculated animals. The dotted line represents the detection limit (2.30). Samples represented on the detection limit are positive in virus isolation, but the viral titre was insufficiently high to determine a viral titre. b) Ct-values of RT-qPCR-positive blood samples. A lower Ct-value indicates a higher amount of viral RNA present in the sample. c) Ct-values of RT-qPCR-positive nasal swabs.

The combined MLE of R_0 for the first and second inoculation was 0.49 (95% CI 0.06; 2.99). Although statistically not significantly different from 1, this point estimate indicated that one typical infectious case introduced in a fully susceptible population would cause on average only 0.49 secondary infections and suggests that infection with TI animals infected with this BVDV-2c strain would theoretically fade out.

Following the first inoculation, the inoculated calves had a loss of appetite for 6 days starting 7 dpi (appetite score = 3). Together with fever between 4 and 11 dpi, loss of appetite was the only clinical sign that was noticed for one inoculated animal ("Inoculated 2", Fig. 1). The two other inoculated animals developed severe watery diarrhoea 8 dpi for 6 days (faeces consistency score = 2). While one of these animals ("Inoculated 3", Fig. 1) completely recovered 14 dpi, the other ("Inoculated 1", Fig. 1) became recumbent 14 dpi (posture score = 2, lying down score = 4) and died 17 dpi. The first and second contact infected animal both developed a haemorrhagic diarrhoea (faeces consistency score = 3) between 21 and 26 dpi and between 30 and 36 dpi, respectively. Both animals also lost appetite (appetite score = 3) during 3 consecutive days, but completely recovered 28 and 38 dpi, respectively.

Following the second inoculation few clinical signs were noticed. Both animals lost appetite for 2 days beginning at 7 dpi, were depressed (posture score = 2) and showed nasal discharge (nasal discharge score = 1) 8 dpi.

All inoculated and contact infected animals showed fever (> 39.0 °C) during infection, with duration of pyrexia varying between 5 and 8 days. Compared to the RT-qPCR-negative calves leucocyte and thrombocyte counts were significantly lower in RT-qPCR-positive animals during both inoculations (Table 1).

Table 1. Comparison of haematology between RT-qPCR-negative and RT-qPCR-positive animals during both inoculations. During the first inoculation 3 inoculated animals infected 2 out of 7 susceptible animals in contact. A second inoculation was performed 63 days later, during which none of the 3 susceptible animals were contact infected by the 2 inoculated animals.

	RT-qPCR-negative		RT-qPCR-positive		*p*-value[a]
	Estimate	95% CI	Estimate	95% CI	
First inoculation					
Leucocytes (1000/µL)	9.0	8.0; 10.1	6.4	5.2; 7.6	< 0.001
Thrombocytes (1000/µL)	486	394; 578	338	240; 436	< 0.001
Packed cell volume (%)	29.7	27.1; 32.3	30.2	27.6; 32.9	0.29
Haemoglobin (g/dL)	11.1	10.1; 12.1	11.3	10.3; 12.3	0.14
Second inoculation					
Leucocytes (1000/µL)	8.8	7.2; 10.3	6.6	4.9; 8.3	< 0.001
Thrombocytes (1000/µL)	487	295; 679	346	115; 547	0.002
Packed cell volume (%)	27.4	25.8; 29.1	26.7	24.8; 28.7	0.33
Haemoglobin (g/dL)	10.5	9.7; 11.3	10.4	9.5; 11.3	0.84

[a] Linear mixed model with "RT-qPCR-positive" (no/yes) and "Animal" as fixed and random effect, respectively (SAS 9.4, SAS Institute).

Discussion

Although pronounced clinical signs such as diarrhoea and death were observed during this study, these clinical observations were not as severe as encountered during the outbreak in Germany (Doll and Holsteg, 2013). Several different BVDV field strains were isolated during this outbreak (Jenckel et al., 2014), which indicates that the BVDV-2c strains were genetically highly variable. Two of these field strains were used in experimental infections. Results from the infection with the NRW 14-13 strain are described in the current study, while Jenckel et al. (2014) experimentally infected 8-week-old calves with NRW 19-13-8. Both strains were considered to have a high level of virulence (Jenckel et al., 2014). During the latter study all animals (n = 8) died acutely or had to be euthanized within 11 days. Given

the high genetic variability in these BVDV-2c strains, changes in the genome with shifts in strain virulence following virus cultivation can possibly explain the difference in morbidity and mortality observed during our study on the one hand and encountered in the field and during the experimental infection of Jenckel et al. (2014) on the other hand. To avoid genetic changes as much as possible, the second passage on cell culture of the isolated virus was used. Another explanation for the observation of more mild clinical signs could be the age of calves, since age appears to have a key influence on the clinical manifestations associated with BVDV infection: more pronounced clinical signs are observed in younger animals (Hamers et al., 2003). While in our study the calves were aged between 16 and 20 weeks and between 25 and 29 weeks at the moment of the first and second inoculation, respectively, the calves in the study of Jenckel et al. (2014) were only 8 weeks old. However, also adult cattle suffered from severe clinical disease during the outbreak in Germany (Doll and Holsteg, 2013).

The clinical signs of the BVDV infected animals in the present study were varying from very mild disease to severe watery and haemorrhagic diarrhoea and death. The severity of the clinical signs was associated with the degree of viraemia. Animals with a higher viral titre (VI) and lower Ct-values (RT-qPCR) showed more severe clinical signs. This result is in agreement with comparable observations made by Walz et al. (2001).

The amount of virus shed is likely to depend on the propensity of a strain to replicate (Bolin and Ridpath, 1992) and could thus also be related to the degree of viraemia. The patterns of the RT-qPCR results of the nasal swabs and blood samples were indeed similar (Fig. 1b-c, Fig. 2b-c).

During the outbreak in Germany TI animals with a high viral titre (genomic load almost equivalent to that in PI animals) and long-lasting virus shedding (up to 8 weeks) were reported (Doll, 2013). Moreover, during the experimental infection executed by Jenckel et al. (2014) very high viral loads in blood and organs (Ct-values < 20 in RT-qPCR) were shown in all animals. In a study comparing Ct-values of PI and TI animals, it has been shown that only 1 out of 57 TI animals obtained a Ct-value below 25, while only 1 out of 17 PI animals had a Ct-value above 25 (Hanon et al., 2014). These results indicate that in the present study, very similar to the observations encountered in the field (Doll, 2013), the animal “Inoculation 1” (Fig. 1) obtained a genomic load 8-9 dpi almost equivalent to that in PI animals, which indicates that this animal, at least for 1-2 days, has substantially contributed to BVDV spread.

At the moment of its death (17 dpi) the animal was still RT-qPCR-positive, but negative in VI, which suggests that viral shed had stopped. The duration of viral shed was thus much shorter than 8 weeks as observed in the field (Doll, 2013). To study the role of these TI animals with a high viral load, the transmission experiment could be repeated using a 'one to one' approach (one infective and one susceptible, repeated five times), since this method is preferred to study the most relevant characteristics of R_0 (Kroese and de Jong, 2001).

Despite the presence of this animal with a high viral titre and four additional inoculated calves during two inoculations, a limited BVDV transmission was demonstrated. Nevertheless, since the nasal swabs of all BVDV infected animals (both inoculated and contact infected) were RT-qPCR-positive, it can be assumed they all had the potential to spread BVDV (Fig. 1c; 2c). This result supports the general assumption that TI cattle, unless infected in early gestation, have little contribution to the overall BVDV spread, as already demonstrated before (Niskanen et al., 2000; Niskanen et al., 2002; Sarrazin et al., 2014).

This result is in contrast with the observations in the field, where it is suggested that BVDV has spread solely through TI animals (Doll, 2013). Nevertheless, thorough evidence is needed to prove that it is not due to PI animals that have escaped identification (Lindberg and Alenius, 1999; Lindberg and Houe, 2005; Laureyns et al., 2010), as this is also suggested for the German outbreak (Jenckel et al., 2014). It might for instance have been the case that PI animals died before they were identified as PI. Furthermore, BVDV can also spread when PI animals are aborted or stillborn (Lindberg et al., 2004). Other factors that may have hampered viral spread, such as the experimental conditions, under which animals are likely to be less challenged by other pathogens and environmental conditions have already been discussed (Sarrazin et al., 2014).

Acknowledgements

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References

Bolin, S.R., Ridpath, J.F., 1992. Differences in virulence between 2 noncytopathic bovine viral diarrhea viruses in calves. American Journal of Veterinary Research 53, 2157-2163.

Doll, K., 2013. BVD-Virus Type 2 – Outbreak in Germany. BVD Day 2013. http://bvd-day2013.eu/conferences2/ (accessed 22 September 2014)

Doll, K., Holsteg, M., 2013. BVD-virus type 2 – An outbreak in Germany. Cattle Practice 21, 216.

Fulton, R.W., Johnson, B.J., Briggs, R.E., Ridpath, J.F., Saliki, J.T., Confer, A.W., Burge, L.J., Step, D.L., Walker, D.A., Payton, M.E., 2006. Challenge with bovine viral diarrhea virus by exposure to persistently infected calves: protection by vaccination and negative results of antigen testing in nonvaccinated acutely infected calves. Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire 70, 121-127.

Hamers, C., Couvreur, B., Dehan, P., Letellier, C., Fischer, L., Brun, A.J., Lewalle, P., Michaux, C., Pastoret, P.P., Kerkhofs, P., 2003. Assessment of the clinical and virological protection provided by a commercial inactivated bovine viral diarrhoea virus genotype 1 vaccine against a BVDV genotype 2 challenge. Veterinary Record 153, 236-240.

Hanon, J.B., Van Der Stede, Y., Antonissen, A., Mullender, C., Tignon, M., Van den Berg, T., Caij, B., 2014. distinction between persistent and transient infection in a bovine viral diarrhoea (BVD) control programme: Appropriate interpretation of real-time RT-PCR and antigen-ELISA test results. Transboundary and Emerging Diseases 61, 156-162.

Jenckel, M., Hoper, D., Schirrmeier, H., Reimann, I., Goller, K.V., Hoffmann, B., Beer, M., 2014. Mixed triple: allied viruses in unique recent isolates of highly virulent type 2 bovine viral diarrhea virus detected by deep sequencing. Journal of Virology 88, 6983-6992.

Kroese, A.H., de Jong, M.C.M., 2001. Design and analysis of transmission experiments. Society for Veterinary Epidemiology and Preventive Medicine, Proceedings, xxi-xxxvii.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. The Veterinary Journal 199, 201-209.

Laureyns, J., Ribbens, S., de Kruif, A., 2010. Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. Veterinary Journal 184, 21-26.

Lindberg, A., Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Veterinary Microbiology 64, 197-222.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Veterinary Record 154, 463-467.

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine-Revue Canadienne de Medecine Comparee 48, 156-161.

Moen, A., 2013. Boviene virus diarree type 2. GD Veterinair 19, 3.

Nickell, J.S., White, B.J., Larson, R.L., Renter, D.G., Roque, J., Hesse, R., Oberst, R., Peddireddi, L., Anderson, G., 2011. Onset and duration of transient infections among antibody- diverse beef calves exposed to a bovine viral diarrhea virus persistently infected calf. International Journal of Applied Research in Veterinary Medicine 9, 29-39.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Veterinaria Scandinavica 41, 93-99.

Niskanen, R., Lindberg, A., Traven, M., 2002. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. The Veterinary Journal 163, 251-259.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterinary Research 41.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Veterinary Microbiology 114, 196-204.

Sarrazin, S., Dewulf, J., Mathijs, E., Laureyns, J., Mostin, L., Cay, A.B., 2014. Virulence comparison and quantification of horizontal bovine viral diarrhoea virus transmission following experimental infection in calves. The Veterinary Journal 202, 244-249.

Velthuis, A.G.J., Bouma, A., Katsma, W.E.A., Nodelijk, G., de Jong, M.C.M., 2007. Design and analysis of small-scale transmission experiments with animals. Epidemiology and Infection 135, 202-217.

Walz, P.W., Bell, T.G., Wells, J.L., Grooms, D.L., Kaiser, L., Maes, R.K., Baker, J.C., 2001. Relationship between degree of viremia and disease manifestations in calves with experimentally induced bovine viral diarrhea virus infection. American Journal of Veterinary Research 62, 1095-1103.

Chapter 5

Biosecurity

A survey on biosecurity and management practices in selected Belgian cattle farms

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Abstract

The shift from cure towards prevention in veterinary medicine involves the implementation of biosecurity, which includes all measures preventing pathogens from entering a herd and reducing the spread of pathogens within a herd. In Belgium no studies have considered the implementation of biosecurity measures in the daily management of cattle farms. Therefore the aim of the study was to map the current application of biosecurity measures in Belgian cattle farms in the prevention of disease transmission within and between farms.

Between March 2011 and April 2013 the data were collected as part of a larger cross-sectional study, conducted to identify risk factors for re-infection with BVDV in cattle herds assumed free from BVDV. Questionnaire data from 33 dairy farms, 16 beef farms and 25 mixed (dairy and beef cattle) farms were analyzed using a combination of a linear scoring system, a categorical principal component analysis and a two-step cluster analysis to differentiate these farms based on their biosecurity level and visit frequencies.

Further enhancement of preventive measures considering external and internal biosecurity was still possible for each farm, as none of the farms obtained an overall high biosecurity level. Three groups of cattle farms were differentiated with a biosecurity level varying from low to high-medium, of which the group with the lowest biosecurity level mainly consisted of mixed farms. Animal-to-animal contacts with cattle from other herds were frequently possible as only 12% of the farmers purchasing cattle quarantined purchased animals at least 3 weeks and contacts over fences on pasture were possible in 70% of the herds. Basic biosecurity measures such as farm-specific protective clothing and boots were present in the majority of the farms, but they were insufficiently or incorrectly used. Cattle farms were very often visited by professional visitors of which the herd veterinarian, the AI technician and the cattle salesman most frequently entered the farm.

It can be concluded that since few biosecurity measures were undertaken by Belgian cattle farmers, they expose their herd to the risk of disease transmission within and between farms. Especially in regions with a high cattle density, small distances to neighbouring farms and high frequencies of professional visits, a farm-specific preventive strategy should be developed, thereby using the facilities often already present on the farm.

Introduction

In modern veterinary medicine, disease prevention at the herd level has become increasingly important in replacing individual animal medicine (Lin et al., 2003; Derks et al., 2013). This shift from treating individuals towards prevention involves the implementation of biosecurity, which includes all measures preventing pathogens from entering a herd (i.e. external biosecurity) and reducing the spread of pathogens within a herd (i.e. internal biosecurity or biocontainment) (Villarroel et al., 2007; Laanen et al., 2013). The implementation of biosecurity measures reduces disease spread and is therefore part of the measures frequently proposed in the control of several infectious diseases. Although biosecurity is usually associated with collective action for disease control in case of large epidemic outbreaks such as foot-and-mouth disease and bovine spongiform encephalopathy (Heffernan et al., 2008), it is also a crucial element in the control of endemic diseases. For the control of bovine viral diarrhoea virus (BVDV) at the herd level, the implementation of biosecurity is even considered the most essential pillar (Lindberg and Houe, 2005). Also for the control of neonatal diarrhoea (Barrington et al., 2002) and respiratory disorders (Callan and Garry, 2002), biosecurity measures are seen as indispensable preventive measures.

Expected consequences of the reduced disease spread and thus indirectly from applying biosecurity measures are improved production characteristics and thus greater profits, better animal welfare, improved immune responses to vaccines and enhanced job satisfaction for farmers (Brennan and Christley, 2012). Recently it was shown in pig production that a higher biosecurity status is linked with a reduction in antimicrobial usage (Laanen et al., 2013).

Although the importance and usefulness of biosecurity is elaborately described, studies demonstrated that most cattle farmers do not implement adequate biosecurity measures (Nöremark et al., 2010; Negron et al.; 2011, Brennan and Christley, 2012; Sayers et al., 2013). In Belgium, an important beef cattle and milk producing country in Europe and a very densely populated livestock area, the application of biosecurity measures in pig and poultry herds has already been studied (Ribbens et al., 2008; Van Steenwinkel et al., 2011). However, for Belgian cattle farms no similar studies have been conducted yet (literature search with keywords ‘biosecurity’ - ‘cattle’ - ‘Belgium’). In order to optimize the use of biosecurity as preventive tool, it is important to understand first if and how such preventive

measures are being used (Brennan and Christley, 2012). Therefore the aim of this study was to map the current application of biosecurity measures in cattle farms in the prevention of disease transmission within and between farms.

Material and methods

Selection of the farms

In Belgium 2,441,319 domestic bovine animals were registered in the animal identification and registration system (SANITEL) in November 2013 (NIS, 2013). This resulted in an average population density at municipality level of 85 animals/km².

The target population for this study comprised all farms with cattle in Belgium. The data were collected as part of a larger cross-sectional study, conducted to identify risk factors for re-infection with BVDV in cattle herds assumed free from BVDV. A non-random sample was obtained based on the following inclusion criteria: having a history of BVDV circulation in the herd and the assumption of being BVDV free at the moment of participation, according to the herd veterinarian's judgement and previous test results. For farm selection, herd veterinarians were contacted to propose cattle farms satisfying the inclusion criteria. Therefore, an e-mail was sent to herd veterinarians in the database of the Institute for Continuing Education at the Faculty of Veterinary Medicine, Ghent University (February 2011). A second call for participation was sent by means of a newsletter of the regional centres for animal disease control “Dierengezondheidszorg Vlaanderen (DGZ)” in Flanders (northern part of Belgium) (November 2011) and “Association Régionale de Santé et d’Identification Animales (ARSIA)” in Wallonia (southern part of Belgium) (January 2012). Proposed farms fulfilling the inclusion criteria were contacted by phone to explain the study objectives and to verify their willingness to participate.

Questionnaire

The questionnaire was designed to collect data regarding BVDV management and control at herd level. According to the control strategy plan of Lindberg and Houe (2005), the questionnaire consisted of four main topics: biosecurity, virus detection, monitoring and vaccination. Most questions were semi-closed. Except for the language (Flanders = Dutch and Wallonia = French), the questionnaires for both parts of the country were identical and checked for consistency by a bilingual researcher. Regarding the biosecurity part, the questionnaire was adapted from an online biosecurity testing tool for pig and poultry herds (www.BioCheck.UGent.be; Laanen et al., 2010). The questionnaire covered several aspects regarding the degree of biosecurity and the different types of contacts between farms. The full questionnaire is available upon request to the first author. The questionnaire was pre-tested regarding content, interpretation of questions and responses. It was emphasised to the farmers that questionnaires would be processed anonymously. The selected farmers were subjected to a face-to-face questionnaire interview. All interviews were conducted by the first author between March 2011 and April 2013.

Data processing

All information was coded numerically to assist analysis, entered into a database worksheet program (Microsoft Excel, 2010) and recoded into categorical data (nominal or ordinal level) for further analysis. Data were exported for analysis into SPSS 22.0 (SPSS Inc., Chicago, IL).

Data analysis

Cattle density map

A map showing the sample distribution and the cattle density at municipality level was produced using Arc Map® version 3.2.1 (ESRI, Redlands, CA, USA). The animal density data were extracted from SANITEL in December 2012.

Creating a biosecurity scoring system

A biosecurity scoring system was created as described by Van Steenwinkel et al. (2011). All variables were coded using values of 1 (biosecurity measure present) or 0 (absent). The variables were divided into groups, each expressing a different aspect of farm biosecurity. Then, for each biosecurity variable group (made up of several measures) the values for each individual variable were added up to generate a biosecurity score. Finally, each group score was equally weighed by scaling from 0 to 10, with a higher score implying a 'better' biosecurity level for the variable group concerned. The following biosecurity groups were considered:

a) Other animals: on-farm and between-farm animal-to-animal contacts.
b) Onto herd movements of animals: purchase of cattle. A farmer was listed as "not purchasing cattle" when the last purchase was at least five years ago.
c) Onto herd movements of persons: frequency of professional visitors entering the herd. Professional visitor frequency was categorized as 'no visit', 'low' (between null and one visit per month), 'medium' (between one visit per month and one visit per week) or 'high' (more than one visit per week). The latter three categories were formed based upon the 33.33 and 66.67 percentile of the number of professional visits in the selected herds, excluding the 'no' visits. A score was defined on a scale from 1 to 4, with a higher score implying lower visit frequencies.
d) Off herd movements: animals and material leaving the herd.
e) Hygiene infrastructure: structures or activities in the daily management to reduce disease spread by the farmer himself, focusing on internal biosecurity.
f) Hygiene visitors: measures taken to prevent disease spread by professional visitors.

Categorical principal component analysis

To analyse the categorical data, a categorical principal component analysis (CATPCA) was performed according to the methodology described by Van Steenwinkel et al. (2011). All variable groups were given an ordinal measurement scale in the analysis. Two supplementary variables were included and attributed a multiple nominal measurement scale: herd type and herd size. Herd size was categorized as 'small' (< 100 animals), 'medium' (between 100 and 150 animals) or 'large' (> 150 animals), based upon the 33.33 and 66.67 percentile of the number of cattle present in the selected farms. The quantification of a supplementary variable

allows the interpretation of its relationship with the result obtained for the other variables, but has no influence on the actual analysis.

The number of dimensions was set to two to allow for a two-dimensional graphical representation. These dimensions represent the principal components to which the variables are reduced and are linear transformations of the original data such that as much original information as possible is retained. Whether a reduction to two dimensions was acceptable, was verified by checking the sum of the percentage of variance accounted for (PVAF), a measure of the amount of retained information and model fit (Linting et al., 2007).

Two-step cluster analysis

The object scores obtained from the CATPCA solutions were then included in a two-step cluster analysis (TSCA, SPSS 22.0) to identify clusters of cattle farms with a similar biosecurity level and farm visit frequencies (Ribbens et al., 2008).

Results

Response

A total of 38 veterinary practices responded. Three of them were from the Netherlands and therefore not included in the study. Six veterinary practices had no farms fulfilling the inclusion criteria after further explanation of the study goals. After receiving further information, another four veterinarians preferred not to participate because of lack of time or compensation. For one veterinarian, willing to participate and having several farms fulfilling the inclusion criteria, it was not appropriate to be included in the study because of a brucellosis outbreak in that region. Eventually, a total of 74 cattle farms from 23 different veterinary practices were visited of which 33 dairy farms, 16 beef farms and 25 mixed (dairy and beef) farms. Also two Dutch dairy farms belonging to the clientele of a Belgian veterinary practice were included in the study. Up to 58% of the participating herds were located in cattle-dense regions (> 100 animals/km²) (Fig. 1).

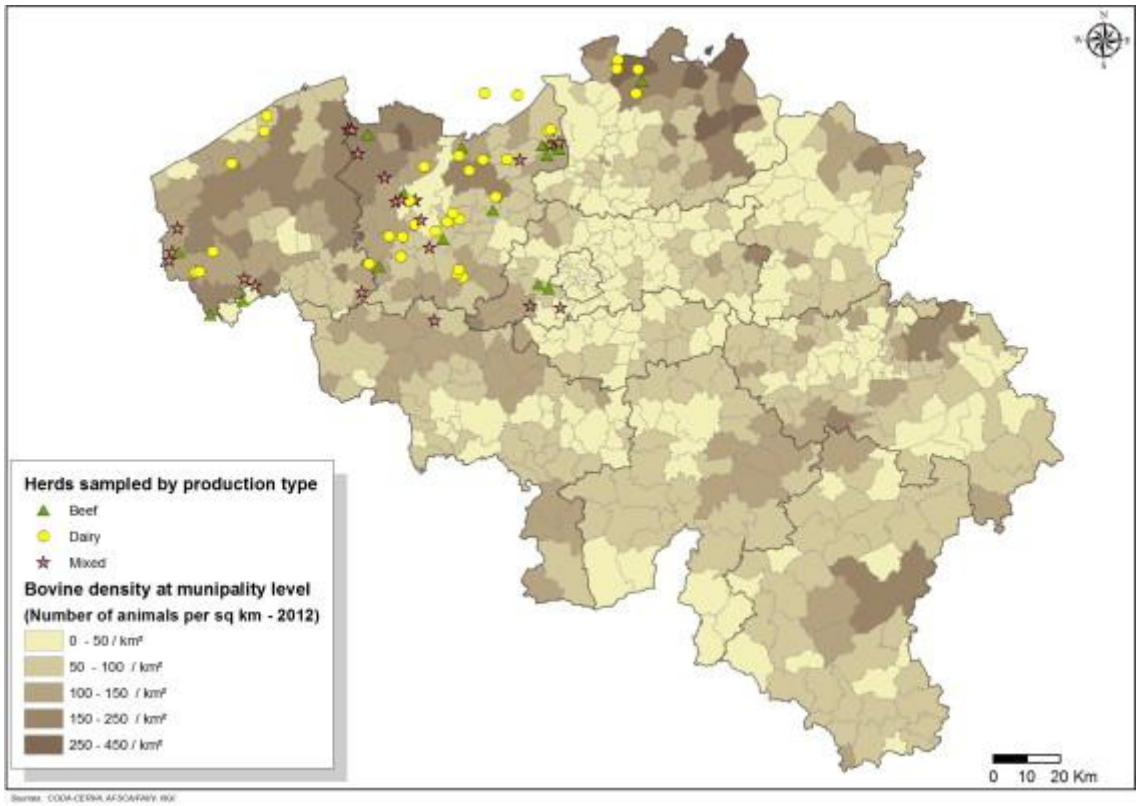

Fig. 1. Production types and municipality level animal densities of the studied cattle farms. A total of 74 cattle farms were studied, of which 16 beef farms, 33 dairy farms and 25 mixed farms (farms containing beef and dairy cattle). Cattle densities at municipality level are displayed in the background.

General characteristics of the studied farms

The high density of cattle farms in Belgium is reflected in a mean distance of only 0.57 km to the nearest cattle farm. For mixed farms this distance is only 0.28 km (Table 1). Also pig herds and sheep premises (from professionals and hobby keepers) were located nearby, with minimum distances of only 0.05 km (data not shown). The average number of cattle present at the studied farms was 138, with mixed farms tending to have slightly more animals.

Table 1. General characteristics of the studied farms (*n* = 74).

