Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
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Epidemiologic studies of bovine respiratory syncytial virus and bovine coronavirus in Norway

Epidemiologiske studier av bovint respiratorisk syncytialvirus og bovint coronavirus i Norge

Ingrid Toftaker

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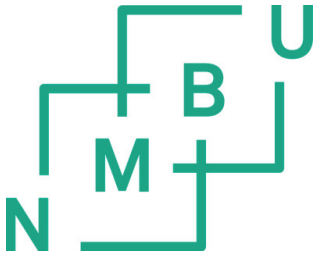

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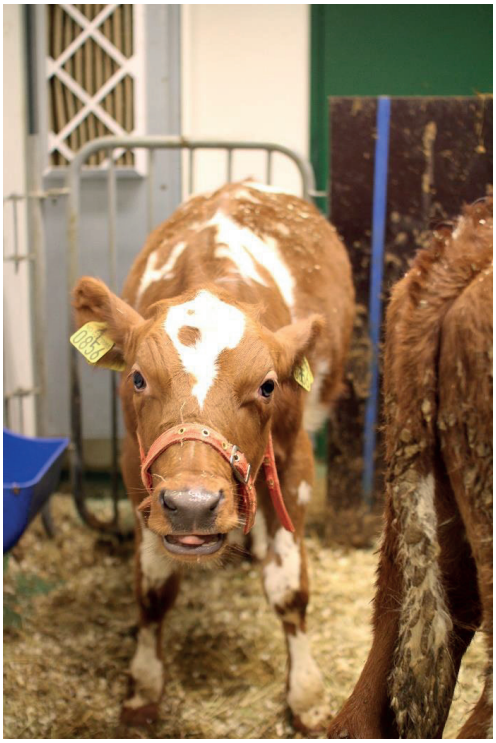

Norwegian Red calf infected with bovine respiratory syncytial virus.

'*Mamma? Universet, er det det samme som universitetet?'*

Tuva, 5 years old.

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Adamstuen, March 2019

Ingrid Toftaker

Abbreviations

BCV	Bovine coronavirus
BLCM	Bayesian latent class models
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BTM	Bulk tank milk
BVDV	Bovine viral diarrhoea virus
CHC	Individual cow health cards
ELISA	Enzyme-linked immunosorbent assay
LCA	Latent class analysis
NDHRS	Norwegian dairy herd recording system
NDPR	Norwegian Direct Payment Register
NPV	Negative predictive value
PPV	Positive predictive value
RT-PCR	Real time polymerase chain reaction
Se	Sensitivity
Sp	Specificity
VNT	Virus neutralization test
WD	Winter dysentery

List of Papers

I. Toftaker, I., Sanchez, J., Stokstad, M., Nødtvedt, A. 2016: Bovine respiratory syncytial virus and bovine coronavirus antibodies in bulk tank milk – risk factors and spatial analysis. Prev. Vet. Med. 133: 73–83 https://doi.org/10.1016/j.prevetmed.2016.09.003

II. Toftaker, I., Holmøy, I.H., Nødtvedt, A., Østerås, O., Stokstad M. 2017: A cohort study of the effect of winter dysentery on herd-level milk production. J. Dairy Sci. 100: 6483-6493 https://doi.org/10.3168/jds.2017-12605.

III. Toftaker, I., Toft, N., Stokstad, M., Sølverød, L., Harkiss, G., Watt, N., O'Brien A., Nødtvedt, A. 2018: Evaluation of a multiplex immunoassay and two indirect ELISAs in bulk milk antibody testing for bovine respiratory syncytial virus and bovine coronavirus using latent class analysis. Prev. Vet. Med. 154: 1-8 https://doi.org/10.1016/j.prevetmed.2018.03.008

IV. Toftaker, I., Ågren, E., Stokstad, M., Nødtvedt, A., Frössling, J. 2018: Herd level estimation of probability of disease freedom applied on the Norwegian control program for bovine respiratory syncytial virus and bovine coronavirus. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2018.07.002

Summary

Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) cause respiratory infection in cattle worldwide. In addition, BCV also causes diarrhoea in calves and contagious diarrhoea in adult cows, i.e. winter dysentery. The occurrence is endemic in the Norwegian cattle population, and the planning of a national control programme against these viral infections prompted the need for more research focused at the herd level. The overall objective of this thesis was to provide knowledge about the epidemiology of BRSV and BCV in the Norwegian cattle population, as a scientific basis for systematic control work. To reach this aim, we utilized register data, bulk tank milk antibody testing and reports of disease outbreaks.

A risk factor analysis showed that large herd size and short distance to neighbours were associated with increased odds of antibody positivity in bulk tank milk for both viruses. Purchase of livestock was an additional risk factor for BCV. Spatial analysis revealed geographic differences in the distribution of prevalence.

A cohort study was performed based on a regional outbreak of winter dysentery. The estimated drop in milk production was 3.6 L/cow at maximum, or 15% for the average herd, showing a notable effect of winter dysentery on milk production at the herd level.

The MVD-Enferplex BCV/BRSV multiplex immunoassay was developed to screen herds for antibodies in bulk tank milk. A diagnostic test evaluation was performed for this application, by comparing it to two commercially available ELISAs, using Bayesian latent class models. Adjusting the configuration and cut-off values of the test resulted in acceptable sensitivity and specificity for both BRSV and BCV.

A framework for herd-level calculations of probability of freedom (*PostPFree*) from BRSV and BCV antibodies over time was developed by use of bulk tank milk testing, geographic information and animal movement data. *PostPFree* was updated every three months, and validation against a subsequent bulk tank milk sample after three years showed that it provided an improved estimate of a herd's antibody status compared to relying on the previous test result.

Altogether, the results have provided insights at the herd level regarding: risk factors for a positive classification, consequences of disease and the uncertainty of herd classification. This knowledge can be directly applied in systematic control work.

Sammendrag (summary in Norwegian)

Bovint respiratorisk syncytialvirus (BRSV) og bovint coronavirus (BCV) er årsak til luftvegslidelser hos storfe over hele verden. I tillegg kan BCV også forårsake kalvediaré og smittsom diaré hos voksne storfe, kalt vinterdysenteri. Forekomsten er endemisk i den norske storfepopulasjonen, og planleggingen av et nasjonalt kontrollprogram mot disse virusinfeksjonene skapte behov for mer forskning på besetningsnivå. Det overordnede målet med avhandlingen var å generere kunnskap om epidemiologien til BRSV og BCV i den norske storfepopulasjonen, som et bidrag til forskningsbasert, systematisk kontrollarbeid. For å nå dette målet brukte vi registerdata, tankmelk-serologi, og rapportering av sykdomsutbrudd.

En risikofaktoranalyse viste at store besetninger og kort avstand til nabogårder, var assosiert med økt odds for antistoffpositivitet i tankmelk for begge virus. I tillegg var kjøp av livdyr en risikofaktor for BCV. Romlig statistisk analyse avdekket geografiske forskjeller i fordelingen av prevalens.

En kohortstudie basert på et regionalt utbrudd av vinterdysenteri viste at det estimerte tapet i melkeproduksjon var 3,6 L/ku på det meste, eller 15% for en gjennomsnittsbesetning. Dette viser at vinterdysenteri har en betydelig effekt på besetningens melkeproduksjon.

En nyutviklet MVD-Enferplex BCV/BRSV multiplex immunoassay har blitt brukt til screening av besetninger for antistoffer i tankmelk. Testen ble evaluert for denne anvendelsen ved å sammenligne den med to kommersielt tilgjengelige ELISA-tester, i Bayesianske latent klasse modeller. Multiplex testen kan, ved å endre konfigurering og cut-off verdier, oppnå akseptabel sensitivitet og spesifisitet for både BRSV og BCV.

En metode for å beregne en besetnings sannsynlighet for frihet (*PostPFree*) fra BRSV og BCV antistoffer over tid ble utviklet ved å inkludere tankmelk-testing, geografisk informasjon og dyreforflytninger. *PostPFree* ble oppdatert hver tredje måned, og ved validering mot ny tankmelktest etter tre år gav den bedre estimater for besetningers antistoffstatus enn det man fikk bare basert på forrige prøvesvar.

Alt i alt har resultatene gitt mer kunnskap på besetningsnivå om: risikofaktorer for positiv klassifisering, konsekvenser av sykdom og usikkerheten rundt klassifisering av besetninger. Denne kunnskapen har direkte anvendelse i systematisk kontrollarbeid.

Introduction

Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) are endemic pathogens in cattle throughout the world (Valarcher and Taylor, 2007; Boileau and Kapil, 2010). Both agents are considered important in the development of bovine respiratory disease, a major challenge to cattle welfare and livestock economy worldwide. Additionally, BCV causes calf diarrhoea and epidemics of diarrhoea in adult cattle, called winter dysentery, resulting in adverse effects on milk production. The livestock industry today is facing a growing public concern regarding the impact on the environment, standards for animal welfare and the use of antimicrobials. At the same time, the industry is forced to retain economic sustainability. Improvement of cattle health through the reduction of endemic diseases is therefore more topical than ever.

The Norwegian cattle population

The Norwegian cattle population consisted of approximately 300,000 cattle in 13,478 herds as of the 1st of January 2017, of which 8331 were dairy and 5147 were beef herds (Statistics Norway, 2017). The industrialized world has experienced a trend over the past few decades of increasing herd sizes in the cattle industry. The same is seen in Norway, although the increase in herd size is modest compared to many other European countries. In 2007, there were 12,740 dairy herds in Norway, with a mean herd size of 18.7 cows. Ten years later (2017), the mean herd size had increased to 26.8 cow-years (TINE Advisory Service, 2018). The milk production is large enough for national self-sufficiency, but export is minimal. The dominating breed is the dual-purpose Norwegian Red (Figure 1), accounting for more than 90% of all milking cows in 2017. The Norwegian Red has traditionally served as a producer of both milk and meat, and in 2017 the average milk production was 7797 kg milk per cow-year (TINE Advisory Service, 2018). In recent decades, the yield has increased, and the same production is therefore reached with fewer cows (Ruud et al., 2013). This has led to a deficit in meat, and a rise of more specialized beef herds. Among dairy herds, tie-stalls is still a common housing system; in 2016 used in 5358 herds, compared to free-stalls in 2716 herds. However, free-stall herds are generally larger, and therefore house more than half the dairy cows. In free-stalls, automatic milking systems are widely used (1659 herds) and account for 55% of the milk produced (TINE Advisory Service, 2017). From the year 2034, free-stalls will be the mandatory housing system in Norway (Forskrift om hold av storfe, 2004).

