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**EPIDEMIOLOGY, IMPACT ON HERD HEALTH
AND CONTROL OF BOVINE HERPESVIRUS 1 IN
ESTONIAN DAIRY CATTLE HERDS**

VEISTE HERPESVIIRUS 1 NAKKUSE EPIDEMIOLOOGIA, MÕJU
KARJA TERVISELE JA TÕRJE EESTI PIIMAVEISE KARJADES

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To my mother

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following four original publications (I-IV). The articles are referred to in the text according to their Roman numerals.

- I** **Raaperi, K.**, Nurmoja, I., Orro, T., Viltrop, A., 2010. Seroepidemiology of bovine herpesvirus 1 (BHV1) infection among Estonian dairy herds and risk factors for the spread within herds. *Preventive Veterinary Medicine* 96, 74-81.
- II** **Raaperi, K.**, Bougeard, S., Aleksejev, A., Orro, T., Viltrop, A., 2012. Association of herd BHV-1 seroprevalence with respiratory disease in youngstock in Estonian dairy cattle. *Research in Veterinary Science* 93, 641-648.
- III** **Raaperi, K.**, Bougeard, S., Aleksejev, A., Orro, T., Viltrop, A., 2012. Association of herd BRSV and BHV-1 seroprevalence with respiratory disease and reproductive performance in adult dairy cattle. *Acta Veterinaria Scandinavica* 54:4.
- IV** **Raaperi, K.**, Aleksejev, A., Orro, T., Viltrop, A., 2012. Dynamics of bovine herpesvirus 1 infection in Estonian dairy herds with and without a control programme. *Veterinary Record*. 171:99.

Authors' contributions to the research papers

Paper	Original idea, study design	Data collection, sample analysis	Data analysis	Writing manuscript
I	AV, KR , TO	KR , IN	KR , AV, TO	KR , AV, TO, IN
II	AV, KR , TO	KR , AA	KR , SB, AV, TO	KR , AV, TO, SB, AA
III	AV, KR , TO	KR , AA	KR , SB, AV, TO	KR , AV, TO, SB, AA
IV	AV, KR , TO	KR , AA	KR , AV, TO	KR , AV, TO, AA

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AA – Annely Aleksejev, SB – Stephanie Bougeard, IN – Imbi Nurmoja

ABBREVIATIONS

AI	Artificial insemination
ARIB	Estonian Agricultural and Information Board
BHV-1	Bovine Herpesvirus 1
BRD	Bovine Respiratory Disease
BRSV	Bovine Respiratory Syncytial Virus
BTM	Bulk tank milk
BVDV	Bovine Viral Diarrhoea Virus
CI	Confidence Interval
CPE	Cytopathic effect
DIVA	Differentiation between infected and vaccinated animals
EARC	Estonian Animal Recording Centre
ELISA	Enzyme-linked immunosorbent assay
ET	Embryo transfer
gE	Glycoprotein E
IBR	Infectious Bovine Rhinotracheitis
IM	Intramuscular
IN	Intranasal
IPB	Infectious pustular balanoposthitis
IPV	Infectious pustular vulvovaginitis
KV	Killed virus
MAb	Monoclonal antibody
MCA	Multiple correspondence analysis
MLV	Modified-live vaccines
NAR	National animal register
PCR	Polymerase chain reaction
PI3	Parainfluenza 3
R	Reproduction ratio
SNLC	Seronegative latent carriers
VNT	Virus neutralization test

1. INTRODUCTION

Bovine herpesvirus 1 has gained a lot of attention worldwide during the last half of a century. The first published report on IBR came from Schroeder and Moys (1954). They described an apparently new upper respiratory disease of dairy cattle that occurred in California in 1953 (Yates, 1982). Madin *et al.* (1956) published a report of the first isolation of the IBR virus in 1956. In 1961, Armstrong *et al.* (1961) suggested that the IBR virus belongs to the Herpesvirus group. Following the apparent emergence of IBR in the USA, it was diagnosed in many countries and is now reported worldwide (Yates, 1982). In Europe the virus was first isolated from cattle with respiratory disease in the United Kingdom in the early 1960's where it remained in a mild form for many years (Yates, 1982). The incidence and severity of infectious bovine rhinotracheitis in Europe increased markedly in the 1970s (Edwards, 1988; Sanco/C3/AH/R20/2000; Ackermann and Engels, 2006). Probably coming from North-America (Sanco/C3/AH/R20/2000) the disease entailed economic losses related to animal morbidity, antimicrobial use and discarded milk. Active research began, to study more about the aetiologic agent of infectious bovine rhinotracheitis/pustular vulvovaginitis/balanoposthitis, its pathogenesis, clinical and diagnostic aspects. Soon research about the epidemiology of the disease started to clarify the extent of the virus spread. Vaccines to protect cattle against IBR have been in existence since the late 1950s (Patel, 2005a). Since the end of the 1970s, conventional vaccines, and especially intranasal live-attenuated vaccines, have effectively contributed to the control of the disease (Lemaire *et al.*, 2000b). Due to the impossibility of the eradication of the virus from endemically infected herds with help of conventional vaccines, gene-deleted marker vaccines were developed. First field trials, and promising results of, using BHV-1 marker vaccines in controlling BHV-1 infections were published in the 1990s (Schlüter *et al.*, 1997). Since the EU allows IBR-free member states to imply restrictions on the import of cattle, semen and embryos from infected countries, more efforts were undertaken to achieve eradication of BHV-1 in the EU in the late 1990s (Ackermann and Engels, 2006). In the 1980s the first countries started eradication of BHV-1. To date six countries have achieved total eradication on a country basis, without using any vaccines, by following a 'test and removal' strategy. Due to the huge spread of BHV-1 in some member states, and the problems encountered in avoiding reinfections, the elimination of the virus from herds could not be made compulsory for owners through EU legislation. However,

the eradication of IBR from artificial insemination and embryo transfer centers was required by EC Directive 92/65/EEC, whereby AI and ET centres had to be BHV-1 free from 1st January 1999 onwards (Sanco/C3/AH/R20/2000). Despite the measures constituted in the EU aiming to avoid virus spread to countries confirmed IBR free, antibody-positive animals are still found in border areas with countries not free from the disease; the main risk factors for the spread of IBR in Switzerland were found to be the purchase and movement of bovines and semen of often unknown IBR status (Ackermann and Engels, 2006; Blickenstorfer *et al.*, 2010). IBR-free countries are endangered as long as IBR-eradication is not a common goal within countries that participate in mutual cattle trade and production of semen for artificial insemination (Ackermann and Engels, 2006). This necessitates a uniform compulsory eradication plan for larger districts. Unfortunately, at present, it is still the decision of each country of how to act. Few countries have started compulsory eradication programmes by combining the ‘test and removal’ method with vaccination with marker vaccines. Some countries have initiated voluntary eradication schemes, however these are not usually expected to lead to the eradication of IBR in the region (Vonk Noordegraaf *et al.*, 1998), leaving the free herds under threat of reinfection. Until the virus is not eliminated from the world, research continues in all aspects of BHV-1.

In Estonia the first reports about BHV-1 originate from the mid-1970s (Aaver and Saar, 1993). Since that time BHV-1 has been diagnosed in many cattle herds, and is related to clinical respiratory disease outbreaks or reproduction problems. In a serological survey conducted in 1993–1995, among 316 randomly selected dairy herds, 43.4% of the herds were seropositive (Viltrop and Alaots, 1997). In 2004, in a national survey for BHV-1 seroprevalence, where 2,912 herds were tested from pooled milk samples the proportion of positive samples was found to be 16.4% (Anonymous, 2004a).

Due to the impact on herd health and export limitations dairy farmers have shown increased interest in the control of BHV-1 infection in Estonia. To develop a disease control programme of good quality knowledge of the current status of the prevalence of the infection is a precondition. To devise the most beneficial and economical control programmes for infected herds it is necessary to know the risk factors associated with virus spread within herds. Efficient diagnostic tools comprise an essential part of every disease control and eradication program, as well as knowledge of performance of these tools in actual conditions. These research questions

have been elaborated in article I. Articles II and III evaluate the impact of BHV-1 on herd health in order to motivate farmers, as well as authorities, to respond to the issue. The efficacy of the vaccination programmes in lowering BHV-1 gE seroprevalence within the herd, as well as the dynamics of the infection in non-vaccinated herds with uninfected heifers, has been studied in article IV.

2. REVIEW OF THE LITERATURE

2.1. Infectious bovine rhinotracheitis and bovine herpesvirus type 1

Infectious bovine rhinotracheitis (IBR), caused by bovine herpesvirus 1 (BHV-1), is a disease of domestic and wild cattle (Turin *et al.*, 1999; OIE, Terrestrial Manual 2008, CH 2.4.13). It is an enzootic disease on the list of the World Animal Health Organisation (OIE, Terrestrial Manual 2008, CH 2.4.13). Although the infection is infrequently life threatening, the introduction of BHV-1 into a cattle farm can cause severe economic losses due to production losses and restrictions in the international trade of livestock (Nandi *et al.*, 2009).

The viral aetiology was first demonstrated in 1928 by Reisinger and Reimann. Until the early nineteen-fifties the manifestation of BHV-1 infections was known as “infectious pustular vulvovaginitis” (IPV) in cows and “infectious pustular balanoposthitis” (IPB) in bulls. At that time, a respiratory form called “infectious bovine rhinotracheitis” arose in North American feedlots (Muylkens *et al.*, 2007; Nandi *et al.*, 2009). The virus also causes a wide variety of other clinical syndromes such as abortion, infertility, conjunctivitis, encephalitis (Nandi *et al.*, 2009), mastitis, enteritis and lesions in the interdigital space (Straub, 2001). Due to breeding synchronization in cattle, BHV-1 may also cause abortion storms (Ackermann and Engels, 2006).

BHV-1 is a member of the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae*, which belongs to the *Herpesviridae* family (International Committee on Taxonomy of Viruses, 2011). The family contains viruses characterized by a relatively short replication cycle and the ability to induce latent infection, mainly in neurons (Muylkens *et al.*, 2007). There is only one antigenic type of BHV-1, irrespective of whether the isolate is derived from cases of IBR or IPV (Nandi *et al.*, 2009). BHV-1 isolates are classified into 3 subtypes by the use of restriction endonuclease analysis: subtypes 1, 2a and 2b (Miller *et al.*, 1991). The BHV-1 subtypes 1 and 2a mainly cause the respiratory form of the disease (IBR), with fever, drop in milk production and abortion (Wentink *et al.*, 1993; Jones and Chowdhury, 2007; Nandi *et al.*, 2009). Subtype 2b is mainly manifested as IPV/IPB (Wentink *et al.*, 1993). Isolates from aborted foetuses have been either subtype 1 or 2a, whereas subtype 2b viruses have not been associated with abortion (Miller *et al.*, 1991). Isolates of BHV-1.1 are more

virulent than are isolates of BHV-1.2b (Nandi *et al.*, 2009). However, there is no specific mucosa tropism of different BHV-1 subtypes, and this phylogenetic subdivision of BHV-1 subtypes could be derived from differences in virulence between strains rather than a difference in mucosa tropism (Steukers *et al.*, 2011). BHV-1.3, which is a neuropathogenic agent, has been reclassified as BHV-5 (Magyar *et al.*, 1993; Meyer *et al.*, 2001; Mahony, 2010).

Subtype 1 strains are prevalent in Europe, North America and South America. Subtype 2a is prevalent in Brazil, and was present in Europe prior to the 1970s. Subtype 2b is frequently isolated in Australia, New Zealand or Europe (Wang *et al.*, 2006; Jones and Chowdhury, 2007). Neither BHV-1.1 nor BHV type 5, are, or have been, found in New Zealand (Wang *et al.*, 2006).

The viral genome consists of double stranded DNA that codes for about 70 proteins, of which 33 structural, and up to 15 nonstructural, proteins have been demonstrated. The viral glycoproteins, which are located in the envelope on the surface of the virion, play an important role in pathogenesis and immunity (OIE, Terrestrial Manual 2008, CH 2.4.13.). The BHV-1 genome encloses ten genes encoding glycoproteins. Among these, six are in the unique long (U_L) region, gK, gC, gB, gH, gM, gL and the four remaining ones are in the unique short (U_S) region, gG, gD, gI and gE (Muylkens *et al.*, 2007). Five envelope proteins (gB, gD, gH, gL and gK) are essential for growth in cultured cells, whereas the remaining seven (gC, gE, gG, gI, gM, gN and Us9) are not essential for growth in cultured cells (Jones and Chowdhury, 2007). Of the nonessential envelope proteins, gE has received a lot of attention because it is required for anterograde neuronal spread *in vivo* and neurovirulence. gE-deleted viruses are transported retrogradely from the nose and eye via the maxillary and ophthalmic branches of the trigeminal ganglia (TG) where they establish latency in sensory neurons. gE-deleted viruses do not reactivate from latency because they cannot spread in an anterograde direction from sensory neurons in the TG to non-neuronal cells in the nose or ocular cavity (Jones and Chowdhury, 2007). The virus produces intranuclear inclusion bodies *in vivo* and *in vitro*. The growth cycle is relatively short, with progeny completed within 12 hours (Yates, 1982).

The virus BHV-1 is resistant to environmental influences. At 4°C, the virus is stable for one month. The virus may survive for more than 30

days in feed, and up to one year in semen frozen at -196°C. The BHV-1 is sensitive to many disinfectants (Nandi *et al.*, 2009).

2.2. Prevalence of BHV-1

IBR occurs in all continents, although there are differences in prevalence and incidence (Ackermann and Engels, 2006). Cattle populations of many countries are endemically infected with BHV-1 (Table 1).

Table 1. Seroprevalence of BHV-1 in different countries

Country	Herds (n)	Animals (n)	Prevalence (%)			References
			Herd	Animal	Animal type	
Europe						
Belgium	556	28,478	65	36	dairy and beef cattle	Boelaert <i>et al.</i> , 2000
southern Italian Apennines	81	948	98.8	77.5	NA	Rinaldi <i>et al.</i> , 2007
England and Wales	341		69		dairy cattle	Paton <i>et al.</i> , 1998
south west England	114	15,736	43.1	42.5	dairy and beef cattle	Woodbine <i>et al.</i> , 2009
Scotland	114	1,152	51	12	dairy and beef cattle	Msolla <i>et al.</i> , 1981
Andalusia (South of Spain)	164	4,035	70.4	45.7	dairy and beef cattle	Gonzales-Garcia <i>et al.</i> , 2009
Galicia (NW Spain)	All	All	50.4	38.4	dairy and beef cattle	Eiras <i>et al.</i> , 2009
Ireland	1,175		74.9		dairy and beef cattle	Cowley <i>et al.</i> , 2011
Lithuania		34,600		14	pedigree cattle breeding farms	Jacevičius <i>et al.</i> , 2008
Hungary	75%		79.3 (LH)	64.1 (LH)	dairy cattle	Tekes <i>et al.</i> , 1999
			13.5 (SH)		dairy cattle	Tekes <i>et al.</i> , 1999

Country	Prevalence (%)					References
	Herds (n)	Animals (n)	Herd	Animal	Animal type	
The Netherlands	33,636		84		dairy and mixed herds	Van Wuijckhuise <i>et al.</i> , 1998
northern Italy	55	6,979	84.3	34.99	dairy cattle	Castrucci <i>et al.</i> , 1997
central Italy			100	38.65	dairy cattle	Castrucci <i>et al.</i> , 1997
Norway	All	All	0	0	dairy and beef cattle	Paisley <i>et al.</i> , 2001
Austria			0	0		OIE, Terrestrial Manual 2008, Ch 2.4.13.
Denmark			0	0		OIE, Terrestrial Manual 2008, Ch 2.4.13.
Finland			0	0		Nuotio <i>et al.</i> , 2007
Switzerland			0	0		OIE, Terrestrial Manual 2008, Ch 2.4.13.
Sweden			0	0		OIE, Terrestrial Manual 2008, Ch 2.4.13.
Italy (Province of Bolzano)			0	0		OIE, Terrestrial Manual 2008, Ch 2.4.13.
Other countries						
Uruguay	230	6,358	99	37	beef cattle	Guarino <i>et al.</i> , 2008
Ecuador	346	2,367	82.1	43.2	dairy and mixed herds	Carbonero <i>et al.</i> , 2011
Parana State of Brazil	2,018	14,803	71.3	59	NA	Dias <i>et al.</i> , 2012
State of Bahia, Brazil		558		56	dairy and beef cattle	Cerqueira <i>et al.</i> , 2000

Country	Prevalence (%)					References
	Herds (n)	Animals (n)	Herd	Animal	Animal type	
Turkey	31	13,011	97	53.2	dairy cattle	Alkan <i>et al.</i> , 2005
Thailand	220		67		dairy cattle	Kampa <i>et al.</i> , 2004
Peru	60		51		dairy cattle	Stähl <i>et al.</i> , 2002
China		2,592		35.8	dairy cattle	Yan <i>et al.</i> , 2008
India		595		60.8	dairy cattle	Trangadia <i>et al.</i> , 2010
southern India		NA		50.9	NA	Renukaradhya <i>et al.</i> , 1996
Mexico		564	97	54.4	beef cattle	Solis-Calderon <i>et al.</i> , 2003
Tecpatan, Chiapas, Mexico		150		62.8	dairy cattle and breeding bulls	Sanchez-Munoz <i>et al.</i> , 2010
Pacific Region of Central Costa Rica	35	496		48	dairy and beef cattle	Raizman <i>et al.</i> , 2011
Southern Province, Zambia		116		48.3	market cattle	Mweene <i>et al.</i> , 2003
Venezuela (Apure State)		615		67	beef cattle	Obando <i>et al.</i> , 1999
Algeria		2,948		20.5	NA	Achour and Moussa, 1996
Tunisia	44	10% ^a		25.9	NA	Ghram and Minocha, 1990
Morocco		524		62.8	NA	Mahin <i>et al.</i> , 1985

^a10% of animals within herd

LH=large herds

SH=small herds

NA=information not available

According to the OIE, 1,461 cases (23 outbreaks) were registered in Russia for the year 2003. A few Far Eastern countries have never reported IBR cases, i.e. the Philippines, Singapore, Sri Lanka, and Vietnam. However,

no official surveillance or eradication programmes exist outside of Europe (Ackermann and Engels, 2006). BHV-1 is endemic in the United States (Godhardt-Cooper *et al.*, 2009) and present in Australian cattle herds (Jordan *et al.*, 2012).

2.3. Dynamics of the infection in populations

BHV-1 seroprevalence varies greatly from herd to herd (Alkan *et al.*, 2005). The polarisation of herds into either strongly positive or virtually negative BHV-1 categories has been found (Paton *et al.*, 1998). BHV-1 comes in contact with herds mainly through the introduction of new animals, either in the acute phase of a primary disease or when latently infected. Even semen from infected bulls and infected embryos are suitable virus carriers (Turin *et al.*, 1999). The prevalence of BHV-1 on dairy farms is dependent on several factors. First, the prevalence is influenced by introductions of BHV-1 on the farms, which is dependent on the risk factors for introduction. Second, the BHV-1 prevalence might also be influenced by reactivation of the virus within the farm, which might be affected by the management of the farm (Van Schaik *et al.*, 1999a; Hage *et al.*, 2003).

An outbreak in a herd is the consequence of virus introduction from outside the herd, or virus reactivation and reexcretion from latently infected cattle within the herd. The spread of the virus within a susceptible herd is often rapid, although small outbreaks may occur (Hage *et al.*, 1996). Once a critical number of susceptible individuals have been infected, the remaining susceptible individuals are unlikely to escape infection (Mollema *et al.*, 2005). It is assumed that 85% of the BHV-1 outbreaks in a fully susceptible population will be major, and lead to complete spread (van Schaik *et al.*, 1999a), however Mollema *et al.* (2005) confirmed the probability of approximately 25% that all susceptible individuals will be infected. Animal husbandry practices may have an influence on virus spread within herds. The slow spread of BHV-1 was seen in a tie-stall housing system (Hage *et al.*, 2003). Close animal contacts (van Nieuwstadt and Verhoeff, 1983; Vonk Noordegraaf *et al.*, 1998; Gungor and Ozkul, 2007) as well as other contacts, such as transmission through fomites, indirect transmission through other species, airborne transmission or minor disease-specific routes such as venereal or iatrogenic transmission (Vonk Noordegraaf *et al.*, 1998) favour the spread of the virus. According

to Hage *et al.* (1996) a BHV-1 infected herd may be infectious for a 7-week period, so after introduction of infectious cattle into susceptible herds, the infectious period of these herds may last up to 10 weeks (Hage *et al.*, 2003).

The prevalence of BHV-1 in youngstock after an outbreak is often independent of the prevalence in the milking herd (van Schaik *et al.*, 1999a). Van Nieuwstadt and Verhoeff (1983) have suggested that the practice of rearing youngstock separated from the dairy herd had probably postponed exposure to the virus in most of these herds. Older animals are more likely to become infected (Hage *et al.*, 1996; Solis-Calderon *et al.*, 2003; Kampa *et al.*, 2004; Boelaert *et al.*, 2005; Woodbine *et al.*, 2009; Carbonero *et al.*, 2011) whereas young animals introduced into the milking herd may be uninfected. In young animals of 6-8 months old a slight antibody response after natural BHV-1 infection was seen, and neutralizing titres fell below protective levels in the 6-8 months after the peak (Gungor and Ozkul, 2007), therefore animals may experience BHV-1 infections several times. After an epidemic outbreak, the herd is not at risk of a new major outbreak for a specific period of time, until the fraction of susceptible animals has passed a critical level (van Nieuwstadt and Verhoeff, 1983; de Koeijer *et al.*, 2008; Keeling and Rohani, 2008). As soon as the herd contains enough susceptible animals to have a considerable chance of seroconversions, the outbreak may recur (van Schaik *et al.*, 1999a).

Four parameters influence the expected time to extinction: the reproduction ratio, the reactivation rate, the population size, and the demographic turn-over in the host population (de Koeijer *et al.*, 2008). The basic reproduction ratio (R_0) is defined as the average number of secondary cases generated by one typical infectious individual, during its full infectious period, in a wholly susceptible population of defined density (Hage *et al.*, 1996; Mollema *et al.*, 2005; de Koeijer *et al.*, 2008). For reactivating viruses, for each infected individual there may be several infectious periods, where the later periods are not induced by a new introduction of the virus (Mollema *et al.*, 2005). Primarily infected, seronegative animals excrete much higher levels of virus over a longer timeperiod than re-infected seropositive animals (Hage *et al.*, 1996). The R_1 of the natural BHV-1 in non-vaccinating herds has been estimated, in several studies, as being from 2.8 to 7 (Hage *et al.*, 1996; Bosch *et al.*, 1998; Mars *et al.*, 2001; Hage *et al.*, 2003). Infection dynamics depends on the virus strain showing different transmission-dynamics (Hage *et al.*, 1996). A low rate of reactivation of latent BHV-1 virus has been demonstrated

(Ståhl *et al.*, 2002; Kampa *et al.*, 2004). A reactivation rate of $p=0.10$ per 13 months, or 0.09 per year, was reported by de Koeijer *et al.* (2008). The infectious agent will become extinct much more quickly in small populations (de Koeijer *et al.*, 2008). Also, the higher the replacement rate of the adult cattle is, the faster the virus-infected animals will be replaced with incoming uninfected heifers.

2.4. Pathogenesis of IBR

The main sources of infection are nasal exudates and cough droplets, genital secretions, semen, foetal fluids and tissues (Nandi *et al.*, 2009; Jones and Chowdhury, 2010). The most preferred method of transmission of BHV-1 between animals is direct nose to nose contact (Turin *et al.*, 1999; Ellis, 2009), however airborne transmission by the aerosol route has been demonstrated over short distances (Mars *et al.*, 1999; Mars *et al.*, 2000a; Ellis, 2009). Housing positive and negative groups under different rooves, or their separation by plastic curtains, have been shown to greatly enhance the feasibility of IBR-eradication in feedlot facilities (Ackermann and Engels, 2006). Although IBR is a respiratory illness, the agent is not easily transmitted by aerosol means. In contrast, salivation onto feed, and the movement of contaminated feed from positive groups to negative groups, have been found to be a major source of BHV-1 transmission in fattening units (Ackermann and Engels, 2006). In general, airborne transmission or spread of the virus by humans is believed to be of minor importance (Wentink *et al.*, 1993; Hage *et al.*, 2003). An indirect route of spread is also possible, by means of contaminated instruments (Turin *et al.*, 1999). Transmission can also occur in the absence of visible lesions and through artificial insemination with semen from sub-clinically infected bulls (Jones and Chowdhury, 2007).

BHV-1 may spread in the infected host by lymphocyte-associated viraemia (Nandi *et al.*, 2009), gaining access to a broader range of tissues and organs, and causing a broader variety of clinical manifestations (Engels and Ackermann, 1996). A hallmark of the herpesvirus biological cycle is its ability to establish latent, life-lasting, and periodically reactivating infections in the host (Turin *et al.*, 1999). After primary infection with BHV-1 (Straub, 1991; Jones and Chowdhury, 2007; Fulton, 2009; Nandi *et al.*, 2009) or vaccination with an attenuated strain (Nandi *et al.*, 2009) cattle become latent carriers. Following primary infection of the eye,

oral cavity, and/or nasal cavity, BHV-1 establishes latency in trigeminal ganglionic (TG) neurons (Winkler *et al.*, 2002). After genital infection, BHV-1 replicates in the mucous membranes of the vagina or prepuce, and becomes latent in the sacral ganglia (Ackermann and Wyler, 1984; Nandi *et al.*, 2009). BHV-1 is thought to infect neurons via the nerve endings in the mucosae, and ascend towards the central nervous system (CNS), whereas the trigeminal route is preferred (Ackermann *et al.*, 1982; Meyer *et al.*, 2001; Muylkens *et al.*, 2007). The neuroinvasion usually does not go further than the first order neuron located in the trigeminal ganglion where the latent infection is established (Ackermann *et al.*, 1982; Muylkens *et al.*, 2007). Although the main site of latency is ganglionic neurons, there is evidence that latency and reactivation occur within germinal centres of the pharyngeal tonsils (Straub, 1991; Winkler *et al.*, 2000; Jones and Chowdhury, 2007; Nandi *et al.*, 2009) and cells of lymphoid origin (Jones and Chowdhury, 2007). During latency, apparently no viral antigens are synthesized, but the genomes of the latent viruses are present in the nuclei of long living cells, such as neurons of the ganglia (Ackermann and Wyler, 1984; Engels and Ackermann, 1996). BHV-1 DNA is consistently detected in tonsils, peripheral blood cells, lymph nodes and spleens of latently infected calves, even when infectious virus is not detected (Jones and Chowdhury, 2007).

Periodically BHV-1 reactivates from latency, the virus is shed, and consequent virus transmission occurs (Jones and Chowdhury, 2007). Therefore the IBR antibody carrier should always be considered as a potential source of infection to other animals (Bitsch, 1973). Reactivation of the virus can occur after natural or corticosteroid-induced stress (Rock *et al.*, 1992; Turin *et al.*, 1999; Winkler *et al.*, 2000; Straub, 2001; Winkler *et al.*, 2002; Jones and Chowdhury, 2007; Fulton, 2009). The reactivation stimulus may occur on several occasions related to stress, such as at parturition (Thiry *et al.*, 1985; Turin *et al.*, 1999), from transport (Thiry *et al.*, 1987; Turin *et al.*, 1999; van Drunen Littel-van den Hurk, 2006; Jones and Chowdhury, 2007; Yoo, 2010), subsequent to the introduction of heifers into a group of dairy cows (Muylkens *et al.*, 2007), moving cattle from one location to another (Jones and Chowdhury, 2010), climatic changes (van Drunen Littel-van den Hurk, 2006; Yoo, 2010), introduction into a new herd, concomitant viral or bacterial infections, poor management conditions, deficient diet (Turin *et al.*, 1999), overcrowding of animals (van Drunen Littel-van den Hurk, 2006) or after superinfection with *Dictyocaulus viviparus* (Msolla *et al.*, 1983). Spontaneous reactivation at irregular intervals has also been observed (Winkler *et al.*, 2002; Nandi

et al., 2009). The reactivated BHV-1 is transported intra-axonally back to the periphery, to the original portal of entry, where it is available for transmission to other susceptible hosts. Viral replication in the course of reactivation may cause recurrence of the disease (Turin *et al.*, 1999).

Consequently, the virus may be shed intermittently into the environment. Once the virus penetrates into the target epithelial cells, it starts to produce new progeny viruses resulting in cell death and a cytopathic effect (CPE) characterized by the cell ballooning, and the rise of intranuclear inclusions indicative of BHV-1 (Gungor and Ozkul, 2007). New progeny viruses are shed in the nasal mucus at high excretion titres, and are responsible for the rapid dissemination of the infection within a cattle herd (Nandi *et al.*, 2009). Regardless of the site of infection, virus shedding can last for 7–10 days after infection (Jones and Chowdhury, 2007).

Genital transmission can occur at mating or through contaminated semen (van Oirschot, 1995). However, in a study by Rana *et al.* (2011), only 19.5% of frozen semen samples from 439 BHV-1 seropositive bulls were found positive for BHV-1 with real-time PCR. In bulls, after intranasal as well as intrapreputial infection, BHV-1 can replicate in the preputial and penile mucosae (van Oirschot, 1995). Bulls can start shedding BHV-1 from the prepuce between 2 and 7 days after primary preputial infection lasting from several days to several weeks (Nandi *et al.*, 2009). Beyond the primary phase of a genital infection, BHV-1 remains latent in the sacral ganglia (Ackermann and Wyler, 1984). As a result of reactivation intermittent virus excretion may follow, where the seminal plasma, rather than the sperm cells, contains the BHV-1 (van Oirschot, 1995).

Following primary infection, non-specific inflammatory and cellular reactions are the first responses to BHV-1 infection (Muylkens *et al.*, 2007). BHV-1 infections via the respiratory route normally induce a considerable, and long-lasting, titre of antibodies in serum after 7 to 10 days (Kramps *et al.*, 1994). The specific humoral antibodies become detectable from day 10 post infection with the ELISA test (Riegel *et al.*, 1987; Turin *et al.*, 1999). Envelope glycoproteins gB, gC, gD and gH are the most potent inducers of virus-neutralizing antibodies (Jones and Chowdhury, 2007). Although antibodies have been correlated with protection and recovery from BHV-1 infection, the cell-mediated immune response is also a critical defence mechanism because cell-to-cell spread occurs before haematogenous spread (Babiuk *et al.*, 1996; van Drunen Littel-van den Hurk, 2007). Therefore, the nonspecific immune responses (mediated

primarily by viral products which induce early cytokines) are amongst the first line of defence in helping clear the infection both directly, as well as indirectly, by stimulating the specific immune response (Babiuk *et al.*, 1996). Cell-mediated immune responses play an important role in killing virus-infected cells that express viral antigens on the cell surface (Jones and Chowdhury, 2007). Furthermore, induction of robust T-cell memory is critical for the long-term duration of immunity (van Drunen Littel-van den Hurk, 2007). Cell-mediated immunity is also important in determining the duration and severity of recurrent infection (Davies and Carmichael, 1973; Rouse and Babiuk, 1978). The cell-mediated immune response is first detected about two days postinfection (pi) and takes place at approximately 8 to 10 days pi (Babiuk *et al.*, 1996; Turin *et al.*, 1999). Mucosal immunity also becomes activated, as shown by IgA found in nasal and genital secretions (Turin *et al.*, 1999).

After infection, antibodies against BHV-1 persist for at least 5.5 years (Chow *et al.*, 1972), while according to Kaashoek *et al.* (1996a), antibodies of latently infected animals could be detected at least three years after infection. Woodbine *et al.* (2009) confirm that age serological profile is consistent with life-long seropositivity. The immune response is presumed to persist for life, although it may fall below the detection limit of some tests (OIE, Terrestrial Manual 2008, CH 2.4.13.).

The passive immunity from colostral antibodies in BHV-1-immune cows is fully efficacious at protecting the neonate against systemic and lethal disease (Mechor *et al.*, 1987; Muylkens *et al.*, 2007). Specific colostral antibodies against IBR virus appear in the nasal secretions of calves as early as the first day after the ingestion of colostrum. The colostral antibodies secreted on the respiratory tract mucosa, which are primarily of the IgG class, persist for 15 to 20 days after birth while serum antibodies may be detected until the calf is four to six months of age (Menanteau-Horta *et al.*, 1985; Mechor *et al.*, 1987; Bradshaw and Edwards, 1996; Woodbine *et al.*, 2009). The mean half-life of BHV-1 maternal antibodies was 21.2 days and the calculated mean time to seronegative status for nonvaccinated calves was 122.9 days (range 0–935) (Fulton *et al.*, 2004). Nevertheless, maternal antibodies can be detected at 9 to 11 months of age (Brar *et al.*, 1978; Cho *et al.*, 2002; OIE, Terrestrial Manual 2008, CH 2.4.13.).

2.5. Impact on animal health

Bovine herpesvirus 1 is primarily associated with clinical syndromes such as rhinotracheitis, pustular vulvovaginitis and balanoposthitis, abortion, infertility, conjunctivitis and encephalitis in bovine species (Nandi *et al.*, 2009). BHV-1-induced immune suppression initiates bovine respiratory disease complex (BRDC), which costs the US cattle industry approximately 3 billion dollars annually (Jones and Chowdhury, 2007; Jones and Chowdhury, 2010). The morbidity in BHV-1 incidents has been estimated to be from 20 to 100 per cent, and mortality may reach 10% (Wiseman *et al.*, 1980; Yates, 1982; Nandi *et al.*, 2009). In general, both of these variables are higher for beef than dairy cattle (Yates, 1982) and considerably higher in neonatal and suckling calves than in adults (van Nieuwstadt and Verhoeff, 1983; Patel, 2005a).

The incubation period varies from a few days until 20 days under natural conditions (Muylkens *et al.*, 2007; Nandi *et al.*, 2009). The introduction of BHV-1 into a previously virus-free farm can result in great damage (Holzhauer *et al.*, 2003) and severe clinical signs such as abortion, reduction in milk production and mortality can be seen (Vonk Noordegraaf *et al.*, 1998; van Schaik *et al.*, 2002). However, gradually the character of the disease has changed from a clinical epidemic to a situation of endemicity, in which the most of the infections reported were subclinical (Vonk Noordegraaf *et al.*, 1998). Many BHV-1 infections are subclinical (Yates, 1982; van Oirschot *et al.*, 1993; Hage *et al.*, 1996; Hage *et al.*, 1998b; Pritchard *et al.*, 2003). The severity of the disease caused by BHV-1 is influenced by several factors, such as the virulence of the BHV-1 strain, resistance factors of the host, especially the age of the host, potential concurrent bacterial infection and the presence of maternal immunity (Muylkens *et al.*, 2007). Also, some extrinsic factors in the animals' environments may affect the outcome and severity of an infection (Yates, 1982).

BHV-1 generally infects cattle over 6 months of age, once maternal immunity has waned (Woodbine *et al.*, 2009). However, Lemaire *et al.* (1999) found typical signs of BHV-1 infection in calves with maternal antibodies of gE negative BHV-1 after inoculation with BHV-1 wild type virus, where the severity of signs was not correlated with the level of maternal antibodies.

The respiratory form of the disease is characterized by pyrexia (40.5-42°C), inappetence and apathy. In adult cows a significant drop in milk yield may

occur at that time. Respiratory signs such as the reddened appearance of nasal mucosa, serous to mucopurulent nasal discharge, and in severe cases heavy breathing at inspiration and coughing, are prevalent. Ocular signs such as unilateral or bilateral lacrimation, conjunctivitis and mucopurulent ocular shedding are also common as a conjunctival form of the disease or occurring concurrently with the respiratory form (Muylkens *et al.*, 2007; Nandi *et al.*, 2009). Conjunctivitis and ocular discharge were a major finding in 13 incidents and, in severely affected cases, conjunctival oedema was seen (Wiseman *et al.*, 1980). Nasal lesions consist of numerous clusters of greyish necrotic foci or yellow-brown diphtheritic plaques (Wiseman *et al.*, 1980; Yates, 1982; Jones and Chowdhury, 2007). Digestive disorders, such as diarrhoea in calves, have been connected with BHV-1 (Wiseman *et al.*, 1980; Yates, 1982; Mechor *et al.*, 1987; Straub, 1991). Acute uncomplicated cases last 5–10 days (Yates, 1982) and the animals recover rapidly, but they remain as carriers, and shed the virus for a considerable period (Nandi *et al.*, 2009). Cattle with uncomplicated BHV-1 infections have upper respiratory disease of variable severity. However, in many, if not most BHV-1-associated BRD cases there is a mixed infection with bacteria, notably *M. haemolytica* and/or *P. multocida*, which results in severe lower respiratory tract disease (Yates, 1982; Alkan *et al.*, 2000; Leite *et al.*, 2002; Patel, 2005a; Patel, 2005b; van Drunen Littel-van den Hurk, 2006; Jacevičius *et al.*, 2008; Ellis, 2009; Nandi *et al.*, 2009). However BHV-1 itself is not very often isolated from lung tissue (Szeredi *et al.*, 2010). Secondary infection may also induce oral erosions, diarrhoea, keratitis, etc. (Yates, 1982). In animals of less than one year of age, in particular in feedlots, BHV-1 is one of the viral pathogens involved in bovine respiratory disease (BRD) complex or “shipping fever” (van Drunen Littel-van den Hurk, 2006). There is evidence that BHV-1 infections can occur simultaneously with bovine virus diarrhoea (BVD) and/or parainfluenza-3 (PI 3) virus (Straub, 1991).

A negative impact on milk production has been confirmed in herds experiencing BHV-1 infection (Wiseman *et al.*, 1980; van Schaik *et al.*, 1999b; van Schaik, 2001a). A diminished average daily growth was found during BHV-1 outbreaks (Janzen *et al.*, 1980; Wiseman *et al.*, 1980). Neurological disease in cattle caused by BHV-1 and BHV-5 infections share similar epidemiological, clinical, and pathological findings, characterized in most cases by nonsuppurative and necrotizing meningoencephalitis (Rissi *et al.*, 2008). Neonatal calves may experience multisystemic infection following congenital infection prior to birth or

early postnatal BHV-1 infection, whereas colostrum-deprived calves are at an increased risk (Muylkens *et al.*, 2007).