	Dairy (*n* = 33)	Beef (*n* = 16)	Mixed[a] (*n* = 25)
Number of animals			
Mean (SE)	135 (10)	129 (21)	147 (14)
95% CI	[115; 155]	[84; 173]	[117; 177]
Size[b] : *n* (% within herd type)			
Small (< 100 animals)	7 (21%)	8 (50%)	9 (36%)
Medium	17 (52%)	3 (19%)	5 (20%)
Large (> 150 animals)	9 (27%)	5 (31%)	11 (44%)
Distance to nearest cattle farm (km)			
Mean (SE)	0.82 (0.18)	0.49 (0.10)	0.28 (0.05)
95% CI	[0.45; 1.20]	[0.29; 0.70]	[0.18; 0.38]
Distance to nearest pig farm (km)			
Mean (SE)	2.54 (0.55)	2.55 (0.84)	1.35 (0.42)
95% CI	[1.41; 3.66]	[0.75; 4.34]	[0.48; 2.22]
Distance to nearest sheep premise[c] (km)			
Mean (SE)	2.12 (0.55)	0.70 (0.19)	0.54 (0.17)
95% CI	[1.00; 3.24]	[0.29; 1.11]	[0.19; 0.90]

SE, standard error; CI, confidence interval

[a] Dairy and beef cattle

[b] 33.33 and 66.67 percentile of the number of cattle present in the selected farms

[c] Professional farms and hobby keepers

Professional visitors entering the farms

In dairy and mixed farms, milk is collected on a regular basis, with milk collection trucks entering the farms every 3 days (Table 2). For one farm, milk was collected daily. Other professional visitors frequently entering the farm, were the artificial insemination (AI) technician, the veterinarian and the cattle salesman. Of the 37 farmers that were making use of the services of an AI technician, beef farms were visited on average less by the AI technician

compared to farms with dairy cattle. On the contrary, the former were visited on average more by the herd veterinarian. A large range in visiting frequencies by the veterinarian was noticed for all herd types. The visiting frequency of rendering company lorries was highly influenced by the possible presence of pigs at the farm.

Table 2. Mean number of monthly professional visits on a cattle farm per herd type.

	Dairy	Beef	Mixed[a]
	Mean (SE) freq./month [95% CI]		
Veterinarian	3.4 (0.4) [2.5; 4.3]	6.6 (1.0) [4.4; 8.7]	5.9 (0.9) [4.0; 7.9]
A.I. technician	9.6 (1.2) [7.2; 12.1]	2.0 (0.9) [0.0; 4.0]	7.5 (1.1) [5.0; 10.0]
Cattle salesman	4.1 (0.6) [2.9; 5.3]	1.7 (0.3) [1.1; 2.4]	4.7 (0.8) [3.0; 6.4]
Feed supplier	1.3 (0.1) [1.0; 1.6]	1.0 (0.1) [0.7; 1.3]	1.6 (0.2) [1.1; 2.1]
Milk collection	10.6 (0.6) [9.4; 11.8]	-	9.6 (0.4) [8.8; 10.4]
Cadaver removal	0.9 (0.1) [0.7; 1.2]	0.6 (0.2) [0.2; 0.9]	0.9 (0.2) [0.5; 1.4]
Other farmers	1.0 (0.5) [0.0; 2.1]	0.3 (0.1) [0.0; 0.6]	0.6 (0.2) [0.2; 1.0]

SE, standard error; CI, confidence interval.
[a] Dairy and beef cattle

Biosecurity measures

Table 3 shows the percentage of farms implementing different types of biosecurity measures for each herd type, listed per biosecurity category. From the category 'Other animals' it was noticed that grazing cattle can be exposed to disease agents, as pasture contacts over fences were frequently possible and manure from other farms very often was dispersed close to the proper farm. Measures related to internal biosecurity ('Hygiene infrastructure'), compartmentalization of young stock and working from young to old (working lines) were in general poorly implicated in cattle farms. The impact of sick animals on herd health was considered limited by the farmers as it appeared that animals which had aborted rarely were isolated and the calving box was used to house sick animals without cleaning and disinfection of this box. Although rendering companies request to collect cadavers at farm entrance, less than half of the farmers provided such a facility at the entrance. A remarkable contradiction was noticed when asking about the lorry of the cattle salesman when animals were transported: most of the farmers considered the lorry was clean while the lorry very often was already loaded with animals from another farm when entering the farm. Highest category scores were obtained for the 'Onto herd movements of animals'. This can be explained by the fact that farmers never purchasing cattle (34%) received the highest score for this category. Farmers purchasing cattle scored very low on the application of good quarantine measures, i.e. using a quarantine stable (no contact with own animals) and a quarantine period of at least 3 weeks. Only 12% of the herds purchasing cattle jointly implemented both quarantine measures. Low category scores were obtained for the hygiene measures for professional visitors. It is remarkable that 88% of the farmers wanted visitors to check in when entering the farm, while an equal percentage of farmers provided direct access to the stables for those visitors. Farm-specific boots, farm-specific clothing for visitors and disinfection footbaths were present in 70%, 66% and 61% of the farms, respectively. Despite their presence in the majority of the farms, only in 20%, 13% and 9% of the studied herds, respectively, these measures were actually used.

Table 3. Percentage of farms implementing different types of biosecurity measures per category and per herd type. Per category a mean score (out of ten) and standard deviation (SD) is given.

Category	Biosecurity components	Dairy (*n*=33)	Beef (*n*=16)	Mixed[a] (*n*=25)
Other animals	No other farm animals present	64	75	48
	No dogs present	39	38	20
	Permanent rodent control	79	94	92
	No pasture contact	48	13	16
	No participation to auctions/competitions	85	94	96
	No dispersion of manure from other herds within 500m of the herd	9	25	12
	Score (mean; SD)	**(5.4; 2.0)**	**(5.6; 1.6)**	**(4.7; 1.4)**
Hygiene infrastructure	Calves and young stock separated from older animals	58	38	32
	Working from young to old animals	15	19	8
	Separated material for young and old animals	21	13	16
	Sick animals treated as last	30	44	28
	Always testing of abortions	42	88	48
	Always isolating animals which aborted	6	13	4
	Cleaning and disinfection of stable after abortion	18	19	8
	Calving takes place in a separated calving box	85	69	56
	No use of calving box to house sick animals	36	69	16
	Cleaning of box after each calving	27	63	28
	Cleaning and disinfection of material used for calving after each calving / use of disposable calving material	58	75	52
	Between age groups clothes are changed	6	6	4
	Between age groups hands are cleaned and disinfected	9	6	0
	Score (mean; SD)	**(3.2; 1.6)**	**(4.0; 2.0)**	**(2.3; 1.4)**

[a] Dairy and beef cattle

Table 3. (*Continued*)

Category	Biosecurity components	Dairy (*n*=33)	Beef (*n*=16)	Mixed[a] (*n*=25)
Off herd movements	Cadaver storage facility at herd entrance	48	50	40
	Cadaver storage facility inaccessible for vermin	21	19	8
	Cadaver storage facility frequently cleaned and disinfected	24	44	36
	Lorry of cattle salesman cleaned when transporting animals	82	100	60
	Lorry of cattle salesman empty when transporting animals	12	44	4
	Cattle salesman does not enter the stables when loading animals	39	6	16
	No material in common use with other farmers	64	63	72
	Score (mean; SD)	**(4.2; 2.0)**	**(4.6; 1.8)**	**(3.4; 1.5)**
Onto herd movements: animals	Purchase of animals	48	94	72
	Purchase of animals in gestation	38	27	33
	Purchasing animals from farms with a higher or equal sanitary status	56	67	17
	Testing of all purchased animals	88	100	94
	Using a quarantine stable when purchasing animals	25	67	17
	Applying a quarantine period of at least 3 weeks when purchasing animals	6	33	17
	Score (mean; SD)	**(7.0; 3.2)**	**(5.9; 2.1)**	**(5.3; 3.4)**
Hygiene visitors	Visitors have to check in when entering the herd	79	81	72
	Visitors have direct access to the stables	73	69	88
	Presence of a hygiene lock	39	6	8
	Stable entry through hygiene lock	3	0	0
	Use of farm-specific boots for visitors	33	13	8
	Use of farm-specific clothing for visitors	24	6	4
	Use of a disinfection footbath	3	19	12
	Feed supplier does not enter stables when supplying feed	85	56	52
	Score (mean; SD)	**(3.1; 2.2)**	**(2.0; 1.5)**	**(1.7; 1.5)**

CATPCA and TSCA

The result of a two-dimensional solution of the CATPCA explained 58.8% of the variance of the scores provided by the 74 herds for the six variables, which means 58.8% of the original information was retained. The PVAF in the first dimension was 32.6% and 26.2% in the second dimension. Fig. 2 shows the plot of component loadings, i.e. the position of the original variables in the two-dimensional space, and the centroid coordinates of the multiple nominal category points (herd types and sizes).

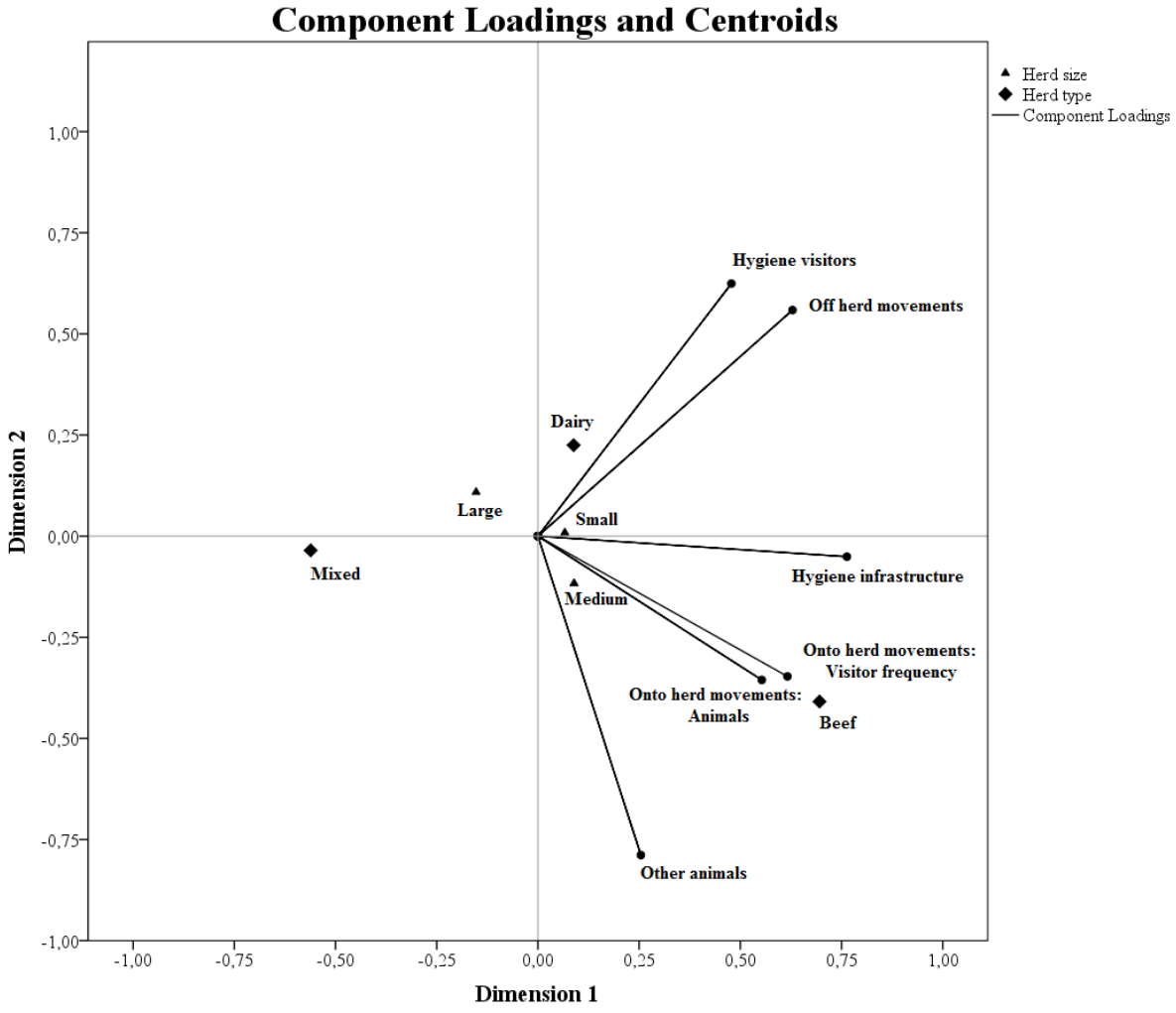

Fig. 2. Biplot of component loadings (the position of the original variables in the two-dimensional space, represented by vectors) and multiple nominal category points (herd types and sizes), resulting from the categorical principal component analysis. The vector of a variable points in the direction of the highest category of the variable, in this case indicating a higher level of biosecurity or lower farm visit frequencies. The first and second dimension distinguish between the production types. Mixed and beef farms tend to have on average the lowest and highest biosecurity scores, respectively, with dairy farms somewhere in between. None of the dimensions distinguishes between different herd sizes, which are all very close to the centre of the plot.

All variables (component loadings, represented by vectors) appear in the upper right and lower right quadrants. The projections of the vectors on each dimension represent the contribution of each variable to that dimension. As their projections on the first dimension point in the same direction and have more or less the same length, they have more or less an equal contribution to the first dimension. The vector of a variable points in the direction of the highest category of the variable, in this case indicating a higher level of biosecurity or lower farm visit frequencies. Beef farms tend to have on average the highest biosecurity scores, as the corresponding category point lies in the direction of the component loading points. The category point of mixed farms is located in the opposite direction of the vectors, which suggests these farms have on average the lowest biosecurity scores. Dairy farms are located somewhere in between. The first dimension thus distinguishes between the production types. Farms having higher scores for the 'Off herd movements' category are likely to also have high scores for the 'Hygiene visitors' category, as both vectors point in the same direction (small angle between both vectors). Both categories have a high contribution to the second dimension, which is opposite to the contribution to the second dimension of the 'Other animals' and to a smaller extent the 'Onto herd movements' categories. This dimension again distinguishes well between dairy and beef farms. Beef farms are likely to have higher scores for the 'Onto herd movements' categories, but lower scores for 'Hygiene visitors' and 'Off herd movements'. None of the dimensions distinguishes between different herd sizes, which are all very close to the centre of the plot.

The object scores obtained from the CATPCA solution, together with the solutions of the TSCA, are presented in Fig. 3. Three clusters were formed.

Cluster 1 (n = 19) – '*low biosecurity status'*: Those herds are all located in the upper left and lower left quadrant, in the opposite direction of all variable groups. This cluster had the lowest scores in all biosecurity groups compared to the other clusters and mainly consists of mixed herds (13 out of 19).

Cluster 2 (n = 31) – '*low-medium biosecurity status*': This cluster had the highest score for the 'Other animals' category compared to the other clusters, but had low scores for 'Off herd movements' (mean score 3.1) and 'Hygiene visitors' (mean score 1.6). The distribution of herd types within this cluster represents the overall distribution of herd types.

Cluster 3 (*n* = 24) – '*high-medium biosecurity status*': This category had the highest scores for all biosecurity groups, except for 'Other animals', compared to the other clusters and scored well for the 'Onto herd movements of animals'. This can be explained by the fact that 10 out of the 25 farms not purchasing animals are represented in this cluster, while only 14 out of the 49 farms purchasing cattle are represented. Except for this variable group (mean score 7.4), the mean scores for the other variable groups varied between 4.1 and 5.8. So, although this cluster had the highest scores for all variable groups except one, these scores were low.

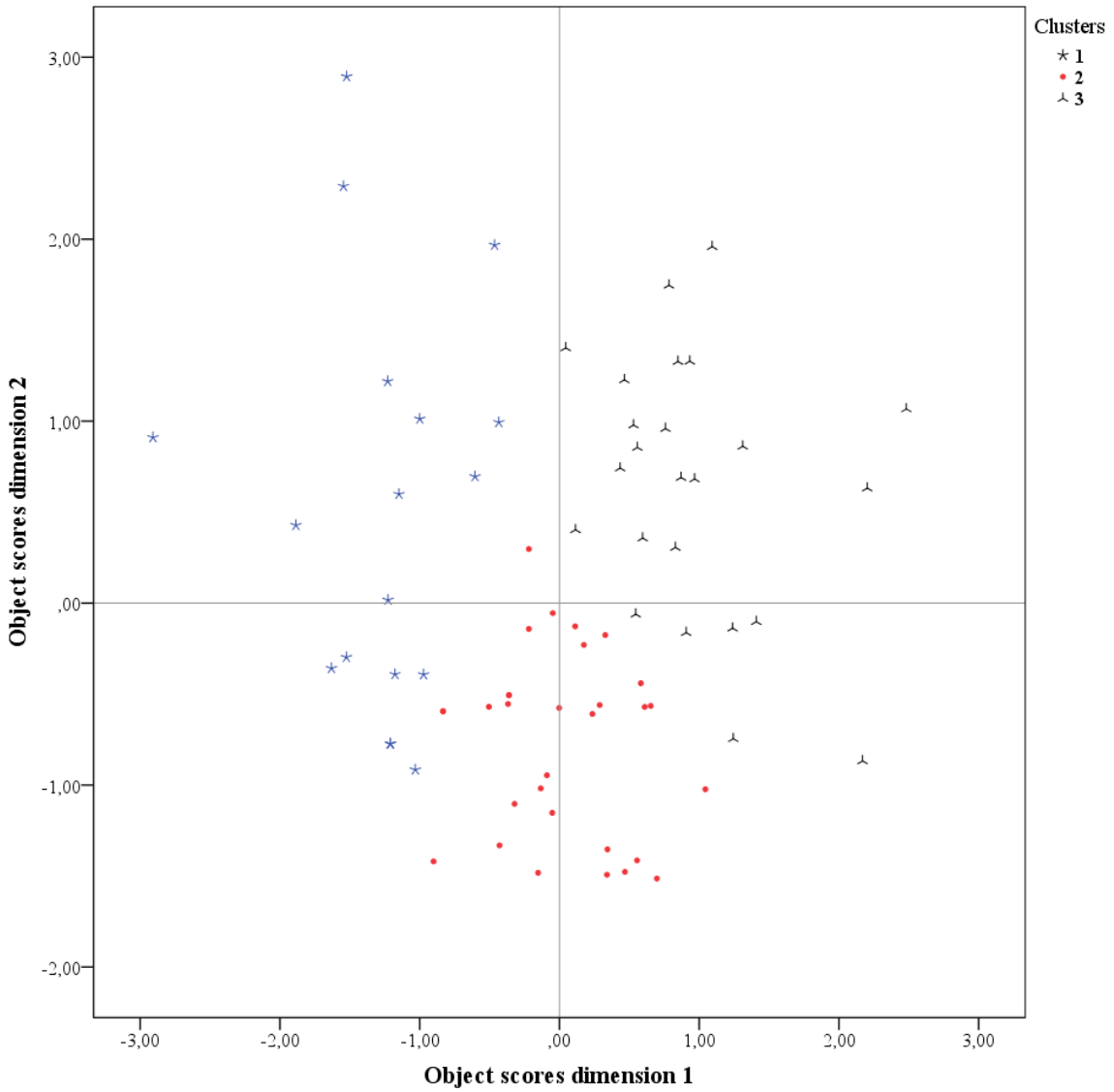

Fig. 3. Object scores of the categorical principal component analysis and two-step cluster solution. Cluster 1 (*n* = 19) is the group with a low biosecurity status, cluster 2 (*n* = 31) is the group with a low-medium biosecurity status and cluster 3 (*n* = 24) is the group with a high-medium biosecurity status.

Discussion

This paper describes the results of a survey on biosecurity and management practices in selected Belgian cattle farms. This is the first Belgian study concerning cattle as other studies described the application of biosecurity measures in pig and poultry farms (Ribbens et al., 2008; Van Steenwinkel et al., 2011). The present study also gives an overview of the frequency of professional visitors entering cattle farms.

A non-random sample of cattle farms was obtained, as the selected farms had a history of BVDV and were assumed BVDV free at the moment of participation. This indicates they had eradicated the virus from their herd and should therefore, in collaboration with the herd veterinarian, be aware of the measures necessary to eradicate the virus and to prevent a possible re-infection. Biosecurity is a crucial pillar in the control of BVDV (Smith and Grotelueschen, 2004; Lindberg and Houe, 2005) and therefore we assumed having selected for farms with a higher implementation of biosecurity measures in their daily management. Notwithstanding this fact, also in these herds a low internal and external biosecurity level was observed.

All participating farms were questioned by means of a face-to-face interview conducted by one person, the first author. By doing so, the purpose of the study and the questions could be explained well, a high participation rate was obtained and interviewer bias was reduced as much as possible. Social desirability response bias, the tendency of some respondents to report a higher biosecurity level than what is really applied (Holbrook et al., 2003), was reduced by checking the answers during a visit of the farm. Another disadvantage of face-to-face interviews is that a smaller sample was obtained than we possibly could have obtained using mailed questionnaires (Smeeth et al., 2001). Face-to-face interviews usually are geographically limited to areas close to the interviewer. Although the studied farms were centred, no farms were excluded from the study because of their geographical location in Belgium. By sending the second call for participation via the newsletter of the regional centres for animal disease control, all Belgian veterinary practices were reached. Very few veterinary practices from the southern part of Belgium responded to the call. Although the call for participation was sent in Dutch and French, a language barrier could be an explanation.

Similar to the study of Van Steenwinkel et al. (2011), the present study aimed at creating a linear scoring system to compare cattle farms relative to each other, rather than developing a risk-based weighted scoring system defining biosecurity in absolute terms. When evaluating the level of biosecurity implementation, three clusters were described based on our interpretation of which variables were more present in each cluster and names were given to each cluster. As none of the studied cattle farms had an overall high integration of biosecurity measures in their management practices, the cluster with the highest variable group scores (cluster 3) only was attributed a 'high-medium biosecurity status' instead of a 'high biosecurity status'. While no clear contrast between herd size was made based on the biosecurity level, a distinction between the herd production types was more explicit. The group with the lowest biosecurity level (cluster 1) mainly consisted of mixed farms. Compared to the other production types mixed farms obtained the lowest scores for each biosecurity group. This suggests that the focus should be on this type of farms when consulting regarding biosecurity is concerned, notwithstanding the fact that further enhancement of preventive measures was still possible for each farm.

Ribbens et al. (2008) and Van Steenwinkel et al. (2011) both reported an "acceptable" biosecurity level in Belgian pig and poultry herds, respectively, while we described a "poor" application of biosecurity measures in cattle herds. In general, measures for visitors entering herds (e.g. herd-specific clothing and disinfection footbaths) were better applied in pig and poultry herds, while further enhancement of preventive measures concerning contact with other animals within and between herds was possible for all types of livestock (pigs, poultry and cattle). Also other studies observed a higher implementation of biosecurity measures in pig herds compared to cattle herds (Nöremark et al., 2010; Sahlström et al., 2014).

Very little attention was paid to the prevention of direct contact with cattle from other herds as high percentages were obtained for pasture fence-line contact and low scores for good quarantine application. The recommended isolation period length varies, but for diseases with a short incubation period preferably 3 to 4 weeks is advised (Barrington et al., 2002; Callan and Garry, 2002; Wells et al., 2002; Villarroel et al., 2007). Even when animals are tested upon arrival, they should be quarantined until test results are available, which very often was not done. One also should realize that cattle can be carriers of other disease agents (e.g. Mortellaro disease, Psoroptes ovis mange, Staphylococcus aureus mastitis) than those few tested for. Moreover, despite the fact the regional centres for animal health in Belgium

offer a cheap 'purchase kit' which tests for endemic diseases such as bovine viral diarrhoea, paratuberculosis and neosporosis, not all farmers used this kit for all purchased cattle.

Contact with other animals besides cattle such as rodents and cats and/or dogs freely moving on the farm, may hamper internal and external biosecurity. Rodent control was generally well applied by means of poison and traps. Additionally, all farmers reported to have cats freely moving on the farm and entering the stables to support rodent control. However, cats have already been identified as a risk factor for the presence of Salmonella (Evans and Davies, 1996) and Q-fever (Schimmer et al., 2011). Furthermore, the role of dogs in the epidemiology of neosporosis is well understood (Almeria and Lopez-Gatius, 2013).

Although animal movements are generally considered as the major cause of disease spread, (professional) visitors and vehicles entering the farm also should be considered when establishing a biosecurity strategy for the farm (Alvarez et al., 2011). The risk of disease transmission depends among other things on the type of vehicle: for instance, rendering company trucks are considered a higher biosecurity risk than feeding company trucks (Ribbens et al., 2009). Nevertheless, only half of the herds provided a cadaver storage facility at herd entrance and the feed supplier had to enter the stables when delivering feed on one third of the farms. Although feed and milk collection trucks are rarely exposed to the animals on a farm (Nöremark et al., 2013), these vehicles should be considered as a biosecurity risk as they visit several herds on the same day (Ribbens et al., 2009). Our study revealed that cattle farms are very often visited by professional visitors, but biosecurity measures concerning visitors (biosecurity group 'Hygiene visitors) were poorly implemented. Similar to the study of Nöremark et al. (2013), the veterinarian, AI technician and cattle salesman were most frequently entering the herd and having direct contact with the animals. The higher visiting frequencies of veterinarians on beef and mixed farms compared to dairy farms in Belgium can be explained by the presence of Belgian Blue cattle on these farms. This is a breed of cattle characterized by a double-muscled phenotype and by consequence a caesarean section for routine management of parturition (Kolkman et al., 2012). When professional visitors frequently enter the herd (and have direct contact with animals), adequate biosecurity measures such as herd-specific protective clothing and boots and/or well-maintained disinfection footbaths should be provided (Villarroel et al., 2007; Nöremark et al., 2013). In this study it was noticed that, despite their presence in the majority of the farms, these basic biosecurity measures were rarely used. Moreover, these measures were not implicated by all

visitors in the same extent: veterinarians used protective clothing and boots more often than AI technicians, followed by cattle salesmen.

A low level of internal biosecurity, described by the biosecurity group 'Hygiene infrastructure', was observed. Analogous with the biosecurity measures for visitors entering the herd, facilities for an adequate internal biosecurity often were present, but they were insufficiently or incorrectly used. For instance, at 64% of the farms with a separate calving box sick animals were sometimes placed in that box. An explanation for a low level of internal biosecurity could be that farmers tend to overlook the implementation of management strategies to reduce disease spread within the herd (Brennan and Christley, 2013). As internal biosecurity involves that measures should be undertaken by the farmers themselves, they may also experience some biosecurity recommendations as time consuming and impractical (Gunn et al., 2008).