Figure 1. Norwegian Red cow in a mountain pasture.

Cattle herds can be found throughout the country, and the county with the highest number of dairy cows is Trøndelag (mid-Norway), followed by Rogaland (in the south-west) (The Norwegian Agricultural Agency, 2017). However, Rogaland is smaller, and therefore has the highest cattle density (i.e. cattle per unit of area). Finnmark (in the north) has the fewest cattle. The spatial distribution of the Norwegian cattle herds is shown in Figure 2.

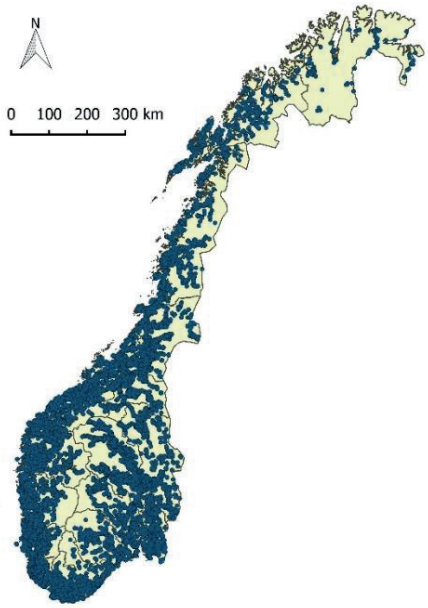

Figure 2. Point map of Norwegian cattle herds (dairy and beef), August 2016, geo-coordinates: The Norwegian Agricultural Agency, background map: diva-gis.org.

The Norwegian cattle population is often promoted as healthy. It is free from many infectious diseases; some have never been detected, and some have been eradicated through successful control and eradication programmes. Some diseases under surveillance, from which the cattle population is currently free, are: bovine viral diarrhoea (BVD), brucellosis, infectious bovine rhinotracheitis and bovine tuberculosis (Sviland et al., 2017; Sviland et al., 2018; Åkerstedt et al., 2018a, b). Furthermore, *Salmonella* spp. and *Mycobacterium avium* subsp. *paratuberculosis* are rarely detected, and *Mycoplasma bovis* and *Coxiella burnetii* have never been detected (Kampen et al., 2012; Heier et al., 2018; Kampen et al., 2018).

Historical background and occurrence of BRSV and BCV

BRSV was first identified in Europe around 1970 (Paccaud and Jacquier, 1970; Wellemans and Leunen, 1971). In Scandinavia, the first BRSV-related outbreak was detected in Norway in 1976 (Ødegaard and Krogsrud, 1977). In the years following, few reports of

BRSV in Norway exist, up until 1995 when a large outbreak involving many cattle herds occurred. An import of beef cattle from Denmark in December 1994 has been suggested as the likely source (Norström et al., 2000). Limited knowledge exists regarding how long BCV has existed in Scandinavia; however, winter dysentery was first described in 1946 in Swedish dairy herds (Hedström and Isaksson, 1951). The etiology was not known at the time, but due to the lack of bacteriologic findings, the authors concluded that the causative agent was most likely a virus. The first descriptions of BCV came from Europe in the seventies, of a 'coronavirus-like agent' as a cause of neonatal calf diarrhoea (Stair et al., 1972; Mebus et al., 1973). BCV was not recognized as the causative agent of winter dysentery until the nineties (Saif, 1990), and the association with respiratory disease was recognized even later (Storz et al., 2000; Saif, 2010; O'Neill et al., 2014). Today, BRSV and BCV are highly prevalent in cattle worldwide (Valarcher and Taylor, 2007; Boileau and Kapil, 2010). The endemic occurrence in Scandinavia is well documented through various serologic studies (Hägglund et al., 2006; Bidokhti et al., 2009; Gulliksen et al., 2009b; Klem et al., 2013; Ohlson et al., 2013). In Norway, Gulliksen et al. (2009b) found a seroprevalence at the calf level of 31.2% for BRSV and 39.3% for BCV, while the proportion of positive herds was 71.1% for BRSV and 80.7% for BCV in a random sample of 135 dairy herds. BRSV and BCV have gained increased attention in Norway and Sweden during recent years as they frequently cause disease outbreaks in a cattle population that is otherwise free of many infectious agents.

Infections with BRSV and BCV

The viruses

BRSV and BCV are enveloped single-stranded RNA viruses. BCV belongs in the order *Nidovirales*, family *Coronaviridae*, genus *Betacoronavirus* and species *Betacoronavirus 1*. BRSV belongs to the order *Mononegavirales*, family *Paramyxoviridae*, genus *Orthopneumovirus* and species *Bovine orthopneumovirus* (International Committee on Taxonomy of Viruses, 2018). Both viruses have cattle as the main reservoir, but serological and experimental studies have shown that other species can be infected (Ismail et al., 2001; Citterio et al., 2003; Kaneshima et al., 2007). Transmission from other species to cattle has never been documented, and is thus likely to be of minor importance. Only a single serotype has been described for both BRSV and BCV; however, antigenic diversity exists (Valarcher and Taylor, 2007; Fulton et al., 2013).

BRSV: Pathogenesis, clinical signs and immune response

BRSV replicates only in the epithelium of the respiratory tract and the virus is primarily shed in nasal discharge (Viuff et al., 1996; Valarcher and Taylor, 2007). In a structured literature review viral shedding was reported from 1 to 14 days after infection, with median time to peak shedding on day 5 (Grissett et al., 2015). In a recent experimental study, detection of viral RNA was reported from days 1 to 27 after exposure, while the infective virus was isolated on days 6 and 13 (Klem et al., 2019). When sentinel calves were introduced to infected calves at day 27 after primary exposure, they did not become infected (Klem et al., 2019). BRSV causes respiratory disease, and does not result in viremia. It is uncertain how long virus can persist in individual animals, but evidence of prolonged persistence or reactivation of virus is lacking. One study reported detection of viral RNA in pulmonary lymph nodes of a calf ten weeks after infection (Valarcher et al., 2001). Van der Poel (1997) suggested a four-fold rise in antibody titres against BRSV after corticosteroid treatment was indicative of reactivation of persistent infection, however re-excretion of virus was not detected.

Infection with BRSV can be limited to the upper respiratory airways, or result in pneumonia with or without secondary bacterial infections (Valarcher and Taylor, 2007). The incubation period is reported to be from two to five days (Valarcher and Taylor, 2007). Clinical signs include cough, nasal discharge, fever and depression, and in severe cases animals may develop dyspnoea (Elvander, 1996; Valarcher and Taylor, 2007). These signs are most evident in young animals, but BRSV can cause disease in cattle of all ages (Poel et al., 1993; Valarcher and Taylor, 2007). The severity of disease varies, from subclinical to fatal (Elvander, 1996; Larsen et al., 2001). Secondary bacterial infections are common; bacterial colonization is facilitated either by direct damage to the respiratory tract and/or by alteration of the host's immune system (Larsen, 2000; Taylor et al., 2010).

The immune response of the host is believed to play an important role in the pathogenesis of BRSV infection (Valarcher and Taylor, 2007; Meyer et al., 2008). Infection of respiratory epithelial cells and alveoli leads to induction of inflammatory chemokines and cytokines which in turn attracts additional inflammatory cells (Valarcher and Taylor, 2007). The inflammatory response leads to necrotizing bronchiolitis and if infection reaches the alveoli, interstitial pneumonia.

Seropositivity after infection is long-lasting; immunoglobulin G (IgG) has been reported detectable at least eight months after infection in naturally infected calves (Schrijver et al., 1996). In adult cows, antibodies can be present for several years (Elvander, 1996). Despite the long-lasting antibody response, the immunological protection is shorter. Reinfection in seropositive animals is regarded as common, often resulting in milder clinical signs (Kimman et al., 1987; Sacco et al., 2014). Reinfection might cause new shedding of viral RNA (Valarcher and Taylor, 2007). As the antibody response in cows is long-lasting, calves often possess maternal antibodies against BRSV. However, maternal antibodies are not considered fully protective (Gershwin, 2012). It has been reported that presence of maternal antibodies can suppress both local and systemic antibody response (Kimman et al., 1987), and the incidence of disease in calves with maternal antibodies can be high (Stott et al., 1980; Kimman et al., 1988). The mean half-life of maternal antibodies against BRSV has been reported to be 36 days, with an estimated time to seronegative status of 187 days in unvaccinated calves (Fulton et al., 2004).

BCV: Pathogenesis, clinical signs and immune response

BCV replicates both in the respiratory tract and in epithelial cells of the gastrointestinal tract, hence virus is shed both in nasal discharge and faeces (Heckert et al., 1991; Park et al., 2007; Oma et al., 2016). Experimental studies have shown shedding of BCV from 1 to 13 days after infection (El-Kanawati et al., 1996; Tråvén et al., 2001; Park et al., 2007). However, in these studies virus isolation was not performed, and hence they did not assess virus infectivity. In an experimental study by Oma et al. (2016), virus infectivity was confirmed in cell culture through day 13 after infection. Viremia has rarely been reported; however, Park et al. (2007) detected viral RNA in blood of infected calves. It is not known how long the virus can persist in individual animals. Oma et al. (2016) found BCV in lymphatic tissue through day 42 after exposure in one calf. A few field studies have found indications of prolonged shedding of BCV in faeces of adult cows (several months); however, reinfection was not excluded (Crouch et al., 1985; Collins et al., 1987). In a recent experimental study, Kanno et al. (2018) detected viral RNA sporadically for 1,085, 700 and 280 days in three calves, and suggested that this demonstrated persistent infection. However, virus sequencing was not performed, and the possibility that infection was caused by reintroduction cannot be completely ruled out. Thus, further studies are warranted to confirm the findings.