IBR is considered to be a causal agent for abortions in cattle (Trangadia *et al.*, 2010; Yang *et al.*, 2012). Abortion is a consequence of a respiratory BHV-1 infection in a seronegative pregnant cow, mostly occurring at between 4 to 8 months of gestation, although embryonic death has also been reported (Miller and Van der Maaten, 1986; Miller and Van der Maaten, 1987; Nandi *et al.*, 2009). The incubation period between inoculation with BHV-1 and abortion is 15 to 64 days (Muylkens *et al.*, 2007). However, abortions can occur at up to 100 days after infection, which is probably due to reactivation from latency (van Nieuwstadt and Verhoeff, 1983; Jones and Chowdhury, 2007). The genital form of BHV-1 infection in female cattle is characterized by IPV and reduced pregnancy rate resulting from a higher number of services per conception (Parsonson and Snowdown, 1975). The minimum dose to infect a cow through artificial insemination may be no more than 32 infectious virus particles (van Oirschot, 1995). Such an infection may lead to fertility disturbances, mainly endometritis (van Oirschot, 1995; Biuk-Rudan *et al.*, 1999; Nandi *et al.*, 2009; Yoo, 2010). Acute IPV usually develops 1–3 days after mating, and frequent micturition and tail swishing are characteristic signs noticed initially. Affected animals develop fever, depression and anorexia; they try to avoid contact of the tail with the vulva. The vulva is swollen and hyperaemic, with small pustules (Yoo, 2010). The pustules usually coalesce to form yellowish white fibrinous membranes that gradually detach to form ulcers (Nandi *et al.*, 2009). Outbreaks of BHV-1 infections in AI centres can run a clinical or subclinical course. Clinical signs vary from mild to severe balanoposthitis, and may be associated with a decrease in semen quality (van Oirschot, 1995). In the case of IPB, lesions similar to those of IPV develop on the mucosa of the penis and prepuce (Nandi *et al.*, 2009). Nevertheless some studies have found no association between herd IBR status and reproductive deficiencies (Obando *et al.*, 2004; Waldner, 2005; Waldner and Kennedy, 2008). Although some forms, particularly respiratory disease and conjunctivitis, often coexist, it is uncommon for respiratory IBR to occur simultaneously with IPV in an individual animal (Yates, 1982).

2.6. Laboratory diagnosis

BHV-1 infections can be diagnosed by detection of virus, or virus components, and antibody by serological tests or by detection of genomic DNA by polymerase chain reaction (PCR), nucleic acid hybridization and sequencing (Nandi *et al.*, 2009). Diagnostic tests recommended for testing BHV-1 by OIE are the virus neutralisation test (VN), enzyme-linked immunosorbent assay (ELISA), agent identification from semen and PCR (OIE, Terrestrial Manual 2008, CH 2.4.13).

BHV-1 can be readily isolated in cell culture of primary or secondary bovine kidney, lungs, testis, turbinate, or trachea, and established cell lines such as Madin–Darby Bovine Kidney (MDBK) cells. The virus can be isolated from nasal swabs, conjunctival swabs, vaginal swabs, preputial washing, placental cotyledons of aborted foetus, foetal liver, lung, spleen, kidney, lymph node, mucous membrane of respiratory tract, tonsils and lungs collected in a virus transport medium (Yoo, 2010; Crook *et al.*, 2012). Raw or frozen semen with preservatives may also be collected for virus isolation. The presence of virus in specimens is detected by a cytopathic effect (CPE). The CPE of BHV-1 is characteristic and usually appears within three days after inoculation (Nandi *et al.*, 2009). In some cases the cytopathic effect was visible at 12 hours post-inoculation, and became characteristic after 36-48 hours (Ruiz-Saenz *et al.*, 2012). The cell culture is passaged at least three times before the sample is considered negative (Nandi *et al.*, 2009).

PCR enables the identification of BHV-1 infected cattle before detectable seroconversion has occurred (Fuchs *et al.*, 1999). It is more sensitive than virus isolation, and is a practical alternative for rapid detection of the virus. The result is available within 12 hours compared to virus isolation which requires seven days (Nandi *et al.*, 2009). Viral DNA is detectable in the peripheral blood of acutely infected animals, as well as in the peripheral blood of subclinically infected cattle (Fuchs *et al.*, 1999). PCR is also considered to be a more sensitive (van Oirschot, 1995; Deka *et al.*, 2005; Wang *et al.*, 2008; Rana *et al.*, 2011) as well as faster, cheaper and easier method to perform for BHV-1 screening from semen in bulls compared to virus-isolation assay (Grom *et al.*, 2006; Wang *et al.*, 2008; Rana *et al.*, 2011). The latter also has limitations in regard to the cytotoxicity of semen to the cells (Rana *et al.*, 2011). The real-time PCR also detects inactivated virus or low titres of virus during the phases of intermittent virus excretion (Rana *et al.*, 2011). The sensitivity and specificity of real-

time PCR, in comparison to virus isolation in cell cultures, was found to be 100% and 90.04%, respectively. PCR is more specific compared to virus isolation (Deka *et al.*, 2005). BHV-1 was detected in the semen of one bull approximately six weeks before seroconversion (de Gee *et al.*, 1996). PCR could be useful in screening breeding bulls or samples of frozen semen prior to use in AI (Kataria *et al.*, 1997; Rocha *et al.*, 1998) as well as in countries endemically infected with BHV-1, to minimize the risk of transmitting virus by semen (de Gee *et al.*, 1996). The gE-specific PCR allows discrimination between wild-type (WT) virus-infected and vaccinated animals in whole-blood samples, which is of importance for control programmes that use the vaccination strategy with a gE-negative virus (Fuchs *et al.*, 1999; Schynts *et al.*, 1999). PCR assay may also be a useful tool for monitoring the spread of live marker vaccine virus and the gE genotype of viral field isolates (Schynts *et al.*, 1999). Previous studies have highlighted the utility of using molecular diagnostic tests such as real-time PCR or semi-nested polymerase chain reaction (SN-PCR) to achieve high sensitivity in the detection of BHV-1 in organ fragments from aborted fetuses and potentially autolyzed tissues (Takiuchi *et al.*, 2005; Crook *et al.*, 2012). Multiplex reverse transcriptase quantitative polymerase chain reaction (mRT-qPCR) used in diagnosing respiratory viruses in humans was recently used in bovines and was confirmed to be more sensitive than virus isolation and the indirect fluorescent antibody test for detection and differentiation of BRSV, BHV-1 and PI3 (Thonur *et al.*, 2012).

As antibodies to primary infection already appear in serum seven days post-infection, serology is a good tool for diagnosing the disease. Paired serum samples taken 2-3 weeks apart will indicate recent infection (Nandi *et al.*, 2009). The persistence of antibodies allows the detection of latently infected cattle (Kaashoek *et al.*, 1996a; Lemaire *et al.*, 2000b). Conventional serological assays cannot distinguish between antigenic subtypes (Spilki *et al.*, 2005; Woodbine *et al.*, 2009). However, an enzyme linked immunosorbent assay is described that allows discrimination between immune responses of the BHV-1.1 and BHV-1.2 subtypes, based on anti-gC monoclonal antibodies (Rijsewijk *et al.*, 1999; Spilki *et al.*, 2005). Both VNT and ELISA have been used for the detection of BHV-1 antibodies, although the latter has advantages over the VNT (Cho and Bohac, 1985; Godhardt-Cooper *et al.*, 2009; Nandi *et al.*, 2009). Riegel *et al.* (1987) demonstrated that with the ELISA, antibodies in sera from experimentally infected cattle were detected sooner after infection, and showed more rapid increases in levels than VNT. A comparison of the

ELISA with the VN tests, by using sera with low levels of antibodies, demonstrated that the ELISA was the most sensitive test (Payment *et al.*, 1979; Riegel *et al.*, 1987). Results can be obtained within a few hours compared to three days for VN testing, therefore ELISA is a more rapid test (Payment *et al.*, 1979; Riegel *et al.*, 1987). In addition, the VN test requires expensive tissue culture systems (Cho and Bohac, 1985). The overall sensitivity of VNT was 0.93. Specificity was 0.96, and became positive from 9 days pi, and had a repeatability of 95.5% (Kramps *et al.*, 2004). gB blocking ELISA is a simple, convenient, specific, and highly sensitive assay for the detection of BHV1-specific antibodies in serum (Cho and Bohac, 1985; Perrin *et al.*, 1993; Kramps *et al.*, 1994). The gB of BHV-1 is required for the penetration of the virus into cells, and is essential for virus infectivity. The protein therefore will never be absent in wild-type virus strains (Kramps *et al.*, 1994). The ability of the selected MAb to recognize all of the 160 different BHV-1 strains indicates that the recognized epitope on gB is conserved and will be present in most, if not all, BHV-1 field strains (Kramps *et al.*, 1994). The diagnostic sensitivity and specificity of different gB-ELISA tests were 96-100% (Riegel *et al.*, 1987; Kramps *et al.*, 1994; de Wit *et al.*, 1998; Nylin *et al.*, 2000; Kramps *et al.*, 2004), became positive from 7-9 days pi (Kramps *et al.*, 1994; Kramps *et al.*, 2004) and had a repeatability of 97.3% (Kramps *et al.*, 2004). As far as quality and standardisation is concerned, the commercially available gB-ELISAs are to be preferred above home-made ones (Kramps *et al.*, 2004). Blocking ELISA is superior to a commercially available indirect ELISA, and to the 24-h virus neutralization test, in detecting low antibody levels in serum. The overall sensitivity of indirect ELISA from serum tests was 87%. Specificity was 99%, became positive from 9 days pi and had repeatability of 95.9% (Kramps *et al.*, 2004). According to Cho *et al.* (2002) a blocking ELISA is superior for the detection of small amounts of passively acquired BHV-1 maternal antibodies compared to indirect ELISA and VNT.

According to Kramps *et al.* (2004) the indirect ELISA has been the tests of choice in laboratories of EU for assaying milk samples for the presence of BHV-1 antibodies. Indirect ELISAs performed best in scoring correctly milk samples compared to gB- and gE-blocking ELISAs (Kramps *et al.*, 2004).

The VNTs, gB-ELISAs and indirect ELISAs cannot differentiate serologically between infected and vaccinated animals (Kramps *et al.*, 2004). Marker ELISA detects antibodies against a glycoprotein that is

lacking in the vaccine (Nandi *et al.*, 2009). So far, only gE-ELISAs are available that enable infected and vaccinated cattle to be differentiated (Van Oirschot *et al.*, 1997; Kramps *et al.*, 2004). The test uses two MAbs directed against two different epitopes on gE, and therefore detects only antibodies against these two or adjacent epitopes (Van Oirschot *et al.*, 1997). Serum antibodies against gE could be detected as early as 11 days after experimental infection and were found to persist at a high and stable level for at least 2–3 years after experimental intranasal or contact infection with either a BHV-1 subtype 1 or BHV-1 subtype 2 strain. In addition, in cattle first vaccinated with a gE-negative vaccine, and then challenged with a field strain of BHV-1, gE-antibodies also persisted for at least 2–3 years (Van Oirschot *et al.*, 1997). Passively immunised gE-negative calves can develop an active and lasting antibody response to gE after infection with BHV-1 (Lemaire *et al.*, 1999). The gE-ELISA is intrinsically less sensitive than the VNTs and gB ELISAs, which may be the consequence of a lower immunogenicity of gE-glycoprotein (Körber *et al.*, 1997; de Wit *et al.*, 1998; Kramps *et al.*, 2004). The sensitivity of different gE ELISA tests has been evaluated as being 72-92.7% and has a specificity of 92-100% (Körber *et al.*, 1997; Van Oirschot *et al.*, 1997; de Wit *et al.*, 1998; Kramps *et al.*, 2004) and a repeatability of 93.5% (Kramps *et al.*, 2004). The sensitivity of gE ELISA from milk samples is 58-84.1% and the specificity is 88-89.1% (Körber *et al.*, 1997; Kramps *et al.*, 2004).

Bulk tank milk (BTM) testing to detect antibodies provides a useful, low cost method for determining the herd BHV-1 status and it is widely used in disease control schemes (Paton *et al.*, 1998). The herd-level relative sensitivity and relative specificity were 55-82 and 99-100%, respectively (Eliot, 1997; Nylin *et al.*, 2000). The Se of the BTM testing can be increased by repeated testing of the herd BTM samples (Eliot, 1997). The herd-level relative sensitivity depended on the within-herd prevalence of seropositive cows and the cut-off value in the assay, but not on the time interval (up to 90 days) between the collection of the bulk-tank milk sample and the individual serum samples. The BHV-1 blocking ELISA on bulk-tank milk could detect seropositive herds (few), with prevalence proportions of as low as one seropositive cow out of 70 cows (Nylin *et al.*, 2000). However, at a cut-off for percent positivity (PP) of 3, expressed as the optical density of the test result, corrected for the optical density of the control, the sensitivity of the indirect BHV-1 BTM ELISA was 100% and the specificity 95% (Kampa *et al.*, 2009). This unusually high sensitivity may arise from the fact that the average size of the herds in that study was nine milking cows (Kampa *et al.*, 2009), therefore the presence in

the milk of at least one seropositive cow in BMT sample results in a high enough concentration of antibodies that cannot be missed with a test.

BTM ELISA testing may be used for the estimation of within-herd prevalence. E.g. in blocking ELISA systems the blocking percentage reflects the concentration of antibodies to BHV-1 in the bulk-tank milk sample, a somewhat linear correlation between the within-herd prevalence and the blocking percentage of the bulk-tank milk samples would be expected. The correlation between the within-herd prevalence and the blocking percentage was from 0.59 (Nylín *et al.*, 2000) to 0.86 (Hartman *et al.*, 1997).

Individual milk samples, which can be collected relatively easily and inexpensively, can be used instead of individual serum samples in the gE-blocking ELISA for the screening of cattle for BHV-1 gE antibodies (Wellenberg *et al.*, 1998). The main problem with the use of milk in the antibody detection tests is the lower concentration of immunoglobulins in comparison with that in serum. However, in comparison to serum, results showed that the gE blocking ELISA (IDEXX, Westbrook, Maine) was highly sensitive for testing of individual milk samples (0.96). In contrast, the modified gE ELISA (with MAb 67 and MAb 75) was less sensitive (0.79). The specificities of the two tests for testing milk samples were very high (1.00 and 0.99, respectively) (Wellenberg *et al.*, 1998).

Defatted milk samples give more reliable results, because there will be fewer false negatives and false positives than with samples containing fat (Wellenberg *et al.*, 1997). In case milk samples will be used in large-scale screening programs, the addition of a preservative to the milk, and storage at -20°C , are necessary, because milk samples cannot always be analyzed on the day of collection. Defatted milk samples can be stored at -20°C for at least 32 days without having any influence on the BHV-1 gE antibodies (Wellenberg *et al.*, 1998).

Pooled serum samples can be used to evaluate herd BHV-1 status. Up to 30 serum samples were used in the serum pools in an Irish study (Cowley *et al.*, 2011), however herd size as well as within-herd prevalence of the infection of the specific population should be taken into account when defining pool sample size.

2.7. Risk factors

The age of an animal is a risk factor for BHV-1 seropositivity (Hage *et al.*, 1996; Kadohira *et al.*, 1997; McDermott *et al.*, 1997; Solis-Calderon *et al.*, 2003; Kampa *et al.*, 2004; Boelaert *et al.*, 2005; Guarino *et al.*, 2008; Jacevičius *et al.*, 2008; Woodbine *et al.*, 2009; Carbonero *et al.*, 2011). Age is a surrogate measure of the amount of exposure-time (Kadohira *et al.*, 1997), and this occurs with diseases that induce life-long seropositivity. Calves have a lower prevalence probably because of reduced contact with cattle in other herds, and shorter duration of exposure (McDermott *et al.*, 1997; Solis-Calderon *et al.*, 2003; Boelaert *et al.*, 2005). However, Segura-Correa *et al.* (2010) found that animals younger than 24 months of age had a higher incidence of seroconversion for BHV-1 than those >24 months of age. The author explained the finding by a higher susceptibility of young animals, or to the practice of culling of unproductive adult cows, some of them probably due to IBR (Segura-Correa *et al.*, 2010). Seroconversions were often restricted to cattle aged between 24–28 months, which is the period in which the youngstock generally enter the milking cow population. Probably the seronegative youngstock became infected with BHV-1 when they were added to the milking cows. Reactivated and re-excreted BHV-1 from latently infected milking cows might have caused these infections (Mars *et al.*, 2001).

The sex of an animal (males are more frequently positive than females) has been shown to be a risk factor for BHV-1 seropositivity (Boelaert *et al.*, 2005). Bulls have more “risky” contacts compared to cows, for example more frequent participation in cattle shows and other risky behaviour, such as escaping and mingling with other cattle (Boelaert *et al.*, 2005). However, contrary results on the prevalence among cows and bulls have been found (Guarino *et al.*, 2008). Using natural service was found to be a risk factor for herd seropositivity in a Brazilian study (Dias *et al.*, 2012).

Farm type is a common risk factor for IBR. Mixed herds (herds with dairy as well as beef/veal animals) have an increased risk of being BHV-1 positive than herds with only dairy cattle (Van Wuijckhuise *et al.*, 1998; Gonzales-Garcia *et al.*, 2009). No significant difference was found between dairy and beef herds in BHV-1 prevalence (Cowley *et al.*, 2011). In a study by Woodbine *et al.* (2009), if the herd was a dairy herd rather than a suckler herd, the rate of seroconversion in adults was greater, possibly due to winter housing or other stressors related to dairy cow management.

However, beef herds had a higher probability of being BHV-1 positive in a study by Dias *et al.* (2012).

A positive association between herd size and BHV-1 seropositivity has frequently been found to be an important risk factor (McDermott *et al.*, 1997; Paton *et al.*, 1998; Van Wuijckhuise *et al.*, 1998; Tekes *et al.*, 1999; Solis-Calderon *et al.*, 2003; Boelaert *et al.*, 2005; Gonzales-Garcia *et al.*, 2009; Woodbine *et al.*, 2009; Segura-Correa *et al.*, 2010) although no association between herd size and herd's BHV-1 status was found by Ståhl *et al.* (2002). In smaller herds there are fewer susceptible animals throughout the year, so that the infection may not be sustained because it is below the epidemic threshold (Van Wuijckhuise *et al.*, 1998; Boelaert *et al.*, 2005). "Herd size" must be considered as a proxy for other risk factors (e.g. purchase of stock and professional visitors, recrudescence of infection through stress, or exposure to more viral types) (Nardelli *et al.*, 2008; Woodbine *et al.*, 2009).

Provision of protective clothing to visitors was a protective factor (van Schaik *et al.*, 1998b; van Schaik, 2001a; van Schaik, 2001b; van Schaik, 2002). The transmission of IBR/IPV virus in imported semen was reported in Switzerland (Kupferschmied, 1986). Kampa *et al.* (2009) believe that seroconversions to BHV-1 in Thailand may have been caused by virus-contaminated semen or by indirect transmission of the virus to the animals by the person performing the inseminations.

Purchase of cattle (Paton *et al.*, 1998; Vonk Noordegraaf *et al.*, 1998; Van Schaik *et al.*, 1999a; Kampa *et al.*, 2004; Vonk Noordegraaf *et al.*, 2004; Dias *et al.*, 2008; Nardelli *et al.*, 2008; Dias *et al.*, 2012) and participation in animal shows (van Schaik *et al.*, 1998a; van Schaik *et al.*, 1998b; Van Wuijckhuise *et al.*, 1998; van Schaik *et al.*, 2002; Boelaert *et al.*, 2005; Dias *et al.*, 2008; Gonzales-Garcia *et al.*, 2009) as well as cattle that escape and mingle with other cattle (van Schaik, 2001a; van Schaik, 2001b) are also important risk factors for the introduction of BHV-1. Direct contacts with other cattle (i.e. allowing cattle to return to the farm when not successfully sold, and grazing cattle at other farms) was found to be a risk factor for BHV-1 introduction in a study conducted by van Schaik *et al.* (2002). Movement and mixing in new herds is stressful for cattle, resulting in recrudescence of virus in infected cattle that then infect susceptible cattle (Woodbine *et al.*, 2009).

Management factors related to the reactivation of BHV-1 on dairy farms were all related to the loose housing system, which incurred an increased risk of reactivation of BHV-1 at the farm (van Schaik *et al.*, 1999a). The reactivation was facilitated when the barn was overcrowded (i.e. more cows than cubicles in the barn). An overcrowded barn leads to higher stress levels of the cows, which might lead to an increased reactivation rate of BHV-1 and more contacts between the cows (van Schaik *et al.*, 1999a).

Grazing (McDermott *et al.*, 1997; Dias *et al.*, 2008) and pasture rental (Dias *et al.*, 2012) were also found to increase the risk of a farm being infected with BHV-1. However, cattle from different farms driven to alpine pastures in Trento region in Italy didn't become infected with BHV-1 (Nardelli *et al.*, 2008). Farm (Van Wuijckhuise *et al.*, 1998; Vonk Noordegraaf *et al.*, 2004) as well as cattle density may increase the risk of BHV-1 introduction (Paton *et al.*, 1998; Vonk Noordegraaf *et al.*, 2004; Muylkens *et al.*, 2007). A higher density of herds in an area increases the potential for the transmission of the virus from herd-to-herd through, for example, visits by animal handlers, windborne aerosols, and contact between animals in the grazing season (van Schaik *et al.*, 1998b; Van Wuijckhuise *et al.*, 1998). Proximity to urban areas was found to be a risk factor for a herd being BHV-1 positive (Gonzales-Garcia *et al.*, 2009). BRSV infection, altitude above the sea level (≤ 1800 m) and average slope ($> 11\%$) were risk factors associated with BHV-1 infection in a study completed in Ecuador, whereas good cleaning of the facilities came out as a protective factor (Carbonero *et al.*, 2011). The presence of wild animals was identified as a risk factor for BHV-1 infection in the West region of Parana State (Dias *et al.*, 2008).

2.9. Control

Since the EU allows IBR-free member states to restrict the import of cattle, semen and embryos from countries not free from IBR, more efforts were undertaken in the late 1990s to achieve eradication of BHV-1 in the EU (Ackermann and Engels, 2006). The main reason to start eradicating BHV-1 in The Netherlands was the restrictions related to BHV-1 for the international trade of cattle, bovine semen and embryos (Vonk Noordegraaf *et al.*, 2002). If BHV-1 eradication is the goal, culling of seropositive animals without vaccination has been the most successful method. However, this can only be considered if the seroprevalence of

BHV-1 is relatively low (Ackermann and Engels, 2006). Vaccination with gE marker vaccines, combined with the detection of gE positive animals, may be considered at the beginning of an eradication program and in countries with a high BHV-1 prevalence and/or with a high risk of BHV-1 occurrence (Vonk Noordegraaf *et al.*, 1998; Nardelli *et al.*, 1999; Alkan *et al.*, 2005; Ackermann and Engels, 2006). When a national prevalence of 5% gE-positive cows is reached, the remaining positive cows in the population have to be detected, so that they can be culled (Vonk Noordegraaf *et al.*, 1998). A voluntary vaccination program is not expected to lead to country-wide eradication of IBR (Bätza, 1997; Vonk Noordegraaf *et al.*, 1998). It appears that a compulsory vaccination program for all herds would be necessary to achieve IBR-free status (Vonk Noordegraaf *et al.*, 1998). An IBR control programme is successful if all the stakeholders are involved, especially the breeders' associations (Bätza, 1997).

2.9.1. Legislation

Estonian legislation establishes requirements for animals introduced and reared in AI centres. All animals introduced to the AI centre must be isolated in their herd of origin, tested and be confirmed negative to BHV-1 antibodies 30 days before movement (Anonymous, 2008). Bulls used for semen collection are tested serologically once a year (Anonymous, 2004b).

The 'Report on Bovine Herpesvirus 1 (BHV-1) marker vaccines and the accompanying diagnostic tests' (Sanco/C3/AH/R20/2000) has been published by the European Commission. The OIE Terrestrial Manual 2010 (Chapter 2.4.13.) covers diagnostic techniques, requirements for vaccines and diagnostic biologicals. EU legislation (Decision 2004/558/CE) defines the requirements to be fulfilled in order to obtain approval for national IBR eradication programmes. Legislation with respect to BHV-1 for the international trade of cattle, bovine semen and embryos is constituted in EU-directives 64/432, 88/407 and 89/556.

2.9.2. Test and removal strategy

A test and removal strategy has been successfully elaborated in several countries. Eradication of BHV-1 with the culling of seropositive animals without vaccination has been the only successful method so far. This

can only be considered if the seroprevalence of BHV-1 is relatively low (Ackermann and Engels, 2006). To accomplish IBR-eradication, it is advisable, first, to create IBR-free breeding stock. In many cases, this can be achieved by gradually removing all seropositive cattle from a conventional breeding lot and replacing them with seronegative progeny (Ackermann and Engels, 2006).

In Finland, the total number of herds with BHV-1 antibody positive animals in 1994 was five, and the total number of seropositive animals was 90. Using the 'test and slaughter' approach and total culling of one herd, the infection was eradicated in 1994. Cattle owners were entitled to financial compensation if animals from their herds were culled for seropositivity, or having the actual disease, and this was a key to the success of the project (Nuotio *et al.*, 2007).

A 'test and removal' policy was also used in Switzerland (Ackermann *et al.*, 1990). Ten years after the first outbreak of IBR in Swiss dairy cows, the national cattle herd was almost free from infection with the BHV-1. The national programme for the eradication of IBR was divided into four phases: (1) Prevention of transmission of the infection by restrictions on the trade of bovines and assessment of the prevalence of cattle with antibodies to BHV-1. (2) Slaughtering animals with antibodies to BHV-1 in order to eradicate BHV-1 from breeding herds. (3) Detection and eradication of further BHV-1 reservoirs (e.g. fattening cattle). (4) Monitoring programme and legal actions in order to maintain the favourable situation. Approximately 50,000 animals were slaughtered in the course of the eradication of IBR (Ackermann *et al.*, 1990). Switzerland has been officially free of IBR since 1994 (Blickenstorfer *et al.*, 2011).

The Danish BHV-1 eradication programme was started in 1984. At the beginning of 1984, the apparent prevalence of seropositive dairy herds was 9%, and a maximum of 11% was reached in 1985. During the campaign, herds were recorded as BHV-1 infected if a bulk-tank milk sample or a serum sample from one or more animals showed a positive reaction. An individual-herd eradication programme was based on serum samples from all animals in the herd. Approximately 4,600 dairy herds out of a total population of about 30,000 dairy herds were recorded BHV-1 infected during the campaign. The eradication of BHV-1 from the Danish cattle population was accomplished by the year 1991 (Nylin *et al.*, 2000).

2.9.3. The DIVA strategy (differentiating infected from vaccinated animals)

The method of depopulation is inefficient for eradication of BHV-1 in countries with high seroprevalence (Ackermann and Engels, 2006; Jacevičius *et al.*, 2008). If there are more than 15-20% BHV-1-positive animals in a population, vaccination is the most realistic strategy to eradicate IBR (Kuijk, 2002). Marker vaccines are vaccines that allow serological differentiation between infected and vaccinated individuals (also called DIVA vaccines). This differentiation is based on the absence of one or more microbial proteins (mostly gE) in the vaccine, that are present in the wild-type micro-organism (van Oirschot *et al.*, 1996b; Van Oirschot *et al.*, 1997). Consequently, after infection, but not after vaccination, an antibody response against that specific protein(s) can be detected (van Oirschot *et al.*, 1996b). The combined use of the marker vaccine and the companion diagnostic test (gE-blocking ELISA) makes it possible to differentiate between vaccinated animals and infected animals (Kaashoek *et al.*, 1995; van Oirschot *et al.*, 1996b; van Oirschot, 1999; Ackermann and Engels, 2006). The use of marker vaccines is important for protecting national herds and is critical as a trading tool once a pathogen has been eradicated in some countries (van Drunen Littel-van den Hurk, 2006).

Although neither conventional nor gE marker vaccines are able to prevent infection and establishment of latency with the wild-type virus, there is general agreement that the circulation of the wild-type virus is significantly diminished in vaccinated herds (Straub, 1991; Ackermann and Engels, 2006; van Drunen Littel-van den Hurk, 2006). Vaccination reduces the risk of BHV1-reactivation on infected farms (Vonk Noordegraaf *et al.*, 2000). Therefore, to eradicate BHV-1, repeated vaccination according to a strict schedule to reduce the possibility of wild virus excretion, and strict management practices are required (van Drunen Littel-van den Hurk, 2006). Immunization of dams with an inactivated vaccine conferred full *in utero* protection against IBR-virus infection. When such calves are reared in isolation they can be used as the basis for a seronegative breeding herd (Pospíšil *et al.*, 1996a; Pospíšil *et al.*, 1996b).

Germany has had guidelines for the voluntary eradication of IBR since 1986. However, this did not result in a substantial decrease in the number of IBR-positive herds (Bätza, 1997). Since 1997 a compulsory eradication programme has been implemented in Germany (Trapp *et al.*, 2003; Truyen *et al.*, 2003). Eradication of the virus is based on two different

concepts: German federal states with a low BHV-1-seroprevalence comply with a selective slaughter programme that incorporates the culling of BHV-1-positive cattle and only exceptional vaccination. States with a high BHV-1-prevalence attempt to eradicate the virus using the marker concept (DIVA strategy) (Trapp *et al.*, 2003). In Bavaria (Germany) a BHV-1 eradication program was initiated in 1986 and was changed to a compulsory program in 1998. The eradication success increased progressively from < 50% in 1986 to 87% of the BHV-1 free farms in 2002 (Truyen *et al.*, 2003).

A compulsory eradication programme was also initiated in the Italian province of Trento (Ackermann and Engels, 2006), as well as in The Netherlands from 1998 (Graat *et al.*, 2001; Vonk Noordegraaf *et al.*, 2004). The compulsory eradication programme in The Netherlands is based on half-yearly vaccination with marker vaccine of all cattle aged >3 months (with the exception of certified BHV-1 free herds) (Vonk Noordegraaf *et al.*, 2004). At the start of the eradication programme, about 25% (7400) of the dairy herds, and 18% (5400) of non-dairy cattle herds, were certified BHV-1 free in The Netherlands (Vonk Noordegraaf *et al.*, 2004). Herds are certified BHV-1-free when all cattle older than one year have no antibodies against BHV-1-gB or gE, as determined with a gB-ELISA or a gE-ELISA, respectively (de Wit *et al.*, 1998). To achieve the IBR free status farmer pays for vaccinations, milk/blood sampling, elimination of the last positive animals and certification. Administration and testing, maintenance model and research will be paid from collective financing (Franken, 1997). In spring 1999, the compulsory eradication programme was postponed due to BVD contamination of the vaccine in The Netherlands (Vonk Noordegraaf *et al.*, 2002). Not a single country that included vaccination in its eradication program has so far succeeded in eradicating IBR (Ackermann and Engels, 2006).

2.9.4. Concerns related to BHV-1 eradication

There are several concerns regarding eradication schemes for BHV-1. Seronegative latent carriers (SNLC) may result after infection of passively immunized calves with a virulent BHV-1 (Hage *et al.*, 1998a; Lemaire *et al.*, 2000a; Lemaire *et al.*, 2000b; Sanco/C3/AH/R20/2000; Lemaire *et al.*, 2001; Geraghty *et al.*, 2012). The existence of SNLCs is of primary economic importance in selection stations, artificial insemination centres,

and BHV-1-free farms or regions, where a virus circulation among free animals can induce a disastrous seroconversion in a significant number of animals (Lemaire *et al.*, 2000b). The PCR protocols allow for the detection of BHV-1 in SNLC cattle (Fuchs *et al.*, 1999; Muylkens *et al.*, 2007). The importance of SNLC for BHV-1-eradication remains unclear. In particular, more epidemiological data of the SNLC reactivation rate in BHV-1-free regions needs to be collected and studied (Sanco/C3/AH/R20/2000).

Another problem is the capacity of BHV-1 to cross the species barrier. Other ruminant species may become infected with BHV-1, however there is so far no evidence that those animals could play a role in the transmission of BHV-1 to cattle (Yates, 1982; Wentink *et al.*, 1993; Hage *et al.*, 1997). BHV-1 infected goats developed mild disease signs during acute infection, and isolation of the virus from nasal secretions (Wafula *et al.*, 1985; Six *et al.*, 2001). Therefore goats should indeed be regarded as a potential BHV-1 reservoir, which must be considered during IBR eradication programs (Six *et al.*, 2001).

A constant concern is the possibility of recombination between the gE-deleted vaccine virus and the wild-type virus, which has been shown to be possible under experimental conditions (Schyns *et al.*, 2003). Wild-type mutant field variants of BHV-1 with a variable response in anti-gE ELISA have been isolated (Egyed *et al.* 2000). The possibility of recombination when using live deleted vaccines is regarded as of minor epidemiological importance for the gE deletion, which is a large deletion of 2.7kb, reversion to virulence is virtually almost impossible because of the size of the deletion. Another problem, linked to recombination, could arise if the recombined vaccine virus should be more virulent than the parent strain, but no evidence has so far been reported that this is possible. However, the possibility of reinsertion could be enhanced by using two different types of live deleted vaccines, and it is therefore advised to use only one type of live deleted vaccine (Sanco/C3/AH/R20/2000). During IBR epidemic events intranasal gE deletion vaccination is frequently carried out and consequently co- and superinfections with wild and gE-negative vaccine strains of BoHV-1 can occur with the possibility to generate virulent viruses from which gE has been deleted. These recombinant viruses could endanger control and eradication programs. Fortunately, there is only a very small window (0 to 6 h) in which superinfection efficient for recombination occurs. The low likelihood of cellular coinfections in natural conditions allows concluding that recombination and its potential

consequences are rare events (Meurens *et al.*, 2004). During a field trial to evaluate the efficacy of repeated vaccinations with BHV-1 marker vaccines, a gE-negative BHV-1 strain was isolated from the nasal secretions of two cows, eight months after vaccination with a gE-negative live-attenuated vaccine, initially given intranasally, then intramuscularly (Dispas *et al.*, 2003). However, BHV-1 strains that do not express a particular gE-epitope in cell culture, can still induce antibodies that are detected in a blocking ELISA, which measures antibodies against that epitope. This adds further support for the use of gE-deleted vaccines in programmes to eradicate BHV-1 infection (van Oirschot *et al.*, 1998). Cattle which had not been vaccinated against BHV-1 were seropositive in a glycoprotein B (gB)-blocking ELISA, but seronegative in a glycoprotein E (gE)-blocking ELISA. However no virus was isolated from those animals (Mars *et al.*, 2000d).

Severe disease problems were diagnosed on four Dutch dairy farms after vaccination with the same batch of BHV-1 marker vaccine. An outbreak of BVDV type 2 infection was caused by the use of a batch of modified live BHV-1 marker vaccine contaminated with BVDV (Barkema *et al.*, 2001).

The Netherlands also has a few feral cattle herds living in extensively managed natural areas. For obvious reasons, in these herds only oral vaccination is allowed, but there is not yet available a useful and effective oral vaccine against BHV-1 (de Koeijer *et al.*, 2008).

2.9.5. Vaccines

Conventional vaccines are widely used to prevent clinical signs of infectious bovine rhinotracheitis. The use of conventional vaccines, however, does not appear to have resulted in a reduction in the prevalence of infection (van Oirschot *et al.*, 1996a). New vaccines containing BHV-1 are being introduced, indicating the market demand to control economic losses due to BHV-1 infections (Patel, 2005b). Vaccination reduces the effective reproduction ratio, the infectious period of an infected animal and the probability that a latently infected animal transmits virus after reactivation (Vonk Noordegraaf *et al.*, 2000). Vaccines reduce the severity of disease and also reduce virus replication and transmission, but they are not able to prevent BHV-1 infection (Castrucci *et al.*, 2002a; Ackermann and Engels, 2006) followed by a state of latency (Straub, 2001). Four kinds of vaccines are available to prevent virus infection: modified live

virus (MLV) vaccines, inactivated vaccines, subunit vaccines, and marker vaccines (Yoo, 2010). Both live and inactivated gE-negative vaccines have shown efficacy under field conditions. The available data seem not to be sufficient to recommend a common vaccination scheme suitable for different kinds of protection (clinical signs, virus shedding and transmission, and reactivation) (Sanco/C3/AH/R20/2000).

The MLV vaccines induce a rapid immune response, long lasting immunity, and result in local and mucosal immunity (Yoo, 2010). MLVs generally induce both humoral and cellular immune responses (Van der Poel *et al.*, 1995; Jones and Chowdhury, 2010). MLV vaccines were able to induce protection against disease by 2–3 days after vaccination, although specific antibodies against BHV-1 were not detected at that time. Therefore live vaccine could be administered in face of an outbreak (Patel, 2005a; Ackermann and Engels, 2006) as suggested by the observation of significant protection as early as seven days by the i.m. route (Patel, 2005a). The protection from infection has been claimed to persist for 6–9 months after vaccination (Ackermann and Engels, 2006). Safety is a concern for MLVs because MLV vaccines, when applied intranasally, were able to establish latency (Whetstone *et al.*, 1986; Castrucci *et al.*, 2002b; Jones and Chowdhury, 2010) with occasional reactivation and shedding (Ackermann and Engels, 2006; van Drunen Littel-van den Hurk, 2007). When transmitted to pregnant cows, infection can lead to abortion (Van der Poel *et al.*, 1995; Jones and Chowdhury, 2010; Yoo, 2010). Unlike the early BHV-1 vaccines, current vaccines are not contraindicated for pregnant cattle, even when given by the i.m. route (Patel, 2005a). MLVs can also be pathogenic in young calves, because their immune system is not fully developed, and most MLVs can be immunosuppressive (Jones and Chowdhury, 2007). However it provides protection from infection with virulent BHV-1, and significantly reduces nasal shedding of the virus after vaccination (Frerichs *et al.*, 1982; Ellis *et al.*, 2005; Patel, 2005a). A single intranasal vaccination affords significant protection in addition to maternally derived antibodies, and the protection can be significantly prolonged by a booster intramuscular vaccination (Patel, 2005a). According to Vonk Noordegraaf *et al.* (2002) preference should be given to live vaccine. The value R of the live vaccine is lower under experimental conditions than that of the inactivated vaccine (Vonk Noordegraaf *et al.*, 1998) shortening the eradication period (Vonk Noordegraaf *et al.*, 2002).