Especially in regions with a high cattle density, small distances to neighbouring herds and high frequencies of professional visits as frequently encountered in Belgium, farmers should be aware of the various ways disease agents can enter and spread within the herd. Nöremark et al. (2010) noticed that even farmers with a poor biosecurity level indicated their on-farm biosecurity was sufficient. This finding and the results from the present study suggest the necessity of informing and guiding farmers in establishing an adequate biosecurity strategy. Another study revealed that most farmers were familiar with the broad concept of biosecurity, but the practical implementation was lacking (Brennan and Christley, 2013). Farmers often argue that the implementation of biosecurity measures is more likely to benefit society (e.g. zoonotic risk, international trade and welfare) rather than the individual farmer, while the direct costs for this purpose are at farm-level (Kristensen and Jakobsen, 2011). Some basic biosecurity measures such as the use of protective clothing, boots and disinfectant footbaths can be implemented without additional costs since these measures were already present in the majority of the studied farms. Newly acquired cattle can easily be isolated on a pasture or a quarantine stable can be installed in a shed, away from the rest of the herd. This illustrates that the implementation of biosecurity measures involves a behavioural change (Ellis-Iversen et al., 2010). Such a preventive strategy is farm-specific and should be developed in collaboration with the herd veterinarian who knows the specific herd structure and can inform the farmer on the critical points (Villarroel et al., 2007; Ellis-Iversen et al.; 2010; Brennan and Christley, 2013). Besides the farmer and the herd veterinarian, all (professional) visitors should be willing to contribute to the biosecurity strategy.

Conclusions

A sample of selected cattle farms with a history of BVDV and assumed BVDV free at the moment of participation was used to map the implementation of biosecurity measures in the daily management practices of Belgian cattle farms. Although we may in this way have selected for farms with better management practices, a low internal and external biosecurity level was observed. Depending on the biosecurity level, three groups of cattle farms were differentiated, but none of them demonstrated an overall high integration of biosecurity measures in their management practices. Especially in regions with a high cattle density, small distances to neighbouring herds and high frequencies of professional visits, measures should be taken to maintain herd health. Such a biosecurity strategy should be developed in collaboration with the herd veterinarian, but all (professional) visitors should contribute to the practical implementation.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

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References

Almeria, S., Lopez-Gatius, F., 2013. Bovine neosporosis: Clinical and practical aspects. Research in Veterinary Science 95, 303-309.

Alvarez, L.G., Webb, C.R., Holmes, M.A., 2011. A novel field-based approach to validate the use of network models for disease spread between dairy herds. Epidemiology & Infection 139, 1863-1874.

Barrington, G.M., Gay, J.M., Evermann, J.F., 2002. Biosecurity for neonatal gastrointestinal diseases. Veterinary Clinics of North America-Food Animal Practice 18, 7-34.

Brennan, M.L., Christley, R.M., 2012. Biosecurity on Cattle Farms: A Study in North-West England. PLoS ONE 7 (1), e28139.

Brennan, M.L., Christley, R.M., 2013. Cattle producers' perceptions of biosecurity. BMC Veterinary Research 9, 71.

Callan, R.J., Garry, F.B., 2002. Biosecurity and bovine respiratory disease. Veterinary Clinics of North America-Food Animal Practice 18, 57-77.

Derks, M., van Werven, T., Hogeveen, H., Kremer, W.D.J., 2013. Veterinary herd health management programs on dairy farms in the Netherlands: Use, execution, and relations to farmer characteristics. Journal of Dairy Science 96, 1623-1637.

Ellis-Iversen, J., Cook, A.J.C., Watson, E., Nielen, M., Larkin, L., Wooldridge, M., Hogeveen, H., 2010. Perceptions, circumstances and motivators that influence implementation of zoonotic control programs on cattle farms. Preventive Veterinary Medicine 93, 276-285.

Evans, S., Davies, R., 1996. Case control study of multiple-resistant Salmonella typhimurium DT104 infection of cattle in Great Britain. Veterinary Record 139, 557-558.

Gunn, G.J., Heffernan, C., Hall, M., McLeod, A., Hovi, M., 2008. Measuring and comparing constraints to improved biosecurity amongst GB farmers, veterinarians and the auxiliary industries. Preventive Veterinary Medicine 84, 310-323.

Heffernan, C., Nielsen, L., Thomson, K., Gunn, G., 2008. An exploration of the drivers to bio-security collective action among a sample of UK cattle and sheep farmers. Preventive Veterinrary Medicine 87, 358-372.

Holbrook, A.L., Green, M.C., Krosnick J.A., 2003. Telephone versus face-to-face interviewing of national probability samples with long questionnaires: comparisons of

respondent satisficing and social desirability response bias. Public Opinion Quarterly 67, 79-125.

Kolkman, I., Hoflack, G., Aerts, S., Laevens, H., Lips, D., Opsomer, G., 2012. Pelvic Dimensions in Phenotypically Double-muscled Belgian Blue cows. Reproduction in Domestic Animals 47, 365-371.

Kristensen, E., Jakobsen, E.B., 2011. Danish dairy farmers' perception of biosecurity. Preventive Veterinary Medicine 99, 122-129.

Laanen, M., Beek, J., Ribbens, S., Vangroenweghe, F., Maes, D., Dewulf, J., 2010. Biosecurity on pig herds: development of an on-line scoring system and the results of the first 99 participating herds. Vlaams Diergeneeskundig Tijdschrift 79, 302-306.

Laanen, M., Persoons, D., Ribbens, S., de Jong, E., Callens, B., Strubbe, M., Maes, D., Dewulf, J., 2013. Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. The Veterinary Journal 198, 508-512.

Lin, J.H., Kaphle, K., Wu, L.S., Yang, N.Y.J., Lu, G., Yu, C., Yamada, H., Rogers, P.A.M., 2003. Sustainable veterinary medicine for the new era. Revue Scientifique et Technique – International Office of Epizootics 22, 949-964.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Linting, M., Meulman, J.J., Groenen, P.J.F., 2007. Nonlinear principal components analysis: introduction and application. Psychological Methods 12, 336–358.

Negron, M., Raizman, E.A., Pogranichniy, R., Hilton, W.M., Levy, M., 2011. Survey on management practices related to the prevention and control of bovine viral diarrhea virus on dairy farms in Indiana, United States. Preventive Veterinary Medicine 99, 130-135.

NIS, 2013. Count of the total number of cattle in Belgium (performed on 15/11/2013). National Institute for Statistics in Belgium. Internet address: http://www.statbel.fgov.be (last accessed March 2014).

Nöremark, M., Frossling, J., Lewerin, S.S., 2010. Application of Routines that Contribute to On-farm Biosecurity as Reported by Swedish Livestock Farmers. Transboundary and Emerging Diseases 57, 225-236.

Nöremark, M., Frossling, J., Lewerin, S.S., 2013. A survey of visitors on Swedish livestock farms with reference to the spread of animal diseases. BMC Veterinary Research 9, 184.

Ribbens, S., Dewulf, J., Koenen, E., Mintiens, K., De Sadeleer, L., de Kruif, A., Maes, D., 2008. A survey on biosecurity and management practices in Belgian pig herds. Preventive Veterinary Medicine 83, 228-241.

Ribbens, S., Dewulf, J., Koenen, F., Mintiens, K., de Kruif, A., Maes, D., 2009. Type and frequency of contacts between Belgian pig herds. Preventive Veterinary Medicine 88, 57-66.

Sahlström, L., Virtanen, T., Kyyrö, J., Lyytikäinen, T., 2014. Biosecurity on Finnish cattle, pig and sheep farms - results from a questionnaire. Preventive Veterinary Medicine 117, 59-67.

Sayers, R.G., Sayers, G.P., Mee, J.F., Good, M., Bermingham, M.L., Grant, J., Dillon, P.G., 2013. Implementing biosecurity measures on dairy farms in Ireland. The Veterinary Journal 197, 259-267.

Schimmer, B., Luttikholt, S., Hautvast, J.L.A., Graat, E.A.M., Vellema, P., van Duynhoven, Y.T.H.P., 2011. Seroprevalence and risk factors of Q fever in goats on commercial dairy goat farms in the Netherlands, 2009-2010. BMC Veterianry Research 7, 81.

Smeeth, L., Fletcher, A.E., Stirling, S., Nunes, M., Breeze, E., Ng, E., Bulpitt, C.J., Jones, D., 2001. Randomised comparison of three methods of administering a screening questionnaire to elderly people: findings from the MRC trial of the assessment and management of older people in the community. BMJ 323, 1403-1407.

Smith, D.R., Grotelueschen, D.M., 2004. Biosecurity and biocontainment of bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 20, 131-149.

Van Steenwinkel, S., Ribbens, S., Ducheyne, E., Goossens, E., Dewulf, J., 2011. Assessing biosecurity practices, movements and densities of poultry sites across Belgium, resulting in different farm risk-groups for infectious disease introduction and spread. Preventive Veterinary Medecine 98, 259-270.

Villarroel, A., Dargatz, D.A., Lane, V.M., McCluskey, B.J., Salman, M.D., 2007. Suggested outline of potential critical control points for biosecurity and biocontainment on large dairy farms. Journal of the American Veterinary Medical Association 230, 808-819.

Wells, S.J., Dee, S., Godden, S., 2002. Biosecurity for gastrointestinal diseases of adult dairy cattle. Veterinary Clinics of North America-Food Animal Practice 18, 35-55.

Chapter 6

Re-infection

A cross-sectional and longitudinal study on the risk of BVDV re-infection in BVDV free cattle herds in Belgium

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Summary

In Belgium, the control of bovine viral diarrhoea virus (BVDV) occurs on a voluntary basis at the herd level. Studies have demonstrated that the essential measures for successful control are poorly implemented and the BVDV prevalence remains high. To better understand the drivers for re-infection with BVDV and to evaluate the risk of re-infection, a cross-sectional and a longitudinal study were conducted.

In the cross-sectional study young stock were sampled for BVDV antibodies and a face-to-face interview with the farmer was conducted on 61 farms with a history of BVDV circulation, but which were assumed BVDV free at the moment of participation. Potential risk factors for a herd being re-infected were identified by a multivariable logistic regression model. In 11 of the 61 herds (18%) at least 20% of the young stock tested BVDV seropositive. Farms monitoring the BVDV status were less likely to be BVDV re-infected (OR 0.09; 95% CI 0.02; 0.55). There was also a decrease in risk when the neighbouring cattle farm was located further away (OR per km 0.06; 95% CI 0.00; 0.96). Re-infection was more likely when farmers participated to auctions and/or competitions (OR 140.27; 95% CI 3.00; 6559.65).

In the longitudinal study the BVDV status of 26 tested BVDV free and non-vaccinating farms was monitored for 18 months by means of a spot test every 6 months. Positive spot tests were detected in 6 farms, corresponding to an incidence risk of re-infection of 15.4% per year. PI animals were detected on four of the re-infected farms. All mothers of the PI animals detected on the re-infected farms were BVDV virus-negative, which indicated that these dams were transiently infected with BVDV during gestation. The purchase of PI cattle was identified as cause of re-infection on one farm.

These results demonstrated a high risk of BVDV re-infection in Belgian cattle herds. Given the violations of essential measures in BVDV control (monitoring and biosecurity) and a high BVDV prevalence, the risk of BVDV re-infection is likely to remain high.

Introduction

Bovine viral diarrhoea virus (BVDV) is worldwide spread and can cause considerable economic losses in cattle (Gunn et al., 2005). The virus is able to infect multiple organ systems, including the uterus of animals in gestation (Baker, 1995). A congenital infection from the second up to the fourth month of gestation may result in the birth of a persistently infected (PI) calf (Peterhans et al., 2010). In a susceptible population BVDV is especially maintained through these PI animals, as they continuously shed BVDV in large quantities once colostrum-derived BVDV antibody titres have declined (Lindberg and Houe, 2005).

Given the economic importance of BVDV, several countries have launched control programmes. A general model for BVDV control was proposed by Lindberg and Houe (2005) and consists of three essential measures: elimination of PI animals to reduce the source of infection, implementation of biosecurity to prevent new BVDV infections and monitoring the BVDV status to detect re-infection. The implementation of vaccination is optional and can be used as an additional biosecurity measure when the risk of re-infection is high, for instance in cattle-dense areas with intense animal trading (Lindberg et al., 2006).

Studies have demonstrated that re-infections occur in regions where BVDV is endemic as well as in regions with a control programme. In the Netherlands, antibodies were detected each year in 5% to 10% of the herds participating in the voluntary BVDV control programme after these herds had been certified as 'BVDV free' (Mars and Van Maanen, 2005). In Denmark about 1% of the cattle herds that previously had been found free, was re-infected during the fourth year of the control and eradication programme (Bitsch et al., 2000).

In Belgium, an important beef cattle and milk producing country in Europe and a very densely populated livestock area, BVDV control occurs on a voluntary basis at the herd level. Although BVDV control strategies are well-described, studies have demonstrated that the essential measures for successful control are poorly implemented in the daily management of Belgian farmers. Many control strategies are based on vaccination alone, without tracking and removing PI animals and without further follow-up of the BVDV status (Laureyns et al., 2013). Furthermore, since few biosecurity measures are undertaken by Belgian cattle farmers, they expose their herd to re-infection with BVDV (Sarrazin et al., 2014). As a consequence, the BVDV prevalence remains high. About one-third of the young stock and half of the cattle

herds are BVDV seropositive in Belgium (Sarrazin et al., 2013). Recently, the outline of the first phase of a Belgian mandatory BVDV control programme has been published (Royal Decree on the control of BVD, 2014). The programme focuses on the detection of PI animals.

Given these conditions a considerable risk of re-infection is expected in Belgian cattle herds. To better understand the drivers for re-infection with BVDV and to evaluate the risk of re-infection, given the current circumstances in Belgian cattle herds, a cross-sectional and a longitudinal study were conducted.

Materials and methods

Selection of the farms

Cross-sectional study

The target population for this study comprised of cattle farms in Belgium. A first inclusion criterion was a history of BVDV circulation in the herd. BVDV circulation in the herd was defined as the detection of at least one PI and/or transiently infected (TI) animal. The second inclusion criterion was the eradication of BVDV from the herd and the assumption of being free from BVDV at the moment of participation. This assumption was based on the herd veterinarian's judgement and previous test results. Farms were selected as described by Sarrazin et al. (2014). In brief, herd veterinarians were contacted to propose cattle farms satisfying the inclusion criteria. Subsequently, proposed farms fulfilling the inclusion criteria were contacted to explain the study objectives and to verify their willingness to participate.

Longitudinal study

The target population for this study comprised of Belgian cattle farms that did not apply BVDV vaccination during the last 3 years. At the onset of the study the farms were assumed BVDV free based upon the herd veterinarian's judgement and previous test results in combination with a negative spot test during the first visit (see further 'Sample collection'). Farms were selected as described for the cross-sectional study.

Sample collection

For each farm included in the cross-sectional study, 10 animals aged 8-15 months were randomly selected and blood sampled for serologic examination (spot test). The spot test is an indirect screening for the presence of PI animals in the herd by testing a limited number of young stock (3-10 animals) for BVDV-specific antibodies (Houe, 1992; 1994). On farms with less than 10 animals in this age range, all animals in this age range (with a minimum of five animals) were sampled. On farms with more than one herd, each was sampled. All blood samples were collected by the first author between March 2011 and November 2013.

Farms considered to be included in the longitudinal study were sampled with a first spot test to verify their BVDV free status. On farms with no seropositive test results in the spot test during this first visit, three additional spot tests were performed every 6 months. All blood samples were collected by the first author between March 2011 and November 2013.

Laboratory analysis

For the detection of BVDV-specific antibodies in the spot test a commercial enzyme-linked immunosorbent assay (ELISA) was used (SERELISA® BVD p80 Ab Mono Blocking, Synbiotics). This ELISA provides a specificity and sensitivity of respectively 97.7% and 95.7% (BVD Technical Brochure). Analyses were done on serum samples according to the instructions provided by the manufacturer. Analyses were performed by the regional centre for animal disease control "Dierengezondheidszorg Vlaanderen (DGZ)".

Data collection

In the cross-sectional study farmers were subjected to a face-to-face questionnaire interview, conducted by the first author. The questionnaire was designed to collect data regarding BVDV management and control at the herd level. According to the control strategy plan of Lindberg and Houe (2005), the questionnaire consisted of 93 questions on four main topics: biosecurity, virus detection, monitoring and vaccination. Most questions were semi-closed. Except for the language (Flanders = Dutch and Wallonia = French), the questionnaires for both parts of the country were identical and checked for consistency by a bilingual researcher. The full questionnaire is available upon request to the first author. The

questionnaire was pre-tested regarding content, interpretation of questions and responses. It was emphasised to the farmers that questionnaires would be processed anonymously.

When during the longitudinal study recirculation of BVDV was suspected following at least one seropositive animal in the spot test, possible causes of infection were explored using a farm-specific questionnaire investigation and additional sample collection if necessary.

Data analysis

Cross-sectional study

A farm was classified as infected with BVDV when more than 20% of the sampled animals tested positive for the presence of BVDV antibodies. The outcome of interest was therefore binary, presence or absence of BVDV infection at the herd level.

First, univariable analysis was performed of each potential risk factor obtained in the questionnaire. For categorical variables the proportion of herds positive within each of the categories was compared using a chi-square test. The association of continuous variables with the outcome of interest was verified by univariable logistic regression. Subsequently, all variables with a p-value < 0.25 were selected.

The correlation between categorical variables was evaluated using a chi-square test ($p < 0.05$) and Pearson and Spearman correlation coefficients were estimated ($r > 0.6$) to verify correlation between continuous variables. The choice of which correlated variable to include in the multivariable model was based on the variable with the smallest p-value in the univariable analysis. Subsequently a multivariable model was built in a stepwise backward manner resulting in a model in which only significant risk factors ($p < 0.05$) were retained based on a likelihood ratio test. Odds ratios, including 95% CI, are reported for all significant variables. Confounding was checked for by monitoring the change in regression parameters and was considered to be present if parameters changed by > 0.25. In the final model all two-way interactions were tested and significant interactions ($p < 0.05$) were taken up in the model. The goodness-of-fit of the final multivariable model was tested using the Hosmer-Lemeshow test (Hosmer and Lemeshow, 2000) with a significance level set at 5% and by examination of the residuals. All statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

Longitudinal study

A farm was classified as infected with BVDV (case) when at least one seropositive animal was detected in the spot test.

Results

Cross-sectional study

A total of 74 farms were willing to participate, of which 13 farms had no documented history of BVDV circulation in the herd. Therefore, the latter farms were excluded from the cross-sectional study. A total of 61 farms were considered for further analysis in the cross-sectional study.

General characteristics of the selected farms

Of the selected farms, 25 were dairy farms, 16 beef farms and 20 mixed farms (farms containing beef and dairy cattle). The average number of cattle present at the studied farms was 140 (SE 9), ranging from 30 to 370. At the moment of the farm visit the farms were believed to be BVDV free (i.e. removal of the last PI animal or detection of a TI animal) for on average 5 years (SE 0.4), ranging from 1 to 15 years. After conducting the spot test, 11 out of 61 farms (18.0%) were indicated as BVDV infected, since more than 20% of the sampled young stock was BVDV seropositive following the spot test.

Risk factor analysis

A total of 15 variables tested had $p < 0.25$, of which seven variables were not included in the multivariable model due to correlation (data not shown). “Purchase of cattle”, a well-described risk factor for BVDV infection, was correlated with “Participation to auctions/competitions” ($p = 0.02$) and since the latter variable had the smallest p-value in the univariable analysis, this variable was included in the multivariable model. In the final multivariable model three factors remained significantly associated with BVDV re-infection

at the herd level: "Monitoring for BVDV", "Distance to the nearest cattle farm" and "Participation to auctions/competitions" (Table 1). No confounding or significant two-way interactions were detected. The model fitted the data sufficiently well, as no significant lack of fit was detected with the Hosmer and Lemeshow test (p = 0.39) and residual analysis. Farms monitoring the BVDV status of the herd had a significant lower probability of being BVDV re-infected, with a more or less 10-fold lower odds compared to farms without BVDV monitoring (p = 0.004). There was also a decrease in risk when the neighbouring cattle farm was located further away: per km to the nearest cattle farm the odds of infection was 17 times lower (p = 0.007). An increase in the probability of BVDV infection was detected when farmers participate to auctions and/or competitions (p < 0.001).

Table 1. Multivariable odds ratios (OR) and 95% confidence intervals (CI) for risk factors associated with a herd to be BVDV re-infected in the cross-sectional study (n = 61).

Risk factor	Category	No. of herds	% positive	*P*	OR	95% CI	
						Lower	Upper
Monitoring BVDV status	No	21	33.3	0.004			
	Yes	40	10.0		0.09	0.02	0.55
Participation to auctions/competitions	No	56	14.3	< 0.001			
	Yes	5	60.0		140.27	3.00	6559.65
Distance to nearest cattle farm (average: 0.6 km; 95% CI 0.4; 0.8)	-	-	-	0.007	0.06	0.00	0.96

Longitudinal study

A total of 36 non-BVDV-vaccinating farms were identified to be potentially included in the longitudinal study. Of these farms, 26 (10 dairy, 5 beef and 11 mixed) had a negative spot test during the first visit and were further monitored in the longitudinal study. Of the farms included in the longitudinal study, 20 farms had a history of BVDV circulation in the herd but were cleared from BVDV at the onset of the study whereas 6 herds had no indications of any previous BVDV infection.

Infection with BVDV was detected on 6 out of 26 farms (23.1% in 18 months), which resulted in an incidence risk of BVDV infection of 15.4% per year. BVDV re-infection was detected in 5 out of 20 (25.0%) farms with a history of BVDV circulation in the herd, while one farm with no previous history of BVDV circulation (16.7%) was BVDV infected. At least one PI animal was detected in four of the infected farms (Table 2). On the two other re-infected farms (case 2 and 6) no virus-positive animals were detected during a screening of the entire herd and no additional seropositive animals were detected in the spot test during the final farm visit.

The cause of re-infection could be conclusively confirmed in one herd (case 3). On this farm an animal in gestation was purchased 2 months before the third spot test. The animal was tested for BVDV upon arrival and calved before the result of the analysis was available. The cow was moved to the lactating group and the calf was housed with other new-born calves. Test results revealed that the purchased cow was PI and by consequence also her calf was PI.

When testing the mothers of the detected PI animals (case 1, 4 and 5) for BVDV viraemia, all dams were virus-negative, which indicated the PI animals resulted from transient BVDV infections of the dams during gestation.

Table 2. Overview of the BVDV infected herds in the longitudinal study (n = 6). Following a negative spot test during the first visit three additional spot tests were performed, each with an interval of 6 months. Per spot test the total number of tested animals and the number of seropositive (+) or non-interpretable (between negative and positive; NI) test results are shown. Additionally, the period during which the farms had been BVDV free at the moment of the first spot test, the number of detected PI animals during the longitudinal study, the herd size and the monitoring strategy applied by the herd veterinarian are presented.

Case	Spot test				Assumed BVDV free since (years)	No. of PI animals detected	Herd size (No. of cattle)	Monitoring strategy applied by the herd veterinarian
	1	2	3	4				
1	0/10 +	3/9 + 1/9 NI	5/8 +	2/10 + 2/10 NI	6	2	190	Virological testing (pools) of calves born during the last year
2	0/10 +	4/10 + 2/10 NI	1/9 NI	0/10 +	4	0	130	Spot test (every year)
3	0/6 +	0/8 +	2/9 + 2/9 NI	0/10 +	1.5	2	92	Spot test (every year)
4	0/8 +	9/10 +	No visit	1/10 NI	6	8	130	Individual virological testing of new-born calves
5	0/10 +	0/9 +	No visit	2/8 +	2	1	210	Virological testing of bulk tank milk (every 6 months)
6	0/8 +	0/10 +	3/11 +	0/10 +	No history	0	75	Spot test (every 6 months)

Discussion

This paper reports on the risk of BVDV re-infection in Belgian cattle herds that previously had been considered free from BVDV. As can expected in a region with a high BVDV prevalence, a high cattle density and no systematic BVDV control, a high frequency of re-infection was demonstrated both in the cross-sectional and the longitudinal study. Similar studies were already performed in a country with (Bitsch et al., 2000) and without systematic BVDV control (Mars and Van Maanen, 2005), but in the present study the risk of re-infection was considerably higher. Furthermore, biologically plausible risk factors for re-infection could be identified despite the limited sample size.

Instead of testing all animals in the herd for BVDV viraemia, the spot test was used to verify the BVDV status of the herd. The spot test is less expensive and time-consuming and is able to detect a recent or current infection when used in young animals (Houe, 1992; Brülisauer et al., 2010; Laureyns et al., 2010). It is obvious that sampling a subset of the herd to obtain information on the entire herd is less sensitive compared to testing every individual animal. Factors that may impair the sensitivity of the spot test have already been discussed (Sarrazin et al., 2013). Nevertheless, the reliability of the spot test increases by repeating it at regular intervals (Houe, 1999), as was clearly demonstrated during the longitudinal study.

In the cross-sectional study a herd was considered BVDV infected when at least 20% of the young stock was BVDV seropositive. However, there is no clear recommendation in the scientific literature regarding a cut-off value (Cuttance and Cuttance, 2014). A similar cut-off was used by Gates et al. (2013a; 2013b), based on the results from Brülisauer et al. (2010). Moreover, no DIVA (Differentiating Infected from Vaccinated Animals) vaccines for BVDV are available and a seropositive test result may thus originate from vaccination (Raue et al., 2011). Finally, given the specificity of 97.7% of the used antibody test and the number of animals tested (n = 559), false positive results are expected. Since the animals were not retested in the cross-sectional study, it is impossible to determine which of the seropositive animals could have been false positive, but it is possible that spot tests with only one single seropositive animal were in fact false positive and these herds (n = 6) were therefore not considered as infected.

The assumption of the selected farms being BVDV free at the moment of participation was based on the herd veterinarian's judgement and previous test results. Farms in the cross-sectional study were believed to be BVDV free for on average 5 years, but 34% (21/61) of these farms did not monitor the BVDV status after elimination of the PI animals (Table 1). As a consequence it is likely that some of the re-infected farms had already been re-infected for a longer period than assumed by the herd veterinarians, who were convinced that the farms they proposed for the study were still BVDV free. For the longitudinal study only farms with a negative spot test following the first farm visit were selected, hereby increasing the probability that the selected farms were actually still BVDV free at the onset of the study.

The importance of monitoring the BVDV status has clearly been demonstrated in this study. First, farms continuing to verify the BVDV status were less likely to be re-infected following the multivariable model. Secondly, six herds having a negative spot test during the first visit eventually became BVDV infected in the longitudinal study. Thirdly, several of the assumed free herds turned out to be not free from BVDV. This result indicates that the BVDV free status should be confirmed on a regular basis by means of diagnostic methods and cannot be based on a clinical evaluation solely. However, in 2 out of 6 farms considered infected following seropositive results in the spot test no virus-positive animals were detected during a screening of the entire herd (case 2 and 6). A possible explanation could be that a PI animal was no longer present at the moment of the screening of the herd (Lindberg and Houe, 2005), e.g. a male calf that was sold for fattening within 2 weeks after birth or a PI animal that died before it was identified as PI. Additionally, BVDV can be spread when PI animals are aborted or stillborn (Lindberg et al., 2004). The detection of animals that have been TI in absence of PI animals could be another explanation. Given the low implementation of biosecurity measures and frequent contact between farms on the one hand (Sarrazin et al., 2014) and a high BVDV prevalence in Belgian cattle herds on the other hand (Sarrazin et al., 2013), transient infections following direct virus transmission (e.g. fence-line pasture contact or during transport) or indirect transmission (e.g. professional visitors with no farm-specific clothing) are likely. To verify whether the virus spread is maintained in the herd the spot test can be repeated 3 months later (Laureyns et al., 2010) or can be repeated with a larger sample, e.g. by including heifers (Pillars and Grooms, 2002; Houe et al., 2006).