Similar to BRSV, seropositivity following BCV infection is long lasting. Under experimental conditions, BCV-specific IgG was detectable in serum and milk from days 9 to 11 after infection of seronegative animals (Tråvén et al., 2001). Antibody production has been shown to last for at least one year after infection (Alenius et al., 1991). However, the protective immunity is believed to be short (Tråvén et al., 1993; Saif, 2010). Two experimental studies reported that calves could be re-infected (new shedding of viral DNA) three weeks after the first challenge, but did not develop clinical signs (El-Kanawati et al., 1996; Cho et al., 2001a). In the study by Oma et al. (2016), sentinel calves were introduced to infected calves after three weeks, but did not become infected. Variation in immunity has been suggested as a reason for variable clinical signs (Tråvén et al., 1993). Calves born to antibody-producing cows often have maternal antibodies. What level of protection they offer is still not fully understood, but in an experimental study, Heckert et al. (1991) found that maternal antibodies delayed and decreased the antibody response in calves, and did not prevent respiratory or enteric infections. A recent longitudinal study following calves from birth to weaning found no association between anti-BCV IgG and incidence of BRD, and no significant difference in antibody titers for calves shedding BCV compared to non-shedders (Workman et al., 2019). A study by Fulton et al. (2011) reported a higher risk of bovine respiratory disease in calves with a low level of BCV-neutralizing antibodies after entering a feedlot. The calves were unvaccinated, and the authors suggest antibodies might be derived from maternal immunity; however as the calves were at least 8 months old it seems more likely that antibodies were a result of previous exposure.

Disease caused by BCV varies in severity from subclinical to severe, and fatalities have been reported (Boileau and Kapil, 2010). Infection with BCV can produce different clinical manifestations, often differentiated into three clinical syndromes in cattle: diarrhoea in calves, respiratory disease and winter dysentery.

Respiratory disease

Respiratory disease caused by BCV occurs in animals of all ages, although clinical signs are more commonly seen in young animals (Clark, 1993). An incubation period of three to eight days is reported (Saif, 2010). Clinical signs include coughing, rhinitis, fever and inappetence (Saif, 2010). Animals might also show concurrent respiratory signs and diarrhoea (Hasoksuz et al., 2002).

Calf diarrhoea

Calf diarrhoea caused by BCV typically occurs between 5 and 30 days of life, with more severe consequences in young animals (Clark, 1993; Boileau and Kapil, 2010). The disease occurs in both dairy and beef herds (Clark, 1993). The pathogenesis involves stunting and fusion of villi in the small intestine along with atrophy of colonic ridges in the colon, leading to malabsorptive diarrhoea (Boileau and Kapil, 2010). After an incubation period of two days, calves become depressed, the suckling reflex is weakened and this in combination with watery diarrhoea leads to dehydration (Gomez and Weese, 2017).

Winter dysentery

Winter dysentery is a contagious disease in adult cattle, characterized by acute onset of diarrhoea often with blood, fever, loss of appetite, depression and a rapid decrease in milk production (Boileau and Kapil, 2010). Enteric lesions are similar to those seen in calf diarrhoea (Durham et al., 1989). Some animals might also show respiratory signs (Tråvén et al., 1993; Cho et al., 2000). Outbreaks of winter dysentery mainly strike adult animals but diarrhoea in calves in the same herd is also reported (Alenius et al., 1991).

Impact

Bovine respiratory disease

What is bovine respiratory disease?

Bovine respiratory disease (BRD) is a general term used for respiratory disease in cattle. The aetiology is multifactorial and several antigens and host, management and environmental factors interact (Edwards, 2010; Murray et al., 2016b). BRD causes problems in both large feedlots and smaller husbandry systems (Smith, 1998; Klem et al., 2014a; Murray et al., 2016b). BRSV and BCV are important agents in the development of BRD. Other viral agents of importance include bovine herpesvirus 1, bovine viral diarrhoea virus (BVDV) and parainfluenza virus type 3, of which only the latter is currently present in Norway. Viral agents act both as primary agents of disease and as predisposing agents for bacterial infections (Griffin, 2010). In feedlots, animals often develop respiratory disease shortly after the introduction of new animals. These respiratory infections in post-weaned beef calves often recorded following stress (e.g. weaning, shipping, commingling) are known as *shipping fever*, whereas infections in dairy or veal calves between two and six months of age are commonly referred to as *enzootic calf pneumonia* (Murray et al., 2016b).

However, these definitions are not strictly followed, and terms are inconsistently used in the literature.

Impact of BRD

The high occurrence, morbidity and mortality of BRD makes it a severe concern for animal welfare (Fulton, 2009). Economic losses following BRD are mainly due to mortality, treatment costs (metaphylactic and/or therapeutic use of antibiotics), vaccination and reduced weight gain (Boileau and Kapil, 2010; Sacco et al., 2014). Long-term effects of BRD have also been shown, and a recent study found reduced milk production during first lactation for cows that were diagnosed with lung consolidation as calves (Dunn et al., 2018). Secondary bacterial infections lead to increased use of antibiotics, and respiratory disease is today considered one of the most important causes of antibiotic treatment in cattle, consequently posing a concern for the emergence of antibiotic resistance. A recent study of Danish herds found that respiratory disorders accounted for 79% of antimicrobials used in veal calves and young bulls (Fertner et al., 2016). The consequences of BRD in smaller husbandry systems are less studied than in feedlots, where BRD constitutes a major challenge with severe impact on animal welfare and profitability (Loneragan et al., 2001; Snowder et al., 2006). However, BRD affects animal health and antimicrobial usage also in other production systems with effects on welfare and profitability.

The role of BRSV and BCV in BRD

A literature review to assess the impact and role of BRSV and BCV in respiratory disease is challenging, as many studies investigate BRD in general. Often, the diagnosis is based on clinical signs without laboratory confirmation, or multiple pathogens are isolated, thus allowing only for assessment of the combined effect. Furthermore, many of these studies are conducted in production systems (for example feedlots) which differ substantially from what is common in Norway. The difference in production systems and the presence of other pathogens, (many of which are not present in Norway), makes direct generalization of findings from international studies to the Norwegian cattle population challenging.

BRSV is known as a major contributor to BRD in most production systems across the world (Valarcher and Taylor, 2007; Brodersen, 2010). Respiratory disease in calves is common in Norway, and in a study by Gulliksen et al. (2009a), calf diarrhoea together with respiratory disease accounted for more than 75% of all reported health events in calves < 180 days. In a Norwegian study on outbreaks of respiratory disease, the most commonly isolated agent was

BRSV (Klem et al., 2014a). Similar findings exist from Denmark, where a small study of 10 outbreaks indicated that BRSV was an important causative agent in calf respiratory disease (Uttenthal et al., 1996). Few studies have assessed the consequences of BRSV infection in Norwegian herds, but one study estimated a loss in production of 0.7 kg milk per cow for 7 days after a BRSV outbreak (compared to > 1 week before outbreak), and hence concluded that the effect on milk production was likely of minor importance (Norström et al., 2001). The milk loss is in concordance with a study from The Netherlands which reported a reduction of approximately 0.6 kg for 5 consecutive days during the infection period (van der Poel et al., 1995). Another Norwegian study describing a BRSV-related outbreak in a bull testing station found reduced weight gain and a reduction in feed conversion rate (Klem et al., 2016).

The role of BCV in BRD is still not fully understood; however, an increasing number of studies emphasize the importance of BCV as an important respiratory pathogen (Lathrop et al., 2000). Several studies from feedlots have shown a negative association between BCV shedding and weight gain (Cho et al., 2001b; Thomas et al., 2006). Pathogenesis and epidemiology of BCV differs across production systems, thus more research is needed to assess the impact of respiratory BCV in smaller husbandry systems and dairy production.

Diarrhoea in calves

Neonatal calf diarrhoea is often reported as the most common cause of morbidity and mortality in pre-weaned dairy calves across the world (Meganck et al., 2014; Gomez and Weese, 2017). BCV, rotavirus, *Escherichia coli* and *Cryptosporidium* spp. are the four most important enteropathogens causing diarrhoea in calves worldwide (Meganck et al., 2014). The relative importance of BCV as a cause of diarrhoea in calves varies between studies. In a Swedish study, diarrhoea was the second most reported disease (after respiratory disease) in calves under 210 days of age (Ortman and Svensson, 2004). Another Swedish study found a higher proportion of diarrhoea in high mortality herds; however, no faecal samples were positive for BCV (Torsein et al., 2011). Similar results exists from Norway, where Gulliksen et al (2009a) reported that the role of BCV as a cause of calf diarrhoea appeared to be minor.

Winter dysentery

In addition to respiratory disease and diarrhoea in calves, BCV is responsible for epidemics of winter dysentery. The disease is most common in dairy herds, but can also appear in beef

cattle (Durham et al., 1989; Cho et al., 2000). Outbreaks are most common during autumn and winter, although outbreaks during summer have been reported (Park et al., 2006; Boileau and Kapil, 2010). The disease is often reported to result in high morbidity and low mortality (Clark, 1993; Cho et al., 2000). During winter dysentery outbreaks, calves are often clinically unaffected (Hedström and Isaksson, 1951; Tråvén et al., 1993). The most evident consequence for the farmer is the associated milk production loss in adult cows. In experimentally infected cows, a drop of 19–56% from the pre-infection level was reported (Tråvén et al., 2001). Jactel et al. (1990) compared milk production to a standardized lactation curve (Wood, 1967), and reported a drop of 6–30% in affected cows from outbreak herds. Other studies also report a milk drop in herds with winter dysentery (Hedström and Isaksson, 1951; Durham et al., 1989; Tråvén et al., 1993). However, quantification based on more than a few herds and longitudinal studies assessing the effect over time is lacking. Estimates of the effect on animal performance is an important part of the total economic impact of the disease, and is thus critical as input for a cost–benefit assessment of systematic control efforts.