Inactivated vaccines are safe for pregnant animals, stable in storage, and cause neither shedding of the virus, nor immunosuppression, abortion

nor latency, although they cannot prevent the development of latency following exposure to the wild-type virus (Patel, 2005b; Yoo, 2010). However, unlike the live BHV-1 vaccines, they are not ideal for use in face of a field BHV-1 infection since at least two doses, usually a month apart, are necessary to confer protection (Patel, 2005b; Jones and Chowdhury, 2007). Also, since they are administered parenterally, they are unlikely to be effective in calves with maternally derived antibodies (Patel, 2005b). KV vaccines usually produce only humoral immunity, but no cellular immune responses and relatively short-term memory (Jones and Chowdhury, 2007; van Drunen Littel-van den Hurk, 2007; Jones and Chowdhury, 2010), however the seroneutralizing response is higher with an inactivated vaccine (Vanopdenbosch and Kerkhofs, 1997). In this case cellular immunity is the dominant mechanism in recovery from primary BHV-1 infection (Patel, 2005b). Conventionally produced inactivated BHV-1 vaccines vary in the quality of protection they afford, both with respect to reduction in virus shedding and clinical signs (Patel, 2005b). Inactivated vaccines have a better protective effect on virus re-excretion, following dexamethasone treatment, than attenuated vaccines (Kerkhofs *et al.*, 2003).

The subunit vaccine contains one or more antigens of the virus (van Oirschot *et al.*, 1996a) that induce protective immunity, and do not contain nucleic acid and other components that might cause unwanted side-effects (Yoo, 2010). The efficacy of the vaccine is a concern as gD-subunit vaccine had R=3.4 compared to placebo group with R=3.2 (Bosch *et al.*, 1998).

A marker vaccine is based on changes in one or more of the non-essential glycoproteins, which allow the differentiation of vaccinated animals from infected ones (Nandi *et al.*, 2009). Glycoproteins gC, gE, gI, gG and gM are nonessential and thus may be deleted with little or no effect on virus production *in vitro* or *in vivo* (van Drunen Littel-van den Hurk, 2006). Numerous viral mutants (gC-, gE-, gG-, Us9-deleted, thymidine kinase (TK)-deleted and LR gene mutant) of the virus have been constructed. Based on recent studies, both gE- and Us9-deleted viruses were found to be safe in calves because they do not reactivate from latency, and they are highly attenuated (Jones and Chowdhury, 2007). gC plays a role in viral attachment and is highly immunogenic, gG, gI and gE have a function in cell to cell spread mechanisms. gE or gI, or gE/gI deleted strains are avirulent, the latter are too less immunogenic and gC and gG deleted viruses preserve a certain degree of virulence (Kaashoek *et al.*, 1998; Sanco/C3/AH/R20/2000). Comparative vaccine efficacy studies

have shown that relative to gC- and gG-deleted viruses, the gE-deleted virus is less efficacious (Jones and Chowdhury, 2007). Regardless of the type of DIVA vaccine, the marker protein needs to be present in all wild virus strains, and induce a strong, long-lasting humoral response, both in unvaccinated and in vaccinated animals (van Drunen Littel-van den Hurk, 2006). Deleting the BHV-1 gE gene has little effect on the immunogenicity of BHV-1, but resulted in a significant reduction of the virulence, a shorter period of viral replication and a reduced spread to the trigeminal ganglia, therefore the gE deletion mutant is a good candidate for a BHV-1 marker vaccine (van Engelenburg *et al.*, 1994; Kaashoek *et al.*, 1998; Belknap *et al.*, 1999). The antibody response against gE has been shown to persist for at least three years at stable levels. In addition, the gE protein is probably present in all wild-type isolates of BHV-1. Consequently, marker vaccines based on gE deletion mutants have been chosen in several European countries to be used in eradication programmes (Kaashoek *et al.*, 1998; Sanco/C3/AH/R20/2000).

Marker vaccines have been shown to be efficacious in: reducing clinical signs after infection, wild-type virus replication after infection, and transmission of the wild-type virus in the laboratory and in the field (Kaashoek and van Oirschot, 1996b; van Oirschot *et al.*, 1996b; Mars *et al.*, 2001; van Drunen Littel-van den Hurk, 2006). The live marker vaccine is safe for breeding cows, bulls, and pregnant cows. It is also effective in the presence of maternal antibodies (Strube *et al.*, 1996; Nandi *et al.*, 2009; Yoo, 2010). Vaccination with marker vaccines does not exclude the establishment of latency of challenge virus (Kaashoek *et al.*, 1998). Live gE-marker vaccines can sometimes be reactivated after dexamethasone treatment. However, vaccine strain transmission in a population is regarded as unlikely (Sanco/C3/AH/R20/2000). Transmission of the vaccine virus has not yet been demonstrated, but may be expected after IN vaccination, no transmission has been reported after IM vaccination. Viral DNA has been detected by PCR in trigeminal ganglia after intranasal vaccination, but not after intramuscular vaccination (Sanco/C3/AH/R20/2000). The BHV-1 gE-negative strain can establish latency not only in seronegative calves, but also in passively immunised calves after only one intranasal inoculation (Sanco/C3/AH/R20/2000). However, after corticosteroid treatments, re-excretion of virus was never detected in cattle that had been inoculated with the gE-negative BHV-1 vaccine strain (Mars *et al.*, 2000c). In a study by Mars *et al.* (2000b), the transmission ratio R_0 of the vaccine strain was estimated to be 0.14, meaning that it is

highly unlikely that vaccine virus transmission occurs (Mars *et al.*, 2000b; Sanco/C3/AH/R20/2000).

Inactivated gE vaccine had reproduction ratio $R=2.28-2.6$ and live marker vaccine had $R=0.92-1.5$ indicating that major outbreaks can still occur with some probability in vaccinating herds (Bosch *et al.*, 1998; Sanco/C3/AH/R20/2000; Mars *et al.*, 2001). The R_1 of the natural BHV-1 in non-vaccination herds has been estimated in several studies to be from 2.8 to 7 (Hage *et al.*, 1996; Bosch *et al.*, 1998; Mars *et al.*, 2001; Hage *et al.*, 2003). Cattle that were first vaccinated twice with gE-negative vaccines, and then challenged with a wild-type BHV-1 strain, had a reduced replication of BHV-1 in nasal mucosae and all developed an antibody response against gE between 7 and 14 days after the challenge (Van Oirschot *et al.*, 1997). Serological testing on farms which used vaccination with marker vaccines showed a high efficacy, with decreasing seroprevalence of BHV-1 gE (Bosch *et al.*, 1998; Makoschey *et al.*, 2007; Vilmos *et al.*, 2007; Jacevičius *et al.*, 2008). None of the marker vaccines prevented excretion of BHV-1 after dexamethasone treatment in any of the animals (Bosch *et al.*, 1997). The attenuated gE-negative vaccine induced the best clinical protection, as evidenced by the total absence of clinical signs and fever in cattle compared to the inactivated gE-negative vaccine and an experimental gD-subunit vaccine (Bosch *et al.*, 1996). Attenuated vaccine reduced the shedding of challenge virus significantly more than the inactivated vaccines (Bosch *et al.*, 1996). However, according to Bosch *et al.* (1997), in cattle vaccinated with the inactivated marker vaccines, the amount, but not duration, of BHV-1 shedding was reduced. No reduction in virus excretion was found in cattle given live marker vaccine (Bosch *et al.*, 1997).

2.10. Surveillance

The goal of surveillance is that a certified IBR free herd that becomes infected is detected in a timely fashion, so that infection of other certified herds is prevented (Graat *et al.*, 2001). What is important is whether the reproduction ratio R , i.e. the average number of certified herds infected by one infected certified herd, can be kept below 1 (Graat *et al.*, 1997; Graat *et al.*, 2001). When the herd level R is below 1, then the surveillance programme is sufficiently good at preventing the spread of infection from herd to herd (Graat *et al.*, 1997; Graat *et al.*, 2001). Otherwise, reintroduction of the virus into BHV-1-free areas can lead to major

outbreaks, thereby causing severe economic losses (Vonk Noordegraaf *et al.*, 2000).

The existing diagnostic tests are sufficiently good for the surveillance of certified herds (Graat *et al.*, 2001). Bulk milk only becomes positive with a minimum of 10-15%, or even 20% positive animals (Hartman *et al.*, 1997; Wellenberg *et al.*, 1997; Sanco/C3/AH/R20/2000; Graat *et al.*, 2001). The dilution effect on pool sensitivity (PSe) will be dependent on the infected cow's concentrations of antibodies in relation to the selected cut-off value for the assay, and her milk production compared with other cows in the pooled sample (Christensen and Gardner, 2000). Therefore bulk milk testing is unable to identify herds containing very small numbers of seroreactors, reducing the sensitivity of the test (Frankena *et al.*, 1997; Hartman *et al.*, 1997; Paton *et al.*, 1998; Van Wuijckhuise *et al.*, 1998). If the infected animals are from young stock, the infected cow is dry, has mastitis or has been treated with antibiotics and is not contributing milk to the bulk tank, then the probability of inclusion in the sample will be zero, and the pooled test result may be negative even though the herd is infected (Christensen and Gardner, 2000; Nylin *et al.*, 2000). However, multiple sample rounds might compensate for this effect (Nylin *et al.*, 2000). In order to increase the sensitivity of BTM ELISA a blocking percentage of <10 was considered to indicate a negative BHV-1 status, samples with blocking percentage between 10 and 50 as weakly positive and percentages >50 were considered positive in a study of Van Wuijckhuise *et al.* (1998). If BHV-1 is detected in the bulk milk, there is a high probability that more than one animal is infected, and that the infection has spread (Frankena *et al.*, 1997). Monitoring by bulk milk samples is the only economical possibility (Eliot, 1997; Hartman *et al.*, 1997; Christensen and Gardner, 2000; Nylin *et al.*, 2000). When used periodically it can be a useful means of providing regular herd surveillance (Paton *et al.*, 1998).

Bulk milk testing can be very useful at the start of an eradication programmes in countries with high BHV-1 prevalence (Hartman *et al.*, 1997). It is useful in discriminating between herds with a high prevalence of BHV-1 and those with low or no prevalence (Sanco/C3/AH/R20/2000). Only in herds with a negative or weak positive bulk milk result is it economically feasible to test the herd individually (Hartman *et al.*, 1997). Bulk milk testing for gE-antibodies has to be repeated several times a year, and the status of farms with a low seroprevalence could be false negative (Sanco/C3/AH/R20/2000).

If herds of 50 cows became free of BHV-1 without vaccination, then the spread of infection between herds might be prevented if animals within herds are sampled once a year (milk or blood samples). This frequency needs to be intensified, at twice a year for larger herds and/or herds with extensive contacts with other herds. When bulk milk is sampled instead, sampling should be done at least every five months and more intensively, monthly, with larger herd sizes and where there is more contact between herds (Graat *et al.*, 2001).

The standards for monitoring IBR-free herds vary by country: in Belgium monitoring is performed by testing blood samples twice a year, in France testing bulk milk samples every six months, in Germany testing blood samples once a year, or milk samples at a higher frequency, in The Netherlands, certified BHV-1 status was monitored through monthly bulk-milk tests of dairy herds (Franken, 1997; Vonk Noordegraaf *et al.*, 2004) and half-yearly serological sampling of non-dairy herds (Vonk Noordegraaf *et al.*, 2004), in Denmark testing bulk milk samples at least every 3 months (Nylin *et al.*, 2000). In Bavaria BHV-1-free farms are controlled by bulk milk serology twice a year, along with blood serology in animals that are negative but from herds where positive field virus infected animals are present. All serological tests are performed with an indirect ELISA test, and all positive results are confirmed by a gB ELISA (Truyen *et al.*, 2003). In Switzerland the yearly surveillance programme is carried out to demonstrate freedom from BHV-1 at a 0.1% (between 2002 and 2004) and at a 0.2% (since 2005) herd prevalence level with a 99% confidence (Reist *et al.*, 2012).

In order to optimise the cost-effectiveness of active surveillance to ensure freedom from BHV-1, a new approach, using targeted sampling of farms was developed and applied in Switzerland. For this, high-risk strata of the animal population were targeted. Relevant risk factors for the introduction of IBR into Swiss cattle farms were identified and their relative risks defined based on a literature review and expert opinion. Taking into account the uneven distribution of the disease risk, a 40% reduction in the full survey costs was achieved, compared to a stratified random sample (Blickenstorfer *et al.*, 2011; Reist *et al.*, 2012).

Monthly bulk milk testing reduces the probability of virus transmission of a dairy farm from 76% to 42% (Vonk Noordegraaf *et al.*, 2000). According to the model of Graat *et al.* (1997), in order to keep the $R_{\text{between herd}} < 1$, the maximum sampling interval must be six months if $R_{\text{within herd}} = 5.6$

(animals are not vaccinated, gB-); 10 months if $R_w=2.6$ (inactivated marker vaccine is used) and two years if $R_w=1.5$ (live marker vaccine is used). Sampling a minimum of 10 animals twice a year is also sufficient to prevent the spread of BHV-1 (Graat *et al.*, 2001).

The R between herds was mainly influenced by the vaccination status, sampling frequency, and contacts between herds. Herd size moderately affected the outcome. Test sensitivity and sample size (with individual sampling), however, were of minor importance (Graat *et al.*, 2001). The major sources for outbreaks in the simulation model of Vonk Noordegraaf *et al.* (1998) were “other contacts” and reactivation of purchased gE-positive cows. The former is controlled through standard biosecurity measures, such as disinfection of visitors’ shoes and agreements with neighbours about pasture use (Vonk Noordegraaf *et al.*, 1998). Prevention of BHV-1 seropositivity should primarily focus on purchase that directly introduces virus into the herds (Vonk Noordegraaf *et al.*, 1998; Boelaert *et al.*, 2005; Ackermann and Engels, 2006; Blickenstorfer *et al.*, 2010). Only certified BHV-1 uninfected cattle should be purchased, and quarantine should be rigorously applied (Vonk Noordegraaf *et al.*, 2004; Boelaert *et al.*, 2005). In addition to the movement of seropositive cattle, trade with BHV-1-positive semen used in artificial insemination is considered to be one of the most important ways that the virus is reintroduced into IBR-free facilities (Ackermann and Engels, 2006). The main risk factors for the outbreak of IBR in Switzerland in 2009 were purchase and movement of bovines, and semen, of often unknown IBR status (Blickenstorfer *et al.*, 2010). Between January 1991 and May 1994, 22 herds became infected with BHV-1 in Denmark, all located close to the German border. Possible transmission routes for the virus were sporadic introduction due to accidental contact with infected cattle near the German border, or airborne transmission of the virus over a longer distance (Nylin *et al.*, 1998). To limit the number of secondarily infected farms, more frequent monitoring on farms with a frequent trade in cattle is an especially important instrument (Vonk Noordegraaf *et al.*, 2000). For beef and veal farms, there is a high probability that, after reintroduction, the virus is not transmitted to other farms (98 and 93%, respectively) (Vonk Noordegraaf *et al.*, 2000).

2.11. Self clearance

The presence of a self-clearance process was indicated in Thailand (Kampa *et al.*, 2004; Kampa *et al.*, 2009). No indication of virus circulation for at least three years was found in eight out of 20 study herds in The Netherlands according to a study of van Nieuwstadt and Verhoeff (1983).

In the process of self clearance in Thailand's dairy herds the seropositive animals were either sold or otherwise removed from the herd as part of normal, commercially motivated, management of the herds. Consecutive replacements of the infected cows with uninfected heifers from within the herd resulted in herds free from the infection. In these herds the infection did not spread, despite the fact that the cows were kept in a hot and humid environment and in close contact with susceptible herd mates. The process of self-clearance is probably dependent on several factors, such as herd size, extent of animal movements within and between herds, and degree of contact (Kampa *et al.*, 2009). Small herds are more likely to support self-clearance than large herds (de Koeijer *et al.*, 2008; Kampa *et al.*, 2009). Self-clearance is probably favoured in regions with low-intensive production systems, i.e. with low levels of stress on the animals (Kampa *et al.*, 2004). Different strains of BHV-1 have a higher tendency for reactivation than others (Kampa *et al.*, 2009). Husbandry practices may affect the epidemiological pattern of BHV-1. Physical contact between animals favours the spread of the virus, and the practice of rearing youngstock separate from the dairy herd probably postponed exposure to the virus (van Nieuwstadt and Verhoeff, 1983). In addition to this, extinction depends on the reproduction ratio, the reactivation rate, and the demographic turnover in the host population (de Koeijer *et al.*, 2008). A relatively short time to extinction can only be achieved if both R_1 and R_0 are below 1, assuming that the reactivation rate is low (Mollema *et al.*, 2005; de Koeijer *et al.*, 2008). The reactivation rate of BHV-1 is generally low, being $p=0.09$ per year, as estimated by de Koeijer *et al.* (2008).

3. AIMS OF THE STUDY

The specific aims covered in this thesis were as follows:

1. Estimate the Se and Sp of the BTM ELISA test (I)
2. Estimate the herd level and within-herd prevalence of BHV-1 infection among Estonian dairy cattle (I)
3. Identify and quantify relevant risk factors for high within-herd seroprevalence in infected herds, and to clarify those factors related to exposure of youngstock to BHV-1 infection (I)
4. Clarify the role of BHV-1 in the incidence of BRD in bovines of different ages (II-III)
5. Ascertain the associations between herd BHV-1 seroprevalence and reproductive performance (III)
6. Detect the efficacy of vaccination programmes in lowering the seroprevalence of BHV-1 gE within a herd as well as to follow the dynamics of the infection in non-vaccinated herds with uninfected heifers (IV)

4. MATERIALS AND METHODS

4.1. Estonian Dairy Cattle Population

The total number of dairy farms in Estonia, according to the Estonian Agricultural and Information Board (ARIB), was about 7,000 in 2007. These herds consisted of 218,000 cattle, including 103,000 dairy cows. Three hundred and thirty-seven medium and large farms, with more than 50 cows, comprised 75% of the total dairy cattle population. In total 1,205 herds delivered their milk produced to dairy companies.

4.2. Study design

A survey estimating the prevalence, risk factors and impact on herd health of BHV-1 (I-III) was conducted between September 2006 and April 2008, and a repeated cross-sectional study (IV) lasted until autumn 2010. Information concerning the dairy cattle herds (location, number of animals) was obtained from the NAR, administered by the ARIB, which is a governmental institution within the Ministry of Agriculture of Estonia.

4.2.1. Study population to estimate the Se and Sp of the BTM ELISA test and impact of BHV-1 on herd health (I-III)

The study population that was used to estimate the Se and Sp of the BTM ELISA test and impact of BHV-1 on herd health consisted of 103 herds with 20 or more dairy cows. Based on a BTM survey conducted in 2004, a proportional number of herds of previously known infection status were selected randomly from four categories of herd size (20–99; 100–199; 200–399; ≥ 400 cows). Herds were randomly selected from the list held by the NAR. In total, 64 herds not vaccinating against IBR, with BTM samples positive for BHV-1 antibodies, and 39 BTM-negative herds, matched by herd size category, were selected for sampling. At least five BTM-negative herds were planned to be sampled in each herd size category, although this was not possible in the largest herd size category as only one non-infected herd was identified in that group.

In each of the selected herds a representative random sample of cows and youngstock older than six months was tested for BHV-1 antibodies in serum. In total, 9,637 serum samples were collected. Simultaneously with the collection of blood samples, BTM samples were collected from each milk tank from 85 herds, and these were tested for BHV-1 antibodies. Two bulk milk tanks were present in 13 farms and three herds had three milk tanks. A total of 104 BTM samples were collected.

4.2.2. Study population to estimate BHV-1 herd prevalence (I)

The source population used to estimate the herd level prevalence of BHV-1 consisted of all 1,205 market-oriented dairy herds. The BTM samples from these herds were obtained from the milk analysis laboratory of the EARC. Five dairy companies out of 20 were willing to identify individual herds, allowing us to record the herd of origin of 328 BTM samples. This enabled us to estimate the herd prevalence of BHV-1 infection in six farm size categories (<20; 20–49; 50–99; 100–199; 200–399; ≥ 400 cows).

4.2.3. Study population to estimate the within-herd prevalence and for risk factor analysis in BHV-1 infected herds (I)

The prevalence and questionnaire data from 64 infected BHV-1 dairy herds was used to estimate the within-herd prevalence and risk factors for high within-herd prevalence, and youngstock being infected in BHV-1 positive herd.

4.2.4. Study population to estimate the dynamics of BHV-1 infection in herds with and without a control programme (IV)

Control programmes to eradicate BHV-1 were devised for seven dairy herds. Descriptive characteristics of the herds, and prevalence of BHV-1 infection among cows and youngstock at the start of the control programmes, are given in Table 1 of Article IV. All the animals of at least three months old were vaccinated twice a year with inactivated BHV-1 gE marker vaccine. Rispoval IBR-Marker inactivatum (Pfizer Animal Health) (farms I, II, III, IV, V and VII) and/or Bovilis IBR

marker inac. (Intervet International) (farms V and VI) were used. In order to evaluate the efficacy of the vaccination programme, serum samples were taken from youngstock, older than six months and born after the first vaccination, and tested for BHV-1 gE antibodies. The first testing (efficacy I in Table 10) was carried out in the first year, and the second (efficacy II in Table 10) two years after the first vaccination. Animals were selected for sampling randomly. In order to monitor changes in prevalence, cross-sectional sampling was performed 1.5 years after the first vaccination in two age groups: cows and youngstock older than six months (prevalence II in Table 10).

Five BHV-1-infected dairy herds, which had uninfected heifers (prevalence up to 5%), were selected to monitor the course of the infection without control strategies. Descriptive characteristics of the herds are given in Table 2 of Article IV. Two longitudinal follow-up samplings (prevalence II and III in Table 11) were performed in order to estimate seroprevalence in cows and heifers. Intervals between the three samplings were of approximately one year. In order to demonstrate active virus circulation in non-vaccinating herds, in each follow-up sampling (prevalence II and III) BHV-1 antibody prevalence was calculated in calves born after the first sampling (prevalence I) and at least six month old at the time of testing (calves >6 month prevalence II and III in Table 11).

4.3. Collection of herd data (I-IV)

During the herd visits questionnaires were filled in to collect herd level data. The information requested included herd size, number of livestock units per farm, employment of a veterinarian and inseminator, frequency of movement of animals between housing units, participation in agricultural shows, purchase history, type of housing (cold/warm barn), housing system for cows and youngstock (loose/tied), management of youngstock (separately from cows/contact with cows for some life period/in the same barn with cows), use of bull for serving cows and heifers, breed(s) of cattle, grazing strategy for cows and/or youngstock, vaccination history, whether employees changed their clothing on the farm and information about disinfection. In addition, questions were asked relating to the history of the peak occurrence of respiratory disease, within the previous two years, of: calves up to three months, 3-16 month old heifers, cows and pregnant heifers. The number of abortions, as

well as the herd's mean insemination index for cows and heifers for the previous year, was recorded if registered on the farm. The insemination index was defined as the number of inseminations per pregnancy.

4.4. Sampling and sample analysis (I-IV)

Blood samples were collected, using disposable needles (0.9 x 38 mm), from the coccygeal vein into 9 ml vacuum tubes (Vacuette, Austria) containing a clotting activator. Serum samples were stored at room temperature for 24 hours before transport to the National Veterinary and Food Laboratory. All serum samples, and defatted milk samples, were tested for BHV-1 antibodies using a commercial BHV-1 gB ELISA test kit, HerdChek* (IDEXX, Switzerland) which has 100% sensitivity and 99.8% specificity. A herd was considered to be infected with IBR if at least one blood test showed a positive result. Suspect antibody test results (samples with a blocking percentage greater than or equal to 45% but less than 55%) were considered to be positive in the data analysis. In vaccinating herds, all serum samples were analysed for BHV-1 gE antibodies with the commercial BHV-1 gE ELISA test kit HerdChek® (IDEXX, USA), with a sensitivity of 100% and a specificity of 99.8%.

The herd BVDV status was established by testing up to 10 serum samples from randomly selected animals, at ages from six months up to age at first calving, for BVDV antibodies as recommended by Houe *et al.* (2006). This enabled detection of a minimum prevalence of 20–28% depending on herd size. The PrioCheck BVDV Ab test kit (Prionics AG, Switzerland) was used for antibody testing. A herd was considered to be infected with BVDV if at least one of the ten serum samples was positive with antibody testing.

The herd BRSV status was established by testing up to 20 (depending on herd size) randomly selected serum samples, from heifers, for BRSV antibodies to allow the detection of at least a 15% prevalence of BRSV antibody carriers in the herd. For BRSV antibodies, the Svanovir ELISA test (Svanova Biotech AB, Sweden) was used.

Depending on herd size up to 25 heifers and 10 cows were tested for *Mycoplasma bovis* antibodies in each herd. This enabled the detection of the prevalence of at least 15% among heifers and 27% among cows with

a 95% level of confidence. BIO K 260 ELISA test (Bio-X Diagnostics, Belgium) was used to measure *M. bovis* antibodies.

4.5. Data analysis

4.5.1. Estimating Se and Sp of BTM ELISA test, and evaluating risk factors for a herd seropositivity, high within-herd prevalence and seropositivity of youngstock (I)

Diagnostic results of individual blood samples collected from the farm were used as a gold standard for the calculation of Se and Sp from the BTM test. The Se and Sp of the BTM ELISA test were estimated, and confidence intervals calculated, using the “Test Evaluation” tool in the computer software Win Episcopo (Thrusfield *et al.*, 2001). The “True Prevalence” tool in Survey Toolbox (AusVet Animal Health Services, 1996), taking into account the Se and Sp of the BTM ELISA, was used to calculate the true prevalence of infected herds by the Rogan-Gladen estimator.

For the assessment of the influence of herd size as a risk factor for BTM being positive for BHV-1 antibodies, a logistic random effects model was used. Given that the 328 herds were not randomly distributed geographically, county was included as a random effect in the model. A likelihood ratio test was performed to control the significance of the contribution of random effect in the model (Dohoo *et al.*, 2009) being significant ($p=0.02$). In order to compare every size group with the next size group variable “herd size” was coded as a hierarchical dummy variable (Dohoo *et al.*, 2009).

Factors potentially related to the within-herd prevalence and seropositivity of youngstock were analyzed using univariable and multivariable logistic regression. For this, the within-herd prevalence was dichotomized at 50%, based on its frequency distribution (Figure 1), while the youngstock population was considered positive when one or more animals in that group tested positive.

4.5.2. Description of the health models and categorization of variables (II-III)

In Model I the dependent variables were related to the history of respiratory disease incidence in calves of up to three months old (called Model I). In Model II the aim was to clarify the risk factors for a high prevalence of respiratory disease in 3–16 month old heifers. Four dependent variables (general disease, nasal discharge, signs of respiratory distress, and lacrimation) were used. On each farm the veterinarian, or farm manager, was questioned about the occurrence of clinical signs of respiratory disease, including general signs (fever, inappetence, dullness), nasal discharge (“red nose”), respiratory distress (cough, dyspnoea), and lacrimation, in two age groups (calves 0–3 months old and youngstock 3–16 months old). The respondents were asked to evaluate the prevalence of the signs described above among animals of each age group during periods of the highest incidence of respiratory disease within the previous two years. The scales used in the estimation were as follows: 1 – no signs or only single cases; 2 – up to 10%; 3 – 10–30%; 4 – over 30%. All four dependent variables in each model were dichotomized. The 10% cut-off point was used for 0–3 month old calves, while for older youngstock the herd was considered to be in the “high frequency” group if anything more than just a single animal showed the mentioned signs concurrently. Descriptive characteristics of the variables included in the models are given in Table 2 of Article II.

The aim of the third model (Model III) was to clarify the association between herd BHV-1 seroprevalence and respiratory disease occurrence in adult dairy cattle (>16 months), as well as the detection of management factors associated with higher BRD occurrence. The respondents in the farm were asked to evaluate the occurrence of clinical signs of respiratory disease, including nasal discharge (“red nose”), respiratory signs (cough, dyspnoea), and lacrimation prevalence among animals of the age group during periods of the highest occurrence of respiratory disease within the previous two years. In order to dichotomize the outcome variables, the value of the outcome variables was taken as 1 if anything more than just a single animal showed the signs concurrently.

As all the respiratory disease symptoms were highly clustered in MCA analysis, one summary variable, describing the level of occurrence of respiratory disease, was created for use in logistic regression analysis. The herd was considered to be in a group of “high frequency of BRD

in calves” (BRDCAL=1), if at least two of the four variables indicating respiratory disease signs among calves were in the “high frequency” category. For older youngstock three variables (GENHEF, RESHEF, NASHEF) were used to create the summary variable “BRD in heifers” (BRDHEF). Lacrimation was excluded, as it was rarely seen among older youngstock (only eight herds belonged to high frequency group LACHEF=1). For heifers, if at least two out of three variables had a value of one, the herd was considered to be in the category of “high frequency of BRD in heifers” (BRDHEF=1). Three variables (RESCOW, NASCOW and LACCOW) were used to create one summary variable in Model III. If at least two out of three variables had a value of one, the herd was considered to be in the category of “high occurrence of BRD in cows and/or pregnant heifers” (BRDCOW=1).

The aim of the fourth model (Model IV) was to detect the linkage between herd BHV-1 seroprevalence and poor reproductive performance in cows and heifers, by taking into account the effect of a possible confounding effect of herd size and other infectious diseases. For this, two outcome variables, dichotomized at their median value (1.3% for the proportion of abortions and 1.9 for insemination index), were used in the logistic regression analysis. Descriptive characteristics of the variables included in the model III and IV are given in Table 2 of Article III.

Multiple correspondence analysis (MCA) was used to obtain an overall view of the associations among variable categories in Models I, II and III, and to avoid problems arising due to multicollinearity. MCA was performed using XLSTAT (Version 2010.4.01; Addinsoft). Logistic regression models were built to quantify estimates of the relationships among outcome and predictor variables by using STATA 11 software (Stata Corporation, Texas, USA).

4.5.3. Models for evaluating BHV-1 infection dynamics in herds with and without a control programme (IV)

Binomial generalised linear models were constructed to estimate the differences between prevalence estimates within farms. For this purpose a three times interaction term between year, farm and animal group (cows/heifers) was included in the models. Multiple comparisons between three prevalence estimates in non-vaccination herds were accomplished with a contrast matrix (Tukey’s all-pair comparisons). In order to evaluate overall

BHV-1 prevalence changes between sampling times in vaccination and non-vaccination herds, one model for each of the datasets was composed to calculate overall estimates for all study herds in both groups. For this purpose interaction terms between animal group (heifers/cows) and year, as well as animal group and farm, were included in the model. R version 2.13.0 (The R Foundation for Statistical Computing) was used for model building.

5. RESULTS

5.1. Se and Sp of the BTM ELISA test (I)

The sensitivity of the ELISA test to detect infected herds using BTM samples was estimated to be 76.5% (95% confidence interval (CI) = 66.4, 86.5) and the specificity 97.2% (95% CI = 91.8, 100). The Spearman correlation coefficient between within-herd prevalence and the blocking percent was 0.89. One herd with a prevalence among cows as low as four per cent (95% CI= 0.5, 8.5%) was detected as positive in the BTM sample. However, a herd with a prevalence among cows of 64% (95% CI= 53.2, 74.8%) was tested negative by BTM ELISA.

5.2. BHV-1 herd prevalence and herd size as risk factor (I)

Nineteen percent of BTM samples collected from the EARC milk analysis laboratory were positive for BHV-1 antibodies. The true prevalence was calculated as 22.0% (95% CI= 20.7, 23.3). The herds providing the 328 BTM samples used for the assessment of herd prevalence originated from all 15 counties in Estonia (samples per counties ranged from 1-118, median 13). The size of the target population, and the proportion of study samples in the different herd size categories, as well as herd prevalence and significance of differences between prevalence of different herd size categories are presented in Table 2.

Table 2. Number of Estonian dairy herds, study sample size and bovine herpesvirus 1 herd prevalence based on bulk milk antibody ELISA results from 328 Estonian dairy herds with respect to herd size, with comparison between categories

Herd size	Size of target population	Study sample (n)	Study sample (%)	Herd prevalence (%)
<20	6,390	118	1.8	3.4 ^a
20-49	255	90	35.3	4.4 ^a
50-99	110	46	41.8	10.9 ^a
100-199	85	31	36.5	41.9 ^b
200-399	83	29	34.9	58.6 ^{bc}
≥400	59	14	23.7	85.7 ^c
Total	6,982	328	4.7	16.8 ¹

^{a, b, c} different superscript letters indicate statistically significant ($p < 0.05$) differences in prevalence between herd size groups, from random effects logistic regression analysis

¹ Herd prevalence based on 328 identified herds

5.3. Within-herd prevalence of BHV-1 (I)

The frequency distribution of BHV-1 seroprevalence over herds is shown in Figure 1.

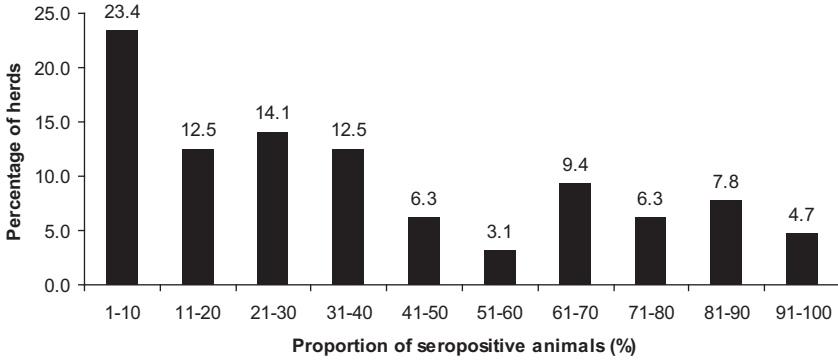


Figure 1. Distribution of the within-herd prevalence of BHV-1 in 64 Estonian dairy herds

In 31.3% of infected herds ($n=20$), total within-herd prevalence was more than 50%. Data describing the within-herd prevalence in different herd size categories are presented in Table 3. The mean seroprevalence in cows was more than twice as high as that in youngstock in every herd size category (Table 3).

Table 3. Within-herd prevalence of bovine herpesvirus 1 in 64 dairy cattle herds of different size categories in Estonia

Herd size	Herds (n)	Prevalence (%)	Prevalence (%)	Prevalence (%)
		Median (range) Mean	(cows) Median (range) Mean	(youngstock) Median (range) Mean
20–99	15	13 (2-76)	28 (3-98)	0 (0-63)
		26.1	34.5	9.4
100–199	14	29 (1-92)	50 (2-96)	2 (0-88)
		32.1	46.1	16.6
200–399	18	34.5 (2-100)	64 (4-100)	5 (0-100)
		39.1	57.1	20.3
≥400	17	56 (1-91)	84 (2-100)	9 (0-91)
		51.2	70.1	31.6
Total	64	31.5 (1-100)	57.5 (2-100)	5 (0-100)
		37.8	52.8	20.1

A high prevalence of infection (>50%) among youngstock was observed in 11 out of 63 herds (in one herd it was not possible to obtain samples from the youngstock) whereas among cows it was detected in 35 out of 64 infected herds. In 71.4% (n=45) of the infected herds the prevalence among youngstock was <20%, and in 36.5% (n=23) of the infected herds all young animals tested negative.

5.4. Risk factor analysis (I)

The overall distribution of within-herd prevalence in our sample population divided herds into two subgroups, with the division at approximately 50% (Figure 1). Results of multivariable logistic regression analysis are given in Table 4.

Table 4. Fixed effects logistic regression model for risk factors for high bovine herpesvirus 1 prevalence (>50%) within infected herds (n=59)

Risk factor	Category	Herds (n)	Prevalence >50% n (% of herds)	OR	p-value	95% CI
Veterinarian employee of the farm	Yes	21	12 (57.1)	6.05	0.03	1.19, 30.62
	No	38	7 (18.4)		-	
Inseminator working only for particular farm	Yes	19	10 (52.6)	5.54	0.04	1.10, 27.91
	No	40	9 (22.5)		-	
BVDV present in herd	Yes	14	8 (57.1)	7.27	0.03	1.24, 42.74
	No	45	11 (24.4)		-	
Herd size						
	20–99	12	3 (25)	1	-	-
	100–199	13	2 (15.4)	0.15	0.14	0.01, 1.85
	200–399	18	5 (27.8)	0.26	0.22	0.03, 2.23
	≥400	16	9 (56.3)	0.23	0.24	0.02, 2.74

^a herd size p=0.495 in multiple Wald test

In the risk factor model for youngstock infected with BHV-1 in an infected herd three risk factors remained in the final model (Table 5).

Table 5. Fixed effects logistic regression model for risk factors for heifers being infected with bovine herpesvirus 1 in infected herds (n=59)

Risk factor	Category	Herds (n)	Heifers infected n (% of herds)	OR	p-value	95% CI
Inseminator working only for particular farm	Yes	19	16 (84.2)	5.8	0.03	1.14, 29.43
	No	40	22 (55.0)			
BVDV present in herd	Yes	14	12 (85.7)	6.5	0.05	0.97, 43.33
	No	45	26 (57.8)			
Herd size						
	0–99	12	5 (41.7)	1	-	-
	100–199	13	7 (53.8)	1.35	0.73	0.25, 7.31
	200–399	18	14 (77.7)	3.02	0.21	0.55, 16.74
	≥400	16	12 (75.0)	1.02	0.99	0.13, 7.62

^a herd size p=0.544 in multiple Wald test

5.5. Risk factors for high occurrence of respiratory disease symptoms in calves and pre-breeding heifers (I)

A high frequency of respiratory disease in calves was associated with a high prevalence of BHV-1 among cows (test value 6.72), the presence of BHV-1 among youngstock (6.10), BRSV prevalence >50% (4.01) and the presence of BVDV in a herd (2.89) in MCA. According to the results of the MCA, several management-related variables, such as the veterinarian (test value 6.79) and inseminator (6.89) being employees of the farm, the inseminator not providing a service to any other farms (4.16) and keeping youngstock separately from cows from six months until pregnancy (4.85) were related to a high prevalence of respiratory disease in calves (Figure 2).