Besides monitoring the BVDV status by serologic evaluation of young stock, herd veterinarians also used pooled samples of non-coagulated blood from a group of calves, ear notch sampling or bulk tank milk samples to directly detect virus-positive animals as

monitoring strategies (Table 2). However, it was noticed that calves destined for fattening and staying on the farm only for a short period of time very often were not tested to reduce the costs. Yet, although PI calves under protection of colostral antibodies shed only low amounts of BVDV (Palfi et al., 1993), all animals should be tested when testing BVDV viraemia to avoid false negative results in the detection of PI animals (Laureyns et al., 2010).

The purchase of cattle was not retained in the multivariable model of the cross-sectional study as this variable was strongly correlated with "Participation to auctions/competitions". However, the purchase of cattle was identified as a cause of re-infection in the longitudinal study (case 3). Furthermore, another re-infected farm (case 5) had also purchased BVDV viraemic animals without putting the animals in quarantine while awaiting the purchase test results, but housed the animals in a shed where contact with other cattle was possible. In both cases the purchased animals were tested for BVDV viraemia, but they were not strictly quarantined until the result of the blood test was known. Strict quarantine includes no direct animal contact and additional hygiene measures such as changing footwear and work clothes before entering and leaving the quarantine area (Villarroel et al., 2007). Furthermore, for diseases with a short incubation period such as BVDV animals should be quarantined for at least 3 weeks (Barrington et al., 2002; Villarroel et al., 2007). As pregnant PI dams invariably transmit the virus to the foetus, resulting in new generations of PI calves (Moennig and Liess, 1995), the calf of the purchased PI heifer (case 3) was also PI, which was rapidly detected following the positive test result of the dam. However, attention must also be paid to the risk of the 'Trojan' cow or heifer, i.e. a non-PI animal that was transiently infected during gestation and that is carrying a PI foetus as a result (Laureyns et al., 2010; Lanyon et al., 2014). When testing such a dam at purchase, she will be virus-negative, but she will introduce BVDV in the herd by giving birth to a PI animal. As a consequence, the calf of a purchased dam should be quarantined until testing for the presence of BVDV. Moreover, purchased animals should calve while they are still in quarantine, as BVDV is transmitted at the birth of PI calves through foetal fluids and uterine lochia, hereby contaminating the environment (Lindberg et al., 2004). In another study investigating re-infection with BVDV the purchase of a pregnant cow or heifer was found to be the cause in 28% of the cases (Bitsch et al., 2000).

Although it is required for cattle to be BVDV free when participating to auctions or competitions in Belgium, this parameter was identified as a risk factor for re-infection and indicates that control measures such as quarantine and testing should also be used for cattle

returning to a farm (Villarroel et al., 2007). Even when every animal is virus-negative at auctions, indirect transmission of BVDV is possible through persons (Ståhl et al., 2005) or cattle trailers. The latter seem a plausible source of infection, as BVDV can persist in the environment (Niskanen and Lindberg, 2003).

Farms were more likely to be BVDV re-infected when the neighbouring farm was located nearby. This risk factor was already described in other studies (Houe, 1999; Ersbøll et al., 2010; Gates et al., 2013b) and is of concern, given the short distances between Belgian cattle farms: the mean distance was 0.6 km (Table 1) and the minimum distance was only 0.04 km (data not shown). A possible route of transmission could be fence-line pasture contact, which is frequently possible on Belgian cattle farms (Sarrazin et al., 2014) and has already been described as a risk factor (Valle et al., 1999). This way of transmission was also hypothesized as cause of re-infection in the longitudinal study (case 1, 2 and 4), but could not be confirmed. Airborne transmission of BVDV has also been described as a local mechanism for BVDV spread, but only for short distances up to 10 m (Niskanen and Lindberg, 2003).

Assumed self-clearance or the elimination of BVDV from the herd without intervention (Lindberg and Houe, 2005) was observed in two herds (case 2 and 6): no virus-positive animals were detected during the screening of the whole herd, no PI animals were born during the year following the positive spot test and/or no additional positive spot test results were obtained. Self-clearance especially tends to occur in smaller herds (Lindberg and Houe, 2005), but was also noticed irrespective of herd size in regions with a high cattle density and high BVDV prevalence (Ståhl et al., 2008). Herd size in case 2 and 6 was 130 and 75, respectively. In case 3 also no additional PI animals were detected and the final spot test was negative. However, ear notch sampling was only used for female calves and the birth of male PI animals that remained unnoticed can therefore not be excluded.

During the longitudinal study, one farmer decided to move a detected PI animal to his father's farm (case 1). Nonetheless, the spot tests remained positive. As the father frequently enters the farm without using farm-specific clothing, these two geographically separated units should be considered as one entity (Laureyns et al., 2010). Given the frequent contacts between these units, indirect BVDV transmission is possible (Ståhl et al., 2005; Niskanen and Lindberg, 2003). Other studies suggest that BVDV can continue to spread for a long time after the removal of PI animals (Moerman et al., 1993; Moen et al., 2005), but also the birth of

additional PI animals in this herd cannot be excluded since only female calves were tested for BVDV.

In four of the herds where re-infection was noticed during follow-up (case 1, 3, 4 and 5) at least one PI animal was detected. Once PI animals are removed, the herd will gradually become seronegative, starting with young calves losing their colostral antibodies (Houe et al., 2006). BVDV re-introduction in such a susceptible population can result in an outbreak as demonstrated in case 4, where no less than eight PI animals were born. In regions with a high risk of re-infection, vaccination can limit the spread of infection within a population and reduce disease severity in an infected animal (Ridpath, 2013). However, for vaccination to succeed, also the three essential measures for systematic BVDV control have to be implemented: biosecurity, removal of PI animals and monitoring (Lindberg and Houe, 2005; Ridpath, 2013).

Conclusions

This paper demonstrated a considerable risk of BVDV re-infection in Belgian cattle herds. Although not monitoring the BVDV status was identified as a risk factor for BVDV re-infection and the fact that the importance of this measure is well-described, it was noticed that monitoring often was not or wrongly performed. Furthermore, nevertheless the purchase of cattle is known to be a frequent cause of infection, essential biosecurity measures to prevent BVDV infection such as quarantine were not implemented. Given these violations of essential measures in BVDV control, the short distances between cattle farms and a high BVDV prevalence, the risk of BVDV re-infection in Belgian cattle herds is likely to remain high.

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Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of this paper.

References

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Veterinary Clinics of North America-Food Animal Practice 11, 425-445.

Barrington, G.M., Gay, J.M., Evermann, J.F., 2002. Biosecurity for neonatal gastrointestinal diseases. Veterinary Clinics of North America-Food Animal Practice 18, 7-34.

Bitsch, V., Hansen, K.E.L., Ronsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994-1998 with special reference to legislation and causes of infection. Veterinary Microbiology 77, 137-143.

Brülisauer, F., Lewis, F.I., Ganser, A.G., McKendrick, I.J., Gunn, G.J., 2010. The prevalence of bovine viral diarrhoea virus infection in beef suckler herds in Scotland. The Veterinary Journal 186, 226-231.

Cuttance, W., Cuttance, E., 2014. Analysis of individual farm investigations into bovine viral diarrhoea in beef herds in the North Island of New Zealand. New Zealand Veterinary Journal 62, 338-342.

Ersbøll, A.K., Ersbøll, B.K., Houe, H., Alban, L., Kjeldsen, A.M., 2010. Spatial modelling of the between-herd infection dynamics of bovine virus diarrhoea virus (BVDV) in dairy herds in Denmark. Preventive Veterinary Medicine 97, 83-89.

Gates, M.C., Humphry, R.W., Gunn, G.J., 2013a. Associations between bovine viral diarrhoea virus (BVDV) seropositivity and performance indicators in beef suckler and dairy herds. The Veterinary Journal 198, 631-637.

Gates, M.C., Woolhouse, M.E.J., Gunn, G.J., Humphry, R.W., 2013b. Relative associations of cattle movements, local spread, and biosecurity with bovine viral diarrhoea virus (BVDV) seropositivity in beef and dairy herds. Preventive Veterinary Medicine 112, 285-295.

Gunn, G.J., Saatkamp, H.W., Humphry, R.W., Stott, A.W., 2005. Assessing economic and social pressure for the control of bovine viral diarrhoea virus. Preventive Veterinary Medicine 72, 149-162.

Hosmer, D.W., Lemeshow, S., 2000. Applied Logistic Regression, 2nd ed. John Wiley and Sons, New York, USA, pp. 143-186.

Houe, H., 1992. Serological analysis of a small herd sample to predict presence or absence of animals persistently infected with bovine viral diarrhea virus (BVDV) in dairy herds. Research in Veterinary Science 53, 320-323.

Houe, H., 1994. Bovine virus diarrhea virus - Detection of Danish dairy herds with persistently infected animals by means of a screening-test of 10 young stock. Preventive Veterinary Medicine 19, 241-248.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterinary Microbiology 64, 89-107.

Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. Journal of Veterinary Diagnostic Investigation 18, 427-436.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. The Veterinary Journal 199, 201-209.

Laureyns, J., Booth, R., Sarrazin, S., Deprez, P., Pfeiffer, D., Dewulf, J., De Vliegher, S., 2013. Assessment of two essential elements of BVDV control on selected Flemish dairy and beef farms. Vlaams Diergeneeskundig Tijdschrift 82, 356-362.

Laureyns, J., Ribbens, S., de Kruif, A., 2010. Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. The Veterinary Journal 184, 21-26.

Lindberg, A., Brownlie, J., Gunn, G.J., Houe, H., Moennig, V., Saatkamp, H.W., Sandvik, T., Valle, P.S., 2006. The control of bovine viral diarrhoea virus in Europe: today and in the future. Revue Scientifique et Technique-Office International des Epizooties 25, 961-979.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Veterinary Record 154, 463-467.

Mars, M.H., Van Maanen, C., 2005. Diagnostic assays applied in BVDV control in The Netherlands. Preventive Veterinary Medicine 72, 43-48.

Moen, A., Sol, J., Sampimon, O., 2005. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Preventive Veterinary Medicine 72, 93-98.

Moennig, V., Liess, B., 1995. Pathogenesis of Intrauterine Infections with Bovine Viral Diarrhea Virus. Veterinary Clinics of North America-Food Animal Practice 11, 477-487.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long-term epidemiologic-study of bovine viral diarrhea infections in a large herd of dairy-cattle. Veterinary Record 132, 622-626.

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. The Veterinary Journal 165, 125-130.

Palfi, V., Houe, H., Philipsen, J., 1993. Studies on the decline of bovine virus diarrhea virus (bvdv) maternal antibodies and detectability of bvdv in persistently infected calves. Acta Veterinaria Scandinavica 34, 105-107.

Peterhans, E., Bachofen, C., Stalder, H., Schweizer, M., 2010. Cytopathic bovine viral diarrhea viruses (BVDV): emerging pestiviruses doomed to extinction. Veterinary Research 41.

Pillars, R.B., Grooms, D.L., 2002. Serologic evaluation of five unvaccinated heifers to detect herds that have cattle persistently infected with bovine viral diarrhea virus. American Journal of Veterinary Research 63, 499-505.

Raue, R., Harmeyer, S.S., Nanjiani, I.A., 2011. Antibody responses to inactivated vaccines and natural infection in cattle using bovine viral diarrhoea virus ELISA kits: Assessment of potential to differentiate infected and vaccinated animals. Veterinary Journal 187, 330-334.

Ridpath, J.F., 2013. Immunology of BVDV vaccines. Biologicals 41, 14-19.

Royal Decree on the control of BVD, 18 June 2014. Published 30 June 2014. www.ejustice.just.fgov.be (Accessed 31 October 2014)

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A survey on biosecurity and management practices in selected Belgian cattle farms. Preventive Veterinary Medicine 117, 129-139.

Sarrazin, S., Veldhuis, A., Meroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Cay, A.B., Piepers, S., Hooyberghs, J., Ribbens, S., Van Der Stede, Y., 2013. Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Preventive Veterinary Medicine 108, 28-37.

Ståhl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-Lopez, J., Belak, S., Alenius, S., Lindberg, A., 2005. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. Preventive Veterinary Medicine 72, 103-108.

Ståhl, K., Lindberg, A., Rivera, H., Ortiz, C., Moreno-López, J., 2008. Self-clearance from BVDV infections - a frequent finding in dairy herds in an endemically infected region in Peru. Preventive Veterinary Medicine 83, 285-296.

Valle, P.S., Martin, S.W., Tremblay, R., Bateman, K., 1999. Factors associated with being a bovine-virus diarrhoea (BVD) seropositive dairy herd in the More and Romsdal County of Norway. Preventive Veterinary Medicine 40, 165-177.

Villarroel, A., Dargatz, D.A., Lane, V.M., McCluskey, B.J., Salman, M.D., 2007. Suggested outline of potential critical control points for biosecurity and biocontainment on large dairy farms. Journal of the American Veterinary Medical Association 230, 808-819.

Chapter 7

General Discussion

Introduction

The control of bovine viral diarrhoea virus (BVDV) in Belgium has been on a voluntary basis at the herd level for about 20 years. Despite the voluntary control programme, this doctoral thesis has demonstrated that the BVDV prevalence in Belgian cattle herds is high (***Chapter 3***). Reasons for this high BVDV prevalence in Belgium will be discussed in the first paragraph of this general discussion.

Currently more efficient diagnostic BVDV tests are available, which can reduce the costs of a BVDV control programme at the national level. Taken into account the high BVDV prevalence in Belgium and the economic importance of BVDV, a mandatory BVDV control programme was launched the 1st of January 2015. The second paragraph introduces the outline of this programme as it is currently designed.

Elimination of PI animals to reduce the source of infection, implementation of biosecurity to prevent new BVDV introductions and monitoring the BVDV status to detect re-infection are described as necessary measures to obtain and maintain a BVDV free status. Assessment how these necessary elements for BVDV control are implemented and whether they are essential to maintain a BVDV free status in Belgian cattle herds, given their current implementation and the high BVDV prevalence is another aim of this doctoral thesis. Therefore, the third paragraph of this general discussion is dedicated to recommendations on each specific level of BVDV control, including vaccination. These recommendations will be discussed in the light of the mandatory Belgian BVDV control programme.

Factors contributing to a high BVDV prevalence in Belgian cattle herds

A first step in the decision process to control a disease at the national level is to verify to what extent the disease poses a problem (Saatkamp et al., 2006), for instance the impact on animal health, its economic consequences and prevalence. The impact of BVDV on animal health and the economic consequences of BVDV infections are important drivers for control

in regions where the virus is endemic (Lindberg et al., 2006) and have already been described (***General Introduction***). It is thus important to have a good insight into the current BVDV prevalence (Brülisauer et al., 2010). Knowledge about this prevalence makes it possible to evaluate whether any progress is made during BVDV control (Lindberg et al., 2006; Barrett et al., 2011). In Belgium, the true prevalence of BVDV-specific antibodies at the herd and animal level (young stock) was estimated to be 47.4% and 32.9%, respectively (***Chapter 3***). This high BVDV prevalence in Belgian cattle herds clearly suggests that the a voluntary approach in BVDV control had little effect and that a more coordinated approach in the form of a mandatory control programme is needed. Moreover, the results also raise questions about the reasons for this high prevalence. Whenever these results are compared with prevalence studies in other countries before initiating a control or eradication programme, caution has to be applied to the target population and the methodology used (e.g. blood samples versus bulk tank milk samples) (Brülisauer et al., 2010). In Switzerland, 22.9% of young stock within the same age category (6-12 months) as in the Belgian prevalence study were BVDV seropositive (Rüfenacht et al., 2000). Another study where young stock was sampled demonstrated that 11.6% to 19.7% of the Scottish beef suckler herds had young stock with a median seroprevalence of 91.9 to 99.8% (Brülisauer et al., 2010). In general, regardless of the target population and the methodology used, BVDV herd seroprevalence in European countries before initiating a mandatory national control or eradication programme varied between 1% (Finland) and 100% (Switzerland) (Rüfenacht et al., 2000; Greiser-Wilke et al., 2003).

Cattle density and herd size

In Belgium 2,441,319 domestic cattle were registered in the animal identification and registration system (SANITEL) in November 2013 (NIS, 2013), which results in an average cattle population density at municipality level of 85 animals/km² (OIE, 2013). Together with the Netherlands, Belgium is one of the most densely populated cattle areas in Europe. It has been shown in this thesis that the average distance to neighbouring farms is small (***Chapter 5***): the mean distance to the nearest cattle farm was only 0.57 km (95% CI 0.39; 0.75). Moreover, it was also shown that the odds for a farm to be BVDV seropositive was 16.7 times higher when the distance to the nearest cattle farm decreased with 1 km (***Chapter 6***).

For several years both the number of cattle farms and heads of cattle have been declining in Belgium. However, the number of cattle farms is decreasing faster than the

number of cattle, which indicates that the average herd size is increasing. In November 2013 the average herd size of Belgian cattle farms was 120 animals (NIS, 2013).

As the transmission rate of an infectious disease is influenced by the number of contacts per unit of time (Lindberg and Houe, 2005), a high cattle density and herd size can facilitate BVDV spread. Studies have shown that both herd size (***Chapter 3***; Ersbøll and Stryhn, 2000; Humphry et al., 2012; Graham et al., 2013; Cuttance and Cuttance, 2014) and herd density (Houe et al., 1995a; Houe, 1999) are associated with the presence of BVDV.

When discussing transmission mechanisms for local BVDV spread, pasture contact is considered a very important route of infection (Gates et al., 2013b). Although zero-grazing is becoming more and more popular for dairy cattle, in Belgium most cattle are grazing and in 70% of the farms fence-line contact with cattle from other farms is possible on pastures (***Chapter 4***). Pasture contacts are shown to be associated with (Valle et al., 1999) or the cause of BVDV infections (Bitsch et al., 2000).

With an increasing herd size it is less likely that self-clearance of BVDV occurs, i.e. the elimination of BVDV from the herd without intervention (Lindberg and Houe, 2005; Ståhl et al., 2008). With reproduction ratios (R) of $+\infty$ (95% CI 1.88; $+\infty$) for PI animals and < 1 (point estimates of 0.24, 0.25 and 0.49 depending on the strain) for TI animals (***Chapter 4***), it is obvious that self-clearance tends to occur when no PI animals are present in the herd, since these data indicate that only PI animals are able to maintain BVDV in a herd. As PI animals originate from infections of susceptible animals in early pregnancy, the probability of self-clearance is likely to be negatively associated with the number of susceptible animals in early pregnancy and thus indirectly with the herd size (Lindberg and Houe, 2005). The importance of transient BVDV infections of cattle in early pregnancy as the source of PI animals was demonstrated in the longitudinal study (***Chapter 6***), where all mothers of the detected PI animals were no PI animals themselves. They had by consequence been infected in early pregnancy. This issue is further discussed below.

Yet, herd size, herd density and initial prevalence are not meaningful predictors for the prospects for successful reduction in BVDV prevalence. It rather is the way in which control activities are organized and implemented that will determine the progress (Lindberg and Houe, 2005).

Contact with cattle through national and international trade

BVDV is still present in all the neighbouring countries of Belgium. In the Netherlands no mandatory control programme at the national level is implemented and BVDV is endemic (Mars and Van Maanen, 2005). In France BVDV is only controlled in specific regions (Holleville, 2013; Maurin, 2013; Potaufeux, 2013), whereas mandatory control and eradication programmes at the national level are on-going in Germany (Ståhl and Alenius, 2012) and Luxembourg (Brownlie and Booth, 2014). Of the approximately 168,000 imported cattle in Belgium in 2011, 95% originated from these neighbouring countries and only 164 animals came from countries where BVDV has been eradicated (FAO, 2014). The majority of the imported cattle are calves destined for fattening, approximately 91% during the period 2005-2009 (Ensoy et al., 2014). Nonetheless, the introduction of BVDV in herds via imported virus-positive cattle was possible, as is was not compulsory to test these animals for BVDV viraemia. Additionally, a same amount of cattle was exported from Belgium in 2011 (FAO, 2014). Due to its favourable central location, there is also a considerable amount of through traffic of cattle in Belgium. In the current Belgian mandatory control programme all imported cattle born after 1 January 2015 has to be tested for BVDV viraemia (see below).

When considering national cattle trade, animals are usually transported over short distances within the same province or to neighbouring provinces (Ensoy et al., 2014). According to the same study the Walloon region (southern part of Belgium) appeared to be an important source of movement towards the rest of the country, with the provinces West and East Flanders as important receivers. A study of Ribbens et al. (2012) demonstrated that almost half of the presumed PI animals (Ag-ELISA or RT-PCR positive) were first being moved to another herd in Flanders (northern part of Belgium) before being classified dead in the animal identification and registration system. This was also observed in the longitudinal study of this thesis (***Chapter 6***). Based on the study of Ribbens et al. (2012) it has been estimated that presumed PI animals had been present in about 6.3% of all active cattle herds in Flanders between October 2009 and October 2011. These results show that, in Belgium, PI animals very often are traded, even when the BVDV status is known, which stresses the need of testing purchased cattle and a more strict legislation concerning the trade of PI animals.

Unawareness of the presence

About 70% to 90% of acute BVDV infections occur with only mild fever and leukopenia (Ames, 1986; Baker, 1995). Although these subclinical infections cause economic losses by affecting animal productivity (Fourichon et al., 2005; Gates et al., 2013a), very often they remain unnoticed by the farmer. In the herds where the presence of a PI animal was assumed based on a positive spot test (≥ 60% seropositive), 83.4% of the farmers stated not to have had any BVDV-related problems (***Chapter 3***). Furthermore, clinical presentations of BVDV infections widely vary, making it a challenge for the farmer and veterinarian to detect the presence of BVDV based on clinical signs (Laureyns, 2014).

Therefore, laboratory tests are needed to confirm the presence of BVDV (Sandvik, 2005). First, the herd status is verified by a spot test or bulk tank milk analysis. When the presence of PI animals is assumed, the BVDV status of all individual animals needs to be tested. As a consequence, analysis costs may mount up which may withhold farmers from performing a screening of the entire herd, even when the presence of a PI animal is suspected (Laureyns et al., 2011).

As BVDV can cause abortion (Grooms, 2004), laboratory investigations into cases of abortion may provide an additional way of identifying (newly) infected herds. However, the annual report of 2013 of the regional centre for animal disease control in the southern part of Belgium (ARSIA) showed that it is assumed that only about 7% of the abortions in cattle are reported (ARSIA, 2013). Moreover, the same report showed that about 29% of the herds with at least one calving per year did not report an abortion for 3 years. Taken into account that Belgian farmers are assumed to report each abortion within the framework of the preservation of the free status of brucellosis and that BVDV analyses on aborted foetuses are performed for free, it is regrettable that this abortion protocol is not used more frequently.

Systematic versus non-systematic BVDV control

BVDV control strategies can be described as being either non-systematic or systematic (Lindberg et al., 2006). A systematic approach implies that there is a goal oriented reduction in the incidence and prevalence of BVDV infections. Three essential elements of systematic control approaches can be identified (Lindberg and Houe, 2005; Lindberg et al., 2006): a) biosecurity measures to prevent (re)introduction, b) elimination of PI animals from infected

herds, and c) monitoring the BVDV status. Non-systematic approaches lack one or more of these elements. In cattle-dense areas with intense cattle trading and a high BVDV prevalence such as Belgium, vaccination as additional biosecurity measure may be advised to avoid infection (Lindberg et al., 2006). For vaccination to succeed, also the three essential measures for systematic BVDV control have to be implemented (Lindberg and Houe, 2005; Ridpath, 2013). Based on this, the general model for BVDV control as proposed by Lindberg and Houe (2005) was slightly modified by Laureyns (2014) (Fig. 1).

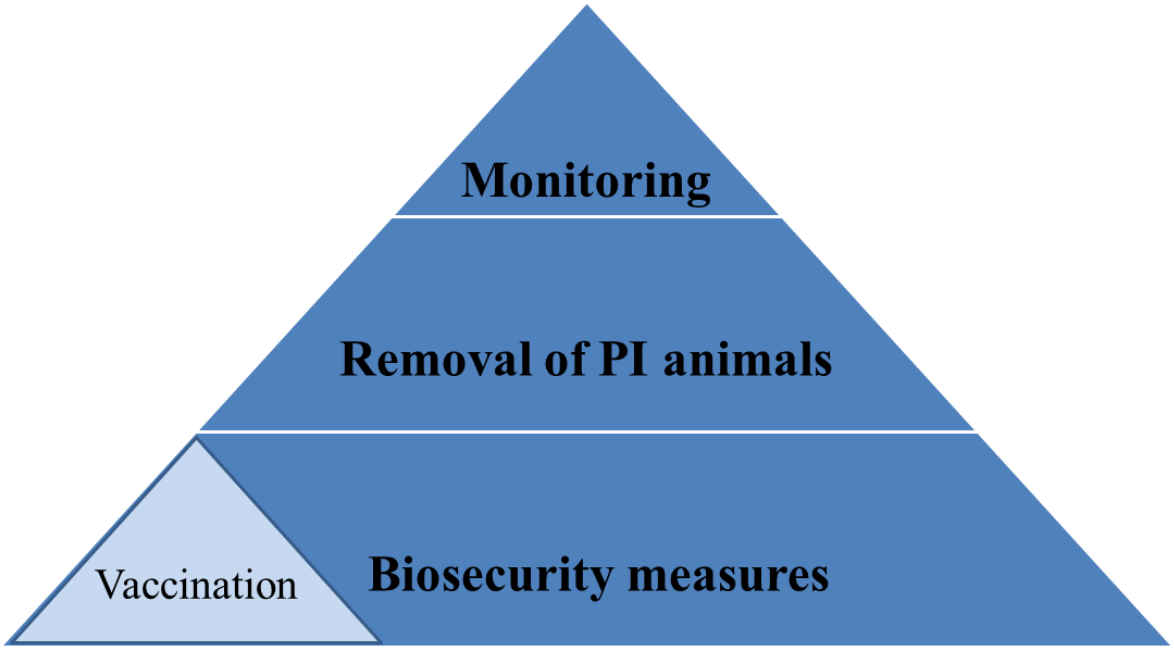


Fig. 1. The general model for BVDV control as proposed by Lauryens (2014) and adapted from Lindberg and Houe (2005) consists of three essential measures: biosecurity measures, removal of PI animals and monitoring. In Belgium, a cattle-dense areas with intense cattle trading and a high BVDV prevalence, vaccination as additional biosecurity measure may be advised to avoid infection.