Transmission

Virus transmission of BRSV and BCV within herds is normally very effective, resulting in a high level of morbidity (Cho et al., 2000; Valarcher and Taylor, 2007; Bidokhti et al., 2009). Both viruses can be transferred during animal-animal contact by the exchange of virus-containing body-secretions; primarily nasal secretion for BRSV and nasal secretions and faeces for BCV. Within herds animals often have extensive contact, and physical contact is likely an important mode of transmission for example from mother to offspring or between calves. A review by La Rosa et al. (2013) describes droplet-transfer of respiratory syncytial virus and coronavirus in humans. Because droplets and aerosols containing virus can travel short distances (depending on droplet size), transmission between animals within a herd does not require physical contact, thus making transmission possible for instance between calves in separate bins. Airborne transmission of BRSV was shown in an experimental study where exposed and sentinel calves were housed in separate isolates connected only through a tube in the wall (Mars et al., 1999). Airborne transmission of BRSV or BCV across longer distances, i.e. between farms, has not been described and is likely of limited importance. Between farms, direct transmission is possible through purchase of animals from other farms. Livestock could carry the infection from the herd of origin or they could be infected during transport if animals from several herds are mixed. Contact between

animals from different farms is also possible on shared pastures. It is not uncommon for herds to co-pasture in Norway.

BRSV and BCV can also be transmitted indirectly via contaminated surfaces, people or fomites. The potential for indirect transmission of virus largely depends on survival outside the host, which is influenced by temperature, humidity, pH and exposure to ultraviolet light (La Rosa et al., 2013). Enveloped respiratory viruses like BCoV and BRSV, are generally regarded fragile outside of the host. However, it has been shown that in a liquid suspension, BCV can remain infective at low temperatures for several weeks (Mullis et al., 2012). Reports of BRSV stability in the environment are scarce in the literature, but survival of the closely related human RS virus was investigated by Hall et al. (1980) who found that virus on average survived 7 and 5 hours in room temperature on countertops and rubber gloves, respectively. Inactivation of RS virus is much slower at low temperatures, and the virus has been reported to be most stable at temperatures below zero degrees Celsius (Hambling, 1964). The potential of indirect transmission of BRSV and BCV was investigated in a recent study by assessing virus survival on human mucosa, boots, coats, wristwatches and stethoscopes (Oma et al., 2018). In two separate experiments, calves were infected with BRSV and BCV, respectively. For both BRSV and BCV, a high load of virus was found on fomites 24 hours after exposure to infected calves. BRSV seemed to lose infectivity more rapidly than BCV as there were no infective BRSV after 24 hours, whereas BCV were still infective. The latter study emphasizes that virus carriage on fomites likely poses an infection risk to cattle. The relative importance of direct transmission between farms through animal trade versus indirect via visitors and fomites is not known.

It is believed that farm outbreaks of disease caused by BRSV and BCV commonly result from new introduction of virus. This is supported by the high rate of rate of self-clearance at the herd level (i.e. clearance of virus from the herd without specific interventions) previously reported (Klem et al., 2013). Similar dynamics have also been reported from Sweden (Ohlson et al., 2013). As the protective immunity acquired after infection is short, reinfection of animals is likely common. Consequently, previous herd-outbreaks do not prevent the herd from infection following reintroduction of virus. Tråvén et al. (1993) reported that among herds with winter dysentery, several had experienced previous outbreaks in the preceding two–four years. Whether chronic infection or reactivation of latent virus occurs is a debated issue in the literature. Some authors suggest that this is possible, and might play a role in sustaining infection in a herd (Van der Poel et al., 1997;

Kanno et al., 2018). Virus circulation for prolonged periods of time is likely possible in large herds if susceptible animals are introduced regularly (De Jong et al., 1996). However, for Norwegian conditions, the small average herd size makes it likely that this is of limited importance compared to new introductions.

Herd-level risk factors

Numerous studies have assessed risk factors for BRSV- and BCV-related disease within herds, typically assessing factors related to host, environment and agent, reflecting the multifactorial nature of the diseases caused by these viruses. However, this section will focus on risk factors where the study unit is the herd, i.e. the outcome is measured at the herd level. The status of a herd is of course related to the animals in the herd (for instance, to the immunity of individual animals). However, risk factors for a positive classification at the herd level will, to a larger extent, reflect the risk of virus introduction into the herd, which in turn is closely linked to transmission routes between farms.

Few studies have estimated the effect of animal purchase as a risk factor for BRSV and BCV. Frössling et al. (2012) found that purchase of livestock was a significant risk factor for herd-level positivity (measured by serology of young stock) for BCV, but not for BRSV. Another Swedish study reported that outbreaks of BRSV-related respiratory disease often started with the purchase of animals (Elvander, 1996). Remarkably, Tråven et al. (1993) found that a lower proportion herds with winter dysentery had purchased cattle during the previous year compared to non-outbreak herds, however the difference was not statistically significant. Large herd size is a frequently reported risk factor for both viruses (Tråvén et al., 1999; Norström et al., 2000; Ohlson et al., 2010b; Frössling et al., 2012). This might be related to more traffic of human visitors in larger herds, leading to higher risk of indirect transmission via fomites. Ohlson et al. (2010b) found that providing boots for visitors had a preventive effect against herd-level antibody positivity (using pooled milk from primiparous cows) for both viruses. Several studies have indicated that the regional cattle density is of importance. In the study by Ohlson et al. (2010b), a short distance to the nearest cattle herd was a significant risk factor for BCV, but not for BRSV, and geographic location was associated with herd status for both viruses. The existence of bordering cattle farms was reported as a risk factor for BRSV positivity among herds in Ecuador (Saa et al., 2012).

A surprising preventive effect of artificial insemination (AI) by external technicians compared to farm personnel was found in two studies from Sweden (Bidokhti et al., 2009;

Ohlson et al., 2010a). Ohlson (2010a) suggests that differences in general biosecurity level and management could be possible explanations. Conflicting findings exist with regard to differences between conventional and organic production. Bidokhti et al. (2009) found that conventionally managed herds had higher odds of positivity compared to organic; however, this could not be verified in a later study with a larger sample size (Wolff et al., 2015). Few studies of risk factors exist from Norway. However, Norström et al. (2000) found that mixed herds (dairy and beef together) had an increased risk of outbreaks of respiratory disease, presumably caused by BRSV, compared to both beef and dairy herds. In temperate climates, outbreaks of respiratory disease caused by BRSV seem to occur more frequently during the early winter season (Stott et al., 1980; van der Poel et al., 1993). Nevertheless, studies from Norway and Sweden found that seroconversion for BRSV at the herd level (i.e. change in status from negative to positive) occurred throughout the year, indicating that virus introduction also happens during the summer (Klem et al., 2013; Ohlson et al., 2013).

Like respiratory disease, winter dysentery also occurs most frequently during the winter. Boileau and Kapil (2010) suggest in a review that this might be due to indoor housing in close confinement combined with increased stability of coronavirus at low ambient temperatures. They state that both host and environmental factors might contribute to the appearance of the disease, such as the age and reproductive status of the animals and various climatic conditions. White et al. (1989) studied risk factors for farmer-diagnosed winter dysentery and found that a large herd size (> 60 cows) and a history of previous winter dysentery outbreaks were risk factors at the herd level, and also found evidence of space–time clustering of the disease. An introduction of virus via people or fomites has been reported as the likely transmission-route in some outbreaks of winter dysentery (Hedström and Isaksson, 1951; Roberts, 1957).

In general, there is a lack of studies on factors affecting the risk of BRSV and BCV introduction to herds.

Disease prevention and control

Finding strategies to control infectious diseases in a way that limits the use of antimicrobials, meets animal welfare requirements and reduces producer and consumer costs is a prioritized issue around the world.

Prevention of disease within herds

Internationally, the control of respiratory disease and diarrhoea largely focuses on limiting the consequences of disease within herds. Limiting between-herd transmission is often difficult due to extensive contact between herds. Given the multifactorial nature of BRD, the preventive approach will often be multi-targeted, including measures to facilitate robust animals, optimize the environment and minimize exposure to pathogens. Preventive measures are often placed in conjunction with introduction of new animals, such as vaccination of calves before shipment, quarantine, preventive or metaphylactic use of antibiotics on arrival. These measures are commonly used, especially in feedlot systems. Vaccination against BRD (both bacterial and viral pathogens) is also widely used. In Norway, metaphylaxis and preventive use of antibiotics is not practiced, and vaccines against BRSV or BCV infection are also of limited use.

Vaccine efficacy

Because the host immune response plays an important role in the pathogenesis of both human RSV and BRSV infection, developing efficacious and safe vaccines has proved a challenging task. In the 1960s formalin adjuvated human RSV vaccines unfortunately resulted in vaccine enhanced disease in infants (Kim et al., 1969). The risk of exacerbating disease has been one of the hampers in the development of RS vaccines in humans, and despite considerable research efforts there is still no approved vaccine available. In veterinary medicine, BRSV vaccines have been on the market for several decades. Disease enhancement after use of inactivated vaccines has been reported in cattle as well (Schreiber et al., 2000), and live vaccines against BRSV can exacerbate disease caused by concurrent infection (Kimman et al., 1989). Another important challenge to the development of efficacious BRSV vaccines is the presence of maternally-derived BRSV neutralizing serum antibodies. Today, both live modified and inactivated vaccines against BRSV are available worldwide, and vaccines exist for both parenteral and intranasal administration. Reports of their efficacy have been summarized in several review articles (Larsen, 2000; Meyer et al., 2008; Ellis, 2017) and recently also in a systematic review and meta-analysis (Theurer et al., 2015). In general, these reports document varying efficacy between studies. However, the meta-analysis of experimental challenge trials evaluating modified live vaccine, found no difference in morbidity or mortality risks between vaccinated and nonvaccinated calves (Theurer et al., 2015). Some authors suggest that mucosal administration is the way forward, and that intranasal vaccines show promising results in terms of immunity and duration

(Valarcher and Taylor, 2007; Ellis, 2017). A general problem with challenge studies for BRSV is the inability to produce clinical disease with a comparable severity to what is seen after natural exposure.