In general, the highest herd size category was related to a higher disease incidence of respiratory signs from the results of the logistic regression analysis (Table 6) and MCA (test value 6.53) (Figure 2).

The results of the logistic regression model are presented in Table 6.

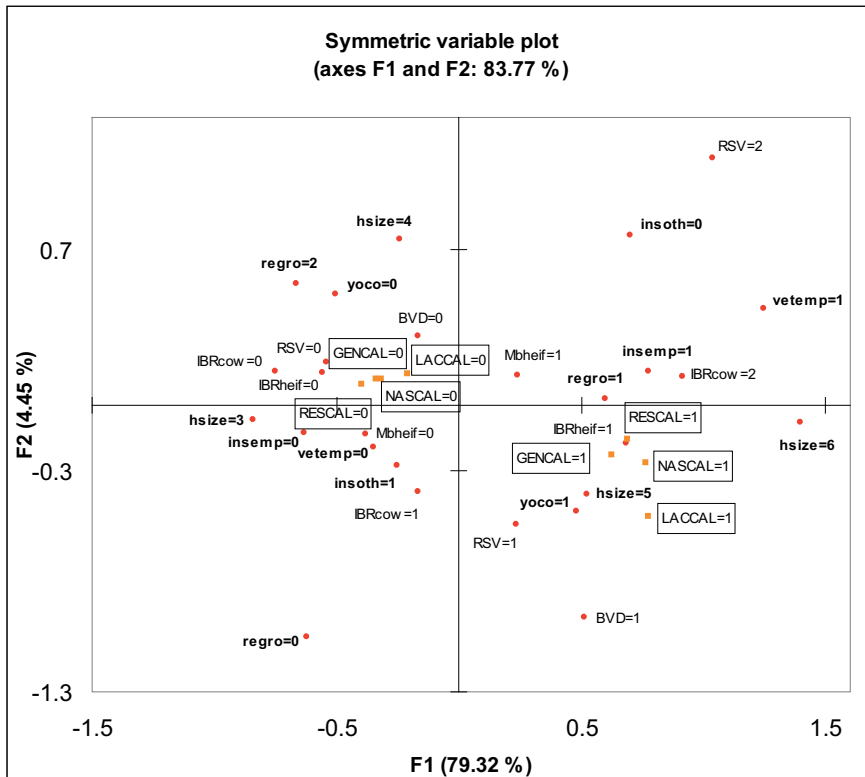


Figure 2. Graphical display of Multiple Correspondence Analysis, with respect to axes 1 and 2 for high incidence of respiratory disease symptoms in calves up to three months old (99 herds).

Table 6. Results of logistic regression analysis for risk factors for high occurrence of respiratory disease in calves up to three months old (99 herds)

Risk factor	Herds (n)	OR	p-value	95% CI
BHV-1 prevalence in cows ^a				
0	38	1	-	-
1-49%	26	14.8	0.005	2.3; 95.5
≥50%	35	19.2	0.002	3.0; 121.8
BVDV in heifers				
0	75	1	-	-
1	24	5.1	0.020	1.3; 20.1
Herd size ^b				
20-99 cows	40	1	-	-
100-199 cows	18	1.7	0.557	0.3; 9.3
200-399 cows	23	8.0	0.008	1.7; 37.3
≥400 cows	18	6.4	0.029	1.2; 33.8

^aBHV-1 prevalence in cows p=0.006 in multiple Wald test

^bHerd size p=0.021 in multiple Wald test

A high prevalence of BHV-1 among cows (test value 6.44) and the presence of BHV-1 among heifers (6.28), positive status for BVDV (3.88) and high prevalence of BRSV (2.98) were all related to a high incidence of respiratory symptoms among older youngstock, according to the MCA. Management factors such as the veterinarian (6.36) and inseminator (6.35) being the employees of the farm, keeping youngstock separately from cows from six months until pregnancy (5.01) and purchasing animals (3.95), were related to a high incidence of respiratory disease in heifers from the results of the MC analysis. In each larger herd size category, the risk of having a high incidence of respiratory disease increased approximately twice with OR=10.2 (CI 1.6; 64.3) in the largest herd size category (test value 6.78 in MCA).

From the logistic regression analysis, only the BVD was significantly associated with a high incidence of respiratory disease among pre-breeding heifers (OR=4.3, CI 1.2; 15.8) (Table 7).

Table 7. Results of logistic regression analysis for risk factors for high occurrence of respiratory disease in heifers three to sixteen months old (99 herds)

Risk factor	Herds (n)	OR	p-value	95% CI
BVDV in heifers				
0	76	1	-	
1	23	4.3	0.027	1.2; 15.8
Herd size ^a				
20-99	40	1	-	-
100-199	19	4.9	0.113	0.7; 34.4
200-399	23	5.3	0.065	0.9; 31.6
≥400	17	8.1	0.022	1.4; 49.1

^aHerd size p=0.137 in multiple Wald test

5.6. Risk factors for a high occurrence of respiratory disease symptoms in cows and pregnant heifers (III)

According to the MCA, the management-related variables significantly related to the high occurrence of respiratory disease symptoms, were the largest herd size category (test value 7.35), loose housing of cows (6.20), the veterinarian (6.61) and inseminator (6.60) being the employees of the farm and the latter not providing a service to other farms (4.21), keeping youngstock separately from cows from 6 months until pregnancy (4.77), and purchasing new animals for the herd (4.51). Infections that were related to a high occurrence of respiratory disease signs in cows and pregnant heifers were: a high prevalence of BHV-1 among cows (6.54), the presence of BHV-1 among heifers (6.15), the presence of BVDV in a herd (3.95), and a high prevalence of BRSV (3.74) (Figure 3).

5.7. BHV-1 as a risk factor for a poor reproduction performance (III)

The results of the logistic regression analysis indicated that, in herds in which BHV-1 is present, among cows the incidence of abortions and the insemination index were higher than those in herds that were negative for BHV-1 (Table 9).

Table 9. Results of logistic regression analysis for risk factors for high abortion and insemination index in cows and heifers (77 herds)

Risk factor	Herds (n)	abortion			insemination index		
		OR	p-value	95% CI	OR	p-value	95% CI
BHV-1 prevalence in cows ^a							
0	25	1	-	-	1	-	-
1-49%	24	7.3	0.003	2.0; 26.9	5.2	0.01	1.5; 18.4
≥50%	28	4.6	0.022	1.2; 16.7	3.4	0.056	1.0; 12.3
Herd size ^b							
20-99 cows	25	1	-	-	1	-	-
100-199 cows	17	1.2	0.754	0.3; 5.0	0.8	0.725	0.2; 3.0
200-399 cows	21	1.7	0.447	0.4; 6.5	1.2	0.83	0.3; 4.2
≥400 cows	14	0.3	0.165	0.1; 1.6	0.9	0.944	0.2; 4.2

^a BHV-1 in cows p=0.009, the Wald test

^a BHV-1 cows p=0.033, the Wald test

^b Herd size p=0.193, the Wald test

^b Herd size p=0.957, the Wald test

5.8. Changes in BHV-1 seroprevalence in vaccinating and non-vaccinating herds (IV)

The results of the BHV-1 gE antibody tests in vaccinating herds are given in Table 10 and the results of the BHV-1 gB antibody testings in non-vaccinating herds are given in Table 11.

Table 10. Proportion of animals positive to bovine herpesvirus type 1 (BHV-1) gE antibodies with 95% CI in vaccinating herds, and the odds of an animal being positive to BHV-1 antibodies in seven test herds compared to prevalence I.

Age group and sampling time	Herds % (95% CI) # posit./all							Overall	OR (95% CI)
	I	II	III	IV	V	VI	VII		
Cows									
Prevalence I	90 (79-96) 54/60	84 (77-91) 98/116	89 (79-96) 58/65	95 (90-97) 172/182	97 (88-100) 58/60	98 (94-100) 120/122	67 (54-78) 42/63	90 (88-92) 602/668	1
Prevalence II	98 (91-100) 61/62	86 (76-94) 57/66	78 (67-88) 51/65	75 (63-85)** 51/68	75 (64-85)* 55/73	98 (91-100) 59/60	22 (12-34)** 13/60	76 (72-80) 347/454	0.3 (0.2; 0.5)**
Heifers									
Prevalence I	38 (24-57) 22/58	24 (16-34) 24/98	91 (81-97) 59/65	89 (84-93) 211/237	60 (47-72) 36/60	44 (32-57) 31/70	2 (0-6) 2/117	55 (51-58) 385/705	1
Prevalence II	1 (0-7)** 1/73	13 (6-23) 9/70	1 (0-7)** 1/80	13 (7-22)** 12/90	4 (1-9)** 4/110	9 (5-16)** 11/120	0 (0-6) 0/60	6 (4-9) 38/603	0.03 (0.02; 0.05)**
Calves >6 months									
Efficacy I	0 (0-7 ^a) 0/48	0 (0-18 ^a) 0/19	0 (0-8 ^a) 0/45	0 (0-14 ^a) 0/25	0 (0-6 ^a) 0/58	0 (0-7 ^a) 0/52	0 (0-9 ^a) 0/41	0 (0-1 ^a) 0/288	
Efficacy II	7 (2-18) 4/54	0 (0-5 ^a) 0/70	0 (0-7 ^a) 0/52	0 (0-6 ^a) 0/60	0 (0-5 ^a) 0/76	0 (0-4 ^a) 0/83	0 (0-7 ^a) 0/51	1 (0.2-2) 4/446	

Prevalence studies are performed at 1.5 year intervals, efficacy is evaluated at one year intervals

* p<0.05 compared to last prevalence estimation in the same category

** p<0.001 compared to last prevalence estimation in the same category

^a one-sided, 97.5% confidence interval

Table 11. Proportion of animals positive to bovine herpesvirus (BHV-1) gB antibodies with 95% CI in nonvaccinating herds and the odds of an animal being positive to BHV-1 antibodies in five test herds compared to prevalence I.

Age group and sampling time	Herds % (95% CI) # posit./all							OR (95% CI)
	VIII	IX	X	XI	XII	Overall		
Cows								
Prevalence I	71 (58-82) 41/58	48 (38-59) 44/91	58 (43-72) 29/50	57 (43-70) 32/56	24 (7-50) 4/17	55 (49-61) 150/272	1	
Prevalence II	53 (39-66) 31/59	34 (20-50) 15/44	86 (73-95)* 38/44	41 (29-55) 24/58	24 (13-38) 12/50	47 (41-53) 120/255	0.8 (0.5; 1.1)	
Prevalence III	64 (51-76) 38/59	21 (10-35)* 10/48	98 (87-100)* 39/40	34 (22-47)* 20/59	0 (0-8) ^c 0/50	42 (36-48) 107/256	0.6 (0.4; 0.9)* ^a	
Heifers								
Prevalence I	3 (0-10) 2/69	0 (0-14) ^c 0/27	4 (0-14) 2/52	0 (0-6) ^c 0/62	0 (0-10) ^c 0/38	2 (0.4-4) 4/248	1	
Prevalence II	2 (0-9) 1/60	2 (0-12) 1/43	24 (13-38)* 12/50	5 (1-14) 3/60	4 (1-15) 2/45	7 (4-11) 19/258	6.6 (2.1; 20.6)* ^a	
Prevalence III	0 (0-7) ^c 0/60	0 (0-8) ^c 0/43	68 (53-80)** ^{ab} 34/50	0 (0-9) ^c 0/40	0 (0-8) ^c 0/50	14 (10-19) 34/243	17.2 (5.6; 52.8)** ^a	
Calves >6 months								
Prevalence II	0 (0-71) ^c 0/3	0 (0-26) ^c 0/12	21 (5-51) 3/14	14 (0.4-58) 1/7	0 (0-16) ^c 0/21	7 (2-17) 4/57		
Prevalence III	0 (0-11) ^c 0/33	0 (0-10) ^c 0/34	68 (53-80) 34/50	0 (0-13) ^c 0/27	0 (0-8) ^c 0/45	18 (13-24) 34/189		

Prevalence studies are performed at one year intervals

* p<0.05 compared to last prevalence estimation in the same category

** p<0.001 compared to last prevalence estimation in the same category

^a difference between the first and third sampling

^b difference between the second and third sampling

^c one-sided, 97.5% confidence interval

6. DISCUSSION

6.1. Se and Sp of the BTM ELISA test (I)

The ELISA test used for the analysis of the BTM samples in this study was not sufficiently sensitive to detect all infected herds – 76.5% of the herds recorded positive from blood testing also had a positive BTM test result. Only one herd recorded negative from blood testing had a positive BTM test result leading to specificity of 97.2%. Nylin et al. (2000) estimated the sensitivity and specificity of the BTM test to be 82% and 100% respectively, with a cut-off value of 30% blocking reaction. The lower sensitivity in this study can partly be explained by the difference in the blocking reaction cut-off which, according to the manufacturer's instructions, was higher in our test. When lowering the cut-off to 30% of blocking reaction in this study the sensitivity of the BTM ELISA test increased to 84.6%, with only a minimal decrease in specificity (97.0%).

The correlation between BHV-1 within-herd prevalence among cows and BTM ELISA blocking percentage was 0.89. A similar result was found in a study by Hartman et al. (1997) where the correlation was 0.86; however a correlation of 0.59 has been reported by Nylin et al. (2000). Differences between these correlations might be due to diversity in the distribution of within-herd prevalence of BHV-1 among different cow populations, the study design (sample size in respect to number of selected herds and animals within herds as well as time interval between collection of BTM and individual samples), the ELISA test and the evaluation of the results, as well as laboratory conditions.

6.2. BHV-1 herd prevalence and herd size as risk factor (I)

The results of this survey indicate that almost a quarter of Estonian market-oriented dairy cattle herds are infected with BHV-1. In a study from 1993–1995 the observed overall herd prevalence was 43.4%. As no systematic control of the infection has been conducted in these herds for twelve years, this substantial decrease in herd prevalence is an indication of self-clearance from BHV-1 infection, particularly in small herds (herd prevalence was 27.6% and 50.0% in herds with <50 and 50-99 cows respectively in the previous study). However self-clearance has not taken place in the larger sized groups of Estonian dairy herds, where the herd

prevalence has remained high through all these years. One hypothetical factor contributing to the self-clearance may be the eradication of the infection from AI centres by the end of 1990s. Secondly, it has been speculated that, in smaller herds, the infection may not be maintained because the number of susceptible animals is smaller throughout the year (Boelaert *et al.*, 2005). In larger herds a continuous influx of a relatively large number of new, susceptible animals predisposes virus circulation.

Herd prevalence of BHV-1 was generally higher in larger herds. As no other herd factors beside “herd size” and geographical location were recorded it was not possible to discover the factors behind that trend. Larger herd size has been found to be a risk factor for BHV-1 herd-seropositivity in previous studies (Van Wuijckhuise *et al.*, 1998; Tekes *et al.*, 1999). Large farms are more likely to have disease introduced, due to the more frequent purchase of new animals (van Schaik *et al.*, 1998b; Van Wuijckhuise *et al.*, 1998) and the tendency to have more visitors (van Schaik *et al.*, 1998b).

6.3. Within-herd prevalence of BHV-1 (I)

In this study the within-herd prevalence was higher on average in larger herds, however the variation within herd size categories was large and the differences are not statistically significant. Seroprevalence among youngstock was lower than that in cows, which is consistent with findings from other studies (Kampa *et al.*, 2004; Guarino *et al.*, 2008; Jacevičius *et al.*, 2008). In more than one-third of infected farms, young animals were seronegative. Only in a few herds (17.5%) was the seroprevalence among youngstock high (>50%). The respiratory subtype BHV-1 is excreted in high titres in nasal secretions, and spreads more efficiently than the other subtypes (Wentink *et al.*, 1993). Genital infection is a problem of breeding animals (Guarino *et al.*, 2008). The viral isolates characterised in the 1990s in Estonia belonged to subtype BHV-1.1 (Saar, 1999). The results of the present study indicate that genital infections might be relevant in the spread of the infection, however the use of a bull for natural mating in cows or heifers was not related to higher within-herd prevalence in the risk factor study. Further studies to clarify the role of different subtypes in the epidemiology of the infection are needed.

6.4. Risk factor analysis (I)

It was found that the presence of BVDV is associated with a higher within-herd seroprevalence and presence of BHV-1 among youngstock. A strong association between a herd concurrently being BVDV and BHV-1 antibody-positive has also been found in other studies (Paton *et al.*, 1998; Kampa *et al.*, 2004). A more effective spread of BHV-1 within an animal infected with BVDV has been demonstrated by Potgieter *et al.* (1984, 1995). Infection with BVDV is known to be immunosuppressive and, particularly in young animals, it is assumed to be a predisposing factor for other viral or bacterial diseases, including BHV-1 (Potgieter, 1995). Regardless of these assumptions it is not possible to make exact cause and effect inferences from these findings due to the cross-sectional study design.

The results of the model also highlighted the significance of indirect iatrogenic transmission of the virus *via* veterinary and insemination equipment, clothes and hands. It is assumed that when the veterinarian and inseminator are employees of the farm, there is a tendency to handle animals more frequently for diagnostic purposes, invasive treatments and heat detection compared to those where these professionals visit the farm on call.

6.5. BHV-1 as a risk factor for high occurrence of respiratory disease and poor reproduction performance (II-III)

BHV-1, BVDV and BRSV were related to a high incidence of respiratory disease in dairy youngstock, according to the results of MCA. The presence of BHV-1 in cows, and BVDV found in a herd, were significantly related to signs of respiratory disease in calves, whereas BRSV was an insignificant factor according to logistic regression analysis. The proportion of BHV-1 seropositive cows reflected the ratio of calves that might receive protective BHV-1 antibodies *via* the colostrum (Mechor *et al.*, 1987). According to the results of this study respiratory disease was observed in calves despite a presumably high level of maternal antibody protection. However, because the occurrence of respiratory disease signs was evaluated retrospectively, herd immunity might have been low during that period, whereas at the time of testing a high prevalence of antibodies might have been the consequence of a recent virus circulation in the herd. On the other

hand, according to the author's experience, calves are rarely provided with adequate colostrum in Estonia. Penny *et al.* (2002) indicated that respiratory disease is more often seen in calves of primiparous cows, probably because of a lack of previous exposure to BHV-1 by their dams. Therefore a number of calves may contract the disease caused by BHV-1 as a result of weak maternal immunity.

BVDV was significantly associated with high respiratory disease incidence in calves up to three months old, as well as in 3–16-month-old heifers, according to logistic regression analysis. In endemically infected herds, young animals losing their maternal immunity are the most susceptible to the virus, and are therefore most prone to experience clinical disease caused by BVDV. BVDV infection in the herd can be a significant risk factor for respiratory disease in calves up to 90 days of age according to Lundborg *et al.* (2005). BVD infection may directly result in respiratory disease, but evidence suggests that BVDV infections potentiate BRD *via* immunosuppression and synergism (Ridpath, 2010).

BHV-1 was not significantly associated with a high incidence of BRD among 3-16 month old heifers in logistic regression analysis. A positive association between a high occurrence of respiratory disease signs and a high prevalence of BHV-1 among cows, as well as the presence of BHV-1 among heifers, was detected with MCA. Although BHV-1 is probably not related to BRD outbreaks in pre-breeding heifers, this cannot be excluded as one of the possible factors in the respiratory disease complex.

BHV-1, BVDV and BRSV are associated with a high occurrence of respiratory disease in Estonian adult dairy cattle, according to the results of the MCA. A high prevalence of BRSV ($\geq 50\%$) was associated with a high occurrence of respiratory disease symptoms in cows and pregnant heifers in the MCA. When combining these three BRD symptoms into one outcome variable in logistic regression analysis, a low to moderate prevalence of BRSV (1-49%) among youngstock was significantly associated with a high occurrence of respiratory disease among cows and pregnant heifers. Sampling antibodies from a small number of young animals that had lost maternal immunity indicates the recent spread of infection (Ohlson *et al.*, 2010). However, some studies have shown that outbreaks of acute respiratory disease associated with BRSV in fully susceptible populations affect adult cattle, pregnant or newly calved cows most severely (Elvander, 1996; Norström *et al.*, 2000). Thereafter the disease remains endemic, manifesting itself among younger animals that

serve as sentinels (Elvander, 1996). Given that the signs of respiratory disease reported in this study were those associated with the occurrence of respiratory disease in the previous two years, cows and pregnant heifers might have experienced disease caused by BRSV at some time previously, following the active spread of the virus among youngstock detected in this study at the time of testing. In a severe outbreak of BRSV in Sweden it was found that concurrent infection with other viruses may affect the expression of disease (Elvander, 1996). In addition to BRSV, BHV-1 and BVDV were associated with a higher occurrence of BRD in MCA. As associations between variables are not adjusted for the effects of other variables, with this method it is not possible to state that BHV-1 and BVDV are direct risk factors for BRD. However, an apparent bivariate association between these variables provides a reason to suggest that BHV-1 and BVDV may participate in the expression of BRD as contributing agents.

BHV-1 increases the risk of a herd having a poor reproductive performance. We can suppose that in herds with a moderate BHV-1 seroprevalence among cows, the level of infection has been low for some time, which enables a susceptible population to evolve, and it is these herds that are the most vulnerable to active virus spread and a higher level of endemic abortions. Abortions due to BHV-1 generally occur between four and eight months of gestation, however the infection can also result in early embryonic death (Givens and Marley, 2008), resulting in a higher insemination index. As reproduction values were registered retrospectively, and present antibodies to BHV-1 reflect virus spread in the past, it is not possible to deduce exact cause-effect relationships. Therefore it is possible that poor fertility, as well as the spread of BHV-1, is influenced by another common factor, e.g. poor management practice. A more thorough study involving farm management practices in addition to infections should be conducted.

The association between BHV-1 and the fertility of cows and heifers has been evaluated previously. In field studies, where the course of BHV-1 infection in previously naive herds was recorded, neither an increase in abortion incidence nor a lower proportion of successful inseminations was found (Cook, 1998; Hage *et al.*, 1998b; Pritchard *et al.*, 2003). The impact of BHV-1 on reproduction performance has also been evaluated indirectly. No associations between the proportion of calves with antibodies against IBR virus and the incidence of abortions, stillbirths, calf death, nor non-pregnancy were found (Waldner and Kennedy, 2008). However a

17-day longer period for successful conception was needed for BHV-1 seropositive rather than seronegative cows (Ata *et al.*, 2006). Differences in the results between studies may arise from differences in study design and discrepancies in other herd characteristics, as well as the BHV-1 strain involved.

6.6. Herd management-related risk factors for the high occurrence of respiratory disease signs (II-III)

Large herd size has been found to be a risk factor for a high incidence of respiratory disease in many studies (Norström *et al.*, 2000; Gay and Barnouin, 2009; Gulliksen *et al.*, 2009). Any infectious agent will establish itself more easily in a large herd because of higher animal density, a greater degree of direct contact between animals, and a larger number of susceptible animals (Gay and Barnouin, 2009; Gulliksen *et al.*, 2009). Large herd size, as well as the presence of BRD, is also associated with increased intra-farm traffic of professional employees such as veterinarians and AI-technicians, as confirmed previously (Norström *et al.*, 2000; Gulliksen *et al.*, 2009). This emphasizes the importance of human-mediated virus spread. An interesting finding was that the housing management for youngstock (loose or tied) was not related to disease incidence in dairy youngstock. This indicates that direct contact is not always obligatory for effective viral spread. Housing youngstock in a separate building from six months until pregnancy also increased the risk of BRD in older youngstock. This may be the effect of relocation and mixing of animals with different infection and immune statuses on the incidence of BRD. This finding also indicates that direct contact with the adult cow population is not always necessary for the maintenance of infection among youngstock.

Loose housing of cows was associated with a higher level of BRD in cows and pregnant heifers. It is suggested that more direct contacts between the animals, and the frequent regrouping of animals in loose housing barns, create greater possibilities for the direct transmission of the infectious agents over the whole farm. Housing youngstock in a separate building, from six months of age until service, was associated with a higher occurrence of BRD. In order to maintain an immunizing infection, the susceptible pool must be replenished *via* recruitment (Keeling and Rohani, 2008). Depending on the pattern of infectious disease epidemiology

within the herd, commingling animals with different immunity status to specific infections may predispose the active circulation of the virus. Newly purchased animals can be a source of BRSV infection, which was confirmed in a Swedish study, in which outbreaks of BRSV occurred most often after the introduction of purchased animals (Elvander, 1996).

6.7. Changes in BHV-1 gE seroprevalence in vaccination herds (IV)

A vaccine intended for the eradication of an infection should effectively stop the circulation of the virus within the herd *via* the reduction of the excretion of the field virus, and reduction of susceptibility of the animals to the infection (Makoschey *et al.*, 2007). The positive effect of a vaccination programme is most obvious among heifers, expressed in the decrease of BHV-1 gE seropositive animals in that age group from 55% (95% CI=51-58) to 6% (95% CI=4-9), suggesting that vaccinating cattle twice a year with a commercial marker vaccine keeps the subsequent generations free of infection (Hage *et al.*, 2003; Vilmos *et al.*, 2007; Rypula *et al.*, 2010). Two years after the beginning of the vaccination programme, gE seropositive animals in this age group were found in only one out of seven study herds (herd I). In this herd deviation from the recommended vaccination scheme took place. In the second year of the programme the farmer decided to begin the vaccination of young-stock from six months of age instead of three, as recommended, to reduce the costs to himself of the programme. Conventionally produced inactivated BHV-1 vaccines vary in the quality of protection and the reduction of virus shedding that they afford (Patel, 2005b). In general, maternal immunity should provide sufficient protection during the first month of life, and vaccination is typically started at the age of three months (Makoschey *et al.*, 2007). Increasing the age at first vaccination leads to a higher number of the susceptible population, enabling the spread of infection in the herd. Seroconversions to BHV-1 were also found in a Polish study, where the beginning of the vaccination of youngstock was moved to the eighth or ninth month of life (Rypula *et al.*, 2010).

Mean BHV-1 prevalence among cows for all seven study herds decreased significantly from 90% (95% CI=88-92) to 76% (95% CI=72-80), $p < 0,001$. However, a significant decrease was observed in only three of the study herds (herds IV, V and VII). The probability of seropositivity to BHV-1

increases with age (Woodbine *et al.*, 2009), and the prevalence among cows can remain high for a couple of years, until the virus-negative replacement heifers substitute for the older infected animals. On the other hand, despite vaccination, circulation of the virus may continue to some extent, whereas the probability of the infection is higher in the cow population, and prevalence among cows will not be reduced within the expected time. In this vaccination study the replacement rate of cows in the herds was 25-30% per year. This means that the cow population was replaced in approximately four years. However, there are always some animals, including infected individuals, that remain in the herd for a longer period, and this adds a couple of years to the time needed for the clearance from infection.

It has been suggested that, in order to keep the reproduction ratio below one, appropriate management practices will have to be included in the BHV-1 eradication programme, i.e. no contact with animals that are not known to be BHV-1 free, culling of animals that are seropositive for the BHV-1 field virus, and hygienic measures for visitors (Hage *et al.*, 1996). All vaccination study herds in the current study were large dairy herds, with more than 400 dairy cows, with high milk yields (above 7,000 kg per cow annually). A higher rate of seroconversion for BHV-1 was found in larger herds by Segura-Correa *et al.* (2010). This may be related to several predisposing management-related factors. According to the first study described in this thesis, the herd-level risk factors were the veterinarian and inseminator, who were employees of the farm, and BVDV was present on the farm, which were related to high within-herd prevalence and BHV-1-infected youngstock. Other characteristics, presented in Table 1 of Article IV, may increase management-related stress for animals, leading to higher susceptibility and a higher probability of infection due to possible reactivation of the virus in latently infected animals; however, a more targeted study is needed to confirm this. Despite this, on the basis of the results of previous studies (Van Schaik *et al.*, 1999a; Hage *et al.*, 2003), control over population density and the use of within-herd biosecurity measures, as well as restraining other immunosuppressive diseases, can improve the outcome of a control programme.

6.8. Changes in BHV-1 prevalence in non-vaccination herds (IV)

The results of this study indicate that the prevalence of BHV-1 gB antibodies among cows decreased slowly in most of the study herds. The probability of a cow being infected is, on average, much lower in a vaccination herd than in a non-vaccination herd, as indicated by a smaller OR and a narrower confidence interval (Table 10 and 11). However, caution should be taken not to overemphasise the difference, due to fact that the high OR in a non-vaccination group is mostly influenced by herd X, where active virus spread took place. The presence of antibodies in calves born after the first sampling, and which had lost their maternal immunity by the time of sampling, indicates that active virus spread has taken place recently. One nine month old animal in herd XI gave a suspect antibody test result in the second sampling. This might have derived from the remnant of maternal immunity.

Herds which have “naturally” uninfected replacements are in a comparable situation with vaccinated herds with the intention to eliminate the virus from the herd *via* replacing the infected animals with uninfected youngstock. In order to achieve self-clearance of BHV-1 infection from the herd, parameters influencing the expected time to extinction should be considered: the reproduction ratio, the reactivation rate, the population size, and the demographic turnover in the host population (de Koeijer *et al.*, 2008).

Mollema *et al.* (2005) indicated that, once the population contains predominantly latently infected animals, it will take a very long time before all latently infected individuals have died and been removed from the population, and conditional on no new outbreaks having taken place. A relatively short time to extinction can only be achieved if both R_1 and R_0 are below 1, assuming that the reactivation rate is low (Mollema *et al.*, 2005; de Koeijer *et al.*, 2008). The reactivation rate of BHV-1 is generally low, being $p=0.09$ per year, as estimated by de Koeijer *et al.* (2008). A low level of reactivation of BHV-1 latent infection leading to progressive self-clearance of the infection was also confirmed by Kampa *et al.* (2004; 2009). Consecutive replacements of the infected cows with uninfected heifers from within the herd have resulted in herds free from BHV-1 infection (Kampa *et al.*, 2009). Absence of the reactivation of BHV-1 can also be suspected in four out of five of the study herds in the current study, suggested by a decrease in seroprevalence among cows and youngstock remaining free of infection (prevalence below 5%). Self-

clearance of BHV-1 may occur under conditions of low levels of stress to the animals (Kampa *et al.*, 2004), avoiding reactivation of BHV-1 in latently infected animals. Several distinctive characteristics could be observed in the current study herds that had BHV-1 uninfected heifers and experienced a reduction in seroprevalence among cows during the two-year study period (Table 11). First, these herds are of medium size and variable levels of milk production. Small herds are probably more likely to experience self-clearance than large herds (de Koeijer *et al.*, 2008; Keeling and Rohani, 2008; Kampa *et al.*, 2009). Secondly, the veterinarian and inseminator are often not employees of the farm, possibly reducing the risk of iatrogenic spread of the virus. Thirdly, most of these farms kept their cows tied, in insulated barns, and grazing was frequently practiced. This may reduce direct animal contacts, reducing the probability of transmission of infection. Fourthly, the farms usually kept their youngstock in separate buildings, although regrouping happened twice: the first time after the calf had finished the colostrum-ingestion period, and the second time before calving. Finally, in general, the herds purchased only single animals, and these were often BVDV negative (Table 2 in Article IV).

7. CONCLUSIONS

- Due to the moderate sensitivity of the BTM ELISA test (76.5%) it should be used with caution when making decisions about the infection status of a herd. A lower cut-off in the ELISA blocking reaction might be justified when using BTM testing in IBR control programmes to be able to discover more infected herds with only a minimal loss in specificity.
- Twenty-two percent of Estonian market-oriented dairy cattle farms are infected with BHV-1. When devising control programmes most of the resources should be focussed on herds with more than 100 cows, as these farms contain the main population infected with BHV-1.
- As within-herd prevalence of BHV-1 is generally high in larger herds, vaccination with gE marker vaccines combined with eradication of gE positive animals is the most appropriate way to eradicate the virus in these herds. Where the within-herd prevalence is low, culling of the seropositive animals without vaccination would be most cost effective (Ackermann and Engels, 2006).
- Youngstock tested negative in 36.5% of the infected herds. These herds are in a more favourable situation when applying an eradication programme because it is possible to raise a BHV-1-free generation from their own herd, and vaccinate youngstock just before introduction to the cow house, as long as the farmer can provide isolation facilities. The duration of the vaccination programme is shorter when replacement animals are free of infection, reducing the cost of eradication.
- In herds under control programmes it is important to be aware of the important risk factors that are related to the spread of the infection within a herd. In BHV-1 positive herds more attention should be paid to possible iatrogenic transmission via the veterinarian and inseminator, and within-herd biosecurity measures should be kept in mind. Infection with BVDV may also be a predisposing factor for more effective viral spread.

- BHV-1 and BVDV had significant impacts on the manifestation of clinical respiratory disease of calves up to three months. BVDV contributed to the occurrence of respiratory disease signs in older youngstock. BRSV was related to clinical respiratory disease in adult dairy cattle. The use of control measures for these viruses may reduce the incidence of respiratory disease and improve herd health. In herds with a poor reproductive performance, BHV-1 should be considered as one of the infectious risk factors, and the eradication of this virus may improve the reproductive performance of the herd.
- In order to reduce the incidence of BRD in dairy youngstock, on-farm biosecurity measures are important in preventing human-mediated spread of the disease. Relocating animals within the farm may predispose to more active viral spread, leading to more BRD cases. Newly introduced animals can serve as the source of infection, therefore quarantine measures should be applied to avoid BRD outbreaks. Direct animal contacts in loose-housing systems may increase the occurrence of BRD in cows and pregnant heifers, perhaps via increased virus transmission. In order to reduce the circulation of infectious agents in the system, animals should be checked for clinical signs of respiratory disease continuously, and those with symptoms separated immediately from healthy animals. If youngstock and cows are kept in separate units, the aetiology of respiratory disease among both animal groups should be ascertained, followed by the application of specific control measures in order to avoid unprotected animals from becoming infected.
- Vaccination with an inactivated marker vaccine is a secure method to inhibit virus circulation within a herd, as long as the vaccination protocol is followed precisely. On the other hand, in some herds, the virus circulation may end by itself for longer periods, which may lead to self-clearance of the herd from the virus. However, aiming for eradication in herds with uninfected youngstock, leaving animals unvaccinated, has an unpredictable outcome – virus circulation may remain retarded or the virus may reactivate and cause an epidemic in the herd.

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9. SUMMARY IN ESTONIAN

Veiste herpesviirus 1 nakkuse epidemioloogia, mõju karja tervisele ja tõrje Eesti piimaveise karjades

Kokkuvõte

Veiste herpesviirus 1 (VHV-1) on veistel nakkava rinotrahheiidi (NRT), pustuloosse vulvovaginiidi (PVV) ja pullidel balanopostiidi (BP) tekitaja. Lisaks hingamisteede patoloogiale ja sigimishäiretele põhjustab viirus ka vasikate suurenenud suremust. Eestis ei rakendata VHV-1 suhtes üleriigilist tõrjeprogrammi. Vaid sperma kogumiseks kasutatavad pullid peavad olema enne seemendusjaama toomist viiruse suhtes testitud ja iga-aastases uuringus negatiivsed. Mitmed Euroopa riigid nagu Soome, Rootsi, Norra, Taani, Šveits ja Austria on ametlikult VHV-1 vabad ning mõned Kesk-Euroopa riigid nagu Saksamaa ja Holland rakendavad kohustuslikku tõrjeprogrammi. Riikidele, kes ei ole VHV-1 vabad, rakenduvad piirangud veiste ekspordile viirusest vabadesse riikidesse, mistõttu viirusevabasuse staatus oleks majanduslikult soodne nii igale farmerile kui ka veisekasvatusektorile kogu riigis.

Eestis diagnoositi haigust esmakordselt 1970ndatel aastatel. 1993-1995. aastal läbiviidud uuringus osutus 316 juhuslikult valitud piimakarja hulgast viirusele seroposiitvuseks 43.4%. Ulatuslikum viiruse üleriigiline levimusuuring viidi läbi 2004. aastal kui VHV-1 antikehadele uuriti 2912 tankipiima proovi ning positiivsete proovide osakaal oli 16.4%. Kuna VHV-1 põhjustab veisekasvatuses majanduslikku kahju nii karja tervist halvendava kui ka loomade ekspordi piirava tegurina, siis on piimakarjakasvatajate hulgast suurenenud huvi viiruse tõrje vastu. Tõrjeprogrammide koostamise eelduseks on viiruse levimuse selgitamine meie veisepopulatsioonis. Nakatunud karjadele efektiivseima tõrjeprogrammi koostamiseks on vaja teada nakkuse levikut soodustavaid riskitegureid. Selleks teostasime uuringu leidmaks riskitegureid, mis on seotud viiruse ulatusliku levikuga nakatunud karjades ning tegureid, mis on seotud nakatunud noorkarja olemasoluga juba VHV-1 positiivses karjas. Parima diagnostilise testi välja selgitamiseks viirusega nakatunud karjade avastamisel hindasime tankipiima ELISA testi tundlikkust ja spetsiifilisust meie veisepopulatsioonis. Et motiveerida nii farmereid kui ka valitsust reageerima antud probleemile, hindasime veel viiruse mõju loomade tervisele erinevates vanusegruppides. Samuti uurisime vaktsineerimisprogrammi efektiivsust VHV-1 glükoproteiin

E (gE) antikehade levimusele ning jälgisime nakkuse dünaamikat mittevaktsineerivates karjades, kus noorkari oli nakatumata.