Systematic control can be implemented either at the herd level or at the regional/national level. In Belgium recommendations for systematic control were proposed by the regional centres for animal health control to be implemented on a voluntary basis at the herd level for about 20 years. This approach has resulted in a low number of cattle farms implementing control strategies and a variety of strategies, of which some are not adequate (Laureyns et al., 2013; ***Chapters 5 and 6***).

The results of this doctoral thesis illustrate that a voluntary approach for BVDV control at the herd level has not been successful in reducing the BVDV prevalence and the

risk of re-infection in Belgium (***Chapters 3 and 6***). The same has been observed before in other countries (O'Rourke, 2002; Lindberg et al., 2006; Ridpath, 2012). Therefore, a mandatory national control programme is needed to achieve a thorough decrease in prevalence. In Belgium, such a control programme was launched in January 2015 with the final aim of eradicating BVDV.

The Belgian BVDV control programme as it is actually designed

The national control programme will be executed in several phases, of which the first phase has started in January 2015 (Royal Decree on the control of BVD, 18 June 2014). During the first phase the programme focuses on the determination of the BVDV status of individual animals by the mandatory testing of new-born calves for BVDV viraemia within 7 days after birth. Although not compulsory, the preferred way of sampling is via ear notch samples, which are obtained when the farmer places the official ear tag. After testing, the BVDV status is mentioned on the official individual identification document. A BVDV-positive animal is considered PI until the contrary is proven in case of retesting. By this concise and straightforward approach, about a third of the Belgian cattle population will be tested every year, given a Belgian cattle population of approximately 2.6 million animals and 950,000 new-born calves per year. This legislation also applies to imported cattle born after 1 January 2015. Secondly, following the test result of the new-born calf, a BVDV status will also be assigned to the mother of the calf. Dams of non-PI calves will be considered "non-PI by descent" while dams of PI calves will have a "PI-suspected" status, which will have to be verified (Table 1). Finally, by testing individual animals, also information about the BVDV status at herd level is obtained.

Depending on the status of the animal specific movement restrictions are imposed. PI animals are not allowed to be grazed or transported, except for slaughter. There is no obligation to cull PI animals from the start of the programme, but PI animals must be quarantined to prevent contact with the rest of the herd. The mandatory testing of cattle with an unknown BVDV status at purchase will start in January 2017. In a next phase a BVDV free status will be assigned to herds of which all individual cattle tested BVDV negative (monitoring and surveillance). Specific directives about how this phase will be implemented are not yet determined and will depend on the progress of the first phase of the programme.

Table 1. Possible BVDV statuses and their implications following the Belgian mandatory BVDV control programme. Source: Dierengezondheidszorg Vlaanderen (DGZ), 2015.

Status	**Meaning**	**Consequence**
PI	- BVDV-positive test result - Calf of dam with PI status	- Only free for slaughter - No grazing - Quarantine
PI-suspected	- Invalid or missing analysis - Dam of a BVDV-positive calf or foetus - Belated analysis of new-born calf	Only free for slaughter while awaiting coming analysis
Non-PI following analysis	- BVDV-negative test result - Valid foreign BVDV certificate	Free for trade
Non-PI by descent	Dam of a non-PI calf	Free for trade
Unknown BVDV status	Born before 2015 and no analysis	Free for trade for the moment

The legal basis of the Belgian BVDV control programme especially focuses on the detection and movement restrictions of PI animals. Since PI animals do not have to be culled immediately after detection, this programme could be considered as incomplete from a scientific point of view since these PI animals may continue to spread virus and therefore maintain an infection in a herd. However, in order to obtain an achievable control programme that was acceptable for all stakeholders in cattle production and economically feasible, some measures of a scientifically optimal control programme were not included in the legal basis of the Belgian BVDV control programme as it is actually designed. Nonetheless, even when a control programme is not optimal from the start, BVDV eradication – the final aim – can be achieved, given it is implemented in a systematic manner (Hult and Lindberg, 2005). Therefore, in addition to this legal basis, non-compulsory guidelines regarding the removal of PI animals and the prevention of re-infection are provided (DGZ, 2015). The herd veterinarians will be closely involved in the recommendation and communication of these guidelines.

The programme provides some financial compensations (DGZ, 2015). First, the postal charges related to the sending of ear notch samples to the laboratories for analysis are for free.

Secondly, when the BVDV status of a mother has to be verified following a BVDV-positive test result of a new-born calf, a financial compensation of €34.62 is provided for the visit of the herd veterinarian, the sampling of the mother and the analysis of the blood sample. Finally, when during this herd visit, the BVDV-positive new-born calf is euthanized, the costs related to the euthanasia are covered (€10).

In the next paragraph each of the four measures for BVDV control, including vaccination (Fig. 1), will be further discussed in the light of the Belgian mandatory control programme. The order in which these measures will be discussed is based on the way the programme is actually designed with first the detection of PI animals and secondly monitoring and surveillance. Finally, it is discussed how biosecurity and vaccination can contribute to the maintenance of a BVDV free status in Belgian cattle herds.

Assessment of the implementation of systematic BVDV control and recommendations to maintain a BVDV free status in Belgian cattle herds

Elimination of BVDV from infected herds

When herds are BVDV infected it is essential to eliminate the virus from the herd. PI and TI animals are well-described sources of BVDV and therefore possible targets in BVDV eradication.

The essentials of PI animals in the epidemiology of BVDV

During the transmission study (***Chapter 4***) a PI animal infected all three remaining susceptible animals in a group of seven seropositive calves within 8 days after its introduction in the group. During the longitudinal study (***Chapter 6***) in one herd young stock (n = 10) was sampled for BVDV antibodies. All but one calf tested BVDV seropositive. This suggested the

presence of a PI animal, which appeared to be the animal that was BVDV seronegative. These results clearly demonstrated that PI animals are very successful virus transmitters and the main source of new infections (Fulton et al., 2006; Nickell et al., 2011) and illustrate why the removal of PI animals is generally considered the key to successful BVDV control (Lindberg and Alenius, 1999; Lindberg and Houe, 2005; Presi and Heim, 2010; Ridpath, 2010; Ståhl and Alenius, 2012).

Therefore, detection and quick removal of PI animals is strongly recommended. The longer PI animals remain present in a herd, the greater the risk of transmitting the virus. In herds with PI animals and where cattle are housed in close contact most animals are undergoing acute infection and usually more than 90% of the animals become seropositive within 3 to 5 months (Houe, 1999). A delayed within-herd transmission is seen when cattle are kept in separate buildings or pens (Houe et al., 1995b; Taylor et al., 1997). The herd infrastructure thus influences the within-herd BVDV spread, but unless PI animals are kept in strict quarantine and meticulous hygienic procedures are implemented, BVDV will spread within the herd (Ezanno et al., 2008). Taken into account the very low level of internal biosecurity in Belgian cattle herds (***Chapter 5***), PI animals should not remain in the herd. However, farmers will tend to fatten such animals, especially when the animals appear to be perfectly healthy (Laureyns et al., 2010). Nevertheless, farmers should be strongly encouraged to cull cattle diagnosed as PI immediately after detection.

Limiting the movements of detected PI animals (no transport or grazing) as foreseen in the programme will likely reduce the between-herd BVDV spread. Nevertheless, indirect transmission remains possible, since PI animals are considered to be the origin of these minor important transmission routes (***General Introduction***; Lindberg and Houe, 2005). When the probability of direct contact with PI animals decreases, relatively more emphasis should be put on preventing infection through indirect contacts (Hult and Lindberg, 2005), which can be achieved by the implementation of biosecurity measures (see below).

In Switzerland and Germany new-born calves are also tested through ear notch sampling. When PI animals are detected, removal from the herd of these BVDV positive animals is mandatory (Switzerland and Germany), completed with testing of all adult cattle (Switzerland) and trading with non-PI animals only (Switzerland and Germany). The mean time elapsed between a positive test result and the slaughter of a virus-positive calf is 12.5 days in Switzerland (Presi et al., 2011), while in Germany confirmed PI animals should be

eliminated without delay. In Germany, this confirmation had to be performed within 60 days after the first positive test result (Schirrmeier, 2014). The proportion of virus-positive new-born calves 2 years after the start of mandatory testing decreased from 1.5% to 0.15% and from 0.55% to 0.17% in Switzerland and Germany, respectively (Presi et al., 2011; Schirrmeier, 2014). The proportion of virus-positive new-born calves 2 years after the start of mandatory testing decreased from 1.5% to 0.15% and from 0.55% to 0.17% in Switzerland and Germany, respectively (Presi et al., 2011; Schirrmeier, 2014). Although a same proportion of virus-positive new-born calves was obtained after 2 years, the decrease was more distinct in Switzerland. Possible explanations could be the quick removal of PI animals and the testing of all cattle to detect PI animals. Taken this into account, farmers and veterinarians should be encouraged to implement the immediate removal of PI animals and to perform an additional screening of the herd when a PI animal is detected.

The essentials of TI animals in the epidemiology of BVDV

TI animals are believed to be of minor importance in the epidemiology of BVDV, because of the short duration of the infection on the one hand and the intermittent shedding of relatively low amounts of virus on the other hand (Lindberg and Houe, 2005). In our experimental studies (***Chapter 4***) the duration of viraemia in TI animals varied between 1 and 9 days with a maximum viral titre of 1.6×10^4 $TCID_{50}$/mL. For comparison, the serum viral titre of the PI calf used in the transmission study was approximately 2.4×10^4 $TCID_{50}$/mL. Although one TI animal thus obtained a viral load almost equivalent to that in PI animals, this was limited in time (2 days) and the viral titres of the other TI animals in the study were below 9.3×10^2 $TCID_{50}$/mL. Some reports nonetheless suggest that BVDV can be maintained in the population only by transient infections (Moerman et al., 1993; Moen et al., 2005; Doll and Holsteg, 2013; Moen, 2013).

The results from our experimental infections (***Chapter 4***) nevertheless support the assumption that TI cattle are of little importance for BVDV spread. When using Belgian field strains in 2 separate transmission experiments only one animal was contact infected during each experiment, which resulted in R_0 of 0.25 (95% CI 0.01; 1.95) and 0.24 (95% CI 0.01; 2.11) (***Chapter 4.1***). Moreover, the blood samples of the contact infected animals became RT-qPCR-positive 10 days after inoculation, while the PI animal already infected two calves within 5 days after the introduction of the PI animal. Even when taking into account the

incubation period (which was 2 days in the transmission study) of the inoculated calves, the PI animal more rapidly transmitted BVDV compared to the TI animals. Furthermore, also when a hypervirulent strain was used, BVDV transmission was insufficient to maintain BVDV in a susceptible population (R_0 of 0.49; 95% CI 0.06; 2.99) (***Chapter 4.2***). These results are in contradiction to the report that suggested that virulent strains can spread explosively through TI animals (Ridpath et al., 2006). Other studies even demonstrated no spread of BVDV by experimentally infected animals (Niskanen et al., 2000; Niskanen et al., 2002). Furthermore, assumed self-clearance of BVDV was noticed in herds where seroconversion (i.e. transient infections) but no PI animals were detected (***Chapter 6***).

Whenever it is claimed that TI animals maintain BVDV in a population, sufficient information is needed to prove that transmission was not due to PI animals that have escaped identification (Lindberg and Alenius, 1999; Lindberg and Houe, 2005). For instance, Moerman et al. (1993) estimated that horizontal BVDV transmission rate by TI cattle had a R_0 of 3.3 (95% CI 2.6; 4.1). However, although separated from the study group, PI animals were present in the herd during this study. In this way they may have attributed to virus spread through indirect transmission (Niskanen and Lindberg, 2003; Lindberg et al., 2004), resulting in an overestimation of R_0. Furthermore it is possible that, for instance during the outbreak in Germany (Doll and Holsteg, 2013), PI animals died before they were identified as PI. Additionally, BVDV can be spread when PI animals are aborted or stillborn (Lindberg et al., 2004).

The major importance of TI animals in BVDV spread is their ability to deliver a PI animal when they are infected in early pregnancy (***Chapter 6***), hereby becoming a Trojan cow (Laureyns et al., 2010; Lanyon et al., 2014). Trojan cows were shown to be a very important cause of re-infection in Danish cattle farms (Bitsch et al., 2000). New-born PI animals will eventually quickly be detected through ear notch sampling, but they will not necessarily immediately be removed and BVDV can also be spread at birth (Lindberg et al., 2004). To minimize the contribution of TI animals in BVDV spread it is therefore recommended to:

a) quarantine purchased pregnant cattle until the test result of the new-born calf is known (see below),
b) prevent contact of pregnant cattle with PI animals by removing PI animals from the herd,
c) pay attention to the calving management (see below).

Test strategy

In the Belgian control strategy, ear notch samples of new-born calves are tested with Ag-ELISA, which detect, depending on the kit, Erns or NS-3 antigen. Ag-ELISA kits that detect Erns antigen are preferred, since this antigen, in contrast with NS-3, remains detectable in ear notch samples even in the presence of colostral antibodies (Fux and Wolf, 2012). The sensitivity and specificity of the Ag-ELISA are reported to be 97.1% and 99.8%, respectively (Presi et al., 2011). The more expensive RT-qPCR could also be used, for which a sensitivity and specificity of 100% and 98%, respectively, are described (Presi et al., 2011). In the Belgian programme, the certification for the Ag-ELISA by the national reference laboratory was attributed to the BVDV Ag/Serum Plus Test (IDEXX) (CODA-CERVA, 2013), for which a specificity of more than 99.7% and a sensitivity of nearly 100% are reported (BVDV Ag/Serum Plus Test Brochure, IDEXX).

Calves with a negative test result receive a non-PI status, which is mentioned on their official identification document. Hence, it is very unlikely that one will retest such animals once they have been declared BVDV free.

From this point of view false negative test results should be avoided as much as possible and the test with the highest sensitivity is preferred, i.e. RT-qPCR. However, RT-qPCR is 4 to 6 times as expensive as Ag-ELISA and even with RT-qPCR false negative test results due to for example an insufficient sample quality or human errors can never be excluded. Instead of the label "non-PI following analysis" for calves with a negative test result, the label "PI-unsuspected" could have been an appropriate alternative to point out that it is possible that some PI animals incorrectly have been assigned a free status. In the programme all stakeholders should therefore be aware that undetected PI animals will be freely moved between herds and be the origin of new introductions in BVDV free herds. In Switzerland, false negative PI animals have accounted for 57% of identified sources of new-born virus-positive calves between October 2009 and December 2011 (Presi et al., 2011).

Given a sensitivity of 97.1% with Ag-ELISA, approximately one million new-born calves per year in Belgium and a PI animal prevalence amongst young calves of approximately 2%, about 580 false negative PI animals are expected during the first year of the programme. These false negative PI animals may result in infections of veal herds (male calves for fattening), prolonged infections in the herd of origin and infections of herds purchasing these cattle (female animals for breeding and lactating purposes). The introduction

of a PI animal in a herd where the majority of the animals are susceptible to infection can result in an outbreak, as demonstrated in this doctoral thesis (***Chapter 6***). When only using ear notch sampling, the presence of such a false negative PI animal will only be detected in a herd about 5 to 6 months after its introduction at the earliest, given that a susceptible animal in early pregnancy is infected immediately after the introduction of the PI animal and gives birth to PI calf. Therefore, to early detect re-infection the implementation of serological monitoring and surveillance could be considered for BVDV free herds, as is argued below.

When PI animals will have to be culled obligatorily, farmers will want to avoid culling TI animals and could therefore opt for a confirmatory test. In the initial phase (October - December 2008) of the Swiss eradication programme the whole bovine population (animals born before the 1st October 2008) was tested. Of the 12,092 virus-positive animals, 7393 (61.1%) had a confirmatory test. With 6229 animals confirmed positive, the confirmation rate was 84.3% (Presi et al., 2011). In a next phase all new-born calves (calves born between 1st October 2008 and 30th September 2009) were tested. Of the 5199 virus-positive animals, 3291 (63.3%) had a confirmatory test and 2,862 (87.0%) were confirmed positive (Presi et al., 2011). This means that the probability to cull a TI animal varied between 13.0% and 15.7% when no confirmatory test is performed. From a farmer's point of view and given the fact that Belgian Blue beef calves are valuable, it thus seems to be worthwhile to perform a confirmatory test. However, the same data also indicate that the probability to have a prolonged stay of a PI animal in the herd is between 84.3% and 87.0% when doing a confirmatory test. Given the young age of the calves, virus excretion of these potential PI calves is partially suppressed by colostral antibodies (Palfi et al., 1993). However, BVDV can still be spread and it has been shown that a PI animal can infect susceptible animals within 5 days (***Chapter 4.1***). Therefore, whenever a confirmatory test is performed, the animal should be quarantined and stringent biosecurity measures should be implemented (see below). Since in Belgium only Ag-ELISA is used, a higher confirmation rate can be expected when performing a confirmation test, compared to Switzerland, where a combination of Ag-ELISA and RT-qPCR is used. As described above, the specificity of RT-qPCR is lower compared to Ag-ELISA and therefore more false positive results are expected with RT-qPCR.

The ultimate goal would be to have one single test to distinguish between PI and TI animals. Studies suggest that this differentiation is possible with one single RT-qPCR (Bhudevi and Weinstock, 2001; Hanon et al., 2014) or Ag-ELISA (Hanon et al., 2014). The distinction is then based on the amount of viral RNA or antigen present in the sample. The

determination of an optimal cut-off value to detect all PI animals and to reduce the detection of TI animals as much as possible would be crucial in the design of such a test. Furthermore, the interpretation of a single quantitative result may be complicated by the variation in levels of viraemia such as intermittent viraemia in PI cattle (Laureyns et al., 2010). During the BVDV-2c outbreak in Germany some TI animals were believed to have obtained a viral and genomic load almost equivalent to that in PI animals, which suggests that these animals have substantially contributed to BVDV spread. As a consequence, such animals would be considered as PI when using one single test and would thus subsequently be eliminated. However, as these animals contribute to virus spread, the removal of these 'supershedding' TI animals could be considered as acceptable.

Follow-up of the BVDV status: monitoring and surveillance

Monitoring and surveillance are defined by the Food and Agriculture Organization (FAO) as "*all activities aimed at detecting changes in the epidemiological parameters of a specified disease*" and "*all regular activities aimed at ascertaining the health status of a given population with the aim of early detection and control of animal diseases*", respectively. In the light of the control programme, monitoring and surveillance will especially be used for the early detection of newly infected herds and the confirmation of the BVDV status in free herds (Lindberg et al., 2006). How monitoring and surveillance will be implemented in the Belgian control programme is not yet completely determined and will depend on the phase of the programme. Considerations concerning the implementation of monitoring and surveillance are discussed.

Detection of disease

During the first phase the detection of disease is conducted by ear notch sampling. This virological testing strategy requires a meticulous administration (Laureyns et al., 2010), which is realised by linking the BVDV test result of the ear notch sample to the official individual identification document of the calf via the animal identification and registration system (SANITEL). This testing system has many advantages such as the fact that all new-born animals are tested, that the test has a relatively high sensitivity and specificity (see

above) and that it is relatively easy to perform. Nonetheless, as every testing system, it is not impeccable. Some drawbacks of the ear notch sampling are that sometimes false negative test results occur (see above). It may also happen that PI foetuses are stillborn or aborted, and thus not tested, but still are able to spread BVDV at the moment of stillbirth or abortion (Lindberg and Alenius, 1999; Lindberg et al., 2004). Although it is compulsory to report and test each abortion, it was shown that this abortion protocol is only rarely used, as described above. Furthermore, PI animals that do not deliver offspring are not detected and a dam-calf relation may be incorrectly registered (Laureyns, 2014). As a consequence, certifying a herd to be BVDV free only based on negative test results with ear notch sampling should not be done too early as some PI animals may slip through the net and remain present in the herd longer than anticipated. Determining the minimal years of delivering BVDV-negative test results following ear notch sampling before a herd can be certified BVDV free is therefore one of the remaining questions to be answered in the years to come.

Towards freedom from disease

The progress made during the first phase of the programme can be evaluated by follow-up of the prevalence of positive ear notch samples both at animal and herd level. In a later phase farms could opt to stop the individual ear notch sampling and switch to a certification of the BVDV status at herd level. This switch is crucial, since it involves that animals will no longer be tested individually and will receive a BVDV status based on the status of the herd. A false negative BVDV status at the herd level could therefore result in the trade of BVDV-positive animals and re-infection of free herds. The certification of the BVDV status at the herd level should therefore be compared from an epidemiological and economical point of view with a continued ear notch sampling of new-born calves. Currently, this option to switch the surveillance from the animal level to the herd level is planned 2 years after the start of the programme (Laureyns, 2014), but in our opinion, the epidemiological and economical optimal moment is likely to be herd-specific and depends on the following parameters:

a) When can a herd be considered BVDV free based on ear notch sampling?
b) What is the risk of re-infection of these BVDV free herds?
c) How is the BVDV free status of a herd monitored?
d) What is the economical trade-off between the different testing systems?

When can a herd be considered BVDV free ?

By testing new-born calves and also assigning a BVDV status to the dam, the proportion of animals with an unknown BVDV status will rapidly decrease. A herd could be assumed to be BVDV free based on ear notch sampling if there were no BVDV-positive test results following ear notch sampling during a number of years (to be determined) and all individual animals in the herd have a non-PI status (by analysis at birth or during an additional screening or by descent). However, as mentioned above, there is always a risk of some PI animals slipping through the net. To avoid the missing of PI animals, this assumed free status should preferably be verified by a serological screening (Houe et al., 2006; Lindberg et al., 2006).

What is the risk of re-infection of these BVDV free herds?

Even when a herd has been certified BVDV free, it can always be re-infected. Given the current BVDV prevalence and limited implementation of biosecurity measures in Belgian cattle herds, an incidence risk of re-infection of 15.4% per year was estimated (***Chapter 6***). In the programme the probability of direct animal-animal contacts with PI animals is reduced by prohibiting the trade and grazing of cattle with a PI status. As a consequence the risk of re-infection will decrease during the progress of the programme in line with the decrease of the number of new-born PI animals. Nonetheless, increased and continuous efforts to prevent re-infection, especially of cattle in early pregnancy, should be undertaken (see below).

How is the BVDV free status of herds monitored?

When moving from individual ear-notch sampling of all new-born calves towards herd level certification, the BVDV detection method will typically be based on serology in young stock (Lindberg et al., 2006). Serological monitoring of young stock to detect the presence of PI animals in a herd is also referred to as the spot test and was first described by Houe (1992; 1994). By testing young stock a recent or on-going infection will be detected, given the young age of the animals (Brülisauer et al., 2010). Furthermore, the interference with vaccination can more easily be avoided in young stock compared to older animals that are used for breeding (see below).

A first prerequisite for a reliable spot test is a sample of young stock representative for the entire herd. When cattle are housed close together in the same building or in buildings nearby, up to 90% of cattle in herds with PI animals are expected to be BVDV seropositive (Houe, 1999). The spot test has proven to be accurate in the distinction of herds with and without PI animals under such conditions (Houe, 1992). However, due to an increasing herd size and the compartmentalization of herds into separate buildings in Belgium, BVDV could spread more slowly with a lower proportion of seropositive animals as a result. A second prerequisite is a sample large enough to substantiate freedom from BVDV. This sample size depends on the herd size and the design prevalence, i.e. the minimum expected prevalence of the BVDV seropositive animals if a herd was infected (Cameron and Baldock, 1998). To substantiate freedom from disease, a low design prevalence should be presumed.

To determine the sample size of young stock needed to substantiate freedom from BVDV, different scenarios were tested using FreeCalc version 2.0, as described by Cameron and Baldock (1998). The modified hypergeometric exact formula was used with type I and II error levels set at 5%. The null hypothesis is that BVDV is present in the herd, at or greater than the design seroprevalence. The alternative hypothesis is that the herd is free from BVDV, or that the seroprevalence is lower than the specified level (Cameron and Baldock, 1998). This calculation assumes random sampling of young stock, which are about one third of the herd size. The following parameters were required:

- Test sensitivity and specificity: set to 95.7% and 97.7%, respectively, based on a commercial Ab-ELISA test kit;
- Number of young stock: was set to vary between 20 and 100 (which represents a total herd size of 60 and 300, respectively);
- Expected within-herd seroprevalence in case of re-infection (design prevalence): was set to vary between 10% and 70%.

Table 2. Sample size of young stock required to substantiate freedom from BVDV depending on the herd size and design seroprevalence. The test sensitivity and specificity were set to 95.7% and 97.7%, respectively (FreeCalc; Cameron and Baldock; 1998).

Design seroprevalence (%)	Number of young stock (one third of the herd size)				
	20	40[a]	50	70	100
10	20[b]	36	46	51	55
20	15	23	24	25	26
30	11	13	13	13	14
40	9	10	10	10	10
50	7	8	8	8	8
60	6	6	6	6	6
70	5	5	5	5	5

[a]In November 2013 the average herd size of Belgian cattle farms was 120 animals (NIS, 2013), which corresponds to a number of young stock of 40.
[b]Even when sampling all young stock, the null hypothesis cannot be rejected and freedom from BVDV cannot be substantiated ($p = 0.07$).

Especially with a low design seroprevalence (10-20%), a large sample of young stock is required to substantiate freedom from BVDV with 95% confidence (Table 2). Once animals are no longer tested individually for BVDV viraemia, the early detection of re-infection should be able to prevent that an untested PI animal is traded, which implies that the spot test should detect re-infection of a herd before a PI animal is born. This means that the infection of cattle in gestation and possibly carrying a PI foetus should be detected, and not the PI animal itself. Since such a re-infection may involve only very few animals, a very low design prevalence should be presumed and by consequence a high sample size is needed. Moreover, it is not clear whether a re-infection of some cattle in gestation will be detected by the spot test in the young stock, given the minor importance of TI animals in BVDV spread (***Chapter 4***). Furthermore, the frequency of substantiating freedom from disease by means of a spot test to prevent the birth of PI animals may be high. To detect in case of re-infection the presence of BVDV using the spot before a PI calf is born, a spot test should be performed every 4 months. This is based on the fact that PI animals result from infection of the foetus between 30 and 125 days of gestation (McClurkin et al., 1984; Blanchard et al., 2010) and that neutralizing antibodies become detectable about 2 weeks after infection (***Chapter 4***). When a spot test is performed every 6 months, a PI calf could be born before it is detected with the

spot test when the infection occurs between 100 and 125 days of gestation and the sampling between 100 and 140 days of gestation.