In Norway one BRSV vaccine was available during the course of this project, a trivalent vaccine for parenteral administration that includes inactivated BRSV, parainfluensa-3-virus (Pi-3-V) and *Mannheimia haemolytica* (Bovilis® Bovipast RSP vet). A few published experimental challenge studies have evaluated the efficacy of this particular vaccine, all of which included calves with maternal antibodies. Two of the studies evaluated the efficacy of a single dose: Mawhinney and Burrows (2005) detected a statistically significant ($p < 0.05$) increase in BRSV-reactive IgG in vaccinated calves, and Van der Sluijs et al. (2010) found that vaccinated calves had significantly less virus shedding in nasal discharge. A third study evaluated the efficacy of two doses of the vaccine given one month apart, and did not detect nasal shedding of virus in vaccinated calves, whereas virus was shed by a variable proportion of unvaccinated calves (Patel, 2004). Significant differences in clinical signs between groups were not detected in any of the above mentioned studies (Patel, 2004; Mawhinney and Burrows, 2005; van der Sluijs et al., 2010).

For BCV there are few vaccines available, and similar to BRSV the development of safe and efficacious vaccines has been difficult. Human vaccines are also lacking, despite efforts to develop vaccines against severe coronaviruses such as SARS-CoV and MERS-CoV. There is no available vaccine labeled for prevention of winter dysentery or BRD caused by BCV. Nevertheless, one report describes reduced risk of BRD treatment among calves in an American feedlot following intranasal administration of a commercially available vaccine against BCV and rotavirus (Plummer et al., 2004). In Norway, there is one trivalent vaccine containing inactivated BCV, rotavirus and *E. coli* available. It is labeled for active immunization of pregnant cows to confer passive transfer of antibodies to calves for protection against neonatal calf diarrhoea (Lactovac, Zoetis). The vaccine has been reported to increase antibody titers in colostrum and serum (Kohara et al., 1997). However, both this study and another field trial failed to find a protective effect of vaccination on occurrence of calf diarrhea (Waltner-Toews et al., 1985; Kohara et al., 1997).

Altogether, the efficacy of available vaccines against BRSV and BCV is questionable. Decisions regarding vaccination also involves considering aspects apart from vaccine efficacy, such management and as type of production. Notably, the BRSV and BCV

vaccines are multivalent and contain other pathogens than the viruses, often bacterial components. Protection against secondary bacterial infection e.g. *Mannheimia haemolytica* could mean that the vaccine provides disease protection in the field regardless of whether it protects against the primary virus infection. This adds to the complexity of decisions regarding whether or not to vaccinate. Theurer et al. (2015) suggests that, ideally, decisions regarding vaccination should be based on data obtained from randomized controlled trials performed in the cattle population in question. To date, no vaccine trials from Norway are available.

Prevention of transmission between herds

Routes of between-herd transmission are not fully understood; however, live animal contact represents a likely transmission pathway for both viruses. As mentioned previously the study by Oma et al. (2018), indicated possible virus carriage through fomites for as long as 24 h after exposure. Implementing a high level of biosecurity and limiting livestock purchase are general measures to reduce the risk of transmission between herds. However, keeping a closed herd is often not possible because of the need to purchase replacement animals. Human traffic is also hard to avoid, especially professional visitors such as veterinarians, AI-technicians and animal transporters. A Swedish study showed that a high proportion of visitors had direct animal contact, and although expected for AI-technicians and veterinarians, it is noteworthy that even animal transporters had animal contact during 41% of visits (Nöremark et al., 2013). Examples of biosecurity measures to reduce the risk of indirect transmission between herds are clothing and footwear available for visitors, washing facilities and use of infectious sluice room for all visitors. In a questionnaire to Swedish farmers, more than 90% of farmers perceived biosecurity as important, nevertheless 64% reported that boots for visitors were not provided or were never used (Ohlson et al., 2010b). This paradox highlights the need to increase awareness of biosecurity routines.

BRSV and BCV have some common features that make them both challenging but possible to control. The high occurrence of both viruses, the potential of both direct and indirect transmission and the high infectivity makes control a challenging task. Limited persistence in the environment and the likely limited importance of wildlife reservoirs favours control. Norwegian herds are small and dispersed throughout the country, suggesting that control of BRSV and BCV could be possible by limiting transmission between herds and regions. Furthermore, the high rate of self-clearance at the herd level (Klem et al., 2013), suggests

that prevention of new introductions of virus could be an effective measure to reduce herd prevalence in the Norwegian cattle population.

Control of BRSV and BCV in Norway

The Norwegian control programme

The Norwegian cattle population is, as mentioned, free from many infectious diseases. This notwithstanding, epidemics of respiratory disease and diarrhoea are common, reducing animal welfare and hampering the economic sustainability of the industry. Against this backdrop, there has been a growing desire over recent years among stakeholders in the Norwegian cattle industry to initiate systematic control of BRSV and BCV in the cattle population. A biosecurity-based control programme was launched by a joint cattle industry in March 2016 after screening nearly all dairy herds for BRSV and BCV antibodies in bulk tank milk (BTM). The planning, design, cost-benefit assessment and implementation was performed by the industry partners in the programme. Based on the initial screening, herds were classified herds as 'red' (antibody positive) or 'green' (antibody negative). Beef herds were included later, and classified based on serum samples of a group of young stock. The current recommendation for dairy herds is for BTM-positive herds to resample either using milk from primiparous cows or serum from a young stock. If the herd is test-positive after sampling of young stock, the advice is to resample when new non-exposed animals are available e.g. after six months. In general, no interventions are recommended for test-positive herds, and the key concept of the programme is to protect test-negative herds against virus introduction via implementation of external biosecurity measures. For a herd to maintain a green status, annual samples are required. Furthermore, it is possible to obtain a 'Healthy herd' certificate by fulfilling the following criteria: a 'green' status, purchase livestock from other test-negative herds only, facilities that allow animals to be loaded onto transport without the truck driver entering the barn, and an approved infectious sluice room. The certificate means the farmer is awarded with premium pricing of young-stock and breeding animals, and additionally receives a discount on livestock insurance (Harald Holm, personal communication, June 3, 2019).

The role of the PhD-project

This PhD-project is part of a larger project established in 2013, funded by the Research Council of Norway, with support from the industry. Altogether, two PhD-students, one engineer and one researcher worked in this larger project, with the aim to increase the knowledge of BRSV and BCV in Norway. Areas of importance for systematic control was

of particular interest. The research group was not directly involved in the decision making behind, or the implementation of, the control programme. However, the initiative to conduct systematic national control of these viruses prompted the need for more research both regarding the distribution and spread of the viruses and regarding testing and classification of herds.

Diagnostic tests and classification of herds

Diagnostics

Diagnostic tests used for demonstration of BRSV and BCV infection include both antigen and antibody tests. Antigen and antibody tests differ in that antigen tests detect the virus itself (or parts of the virus, e.g. RNA) and antibody tests detect specific antibodies against the virus. Antigen detection can be performed on a variety of sample material: tissue samples for post-mortem diagnosis (tissue from the respiratory tract, lungs or distal small intestine and colon), ante-mortem samples including nasal swabs, lung lavage or tracheal washes, and additionally faecal samples for BCV infection (Larsen, 2000; Boileau and Kapil, 2010; Saif, 2010). The most common antigen test used for BRSV and BCV is reverse transcription polymerase chain reaction (RT-PCR), but other antigen tests exist, including haemagglutination assays, immunofluorescence techniques, immune electron microscopy, and antigen ELISAs (Schoenthaler and Kapil, 1999; Larsen, 2000; Boileau and Kapil, 2010; Saif, 2010). Methods for antibody detection include different ELISAs, as well as virus neutralization tests (VNT) (Alenius et al., 1991; Larsen, 2000).

Antibody detection is performed on either milk or serum samples. The purpose of testing is of importance for the choice of test. For outbreak investigations, the method of choice will often be either antigen detection or antibody testing using paired serum samples for detection of titre increase. Because it is cheaper and more practical to perform antibody testing is commonly used for screening, when sampling of many herds or animals is necessary.

Evaluating diagnostic tests

Put simply, the purpose of a diagnostic test is most commonly to detect or rule out disease. Although diagnostic tests are usually thought of as a laboratory procedure, the term has a wider meaning. According to Dohoo et al. (2009, p. 92) ‘A test, more generally, is any device or process designed to detect, or quantify, a sign, substance, tissue change, or body response in an animal. Tests can also be applied at the herd, or other level of aggregation’.

Ideally, a diagnostic test would always answer one's diagnostic question without error, and unequivocally indicate presence or absence of disease. However, perfect tests are rare. Thus, there is a need to quantify one's trust in the test result. Preferably, a test should be precise and accurate. High precision, or consistency, means the test results show little variation between batches, runs, days and even between different labs (especially if results from different laboratories are used). The accuracy of a diagnostic test is commonly measured by its sensitivity (Se) and its specificity (Sp). The diagnostic Se refers to the ability of the test to correctly classify diseased (or seropositive, infected etc.) animals (or other study units); and the diagnostic Sp refers to the ability of the test to correctly identify healthy (i.e. seronegative, non-infected etc.) animals. This differs from the analytic sensitivity and specificity used in laboratory settings to describe the ability of a test to correctly identify the presence of a particular analyte (Saah and Hoover, 1997). Throughout this thesis, Se and Sp will refer to the diagnostic sensitivity and specificity, respectively.