Viiruse antikehade levimusuuring viidi läbi aastatel 2006 kuni 2008. Tankipiima ELISA testi omaduste, viiruse karjasisesel levimuse, riskitegurite ning tervise mõjude hindamiseks valisime uuringupopulatsiooni 103 piimaveise karja, kus oli enam kui 20 lehma. Võttes arvesse eelnevalt teadaoleva karja tankipiima VHV-1 antikehade staatust, valiti karjad erinevatest suurusgruppidest (20–99; 100–199; 200–399; ≥ 400 lehma vastavalt). Uuringu gruppi kuulus seega 64 VHV-1 nakatunud ja 39 nakatumata karja, kus ei rakendatud viirusevastast vaktsineerimist. Igas karjas uuriti esinduslikku valimit lehmi ja üle 6-kuu vanuseid mullikaid VHV-1 antikehade suhtes. Kokku uuriti 9637 seerumi proovi. Samaaegselt seerumi proovide kogumisega võeti uuringualustest karjadest ka tankipiima proovid (kokku 104 proovi). Viiruse levimuse selgitamiseks karja tasandil moodustati lähtepopulatsioon kõikidest piima turustavatest piimaveise karjadest. Kokku uuriti 1205 karja tankipiima proovi VHV-1 antikehade suhtes. Neist 328 karja õnnestus piimatööstuste andmestike alusel tuvastada, mis võimaldas meil hinnata VHV-1 levimust erineva suurusega karjades.

Pärast 2006-2008. aastal läbi viidud VHV-1 levimusuuringut koostati seitsmele piimaveise karjale VHV-1 tõrjeprogrammid. Vaktsineerimisskeem nägi ette kõigi vähemalt 3-kuu vanuste loomade vaktsineerimise inaktiveeritud gE markervaktsiiniga. Vaktsineerimise efektiivsuse monitooringuks kontrollprogrammi ajal jälgiti muutusi viiruse levimuses. Selleks võeti seerumi proove noorloomadelt, kes on vanemad kui 6 kuud ja sündinud pärast esimest vaktsineerimist ning testiti VHV-1 gE antikehade suhtes. Antud vanuses loomad on kaotanud ternespiimaga saadud immuunsuse ning on seetõttu heaks uuringugrupiks viiruse leviku tuvastamiseks karjas. Esimene testimine viidi läbi aasta ning teine 2 aastat pärast esmakordset vaktsineerimist. Viiruse levimuse muutuste jälgimiseks tehti 1.5 aastat pärast esimest vaktsineerimist kahes vanuserühmas: lehmadel ja üle 6 kuu vanustel mullikatel läbilõikeuuring. Viiruse leviku jälgimiseks valiti tõrjestrategiaid mittekasutatavates karjades viis VHV-1 positiivset karja, kus noorkari oli nakatumata (viiruse levimus alla 5%). Viiruse levimuse muutuse hindamiseks lehmade ja mullikate seas viidi läbi kaks läbilõikeuuringut. Kolme uuringu ajaline vahe oli umbes 1 aasta. Viiruse aktiivse leviku kindlakstegemiseks igal uuringukorral uuriti VHV-1 levimust vasikatel, kes olid sündinud pärast esimest uuringut ning

proovide kogumise ajal vähemalt 6 kuud vanad. Andmete kogumiseks karjast küsitleti esimesel uuringul loomaarsti või loomakasvatusjuhti.

Kõiki seerumi proove analüüsi VHV-1 antikehadele kasutades VHV-1 gB ELISA testi HerdChek* (IDEXX, Šveits). Kari loeti VHV-1 suhtes positiivseks kui vähemalt ühes seerumi proovis avastati viirusevastased antikehad. Vaktsineerivates karjades uuriti kõiki järeluuringu käigus kogutud proove VHV-1 gE ELISA kommertsiaalse testi HerdChek®-ga (IDEXX, USA). Karja veiste viirusdiarröa (VVDV) staatuse määratlemiseks uuriti igas karjas kümme seerumi proovi viiruse antikehade suhtes kasutades selleks PrioCheck VVDV Ab testi (Prionics AG, Šveits). Kari loeti VVDV suhtes positiivseks kui vähemalt ühes proovis tuvastati viirusevastased antikehad. Karja respiratoorsüntsütsiaal viirusega (RSV) nakatatus määratlemiseks uuriti kuni 20 (olenevalt karja suuruselt) mullika seerumi proovi viirusele tekkinud antikehade suhtes kasutades selleks Svanovir ELISA testi (Svanova Biotech AB, Rootsi). Olenevalt karja suuruselt testiti kuni 25 mullikat ning 10 lehma *Mycoplasma bovis* antikehadele BIO K 260 ELISA testiga (Bio-X Diagnostics, Belgia).

Tankipiima proovide ELISA testi tundlikkuse ja spetsiifilisuse hindamiseks võeti kuldstandardiks antud farmi seerumi proovide analüüsi tulemused. Karja suuruse kui riskiteguri hindamiseks tankipiima VHV-1 antikehade staatusele kasutati logistilist segamudelit ning karjasisesel kõrge VHV-1 antikehade levimuse ning nakatunud noorkarja riskitegurite kindlakstegemiseks teostati logistiline regressioonanalüüs. Viiruse mõju karja tervisele hindamiseks rakendati nii mitmest korrespondentsanalüüsi (MKA) kui ka logistilist regressioonanalüüsi. Regressioonanalüüs teostati kasutades selleks statistikaprogrammi Stata IC 10 ning korrespondentsanalüüsiks kasutati tarkvara XLSTAT versiooni 2010.4.01. Viiruse levimushinnangute muutuste hindamiseks nii tõrjealustes kui ka mittevaktsineerivates karjades kasutati binoomset lineaarset mudelit. Statistiliseks andmeanalüüsiks kasutati R tarkvara versiooni 2.13.0.

Tankipiima proovide uurimiseks kasutatud ELISA test ei olnud piisavalt tundlik avastamiseks kõiki nakatunud karju – 76.5% karjadest, mis olid ka seerumi proovides VHV-1 antikehadele positiivsed, oli võimalik tankipiima testiga tuvastada kui nakatunud karjad. Vaid üks seerumi proovides negatiivne kari andis tankipiima testimisel positiivse tulemuse, mistõttu testi spetsiifilisus oli 97.2%. Sellest tulenevalt võib tõrjeprogrammide rakendamisel nakatunud karjade paremaks avastamiseks alandada positiivse proovi määratlemise piirväärtust. Langetades

blokeerimisreaktsiooni piiri 30%-le senise 45% asemel, tõuseks testi tundlikkus meie andmetel 84.6%-ni, kusjuures spetsiifilisus väheneks minimaalselt (97.0%). Korrelatsioon lehmade VHV-1 karjasisese levimuse ning tankipiima ELISA testi blokeerimisprotsendi vahel oli 0.89. Seega annab blokeerimisprotsent kaudse ülevaate VHV-1 karjasisesest levimusest lüpsvate lehmade hulgas.

Üheksateist protsenti piima turustavate karjade tankipiima proovidest andis VHV-1 antikehade suhtes positiivse tulemuse. Võttes arvesse testi omadusi, saame tõeliseks karja tasemel levimuseks 22.0% (95% CI= 20.7, 23.3). 1993-1995. aastal läbiviidud uuringus oli karja tasemel levimus 43.4%. Kuna antud viiruse suhtes ei ole rakendatud nende vahepealsete aastate jooksul süstemaatilist tõrjet, siis viitab levimuse alanemine karjade isevabanemisele nakkusest. Andmed viitavad, et see on toimunud eelkõige väikeste karjade hulgas. Suurtes karjades on aga viiruse levimus jäänud kõrgeks. Üheks hüpoteetiliseks teguriks, mis on isevabanemisele kaasa aidanud, on tõenäoliselt seemendusjaamade vabanemine viirusest 1990ndate aastate lõpuks. Lisaks on väikestes karjades vastuvõtlike loomade arv väiksem ning suletud süsteemi puhul, kus uusi loomi karja ei tooda ning viiruse reaktiveerumist ei toimu, võib kari teatud aja möödudes viirusest ise vabaneda. Suurtes karjades aga tuuakse põhikarja pidevalt juurde uusi viirusele vastuvõtlikke loomi, mis soodustab omakorda viiruse tsirkulatsiooni.

Viiruse levimus karja tasemel oli suurte karjade puhul suurem. Kahjuks ei olnud võimalik uuringualuste karjade kohta registreerida karju iseloomustavaid teisi tegureid peale karja suuruse ning geograafilise asukoha, mistõttu osutus võimatuks kindlaks määrata antud nähtuse võimalikud põhjused. Suured farmid on enam ohustatud viiruse karja toomisest ostetavate loomadega ning sagedasemate väliskontaktide tõttu.

Viiruse karjasisene levimus osutus kõrgemaks küll suuremates karjades, kuid karja suurusgruppide vahel statistiliselt olulist erinevust selle näitaja osas ei tuvastatud. Viirusele tekkinud antikehade levimus oli võrreldes lehmadega väiksem noorloomade hulgas. Enam kui kolmandikus nakatunud karjades oli noorkari seronegatiivne. Vaid üksikutes karjades (17.5%) oli antikehade levimus mullikate hulgas kõrge (>50%). Võib oletada, et neis karjades oli levinud viiruse respiratoorne alamtüüp, mis levib kiiresti aerogeensel teel vastuvõtlike loomade seas. 1990ndatel aastatel Eestis isoleeritud viiruse isolaadid kuulusid VHV-1.1 alamtüübi hulka. Siiski võib oletada, et genitaalne nakatumine on viiruse levikul meie

karjades oluline vaatamata sellele, et pulli otsene kasutamine lehmade või mullikate tiinestamiseks ei olnud meie uuringus seotud viiruse kõrgema karjasisese levimusega. Antud küsimuse selgitamiseks on vaja läbi viia täiendavaid uuringuid.

Meie uuringu tulemused näitavad, et VVDV olemasolu karjas on seotud kõrge (>50%) VHV-1 karjasisese levimusega ning VHV-1 olemasoluga noorkarja hulgas. Eelnevates uuringutes on tuvastatud tugev positiivne seos karja VVDV ja VHV-1 staatuse vahel. VVDV nakkus on loomale immuunsupressiivne ning loob eelsoodumuse teiste viirus- ja bakternakkuste, k.a. VHV-1 levikuks. Vaatamata neile eeldustele on antud läbilõikeuuringuga võimatu tuvastada põhjus-tagajärg seoseid. Meie uuringus selgus, et kaudne iatrogenne VHV-1 nakkuse ülekanne loomaarsti ja seemendaja vahendusel on võimalik. Me eeldame, et kui loomaarst ja seemendaja on farmi töötajad, on selles farmis enam kontakte antud personali ja loomade vahel võrreldes nende karjadega, kus loomaarst ja seemendaja külastavad farmi vaid kutsel. Pideva kohaloleku tõttu teostavad nad sagedamini diagnostilisi protseduure, ravimenetlusi ning vahetut inna avastamist, soodustades sellega viiruse vahendatud ülekannet.

VHV-1, VVDV ja RSV olid MKAs oluliselt seotud respiratoorhaiguse sagedasema esinemisega kõigis vanusegruppides. Logistilise regressioonanalüüsi tulemused viitavad VHV-1 ja VVDV olulisele rollile vasikate hingamisteede haigestumises, kus VHV-1 kõrge levimus lehmadel ja VVDV olemasolu karjas olid seotud respiratoorhaiguse sagedasema esinemisega. VHV-1 seropositiivsete lehmade proportsioon annab kaudselt aimu sellest, kui suur osa vasikatest saavad ternespiimaga VHV-1 vastaseid antikehi. Meie uuringu tulemused viitavad aga sellele, et vaatamata eeldatavalt tugevale kolostraalimmuunsusele, tekib vasikatel siiski respiratoorne haigestumine. Kuna respiratoorhaiguse tunnuste esinemist hinnati retrospektiivselt, võis karja immuunsus olla tol hetkel madal, kusjuures proovide kogumise hetkel peegeldas antikehade kõrge tase hiljutist viiruse tsirkuleerimist karjas. Teisalt võib olla tegemist ka ternespiima puuduliku jootmisega. Kuna esmaspoegijad ei ole tihtipeale viirusega kokku puutunud, on ka ternespiimaga antav immunoloogiline kaitse nende loomade vasikatel nõrgem. Seega võis haigus avalduda peamiselt just nendel vasikatel, kellel maternaalne kaitse oli nõrk.

VVDV oli oluliselt seotud respiratoorse haigestumisega kuni 3-kuu vanustel vasikatel ja 3-16-kuu vanustel mullikatel. Endeemiliselt nakatunud karjades avaldub viirusinfektsioon kõige enam noorloomadel, kes

on vabanenud maternaalsest immuunsusest ning muutunud seetõttu viirusele vastuvõtlikuks. Siiski on VVDV nakkust tuvastatud kui olulist riskitegurit ka kuni 3-kuu vanustel vasikatel. VVDV nakkus võib põhjustada respiratoorset haigestumist otseselt või soodustada haiguse teket immuunsupressiivse ning sünergilise toime tõttu.

Logistilises regressioonianalüüsis ei olnud VHV-1 oluliselt seotud respiratoorse haigestumisega 3-16-kuu vanustel mullikatel ega täiskasvanud veistel, kuid olulisele positiivsele seosele viitasid MKA tulemused. Seega ei saa me täielikult välistada VHV-1 rolli respiratoorses haigestumises antud vanusegrupis.

Madal kuni mõõdukas RSV levimus (1-49%) mullikate hulgas oli oluliselt seotud respiratoorse haigestumise sagedasema esinemisega lehmadel ja tiinetel mullikatel. Hinnates antikehade esinemist maternaalse immuunsuse kaotanud noorloomadel on võimalik kindlaks teha viiruse hiljutist ringlemist karjas. RSV esmakordsel karja toomisel kulgeb viirusinfektsioon kõige ägedamalt täiskasvanud loomade hulgas ning jääb seejärel endemiliseks avaldudes järeltuleval põlvkonnal. Kuna meie hindasime respiratoorse haigestumise esinemist kahe eelneva aasta jooksul, võib antikehade olemasolu noorkarja hulgas peegeldada eelnevat RSVst tingitud viirusinfektsiooni lehmade ja tiinete mullikate seas.

VHV-1 madal kuni mõõdukas levimus (1-49%) oli seotud karja halva sigimisstaatusega. Võime eeldada, et karjades, kus VHV-1 levimus lehmade hulgas on mõõdukas, on nakkuse levik olnud mõnda aega soikeseisundis võimaldades välja kujuneda vastuvõtlikul populatsioonil. Need on aga karjad, kus on kõige soodsamad tingimused aktiivsele viiruse levikule ja abortide sagedasemale esinemisele. VHV-1 nakkusest tingitud abordid tekivad peamiselt tiinuse neljandast kaheksanda kuuni, kuid nakkus võib põhjustada ka embrüonaalset suremust suurendades sel viisil karja seemendusindeksit. Kuna sigimisinäitajaid hinnati retrospektiivselt ning antikehade olemasolu viitab viiruse levikule minevikus, ei ole võimalik hinnata täpselt põhjus-tagajärje vahelisi seoseid. Halb sigivus ning VHV-1 levik võivad olla mõjutatud mingi teise ühise faktori poolt nagu näiteks halvad pidamistingimused.

MKA analüüsiga tuvastati mitmeid potentsiaalseid pidamisega seotud riskitegureid, mis on seotud respiratoorse haigestumise sagedasema esinemisega. Karja suurus osutus oluliseks riskiteguriks loomade hingamisteede haigestumise esinemisel. Nakkus püsib suurtes

karjades paremini loomade suurema asustustiheduse, loomadevaheliste efektiivsemate kontaktivõimaluste ning vastuvõtlike loomade suurema arvu tõttu. Suuremas karjas on ka enam personali farmisisesest liikumist, mis võib osutada nakatise ülekande üheks võimaluseks. Kuna noorloomade pidamisviis (vabalt või lõas) ei osutunud analüüsis oluliseks, viitab see sellele, et otsesed loomadevahelised kontaktid ei pruugi alati olla obligatoorsed nakkuse ülekandeks. Noorloomade pidamine eraldi hoones kuuendast elukuust kuni tiinestumiseni oli samuti seotud kõrgema riskiga noorloomade haigestumiseks hingamisteede haigusse. Selle põhjuseks võib olla loomade ümbergrupeerimise ja erineva nakatatusena ning immuunstaatusena loomade kokku koondamine. Antud tulemused viitavad ka sellele, et otsesed kontaktid lehmadega ei ole vajalikud nakkuse levimiseks noorloomade hulgas.

Lehmade vabapidamise korral oli lehmade ja tiinete mullikate haigestumine hingamisteede haigusse sagedasem. Võime eeldada, et suurem otsekontaktide võimalus loomade vahel ning sagedased ümbergrupeerimised vabapidamislauas loovad eeldused nakkuste efektiivsemaks levikuks farmis. Loomade ostmine oli samuti riskiteguriks lehmade ja tiinete mullikate hingamisteede haiguse esinemisel. Karja ostetud loomad võivad olla otsesed viiruse sissetoojad, misjärel tekib haiguse puhang vastuvõtlike loomade seas.

Viiruse tõrjeks kasutatav vaktsiin peab efektiivselt peatama viiruse tsirkulatsiooni karjas vähendades viiruse eritamist latentselt nakatunud loomadel ning vähendades loomade vastuvõtlikkust nakkusele. Vaktsineerimise efekti näeme seetõttu kõige ilmekamalt vaadeldes muutusi viiruse levimuses noorkarja hulgas. Seitsme uuringu all olnud karja keskmine VHV-1 levimus mullikate hulgas alanes 1.5 aastaga 55%lt (95% CI 51-58) 6%ni (95% CI 4-9). VHV-1 gE seropositiivsete mullikate proportsioon alanes statistiliselt oluliselt viies uuritud karjas 1.5 aastat pärast vaktsineerimise algust, kuid levimuse vähenemise trendi võis märgata kõigis vaktsineerivates karjades.

Aasta pärast vaktsineerimisprogrammiga alustamist olid >6-kuused vasikad VHV-1 gE antikehadele kõikides karjades negatiivsed. Teisel testimisel 2 aastat pärast esimest vaktsineerimist oli sama loomapopulatsioon endiselt negatiivne karjades II kuni VII, kusjuures karjas I osutusid neli 10-kuu vanust looma viirusele tekkinud antikehadele positiivseteks, mistõttu antud loomade hulgas oli viiruse levimus 7.4%. Antud juhul oli tegemist farmeri teadliku kõrvalekaldega vaktsineerimisskeemist.

Nimelt otsustati vaksineerimiskulude kokkuhoiu eesmärgil hakata loomi vaksineerima alates 6. elukuust ettenähtud 3. elukuu asemel. Tavaliselt annavad kolostraalsed antikehad vasikale kaitse esimesteks elukuudeks ning vaksineerimisega alustatakse 3. elukuust. Lükates aga esimest vaksineerimist edasi, tekib nakkusele vastuvõtlik loomade populatsioon, mis võimaldab viirusel karjas levida.

Enne vaksineerimisprogrammiga alustamist oli seitsme uuritud karja VHV-1 keskmine levimus lehmade seas 90% (95% CI 88-92) ning see alanes 1.5 aastaga 76%ni (95% CI 72-80). Statistiliselt olulist levimuse alanemist võis täheldada kolmes uuritud karjas (karjad IV, V and VII), teistes karjades ei olnud muutused viiruse levimuses olulised. Vanemad loomad on tavaliselt enam nakatunud, mistõttu viiruse levimus lehmade hulgas võib jääda vaatamata vaksineerimisele veel mõneks ajaks kõrgeks. Lehmade hulgas hakkab viiruse levimus alanema siis, kui viirus-negatiivsed mullikad tulevad põhikarja ning vahetavad välja vanemaid viirusega nakatunud loomi. Vaatamata vaksineerimisele võib viiruse levik teatud määral karjas jätkuda ka vaksineerimisprogrammi rakendamise ajal, kusjuures selle tõenäosus on suurem lehmade hulgas. Meie uuringualustes karjades oli põhikarja asendumäär 25-30% aastas. See tähendab, et lehmade populatsioon vahetub karjas välja umbes 4 aastaga. Siiski jääb alati karja teatud hulk vanu loomi, kes on suure tõenäosusega viiruse kandjad, mistõttu haigusest vabanemiseks kulub lisaks veel paar aastat.

Kõik meie uuringus osalenud vaksineerimist rakendavad karjad olid suured karjad, kus oli enam kui 400 lehma ning kõrge piimatoodang (üle 7000 kg lehma kohta aastas). Suurtes karjades on VHV-1 levik soodustatud teatud pidamisega seotud faktorite olemasolu tõttu. Meie uuringud on näidanud, et karja tasandi riskitegurid nagu loomaarsti ja seemendaja olemasolu farmi töolistena ja VVDV olemasolu farmis on seotud suurema VHV-1 karjasisesega levimusega ning sellega, et noorkari on nakatunud. Seega peaks viiruse reaktiveerumise ärahoidmiseks ja tõrje edukuse tagamiseks vältima farmis üleasustust, rakendama karjasiseseid bioturvalisuse võtteid ning ohjama teisi immuunsupressiivseid haigusi. Vaksineerimine üksi ei pruugi viia viiruse elimineerumiseni karjast kui ei rakendata karjatervise võtteid, mis piiraksid viiruse levikut.

Karjad, kus on “loomulikult” nakatumata noorkari on võrreldavas situatsioonis karjadega, kes vaksineerivad oma loomi eesmärgiga hoida järeltulev põlvkond nakatumata ning asendada nendega põhikari. Karjades IX ja XI alanes viiruse levimus lehmade hulgas iga uuringu korruga,

kusjuures statistiliselt oluline langus oli 2 aastat pärast esimest uuringut ($p < 0.05$). Karjas X suurenes viiruse vastaste antikehade levimus lehmade hulgas 1 ja 2 aastat pärast esimest uuringut. Karjas XII jäi viiruse levimus teisel uuringukorral samaks ning kolmandal korral osutusid kõik uuritud lehmad viiruse suhtes negatiivseteks. Kahe aastaga alanes viiruse keskmine levimus lehmade seas 55%lt (95% CI 49-61) to 42%ni (95% CI 36-48), $OR=0.6$ (95% CI 0.4-0.9, $p < 0.05$). Uuringu tulemused näitavad, et viiruse levimus lehmade seas alanes aeglaselt enamikus karjades. Viiruse levimus lehmade seas alanes teisel uuringukorral enam vaktsineerivates karjades võrreldes mittevaktsineerivate karjadega, millele viitab väiksem OR väärtus ning fakt, et levimuse muutus mittevaktsineerivates karjades ei olnud statistiliselt oluline. Siiski tuleb silmas pidada, et kõrge OR väärtus mittevaktsineerivates karjades on suuresti mõjutatud karja X poolt, kus uuringuperioodi ajal toimus viiruse aktiivne levik.

VHV-1 levimus mullikate seas jäi alla 5% karjades VIII, IX, XI ja XII. Karjas X suurenes viirusevastaste antikehadega mullikate proportsioon oluliselt iga uuringu korraga, olles kolmandas uuringus 68% (95% CI = 53–80). Kahe aastaga suurenes keskmine VHV-1 levimus mullikate hulgas 2%lt (95% CI 0.4-4) 14%ni (95% CI 10-19), $OR=17.2$ (95% CI 5.6-52.8, $p < 0.001$). Viirusevastaste antikehade olemasolu vasikatel, kes on sündinud pärast esimest uuringut ja on uuringu läbiviimise ajal vähemalt 6 kuud vanad, näitab, et viirus on karjas hiljuti tsirkuleerinud. Meie uuringus osutus antud populatsioon antikehade suhtes negatiivseks kolmes karjas mõlemas järeluuringus. Karjas X oli VHV-1-le antikehade levimus 21% (95% CI 5-51) (teine uuring) ja 68% (95% CI 53-80) (kolmas uuring). Karjas XI osutus üks loom antud vanuserühmast teises uuringus VHV-1 antikehade suhtes kahtlaseks. Viimasel juhul oli tegemist 9-kuu vanuse vasikaga, kelle puhul võib oletada, et test tuvastas ternespiimaga saadud jäänukantikehad.

Kuna varasemates uuringutes on kirjeldatud karjade isevabanemist VHV-1st, oli meie eesmärk uurida, kas see on võimalik ka meie karjades. Nakkusest isevabanemise eelduseks on see, et latentselt nakatunud looma(de)l viirus ei aktiveeru ning uusi nakkusi ei teki. Üldiselt on viiruse reaktiveerumine harv protsess ning nakatunud lehmade asendamine viirusest vabade mullikatega on tinginud karjade isevabanemise viirusest. VHV-1 reaktiveerumise puudumist võib oletada neljas meie uuringu all olevas karjas, kus viiruse levimus lehmade hulgas vähenes ning noorkari jäi nakatumata (levimus alla 5%). Isevabanemine on võimalik siis, kui loomade stressi tase on madal vältides viiruse reaktiveerumist latentselt

nakatunud loomadel. Meie poolt uuritud karjades võib täheldada mitmeid tunnuseid, mis on tõenäoliselt tinginud olukorra, kus viiruse levikut ei ole mõnda aega toimunud. Esiteks on tegemist keskmise suurusega karjadega. Uuringud on näidanud, et väiksemates karjades on isevabanemine tõenäolisem kui suurtes. Teiseks ei ole nendes karjades loomaarst ega seemendaja farmi palgalised töölised, millega tõenäoliselt väheneb viiruse iatrogenne levik. Kolmandaks on need enamasti karjad, kus lehmad on soojas laudas lõaspidamisel ning lehma karjatatakse. Nii vähenevad loomadevahelised kontaktid ning väheneb viiruse levik. Neljandaks, nendes karjades hoitakse noorkarja eraldi loomapidamishoones ja loomi grupeeritakse ümber vaid kaks korda: esimest korda pärast ternespiima perioodi lõppu ning teine kord enne poegimist. Samuti ostavad need karjad vaid üksikuid loomi ja on tihti VVDV negatiivsed.

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Seroepidemiology of bovine herpesvirus 1 (BHV1) infection among Estonian dairy herds and risk factors for the spread within herds

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ABSTRACT

The objectives of this study were to reassess the herd level and within-herd prevalence of bovine herpesvirus 1 (BHV1) infection in Estonian dairy cattle, estimate the sensitivity and specificity of the enzyme-linked immunosorbent assay (ELISA) for bulk tank milk (BTM) testing and determine the risk factors related to high prevalence of the infection in herds.

To estimate the herd prevalence, BTM samples from each of the 1,205 herds that sell milk to dairy companies were analysed for BHV1 antibodies. One hundred and three herds with known BHV1 infection status were selected to estimate within-herd prevalence and to calculate the sensitivity and specificity of BTM ELISA. In these herds serum samples were collected from cows and youngstock, together with BTM samples. A commercial blocking ELISA test was used to analyse samples for antibodies against BHV1. A questionnaire was completed to collect herd data.

The sensitivity and specificity of the BTM ELISA were 76.5% and 97.2%, respectively, and the true herd prevalence of BHV1 was calculated to be 22.0%. The herd prevalence increased significantly with herd size, being 3.4% in the smallest category (less than 20 cows) and 85.7% in herds of size over 400. The mean within-herd prevalence was 37.8% (range 1–100, median 31.5). The mean within-herd prevalence increased with herd size.

Data from 59 infected herds was used to determine the risk factors associated with high within-herd prevalence (>50%) of BHV1, using logistic regression analysis. As, in some infected herds, the youngstock were uninfected, risk factors for the presence of BHV1 among youngstock from 6 months until calving were analysed. The results indicate the importance of iatrogenic spread of the virus, since the overall within-herd prevalence was higher in those herds in which a veterinarian was an employee of the farm and an inseminator worked only for the particular farm. The presence of bovine viral diarrhoea virus (BVDV) in a herd was associated with a higher prevalence of BHV1.

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1. Introduction

Bovine herpesvirus type 1 (BHV1), a member of the Alphaherpesvirus family, is the causative agent of infectious bovine rhinotracheitis (IBR), abortion, infectious pustular vulvovaginitis in cows and heifers and respira-

tory disease and systemic infection in calves. The virus may cause latent infection while stress can induce reactivation and intermittent excretion of the virus into the environment (Muyllkens et al., 2007). Although the disease caused by the infection is infrequently life threatening, it can cause severe economic losses due to production losses and restrictions in the trade of livestock (Nandi et al., 2009). The disease is common in many countries, although there are differences in prevalence (Ackermann and Engels, 2006). Many countries have implemented national or regional, compulsory or voluntary, eradi-

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cation programmes in recent years (Muylkens et al., 2007).

Infection with BHV1 is recognised as an important health problem in Estonian dairy herds. IBR was first diagnosed in Estonia in the mid-1970s. At the beginning of the 1990s, BHV1 was isolated in nine out of 15 counties among herds that suffered from respiratory diseases or reproductive disorders (Aaver and Saar, 1993). Until the early-1990s, several outbreaks occurred in vaccinated bulls in artificial insemination (AI) centres (Saar, 1999). In a serological survey conducted in 1993–1995, among 316 randomly selected dairy herds 43.4% of the herds were seropositive (Viltrop and Alaots, 1997). In a national survey for IBR, using herd pools of milk samples, conducted in 2004 which included 2912 herds of all categories, the proportion of positive samples was 16.4% (Anonymous, 2004a).

Although BHV1 infections have been registered in Estonian cattle herds for years, no systematic control programs exist against IBR, except for bulls used for semen collection in AI centres. All animals introduced to the AI centre must be isolated in their herd of origin, tested and be confirmed negative to BHV1 antibodies 30 days before movement (Anonymous, 2008). Bulls used for semen collection are tested serologically once a year (Anonymous, 2004b). Farmers and cattle breeders, as well as veterinary authorities, have, in recent years, shown an increased interest in the control of BHV1 infection. For developing a sound disease control program for BHV1 knowledge about the current status of BHV1 prevalence is required as well as an adequate diagnostic tool.

Antibodies against BHV1 in animals can be detected with an enzyme-linked immunosorbent assay (ELISA) test as soon as 9 days after infection (Kramps et al., 1994, 2004), whereas infected animals remain seropositive all their lives (Muylkens et al., 2007). To establish the herd infection status, bulk tank milk (BTM) antibody testing is often used in disease control programmes (Paton et al., 1998). If antibodies are detected in the BTM there is a high probability that more than one animal within the farm is infected, and that the infection has spread (Frankena et al., 1997; Nandi et al., 2009). The ELISA is a rapid, inexpensive and simple method for screening a large number of BTM samples for BHV1 antibodies (Nylén et al., 2000). Greiner and Gardner (2000) claimed that the diagnostic sensitivity and specificity of the test vary among studies and this variation is mainly attributable to differences among the reference populations and sampling strategies, as well as technical variation of the test characteristics, laboratory proficiency, the choice of gold standard and cut-off value for interpretation, and handling of intermediate or uninterpretable results.

To devise the most beneficial and economical control programmes for infected herds it is necessary to know the risk factors associated with virus spread within herds. Most studies that have been performed so far have investigated risk factors for a herd infected with BHV1 (Van Wuijckhuise et al., 1998; Van Schaik et al., 2002; Vonk Noordegraaf et al., 2004) or determined risk factors at the individual animal level (Solis-Calderon et al., 2003; Boelaert et al., 2005; Woodbine et al., 2009). Van Schaik et al. (1999) and

Segura-Correa et al. (2009) investigated risk factors related to seroconversion of BHV1 in dairy farms.

The main objectives of our study were to estimate the herd level and within-herd prevalence of BHV1 infection among Estonian dairy cattle, and to estimate the Se and Sp of the BTM ELISA test. We also aimed to identify and quantify relevant risk factors for high within-herd seroprevalence in infected herds and to clarify those factors related to exposure of young stock to BHV1 infection.

2. Materials and methods

2.1. Estonian dairy cattle population

The total number of dairy farms in Estonia, according to the Estonian Agricultural and Information Board (ARIB), was about 7,000 in 2007 (Table 1). These herds consisted of 218,000 cattle, including 103,000 dairy cows. Three hundred and thirty-seven medium and large farms with more than 50 cows comprised 75% of the total dairy cattle population. In total 1,205 herds delivered their milk produced to dairy companies.

2.2. Study design

The survey was conducted between September 2006 and April 2008.

Information concerning the dairy cattle herds (location, number of animals) was obtained from the national animal register (NAR) administered by the ARIB, which is a governmental institution within the Ministry of Agriculture.

2.2.1. Study population to estimate the Se and Sp of BTM ELISA test

The study population that was used to estimate the Se and Sp of the BTM ELISA test consisted of 103 herds with 20 or more dairy cows (herds of the source population contain 86.9% of all dairy cows in Estonia). Based on a BTM survey conducted in 2004, a proportional number of herds of previously known infection status were selected randomly from four categories of herd size (20–99; 100–199; 200–399; >400 cows). Herds were selected from the list held by NAR using a random number generator. In total, 64 herds not vaccinating against IBR with BTM samples positive for BHV1 antibodies and 39 BTM negative herds matched by herd size category were selected for sampling. At least five BTM negative herds were intended to sample in each herd size category although it was not possible in the largest herd size category as only one non-infected herd was identified in that group.

In each of the selected herds a representative random sample of cows and youngstock older than 6 months was tested for BHV1 antibodies in serum. Calculations to determine the sample size used the actual number of the animals present in a farm. Previous information about infection status was used, if available, in presumed infected herds to consider expected prevalence. In herds without this information available the expected prevalence was presumed to be 50% in youngstock and 75% in cows. To confirm freedom of disease in a BTM negative herd the minimum expected prevalence of 5%, a confidence interval of 95% and an accepted error of 10% was used to calculate the sam-

Table 1

Number of Estonian dairy herds, study sample and bovine herpesvirus 1 herd prevalence based on bulk milk antibody ELISA results from 328 Estonian dairy herds with respect to herd size with comparison between categories.

Herd size	Size of target population	Study sample (n)	Study sample (%)	Herd prevalence (%)
<20	6,390	118	1.8	3.4 a
20–49	255	90	35.3	4.4 a
50–99	110	46	41.8	10.9 a
100–199	85	31	36.5	41.9 b
200–399	83	29	34.9	58.6 bc
≥400	59	14	23.7	85.7 c
Total	6,982	328	4.7	16.8 ^a

a,b,c: different letters indicate statistically significant ($p < 0.05$) difference in prevalence between herd size groups in random effects logistic regression analysis.

^a Herd prevalence based on 328 identified herds.

ple size. A random number generator was used to select the animals for sampling in tie-stall systems, whereas in loose housed systems the sample was divided between pens (groups) in the barn, and animals were selected for sampling randomly within the groups. In total, 9,637 serum samples were collected. Simultaneously with the collection of blood samples, BTM samples were collected from each milk tank from 85 herds and these were tested for BHV1 antibodies. Two bulk milk tanks were present in 13 farms and three herds had three milk tanks. A total of 104 BTM samples were collected.

2.2.2. Study population to estimate BHV1 herd prevalence

The source population used to estimate the herd level prevalence of BHV1 consisted of all 1,205 market-oriented dairy herds. The BTM samples from these herds were obtained from the milk analysis laboratory of the Estonian Animal Recording Centre (EARC). Five dairy companies out of 20 were willing to identify individual herds allowing us to record the herd of origin of 328 BTM samples. This enabled us to estimate the herd prevalence of BHV1 infection in six farm size categories (<20; 20–49; 50–99; 100–199; 200–399; >400 cows).

2.2.3. Study population to estimate the within-herd prevalence and for risk factor analysis

The prevalence and questionnaire data from 64 infected dairy herds described in Section 2.2.1 was used to estimate the within-herd prevalence and risk factors for high within-herd prevalence and youngstock being infected in BHV1 positive herd.

As recommended by Houe et al. (2006) “spot testing” was used to confirm the BVDV infection status of a herd. Accordingly, 10 serum samples from randomly selected young animals older than 6 months to calving were analysed for BVDV antibodies to clarify the herd infection status, enabling the detection of a minimum prevalence of 20–28% depending on herd size.

2.3. Collection of herd data

During the herd visits questionnaires were filled in to collect data on herd level risk factors. In order to reduce possible bias all the questionnaires were filled in and interpreted by a single trained person. The requested infor-

mation included herd size, number of livestock units per farm, employment of the veterinarian and inseminator, frequency of movement of animals between barns, participation in agricultural shows, purchase history, type of housing (cold/warm barn), keeping system for cows and youngstock (loose/tied), management of youngstock (separately from cows/contact with cows for some life period/in the same barn with cows), use of bull for serving cows and heifers, breed(s) of cattle, grazing of cows and/or youngstock, vaccination history, whether employees change clothes on the farm and information about disinfection.

2.4. Sampling and sample analysis

Blood samples were collected from the coccygeal vein into 9 ml vacuum tubes (Vacuette, Austria) containing a clotting activator, using disposable needles (0.9 mm × 38 mm). Serum samples were stored at room temperature for 24 h before transport to the National Veterinary and Food Laboratory. The serum and defatted milk samples were analysed for BHV1 antibodies using a commercial HerdChek[®] IBR gB ELISA test kit (IDEXX, Switzerland), and the PrioCheck BVDV Ab test kit (Prionics AG, Switzerland) was used to analyse serum samples for BVDV antibodies.

A herd was considered to be infected with IBR if at least one blood test showed a positive result. According to test instructions bulk milk was considered to be “negative” if the blocking percentage was less than 45%, “suspect” between 45 and 55% and “positive” when over 55% (IDEXX, Switzerland). All suspect results were considered to be positive in the data analysis. A herd was considered to be infected with BVDV if at least one of the 10 serum samples was positive with antibody testing. The herd was considered to be suspect of containing persistently infected animals if at least six out of 10 young animals tested positive for BVDV antibodies (Houe, 1992).

2.5. Data analysis

Diagnostic results of individual blood samples collected from the farm were used as a gold standard for calculating Se and Sp of the BTM test. The Se and Sp of the BTM ELISA test were estimated and confidence intervals calculated using the “test evaluation” tool in the computer

software Win Episcope (Thrusfield et al., 2001). The “true prevalence” tool in Survey Toolbox (AusVet Animal Health Services, 1996), taking into account the Se and Sp of the BTM ELISA, was used to calculate the true prevalence of infected herds by the Rogan–Gladden estimator. Given that the sensitivity and specificity of gB blocking ELISA systems with individual serum samples is relatively high, having been reported as 99% (Kramps et al., 1994) to 100% (Nylén et al., 2000) and 96% (Kramps et al., 1994) to 99.7% (de Wit et al., 1998), respectively, the within-herd prevalence estimates were not corrected for the Se and Sp of the test.