To minimize the risk of trading PI animals, the spot test could be combined with the individual testing of cattle for trade. In this way the design seroprevalence could be raised to reduce the sample size without losing information about the BVDV status at herd level and risking the trade of PI cattle.

Once the final aim, eradicating BVDV in Belgium, is obtained and freedom from BVDV is substantiated in every farm, the individual testing of animals for trade will become redundant and the BVDV free status can be verified by means of a serological surveillance.

What is the economical trade-off between the different testing systems?

Several factors will determine whether a switch to certification at herd level is economically favourable in a specific case. A first factor is the herd size, which determines the number of new-born calves to be tested (if ear notch sampling is used) and the number of young stock to be included in the spot test. In the case of ear notch sampling, the number of animals to be tested is increasing with the herd size whereas in the spot test the additional number of young stock to be included is proportionally decreasing with an increasing herd size as shown in Table 2. Therefore, the spot test will become increasingly beneficial as the herd size increases. Yet, the sample size of the spot test is also strongly influenced by the defined design seroprevalence. If this is set at a relatively low prevalence the sample size will always be relatively large, also in large herds. Furthermore, the frequency of substantiating freedom from disease by means of a spot test will influence the costs. Finally, when it appears that serology in itself is insufficient to minimize the risk of trading PI cattle, additional testing of animals for trade is necessary, which increases the number of animals to test for BVDV viraemia, but could decrease the sample size for the spot test. To be able to answer all these questions, it would be advisable to start a pilot study in some herds that have been shown to be BVDV free for some years already.

Reducing the risk of BVDV (re-)introduction: biosecurity

Although the implementation of biosecurity measures in the daily management of cattle farms is considered the most essential pillar in systematic BVDV control (Lindberg and Houe, 2005), a very low biosecurity level was noticed in Belgian cattle farms (***Chapter 5***). In a study asking Belgian farmers about their perspectives towards biosecurity, they indicated that little to no barriers are present for taking preventive measures (Laanen et al., 2014). This suggests that the limited implementation of biosecurity and disease prevention measures is most likely due to insufficient motivation. The following reasons might explain this low motivation:

- There is insufficient information on which measures should be taken;
- There is insufficient information on the costs and benefits;
- Biosecurity should be considered a collective responsibility rather than an individual responsibility.

There is insufficient information on which measures should be taken.

Biosecurity includes a very broad range of specific measures preventing pathogens from entering a herd and reducing the spread of pathogens within a herd (Villarroel et al., 2007). Additionally, a preventive strategy is farm-specific and some measures may be of high value for some farmers, while the same measures might be less useful for others. As a consequence, it is difficult for farmers to choose the most appropriate strategy.

Cattle farmers prefer to obtain information on biosecurity from their herd veterinarian (Brennan and Christley, 2013; Laanen et al., 2014). A farm-specific preventive strategy should therefore be developed in collaboration with the herd veterinarian who knows the specific herd structure and can inform the farmer on the critical points (Villarroel et al., 2007; Ellis-Iversen et al., 2010; Brennan and Christley, 2013).

For pig and poultry herds, an online risk based biosecurity testing tool is made available (www.BioCheck.UGent.be; Laanen et al., 2010; Gelaude et al., 2014). By using this tool, farmers can verify their biosecurity level and find out at which level biosecurity can be improved in their herd. Secondly, farmers can compare their biosecurity level with other farms. A similar testing tool for cattle farms is not yet available, but could be very useful to

help cattle farmers to improve their biosecurity level. In this way, information is delivered from a neutral source and as a result cattle farmers might be more willing to accept this information (Heffernan et al., 2008) and might become more motivated to implement these measures.

There is insufficient information on the costs and benefits.

Belgian cattle farmers consider many biosecurity measures too expensive and are therefore not willing to implement them (Laanen et al., 2014). Nevertheless, financial contributions by other stakeholders in cattle production toward farmers are made to improve biosecurity. For instance, the regional centres for animal disease control offer farmers the opportunity to test purchased animals for endemic diseases such as neosporosis, paratuberculosis and BVD at reduced price. As mentioned above, basic analyses on aborted and stillborn foetuses are performed for free. Moreover, basic biosecurity measures such as the use of protective clothing, boots and disinfectant footbaths are already present in many cattle farms and can by consequence be implemented without additional material costs. Although the implementation of biosecurity also involves an investment in time, farmers nonetheless believe that biosecurity is more time efficient than treating disease on-farm (Brennan and Christley, 2013).

The costs of an average BVDV outbreak in a dairy herd are estimated to lie between €21 ($25) and €135 ($160) per cow per year (Houe, 2003; Lindberg et al., 2006). Cost-benefit analyses for systematic BVDV control, which include the implementation of biosecurity, showed that it is economically favourable to control BVDV (Valle et al., 2005; Reichel et al., 2008). However, these studies are usually performed at the national level and further research to obtain information on costs and benefits of investing in biosecurity in general (i.e. not focusing on BVDV alone) for different cattle production types is currently lacking and could be very useful in convincing farmers to implement biosecurity measures. Belgian farmers indicated “more information on the economic benefits of measures” as the primary interest for taking measures in disease prevention (Laanen et al., 2014).

Although quantitative results of implementing biosecurity in disease prevention are currently lacking, expected consequences of applying biosecurity measures are improved production characteristics, better animal welfare, improved immune responses to vaccines and enhanced job satisfaction for farmers (Brennan and Christley, 2012). Moreover, it was shown

in pig production that a higher biosecurity status is linked with a reduction in antimicrobial usage (Laanen et al., 2013).

Biosecurity should be considered a collective responsibility rather than an individual responsibility.

When considering who should be responsible for the implementation of biosecurity, stakeholders in cattle production typically point at each other. In case of epidemic threats farmers consider biosecurity the government's responsibility (Heffernan et al., 2008). Furthermore farmers indicate that the government should make a greater contribution towards biosecurity in Great Britain (Gunn et al., 2008). From a farmer's point of view, endemic diseases are considered to be an individual problem for farmers with a 'bad' herd management (Heffernan et al., 2008). Hovi et al. (2005) showed that farmers are more prone to implement measures for disease control when also other farmers would join. Although farmers consider the herd veterinarian the preferred source of information regarding biosecurity (Gunn et al., 2008; Heffernan et al., 2008; Brennan and Christley, 2013; Laanen et al., 2014), the veterinarians' advise appears to be a poor motivator to implement biosecurity (Laanen et al., 2014). Conversely, veterinarians considered the farmers' ability or willingness to invest in biosecurity measures a major constraint (Gunn et al., 2008).

These attitudes suggest that as long as biosecurity measures, or any action in general, are not mandatory, a low degree of participation can be expected (Laanen et al., 2014). However, mandatory biosecurity measures appear to be a poor motivator to implement biosecurity (Laanen et al., 2014). This can be illustrated with the discrepancy between the presence and the use of a disinfection footbath in Belgian dairy farms (***Chapter 5***). A disinfection footbath was present in 64% of the farms with dairy cattle, but was only used in 7% of these farms. For dairy farms the use of a disinfection footbath is required in order to fulfil the specifications of a milk quality system. This suggests that farmers only acquired a disinfection footbath to avoid penalties. These results clearly show that the mandatory implementation of biosecurity alone will not be sufficient and will also involve a behavioural change.

When a farmer invests time and money in eradicating BVDV and the neighbouring farm does not undertake any action, re-infection is very likely to occur in Belgium: 6 out of 26 farms (23.1%) were re-infected within 18 months (***Chapter 6***). As a consequence, it is

indeed quite obvious that some farmers are less motivated to continue their efforts. Furthermore, all stakeholders should be willing to contribute to an improved biosecurity level. During the farm visits that were performed for the studies in this doctoral thesis some remarkable excerpts about the perception regarding biosecurity were noticed. For instance, on farms with both pigs and cattle, the herd veterinarian responsible for the pigs always had to wear farm-specific clothing and boots because "everyone is doing this", while the herd veterinarian for cattle did not need to change clothes because "nobody is doing this". Another example is that the herd veterinarian always had to wear farm-specific clothing and boots, while the AI technician was not required to do this because "he is a very busy man and does not have the time for it". Again, it is quite obvious that farmers are less motivated when not everyone is implementing these measures. Conversely, as mentioned above, veterinarians considered the farmers' ability or willingness to invest in biosecurity measures a major constraint (Gunn et al., 2008).

With respect to systematic BVDV control voluntary or compulsory regulations should provide a general framework to improve the biosecurity levels in cattle farms in order to prevent BVDV (re-)infection. These recommendations should be formulated for all stakeholders who are contributing to animal health, hereby focusing on the herd veterinarians, as they are familiar with the specific herd structure and have the knowledge to implement biosecurity measures. It may however be difficult to impose and control the implementation of these preventive measures in a mandatory programme (Laureyns, 2014). Nonetheless, when focusing on the main routes of re-infection as encountered in this doctoral thesis and described in literature, it should be possible to improve the low implementation of biosecurity as currently observed in Belgian cattle farms. It is believed that the mandatory BVDV control programme can be a first move in the right direction toward collective behaviour regarding biosecurity in cattle farms. The following factors are considered to be of primary interest:

- purchase of cattle,
- contact on pastures,
- calving management,
- professional visitors entering the farm.

Purchase of cattle

During the first 2 years of the Belgian BVDV control programme, adult cattle can still be freely moved. Furthermore, as mentioned above, PI animals incorrectly considered as BVDV free at birth can also be transported between herds. It has been noticed in this thesis that cattle originating from different herds often are transported together: only 16% of the trucks of cattle salesmen were empty upon arrival at the farm (***Chapter 5***). False negative PI animals could thus infect susceptible cattle during transport.

The purchase of cattle has been shown to be a very important route of BVDV transmission and therefore, farmers should test and quarantine all purchased animals from the start of the programme. Testing of purchased animals should always be combined with quarantine for all cattle entering the herd, including animals that are re-introduced following exhibitions, competitions or auctions. The correct application of quarantine measures, i.e. no direct or indirect contact with other animals during at least 3 weeks, should be the basis, with testing as an additional measure. In this way, purchased cattle are also monitored for other disease agents than those tested for. Moreover, if newly introduced animals are only infected shortly before arrival at the herd (e.g. during transport) they might have a negative test result when entering the herd and therefore be false negative. If these TI animals are kept in quarantine for 3 weeks they are likely to be virus-negative at the end of the quarantine period according to the results of our transmission studies (***Chapter 4***).

Contact on pastures

Fence-line contact on pastures with cattle of neighbouring farms is another important source of potential BVDV (re-)infection (Bitsch et al., 2000). A distance of at least 3 m between pasture fences is advised, but difficult to realize. A more suitable recommendation is to avoid that cattle in gestation graze where contact with cattle of neighbouring farms is possible. Additionally, when cattle in gestation are grazed, special attention should be paid at calving as described above.

Calving management

In addition to purchased and grazed cattle in gestation, all cattle should calve in a separate pen. This pen should only be used for this purpose and may never house diseased

animals (Villarroel et al., 2007). The dam and the calf should be separated from the rest of the herd until the BVDV test result of the calf is known.

Professional visitors entering the farm

As BVDV spread by persons involves indirect transmission, this route of infection was considered to be of minor importance (Lindberg and Alenius, 1999). However, this transmission route should not be ignored (Ståhl et al., 2005) and attention should be paid to indirect BVDV transmission as the programme proceeds (Hult and Lindberg, 2005). It is of interest to already implement these preventive measures from the start of the Belgian programme for the following reasons. First, biosecurity tools to prevent disease spread by visitors such as the use of protective clothing, boots and disinfectant footbaths are already present in the majority of the Belgian cattle farms (***Chapter 5***). Secondly, the implementation of such biosecurity measures will involve a behavioural change (Ellis-Iversen et al., 2010), which is needed to integrate more preventive measures in the daily management of Belgian farmers (Laanen et al., 2014).

An additional biosecurity measure: vaccination

Given the current poor implementation of the necessary elements for systematic BVDV control and the high risk of (re-)infection in Belgian cattle herds, the use of vaccination seems to be of interest. However, it is not unlikely that because of the frequent use of vaccination the previously listed essential measures are ignored, as described by Laureyns et al. (2013). Vaccination is often used as the single control measure and it is generally accepted that this approach does not result in a decrease of BVDV prevalence (O'Rourke, 2002). Moreover, due to incorrect use of vaccines cattle may not be fully protected (Meadows, 2010). Nonetheless, BVDV vaccination may be useful as an additional biosecurity measure in a systematic control scheme (Lindberg et al., 2006). How BVDV vaccination can contribute in BVDV control and subsequently how vaccination can be implemented for this purpose in the Belgian control programme should therefore be well communicated to all stakeholders. A more correct use of vaccines (e.g. when should animals

be vaccinated?) may result from additional information on the goals of vaccination (Lindberg et al., 2006; Ridpath, 2013):

- limiting the spread of infection within a population by protection of foetal infections,
- reducing the extent of clinical disease in infected animals.

Limiting the spread of infection within a population by protection of foetal infections

The major importance of TI cattle in BVDV transmission is when these animals are infected during early pregnancy, with birth of PI animals as a result. In Belgium, currently two commercial BVDV vaccines are available, of which one claims to be able to prevent foetal infection when female cattle is immunised 4 weeks before pregnancy (BCFI, 2014). However, even when using artificial insemination, where the moment of conception is thus known, vaccination should be used with caution. First, in case of repeat breeding the optimal moment of immunization is lost. Secondly, the BVDV vaccines commercially available in Belgium contain at least five doses per bottle. When opened, the vaccine should be used within maximal 10 h. In case farmers cannot use an entire bottle when not having five animals that need to be inseminated, they often store the vaccine until another animal needs to be vaccinated. As a result, the efficacy of the vaccine cannot be guaranteed anymore. Instead of vaccinating animals individually, vaccination of the entire herd (or female breeding cattle) every 6 months is proposed as an alternative. However, even when using this strategy the primary vaccination should preferably be performed just before the first pregnancy to obtain maximal efficacy. Recently, the European Medicines Agency (EMA) has approved the marketing authorisation of a new BVDV modified live vaccine (MLV) that claims to be able to prevent foetal infection (EMA, 2014). The vaccine contains both a BVDV-1 and a BVDV-2 strain and its launch is planned for spring 2015 (Boehringer-Ingelheim, 2014). This vaccine could eliminate the incomplete foetal protection against BVDV-2 strains in animals vaccinated with BVDV-1 due to the differences in antigenic cross reactivity between BVDV-1 and BVDV-2 (Ridpath, 2013).

These recommendations for good vaccination show that a meticulous record keeping of when and which animals should be vaccinated is necessary (Laureyns, 2014). When bearing these guidelines in mind, vaccination could be effective at the herd level through the prevention of intra uterine infection, notwithstanding the fact it may not be fully effective in every individual animal (Ridpath, 2013). Therefore, vaccination on its own is not a valuable

method for BVDV control. It rather is an additional measure of biosecurity (Laureyns et al., 2013). Again, this stresses the necessity of the essential measures in BVDV control to counter possible vaccination failure.

Reducing the extent of clinical disease in infected animals

During the progress of a control programme the proportion of seronegative and thus susceptible animals increases. As described above, BVDV introduction in susceptible herds can result in an outbreak and birth of many PI animals (***Chapter 6***). When it concerns a virulent BVDV strain, BVDV introduction in a susceptible population can lead to high mortality rates. During the BVDV-2c outbreak in Germany infected cattle herds were restricted and cattle received emergency vaccinations with a commercial BVDV-1 MLV (Doll and Holsteg, 2013). However, the effectiveness of these emergency vaccinations was variable, since in some cases the disease appears to have subsided, while in others the losses continued unchanged. Following an experimental study using a BVDV field strain isolated during the outbreak in Germany an emergency vaccination scenario was simulated by vaccinating animals at day 5 post-infection with a single dose of a BVDV MLV. This post-inoculation did not influence the clinical manifestations: all animals died acutely or had to be euthanized (Jenckel et al., 2014).

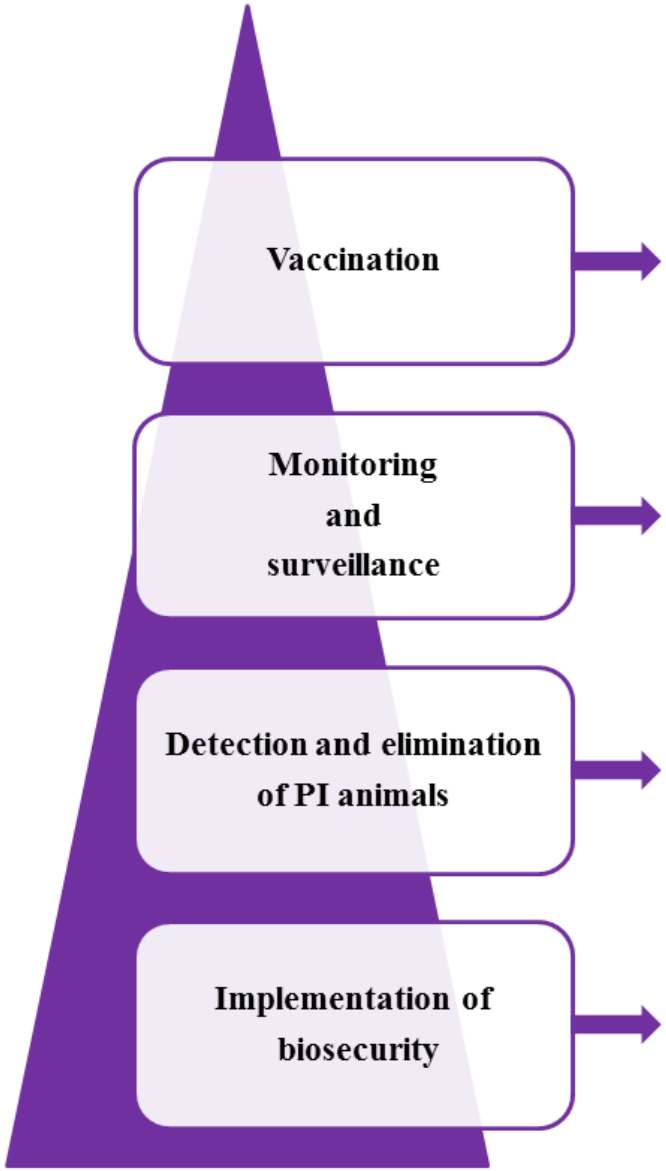
When using BVDV vaccination to reduce the extent of clinical manifestations, cattle of all ages are likely to be vaccinated. As a result, vaccination interferes with serological tests and thus with monitoring and surveillance, since no DIVA vaccines are available. Therefore, vaccination was not allowed in the Scandinavian control programmes (Houe et al., 2006).

Vaccination in the Belgian programme

A vaccination ban is not advised in the early phase of the Belgian programme. Therefore strategies to combine vaccination and monitoring should be used to avoid false positive spot tests due to the detection of vaccination antibodies. Given the fact that vaccines to prevent bovine respiratory disease in young calves without a BVDV component are available and that the main goal of vaccination should be the prevention of foetal infections in breeding cattle, the use of unvaccinated sentinel young stock for serological monitoring is appropriate. These animals could be kept unvaccinated until the age of first pregnancy.

Whether or when a vaccination ban could be imposed in Belgium will become clear during the programme. When considering the currently high risk and the tremendous effects of a BVDV re-introduction in a fully susceptible population, a vaccination ban is not advised at the moment. On the other hand, to optimise the use of monitoring and surveillance, it would be of interest to monitor all age groups in a herd. As a consequence, serological analysis of older animals (bulk tank milk for dairy herds, blood sampling for beef herds) is also needed. Therefore, further research to develop BVDV marker (DIVA) vaccines is necessary. Furthermore, one should always bear in mind the risks associated with the use of vaccination. For instance, a strong association between the use of a specific BVDV vaccine and bovine neonatal pancytopenia was demonstrated (Sauter-Louis et al., 2012). Contamination of a bovine herpesvirus marker vaccine with BVDV resulted in an outbreak on Dutch dairy farms (Barkema et al., 2001).

A final overview of the recommendations made in this doctoral thesis to obtain a systematic approach in the BVDV control programme in Belgium is given below (Fig. 2).



Current design of the programme		Recommendations	
Measures	Date	Measures	Date
No ban of vaccination	-	- Vaccination of breeding cattle - No vaccination of young stock until sampled for serological monitoring	2015
Monitoring and surveillance: specific approach to be determined	2017	Pilot study to determine an optimal monitoring and surveillance strategy	2015
- Ear notch sampling of new-born calves - Testing of transported animals	2015 2017	- Direct elimination of PI animals - Screening of the enitre herd when having BVDV-positive new-born calf - Testing of transported animals	2015
- No transport of PI animals - No grazing of PI animals - PI animals in quarantine	2015	- Quarantine for all cattle entering the herd - Avoid contact on pastures - Calving management - Farm-specific clothing for professional visitors	2015

Fig. 2. This figure provides an overview of the Belgian BVDV control programme as it is actually designed and the recommendations to obtain a systematic approach in BVDV control based on the results of this doctoral thesis.

Conclusions

In the light of the mandatory Belgian BVDV control programme and bearing in mind the general model for systematic BVDV control, the following conclusions can be formulated:

Considering ***biosecurity***, all stakeholders contributing to animal production should be informed on the need of the implementation of biosecurity measures in the daily management. Basic biosecurity measures focusing on the gaps of the control programme as it is currently designed should be introduced. Testing and quarantining of all purchased and re-introduced cattle, with special attention to the Trojan cow, awareness of the risk of pasture contacts, attention to the calving management and farm-specific clothing and boots for professional visitors are basic but essential measures that need to be implemented.

Considering the ***removal of virus sources***, the mandatory testing of all new-born calves is a step forward. Nevertheless, cattle diagnosed as PI should be removed immediately after detection. Furthermore, other routes of PI introduction, such as purchase of cattle and contact on pastures should not be ignored.

Considering ***monitoring and surveillance***, recommendations regarding this essential measure should be formulated as soon as possible, as evaluation of the progress made in control or eradication programmes can stimulate stakeholders to continue and to fully collaborate. A pilot study in some herds that have been shown to be BVDV free for some years already could be performed to examine which strategy is favourable in a later phase of the programme.

Considering ***vaccination***, all stakeholders should be informed both on the power and incapacities of vaccination. With good biosecurity, removal of PI animals and monitoring, vaccination as an additional biosecurity measure, can be helpful to reduce BVDV prevalence in the early phase of the programme. The further need of vaccination during the programme has to be evaluated regularly and may depend on the development of a DIVA vaccine.

References

Ames, T.R., 1986. The causative agent of BVD - its epidemiology and pathogenesis. Veterinary Medicine 81, 848-869.

ARSIA, 2013. Association Régionale de Santé et d'Identification Animales. Rapport annuel. http://www.arsia.be/wp-content/uploads/2014/07/Arsia-RA-2013-light.pdf (accessed 25 September 2014).

Baker, J.C., 1995. The clinical manifestations of bovine viral diarrhea infection. Veterinary Clinics of North America-Food Animal Practice 11, 425-445.

Barkema, H.W., Bartels, C.J.M., van Wuijckhuise, L., Hesselink, J.W., Holzhauer, M., Weber, M.F., Franken, P., Kock, P.A., Bruschke, C.J.M., Zimmer, G.M., 2001. Outbreak of bovine viral diarrhoea on Dutch dairy farms induced by a BHV1 marker vaccine contaminated with BVDV type 2. Tijdschrift Voor Diergeneeskunde 126, 158-165.

Barrett, D.J., More, S.J., Graham, D.A., O'Flaherty, J., Doherty, M.L., Gunn, H.M., 2011. Considerations on BVD eradication for the Irish livestock industry. Irish Veterinary Journal 64, 1-10.

BCFI, 2014. Belgisch Centrum voor Farmacotherapeutische Informatie. Instruction leaflet Bovilis BVD®, MSD Animal Health.
http://www.cbip-vet.be/nl/texts/NRUOOOL1AL2o.php#diarree
(Accessed 26 September 2014).

Bhudevi, B., Weinstock, D., 2001. Fluorogenic RT-PCR assay (TaqMan) for detection and classification of bovine viral diarrhea virus. Veterinary Microbiology 83, 1-10.

Bitsch, V., Hansen, K.E.L., Ronsholt, L., 2000. Experiences from the Danish programme for eradication of bovine virus diarrhoea (BVD) 1994-1998 with special reference to legislation and causes of infection. Veterinary Microbiology 77, 137-143.

Blanchard, P.C., Ridpath, J.F., Walker, J.B., Hietala, S.K., 2010. An outbreak of late-term abortions, premature births, and congenital deformities associated with a Bovine viral diarrhea virus 1 subtype b that induces thrombocytopenia. Journal of Veterinary Diagnostic Investigation 22, 128-131.

Boehringer-Ingelheim, 2014. New vaccine against Bovine Viral Diarrhea: Boehringer Ingelheim receives European marketing authorization for Bovela®.
www.boehringer-ingelheim.com (Accessed 30 December 2014)

Brennan, M.L., Christley, R.M., 2012. Biosecurity on cattle farms: A study in North-West England. Plos One 7, e28139.

Brennan, M.L., Christley, R.M., 2013. Cattle producers' perceptions of biosecurity. BMC Veterinary Research 9, 71.

Brownlie, J., Booth, R., 2014. Bovine viral diarrhea: update on disease and its control. Veterinary Times. http://www.rvc.ac.uk/BVD/documents/VetTimesBVD19May2014.pdf (accessed 15 September 2014).

Brülisauer, F., Lewis, F.I., Ganser, A.G., McKendrick, I.J., Gunn, G.J., 2010. The prevalence of bovine viral diarrhoea virus infection in beef suckler herds in Scotland. The Veterinary Journal 186, 226-231.

BVDV Ag/Serum Plus Test Brochure, IDEXX.

www.idexx.com/pubwebresources/pdf/en_us/livestock-poultry/bvdv-ag-serum-plus-test-sheet.pdf (accessed 9 November 2014)

Cameron, A.R., Baldock, F.C., 1998. A new probability formula for surveys to substantiate freedom from disease. Preventive Veterinary Medicine 34, 1-17.

CODA-CERVA, 2013. Certification for diagnostic reagents to be used in Belgian official animal disease control programmes: ELISA kit for the detection of specific BVDV antigens in bovine ear notch, serum and full blood samples. Final report.

Cuttance, W., Cuttance, E., 2014. Analysis of individual farm investigations into bovine viral diarrhoea in beef herds in the North Island of New Zealand. New Zealand Veterinary Journal 62, 338-342.

Dierengezondheidszorg Vlaanderen (DGZ), 2015. Stop BVD! http://www.dgz.be/programma/stop-bvd (Accessed 2 January 2015)

Doll, K., Holsteg, M., 2013. BVD-virus type 2 – An outbreak in Germany. Cattle Practice 21, 216.