Diagnostic test evaluation against a perfect reference procedure

The Se and Sp are characteristics of the test. However, in a clinical setting, calculating predictive values is often more informative. The positive predictive value (PPV) is the probability that, given a positive test result, the test subject truly has the diagnosis in question. The negative predictive value (NPV) is the probability that, given a negative test, the subject does not have the diagnosis in question. Traditionally, evaluation of diagnostic tests has been performed against a perfect reference procedure, i.e. a 'gold standard'. In this scenario, the Se, Sp and predictive values can be calculated directly from cross-tabulating the results. When test accuracy is known, the predictive values can also be calculated from the Se, Sp and prior probability of positivity, i.e. the prevalence in the tested population. The concept of evaluating a diagnostic test against a gold standard method is illustrated in the 2x2 table shown below (Table 1).

Table 1. Data layout for diagnostic test evaluation against a perfect reference procedure (gold standard).

	True diagnosis (gold standard)		
Test result	Positive (diseased)	Negative (healthy)	Total
Positive	a	b	$a+b$
Negative	c	d	$c+d$
Total	$a+c$	$b+d$	n

Sensitivity = $a/(a+c)$ Positive predictive value = $a/(a+b)$

Specificity = $d/(b+d)$ Negative predictive value = $c/(c+d)$

Diagnostic test evaluation in the absence of a gold standard

Unfortunately, a perfect reference procedure is rarely available, and the true status of the test subjects is therefore hard to verify. Thus, statistical methods have been developed over the past several decades for the evaluation of diagnostic tests in the absence of a gold standard: namely, latent class models. These models allow for estimation of test accuracy and prevalence by treating disease as a latent (unknown) variable. A problem that arises when using two tests, where neither test is assumed perfect, is that there are altogether five parameters to be estimated: the Se and Sp of both tests, in addition to the population prevalence. This means that there are more unknown parameters than available degrees of freedom, i.e. the parameters cannot be uniquely estimated from the data alone, resulting in what is called a non-identifiable model. Hui and Walter (1980) presented a solution to this problem by using two tests in two populations. As introducing a second population will add only one new parameter to be estimated (i.e. the prevalence of the new population), while increasing the available degrees of freedom by three, the model becomes identifiable. The two tests, two populations model has since been widely used for diagnostic test evaluations, and many extensions of the model have been developed.

BCV/BRSV commercial ELISA

The Svanovir® BRSV-Ab and the Svanovir® BCV-Ab are commercially available ELISA tests, used in many previous studies both in Norway and Sweden (Beaudeau et al., 2010; Ohlson et al., 2010a; Ohlson et al., 2010b; Klem et al., 2013). The Svanovir® BRSV-Ab is currently used in Swedish herds in a voluntary sampling programme for dairy operations as

part of the control initiative called ‘safe animal trade’ run by Växa Sverige (the largest dairy cooperation in Sweden).

For the Svanovir® BRSV-Ab the parameters provided by the manufacturer are Se 94% and Sp 100%. They originate from a validation study where the Svanovir® BRSV-Ab was compared to another indirect ELISA in use at the Central Veterinary Laboratory (CVL), Weybridge (Elvander et al., 1995). The study design was complex, but in one part both tests were used on 174 serum samples, of which 17 were excluded due to absorbance values in the “grey zone” for the CVL ELISA, leaving 157 samples for cross-tabulation of results. The CVL ELISA was used as a gold standard, and relative estimates of Se and Sp were obtained for the Svanovir® BRSV-Ab. The gold standard approach poses a limitation to the study, as it can be argued that neither of the tests are likely to be perfect. An erroneous assumption of a perfect reference test can introduce bias in the accuracy parameters (Lijmer et al., 1999). Furthermore, using a gold standard that produces three categories (positive, negative and inconclusive) seems particularly problematic, as many samples had to be excluded from the analysis, effectively introducing a source of selection bias. Different cut-off values were not assessed, and the option of altering cut-off values is not discussed in the paper (Elvander et al., 1995).

For the Svanovir® BCV-Ab the test parameters provided by the manufacturer are Se 84.6% and Sp 100%, with a reference to a study conducted in 1991 (Alenius et al., 1991). The aim of this study was not to validate the ELISA test, but it included a serologic investigation, and thus knowledge of the performance of the test was required. The ELISA was compared to a VNT in a subset of 91 serum samples. Traditionally, VNT has been used as a gold standard test to which a new antibody test can be compared. Nevertheless, the assumption that the reference test is error free, is questionable. VNTs are often considered highly sensitive and could also be highly specific, but might be subject to cross-reactivity, as well as inter-laboratory and inter-assay differences. Moreover, they require the growth of virus in cell culture, and can thus be sensitive to the choice of virus and cell line, and highly skilled people are needed for assessment of the cytopathic effect. A cross-tabulation of results is not provided in the paper and estimates of Se and Sp are not stated, probably reflecting that estimation of test parameters was not the objective of this study. The limited information provided in the paper makes it difficult to reproduce the test parameters.

Both the BRSV and BCV ELISA have test parameters obtained for serum samples only; however, the tests are in use for individual milk samples and BTM as well. The documentation of test parameters are based on a single study for each test, with limitations as discussed above. Therefore, more evidence to substantiate the test parameters for serum samples as well as estimation of test accuracy for other applications would provide useful information to test users.

BCV/BRSV multiplex immunoassay

Because a diagnostic test can perform differently in different populations, each diagnostic test should be evaluated for the intended application, in the population where it is going to be used. In conjunction with the planning of the national BRSV/BCV control programme, a new diagnostic test, the MVD-Enferplex BCV/BRSV multiplex immunoassay (Enfer Scientific, Naas, Ireland), was developed for detection of antibodies in BTM. This test will hereafter be referred to as the multiplex. The test has several antigens for each virus printed at the bottom of the same ELISA well; three antigens for BCV and four for BRSV, thus enabling simultaneous detection of antibodies against both viruses. A positive reaction is indicated by chemiluminescence, which is captured by a camera. As the test was new, a validation for the intended application in the Norwegian cattle population was necessary.

Classification of herds

Herd testing

Christensen and Gardner (2000) define a herd test as 'an evaluation of a sample of (or all) animals from a herd and the application of decision rules that classify the herd as positive or negative based on the test results from individual animals'. When a diagnostic test is used as a herd test, it is the herd-sensitivity (HSe) and -specificity (HSp) that are of interest. The HSe is the probability that a positive (e.g. seropositive) herd yields a positive herd test result, and herd-level specificity (HSp) is the probability that a negative (e.g. seronegative) herd yields a negative herd test result (Martin et al., 1992). A herd diagnosis can be based on a number of individual samples or various pooling schemes, including BTM.

Herd classification is a key component of any herd-level control programme, hence there is a need for herd-level diagnostic tools that accurately classify the herds in a cost-efficient manner. For BRSV and BCV, antibody ELISAs are readily available and cheap and easy to use on a large scale, thus detection of antibodies has been used to classify herds (Ohlson et al., 2010a; Klem et al., 2013; Wolff et al., 2015). For both viruses, studies from Sweden

have shown a high correlation between antibodies in milk and serum, indicating that serum samples can be replaced with milk samples in dairy herds (Elvander et al., 1995; Ohlson et al., 2014). Samples that have been used for herd testing of BRSV and BCV include milk from primiparous cows, serum from young stock and BTM (Beaudeau et al., 2010; Ohlson et al., 2013; Klem et al., 2014b). Because of the long-lasting antibody response the time period reflected by a positive test result will depend on the age of the tested animals, i.e. reflecting a period approximately equal to their lifetime. Testing milk from primiparous cows will express what has been circulating in the herd over the previous two years, whereas the time period reflected by serum from young stock is shorter. Serology of animals younger than six months is usually avoided due to unwanted interference of maternal antibodies (Larsen, 2000).

Bulk tank milk testing

A special case of herd testing, where all lactating cows are included in the pooled sample, is BTM testing. BTM testing is convenient for sampling a large number of herds and is economical, as only a single sample is required to classify the herd. It has been used in several observational studies and surveillance and control programmes (Lindberg and Alenius, 1999; Nuotio et al., 2003; Løken and Nyberg, 2013; Ågren et al., 2016). For BRSV and BCV, the bulk tank milk can be antibody positive for a long time, possibly many years after virus has circulated in the herd (Alenius et al., 1991; Klem et al., 2014b). A positive BTM antibody test does not equal herd immunity. As antibody producing cows can be re-infected, detection of antibodies in BTM should not be used for inference about the level of herd protection. Altogether, the inference that can be made from a positive BTM test is limited for the individual herd, and follow-up testing of primiparous cows or young stock can be used for a more up-to-date herd diagnosis. An elaboration of the interpretation of BTM tests for BRSV and BCV is presented in the discussion.

Probability of freedom at the herd level

When using a diagnostic test for decision-making at the individual animal level, test results are commonly integrated with prior knowledge about how common the disease is, anamnestic information, clinical signs and other factors that might influence the probability of disease. Veterinary practitioners do this either intuitively or in a more quantitative manner, for instance by calculating predictive values. This can also be done for herd tests, and the trust one has in a negative test result can be quantified by the NPV. As mentioned, the NPV depends on the accuracy of the test and the prior probability of positivity, which

for herd tests is the herd prevalence in the population. However, herd testing is often used in surveillance or systematic control: Here, it might be useful to also account for how old the test result is. It is intuitively obvious that our confidence in a negative test decreases with the time elapsed since sampling, as long as there is a risk of reintroduction of virus. In the BRSV/BCV control programme, a negative test result is valid for one year before re-testing is required, but because virus is continuously circulating in the population, the assumption of a long-lasting negative status is questionable. Furthermore, there are likely differences in the risk of introduction between different herd types, even for herds with equal starting points (i.e. a negative test). Therefore, the decrease in one's confidence in a negative status over time should reflect the herd's known risk factors for virus introduction. For instance, purchase of livestock should entail a decreased probability of freedom (continued seronegativity) compared to no purchases.