For the assessment of the influence of herd size as a risk factor for BTM to be positive for BHV1 antibodies, a logistic random effects model was used. Given that the 328 herds were not randomly distributed geographically, county was included as a random effect in our model. A likelihood ratio test was performed to control the significance of the contribution of random effect in the model (Dohoo et al., 2009) being significant ($p=0.02$). In order to compare every size group with next size group variable “herd size” was coded as a hierarchical dummy variable (Dohoo et al., 2009).

Factors potentially related to the within-herd prevalence and seropositivity of youngstock were analysed using univariable and multivariable logistic regression. For this, the within-herd prevalence was dichotomized at 50% based on its frequency distribution while the youngstock population was considered positive when one or more animals in that group tested positive.

Logistic regression modelling started with univariable analyses and all variables with a p -value <0.15 were included in the multivariable model. If collinearity was suspected based on coinciding meaning, a chi-square test was used pairwise on the predictor variables for evaluation of the association, and the variable with the smaller p -value in the univariable analysis, and greater biological significance, was included in the model.

All variables selected in the univariable step were incorporated in a multivariable model, and single variables were excluded one by one based on their p -value. The change in the regression coefficients was followed to detect any confounding between variables whereas change in coefficients of more than 20% between crude and adjusted values was considered to be important (Dohoo et al., 2009). Where interaction was suspected the interaction term was included and controlled for significance. Risk factors with p -values ≤ 0.05 were retained in the model. Herd size was included in the models as a potential confounder, although it was not statistically significant. The final model fit was assessed with Hosmer–Lemeshow goodness-of-fit test (Dohoo et al., 2009). The data analysis was performed by using StataC 10 and procedure xtlogit with population-averaged estimator was used for implementing random effect logistic regression model (Stata Corporation, Texas, USA).

3. Results

3.1. Se and Sp of the BTM ELISA test

The sensitivity of the ELISA test to detect infected herds using BTM samples was estimated as 76.5% (95% confi-

dence interval (CI)=66.4, 86.5) and the specificity was 97.2% (95% CI=91.8, 100). The Spearman correlation coefficient between within-herd prevalence and the blocking percent was 0.89. One herd with a prevalence among cows as low as four per cent (95% CI=0.5, 8.5%) was detected as positive in BTM sample. However, a herd with a prevalence among cows of 64% (95% CI=53.2, 74.8%) was tested negative in BTM ELISA.

3.2. BHV1 herd prevalence and herd size as risk factor

Nineteen percent of BTM samples collected from the EARC milk analysis laboratory were positive for BHV1 antibodies. The true prevalence was calculated as 22.0% (95% CI=20.7, 23.3). The herds delivering the 328 BTM samples used for assessment of herd prevalence originated from all 15 counties in Estonia (samples per counties ranged from 1 to 118, median 13). The size of the target population, and the proportion of study samples in the different herd size categories, as well as herd prevalence and significance of differences between prevalence of different herd size categories are presented in Table 1. The herd level prevalence was different among herds of different size categories, although this was not statistically significant between three lowest herd size categories nor between the categories with 100–199 and 200–399 cows. When each herd size group was compared with the next size hierarchically, the prevalence differed significantly between herd size categories with 50–99 and 100–199 cows (OR 5.5, 95% CI=1.7, 17.6, $p=0.004$). A significant increase in prevalence estimates was also detected between herd size groups of 100–199 and ≥ 400 cows (OR 7.8, 95% CI=1.5, 39.4, $p=0.014$).

3.3. Within-herd prevalence of BHV1

The frequency distribution of BHV1 seroprevalence over herds is shown in Fig. 1. In 31.3% of infected herds ($n=20$) total within-herd prevalence was more than 50%. Data describing the within-herd prevalence in different herd size categories are presented in Table 2. The mean within-herd prevalence in investigated infected herds was 37.8%. The mean seroprevalence in cows was more than twice as high as that in youngstock in every herd size category (Table 2).

A high prevalence of infection ($>50\%$) among youngstock was observed in 11 herds out of 63 (in one herd it was not possible to obtain samples from youngstock) whereas among cows it was detected in 35 out of 64 infected herds. In 71.4% ($n=45$) of the infected herds the prevalence among youngstock was $<20\%$, and in 36.5% ($n=23$) of the infected herds all young animals tested negative.

In herds with more than one bulk milk tank there were no differences in the antibody status of BTM samples within farms.

3.4. Risk factor analysis

The overall distribution of within-herd prevalence in our sample population divided herds into two subgroups, with a division at approximately 50% (Fig. 1). Nine risk

Table 2

Within-herd prevalence of bovine herpesvirus 1 in 64 dairy cattle herds of different size categories in Estonia.

Herd size	Herds (n)	Prevalence (%) Median (range) Mean	Prevalence (%) (cows) Median (range) Mean	Prevalence (%) (youngstock) Median (range) Mean
20–99	15	13 (2–76) 26.1	28 (3–98) 34.5	0 (0–63) 9.4
100–199	14	29 (1–92) 32.1	50 (2–96) 46.1	2 (0–88) 16.6
200–399	18	34.5 (2–100) 39.1	64 (4–100) 57.1	5 (0–100) 20.3
>400	17	56 (1–91) 51.2	84 (2–100) 70.1	9 (0–91) 31.6
Total	64	31.5 (1–100) 37.8	57.5 (2–100) 52.8	5 (0–100) 20.1

Table 3

Fixed effects logistic regression model for risk factors for high bovine herpesvirus 1 prevalence (>50%) within infected herds (n=59).

Risk factor	Category	Herds (n)	Prevalence >50% n (% of herds)	OR	p-Value	95% CI
Veterinarian employee of the farm	Yes	21	12 (57.1)	6.05	0.03	1.19, 30.62
	No	38	7 (18.4)			
Inseminator working only for particular farm	Yes	19	10 (52.6)	5.54	0.04	1.10, 27.91
	No	40	9 (22.5)			
BVDV present in herd	Yes	14	8 (57.1)	7.27	0.03	1.24, 42.74
	No	45	11 (24.4)			
Herd size ^a	20–99	12	3 (25)	1	–	–
	100–199	13	2 (15.4)	0.15	0.14	0.01, 1.85
	200–399	18	5 (27.8)	0.26	0.22	0.03, 2.23
	>400	16	9 (56.3)	0.23	0.24	0.02, 2.74

^a Herd size $p=0.495$ in multiple Wald test.

factors were included into multivariable model after univariable analysis ($p < 0.15$). In the multivariable analysis procedure four variables remained significant (Table 3). Variables that dropped out of the model were: owner has more than one holding in different locations (yes/no), purchase of animals within previous 3 years (yes/no), cow management (loose/tied), grazing cows (yes/no), vaccination for diseases other than IBR or BVD (yes/no). BVDV present in a herd significantly increased the probability for a herd having within-herd prevalence of BHV1 over 50% (OR = 7.27). If the veterinarian was the employee of the farm and inseminator worked only for particular farm the probability for high within-herd prevalence was higher compared to those herds where these professional employees were not engaged with only one farm (OR = 6.05 and OR = 5.54, respectively) (Table 3).

In the risk factor model for youngstock infected with BHV1 in an infected herd three risk factors remained in the final model. Variables excluded from the model were: veterinarian working on farm (yes/no), number of livestock units on a farm (one/more than one), attending animal shows (yes/no), cow management (loose/tied), employees change clothes in farm (yes/no), vaccination for diseases other than IBR or BVD (yes/no). In both models five herds were excluded from the analysis because of missing data in any of the predictors. BVDV was borderline significant in the final logistic regression model increasing the probability of youngstock to be infected with BHV1 (OR = 6.5). When the inseminator was working in only one particular farm the risk of youngstock to be infected was higher than in those where the person provided service to many herds (OR = 5.8) (Table 4).

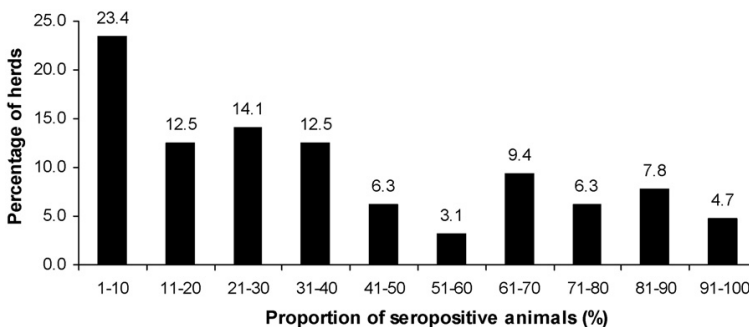
**Fig. 1.** Distribution of the within-herd prevalence of BHV1 in 64 Estonian dairy herds.

Table 4
Fixed effects logistic regression model for risk factors for heifers being infected with bovine herpesvirus 1 in infected herds ($n = 59$).

Risk factor	Category	Herds (n)	Heifers infected n (% of herds)	OR	p -Value	95% CI
Inseminator working only for particular farm	Yes	19	16 (84.2)	5.8	0.03	1.14, 29.43
	No	40	22 (55.0)			
BVDV present in herd	Yes	14	12 (85.7)	6.5	0.05	0.97, 43.33
	No	45	26 (57.8)			
Herd size ^a	20–99	12	5 (41.7)	1	–	–
	100–199	13	7 (53.8)	1.35	0.73	0.25, 7.31
	200–399	18	14 (77.7)	3.02	0.21	0.55, 16.74
	>400	16	12 (75.0)	1.02	0.99	0.13, 7.62

^a Herd size $p = 0.544$ in multiple Wald test.

4. Discussion

4.1. *Se and Sp of the BTM ELISA test*

The ELISA test used for the analysis of the BTM samples in this study was not sufficiently sensitive to detect all infected herds—76.5% of the herds positive in blood testing also had a positive BTM test result. Only one herd negative in blood testing had positive BTM test result leading to specificity of 97.2%. Nylin et al. (2000) estimated the sensitivity and specificity of the BTM test to be 82% and 100%, respectively, with a cut-off value of 30% blocking reaction. The lower sensitivity in our study can partly be explained by the difference in blocking reaction cut-off, which according to the manufacturer's instructions was higher in our test. When lowering the cut-off to 30% of blocking reaction in our study the sensitivity of the BTM ELISA test increased to 84.6% with only a minimal decrease in specificity (97.0%).

The correlation between BHV1 within-herd prevalence among cows and BTM ELISA blocking percent was 0.89. A similar result was found in a study of Hartman et al. (1997) where the correlation was 0.86; however, a correlation of 0.59 has been reported by Nylin et al. (2000). Differences between these correlations might be due to diversity in the distribution of within-herd prevalence of BHV1 among different cow populations, study design (sample size in respect to number of selected herds as well as animals within herds), ELISA test and the evaluation of the results as well as laboratory conditions.

4.2. *BHV1 herd prevalence and herd size as risk factor*

The results of this survey indicate that almost a quarter of Estonian market-oriented dairy cattle herds are infected with BHV1. In a study from 1993 to 1995 the observed overall herd prevalence was 43.4%. As no systematic control of the infection has been conducted in these herds during 12 years this substantial decrease in herd prevalence is an indication of self-clearance from BHV1 infection, particularly in small herds (herd prevalence was 27.6% and 50.0% in herds with <50 and 50–99 cows, respectively, in previous study). However, the self-clearance has not taken place in larger size groups of Estonian dairy herds where the herd prevalence has remained high through all these years. One

hypothetical factor contributing to the self-clearance may be eradication of the infection from AI centres by the end of 1990s. Second, it has been speculated that, in smaller herds, the infection may not be maintained because the number of susceptible animals is smaller throughout the year (Boelaert et al., 2005). In larger herds continuous influx of a relatively large number of new, susceptible animals predisposes virus circulation.

Herd prevalence of BHV1 was generally higher in larger herds. As no other herd factors beside "herd size" and geographical location were recorded it was not possible to discover the factors behind that trend. Larger herd size has been found to be a risk factor for BHV1 herd-seropositivity in previous studies (Tekes et al., 1999; Van Wuijckhuise et al., 1998), although it was not significant in determining the BHV1 status of a herd in a study of Van Schaik et al. (1998). Large farms are more likely to have a disease introduced, due to the more frequent purchase of new animals (Van Schaik et al., 1998; Van Wuijckhuise et al., 1998) and the tendency to have more visitors (Van Schaik et al., 1998).

4.3. *Within-herd prevalence of BHV1*

In our study the within-herd prevalence on average was higher in larger herds, however, the variation within-herd size categories was large and the differences are not statistically significant. Seroprevalence among youngstock was lower than that in cows, which is consistent with other studies (Guarino et al., 2008; Jacevičius et al., 2008; Kampa et al., 2004). In more than one-third of infected farms, young animals were seronegative. Only in few herds (17.5%) the seroprevalence among youngstock was high (>50%). Respiratory subtype of BHV1 is excreted in high titres in nasal secretions and spreads more efficiently than the other subtypes (Wentink et al., 1993), genital infection is a problem of breeding animals (Guarino et al., 2008). The viral isolates characterised in 1990s in Estonia belonged to subtype BHV-1.1 (Saar, 1999). The results of the present study indicate that genital infections might be relevant in the spread of the infection, however, use of bull for natural mating in cows or heifers was not related to higher within-herd prevalence in risk factor study. Further studies to clarify the role of different subtypes in epidemiology of the infection are needed.

4.4. Risk factor analysis

We found that the presence of BVDV is associated with a higher within-herd seroprevalence. A strong association between a herd concurrently being BVDV and BHV1 antibody-positive has been found also in other studies (Kampa et al., 2004; Paton et al., 1998). A more effective spread of BHV1 within an animal infected with BVDV has been demonstrated by Potgieter and co-workers (1984, 1995). Infection with BVDV is known to be immunosuppressive and, particularly in young animals, it is assumed to be a predisposing factor for other viral or bacterial diseases, including BHV1 (Potgieter, 1995). Regardless of these assumptions it is not possible to make exact cause and effect inferences from our findings due to the cross-sectional study design.

The results of the model also highlighted the significance of indirect iatrogenic transmission of the virus via veterinary and insemination equipment, clothes and hands. We can assume that when the veterinarian and inseminator are employees of the farm, there is a tendency to handle animals more frequently for diagnostic purposes, invasive treatments and heat detection compared to those where these professionals visit the farm on call.

Although the average BHV1 within-herd prevalence was higher in larger herds "herd size" was not a significant predictor but acted as a confounder in the model for risk factors for high within-herd prevalence. Significant effects might have been missed because of relatively small sample size or some other unrecorded risk factors related to herd size might influence the prevalence.

A loose housing system has been found to be an important risk factor in reactivation of BHV1 at the farm (Van Schaik et al., 1999). In our study none of the housing systems for cows or youngstock were significant in the model detecting risk factors for high within-herd prevalence demonstrating that close animal contacts may not always be important for BHV1 transmission within a herd.

The results of the model analysing the risk factors for presence of BHV1 among youngstock, indicated that it is necessary to be aware of the important role of the inseminator as a possible transmitter of the virus and BVDV as a possible predisposing factor for virus spread. Most surprisingly keeping youngstock together with cows was not significantly associated with the BHV1 infection status of young animals. Although BHV1 is respiratory illness, the agent is not easily transmitted by aerosol (Ackermann and Engels, 2006). Youngstock kept in the same building with cows are usually located in separate group or they are kept fixated in the barn distant from cows. Therefore the spread of the virus to youngstock is more dependent on indirect viral transmission routes related to herd management. Different subtypes of the BHV1 may also be of importance.

5. Conclusions

Due to the moderate sensitivity of the BTM ELISA test (76.5%) it should be used with caution when making decisions about the infection status of a herd. Lower cut-off in the ELISA blocking reaction might be justified when using

BTM testing in IBR control programmes to be able to discover more infected herds with only a minimal loss in specificity.

Twenty-two percent of Estonian market-oriented dairy cattle farms are infected with BHV1. When elaborating control programmes most of the resources should be directed to herds with more than 100 cows as those farms contain the main population infected with BHV1.

As within-herd prevalence of BHV1 is generally high in larger herds vaccination with gE marker vaccines combined with eradication of gE positive animals is the most appropriate way to eradicate the virus in those herds. Where the within-herd prevalence is low culling of the seropositive animals without vaccination would be most cost effective (Ackermann and Engels, 2006).

Youngstock tested negative in 36.5% of infected herds. These herds are in more favourable situation when applying an eradication programme because it is possible to raise a BHV1-free generation from their own herd and vaccinate youngstock just before introduction to the cow house, as long as the farmer can provide isolation facilities. The duration of vaccination programme is shorter when replacement animals are free of infection reducing the cost of eradication.

In herds under control programmes it is important to be aware of the important risk factors that are related to spread of the infection within a herd, because vaccination does not prevent the spread of the virus totally (Bosch et al., 1997). In BHV1 positive herds more attention should be paid to possible iatrogenic transmission via the veterinarian and inseminator, and within-herd biosecurity measures should be kept in mind. Infection with BVDV may also be a predisposing factor for more effective viral spread.

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Association of herd BHV-1 seroprevalence with respiratory disease in youngstock in Estonian dairy cattle

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ABSTRACT

The associations between herd bovine herpesvirus 1 (BHV-1) seroprevalence, along with other infectious and farm management factors with bovine respiratory disease (BRD) in dairy calves and heifers, were investigated. Serum samples from 103 dairy cattle herds were analyzed for antibodies against BHV-1, bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), and *Mycoplasma bovis* (*M. bovis*). A questionnaire was used to record herd management practices.

A high occurrence of respiratory disease in unweaned calves was associated with low to moderate and high prevalence of BHV-1 among cows (OR = 14.8, $p = 0.005$ and OR = 19.2, $p = 0.002$, respectively) and positive BVDV status of a herd (OR = 5.1, $p = 0.02$). The presence of BVDV in a herd was related to a high incidence of respiratory disease in heifers 3–16 months old (OR = 4.3, $p = 0.027$). Based on the results of multiple correspondence analysis, holding youngstock separately from cows until pregnancy, introducing new animals and the activities of on-farm employees may contribute to a higher incidence of BRD.

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1. Introduction

Respiratory disease is one of the foremost cattle health concerns (Callan and Garry, 2002) and is estimated to cause significant economic losses to the dairy industry, due to increased morbidity and mortality, and negative long-term consequences to herd health and productivity including poor growth, hampered reproductive performance, lower milk production, and reduced longevity (Gay and Barnouin, 2009; Poulsen and McGuirk, 2009; Van der Fels-Klerx et al., 2002a). Bovine respiratory disease (BRD) is usually of multifactorial origin, involving infectious, environmental and management-related factors (Gay and Barnouin, 2009; Lundborg et al., 2005; Van der Fels-Klerx et al., 2000) as well as those related to stress and the immunity of the animal (Gorden and Plummer, 2010). Bovine herpesvirus 1 (BHV-1) is considered an important component of the BRD disease complex in cattle. However, controversial results can be found from previous field studies detecting the role of BHV-1 among calves and dairy youngstock. Some studies have confirmed that BHV-1 did not contribute significantly to the occurrence of respiratory disease in feedlot calves (Martin et al., 1989; Allen et al., 1992), but Penny et al. (2002) ascertained severe multisystemic form of the disease in neonatal beef calves. In endemically infected herds, BHV-1 is presumed to be an

uncommon agent causing BRD in young calves due to the high percentage of seropositive dams and the protective effect of colostral antibodies (Mechor et al., 1987), and so BHV-1 is most commonly seen in housed cattle from 6 to 18 months of age (Penny et al., 2002). In calves the significant contributors to the incidence of respiratory disease are bovine viral diarrhoea virus (BVDV) and bovine respiratory syncytial virus (BRSV), as has been demonstrated in previous studies (Allen et al., 1992; Elvander, 1996; Fulton et al., 2000; Martin et al., 1989). Recent field studies detecting the role of infectious agents in BRD in young dairy calves have identified that Nordic countries are free of BHV-1 and have a low prevalence of BVDV (Angen et al., 2009; Autio et al., 2007; Lundborg et al., 2005). In Estonia, a high proportion of dairy herds are endemically infected with BHV-1 (Raaperi et al., 2010) and also with BVDV (Viltrop et al., 2002). Consequently the etiology of BRD can differ in different regions due to dissimilar epidemiological situations. Thus the first aim of the study was to clarify the role of BHV-1 in the incidence of BRD in calves as well as in older youngstock in Estonia, taking into account the impact of other respiratory pathogens such as BRSV, BVDV and *Mycoplasma bovis*.

Several environmental and management factors also influence the development and the severity of disease during infections (Gulliksen et al., 2009). Previous studies have mainly involved factors related to calving management (Svensson et al., 2003), characteristics of the calf pen (Van der Fels-Klerx et al., 2002b; Lundborg et al., 2005; Svensson et al., 2003), microclimate (Assie et al., 2009; Van der Fels-Klerx et al., 2000) and colostrum feeding

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strategies (Virtala et al., 1999; Svensson et al., 2003). Herd level variables such as larger herd size (Assie et al., 2009; Gulliksen et al., 2009) and the purchase and introduction of new cattle into a herd (Van der Fels-Klerx et al., 2000) have been found to be significant risk factors for acute respiratory disease in young calves. The second aim was to evaluate the impact of factors related to general farm practices in Estonia on the incidence of respiratory disease in pre-weaning dairy calves and older youngstock.

2. Materials and methods

2.1. Study design

The target population in this study was Estonian commercial dairy cattle herds of more than 20 cows. A detailed description of the method of herd selection and sampling of animals has been described in Raaperi et al. (2010) Sections 2.2 and 2.3. Briefly, a stratified random sample of non-vaccinating BHV-1 antibody-positive and negative herds was selected from amongst herds of different size categories. Thereof herds' infection statuses were first defined based on the results of a prior bulk tank milk (BTM) survey from the year 2004; however, results of individual samples collected during the present study was used to define the herd true infection status. In each of the selected herds, serum samples from a number of randomly selected cows, and youngstock older than 6 months, were analyzed for BHV-1 antibodies. The sample size for prevalence estimation in BHV-1 BTM-positive herds was calculated at a 95% confidence and a 5% error level assuming 75% prevalence among cows and 50% in youngstock. Freedom from BHV-1 antibody carriers in BHV-1 BTM-negative herds was determined at 5% prevalence and 95% confidence level. The numbers of BHV-1 positive and negative herds in the different size categories included in this study are presented in Table 1.

For additional information, a questionnaire to record herd-level data was completed for every herd. The information requested included herd size, farm management and biosecurity practices and vaccination history. In addition, questions relating to the history of the incidence of respiratory disease in the previous two years, among calves up to 3 months of age and 3–16 months old youngstock, were asked. All samples and questionnaires were collected between September 2006 and April 2008.

2.2. Sample analysis

Blood was collected from the coccygeal vein of each animal by using disposable needles and 9 ml vacuum tubes containing clotting activator (Vacuette, Austria). After collection, serum samples were stored in room temperature for 24 h and transported to the National Veterinary and Food Laboratory for immediate serology.

All serum samples were tested for BHV-1 antibodies using a commercial BHV-1 gB ELISA test kit, HerdChek® (IDEXX, Switzerland). Suspicious antibody test results were considered as positive in the data analysis. In each of the herds certain number of animals was tested additionally for BVDV, BRSV and *M. bovis* antibodies.

Thereby a random subsample amongst BHV-1 tested animals was selected for analysis.

The herd BVDV status was established by testing up to 10 serum samples from randomly selected animals, at ages from 6 months up to age at first calving, for BVDV antibodies as recommended by Houe et al. (2006). This sample size enabled detection of a minimum prevalence of 20–28% depending on herd size at a 95% confidence level. The PrioCheck BVDV Ab test kit (Prionics AG, Switzerland) was used for analysis.

The herd BRSV status was established by testing up to 20 (depending on herd size) randomly selected serum samples from heifers for BRSV antibodies to allow detection of at least a 15% prevalence of BRSV antibody carriers in the herd at a 95% confidence level assuming 94.6% sensitivity and 100% specificity of the test. For BRSV antibodies, the Svanovir ELISA test (Svanova Biotech AB, Sweden) was used.

Depending on herd size up to 25 heifers and 10 cows were tested for *M. bovis* antibodies in each herd. This enabled to detect the prevalence of at least 15% among heifers and 27% among cows with 95% level of confidence. BIO K 260 ELISA test (Bio-X Diagnostics, Belgium) was used to measure *M. bovis* antibodies.

2.3. Description of the models and variable coding

In Model I the dependent variables were related to the history of respiratory disease incidence in calves of up to 3 months old. In Model II the aim was to clarify the risk factors for a high prevalence of respiratory disease in 3–16-month-old heifers. Four dependent variables (general disease, nasal discharge, signs of respiratory distress, and lacrimation) were used (Table 2).

On each farm the veterinarian or farm manager was questioned about the occurrence of clinical signs of respiratory disease, including general signs (fever, inappetence, dullness), nasal discharge ("red nose"), respiratory signs (cough, dyspnoea), and lacrimation, in two age groups (calves 0–3 months old and youngstock 3–16 months old). The respondents were asked to evaluate the prevalence of the signs described above among animals of each age group during periods of the highest incidence of respiratory disease the previous two years. The scales used in the estimation were as follows: 1 – no signs or only single case; 2 – up to 10%; 3 – 10–30%; 4 – over 30%. All four dependent variables in each model were dichotomized. The 10% cut-off point was used for 0–3-month-old calves while for older youngstock the herd was considered to be in the "high frequency" group if more than just a single animal showed the mentioned signs concurrently (Table 2).

As all the respiratory disease signs were found to be highly clustered from multiple correspondence analysis (MCA) (discussed in Section 3), one summary variable describing the level of incidence of respiratory disease for each age group was created to be used in logistic regression analysis. The herd was considered to be in a group of "high frequency of BRD in calves" (BRDCAL = 1), if at least two of the four variables indicating respiratory disease signs among calves were in the "high frequency" category. For older

Table 1
Number of herds in Estonia in 2007 and study sample size.

Herd size	Number of herds in Estonia in 2007	Study sample (BHV-1 antibody-positive herds)	Study sample (BHV-1 antibody-negative herds)	Study sample in total
20–49	255	9	17	26
50–99	110	7	9	16
100–199	85	14	5	19
200–399	83	18	6	24
≥400	59	17	1	18
Total	592	65	38	103

Table 2
Descriptive characteristics of the variables (99 herds).

Variable	Definition of the categories of the variable	Number of herds	
		Model I	Model II
General disease (fever, inappetence, dullness) in calves 0–3 months old (GENCAL)	0 – present in less than 10% concurrently in some time point during recent years	66	
	1 – present in more than 10% concurrently in some time point during recent years	33	
Nasal discharge (“red nose”) in calves 0–3 months old (NASCAL)	0 – present in less than 10% concurrently in some time point during recent years	69	
	1 – present in more than 10% concurrently in some time point during recent years	30	
Respiratory signs (cough, dyspnoe) in calves 0–3 months old (RESCAL)	0 – present in less than 10% concurrently in some time point during recent years	63	
	1 – present in more than 10% concurrently in some time point during recent years	36	
Lacrimation in calves 0–3 months old (LACCAL)	0 – present in less than 10% concurrently in some time point during recent years	78	
	1 – present in more than 10% concurrently in some time point during recent years	21	
Respiratory disease occurrence in calves 0–3 months old (BRDCAL)	0 – less than two respiratory disease signs are present	65	
	1 – at least two respiratory disease signs are present	34	
General disease (fever, inappetence, dullness) in heifers 3–16 months old (GENHEF)	0 – not present at all or was shown only as single cases in some time point during recent years		89
	1 – present in more than just single cases in some time point during recent years		10
Nasal discharge (“red nose”) in heifers 3–16 months old (NASHEF)	0 – not present at all or was shown only as single cases in some time point during recent years		84
	1 – present in more than just single cases in some time point during recent years		15
Respiratory signs (cough, dyspnoe) in heifers 3–16 months old (RESHEF)	0 – not present at all or was shown only as single cases in some time point during recent years		80
	1 – present in more than just single cases in some time point during recent years		19
Lacrimation in heifers 3–16 months old (LACHEF)	0 – not present at all or was shown only as single cases in some time point during recent years		91
	1 – present in more than just single cases in some time point during recent years		8
Respiratory disease occurrence in heifers 3–16 months old (BRDHEF)	0 – less than two respiratory disease signs are present		82
	1 – at least two respiratory disease signs are present		17
Relocating animals between the barns (regro)	0 – one to several times a year	17	17
	1 – every month	51	50
	2 – only one unit	31	32
Veterinarian being the employee of the farm (vetemp)	0 – no	76	77
	1 – yes	23	22
Inseminator being employee of the farm (insemp)	0 – no	55	55
	1 – yes	44	44
Keeping youngstock together with cows (yoco)	0 – together	49	51
	1 – in separate building from 6 months until pregnancy	50	48
Keeping management for youngstock (keyo)	1 – tied	23	
	2 – loose	30	
	3 – some period of life tied, some period loose	46	
Number of livestock units within the farm (units)	0 – farm has only one livestock unit	31	32
	1 – farm has more than one livestock unit	68	67
Herd size (hsize)	3 – 20–99 cows	40	40
	4 – 100–199 cows	18	19
	5 – 200–399 cows	23	23
	4 – >400 cows	18	17
Has the farm purchased new animals within three previous years (purc)	0 – no	48	48
	1 – yes	51	51
Does the inseminator give service to other farms (insoth)	0 – no	26	
	1 – yes	73	
Using bull to inseminate heifers (bullh)	0 – no	62	61
	1 – yes	39	38
BVDV present in the herd (BVD)	0 – no	75	76
	1 – yes (at least one tested positive animal)	24	23

(continued on next page)

Table 2 (continued)

Variable	Definition of the categories of the variable	Number of herds	
		Model I	Model II
RSV prevalence (RSV)	0 – negative	44	43
	1 – 1–50%	42	43
	2 – >50%	13	13
<i>Mycoplasma bovis</i> prevalence in heifers (MBheif)	0 – <50% (median for cut-off)	41	42
	1 – >50%	58	57
<i>Mycoplasma bovis</i> prevalence in cows (MBCow)	0 – <30% (median for cut-off)	48	47
	1 – >30%	51	52
BHV-1 prevalence in cows (BHVcow)	0 – negative	38	38
	1 – 1–50%	26	28
	2 – >50%	35	33
BHV-1 prevalence in heifers (BHVheif)	0 – negative	55	57
	1 – positive	44	42

youngstock three variables (GENHEF, RESHEF, NASHEF) were used to create the summary variable “BRD in heifers” (BRDHEF). Lactration was excluded, as it was rarely seen among older youngstock (only eight herds belonged to high frequency group LACHEF = 1). For heifers, if at least two out of three variables had a value of one, the herd was considered to be in the category of “high frequency of BRD in heifers” (BRDHEF = 1) (Table 2).

To obtain more variability in the dependent variables in the smallest herd size category, herds of 20–49 and 50–99 cows were merged into one herd size category (20–99 cows). All the continuous independent variables were transformed into categorical variables in order to avoid violating the assumption of linearity in logistic regression analysis and to carry out a MCA (see Table 2).

2.4. Data processing

In order to select predictor variables to include in the model, univariable logistic regression analysis was performed. Only variables with a *p*-value lower than 0.20 for any of the outcome variables were included in the data analysis. Collinearity among all the dependent and explanatory variables was checked with the chi-square test.

Two herds were excluded from data analysis due to missing values for some of the outcome variables.

In order to avoid a reduction in the number of herds in the statistical analysis because of the absence of single values in dependent or predictor variables, the original dataset was completed using an imputation technique using Stata 11 software (Stata Corporation, TX, USA). The missing values were replaced using linear regression for multiple imputation for continuous variables and using logistic regression for binary variables (command `mi impute`). For each missing data point, five imputation values were generated and the mean was calculated (Allison, 2002).

Imputed values were created for four herds for the variable “BHV-1 prevalence in cows”, for six herds for the variables “BVDV prevalence” and “*M. bovis* prevalence in heifers”, for eight herds for the variable “BRSV prevalence in heifers”, and for one herd for “*M. bovis* prevalence in cows”.

MCA was used to obtain an overview of the associations among variable categories and to reduce multicollinearity (Ribbens et al., 2008; Thomsen et al., 2007) where there are a large number of explanatory variables. The main objective of MCA is to summarize the associations within a set of categorical variables in a small number of dimensions, and to interpret a low-dimensional (often a two-dimensional) graphical representation of these associations (Dohoo et al., 1996). The main advantage of MCA is that it takes into account two-way interaction effects in determining the

solution, even if the number of variables is large. The test values are considered the standardized coordinates, and are used to interpret the significant variable categories to build each component, i.e. with absolute test values higher than the threshold value of 1.96 (Lebart, 2006). The test values are interpreted as the number of standard deviations from the center of gravity of the analysis. The MCA was performed using XLSTAT (Version 2010.4.01; Addinsoft). All the explanatory variables were incorporated as active variables in the analysis, whereas the dependent variables were projected as supplementary illustrative variables on the already existing configuration (Greenacre and Blasius, 2006). Cumulative contributions of the variables were also calculated, and variables with the lowest cumulative contributions were not included in the final MCA.

Logistic regression models were constructed to quantify estimates of the relationships among dependent and predictor variables using the logit function of Stata 11. All the variables selected in the univariable analysis, and not highly associated in the MCA display, were included in the multivariable logistic regression model. Non-significant variables were removed from the model with a backward elimination procedure. The change in regression coefficients was noted to identify important confounding factors, and if interaction was suspected the interaction term was included and controlled for significance. The fit of the model was evaluated with the Hosmer–Lemeshow goodness-of-fit test (Dohoo et al., 2009).

3. Results

Fig. 1 shows the relationships among the various dependent and explanatory variables for Model I. All four dependent variables were correlated with each other. Fifty-four percent of the pairs of explanatory variables belonging to the group of infections, and 69% from the management factor group, had significant collinearity with the dependent variables. The within-group correlation was 27% for the block that comprised variables related to infections, and 61% for the management block. In addition, 43% of the variables from these two explanatory blocks had significant correlations with each other.

In Model II, all four dependent variables were significantly correlated with each other according to the chi-square analysis. Overall, 42% of the variables related to management and 50% of those related to infections had a *p*-value of less than 0.05 using the chi-square test with dependent variables.

The results of the MCA for Model I, highlighting the most relevant relationships among the sets of variables, are given in Fig. 2. Owing to relatively low collinearity with other variables (Fig. 1)

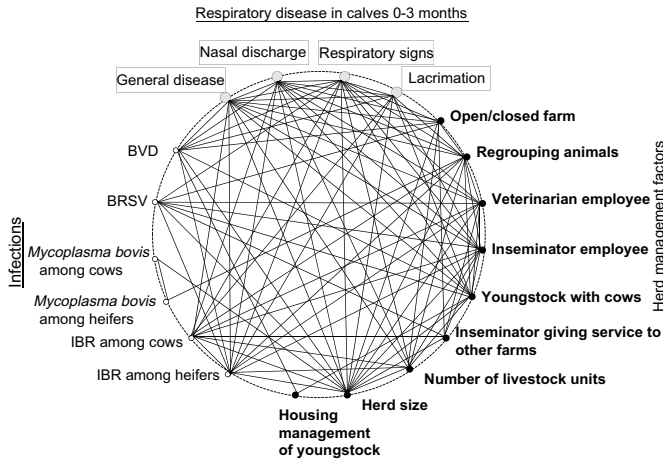


Fig. 1. Relationship between all the dependent and independent variables checked using chi-square test in Model 1 (threshold value 5% of significance level).

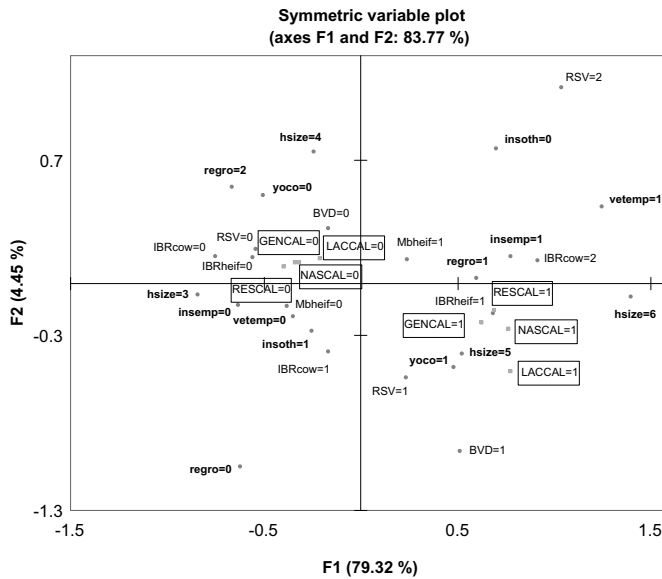


Fig. 2. Graphical display of multiple correspondence analysis, with respect to axes 1 and 2 for high incidence of respiratory disease signs in calves up to 3 months old (99 herds).

and/or low cumulative contribution in the MCA analysis, four variables were excluded from the final analysis (*M. bovis* prevalence in cows, number of units, management for youngstock, and purchase of animals). The cumulative inertia calculated from the eigenvalues of the first two axes accounted for 83.19% of the total variance of the dataset (78.08% and 5.10% for dimensions 1 and 2, respectively). All the dependent variables were highly clustered on the graphical display. The first (horizontal) axis reflected a clear subdivision of the responses towards the dependent variables giving corroborative information. In the text which follows, the term

“high frequency of BRD in calves” is used in relation to one category for each of the four dependent variables, i.e. positive variable coordinates on the first MCA axis. The interpretation of the two-dimensional plot of MCA is based on the proximities of the points which correspond to the variables categories. The distance of a variable from the origin of the plot reflects the variation from the “average response pattern”. Therefore, categories corresponding to the most frequent categories lie near the origin of the axes; however, categories with unique characteristics are located far from the intersection area. Variable categories that

Table 3
Results of logistic regression analysis for risk factors for high occurrence of respiratory disease in calves until 3 months old (99 herds).