Ellis-Iversen, J., Cook, A.J.C., Watson, E., Nielen, M., Larkin, L., Wooldridge, M., Hogeveen, H., 2010. Perceptions, circumstances and motivators that influence implementation of zoonotic control programs on cattle farms. Preventive Veterinary Medicine 93, 276-285.

Ensoy, C., Faes, C., Welby, S., Van der Stede, Y., Aerts, M., 2014. Exploring cattle movements in Belgium. Preventive Veterinary Medicine 116, 89-101.

Ersbøll, A.K., Stryhn, H., 2000. Epidemiological modelling of infectious disease in animals: Bovine virus diarrhoea in Danish dairy herds. In: Proceedings of the 9th International

Symposium on Veterinary Epidemiology and Economics. Breckenridge, Colorado, USA, 520-522.

European Medicines Agency (EMA), 2014. CVMP summary of positive opinion for Bovela. http://www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/veterinary/003703/WC500175224.pdf (Accessed 30 December 2014)

Ezanno, P., Fourichon, C., Seegers, H., 2008. Influence of herd structure and type of virus introduction on the spread of bovine viral diarrhoea virus (BVDV) within a dairy herd. Veterinary Research 39, 39.

FAOSTAT, Food and Agriculture organization of the United Nations Statistics Division, 2014. Trade of live animals. http://faostat3.fao.org/faostat-gateway/go/to/home/E (accessed 15 September 2014).

Fourichon, C., Beaudeau, F., Bareille, N., Seegers, H., 2005. Quantification of economic losses consecutive to infection of a dairy herd with bovine viral diarrhoea virus. Preventive Veterinary Medicine 72, 177-181.

Fulton, R.W., Johnson, B.J., Briggs, R.E., Ridpath, J.F., Saliki, J.T., Confer, A.W., Burge, L.J., Step, D.L., Walker, D.A., Payton, M.E., 2006. Challenge with bovine viral diarrhea virus by exposure to persistently infected calves: protection by vaccination and negative results of antigen testing in nonvaccinated acutely infected calves. Canadian Journal of Veterinary Research-Revue Canadienne de Recherche Veterinaire 70, 121-127.

Fux, R., Wolf, G., 2012. Transient elimination of circulating bovine viral diarrhoea virus by colostral antibodies in persistently infected calves: a pitfall for BVDV-eradication programs? Veterinary Microbiology 161, 13-19.

Gates, M.C., Humphry, R.W., Gunn, G.J., 2013a. Associations between bovine viral diarrhoea virus (BVDV) seropositivity and performance indicators in beef suckler and dairy herds. The Veterinary Journal 198, 631-637.

Gates, M.C., Woolhouse, M.E.J., Gunn, G.J., Humphry, R.W., 2013b. Relative associations of cattle movements, local spread, and biosecurity with bovine viral diarrhoea virus (BVDV) seropositivity in beef and dairy herds. Preventive Veterinary Medicine 112, 285-295.

Gelaude, P., Schlepers, M., Verlinden, M., Laanen, M., Dewulf, J., 2014. Biocheck.UGent: A quantitative tool to measure biosecurity at broiler farms and the relationship with technical performances and antimicrobial use. Poultry Science.

doi: 10.3382/ps.2014-04002

Graham, D.A., Clegg, T.A., Lynch, M., More, S.J., 2013. Herd-level factors associated with the presence of bovine viral diarrhoea virus in herds participating in the voluntary phase of the Irish national eradication programme. Preventive Veterinary Medicine 112, 99-108.

Greiser-Wilke, I., Grummer, B., Moennig, V., 2003. Bovine viral diarrhoea eradication and control programmes in Europe. Biologicals 31, 113-118.

Grooms, D.L., 2004. Reproductive consequences of infection with bovine viral diarrhea virus. Veterinary Clinics of North America-Food Animal Practice 20, 5-19.

Gunn, G.J., Heffernan, C., Hall, M., McLeod, A., Hovi, M., 2008. Measuring and comparing constraints to improved biosecurity amongst GB farmers, veterinarians and the auxiliary industries. Preventive Veterinary Medicine 84, 310-323.

Hanon, J.B., Van Der Stede, Y., Antonissen, A., Mullender, C., Tignon, M., Van den Berg, T., Cay, A.B., 2014. Distinction between persistent and transient infection in a bovine viral diarrhoea (BVD) control programme: appropriate interpretation of real-time RT-PCR and antigen-ELISA test results. Transboundary and Emerging Diseases 61, 156-162.

Heffernan, C., Nielsen, L., Thomson, K., Gunn, G., 2008. An exploration of the drivers to bio-security collective action among a sample of UK cattle and sheep farmers. Preventive Veterinary Medicine 87, 358-372.

Holleville, P., 2013. Limit new infections: constraints and means in Pays de la Loire. BVD Day 2013. http://bvd-day2013.eu/conferences2/ (accessed 18 Aug. 2014)

Houe, H., 1992. Serological analysis of a small herd sample to predict presence or absence of animals persistently infected with bovine viral diarrhea virus (BVDV) in dairy herds. Research in Veterinary Science 53, 320-323.

Houe, H., 1994. Bovine virus diarrhea virus - Detection of Danish dairy herds with persistently infected animals by means of a screening-test of 10 young stock. Preventive Veterinary Medicine 19, 241-248.

Houe, H., 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterinary Microbiology 64, 89-107.

Houe, H., 2003. Economic impact of BVDV infection in dairies. Biologicals 31, 137-143.

Houe, H., Baker, J.C., Maes, R.K., Lloyd, J.W., Enevoldsen, C., 1995a. Comparison of the prevalence and incidence of infection with bovine virus diarrhoea virus (BVDV) in

Denmark and Michigan and association with possible risk factors. Acta Veterinaria Scandinavica 36, 521-531.

Houe, H., Baker, J.C., Maes, R.K., Wuryastuti, H., Wasito, R., Ruegg, P.L., Lloyd, J.W., 1995b. Prevalence of cattle persistently infected with bovine viral diarrhea virus in 20 dairy herds in 2 counties in central Michigan and comparison of prevalence of antibody-positive cattle among herds with different infection and vaccination status. Journal of Veterinary Diagnostic Investigation 7, 321-326.

Houe, H., Lindberg, A., Moennig, V., 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. Journal of Veterinary Diagnostic Investigation 18, 427-436.

Hovi, M., McLeod, A., Gunn, G., 2005. Assessing UK farmer attitudes to biosecurity on sheep and cattle farms. Research in Veterinary Science 78, 24 (Suppl. 1).

Hult, L., Lindberg, A., 2005. Experiences from BVDV control in Sweden. Preventive Veterinary Medicine 72, 143-148.

Humphry, R.W., Brülisauer, F., McKendrick, I.J., Nettleton, P.F., Gunn, G.J., 2012. Prevalence of antibodies to bovine viral diarrhoea virus in bulk tank milk and associated risk factors in Scottish dairy herds. Veterinary Record 171.

Jenckel, M., Hoper, D., Schirrmeier, H., Reimann, I., Goller, K.V., Hoffmann, B., Beer, M., 2014. Mixed triple: allied viruses in unique recent isolates of highly virulent type 2 bovine viral diarrhea virus detected by deep sequencing. Journal of Virology 88, 6983-6992.

Laanen, M., Beek, J., Ribbens, S., Vangroenweghe, F., Maes, D., Dewulf, J., 2010. Biosecurity on pig herds: development of an on-line scoring system and the results of the first 99 participating herds. Vlaams Diergeneeskundig Tijdschrift 79, 302-306.

Laanen, M., Maes, D., Hendriksen, C., Gelaude, P., De Vliegher, S., Rosseel, Y., Dewulf, J., 2014. Pig, cattle and poultry farmers with a known interest in research have comparable perspectives on disease prevention and on-farm biosecurity. Preventive Veterinary Medicine 115, 1-9.

Laanen, M., Persoons, D., Ribbens, S., de Jong, E., Callens, B., Strubbe, M., Maes, D., Dewulf, J., 2013. Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. The Veterinary Journal 198, 508-512.

Lanyon, S.R., Hill, F.I., Reichel, M.P., Brownlie, J., 2014. Bovine viral diarrhoea: Pathogenesis and diagnosis. The Veterinary Journal 199, 201-209.

Laureyns, J., 2014. "Challenges in the Control of Bovine Viral Diarrhoea Virus: Implications for a Belgian Eradication Programme". Merelbeke, Belgium: Ghent University. Faculty of Veterinary Medicine. http://hdl.handle.net/1854/LU-4231850

Laureyns, J., Booth, R., Sarrazin, S., Deprez, P., Pfeiffer, D., Dewulf, J., De Vliegher, S., 2013. Assessment of two essential elements of BVDV control on selected Flemish dairy and beef farms. Vlaams Diergeneeskundig Tijdschrift 82, 356-362.

Laureyns, J., Pardon, B., Letellier, C., Deprez, P., 2011. Periparturient infection with bovine viral diarrhea virus type 1 causes hemorrhagic proctocolitis in a cow. Canadian Veterinary Journal-Revue Veterinaire Canadienne 52, 1135-1139.

Laureyns, J., Ribbens, S., de Kruif, A., 2010. Control of bovine virus diarrhoea at the herd level: Reducing the risk of false negatives in the detection of persistently infected cattle. Veterinary Journal 184, 21-26.

Lindberg, A., Alenius, S., 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Veterinary Microbiology 64, 197-222.

Lindberg, A., Brownlie, J., Gunn, G.J., Houe, H., Moennig, V., Saatkamp, H.W., Sandvik, T., Valle, P.S., 2006. The control of bovine viral diarrhoea virus in Europe: today and in the future. Revue Scientifique et Technique-Office International des Epizooties 25, 961-979.

Lindberg, A., Houe, H., 2005. Characteristics in the epidemiology of bovine viral diarrhea virus (BVDV) of relevance to control. Preventive Veterinary Medicine 72, 55-73.

Lindberg, A., Stokstad, M., Loken, T., Alenius, S., Niskanen, R., 2004. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. Veterinary Record 154, 463-467.

Mars, M.H., Van Maanen, C., 2005. Diagnostic assays applied in BVDV control in The Netherlands. Preventive Veterinary Medicine 72, 43-48.

Maurin L., 2013. BVD eradication in Brittany: adapting methodologies to epidemiology, results, perspectives. BVD Day 2013. http://bvd-day2013.eu/conferences2/ (accessed 18 Aug. 2014)

McClurkin, A.W., Littledike, E.T., Cutlip, R.C., Frank, G.H., Coria, M.F., Bolin, S.R., 1984. Production of cattle immunotolerant to bovine viral diarrhea virus. Canadian Journal of Comparative Medicine-Revue Canadienne de Medecine Comparee 48, 156-161.

Meadows, D., 2010. A Study to Investigate the Use and Application of BVDV Vaccine in UK Cattle. Cattle Practice 18, 202-215.

Moen, A., 2013. Boviene virus diarree type 2. GD Veterinair 19, 3.

Moen, A., Sol, J., Sampimon, O., 2005. Indication of transmission of BVDV in the absence of persistently infected (PI) animals. Preventive Veterinary Medicine 72, 93-98.

Moerman, A., Straver, P.J., de Jong, M.C.M., Quak, J., Baanvinger, T., van Oirschot, J.T., 1993. A long-term epidemiologic-study of bovine viral diarrhea infections in a large herd of dairy-cattle. Veterinary Record 132, 622-626.

Nickell, J.S., White, B.J., Larson, R.L., Renter, D.G., Roque, J., Hesse, R., Oberst, R., Peddireddi, L., Anderson, G., 2011. Onset and duration of transient infections among antibody- diverse beef calves exposed to a bovine viral diarrhea virus persistently infected calf. International Journal of Applied Research in Veterinary Medicine 9, 29-39.

NIS, 2013. Count of the total number of cattle in Belgium (performed on 15/11/2013). National Institute for Statistics in Belgium. http://www.statbel.fgov.be (Accessed March 2014).

Niskanen, R., Lindberg, A., 2003. Transmission of bovine viral diarrhoea virus by unhygienic vaccination procedures, ambient air, and from contaminated pens. The Veterinary Journal 165, 125-130.

Niskanen, R., Lindberg, A., Larsson, B., Alenius, S., 2000. Lack of virus transmission from bovine viral diarrhoea virus infected calves to susceptible peers. Acta Veterinaria Scandinavica 41, 93-99.

Niskanen, R., Lindberg, A., Traven, M., 2002. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. The Veterinary Journal 163, 251-259.

O'Rourke, K., 2002. BVDV: 40 years of effort and the disease still has a firm hold. Journal of the American Veterinary Medical Association 220, 1770-1773.

OIE, 2013. OIE World Animal Health Information System, info by country, animal population. http://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Animalpopulation (Accessed 11 September 2014).

Palfi, V., Houe, H., Philipsen, J., 1993. Studies on the decline of bovine virus diarrhea virus (BVDV) maternal antibodies and detectability of BVDV in persistently infected calves. Acta Veterinaria Scandinavica 34, 105-107.

Potaufeux, V., 2013. BVDV in Eastern France. Targeting 2015: a collective control scheme running. BVD Day 2013. http://bvd-day2013.eu/conferences2/ (accessed 18 Aug. 2014)

Presi, P., Heim, D., 2010. BVD eradication in Switzerland-A new approach. Veterinary Microbiology 142, 137-142.

Presi, P., Struchen, R., Knight-Jones, T., Scholl, S., Heim, D., 2011. Bovine viral diarrhea (BVD) eradication in Switzerland-Experiences of the first two years. Preventive Veterinary Medicine 99, 112-121.

Reichel, M.P., Hill, F.I., Voges, H., 2008. Does control of bovine viral diarrhoea infection make economic sense? New Zealand Veterinary Journal 56, 60-66.

Ribbens, S., De Bleecker, K., Van Driessche, E., Antonissen, A., Laureyns, J., Sarrazin, S., Dewulf, J., Mijten, E., Stoop, S., 2012. Diagnosed BVDV PI-animals in Northern-Belgium: where do they go? 13th International Symposium on Veterinary Epidemiology and Economics (ISVEE). Maastricht, The Netherlands. 20-24 August 2012.

Ridpath, J.F., 2010. Bovine viral diarrhea virus: Global status. Veterinary Clinics of North America-Food Animal Practice 26, 105-121.

Ridpath, J.F., 2012. Preventive strategy for BVDV infection in North America. Japanese Journal of Veterinary Research 60, S41-S49.

Ridpath, J.F., 2013. Immunology of BVDV vaccines. Biologicals 41, 14-19.

Ridpath, J.F., Neill, J.D., Vilcek, S., Dubovi, E.J., Carman, S., 2006. Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Veterinary Microbiology 114, 196-204.

Royal Decree on the control of BVD, 18 June 2014. Published 30 June 2014. www.ejustice.just.fgov.be (Accessed 31 October 2014)

Rüfenacht, J., Schaller, P., Audige, L., Strasser, M., Peterhans, E., 2000. Prevalence of cattle infected with bovine viral diarrhoea virus in Switzerland. Veterinary Record 147, 413-417.

Saatkamp, H.W., Gunn, G.J., Stott, A.W., Humphry, R.W., 2006. Position paper: socio-economic aspects on BVDV control. EU Thematic Network on BVDV control. http://www.afbini.gov.uk/chs-thematic-network-position-paper-on-bvd-control.pdf (Accessed 11 September 14)

Sandvik, T., 2005. Selection and use of laboratory diagnostic assays in BVD control programmes. Preventive Veterinary Medicine 72, 3-16.

Sauter-Louis, C., Carlin, A., Friedrich, A., Assad, A., Reichmann, F., Rademacher, G., Heuer, C., Klee, W., 2012. Case control study to investigate risk factors for bovine neonatal

pancytopenia (BNP) in young calves in southern Germany. Preventive Veterinary Medicine 105, 49-58.

Schirrmeier, H., 2014. Three years of mandatory BVD control in Germany – Lessons to be learned. Federal Research Institute of Animal Health. http://www.fli.bund.de/fileadmin/dam_uploads/IVD/SD20146806.pdf (Accessed 29 October 2014)

Ståhl, K., Alenius, S., 2012. BVDV control and eradication in Europe - an update. Japanese Journal of Veterinary Research 60 , S31-S39.

Ståhl, K., Kampa, J., Baule, C., Isaksson, M., Moreno-Lopez, J., Belak, S., Alenius, S., Lindberg, A., 2005. Molecular epidemiology of bovine viral diarrhoea during the final phase of the Swedish BVD-eradication programme. Preventive Veterinary Medicine 72, 103-108.

Ståhl, K., Lindberg, A., Rivera, H., Ortiz, C., Moreno-Lopez, J., 2008. Self-clearance from BVDV infections - A frequent finding in dairy herds in an endemically infected region in Peru. Preventive Veterinary Medicine 83, 285-296.

Taylor, L.F., Janzen, E.D., Ellis, J.A., van den Hurk, J.V., Ward, P., 1997. Performance, survival, necropsy, and virological findings from calves persistently infected with the bovine viral diarrhea virus originating from a single Saskatchewan beef herd. Canadian Veterinary Journal-Revue Veterinaire Canadienne 38, 29-37.

Valle, P.S., Martin, S.W., Tremblay, R., Bateman, K., 1999. Factors associated with being a bovine-virus diarrhoea (BVD) seropositive dairy herd in the More and Romsdal County of Norway. Preventive Veterinary Medicine 40, 165-177.

Valle, P.S., Skjerve, E., Martin, S.W., Larssen, R.B., Osteras, O., Nyberg, O., 2005. Ten years of bovine virus diarrhoea virus (BVDV) control in Norway: a cost-benefit analysis. Preventive Veterinary Medicine 72, 189-207.

Villarroel, A., Dargatz, D.A., Lane, V.M., McCluskey, B.J., Salman, M.D., 2007. Suggested outline of potential critical control points for biosecurity and biocontainment on large dairy farms. Journal of the American Veterinary Medical Association 230, 808-819.

Summary

The first reports on BVDV date back to 1946. An apparently new transmissible disease in cattle with varying clinical signs was described and is nowadays worldwide spread. Up to date many studies have been published and are still being performed to understand the complex pathogenesis and epidemiology of the virus, but the considerable economic impact on cattle production is already generally recognized. Convinced by the economic importance of BVDV, several European countries launched an eradication or control campaign. This doctoral thesis aimed at gaining insight into the current BVDV approach in Belgian cattle herds.

As a general introduction (***Chapter 1***), an overview of BVDV, being relevant for this thesis is given. BVDV consists of a group of many genetic variants and causes a broad range of clinical signs, which vary from subclinical infections to severe outbreaks with high mortality. From this, the impact of BVDV on cattle production logically follows. The epidemiology of BVDV is highlighted. First, the transmission of BVDV, through PI and TI animals or indirectly, is described. Furthermore, risk factors for the occurrence of BVDV infections are introduced. The diagnosis of BVDV based upon clinical signs is not obvious and should therefore be supported by laboratory tests. Finally, the elements for systematic BVDV control are introduced: biosecurity, elimination of virus sources, monitoring and finally vaccination as an optional measure.

In Belgium recommendations for systematic control were proposed by the regional centres for animal health control to be implemented on a voluntary basis at the herd level. Few recent data on the effect of this approach on the BVDV situation were available. However, such information can be very useful for the Belgian mandatory BVDV control programme that has been launched in January 2015. Therefore, the overall objectives of this doctoral thesis were to estimate the BVDV prevalence, to gain insight into the current implementation of essential measures in BVDV control and to evaluate the risk of (re-)infection in Belgian cattle herds (***Chapter 2***). The results are used to provide recommendations for further improvement of the Belgian mandatory control programme.

In Belgium only limited and outdated data on the prevalence of BVDV were available. Therefore, the first objective was to estimate both the virological and the serological BVDV prevalence in Belgium using a large representative sample. Additionally, potential risk factors for herds to be BVDV seropositive were studied (***Chapter 3***). For this, young stock aged between 6 and 12 months from randomly selected Belgian cattle herds were tested for BVDV-

specific antibodies and antigen. Furthermore a questionnaire including different herd management topics and questions about participation in animal health programmes, including BVDV, was sent to Belgian cattle farmers. The true prevalence of BVDV-specific antibodies and antigen at the herd level was respectively 47.4% and 4.4%, while at the animal level this was respectively 32.9% and 0.3%. In 44.4% of the herds where BVDV-specific antibodies were detected at least 60% of the sampled young stock was BVDV seropositive, which suggested the presence of PI animals. Interestingly, 83.4% of these farmers stated not to have suffered from problems related to BVDV. Moreover, only 8.4% of all farmers who completed the questionnaire reported problems possibly related to BVDV the past 3 years. This demonstrated that farmers are often unaware of the presence of BVDV in their herd. Risk factors for a herd to be BVDV seropositive were identified by means of a multivariable logistic regression model. Large herds were significantly more likely to be BVDV seropositive (OR = 1.004, $p < 0.01$). The interaction between "Antigen positive animal detected in this study" and "BVDV vaccination in 2009" was significant ($p < 0.01$). In non-vaccinating herds, the detection of antigen positive animals was significantly associated with BVDV seropositive herds (OR = 13.8, $p < 0.01$). In herds with no antigen positive animals detected, vaccination resulted in a significant risk factor to be BVDV seropositive compared to non-vaccinating herds (OR = 3.4, $p < 0.01$). Herds reporting BVDV-related problems the past 3 years were more likely to be BVDV seropositive (OR = 1.9, $p < 0.05$). This relation became non-significant (OR = 1.8, $p = 0.08$) when only a subset of herds with no vaccination of animals < 12 months was taken into account. The main conclusion was that there is an active circulation of BVDV in a considerable number of Belgian cattle herds.

Next, the importance of TI cattle in the epidemiology of BVDV was assessed (Chapters 4.1 and 4.2). In general TI animals are considered to be of minor importance, but a recent BVDV-2c outbreak in Germany and the Netherlands has challenged this assumption.

In a first transmission study (***Chapter 4.1***), two experimental infections were performed to determine basic reproduction ratios (R_0). In each experiment three calves were infected via intranasal inoculation and housed together with seven susceptible animals. Two strains isolated in Belgium were used, a virulent BVDV-1b and a virulent BVDV-2a field isolate, resulting in an R_0 of 0.25 (95% CI 0.01; 1.95) and 0.24 (95% CI 0.01; 2.11), respectively. A PI animal was then introduced to the remaining uninfected animals and produced an R of $+\infty$ (95% CI 1.88; $+\infty$). The results supported the suggestion that TI animals, compared to PI animals, contribute only a limited amount to BVDV spread.

Additionally, the severe clinical symptoms observed in the field with these isolates could not be reproduced during these experiments suggesting that other factors besides strain virulence influence the clinical manifestations evoked by BVDV.

In a second transmission study (***Chapter 4.2***), an experimental infection was performed using a hypervirulent BVDV-2c field strain isolated during a severe BVDV outbreak in Germany to determine R_0 and to describe the clinical signs caused by such an infection. Three calves were intranasally infected and housed together with seven susceptible animals. The clinical signs of the BVDV infected animals varied from very mild disease (fever, loss of appetite) to severe watery and haemorrhagic diarrhoea and death. The clinical signs and the level of BVDV excretion depended on the degree of viraemia. R_0 was estimated to be 0.49 (95% CI 0.06; 2.99) suggesting a limited viral spread using the BVDV-2c strain. It was concluded that also this BVDV-2 infection in TI animals resulted in limited transmission towards other animals.

In modern veterinary medicine, disease prevention at the herd level has become increasingly important in replacing individual animal medicine. This shift involves the implementation of biosecurity. At present, no studies have considered the implementation of biosecurity measures in the daily management of Belgian cattle farms. Therefore another aim of the thesis was to map the current application of biosecurity measures in Belgian cattle farms in the prevention of disease transmission within and between farms (***Chapter 5***). The data were collected as part of a larger cross-sectional study (Chapter 6), conducted to identify risk factors for re-infection with BVDV in cattle herds assumed free from BVDV. Questionnaire data from 33 dairy farms, 16 beef farms and 25 mixed (dairy and beef cattle) farms were analysed using a combination of a linear scoring system, a categorical principal component analysis and a two-step cluster analysis to differentiate these farms based on their biosecurity level and visit frequencies. Further enhancement of preventive measures considering external and internal biosecurity was still possible for each farm, as none of the farms obtained an overall high biosecurity level. Three groups of cattle farms were differentiated with a biosecurity level varying from low to high-medium, of which the group with the lowest biosecurity level mainly consisted of mixed farms. Animal-to-animal contacts with cattle from other herds were frequently possible as only 12% of the farmers purchasing cattle quarantined purchased animals at least 3 weeks and contacts over fences on pasture were possible in 70% of the herds. Basic biosecurity measures such as farm-specific protective clothing and boots were present in the majority of the farms, but they were

insufficiently or incorrectly used. Cattle farms were very often visited by professional visitors of which the herd veterinarian, the AI technician and the cattle salesman most frequently entered the farm. The main conclusion was that since few biosecurity measures were undertaken by Belgian cattle farmers, they expose their herd to the risk of disease transmission within and between farms.

In a final study (***Chapter 6***), a cross-sectional and a longitudinal study were conducted to better understand the drivers for re-infection with BVDV and to evaluate the risk of re-infection in Belgian cattle herds, given the current high BVDV prevalence and poor implementation of biosecurity measures. In the cross-sectional study young stock were sampled for BVDV antibodies and a face-to-face interview with the farmer was conducted on 61 farms. Potential risk factors for a herd being re-infected were identified by a multivariable logistic regression model. In 11 of the 61 herds (18%) at least 20% of the young stock tested BVDV seropositive. Farms monitoring the BVDV status were less likely to be BVDV re-infected (OR 0.09; 95% CI 0.02; 0.55). There was also a decrease in risk when the neighbouring cattle farm was located further away (OR per km 0.06; 95% CI 0.00; 0.96). Re-infection was more likely when farmers participated to auctions and/or competitions (OR 140.27; 95% CI 3.00; 6559.65). In the longitudinal study the BVDV status of 26 non-BVDV-vaccinating farms was monitored for 18 months. An incidence risk of re-infection of 15.4% per year was estimated. On 4 of 6 re-infected farms at least one PI animal was detected. The purchase of cattle was identified as cause of re-infection on one farm. These results demonstrated a high risk of BVDV re-infection in Belgian cattle herds. The main conclusion was that the risk of BVDV re-infection is likely to remain high, given the high BVDV prevalence and the violations of essential measures in BVDV control such as monitoring and biosecurity.