Estimations of probability of freedom are commonly performed to document freedom from disease at a national level, as this is often necessary for international trade purposes (Alban et al., 2008; Murphy et al., 2012; Frössling et al., 2013). However, a few recent studies have used the same methodology with an increased focus at the herd level (More et al., 2013; Veldhuis et al., 2017; Ågren et al., 2018). Calculations of probability of freedom can be utilized as a quantitative approach to estimate the decrease in confidence of a herd's negative status over time. In the control of BRSV and BCV, the probability that a herd is antibody negative over time could be useful for classifying herds into different risk categories, which in turn could be used for risk-based sampling or as a risk assessment tool in the organization of animal trade. Reduced costs and faster progress of the control programme would be the desired result. How integration of herd-level data can be used in BRSV and/or BCV surveillance and control is not yet known.

Knowledge gaps

Studies on risk factors for disease caused by BRSV or BCV often measure the outcome at the individual level, and thus focus on factors related to the animal or the environment, including housing and management factors. This is undoubtedly important knowledge, as optimizing the environment and creating robust animals has commonly been the target of preventive measures. Internationally, vaccination and mass medication are widely used to control disease associated with these viruses. Nevertheless, they continue to cause problems. The effect of vaccination is questionable, and mass medication is in conflict with the

prudent use of antibiotics endorsed by most countries. Reducing the consequences of diseases caused by these viruses by preventing introduction to herds, could be the key to a more sustainable control strategy. But how can transmission between herds be prevented? What is the relative importance of different transmission routes? What are the risk factors for virus introduction? An identification of risk factors for virus introduction under Norwegian conditions had still not been performed at the start of this project, and was important to provide key information in the design of control strategies. This led to the following research question to be answered in this PhD-project:

- What are the risk factors for a positive herd status for BRSV and BCV based on detection of antibodies in BTM?

Furthermore, the spatial distribution of these viruses has been explored to a very limited extent in Norway. Are negative and positive herds mixed together? Are there any spatial clusters of positive herds? Potential hot spots might require special attention, or a different control strategy compared to low prevalence areas. Based on previous studies from Scandinavia it was hypothesised that prevalence was likely to vary, but what was the prevalence of positive herds nationally and in different regions, and what did the spatial distribution in Norway look like? To gain some insight into this, the second research questions was formulated for one region in western Norway:

- What is the spatial distribution of BRSV- and BCV-positive herds based on detection of antibodies in BTM in a region in western Norway?

Similar to risk factor studies, most studies on the consequences of disease are performed at the individual level. Quantifying the total cost of these infections at the herd level, not to mention the national level, is complex. The different clinical manifestations; BRD, calf diarrhoea and winter dysentery all have different impact. The consequences can furthermore be divided into effects on animal health and welfare, effects on antibiotic usage and effects on production e.g. reproductive performance, growth rate and milk production. Therefore, a decomposition of the problem is necessary. For winter dysentery, only a few studies aiming to quantify the associated drop in milk production exist at the herd level, and these include only a few herds (Durham et al., 1989; Jactel et al., 1990). Because a herd often consists of cows with differing susceptibility to disease and the severity of infection varies, it is problematic to draw inference at the herd level from studies performed at the cow level. The impact at the herd level is of utmost interest for the farmer, whose main concern is the effect

on net income. Furthermore, a quantification of milk production loss is needed as input in the cost–benefit analysis that is an essential step in the planning of a control programme. This led to the following research question:

- What is the effect of winter dysentery on herd level milk production?

Herd level control programmes generally require classification of herds with respect to the risk of harbouring infection. Identifying a cost-efficient procedure with acceptable accuracy is necessary. For BRSV and BCV, some practical questions arose: Should BTM samples or individual samples be used? If using individual milk or serum samples, can they be pooled, and how many samples should be included in the pool? How are antibodies in BTM associated to the within-herd prevalence? The owners of the control programme had decided to initiate systematic control by screening all dairy herds using a new multiplex immunoassay on BTM. The diagnostic accuracy for this application were therefore of particular interest, and this led to the following research question:

- What is the diagnostic test sensitivity and specificity of the BCV/BRSV multiplex test for detection of antibodies in BTM?

Because of the endemic occurrence of BRSV and BCV, there is a continuous risk of reintroduction of virus to all herds. The uncertainty of a herd's status at a given time therefore depends not only on the accuracy of the test, but also the risk of virus introduction. For all herds, the confidence in a disease-free status will decrease over time since the last negative test, however, the risk likely differs between herd types. Available data on test accuracy, herd location and movements might be useful to better predict the probability of an antibody negative status over time. This led to the research question:

- How can test sensitivity, location and animal movements be used to predict the herd level probability of freedom from antibodies over time?

Aims of the Study

The overall aim of this thesis was to provide knowledge of the epidemiology of BRSV and BCV in the Norwegian cattle population. To answer the questions identified under knowledge gaps, the specific aims of the thesis were to:

1) Identify risk factors for and spatial distribution of BRSV- and BCV-positive herds based on detection of antibodies in bulk tank milk.
2) Quantify the effect of winter dysentery on herd-level milk production.
3) Validate the new multiplex immunoassay used in the Norwegian BRSV and BCV control programme.
4) Develop a framework for estimation of the herd-level probability of freedom from BRSV and BCV antibodies over time.

Materials and methods

This section gives a brief overview of the material and methods used in Papers I to IV. It starts with a description of the study sample, i.e. the included herds, of each paper. Then, a brief description of the sources of secondary data follows, after which the methods are described. The different methods are sectioned into regression models, spatial analysis, diagnostic tests and classification of herds, and finally calculations of probability of freedom. An overview of study design, study aims, sources of secondary data and statistical methods are presented in Table 2. Further details are provided in the papers, attached as Appendix I to IV. Methodological considerations are addressed in the discussion.

Study samples and register data

The study unit was the herd in all four papers in the present thesis. None of the studies were national, but were conducted in different regions of the country. A brief description of the study sample of each paper follows.

Paper I was a cross-sectional study in which the study sample consisted of dairy herds in two counties in western Norway, namely Møre og Romsdal and Sogn og Fjordane. Of the 1854 dairy herds that shipped milk in the study region at the time of sampling, 1347 delivered a BTM sample and were included in the study. Note that different subsets of herds were included in different parts of the analysis (see Paper I for details).

Paper II was based on reports from veterinarians and producers of outbreaks of winter dysentery to a 'hotline' operated by the advisory service of the largest dairy company in Norway (TINE SA). From the 4th of November 2011 to the 13th of March 2012, 241 herd outbreaks were reported from 7 counties in eastern Norway. A retrospective cohort study was conducted, and herds with a reported outbreak were considered exposed (WD+), and were compared to non-exposed herds (WD-), consisting of all other herds that were members of the Norwegian dairy herd recording system (NDHRS) located in the same area. After data-cleaning, 2317 study herds (224 WD+ and 2093 WD-) remained for analysis of milk production and 1539 (167 WD+ and 1372 WD-) herds for analysis of milk composition.

In Paper III, the study sample was selected from herds that submitted a bulk tank milk sample for the national screening in 2016. A diagnostic test evaluation was performed using two tests in two populations with an expected difference in prevalence, and based on this

assumption, samples from two different counties were randomly selected. As a result of Paper I, we knew that prevalence in Sogn og Fjordane (in western Norway) was likely to be low, and we therefore selected 360 herds from this county and 360 herds from Oppland (in eastern Norway), where prevalence was expected to be higher based on field experience and the knowledge of outbreaks, as described in Paper II.

The study sample in Paper IV consisted of 1148 herds, of which all were also included in Paper I. A subset of the herds from Paper I was used because this study included previous test results in addition to new BTM samples, collected one year and three years later. It is worth noting that only the initially test-negative herds were included in the probability of freedom calculations (n=676 for BRSV, and n=333 for BCV). The relationship between the study sample of the three papers that include BTM results (Paper I, Paper III and Paper IV) are visualized in Figure 3. The study sample of Paper II is not included in this figure, as these herds were not sampled and were never matched against the herds in the datasets for the other studies. However, there is likely to be an overlap with some herds in Paper III, as these herds were selected from the same region as the 'outbreak region' of Paper II.

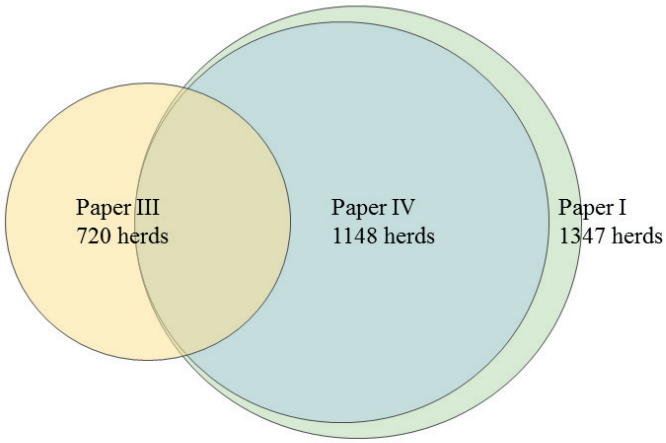

Figure 3. Venn diagram illustrating the relationship between the study samples in Papers I, III and IV.

Register data

There are several national databases for livestock in Norway. Register data were used in all four studies included in this thesis, and a brief description of these registries follows.

Norwegian dairy herd recording system

The Norwegian dairy herd recording system is a database owned by TINE SA. Membership is voluntary, and in 2017 the NDHRS contained records from 96% of all Norwegian dairy herds (TINE Advisory Service, 2018). The NDHRS is a relational database containing tables on herd characteristics, health events (disease and treatment), lactation data (production and milk composition), data on calving and reproductive performance and more. Data on health events are recorded on farm by the veterinarian, using individual cow health cards (CHC), and are later transferred to the NDHRS. Data from the NDHRS have been frequently used for research purposes (Norström et al., 2001; Gulliksen et al., 2009a; Klem et al., 2013). For studies included in this thesis, NDHRS records were retrieved for various herd-level variables (e.g. herd size, milking system, and production type) in Paper I, Paper II and Paper IV (see the respective papers for details).