Risk factor	Number of herds	OR	p-Value	95% CI
<i>BHV-1 prevalence in cows^a</i>				
0	38	1	–	–
1–49%	26	14.8	0.005	2.3; 95.5
≥50%	35	19.2	0.002	3.0; 121.8
<i>BVDV in heifers</i>				
0	75	1	–	–
1	24	5.1	0.020	1.3; 20.1
<i>Herd size^b</i>				
20–99 cows	40	1	–	–
100–199 cows	18	1.7	0.557	0.3; 9.3
200–399 cows	23	8.0	0.008	1.7; 37.3
≥400 cows	18	6.4	0.029	1.2; 33.8

^a BHV-1 prevalence in cows $p = 0.006$ in multiple Wald test.

^b Herd size $p = 0.021$ in multiple Wald test.

occur predominantly together are plotted close to each other, whereas variable categories, which are rarely associated with each other, are plotted far apart (Ribbens et al., 2008). The dependent variables are mainly associated with the first axis, whereas the second (vertical) MCA axis reflects the outlying position of the variable categories. It means that the variables associated with the second axis are important to split up herds but have no specific relation with the outcome under study.

In Model II, the two MCA axes accounted for 82.70% of the data variation (79.85% and 2.85% for dimensions 1 and 2, respectively). Two variables (using a bull to inseminate heifers and *M. bovis* prevalence in cows) had low partial contribution values and the variable “number of units” showing high collinearity between variable “regrouping animals” were not included in the final MCA.

In logistic regression analysis, the variable “herd size” acted as a confounder and was therefore retained in the models.

3.1. BHV-1 as a risk factor for occurrence of respiratory disease in calves and pre-breeding heifers

A high frequency of respiratory disease in calves was associated with a high prevalence of BHV-1 among cows (test value 6.715), and the presence of BHV-1 among youngstock (6.097), BRSV prevalence > 50% (4.006) and the presence of BVDV in a herd (2.885) (Fig. 2). The results of the logistic regression model are presented (Table 3). Moderate (1–49%) and high (≥50%) seroprevalences of BHV-1 among cows were both related to a high frequency of respiratory disease in calves (OR = 14.8, CI 2.3; 95.5 and OR = 19.2, CI 3.0; 121.8, respectively). The presence of BVDV in the herd was also associated significantly with a high incidence of respiratory disease among calves (OR = 5.1, CI 1.2; 20.1).

A high prevalence of BHV-1 among cows (test value 6.440) and presence of BHV-1 among heifers (6.278), positive status for BVDV (3.881) and high prevalence of BRSV (2.984) were all related to a high incidence of respiratory signs among older youngstock according to the MCA. In the logistic regression analysis, only the BVD was significantly associated with a high incidence of respiratory disease among pre-breeding heifers (OR = 4.3, CI 1.2; 15.8) (Table 4).

3.2. Management risk factors related to a high incidence of respiratory disease in calves and pre-breeding heifers

According to the results of the MCA, several management-related variables, such as the veterinarian (test value 6.790) and inseminator (6.885) being employees of the farm, the inseminator

Table 4
Results of logistic regression analysis for risk factors for high occurrence of respiratory disease in heifers 3–16 months old (99 herds).

Risk factor	Number of herds	OR	p-Value	95% CI
<i>BVDV in heifers</i>				
0	76	1	–	–
1	23	4.3	0.027	1.2; 15.8
<i>Herd size^a</i>				
20–99	40	1	–	–
100–199	19	4.9	0.113	0.7; 34.4
200–399	23	5.3	0.065	0.9; 31.6
≥400	17	8.1	0.022	1.4; 49.1

^a Herd size $p = 0.137$ in multiple Wald test.

not providing a service to any other farms (4.157) and keeping youngstock separately from cows from six months until pregnancy (4.854) were related to a high prevalence of respiratory disease in calves (Fig. 2). In general, the highest herd size category was related to a higher disease incidence of respiratory signs from the results of the logistic regression analysis (Table 3) and MCA (test value 6.529) (Fig. 2). The second (vertical) MCA axis reflects the outlying position of the variable categories for “relocating animals between the barns”. This means that this variable is important when dividing herds into groups, but has no specific relationship with the disease signs studied.

Management factors such as the veterinarian (6.355) and inseminator (6.345) being the employees of the farm, keeping youngstock separately from cows from six months until pregnancy (5.009) and purchasing animals (3.948), were related to a high incidence of respiratory disease in heifers from the results of the MC analysis. In each larger herd size category, the risk of having a high incidence of respiratory disease increased approximately twice with OR = 10.2 (CI 1.6; 64.3) in the largest herd size category (test value 6.783 in MCA).

4. Discussion

4.1. Contribution of statistical methods

A high level of collinearity was detected between the different variables and between the different variable blocks when analyzed with the chi-square test. Multicollinearity is a frequent problem in epidemiological studies that evaluate a large number of risk factors simultaneously (Dohoo et al., 1996). Dohoo et al. (1996) suggested that correspondence analysis can be an alternative when analyzing complex relationships among variables. Various types of information can be obtained from the MCA. Although we had three to four different dependent variables, all outcome variables within the same category were closely linked. This enabled us to divide the herds into two groups when interpreting the results: herds with high and low occurrence of clinical signs of respiratory disease. Secondly, we could detect possible risk factors closely linked with the dependent variables. In addition, the associations among different independent variables could be detected.

Logistic regression analysis was performed in order to identify significant risk factors with exact quantified estimates, and to obtain additional information about confounding effects. Therefore, the results of both statistical methods are necessary for the interpretation of the data.

4.2. BHV-1 as a risk factor for high occurrence of respiratory disease in calves and pre-breeding heifers

Respiratory disease incidence was measured retrospectively. For this reason it is not possible to confirm the exact role of all of the studied infections in the incidence of respiratory disease in

dairy herds but it is possible to make some conclusions about the contribution of these infections to BRD incidence among calves and heifers.

BHV-1, BVDV and BRSV were related to a high incidence of respiratory disease in dairy youngstock, according to the results of MCA. The presence of BHV-1 in cows and BVDV existing in a herd were significantly related to signs of respiratory disease in calves, whereas BRSV was an insignificant factor according to logistic regression analysis. The proportion of BHV-1 seropositive cows reflected the ratio of calves that might receive protective BHV-1 antibodies via the colostrum (Mechor et al., 1987). According to the results of this study respiratory disease was observed in calves despite a presumably high level of maternal antibody protection. However, because the occurrence of respiratory disease signs was evaluated retrospectively, herd immunity might have been low during that period, whereas at the time of testing a high prevalence of antibodies might have been the consequence of a recent virus circulation in the herd. On the other hand, according to the authors' experience, calves are rarely provided adequate colostrum in Estonia. Penny et al. (2002) indicated that respiratory disease is more often seen in calves of primiparous cows, probably because of a lack of previous exposure to BHV-1 by their dams. Therefore a number of calves may contract the disease caused by BHV-1 as a result of weak maternal immunity.

BVDV was significantly associated with high respiratory disease incidence in calves until three months old as well as in 3–16-month-old heifers according to the logistic regression analysis. In endemically infected herds, young animals losing their maternal immunity are most susceptible to the virus, and are therefore most prone to experience clinical disease caused by BVDV. However, BVDV infection in the herd can be a significant risk factor for respiratory disease in calves up to 90 days of age (Lundborg et al., 2005). BVD infection may directly result in respiratory disease, but evidence suggests that BVDV infections potentiate BRD via immunosuppression and synergism (Ridpath, 2010).

BHV-1 was not significantly associated with a high incidence of BRD among 3–16-month-old heifers in logistic regression analysis. A positive association between a high occurrence of respiratory disease signs and a high prevalence of BHV-1 among cows as well as the presence of BHV-1 among heifers was detected with MCA. Although BHV-1 is probably not related with BRD outbreaks in pre-breeding heifers; however, it cannot be excluded as one of the possible factors in the respiratory disease complex. It is common that pre-breeding heifers are the age group that receives the least human attention in the herd; therefore, the disease incidence might be underestimated. This may be the reason why a relatively low prevalence of BRD among heifers was found and significant associations with other respiratory infections were probably missed.

The high prevalence of BRSV among dairy youngstock has been found to be related to an increased risk of respiratory disease among young calves (Gulliksen et al., 2009). When BRSV initially infects a herd, respiratory disease can be observed in all age groups (Elvander, 1996), whereas in endemically infected herds the disease manifests itself mainly among naive calves (Valarcher and Taylor, 2007). In the MCA we detected significant association between high prevalence of BRSV among dairy heifers and high occurrence of respiratory disease signs among dairy calves and pre-breeding heifers. As BRSV was not a significant factor in the composite outcome of the high incidence of BRD in the logistic regression analysis, we may presume that slow endemic spread, rather than an acute BRD outbreak, is typically the course of BRSV among youngstock. As maternal antibodies are only partially protective after infection with BRSV (Valarcher and Taylor, 2007), the disease may manifest itself even in very young calves.

Serological testing for *M. bovis* is generally presumed to be effective in indicating exposure to the agent, and can be used to identify groups of cattle free of infection (Caswell and Archambault, 2008; Maunsell and Donovan, 2009). In contrast to BHV-1, BVDV and BRSV (Elvander, 1996; Houe, 1994; Muylkens et al., 2007), antibodies to *M. bovis* post infection do not persist life-long (Maunsell and Donovan, 2009). Given that maternal antibodies against *M. bovis* probably have no protective effect against infection, young calves can be infected at a very early age (Maunsell and Donovan, 2009). However, our results indicate that the presence of *M. bovis* infection in older animals (above 6 months of age) does not necessarily have an impact on the incidence of BRD in calves. Further research using clinical specimens is needed to determine the role of *M. bovis* in the pathogenesis of BRD in young dairy calves.

4.3. Herd management-related risk factors for the high occurrence of respiratory disease signs

Large herd size has been found to be a risk factor for a high incidence of respiratory disease in many studies (Gay and Barnouin, 2009; Gulliksen et al., 2009; Norström et al., 2000). Any infectious agent will establish itself more easily in a large herd because of higher animal density, a greater degree of direct contact between animals, and a larger number of susceptible animals (Gay and Barnouin, 2009; Gulliksen et al., 2009; Raaperi et al., 2010). Large herd size, as well as the presence of BRD, is also associated with increased intra-farm traffic of professional employees such as veterinarians and AI-technicians, as confirmed previously (Gulliksen et al., 2009; Norström et al., 2000; Raaperi et al., 2010). This emphasizes the importance of human-mediated virus spread. An interesting finding was that the housing management for youngstock (loose or tied) was not related to disease incidence. This indicates that direct contact is not always obligatory for effective viral spread. Housing youngstock in a separate building from 6 months until pregnancy also increased the risk of BRD in older youngstock. This may be the effect of relocation and mixing of animals with different infection and immune statuses on the incidence of BRD. This finding also indicates that direct contact with the adult cow population is not always necessary for the maintenance of infection among youngstock.

5. Conclusions

The results of this study demonstrate that although several infectious and management-related factors are associated with BRD in dairy calves and heifers, BHV-1 and BVDV had significant impact on the manifestation of clinical respiratory disease. BVDV contributed to the occurrence of respiratory disease signs in older youngstock. The use of control measures for these viruses may reduce the incidence of respiratory disease and improve herd health.

In order to reduce the incidence of BRD in dairy youngstock, on-farm biosecurity measures are important to prevent human-mediated spread of the disease. Relocating animals within the farm may predispose to more active viral spread, leading to more BRD cases. Newly introduced animals can serve as the source of infection; therefore, quarantine measures should be applied to avoid BRD outbreaks.

Conflict of interest statement

Authors of this research paper have no financial or personal interests that could have influenced the output of this paper.

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RESEARCH

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Association of herd BRSV and BHV-1 seroprevalence with respiratory disease and reproductive performance in adult dairy cattle

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Abstract

Background: The aim of this study was to detect the associations between bovine herpesvirus 1 (BHV-1) status of a herd and respiratory disease (BRD) occurrence and reproductive performance in pregnant heifers and cows. The association between management-related factors and higher BRD occurrence was also estimated.

Methods: Serum samples, collected from cows and youngstock from 103 dairy cattle herds, were analyzed for antibodies against BHV-1, bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), and *Mycoplasma bovis*. A questionnaire was used to collect data concerning herd management factors and reproductive performance, as well as the occurrence of clinical signs of respiratory disease in the last two years, as evaluated by the veterinarian or farm manager. Multiple correspondence analysis (MCA) and logistic regression analysis were performed to identify and quantify the risk factors.

Results: A low to moderate prevalence (1–49%) of BRSV antibodies among youngstock was associated with a high occurrence of respiratory disease (OR = 6.2, $p = 0.010$) in cows and in-calf heifers. Employees of the farm may participate in the spread of such disease. Larger herd size, loose-housing of cows, housing youngstock separately from cows until pregnancy, and purchasing new animals were factors possibly related to a high occurrence of respiratory disease symptoms in pregnant heifers and cows. The highest risk of abortions (> 1.3%) and increased insemination index (number of inseminations per pregnancy) (> 1.9) occurred in herds with a moderate prevalence of BHV-1 antibodies (1–49%) in cows.

Conclusions: BHV-1 was not associated with acute respiratory disease in adult dairy cattle, however was significantly related to reproductive performance. BRSV possesses the main role in respiratory disease complex in adult dairy cattle.

Keywords: Bovine respiratory disease, reproduction, dairy cattle, bovine herpesvirus 1, bovine respiratory syncytial virus

Background

Bovine respiratory disease (BRD) incorporates all possible respiratory diseases in cattle and is characterised by abnormal clinical signs of the respiratory tract [1]. Bovine respiratory disease refers to bacterial bronchopneumonia that may be complicated by previous, or concurrent, viral or *Mycoplasma* infection [2]. The principal viruses involved in BRD include bovine herpesvirus 1

(BHV-1), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (PI-3) and bovine viral diarrhoea virus (BVDV) [2]. Despite advances in veterinary medicine, animal husbandry, and animal welfare, respiratory disease among dairy cattle continues to be a major problem in the dairy industry [3]. In addition to enzootic calf pneumonia, outbreaks of respiratory disease in adult animals can have devastating economic outcomes for dairy owners [3].

Many studies have been performed to detect animal-level risk factors for respiratory disease in young calves, whereas the literature concerning BRD in adult dairy

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cattle is deficient [1,3]. In adult dairy cattle, respiratory disease is less important than mastitis, lameness, or reproductive disorders as a cause of morbidity [2]. According to the Annual Report of the Estonian Animal Recording Centre (EARC, 2009), BRD was the reason for culling dairy cows in 0.7% of cases. According to our experience, in most herds BRD occurs as a sporadic disease in adult dairy cattle. However, epidemic outbreaks occur with high morbidity accompanied with dramatic economic losses due to medication use and discarded milk, as well as cow fatalities.

The subclinical course of BHV-1 infection has been observed after the introduction of the virus to a naive herd [4,5], however high morbidity of BHV-1 outbreaks involving respiratory disease symptoms (lethargy, coughing, conjunctivitis and oculonasal discharge) was seen on a number of occasions [6]. Outbreaks of severe respiratory disease due to bovine respiratory syncytial virus (BRSV) have been observed in dairy herds throughout Sweden, where adult cattle were most severely affected [7]. Risk factors associated with acute bovine respiratory disease, especially with BRSV outbreaks, were larger herd size, as well as the type of the production with a higher risk in dairy herds compared to beef herds [8,9]. Acute BRD has been found to occur mainly during cold months, with an epidemic peak in December [8]. Despite the multifactorial nature of BRD [3], only limited research data is available on herd management-related risk factors for respiratory disease in adult dairy cattle.

Poor fertility is the leading cause of culling cows in Estonia (EACR, 2009). Problems associated with reduced fertility in dairy cattle are related to: diseases of the reproductive tract of the cow, bull fertility, breeding management, and the environment [10], as well as nutrition [11]. Several infectious diseases are related to abortion in cattle, and BHV-1, BVDV and *Neospora caninum* are often diagnosed as causes of abortion in cattle world-wide [12]. However, field studies estimating the effect of BHV-1 on herd level reproductive performance have given contrary results. Previous studies [13,14] found no association between the proportion of calves with antibodies against BVDV or BHV-1 virus and reproductive performance in beef herds. A somewhat higher mean open days period was found in cows that were serologically positive for BHV-1 than in seronegative dairy cows [15], however no decrease in reproduction performance was found to occur during an outbreak of BHV-1 in a dairy herd [4]. To our knowledge no epidemiological studies have been published to identify and quantify the association between herd BHV-1 seroprevalence and farm-level reproductive performance in dairy cattle.

The objective of this study was to ascertain the associations between herd BHV-1 seroprevalence and the occurrence of acute respiratory disease and reproductive performance in adult dairy cattle. The association between management-related factors and higher BRD occurrence was also estimated.

Methods

Study design

The survey was conducted between September 2006 and April 2008. The target population in our study was dairy cattle herds with more than 20 cows. Herds were stratified according to number of cows into five classes: 20-49, 50-99, 100-199, 200-399 and > 400 cows. As the main interest of the study was to investigate the influence of BHV-1 infection on herd health, the herds were included in the study depending on their BHV-1 antibody status. For that, results of the bulk tank milk survey of BHV-1 antibodies of all dairy herds in Estonia completed in 2004 were used. Herds were selected randomly from the list by using random number generator. In total, 65 BHV-1 seropositive and 38 BHV-1 seronegative herds, matched by herd size, were selected for the study. Distribution of herds included in the study as well as information about the source population is given in Table 1.

In each of the selected herds, serum samples from a representative number of randomly selected cows, and youngstock older than six months, were analyzed. In total, 9,637 serum samples were collected. A precise description about the selection of herds, and animals within herds, has been reported in Raaperi et al. [16].

A questionnaire that recorded herd-level data was completed for every herd. The information requested included: herd size, number of livestock units per farm, deployment of the veterinarian and inseminator, frequency of movement of animals between barns, participation in agricultural shows, purchase history of cows, type of housing (cold/warm barn), housing system for cows and youngstock (loose/tied), management of youngstock (separately from cows/contact with cows for some period/in the same barn with cows), use of bull for serving cows and heifers, breed(s) of cattle, grazing management of cows and/or youngstock, vaccination history, whether employees change clothes on the farm, and information about disinfection. In addition, questions were asked relating to the history of the peak occurrence of respiratory disease, within the previous two years, of cows and pregnant heifers. The number of abortions, as well as the herd's average insemination index for cows and heifers for the previous year was recorded if registered in farm. Insemination index is defined as number of inseminations per pregnancy.

Table 1 Number of herds in Estonia in 2007 and study sample size

Herd size	Number of herds in Estonia in 2007	Study sample (BHV-1 antibody positive herds)	Study sample (BHV-1 antibody negative herds)	Study sample in total
20-49	255	9	17	26
50-99	110	7	9	16
100-199	85	14	5	19
200-399	83	18	6	24
≥400	59	17	1	18
Total	592	65	38	103

Sample analysis

All serum samples were tested for BHV-1 antibodies using a commercial BHV-1 gB ELISA test kit, Herd-Chek® (IDEXX, Switzerland) having 100% sensitivity and 99.8% specificity. Suspect antibody test results (samples with blocking % greater than or equal to 45% but less than 55%) were considered as positive in the data analysis.

The herd BVDV status was established by testing up to 10 serum samples from randomly selected animals, at ages from six months up to age at first calving, for BVDV antibodies as recommended by Houe et al. [17]. This enabled detection of a minimum prevalence of 20-28% depending on herd size. The PrioCheck BVDV Ab test kit (Prionics AG, Switzerland) was used for antibody testing. The test has a relative sensitivity and specificity of approximately 98% and 99%, respectively, compared to a virus neutralization test [18].

The herd BRSV status was established by testing up to 20 (depending on herd size) randomly selected serum samples from heifers for BRSV antibodies to allow detection of at least a 15% prevalence of BRSV antibody carriers in the herd at a 95% confidence level assuming 94.6% sensitivity and 100% specificity of the test. For BRSV antibodies, the Svanovir ELISA test (Svanova Biotech AB, Sweden) was used.

Depending on herd size up to 25 heifers and 10 cows were tested for *Mycoplasma bovis* antibodies in each herd. This enabled to detect the prevalence of at least 15% among heifers and 27% among cows with 95% level of confidence. BIO K 260 ELISA test (Bio-X Diagnostics, Belgium) with sensitivity and specificity of 100% in 10% cut-off of optical density of the positive control was used to measure *M. bovis* antibodies.

Description of the models and categorization of variables

The aim of the first model (Model I) was to clarify the association between herd BHV-1 seroprevalence and respiratory disease occurrence in adult dairy cattle, as well as the detection of management factors associated with higher BRD occurrence. On each farm the veterinarian or farm manager was questioned about the occurrence of clinical signs of respiratory disease,

including nasal discharge ("red nose"), respiratory signs (cough, dyspnoea), and lacrimation. The respondents were asked to evaluate the prevalence of these signs among animals of each age group during periods of the highest occurrence of respiratory disease in the previous two years. The scale of the estimation was presented as follows: 1 - no signs or only single cases; 2 - up to 10%; 3 - 10-30%; 4 - over 30%. In order to dichotomize the outcome variables the value of the outcome variables was taken as 1 if more than just a single animal showed the signs concurrently. All of the three variables (see Table 2) were chosen as supplementary variables in Model I when multiple correspondence analysis (MCA) was carried out.

As all the respiratory disease symptoms were highly clustered in the MCA analysis (see Results section), one summary variable, describing the level of occurrence of respiratory disease, was created for use in logistic regression analysis. Three variables (RESCOW, NASCOW and LACCOW) were used to create one summary variable. If at least two out of three variables had a value of one, the herd was considered to be in the category of "high occurrence of BRD in cows and/or pregnant heifers" (BRDCOW = 1) (Table 2).

The aim of the second model (Model II) was to detect the linkage between herd BHV-1 seroprevalence and poor reproductive performance in cows and heifers, by taking into account the effect of a possible confounding effect of herd size and other infectious diseases. For that, two outcome variables dichotomized at their median value (1.3% for the proportion of abortions and 1.9 for insemination index) were used in the logistic regression analysis (Table 2).

To obtain more variability in the outcome variables in the smallest category of herd size, herds with 20-49 and those with 50-99 cattle were merged into one smallest herd size category (20-99 cows). All the continuous independent variables were transformed into categorical variables in order to avoid violating the assumption of linearity in logistic regression analysis, and to carry out MCA. The categorization and descriptive statistics of the variables used for the statistical analyses in the models are shown in Table 2.

Table 2 Descriptive characteristics of the variables included in the models (100 herds in Model I and 77 herds in Model II)

Variable	Definition of the categories of the variable	Number of herds	
		Model I	Model II
Nasal discharge ("red nose") in cows and/or pregnant heifers (NASCOW)	0 - not present at all or was shown only as single cases at some point during the last two years	82	
	1 - present in more than just single cases at some point during the last two years	18	
Respiratory symptoms (cough, dyspnoea) in cows and/or pregnant heifers (RESCOW)	0 - not present at all or was shown only as single cases at some time point during the last two years	80	
	1 - present in more than just single cases at some point during the last two years	20	
Lacrimation in cows and/or pregnant heifers (LACCOW)	0 - not present at all or was shown only as single cases at some point during the last two years	88	
	1 - present in more than just single cases at some point during the last two years	12	
Respiratory disease occurrence in cows and/or pregnant heifers (BRDCOW)	0 - less than two respiratory disease symptoms were present in more than a single case at some time during the last two years	81	
	1 - at least two respiratory disease symptoms were present in more than a single case at some time during the last two years	19	
Incidence of abortions in the herd (ABORT)	0 - < 1.3% in a herd (median for cut-off value)		37
	1 - ≥ 1.3% in a herd		40
Insemination index for cows and heifers (INSIN)	0 - < 1.9 in a herd (median for cut-off value)		38
	1 - ≥ 1.9 in a herd		39
Herd size (hsize)	3 - 20-99 cows	40	25
	4 - 100-199 cows	19	17
	5 - 200-399 cows	23	21
	6 - > 400 cows	18	14
Veterinarian an employee of the farm (vetemp)	0 - no	77	
	1 - yes	23	
Does the inseminator give service to other farms (insoth)	0 - no	28	
	1 - yes	72	
Inseminator an employee of the farm (insemp)	0 - no	56	
	1 - yes	44	
Using bull to inseminate heifers (bullh)	0 - no	61	
	1 - yes	39	
Keeping youngstock together with cows (yoco)	0 - together	51	
	1 - in separate building from 6 months until pregnancy	49	
Housing for youngstock (keyo)	1 - tied	23	
	2 - loose	30	
	3 - some period of life tied, some period loose	47	
	1 - tied	71	
Housing for cows (keco)	2 - loose	29	
	0 - no	47	
Has the farm purchased new animals within the last three years (purc)	0 - no	47	
	1 - yes	53	
BVDV present in the herd (BVD)	0 - no	77	57
	1 - yes (at least one animal tested positive)	23	20
RSV prevalence (BRSV)	0 - negative	46	34
	1 - 1 - 49%	40	33
	2 - ≥ 50%	14	10
BHV-1 prevalence in cows (BHVcow)	0 - negative	37	25
	1 - 1 - 49%	28	24
	2 - ≥ 50%	35	28
BHV-1 prevalence in heifers (BHVheif)	0 - negative	56	40
	1 - positive	44	37

Data analysis

In order to select predictor variables to include in the model, univariable logistic regression analysis was performed. Only variables with a p-value lower than 0.2 for any of the outcome variables were included in the data analysis (Table 2). Variables that were not associated with dependent variables in a p-value of ≤ 0.2 and not included in the model were '*Mycoplasma bovis* prevalence in cows', '*Mycoplasma bovis* prevalence in heifers', 'relocating animals between the barns', 'frequency of overgrouping animals in the farm', 'using bull to inseminate cows', 'grazing youngstock', 'grazing cows' and 'number of livestock units within the farm'. Collinearity among all the outcome and explanatory variables was checked with the chi-square test. Variables 'housing of cows' (tied/loose) and 'barn type' (cold/warm) were highly collinear and had the same explanation so only the former was included in the models having better explaining capacity. Two herds were excluded from the data analysis owing to a large number of missing values. In order to avoid a reduction in the number of herds in the statistical analysis because of the absence of single values in some of the predictor variables, the original dataset was completed using an imputation technique in Stata 11 software (Stata Corporation, Texas, USA). The missing values were replaced using linear regression for multiple imputation for continuous variables and using logistic regression for binary variables (command *mi impute*). For each missing data point, five imputation values were generated and the mean was calculated [19].

Imputed values were created for four herds for the variable "BHV-1 prevalence in heifers" (4%), for six herds for the variables "BVDV prevalence" and "*Mycoplasma bovis* prevalence in heifers" (6%), for eight herds for the variable "BRSV prevalence in heifers" (8%), and for one herd for "*Mycoplasma bovis* prevalence in cows" (1%) in the initial dataset. In Model I, values were missing for some outcome variables for one herd and this herd was excluded from the data analysis. In Model II 24 herds were excluded from the analysis owing to missing values in either of the two outcome variables.

Multiple correspondence analysis (MCA) was used to obtain an overall view of the associations among variable categories in Model I, and to avoid problems arising due to multicollinearity. The test values are considered the standardized coordinates, and are used to interpret the significant variable categories to build each component, *i.e.* with absolute test values higher than the threshold value of 1.96 [20]. The test values are interpreted as the number of standard deviations from the centre of gravity of the analysis. The MCA was performed using XLSTAT (Version 2010.4.01; Addinsoft).

Logistic regression models were built to quantify estimates of the relationships among outcome and predictor

variables. All the variables with p-value < 0.2 selected in the univariable logistic regression analysis [21] were included in the multivariable logistic regression model. For the final multivariable model variables with p-value > 0.05 were excluded with backward elimination procedure [21]. The *logit* function of Stata 11 (Stata Corporation, Texas, USA) was applied for logistic regression analysis. The change in regression coefficients was noted to identify important confounding factors (change in regression coefficients between the crude and adjusted value of $> 20\%$ [21]), and biologically meaningful interaction were tested. The fit of the model was evaluated with the Hosmer-Lemeshow goodness-of-fit test [21]. There was no indication of lack of fit in the logistic regression models.

Results

Risk factors for a high occurrence of respiratory disease symptoms in cows and pregnant heifers (Model I)

The three outcome variables NASCOW, RESCOW and LACCOW were significantly related to each other in the chi-square test. Overall, 33% of the variables related to infections, and 19% of those related to herd management were significantly associated with the outcome variables. Variables describing *Mycoplasma bovis* prevalence in cows and youngstock were not related to any of the outcome variables in the univariable analysis.

The total cumulative inertia for the first two axes in the MCA was 80.39% (75.01% and 5.38% for axes 1 and 2). According to the MCA, the management-related variables linked to the first axis, and significantly related to the high occurrence of respiratory disease symptoms, were the largest herd size category (test value 7.35), loose housing of cows (6.20), the veterinarian (6.61) and inseminator (6.60) being the employees of the farm and the latter not providing a service to other farms (4.21), keeping youngstock separately from cows from 6 months until pregnancy (4.77), and purchasing new animals for the herd (4.51) (Figure 1). Infections that were related to a high occurrence of respiratory disease signs in cows and pregnant heifers were: a high prevalence of BHV-1 among cows (6.54) and the presence of BHV-1 among heifers (6.15), the presence of BVDV in a herd (3.95), and a high prevalence of BRSV (3.74).

A low to moderate prevalence of BRSV measured in youngstock (1-49%) was significantly related to a higher occurrence of respiratory disease (OR = 6.2, CI 1.6; 25.0, $p = 0.010$) among cows and pregnant heifers, according to the results of the logistic regression analysis. Herd size was an insignificant variable in the model, but acted as a confounder and was therefore retained (Table 3).

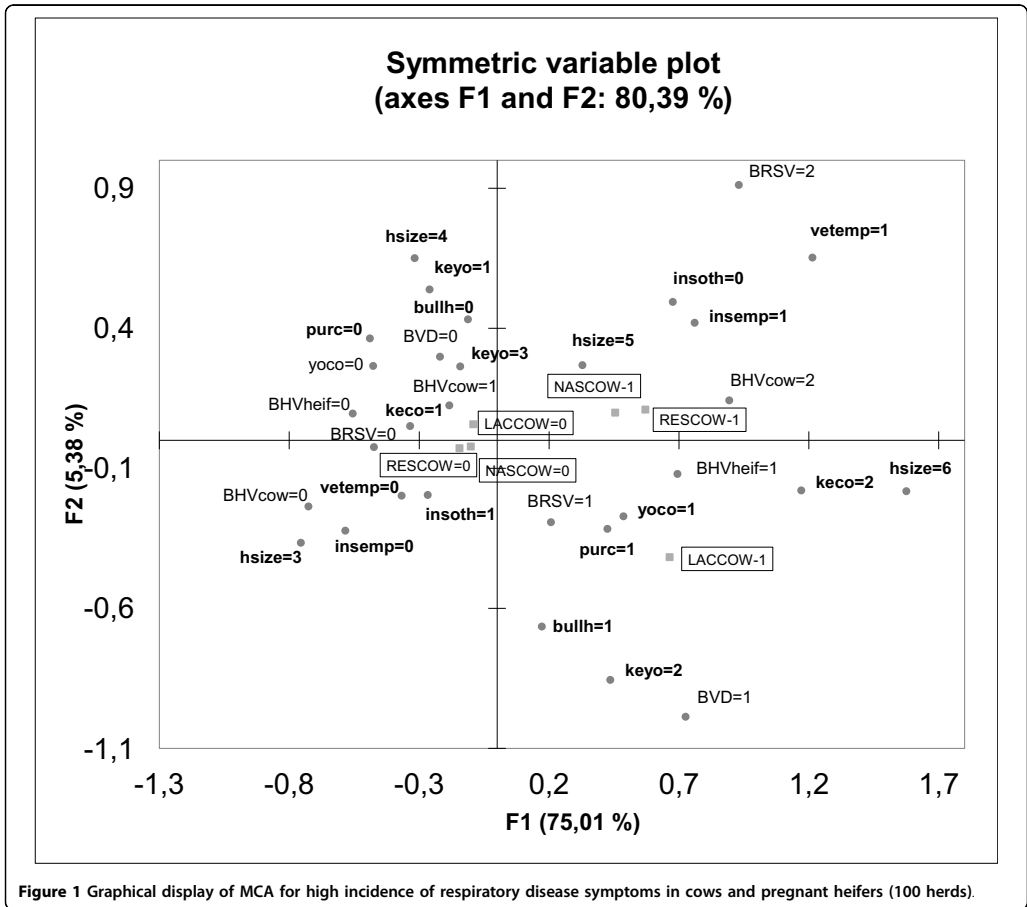


Table 3 Results of logistic regression analysis for risk factors for high occurrence of respiratory disease in pregnant heifers and cows (100 herds)

Risk factor	Herds (n)	OR	p	95% CI
BRSV prevalence in heifers ^a				
0	46	1	-	-
1-49%	40	6.2	0.010	1.6; 25.0
≥50%	14	1.3	0.769	0.2; 8.2
Herd size ^b				
20-99 cows	40	1	-	-
100-199 cows	19	1.3	0.81	0.2; 8.2
200-399 cows	23	2.8	0.160	0.7; 11.8
> 400 cows	18	4.7	0.052	1.0; 22.8

^aBRSV prevalence in cows p = 0.017, the Wald test

^bHerd size p = 0.208, the Wald test

BHV-1 as a risk factor for a high incidence of abortions and high insemination index in breeding animals (Model II)

The results of the logistic regression analysis indicated that, in herds in which BHV-1 is present, among cows the incidence of abortions and the insemination index were higher than those in herds negative for BHV-1. A low to moderate prevalence of BHV-1 among cows (1-49%) was related to the highest risk of a higher incidence of abortions (OR = 7.3, CI 2.0; 26.9, p = 0.003) and an increased insemination index (OR = 5.2, CI 1.5; 18.4, p = 0.010) in a herd. Herd size, as a confounding variable, was also controlled in the model (Table 4).

Table 4 Results of logistic regression analysis for risk factors for high abortion and insemination index in cows and heifers (77 herds)

Risk factor	Herds (n)	abortion			insemination index		
		OR	p	95% CI	OR	p	95% CI
BHV-1 prevalence in cows ^a							
0	25	1	-	-	1	-	-
1-49%	24	7.3	0.003	2.0; 26.9	5.2	0.01	1.5; 18.4
≥50%	28	4.6	0.022	1.2; 16.7	3.4	0.056	1.0; 12.3
Herd size ^b							
20-99 cows	25	1	-	-	1	-	-
100-199 cows	17	1.2	0.754	0.3; 5.0	0.8	0.725	0.2; 3.0
200-399 cows	21	1.7	0.447	0.4; 6.5	1.2	0.83	0.3; 4.2
> 400 cows	14	0.3	0.165	0.1; 1.6	0.9	0.944	0.2; 4.2
				^a BHV-1 in cows p = 0.009, the Wald test		^a BHV-1 cows p = 0.033, the Wald test	
				^b Herd size p = 0.193, the Wald test		^b Herd size p = 0.957, the Wald test	

Discussion

Risk factors for a high occurrence of respiratory disease symptoms in cows and pregnant heifers

Three symptoms most commonly related to respiratory disease were asked to evaluate by the respondents. As relatively small number of herds had values equal or higher than 'up to 10%', the variables were dichotomised separating herds with high or low occurrence of that symptom. In the graphical display of MCA all three respiratory disease symptoms were closely linked meaning that those were present concurrently in most of the herds. This encouraged us to create one summary outcome variable describing the occurrence of respiratory disease. We find that asking more precise preliminary information from the respondents and controlling the explanation capability of the variables before combining those into one outcome variable has decreased the recall bias and gives easily interpretable results.

BHV-1, BVDV and BRSV are associated with a high occurrence of respiratory disease in Estonian adult dairy cattle, according to the results of the MCA. A high prevalence of BRSV (≥50%) was associated with a high occurrence of respiratory disease symptoms in cows and pregnant heifers in the MCA. When combining these three BRD symptoms into one outcome variable in logistic regression analysis, a low to moderate prevalence of BRSV (1-49%) among youngstock was significantly associated with a high occurrence of respiratory disease among cows and pregnant heifers. This discrepancy may arise from the fact that relatively small number of herds (n = 14) belong to the highest BRSV prevalence group. This may result in larger standard errors of the estimates in the logistic regression analysis affecting also the p-value of the predictor. MCA on the other hand is not as sensitive to sample size as conditional methods. Sampling antibodies from a small number of young animals that have lost maternal immunity indicates the recent spread

of infection [22]. However, some studies have shown that outbreaks of acute respiratory disease associated with BRSV in fully susceptible populations affect adult cattle, pregnant or newly calved cows, most severely [7,9]. Thereafter the disease remains endemic, manifesting itself among younger animals that serve as sentinels [7]. Given that the signs of respiratory disease reported in this study were those associated with the occurrence of respiratory disease in the previous two years, cows and pregnant heifers might have experienced disease caused by BRSV some time previously, following the active spread of the virus among youngstock detected in this study at the time of testing. In a severe outbreak of BRSV in Sweden it was found that concurrent infection with other viruses may affect the expression of disease [7]. In addition to BRSV BHV-1 and BVDV were associated with higher occurrence of BRD in MCA. As associations between variables are not adjusted for the effects of other variables with this method we can't state that BHV-1 and BVDV are direct risk factors for BRD. However, apparent bivariate association between these variable gives a reason to suggest that BHV-1 and BVDV may participate in the expression of BRD rather as contributing agents.

Large herd size has been found to be a risk factor for the high occurrence of respiratory disease in many studies [8,9,23]. Elvander [7] has shown that BRSV spreads rapidly within the herd. In larger dairy herds there are more numerous between-animal contacts [8], increased inter- and intra-farm traffic by farm employees such as veterinarians and AI-technicians [8,9,16,23] as well as potential higher animal densities [8] allowing the more efficient spread of infectious agents. The number of animals susceptible to infections in large herds is also higher than in small herds contributing maintenance of infections within a herd over extended periods [23].

MCA has spotlighted several other management practices as possible risk factors for BRD in adult dairy

cattle. Although these associations are not conclusive due to the limitations of MCA, they are worthwhile to mention here as factors likely contributing to the disease and requiring attention. First, loose housing of cows was associated with a higher level of BRD in cows and pregnant heifers. We may suggest that more direct contacts between the animals, and the frequent regrouping of animals in loose housing barns, create greater possibilities for the direct transmission of the infectious agents over the whole farm.