In the general discussion (***Chapter 7***), first, the factors contributing to the high BVDV prevalence in Belgian cattle herds as described in Chapter 3 are discussed. A high cattle density together with an increasing herd size, the frequent contact with cattle through national and international trade, the fact that farmers often are unaware of the presence of BVDV and especially the absence of a systematic approach for BVDV control were considered to be important influencing parameters. Therefore, a mandatory national control programme has been launched in January 2015 with the final aim of eradicating BVDV. Secondly, the current design of this Belgian control programme is introduced. In an early phase the programme focuses on the detection of PI animals by the mandatory testing of new-born calves. The

preferred way of sampling is via ear notch samples, which are obtained when the farmer places the official ear tag. Following the test result of the new-born calf, a BVDV status will also be assigned to the mother of the calf. Depending on the status of the animal specific movement restrictions are imposed. PI animals are not allowed to be grazed or transported, except for slaughter. In addition to this legal basis, non-compulsory guidelines regarding the removal of PI animals and the prevention of re-infection are provided by the regional centres for animal disease control. In the final paragraph of the general discussion, each of the measures for systematic BVDV control (removal of virus sources, biosecurity and monitoring) are discussed in the light of the Belgian mandatory control programme. Regarding the removal of virus sources, the focus should be on the detection and elimination of PI animals, while the major importance of TI animals in BVDV spread is their ability to deliver a PI animal when they are infected in early pregnancy. The mandatory testing of all new-born calves is a step forward. Nevertheless, cattle diagnosed as PI should be removed immediately after detection to avoid infection of susceptible cattle in early pregnancy. Regarding the prevention of infection of cattle in early pregnancy, the risk of infection, which is high at the moment, will decrease during the progress of the programme in line with the decrease of the number of new-born PI animals. Nonetheless, increased and continuous efforts to prevent re-infection should be undertaken. Specific recommendations to prevent infection of cattle in early pregnancy are quarantining of purchased cattle, avoiding contact on pastures, separating a new-born calf and its dam until the test result is known and finally providing herd-specific clothing for professional visitors entering the farm. Given the high BVDV prevalence and high risk of infection at the start of the programme, vaccination as an additional biosecurity measure to prevent foetal infections may be advised. In a next phase a BVDV free status will be assigned to herds of which all individual cattle tested BVDV negative. Specific directives about how this phase will be implemented are not yet determined and will depend on the progress of the first phase of the programme. Regarding the transition to this next phase, it would be advisable to start a pilot study in some herds that have been shown to be BVDV free for some years already to examine whether a continued ear notch sampling is economically favourable compared to the individual testing of animals for trade in combination with a serological screening of young stock.

Samenvatting

De eerste publicaties over BVDV dateren van 1946. Toen werd een schijnbaar nieuwe overdraagbare runderziekte met variabele klinische symptomen beschreven. BVDV is heden ten dage wereldwijd verspreid. Tot op vandaag zijn reeds talloze studies gepubliceerd en worden nog steeds uitgevoerd om de complexe pathogenese en epidemiologie van het virus beter te begrijpen. De aanzienlijke economische impact van BVD is echter algemeen erkend. Gezien het economische belang van BVDV zijn in verschillende Europese landen nationale bestrijdingsprogramma's van kracht. Dit doctoraatsonderzoek is gewijd aan het in kaart brengen van de huidige BVDV situatie in Belgische rundveebedrijven.

In de algemene inleiding (***Hoofdstuk 1***) worden de aspecten van BVDV die van belang zijn voor deze thesis aangekaart. BVDV omvat een groep van vele genetische varianten en veroorzaakt een brede waaier aan klinische symptomen, gaande van subklinische infecties tot ernstige uitbraken met hoge mortaliteit. Hierdoor wordt het economische belang van BVDV duidelijk. De epidemiologie van BVDV wordt grondig besproken. Eerst worden de verschillende verspreidingsmechanismen, via PI en TI dieren of indirect, beschreven. Verder worden risicofactoren voor infecties met BVDV aangekaart. Het stellen van de diagnose BVDV op basis van enkel de klinische symptomen is niet eenvoudig en daarom zijn laboratoriumtesten nodig. Tot slot worden de verschillende maatregelen voor een systematische BVDV controle ingeleid: bioveiligheid, verwijderen van de virusbronnen, monitoring en tot slot vaccinatie als optioneel onderdeel.

In België werden aanbevelingen voor een systematische BVDV controle gegeven door de regionale dierengezondheidscentra. Deze aanbevelingen konden op vrijwillige basis toegepast worden op bedrijfsniveau. Weinig recente gegevens over het effect van deze aanpak op de BVDV situatie zijn voorhanden. Nochtans zijn dergelijke gegevens zeer nuttig voor het Belgische verplichte nationale BVDV bestrijdingsprogramma dat vanaf januari 2015 van start is gegaan. Daarom waren de doelstellingen van dit doctoraatsonderzoek om de BVDV prevalentie te schatten, inzicht te krijgen in de huidige toepassing van de essentiële onderdelen voor een systematische BVDV controle en om het risico op (her)besmetting met BVDV in Belgische rundveebedrijven in te schatten (***Hoofdstuk 2***). De bekomen resultaten worden gebruikt om aanbevelingen te doen voor het Belgische bestrijdingsprogramma.

In België zijn slechts beperkte en gedateerde gegevens over de prevalentie beschikbaar. Daarom was de eerste doelstelling om zowel de virologische als serologische prevalentie van BVDV in België te schatten, hierbij gebruikmakend van een grote

representatieve steekproef. Bijkomend werden risicofactoren voor het BVDV seropositief zijn van rundveebedrijven bestudeerd (***Hoofdstuk 3***). Hiervoor werd jongvee tussen 6 en 12 maanden oud van willekeurig geselecteerde Belgische rundveebedrijven getest voor de aanwezigheid van BVDV antistoffen en antigen. Verder werd een enquête over verscheidene onderdelen van het bedrijfsmanagement en over deelname aan dierengezondheids-programma's, waaronder BVDV, verstuurd naar Belgische rundveehouders. De ware prevalentie van respectievelijk BVDV antistoffen en antigen op bedrijfsniveau was 47.4% en 4.4%. Op dierniveau was dit respectievelijk 32.9% en 0.3%. Bij 44.4% van de bedrijven waar BVDV antistoffen werden gedetecteerd, was minstens 60% van het onderzochte jongvee seropositief. Dit deed de aanwezigheid van PI dieren vermoeden. Opmerkelijk was dat 83.4% van deze rundveehouders verklaarde geen BVDV-gerelateerde problemen te hebben gehad. Verder meldde slechts 8.4% van de rundveehouders die de enquête invulden, mogelijk BVDV-gerelateerde problemen te hebben gehad gedurende de afgelopen 3 jaar. Dit toonde aan dat rundveehouders zich vaak niet bewust zijn van de aanwezigheid van BVDV op hun bedrijf. Risicofactoren voor het BVDV seropositief zijn op bedrijfsniveau werden nagegaan aan de hand van een multivariabel logistisch regressiemodel. Grotere bedrijven hadden meer kans om seropositief te zijn (OR = 1.004, $p < 0.01$). De interactie tussen de variabelen "Antigen positief dier gedetecteerd tijdens deze studie" en "BVDV vaccinatie in 2009" was significant ($p < 0.01$). Op niet-vaccinerende bedrijven was het detecteren van antigen positieve dieren significant geassocieerd met het seropositief zijn op bedrijfsniveau (OR = 13.8, $p < 0.01$). Op bedrijven zonder detectie van antigen positieve dieren was vaccinatie een risicofactor voor het seropositief zijn in vergelijking met niet-vaccinerende bedrijven (OR = 3.4, $p < 0.01$). Bedrijven die BVDV-gerelateerde problemen gedurende de laatste 3 jaar rapporteerden, hadden meer kans om seropositief te zijn (OR = 1.9, $p < 0.05$). Dit verband was niet significant wanneer enkel de bedrijven die geen dieren jonger dan 12 maanden vaccineerden, werden beschouwd (OR = 1.8, $p = 0.08$). De voornaamste conclusie was dat er een actieve circulatie van BVDV is op een aanzienlijk deel van de Belgische rundveebedrijven.

Vervolgens werd het belang van TI dieren in de epidemiologie van BVDV nagegaan (Hoofdstukken 4.1 en 4.2). TI dieren worden algemeen van weinig belang beschouwd, maar een recente BVDV-2c uitbraak in Duitsland en Nederland heeft deze assumptie in vraag gesteld.

Tijdens een eerste transmissiestudie (***Hoofdstuk 4.1***) werden twee experimentele infecties uitgevoerd om de basis reproductie ratio (R_0) na te gaan. Tijdens elke infectie werden drie kalveren intranasaal geïnoculeerd en samen gehuisvest met zeven gevoelige dieren. Twee stammen die werden geïsoleerd in België werden hiervoor gebruikt, een virulente BVDV-1b en een virulente BVDV-2a veldstam. Dit resulteerde in een R_0 van respectievelijk 0.25 (95% CI 0.01; 1.95) en 0.24 (95% CI 0.01; 2.11). Een PI dier werd vervolgens bij deze kalveren geplaatst en dit resulteerde in een R van $+\infty$ (95% CI 1.88; $+\infty$). Deze resultaten onderbouwen de veronderstelling dat TI dieren, in vergelijking met PI dieren, slechts in beperkte mate bijdragen tot het verspreiden van BVDV. Bijkomend konden de ernstige klinische symptomen die met deze stammen in het veld werden vastgesteld niet worden gereproduceerd, wat erop wijst dat andere factoren, naast de virulentie van stammen, een invloed hebben op de klinische symptomen die gepaard gaan met BVDV infecties.

Tijdens een tweede transmissiestudie (***Hoofdstuk 4.2***) werd een experimentele infectie uitgevoerd met een hypervirulente BVDV-2c veldstam die werd geïsoleerd tijdens een ernstige BVDV uitbraak in Duitsland. De doelstellingen waren om R_0 te bepalen en de klinische symptomen die gepaard gaan met een dergelijke infectie te beschrijven. Drie kalveren werden intranasaal geïnfecteerd en samen gehuisvest met zeven gevoelige kalveren. De klinische symptomen gepaard gaande met de infectie waren variabel, gaande van mild (koorts en verminderde eetlust) tot ernstige waterige en bloederige diarree en sterfte. De klinische symptomen en de graad van BVDV uitscheiding waren afhankelijk van de graad van viremie. R_0 werd geschat op 0.49 (95% CI 0.06; 2.99), wat wijst op een beperkte virusverspreiding met de BVDV-2c stam. Het besluit was dat ook met deze BVDV-2c infectie TI dieren slechts beperkt het virus verspreiden naar andere dieren.

In de huidige diergeneeskunde neemt het belang van ziektepreventie op bedrijfsniveau ter vervanging van individuele geneeskunde op dierniveau toe. Bioveiligheid maakt onderdeel uit van deze ziektepreventie. Tot op heden zijn er geen studies uitgevoerd die de toepassing van bioveiligheidsmaatregelen in de dagelijkse bedrijfsvoering van Belgische rundveebedrijven nagaan. Een volgende doelstelling van deze thesis was dan ook om de huidige toepassing van bioveiligheidsmaatregelen op Belgische rundveebedrijven ter preventie van ziekteverspreiding tussen en op bedrijven in kaart te brengen (***Hoofdstuk 5***). Gegevens werden verzameld als onderdeel van een dwarsdoorsnede onderzoek (Hoofdstuk 6), dat werd uitgevoerd om risicofactoren voor de herinfectie met BVDV op verondersteld BVDV-vrije rundveebedrijven te identificeren. Gegevens van 33 melkvee-, 16 vleesvee- en

25 gemengde (melkvee en vleesvee) bedrijven werden verzameld en geanalyseerd met behulp van een lineair scoresysteem, een categorische hoofdcomponentenanalyse en een clusteranalyse om deze bedrijven te kunnen onderscheiden op basis van hun bioveiligheidsniveau en bezoekfrequenties. Verbetering van preventieve maatregelen aangaande externe en interne bioveiligheid was mogelijk voor elk bedrijf, aangezien geen enkel bedrijf een hoog bioveiligheidsniveau behaalde. Drie groepen rundveebedrijven werden onderscheiden met een bioveiligheidsniveau gaande van laag tot gemiddeld-hoog, waarvan de groep met het laagste niveau vooral uit gemengde bedrijven bestond. Dier-diercontacten met runderen van andere bedrijven waren zeer plausibel aangezien slechts 12% van de rundveehouders die runderen aankochten de aangekochte dieren minstens 3 weken in quarantaine plaatsten en weidecontact mogelijk was in 70% van de bedrijven. Elementaire bioveiligheidsmaatregelen zoals bedrijfskledij en laarzen waren aanwezig in de meerderheid van de bedrijven, maar ze werden onvoldoende of verkeerd gebruikt. De rundveebedrijven werden zeer vaak bezocht door professionele bezoekers, waarvan de bedrijfsdierenarts, de inseminator en veehandelaar het vaakst het bedrijf betraden. De belangrijkste conclusie was dat slechts weinig bioveiligheidsmaatregelen in acht worden genomen door Belgische rundveehouders. Op deze manier stellen ze hun dieren bloot aan het risico op ziektetransmissie binnenin en tussen bedrijven.

In een laatste onderzoek (***Hoofdstuk 6***) werden een dwarsdoorsnede en een longitudinale studie uitgevoerd om de factoren die herinfectie met BVDV in de hand werken en het risico op herinfectie in Belgische rundveebedrijven na te gaan onder de huidige hoge BVDV prevalentie en beperkte toepassing van bioveiligheidsmaatregelen. Tijdens het dwarsdoorsnede onderzoek werd jongvee bemonsterd voor serologie en werd een enquête afgenomen met de veehouder op 61 bedrijven. Mogelijke risicofactoren voor herinfectie met BVDV werden onderzocht met behulp van een multivariabel logistisch regressiemodel. In 11 van 61 bedrijven (18%) testte minstens 20% van het onderzochte jongvee seropositief. Bedrijven die de BVDV status monitoren, hadden minder kans op herinfectie (OR 0.09; 95% CI 0.02; 0.55). Het risico op herinfectie daalde ook wanneer de afstand tot het dichtstbijzijnde rundveebedrijf verhoogde (OR per km 0.06; 95% CI 0.00; 0.96). De kans op herinfectie was groter wanneer rundveehouders deelnamen aan prijskampen en/of veilingen (OR 140.27; 95% CI 3.00; 6559.65). Tijdens de longitudinale studie werd de BVDV status van 26 niet-vaccinerende bedrijven opgevolgd gedurende 18 maanden. Een cumulatieve incidentie van herinfectie van 15.4% per jaar werd geschat. Op 4 van de 6 opnieuw geïnfecteerde bedrijven

werd de geboorte van minstens één PI dier vastgesteld. De aankoop van rundvee werd geïdentificeerd als oorzaak van herinfectie op één bedrijf. Deze resultaten toonden een groot risico op herinfectie met BVDV op Belgische rundveebedrijven aan. De voornaamste conclusie was dat dit risico op herinfectie waarschijnlijk hoog blijft zolang de BVDV prevalentie hoog blijft en er fouten worden gemaakt tegen de essentiële onderdelen van de BVDV controle, zoals bioveiligheid en monitoring.

In de algemene discussie (***Hoofdstuk 7***) worden eerst de factoren die bijdragen tot een hoge BVDV prevalentie op Belgische rundveebedrijven besproken. Een hoge rundveedensiteit in combinatie met een toenemende bedrijfsgrootte, frequente contacten met rundvee tijdens nationale en internationale handel, het feit dat rundveehouders zich vaak niet bewust zijn van de aanwezigheid van BVDV op hun bedrijf en vooral het ontbreken van een systematische aanpak van BVDV worden als belangrijke factoren beschouwd. Daarom is in januari 2015 een nationaal verplicht bestrijdingsprogramma van kracht gegaan dat als ultieme doelstelling het verwijderen van BVDV in België heeft. In een tweede paragraaf wordt dan ook het Belgische bestrijdingsprogramma besproken. In een eerste fase focust dit programma zich op het detecteren van PI dieren door het verplicht testen van pasgeboren kalveren. Het gebruik van oorweefselstalen, die worden bekomen bij het plaatsen van het oormerk, is hiervoor de aangewezen methode. Op basis van het testresultaat van het kalf wordt eveneens een BVDV status toegekend aan het moederdier. Afhankelijk van het resultaat wordt de beweging van dieren beperkt. PI dieren mogen niet op de weide en mogen het bedrijf niet verlaten, tenzij voor de slacht. Als aanvulling op deze wettelijk vastgelegde maatregelen worden eveneens richtlijnen aangaande het verwijderen van PI dieren en het voorkomen van herinfectie verstrekt door de regionale diergezondheidscentra. In de laatste paragraaf van de algemene discussie worden de onderdelen voor een systematische aanpak van BVDV (verwijderen van virusbronnen, bioveiligheid en monitoring) besproken in het kader van het Belgisch verplicht nationaal bestrijdingsprogramma. Aangaande het verwijderen van virusbronnen dient de focus gericht te zijn op het opsporen en opruimen van PI dieren, terwijl het belang van TI dieren vooral het voortbrengen van PI dieren betreft, wanneer TI dieren worden geïnfecteerd in een vroeg stadium van de dracht. Het verplicht testen van alle nieuwgeboren kalveren is een grote stap vooruit. Nochtans zouden runderen die als PI worden geïdentificeerd onmiddellijk dienen te worden opgeruimd. Wat het voorkomen van herinfectie betreft, zal het risico op herinfectie, dat momenteel hoog is, afnemen naar mate het programma vordert en dit in lijn met een daling van het aantal nieuwgeboren PI dieren.

Nochtans zou een verhoogde en continue toepassing van bioveiligheidsmaatregelen de norm moeten zijn. Specifieke aanbevelingen betreffende bioveiligheid om infectie van dieren in een vroeg drachtstadium te voorkomen, zijn het in quarantaine plaatsen van aangekochte dieren, het verhinderen van contact met runderen van een ander bedrijf op de weide, het apart houden van een pasgeboren kalf en het moederdier tot het testresultaat bekend is en het voorzien van bedrijfskledij voor professionele bezoekers. Gezien de hoge BVDV prevalentie en het risico op herinfectie bij aanvang van het programma, kan vaccinatie als bijkomende bioveiligheidsmaatregel, aangewezen zijn om infectie van de foetus te voorkomen. In een latere fase wordt een status toegekend aan bedrijven waar alle individuele runderen een BVDV status hebben. Specifieke richtlijnen over hoe deze fase zal worden uitgevoerd zijn nog niet voorhanden en zijn afhankelijk van de voortgang van de eerste fase van het programma. Aangaande de overgang van de eerste fase van de bestrijding naar het toekennen van een status aan het bedrijf, kan een pilootstudie in bedrijven die reeds een tijd vrij zijn van BVDV aangewezen zijn om een verderzetting van het bemonsteren van oorweefselstalen economisch en epidemiologisch af te wegen ten opzichte van het individueel testen van dieren bestemd voor handel in combinatie met een serologische screening van jongvee.

Curriculum vitae

Steven Sarrazin werd geboren op 25 april 1986 te Menen. Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Janscollege te Poperinge (Wetenschappen-Wiskunde) werd de studie Diergeneeskunde aangevat aan de Universiteit Gent. Hij behaalde in 2010 het diploma Master in de diergeneeskunde (optie herkauwers) met de grootste onderscheiding.

In oktober 2010 trad hij als doctoraatsbursaal in dienst van de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde. In samenwerking met de eenheid voor Endemische en (her)Opduikende Virale Ziekten van het Centrum voor Onderzoek in de Diergeneeskunde en Agrochemie (CODA) verrichtte hij onderzoek naar de impact van horizontale Boviene Virale Diarree virustransmissie in de haalbaarheid van een bestrijdingsplan op bedrijfsniveau. Dit driejarig mandaat werd gefinancierd door de Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu. Vanaf 15 december 2013 tot op heden was hij in dienst van de Universiteit Gent.

Gedurende de vier jaar die hij besteedde aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde stond hij mee in voor de opleiding van de studenten en nam hij deel aan de werking van de kliniek verloskunde rund.

Steven is auteur of medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en presenteerde zijn onderzoeksresultaten op verschillende nationale en internationale congressen.

Bibliography

Publications

Sarrazin, S., Veldhuis, A., Méroc, E., Vangeel, I., Laureyns, J., Dewulf, J., Cay, A.B., Piepers, S., Hooyberghs, J., Ribbens, S., Van Der Stede, Y., 2013. Serological and virological BVDV prevalence and risk factor analysis for herds to be BVDV seropositive in Belgian cattle herds. Preventive Veterinary Medicine 108, 28-37.

Laureyns, J., Piepers, S., Ribbens, S., **Sarrazin, S.**, De Vliegher, S., Van Crombrugge, J.M., Dewulf, J., 2013. Association between herd exposure to BVDV-infection and bulk milk somatic cell count of Flemish dairy farms. Preventive Veterinary Medicine 109, 148-151.

Laureyns, J., Pardon, B., Cay, A.B., **Sarrazin, S.**, Deprez, P., 2013. Spontaneous bleeding in a neonatal calf persistently infected with BVDV1b. Vlaams Diergeneeskundig Tijdschrift 82, 87-89.

Laureyns, J., Booth, R., **Sarrazin, S.**, Deprez, P., Pfeiffer, D., Dewulf, J., De Vliegher, S., 2013. Assessment of two essential elements of BVDV control on selected Flemish dairy and beef farms. Vlaams Diergeneeskundig Tijdschrift 82, 356-362.

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A survey on biosecurity and management practices in selected Belgian cattle herds. Preventive Veterinary Medicine 117, 129-139.

Sarrazin, S., Dewulf, J., Mathijs, E., Laureyns, J., Mostin, L., Cay, A.B., 2014. Virulence comparison and quantification of horizontal bovine viral diarrhoea virus transmission following experimental infection in calves. The Veterinary Journal 202, 244-249.

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A cross-sectional and longitudinal study on the risk of BVDV re-infection in BVDV-free cattle herds in Belgium. BMC Veterinary Research. *Submitted*.

Sarrazin, S., Dewulf, J., Laureyns, J., Mostin, L., Cay, A.B., 2014. A transmission experiment in calves infected with a recently discovered hypervirulent BVDV-2c strain. Transboundary and Emerging Diseases. *Under review*.

Oral presentations

Sarrazin, S., Veldhuis, A., Meroc, E., Laureyns, J., Dewulf, J., Cay, A.B., Hooyberghs, J., Van Der Stede, Y., 2011. Serological an virological BVDV prevalence in Belgian cattle herds. 6th European Congress of Bovine Health Management, 7-9 September 2011, Liège, Belgium.

Sarrazin, S., Veldhuis, A., Meroc, E., Laureyns, J., Dewulf, J., Cay, A.B., Hooyberghs, J., Van Der Stede, Y., 2011. Serological an virological BVDV prevalence in Belgian cattle herds. 19th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics, 27 October 2011, Sint-Niklaas, Belgium.

Ribbens, S., De Bleecker, K., Van Driessche, E., Antonissen, A., Laureyns, J., **Sarrazin, S.**, Dewulf, J., Mijten, E. and Stoop S., 2012. Diagnosed BVDV PI-animals in Northern-Belgium: where do they go? 27th World Buiatric Congress, 3-8 June 2012, Lisbon, Portugal.

Ribbens, S., De Bleecker, K., Van Driessche, E., Antonissen, A., Laureyns, J., **Sarrazin, S.**, Dewulf, J., Mijten, E. and Stoop S., 2012. Diagnosed BVDV PI-animals in Northern-Belgium: where do they go? 13th International Society for Veterinary Epidemiology and Economics Congress, 20-24 August 2012, Maastricht, The Netherlands.

Sarrazin, S., Dewulf, J., Mathijs, E., Laureyns, J., Mostin, L., Cay, A.B., 2013. Virulence comparison and quantification of BVDV transmission following experimental infection in calves. 7th EPIZONE annual meeting, 1-4 October 2013, Brussels, Belgium.

Sarrazin, S., Schirrmeier, H., Dewulf, J., Laureyns, J., Mostin, L., Beer, M., Cay, A.B., 2013. Virus transmission and clinical symptoms in calves experimentally infected with a recently discovered hypervirulent BVDV-2c strain. 7th EPIZONE annual meeting, 1-4 October 2013, Brussels, Belgium.

Sarrazin, S., 2013. Practical implementations of biosecurity measures in cattle herds. 2nd Center of expertise on Antimicrobial Consumption and Resistance in Animals (AMCRA) Seminar: Vaccination, biosecurity and management as tools for a reduced antimicrobial usage. 10-11 October 2013, Brussels, Belgium. (invited)

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2013. A descriptive study on biosecurity measures in selected Belgian cattle herds. 20th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics, 8 November 2013, Drongen, Belgium.

Poster presentations

Sarrazin, S., Veldhuis, A., Meroc, E., Laureyns, J., Dewulf, J., Caij, A.B., Hooyberghs, J., Van Der Stede, Y., 2011. Serological an virological BVDV prevalence in Belgian cattle herds. 8th ESVV Pestivirus Symposium, 25-28 September 2011, Hannover, Germany.

Laureyns, J., Ribbens, S., **Sarrazin, S.**, De Vliegher, S., Van Crombrugge, J.M., De Bleecker, K., Dewulf, J., 2012. The relationship between herd exposure to BVDV-infection and bulk milk somatic cell count of dairy farms. 27th World Buiatric Congress, 3-8 June 2012, Lisbon, Portugal.

Sarrazin, S., Hanon, J.B., Dernelle, E., Dewulf, J., Cay, A.B., 2012. Necessity of a strict biosecurity to maintain BVDV-free status in artificial insemination centers. 13th International Society for Veterinary Epidemiology and Economics Congress, 20-24 August 2012, Maastricht, The Netherlands.

Sarrazin, S., Laureyns, J., Cay, A.B., Dewulf, J., 2012. Lack of monitoring and biosecurity measures to maintain a BVDV-free status in cattle herds. 13th International Society for Veterinary Epidemiology and Economics Congress, 20-24 August 2012, Maastricht, The Netherlands.

Sarrazin, S., Dewulf, J., Mathijs, E., Laureyns, J., Mostin, L., Cay, A.B., 2013. Determination of virulence and horizontal BVDV transmission following experimental infection in calves**.** 20th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics, 8 November 2013, Drongen, Belgium.

Sarrazin, S., Schirrmeier, H., Dewulf, J., Laureyns, J., Mostin, L., Beer, M., Cay, A.B., 2013. Virus transmission and clinical symptoms in calves experimentally infected with a recently discovered hypervirulent BVDV-2c strain**.** 20th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics, 8 November 2013, Drongen, Belgium.

Sarrazin, S., Cay, A.B., Laureyns, J., Dewulf, J., 2014. A cross-sectional and longitudinal study on the risk of BVDV re-infection in BVDV-free cattle herds in Belgium. 21th Annual Conference of the Flemish Society for Veterinary Epidemiology and Economics in collaboration with AESA, 24 Ocotber 2014, Brussels, Belgium.

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