Second, housing youngstock in a separate building from six months of age until service was associated with a higher occurrence of BRD. In order to maintain an immunizing infection, the susceptible pool must be replenished via recruitment [24]. Depending on the pattern of infectious disease epidemiology within the herd, commingling animals with different immunity status to specific infections may predispose the active circulation of the virus.

Newly purchased animals can be the source of BRSV infection, which was confirmed in a Swedish study in which outbreaks of BRSV occurred most often after the introduction of purchased animals [7].

High occurrence of respiratory disease was present in 19% of herds included in this study. Due to sporadic nature of the disease the sample size evaluating risk factors for high occurrence of BRD in adult dairy cattle should be larger. Therefore the results of this study give first insight about the risk factors associated with the disease and some factors might have been missed as significant influencing factors.

BHV-1 as a risk factor for a high incidence of abortions and increased insemination index in breeding animals

BHV-1 increases the risk of a herd having a poor reproductive performance. We can suppose that in herds with a moderate BHV-1 seroprevalence among cows, the level of infection has been low for some time, which enables a susceptible population to evolve, and it is these herds that are most vulnerable to active virus spread and a higher level of endemic abortions. Abortions due to BHV-1 generally occur between four and eight months of gestation, however the infection can also result in early embryonic death [25] resulting in a higher insemination index. As reproduction values were registered retrospectively and antibodies present to BHV-1 reflect virus spread in the past, it is not possible to draw exact cause-effect relationships. Therefore it is possible that poor fertility as well as spread of BHV-1 is influenced by another common factor e.g. poor management practice. A more thorough study involving farm management practices in addition to infections should be conducted.

Association between BHV-1 and fertility of cows and pregnant heifers has been evaluated previously. In field

studies, where the course of BHV-1 infection in previously naive herds was recorded, neither an increase in abortion incidence nor a lower proportion of successful inseminations was found [4,5,26]. The impact of BHV-1 on reproduction performance has also been evaluated indirectly. No associations between the proportion of calves with antibodies against IBR virus and incidence of abortions, stillbirths, calf death, nor non-pregnancy were ascertained [14]. However a 17 day longer period for successful conception was required for BHV-1 seropositive rather than seronegative cows [15]. Differences in the results between studies may arise from differences in study design and discrepancies in other herd characteristics as well as the BHV-1 strain involved.

BVDV was also included in this study as a possible confounder for BHV-1, or directly related to fertility. The presence of BVDV was not associated with reproductive performance in this study. A negative impact of BVDV on reproductive efficiency of the herd has likewise not been found in previous studies [14,27,28], however significant relationships might have been missed in our study because of the relatively small sample size (only 20 BVDV-positive herds). As *Neospora caninum* is the pathogen often diagnosed as a cause of abortions in cattle [12] and related to a higher risk of non-pregnancy and abortions [13], it might be important to investigate to obtain more accurate results.

Contribution of statistical methods

Correspondence analysis is an exploratory multivariate technique for the graphical and numerical analysis [29] designed to analyse the relationships among a set of categorical variables [21]. The result is a scatterplot which identifies clusters of predictors that are closely associated, with clusters farther from the intersection of the axes having stronger associations. The values of the outcome variables were also projected on the same axes to determine which clusters of predictor variable values were associated with the outcomes of interest [21]. A high level of multicollinearity was found using chi-square analysis. Multiple correspondence analysis can be an alternative tool when analysing relationships between different variables in terms of multicollinearity [30]. MCA showed that all supplementary variables belonging to the same category were highly related; this result was used as a justification to generate one outcome variable describing high/low respiratory disease occurrence in cows and pregnant heifers. The latter variable was used as an outcome variable in the logistic regression analysis. MCA also gave an insight into associations between outcome and predictor variables, as well as associations between different predictor variables. As these associations are not adjusted for other variables in the analysis we discuss those as possibly relevant associations. MCA

is used as a preliminary analysis to have a clear and reliable view of the variable links in presence of multicollinearity. Our final conclusions confirming the risk factor status of each variable rely on the results of the logistic regression analysis.

Conclusions

The results of this study demonstrate the significant role of BRSV in the aetiology of BRD in Estonian adult dairy cattle. The presence of neither BHV-1 nor BVDV were associated with acute respiratory disease in adult dairy cattle, however these may not be excluded as possible contributors to the disease. Therefore, precautions to prevent the introduction of the BRS virus into herds should be implemented. In order to reduce the incidence of BRD in dairy cattle, on-farm biosecurity measures may be important as well to prevent human-mediated spread of the infections. As indicated in MCA direct animal contacts in loose-housing systems may increase the occurrence of BRD in cows and pregnant heifers, perhaps *via* increased virus transmission. In order to reduce the circulation of infectious agents in the system, animals should be checked for clinical signs of respiratory disease continuously, and those with symptoms separated immediately from healthy animals. If youngstock and cows are kept in separate units, the aetiology of respiratory disease among both animal groups should be ascertained, followed by the application of specific control measures in order to avoid unprotected animals becoming infected.

In herds with poor reproductive performance, BHV-1 should be considered as one of the infectious risk factors, and the eradication of this virus may improve the reproductive performance of the herd.

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Authors' contributions

KR was involved in the developing the study design, performing the field study, analysing data and writing the manuscript. SB helped analysing data and revising the manuscript. AA participated in designing the study and performing sample analysis and storage of samples. TO and AV applied for funding, attended in designing the study, analysing data and drafting the manuscript. All authors have read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Paper

Dynamics of bovine herpesvirus type 1 infection in Estonian dairy herds with and without a control programme

K. Raaperi, A. Aleksejev, T. Orro, A. Viltrop

Bovine herpesvirus type 1 (BHV-1) is an important bovine pathogen, exacerbating poor health and the productivity of cattle. The aims of this study were to detect the efficacy of vaccination programmes in lowering the seroprevalence of BHV-1 gE within the dairy herd and to follow the dynamics of the infection in non-vaccinated herds with uninfected heifers. A two-year longitudinal study was carried out on seven herds that were vaccinated, and in five herds with uninfected heifers without applying a control programme. After the start of the vaccination programme, calves born remained free from the virus. However, in one herd, 7 per cent (95 per cent CI 2 to 18) of these animals showed antibodies to BHV-1 two years after the first vaccination. A decline in BHV-1 antibody prevalence was found in vaccinating herds. Among the five herds not under the control programme, one experienced active virus spread, although one herd experienced self-clearance of the virus. In the herds with high BHV-1 prevalence, vaccinating all cattle from three months of age twice a year with a commercial inactivated marker vaccine efficiently protected offspring from becoming infected, and lowered the prevalence of BHV-1 within the herd. A small proportion of herds may experience self-clearance of the virus.

BOVINE herpesvirus type 1 (BHV-1) is widespread, and is an important pathogen of cattle that causes substantial economic losses due to reproductive failure and increased calf mortality, as well as respiratory disease (Kampa and others 2004). In Estonia, 22 per cent of the dairy cattle herds are BHV-1 positive, and within-herd prevalence increases with herd size (Raaperi and others 2010). These BHV-1 infected herds could benefit economically by improving the health status of the animals by eradicating the virus from the herd. In Estonia, no systematic control programmes have been applied against BHV-1, except for bulls used for semen collection in artificial insemination (AI) centres. All animals introduced to the AI centre must be isolated in their herd of origin, tested and be confirmed negative to BHV-1 antibodies 30 days before movement (Anon 2008). Bulls used for semen collection are tested serologically once a year (Anon 2004). Several countries in Europe as Austria, Denmark, Norway, Sweden, Finland and Switzerland are BHV-1 free, and certain countries have started programmes with the aim of eradicating BHV-1 (Ackermann and Engels 2006). According to the OIE Terrestrial Animal Health Code (2011), there is an increased demand for cattle, semen and embryos from

countries or zones free from BHV-1 infection. This causes export limitations for countries or herds not free from BHV-1, and therefore eradication of the infection may have direct economic benefits for the farm and for the cattle industry of the whole country.

Culling of seropositive animals without vaccination has been shown to be the best and fastest method to achieve eradication of BHV-1 (Ackermann and Engels 2006). Vaccination with gE marker vaccines, enabling discrimination between infected and vaccinated animals, combined with eradication of gE-positive animals, is the strategy of choice in populations with high seroprevalence (Bosch and others 1998, Ackermann and Engels 2006). Vaccination will significantly reduce the BHV-1 re-excretion of latently infected cattle (Bosch and others 1997) and therefore the youngstock remain free of the virus. Vaccination can induce eradication of the infection from a herd within a few decades depending on herd size (de Koeijer and others 2008). The eradication process can be enhanced by increased removal of seropositive animals from the herd once the prevalence of virus-positive animals has become low (de Koeijer and others 2008). Marker vaccines have been shown to be effective in reducing the within-herd prevalence of BHV-1 gE in longitudinal field studies (Makoschey and others 2007, Vilmos and others 2007, Jacevičius and others 2008). However, due to differences in population characteristics, the vaccines used (live or killed vaccines) and the vaccination protocols conducted in those studies, leading to different immune response and virological protection (Kerkhofs and others 2003), it is not possible to extrapolate the results of these studies to our population. Therefore, the authors' first aim was to determine the efficacy of the proposed vaccination programme by using an inactivated marker vaccine, according to the manufacturers' instructions, in lowering the seroprevalence of BHV-1 gE within the herd.

Self-clearance of some bovine viral infections from the herd has previously been reported, such as for bovine viral diarrhoea virus

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TABLE 1: Description of herds under control programme

	Farm	I	II	III	IV	V	VI	VII
Farm characteristics	Number of cows	402	1000	530	1820	2187	674	619
	Year milk production (kg/cow)	7523	8369	7000	8416	8325	8300	8085
Farm management	Number of barns (0 – one barn, 1 – more than 1)	0	1	1	1	1	1	1
	Veterinarian is the employee of the farm (0 – no, 1 – yes)	1	1	1	1	1	1	0
	Inseminator is the employee of the farm (0 – no, 1 – yes)	1	1	1	1	1	1	1
	Using bull (0 – no, 1 – heifers, 2 – cows, 3 – both)	0	0	2	1	0	0	0
Farm biosecurity	Inseminator give service to other farms	0	0	0	1	1	1	1
	Purchase in 3 years (0 – closed, 1 – <2 animals, 2 – ≥2 animals)	2	2	2	0	2	2	2
Cow management	Barn type (1 – warm, 2 – cold)	2	2	2	2	2	1	1
	Keeping system (1 – tied, 2 – loose)	2	2	2	2	2	1	1
	Grazing (0 – no, 1 – yes)	1	0	0	0	0	1	1
Youngstock management	Keeping (1 – with cows, 2 – some period separately, 3 – separately)	1	3	3	3	3	3	3
	Keeping system (1 – fixed, 2 – loose, 3 – mixed)	3	3	3	3	3	2	1
	Relocating animals between the barns (0 – no relocating between barns, 1 – once a year, 2 – twice a year, 3 – more than twice a year)	0	3	2	3	3	3	3
	Grazing (0 – no, 1 – yes)	1	0	1	1	1	1	1
	Infection status	BVD (0 – negative, 1 – positive)	1	1	1	1	1	1
	IBR prevalence in cows	90	84	89	95	97	98	67
	IBR prevalence in youngstock	38	24	91	89	60	44	2

(BVDV) (Lindberg and Alenius 1999, Viltrop and others 2002, Kampa and others 2009), bovine respiratory syncytial virus (BRSV) (Ohlson 2010) and BHV-1 infection (Kampa and others 2004, Kampa and others 2009). Kampa and others (2009) demonstrated that four out of seven infected herds, where all animals were sampled, were free from BHV-1, without any intervention, over a three-year period. However, time to extinction of BHV-1 in a small cattle population with 10 animals without any intervention was calculated by Mollema and others (2005) to be 100 years. In more than one-third of Estonian BHV-1-infected farms participating in the study of Raaperi and others (2010), young animals were seronegative. Knowing that the reactivation rate of BHV-1 (virus reactivation probability per unit of time among all animals previously infected) is generally low (de Koeijer and others 2008), self-clearance of the virus from the herd may occur.

The aims of this study were to assess the efficacy of vaccination programmes in lowering the seroprevalence of BHV-1 gE within the herd as well as to follow the dynamics of the infection in non-vaccinating herds with uninfected replacement heifers and to detect whether self-clearance of the BHV-1 infection may occur in those herds.

Materials and methods

Survey design and study population

After a prevalence study conducted from 2006 to 2008 (Raaperi and others 2010), control programmes to eradicate BHV-1 were elaborated for seven dairy herds. These were mainly large herds with high milk yields applying loose housing in cold barns. The farms have usually several animal keeping units (barns) and youngstock is kept in a separate barn from older animals. Young animals are frequently relocated between different barns. All farms had purchased animals from other herds recently before the start of the vaccination programme. The farms employed their own veterinarians and inseminators. The herds were all seropositive for BVDV and the prevalence of BHV-1 antibodies was high, especially among cows. Descriptive characteristics of the herds, and prevalence of BHV-1 infection among cows and youngstock at the start of the control programmes, are given in Table 1. As the BHV-1 seroprevalence of the cattle herds was high, vaccination with a marker vaccine was considered to be the best strategy (Ackermann and Engels 2006). All the animals of at least three months old were vaccinated

twice a year with inactivated BHV-1 gE marker vaccine. Rispoval IBR-Marker inactivatum (Pfizer Animal Health) (farms I, II, III, IV, V and VII) and/or Bovilis IBR marker inac. (Intervet International) (farms V and VI) were used. The programme determined that vaccination should be undertaken until the prevalence of BHV-1 gE seropositive animals within the herd decreased below a level of 10 per cent. After this, all the animals were tested for BHV-1 gE antibodies to discover the virus-infected animals, which were eliminated from the herd, with follow-up sampling to confirm the negative status of a herd.

To monitor the efficacy of the vaccination, and changes in seroprevalence during the control programme, the authors devised a longitudinal surveillance programme for the herds. In order to evaluate the efficacy of the vaccination programme, serum samples were taken from youngstock older than six months and born after the first vaccination and tested for BHV-1 gE antibodies. This is the age group that has lost their maternal immunity (Woodbine and others 2009), serving as sentinels to monitor virus circulation within the herd. If the herd is effectively protected by vaccination, and virus circulation has ceased, these animals should be free from wild virus antibodies (gE-negative). The first testing (efficacy I) was carried out in the first year and the second (efficacy II) two years after the first vaccination. An expected prevalence of 5 per cent and a confidence level of 95 per cent were used to calculate the sample size, using the software W in Episcopo 2.0 (Thrusfield and others 2001). A random number generator in Survey Toolbox (AusVet Animal Health Services 1996) was used to select the animals for sampling in tie-stall systems. In loose-housed systems, the sample was randomly divided between pens (groups) in the barn. Animals were selected for sampling randomly within the groups.

In order to monitor changes in prevalence, cross-sectional sampling was performed 1.5 years after the first vaccination in two age groups: cows and youngstock older than six months (prevalence II). The expected prevalence was presumed to be 50 per cent in youngstock and 75 per cent in cows when calculating the sample size. A confidence level of 95 per cent and an accepted error of 10 per cent were used to calculate the sample size.

Five BHV-1-infected dairy herds, which had uninfected heifers (prevalence up to 5 per cent), were selected to monitor the course of the infection without control strategies. Herds included in this study were medium-sized herds with variable milk production. Cows of four herds were kept in warm barns and of one herd in cold barn and grazing is applied during summer. Animals were kept in several barns

Table 2. Description of herds not under control programme

	Farm	VIII	IX	X	XI	XII
Farm characteristics	Number of cows	274	112	158	300	166
	Year milk production (kg/cow)	6600	4200	4738	7500	8400
Farm management	Number of barns (0 – one barn, 1 – more than 1)	1	1	1	1	1
	Veterinarian is the employee of the farm (0 – no, 1 – yes)	0	0	0	1	0
	Inseminator is the employee of the farm (0 – no, 1 – yes)	1	bull only	0	1	0
	Using bull (0 – no, 1 – heifers, 2 – cows, 3 – both)	0	3	1	0	1
Farm biosecurity	Inseminator give service to other farms	1	bull only	1	1	1
	Purchase in 3 years (0 – closed, 1 – <2 animals, 2 – ≥2 animals)	0	1	2	0	2
Cow management	Barn type (1 – warm, 2 – cold)	1	1	1	1	2
	Keeping system (1 – tied, 2 – loose)	1	1	2	1	2
	Grazing (0 – no, 1 – yes)	1	1	1	1	1
Youngstock management	Keeping (1 – with cows, 2 – some period separately, 3 – separately)	2	1	3	3	2
	Keeping system (1 – fixed, 2 – loose, 3 – mixed)	3	1	1	3	2
	Relocating animals between the barns (0 – no relocating between barns, 1 – once a year, 2 – twice a year, 3 – more than twice a year)	2	1	2	3	2
	Grazing (0 – no, 1 – yes)	1	1	1	1	1
Infection status	BVD (0 – negative, 1 – positive)	1	0	0	0	1
	IBR prevalence in cows	71	48	58	57	24
	IBR prevalence in youngstock	3	0	4	0	0

and youngstock was relocated between different barns at least once a year. The veterinarians and inseminators were not usually employees of the farm. Two of the farms had not purchased animals during the previous three years, and the others had bought less than two animals. Two of the study herds were positive for BVDV antibodies, whereas in three of the farms infection was not found. BHV-1 prevalence among cows was moderate (Table 2). Two longitudinal follow-up samplings (prevalence II and III in Table 3) were performed in order to estimate seroprevalence in cows and heifers. Intervals between the three samplings were approximately of one year. Calculation of the sample size was performed in the same way as the prevalence was estimated in the vaccination group. In order to demonstrate active virus circulation in non-vaccinating herds, in each follow-up sampling (prevalence II and III) BHV-1 antibody prevalence was calculated in calves born after the first sampling (prevalence I) and at least six months old at the time of testing (calves > six months prevalence II and III in Table 3).

In order to collect herd data, the veterinarian or farm manager was questioned at the time of the first sampling. Information concerning herd characteristics, farm management and biosecurity practices, and the keeping systems of cows and young-stock, was collected to describe the herds (Tables 1 and 2).

Sampling and sample analysis

In vaccinating herds all serum samples were analysed for BHV-1 gE antibodies with the commercial BHV-1 gE ELISA test kit HerdChek (IDEXX), with a sensitivity of 100 per cent and a specificity of 99.8 per cent. In herds without a control programme, the BHV-1 gB ELISA test kit HerdChek (IDEXX), with a 100 per cent sensitivity and 99.8 per cent specificity, was used. Samples were analysed according to the manufacturer's instructions. All suspect results were considered to be positive in the data analysis. Ten serum samples from randomly selected young animals older than six months to calving were analysed for BVDV antibodies using the PrioCHECK BVDV Ab test kit (Prionics AG) to clarify the herd infection status (Houe and others 2006). The test has a relative sensitivity and specificity of approximately 98 per cent and 99 per cent, respectively, compared with a virus neutralisation test (Kramps and others 1999).

Data analysis

The prevalence of BHV-1 gE antibodies in vaccination herds, and gB antibodies in non-vaccination herds, was calculated separately for three

groups of animals within the herd: cows, young stock from age six months until calving and calves > six months born after the first vaccination in the vaccination group or born after the first sampling in non-vaccinating group. The number of seropositive animals was divided by the number of animals sampled in that age group, and 95 per cent exact CI (Dohoo and others 2009) were calculated for prevalence estimates by using Stata 11 software (Stata Corporation). Binomial generalised linear models were constructed to estimate the differences between prevalence estimates within farms. For this purpose a three way interaction term between year, farm and animal group (cows/heifers) was included in the models. Multiple comparisons between three prevalence estimates in non-vaccination herds was accomplished with a contrast matrix (Tukey's all-pair comparisons). In order to evaluate overall BHV-1 prevalence changes between sampling times in vaccination and non-vaccination herds, one model for each of the datasets was composed to calculate overall estimates for all study herds in both groups. For this purpose interaction terms between animal group (heifers/cows) and year as well as animal group and farm were included in the model. R version 2.13.0 (The R Foundation for Statistical Computing) was used for model building.

Results

Changes in BHV-1 gE seroprevalence in vaccination herds

One year after the start of the vaccination programme, the sample of sentinel group of animals (efficacy I, ie, calves born after the first vaccination) was negative to BHV-1 gE antibodies in all herds. In the second testing, conducted two years after the first vaccination (efficacy II), the sample of animals born after the first vaccination was negative to BHV-1 gE antibodies in herds II to VII, whereas four out of 54 animals in the 10-month age group showed antibodies against gE in herd I giving a prevalence of 7.4 per cent (Table 4).

In herd I, the proportion of gE-seropositive cows sampled 1.5 years after the first vaccination (prevalence II) was larger compared with the first testing, however the increase was not significant. In three herds, the prevalence among cows decreased significantly compared with the first testing.

A significant decline in the prevalence of BHV-1 gE seropositive heifers was detected in five out of seven herds 1.5 years after the start of vaccination (prevalence II), but the trend towards lower prevalence was observed in all vaccinating herds (Table 4).

Within 1.5 years after the start of the vaccination programme, the mean prevalence among cows decreased from 90 per cent (95 per

TABLE 3: Proportion of animals positive to bovine herpesvirus type 1 (BHV-1) gE antibodies with 95% CI in vaccinating herds and odds of an animal to be positive to BHV-1 antibodies in seven test herds compared with prevalence I

Age group and sampling time	Herds							Overall	OR (95% CI)
	I	II	III	IV	V	VI	VII		
Cows									
Prevalence I	90 (79-96)	84 (77-91)	89 (79-96)	95 (90-97)	97 (88-100)	98 (94-100)	67 (54-78)	90 (88-92)	1
	54/60	98/116	58/65	172/182	58/60	120/122	42/63	602/668	
Prevalence II	98 (91-100)	86 (76-94)	78 (67-88)	75 (63-85)†	75 (64-85)*	98 (91-100)	22 (12-34)†	76 (72-80)	0.3 (0.2, 0.5)†
	61/62	57/66	51/65	51/68	55/73	59/60	13/60	347/454	
Heifers									
Prevalence I	38 (24-57)	24 (16-34)	91 (81-97)	89 (84-93)	60 (47-72)	44 (32-57)	2 (0-6)	55 (51-58)	1
	22/58	24/98	59/65	211/237	36/60	31/70	2/117	385/705	
Prevalence II	1 (0-7)†	13 (6-23)	1 (0-7)†	13 (7-22)†	4 (1-9)†	9 (5-16)†	0 (0-6)	6 (4-9)	0.03 (0.02, 0.05)†
	1/73	9/70	1/80	12/90	4/110	11/120	0/60	38/603	
Calves >6 months									
Efficacy I	0 (0-7)	0 (0-18)‡	0 (0-8)‡	0 (0-14)‡	0 (0-6)‡	0 (0-7)‡	0 (0-9)‡	0 (0-1)‡	
	0/48	0/19	0/45	0/25	0/58	0/52	0/41	0/288	
Efficacy II	7 (2-18)	0 (0-5)‡	0 (0-7)‡	0 (0-6)‡	0 (0-5)‡	0 (0-4)‡	0 (0-7)‡	1 (0.2-2)	
	4/54	0/70	0/52	0/60	0/76	0/83	0/51	4/446	

Prevalence studies are performed in 1.5 year interval, efficacy is evaluated in one year interval
* P<0.05 compared with last prevalence estimation in the same category
† P<0.001 compared with last prevalence estimation in the same category
‡ one-sided, 97.5% CI
n posit/all Number of positive samples/total number of samples

cent CI 88 to 92) to 76 per cent (95 per cent CI 72 to 80) compared with prevalence estimated before the introduction of vaccination programme, giving OR=0.3 (95 per cent CI 0.2 to 0.5, $P<0.001$). Mean prevalence among heifers decreased from 55 per cent (95 per cent CI 51 to 58) to 6 per cent (95 per cent CI 4 to 9) with OR=0.03 (95 per cent CI 0.02 to 0.05, $P<0.001$).

Changes in BHV-1 seroprevalence in non-vaccinating herds

In herds IX and XI, the seroprevalence among cows decreased with each sampling, whereas a significant decline was observed two years after the first sampling ($P<0.05$). In herd X, seroprevalence among cows was significantly higher one and two years after the first sampling, compared with the first prevalence estimate. In herd XII, the prevalence remained constant in the second sampling followed with a zero prevalence of BHV-1 antibodies among cows in the third sampling. Within two years, the mean BHV-1 antibody prevalence among cows decreased from 55 per cent (95 per cent CI 49 to 61) to 42 per cent (95 per cent CI 36 to 48) with OR=0.6 (95 per cent CI 0.4 to 0.9, $P<0.05$).

Prevalence among young stock remained below 5 per cent in herds VIII, IX, XI and XII and no statistically significant changes could be found. In herd X, the seroprevalence to BHV-1 antibodies among young stock increased significantly with each sampling, resulting in 68 per cent (95 per cent CI 53 to 80) in the third testing. In two years, the mean BHV-1 seroprevalence among heifers increased from 2 per cent (95 per cent CI 0.4 to 4) to 14 per cent (95 per cent CI 10 to 19), OR=17.2 (95 per cent CI 5.6 to 52.8, $P<0.001$) (Table 3).

The prevalence of BHV-1 gB antibodies among calves born after the first sampling, and at least six months old at the time of testing, was zero in three herds at both sampling times. However, 21 per cent (95 per cent CI 5 to 51) (second sampling) and 68 per cent (95 per cent CI 53 to 80) (third sampling) seroprevalence among these animals was found in herd X. In herd XI, one animal in this age group showed antibodies for BHV-1 at the second sampling (Table 3).

Discussion

Changes in BHV-1 gE seroprevalence in vaccination herds

A vaccine intended for the eradication of an infection should effectively stop the circulation of the virus within the herd via reduction of the excretion of field virus and reduction of susceptibility of the animals to infection (Makoschey and others 2007). The positive

effect of a vaccination programme is most obvious among heifers, expressed in the decrease of BHV-1 gE seropositive animals in that age group from 55 per cent (95 per cent CI 51 to 58) to 6 per cent (95 per cent CI 4 to 9), suggesting that vaccinating cattle twice a year with a commercial marker vaccine keeps the forthcoming generation free of infection (Hage and others 2003, Vilmos and others 2007, Rypula and others 2010). Two years after the beginning of the vaccination programme, gE seropositive animals in this age group were found in only one out of seven study herds (herd I). In this herd deviation from the recommended vaccination scheme took place. In the second year of the programme, the farmer decided to begin the vaccination of young stock from six months of age instead of three as recommended, to reduce the costs to himself of the programme. Conventionally, produced inactivated BHV-1 vaccines vary in the quality of protection and reduction of virus shedding that they afford (Patel 2005). In general, maternal immunity should provide sufficient protection during the first month of life, and vaccination is typically started at the age of three months (Makoschey and others 2007). Increasing the age of the first vaccination leads to the evolution of a higher number of the susceptible population, enabling the spread of infection in the herd. Seroconversions to BHV-1 were also found in a Polish study when the beginning of the vaccination of young stock was moved to the eighth or ninth month of life (Rypula and others 2010).

Mean BHV-1 prevalence among cows for all seven study herds decreased significantly from 90 per cent (95 per cent CI 88 to 92) to 76 per cent (95 per cent CI 72 to 80), $P<0.001$. However, a significant decrease was observed in only three of the study herds (herds IV, V and VII). The probability of seropositivity to BHV-1 increases with age (Woodbine and others 2009) and the prevalence among cows can remain high for a couple of years until the virus-negative replacement heifers substitute older infected animals. On the other hand, despite the vaccination, circulation of the virus may continue to some extent, whereas the probability of the infection is higher in the cow population and prevalence among cows will not reduce in the expected time. In this vaccination study the replacement rate of cows in the herds was 25 to 30 per cent per year. This means that the cow population was replaced in approximately four years. However, there are always animals, including infected individuals, that remain in the herd for a longer period, which adds a couple of years to the time needed for the clearance from infection.

TABLE 4: Proportion of animals positive to bovine herpesvirus type 1 (BHV-1) gB antibodies with 95% CI in nonvaccinating herds and odds of an animal to be positive to BHV-1 antibodies in five test herds compared with prevalence I

Age group and sampling time	Herds					Overall	OR (95% CI)
	VIII	IX	X	XI	XII		
Cows							
Prevalence I	71 (58-82)	48 (38-59)	58 (43-72)	57 (43-70)	24 (7-50)	55 (49-61)	1
	41/58	44/91	29/50	32/56	4/17	150/272	
Prevalence II	53 (39-66)	34 (20-50)	86 (73-95) [†]	41 (29-55)	24 (13-38)	47 (41-53)	0.8 (0.5 to 1.1)
	31/59	15/44	38/44	24/58	12/50	120/255	
Prevalence III	64 (51-76)	21 (10-35) ^{*‡}	98 (87-100) ^{*‡}	34 (22-47) ^{*‡}	0 (0-8) [†]	42 (36-48)	0.6 (0.4 to 0.9) ^{*‡}
	38/59	10/48	39/40	20/59	0/50	107/256	
Heifers							
Prevalence I	3 (0-10)	0 (0-14) [†]	4 (0-14)	0 (0-6) [†]	0 (0-10) [†]	2 (0.4-4)	1
	2/69	0/27	2/52	0/62	0/38	4/248	
Prevalence II	2 (0-9)	2 (0-12)	24 (13-38) [†]	5 (1-14)	4 (1-15)	7 (4-11)	6.6 (2.1; 20.6) ^{*‡}
	1/60	1/43	12/50	3/60	2/45	19/258	
Prevalence III	0 (0-7) [†]	0 (0-8) [†]	68 (53-80) ^{†‡§}	0 (0-9) [†]	0 (0-8) [†]	14 (10-19)	17.2 (5.6; 52.8) ^{†‡}
	0/60	0/43	34/50	0/40	0/50	34/243	
Calves >6 months							
Prevalence II	0 (0-71) [†]	0 (0-26) [†]	21 (5-51)	14 (0.4-58)	0 (0-16) [†]	7 (2-17)	
	0/3	0/12	3/14	1/7	0/21	4/57	
Prevalence III	0 (0-11) [†]	0 (0-10) [†]	68 (53-80)	0 (0-13) [†]	0 (0-8) [†]	18 (13-24)	
	0/33	0/34	34/50	0/27	0/45	34/189	

Prevalence studies are performed in one year interval
^{*} P<0.05 compared with last prevalence estimation in the same category
[†] P<0.001 compared with last prevalence estimation in the same category
[‡] Difference between the first and third sampling
[§] Difference between the second and third sampling
[#] one-sided, 97.5% CI
n posit/all Number of positive samples/total number of samples

A mean within-herd reproduction ratio R_1 (R-effective), defined as the mean number of secondary cases generated by one infectious animal, should be smaller than one in order to achieve eradication of the infection from the herd (Bosch and others 1998). This may be achieved by vaccination resulting in a decrease in the number of reactivation events per host lifetime (Mollema and others 2005). The R_1 of the natural BHV-1 in non-vaccination herds has been estimated in several studies as being from 2.8 to 7 (Hage and others 1996, Bosch and others 1998, Mars and others 2001, Hage and others 2003). In vaccination herds, R_1 has been estimated to be from 1.5 (Mars and others 2001) up to 2.4 when using inactivated gE-negative BHV-1 vaccine (Bosch and others 1998). Although this reproduction ratio is above one, the eradication of the infection from a herd within a few decades is still possible (de Koeijer and others 2008).

It has been suggested that, in order to keep the reproduction ratio below one, appropriate management practices will have to be included in the BHV-1 eradication programme, that is, no contact with animals that are not known to be BHV-1 free, culling of animals seropositive for BHV-1 field virus, and hygienic measures for visitors (Hage and others 1996). All our vaccination study herds were large dairy herds with more than 400 dairy cows with high milk yields (above 7000 kg per cow annually). A higher rate of seroconversion for BHV-1 was found in larger herds by Segura-Correa and others (2010). This may be related to several predisposing management-related factors. According to a previous study (Raaperi and others 2010), the herd-level risk factors were the veterinarian and inseminator, who were employees of the farm, and BVDV being present on the farm, relating to high within-herd prevalence and BHV-1-infected young stock. Other characteristics given in Table 1 may increase management-related stress for animals, leading to higher susceptibility and higher probability of infection due to possible reactivation of the virus in latently infected animals; however, a more targeted study is needed to confirm this. Despite this, relying on the results of previous studies (Van Schaik and others 1999, Hage and others 2003, Raaperi and others 2010), control over population density and the use of within-herd biosecurity measures, as well as restraining other immunosuppressive diseases,

can improve the outcome of a control programme.

In this study, all the vaccination herds used inactivated gE negative BHV-1 vaccines. Confusing results about the efficacy of different vaccines has been reported in previous studies. According to Vonk Noordegraaf and others (1998) R_{ind} (R effective within herds) of the live vaccine is lower than that of an inactivated marker vaccine, indicating that preference should be given to the live vaccine. However, Bosch and others (1997) confirmed that inactivated vaccines reduce virus excretion more efficiently than live vaccines. Based on the report of the European Commission on BHV-1 marker vaccines (Sanco/CS/AH/R20/2000), no clear benefit could be ascertained as to one precise vaccine or immunisation scheme. The results of the current study indicate that using inactivated vaccines, without deviation from the vaccination protocol, may lead to rapid reduction in gE seroprevalence among young stock and cows in herds with high seroprevalence, giving good grounds for the possibility of eradication of BHV-1 from herds. However due to the relatively small number of study herds care should be taken not to overemphasise the results of this study. Also, a longer observation period until eradication of the virus from the study herds is needed to confirm the benefit of the vaccination protocol. Vaccination alone will not necessarily lead to elimination of the virus unless other herd health measures are instituted to reduce the risk of virus spread.

Changes in BHV-1 prevalence in non-vaccination herds

The results of this study indicate that the prevalence of BHV-1 gB antibodies among cows decreased slowly in most of the study herds. The prevalence amongst cows between the first and second sample decreased more in vaccination herds than non-vaccination herds as evidenced by the smaller OR, and the fact that the change in unvaccinated herds was not statistically significant at the 5 per cent level (95 per cent CI includes 1) (Tables 3 and 4). However, caution should be made not to overemphasise the difference due to the fact that the high OR in a non-vaccination group is mostly influenced by herd X, where active virus spread took place. The presence of antibodies in calves born after the first sampling, and which had lost their maternal

immunity by the time of sampling, indicates that active virus spread has taken place recently. One nine-month-old animal in herd XI gave a suspect antibody test result in the second sampling. This might derive from the remnant of maternal immunity.

Herds which have 'naturally' uninfected replacements are in a comparable situation with vaccinated herds with the intention to eliminate the virus from the herd via replacing the infected animals with uninfected young stock. In order to achieve self-clearance of the BHV-1 infection from the herd, parameters influencing the expected time to extinction should be considered: the reproduction ratio, the reactivation rate, the population size and the demographic turnover in the host population (de Koeijer and others 2008).

Mollema and others (2005) indicated that, once the population contains predominantly latently infected animals, it will take a very long time before all latently infected individuals have died in the population, conditional on no new outbreaks having taken place. A relatively short time to extinction can only be achieved if both R_0 and R_0 are below 1, assuming that the reactivation rate is low (Mollema and others 2005, de Koeijer and others 2008). The reactivation rate of BHV-1 is generally low, being $P=0.09$ per year, as estimated by de Koeijer and others (2008). A low level of reactivation of BHV-1 latent infection leading to progressive self-clearance of the infection was also confirmed by Kampa and others (2004, 2009). Consecutive replacements of the infected cows with uninfected heifers from within the herd have resulted in herds free from BHV-1 infection (Kampa and others 2009). Absence of the reactivation of BHV-1 can also be suspected in four out of five of the study herds in the current study, suggested by a decrease in seroprevalence among cows and young stock remaining free of infection (prevalence below 5 per cent). Self-clearance of BHV-1 may occur in conditions of low levels of stress to the animals (Kampa and others 2004) avoiding reactivation of BHV-1 in latently infected animals. Several distinctive characteristics could be observed in the current study herds having BHV-1 uninfected heifers and experiencing a reduction in seroprevalence among cows during the two-year study period (Table 2). First, these herds are of medium size and variable levels of milk production. Small herds are probably more likely to experience self-clearance than large herds (de Koeijer and others 2008, Keeling and Rohani 2008, Kampa and others 2009). The time to extinction of BHV-1 increases with population size and is dependent on R_0 and R_0 . If $R_0 > 1$, the time to extinction increases exponentially with increasing herd size up to millions of years in larger populations ($n=50$), whereas in cases of $R_0 < 1$, the infectious individual will infect only a few susceptible individuals, and the time to extinction is hardly affected by population size (Mollema and others 2005, de Koeijer and others 2008). Second, veterinarian and inseminator are often not employees of the farm, possibly avoiding the iatrogenic spread of the virus (Raaperi and others 2010). Third, most of these farms keep their cows tied in insulated barns and grazing is frequently applied. This may reduce direct animal contacts, reducing the infection transmission probability. Fourthly, the farms usually keep their young stock in separate buildings, although regrouping is performed twice: the first time after the calf has finished the colostrum-ingestion period, and the second time before calving. Finally, in general, the herds purchased only single animals, and these were often BVDV negative (Table 2).

In conclusion, the authors' results indicate that vaccination is a secure method to stop BHV-1 circulation within a herd as long as the vaccination protocol is followed precisely. On the other hand, in some herds the virus circulation may end by itself for longer periods, which may lead to self-clearance of the herd from the virus. However, aiming for eradication in herds with uninfected young stock leaving animals unvaccinated has an unpredictable outcome – virus circulation may remain retarded or the virus may reactivate and cause an epidemic in the herd.

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Dynamics of bovine herpesvirus type 1 infection in Estonian dairy herds with and without a control programme

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1.1. Scholarly articles indexed by Thomson Reuters Web of Science

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3.4. Articles/presentations published in conference proceedings

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3.5. Articles/presentations published in local conference proceedings

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5.2. Conference abstracts

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