Milk shipment data

Milk shipment data refers to the routinely recorded milk production data collected at each bulk tank milk shipment. Milk shipment data are rarely used for research purposes. An extraction from the largest milk shipment database owned by TINE SA was used in Paper II. The retrieved records contained the following variables: volume of milk shipped, somatic cell count, freezing point and milk components (fat, protein, lactose, urea and free fatty acids).

Norwegian livestock register

The Norwegian livestock register is managed by the food safety authorities. The main purpose of this register is to enable traceability and tracking of all livestock in Norway. There are three events that trigger a mandatory report for all farmers owning cattle: births, deaths and movements (Forskrift om sporbarhet og merking av storfe, 2010). Births are registered in conjunction with the ear tagging of new-born calves, and the report includes the animal identification, herd identification, date of birth, sex, breed and identification of the mother. Recorded animal movements include purchased animals, sold animals, animals sent to slaughter and other reasons for movements. Data on animal movements have been used to a limited extent for research in Norway, but their use has been explored in other

countries, for instance Sweden (Widgren and Frössling, 2010; Frössling et al., 2012). Records of livestock movements retrieved from the Norwegian livestock register were used in Paper I and Paper IV.

Norwegian Direct Payment Register

The Norwegian Direct Payment Register (NDPR) is owned by the Norwegian Agricultural Agency, and contains records on all Norwegian herds that have applied for subsidies. All cattle farms (dairy and beef) in Norway are entitled to receive financial support from the government. The database contains records on the number of animals (beef and dairy) in different age-groups and the geocoded location of the herd, and is updated every six months. The figures recorded in the NDPR are used for official statistics, and are estimated to be the census population of herds. Geo-coordinates from the NDPR were used in Paper IV. Geo-coordinates were also used in Papers I and II, however these were provided by the industry (TINE SA).

Table 2. Aim, study design, sources of secondary data and statistical methods used in Papers I–IV.

	Aim	Study design	Sources of secondary data	Statistical methods
Paper I	Identify risk factors for and spatial distribution of BRSV- and BCV-positive herds based on detection of antibodies in bulk tank milk.	Cross-sectional study	NDHRS[1], The Norwegian livestock register	Logistic regression Spatial cluster analysis
Paper II	Quantify the effect of winter dysentery on herd-level milk production.	Cohort study	NDHRS[1], Milk shipment data	Mixed linear regression
Paper III	Validate the new multiplex immunoassay used in the Norwegian control programme against BRSV and BCV.	Diagnostic test evaluation	None	Latent class analysis
Paper IV	Develop a framework for estimation of the herd-level probability of freedom from BRSV and BCV antibodies over time.	Simulation study	NDHRS[1], NDPR[2], The Norwegian livestock register	Probability of freedom estimation

[1]Norwegian Dairy Herd Recording System

[2]Norwegian Direct Payment Register

Multivariable regression models

Regression models were used in Paper I and Paper II.

Logistic regression

In Paper I, one part of the aim was to assess risk factors for antibody positivity at herd level. There were two outcome variables: the BTM antibody test result for BRSV and BCV, each assessed separately. The test results were dichotomized and herds were classified as either negative or positive for each virus. As the outcome was binary, logistic regression was used to obtain estimates for the effect of different risk factors on antibody positivity. Univariable analysis using simple logistic regression was performed for a set of 11 explanatory variables assessing a possible association with the outcome. Variables were included in the following multivariable model-building process if the p-value < 0.2. A total of four multivariable logistic regression models were built: two for each virus, one including only the x- and y-coordinates to account for large geographic trends (i.e. first-order spatial effects), and one also including all other risk factors retained in the model building process. This was performed to allow for comparison of the spatial distribution of high values of residuals from models adjusting for risk factors like herd size and distance to neighbours and models without such adjustment, further explained under spatial analysis. Post-estimation assessment of residuals was performed to evaluate model performance and goodness of fit.

Repeated measures

The primary objective of study II was to estimate the effect of a clinical outbreak of farmer-diagnosed winter dysentery on herd-level milk production and milk composition. Thus, the main explanatory variable was whether or not the herd had reported an outbreak of winter dysentery. The outcome of the main analysis was herd-level milk production, measured by volume of milk (L) per cow per day. Secondary outcomes were somatic cell count (1000 cells/mL), free fatty acids (millimoles/L) and contents of fat (%), protein (%), fat/protein ratio, urea (%) and lactose (%). All outcomes were continuous and hence linear regression models were used. The average production was higher in WD+ than WD- herds in the time period before outbreak was reported. This was analytically adjusted for by including the pre-outbreak milk production in the model. For the analysis on milk production, we had repeated milk shipment records for each herd. Dependency between measurements was accounted for by including a herd random effect (random intercept) with a correlation structure. Several different correlation structures were considered, and a Toeplitz six correlation structure was chosen based on Akaike's information criteria (AIC). An

interaction term between WD and time since outbreak was included to allow for a time-dependent effect, modelled as a cubic spline to allow for a non-linear change in effect of WD on milk yield over time. The milk component analyses were simpler: Due to fewer measurements, we only used the first available milk component analysis within 20 days after reporting of the outbreak as the outcome, thus there was no need to account for repeated measurements in these analyses.

Spatial analysis

Maps

Simple point maps of study herds were made in Paper I and Paper II. In Paper II, the map showed the spatial distribution of herds that had reported, and not reported, winter dysentery during the study period. In Paper I, the point map visualized the spatial distribution of BTM-positive and -negative herds. Explorative spatial analysis was performed in Paper I, and the prevalence risk distribution was visualized on isopleth maps showing prevalence on a smooth continuous surface with a colour scale ranging from blue (low prevalence) to red (high prevalence). The isopleth maps were generated using Kernel density estimation, which is a weighted moving average method used to estimate the intensity, or mean function, of point processes (Berke, 2005). To compare the density of cases to the density of the background population (i.e. to achieve prevalence risk), the Kernel density raster layer for the cases was divided by the Kernel density raster layer for the background population.

Spatial cluster analysis

Spatial cluster analysis of test-positive herds was performed in Paper I using the spatial scan statistic (SaTScan version 8.1.1) (Kulldorff, 2009). In brief, spatial clusters are detected as the software scans the area using circular windows with varying diameters. The number of observed vs. the number of expected observations inside the window are compared for each location and size, and the likelihood function is calculated for each window (Kulldorff, 2015). As recommended for geocoded point data, detection of clusters of BTM-positive herds was performed under the Bernoulli model, which means that the clustering of case location distribution is compared to that of the controls. In this study, the cases were the BTM-positive herds and controls were the BTM-negative herds. Furthermore, residuals from the multivariable regression models were analysed using the spatial scan test under the normal probability model, which can be used for continuous data. We compared the spatial distribution of high values of residuals from models adjusting only for first-order effects (x-

and y-coordinates included in the model) with the final models including all the risk factors retained in the model building process. This was done to explore whether including significant risk factors would alter the spatial distribution of risk, and hence the appearance of spatial clusters of high values of residuals. All cluster analyses were performed with a maximum spatial cluster size of 20% of population at risk, with no allowed overlap between clusters, and we only reported statistically significant clusters ($p<0.05$). Statistical significance of the results was tested using Monte Carlo simulation (999 iterations).

Diagnostic tests and classification of herds

In Paper II, herds were classified as WD+ or WD- (exposed and non-exposed, respectively), based on whether or not the farmer had reported an outbreak of winter dysentery. Under-reporting of outbreaks was expected due to the voluntary reporting system. We therefore simulated outbreak dates for all herds in the WD- group, thus restricting the analysis to a comparable time window and lowering the chance of a real (but not reported) WD outbreak within the modelled time period.

Study herds in Papers I and III were classified based on bulk tank milk testing as antibody-negative or -positive for BRSV and BCV, respectively. In Paper I, BTM samples were analysed using commercially available ELISAs, and herds were classified based on the dichotomized test results. Apparent prevalence and estimates of true prevalence were calculated for the study region. True prevalence estimates were based on test accuracy parameters presented by the manufacturer, and calculated using the Rogan-Gladen estimator of true prevalence (Rogan and Gladen, 1978). In Paper III, the same test was used as a reference test in the evaluation of the new multiplex immunoassay. Study herds were therefore cross-classified according to two different tests for BRSV, and then the same was done for BCV. Estimation of accuracy parameters was performed using latent class analysis, thus allowing for imperfect classification of both tests, as described in the next section.

In Paper IV, herd classification was explored further and in addition to test results we used information on herd size, geographic information and animal movement data to achieve an updated estimate of herd status for the initially test-negative herds. An overview of study design, diagnostic tests, herd classification and actions taken to correct for the uncertainty in herd classification is shown in Table 3.

Table 3. For Papers I–IV: Diagnostic tests, herd classification and actions taken to correct for uncertainty in herd classification.

	Diagnostic tests	Herd classification	Actions taken to correct for uncertainty in herd classification
Paper I	Bulk tank milk (BTM) ELISA[1]	BTM+/BTM- , binary	Rogan-Gladen estimator of true prevalence
Paper II	Owner-reported winter dysentery (WD)	WD+/WD-, binary	Simulation of pseudo-outbreak dates for WD- herds
Paper III	BTM ELISA[1] BTM multiplex immunoassay[2]	Combination of paired test outcomes: +/+ +/− −/+ −/−	Estimation of test accuracy parameters for BTM application, allowing for imperfect tests
Paper IV	BTM ELISA[1] BTM multiplex immunoassay[2]	Probability of freedom, continuous	Incorporating time from last negative sample and data on risk factors, in herd classification

[1]SVANOVIR® BRSV-ab and BCV-ab, Svanova Biotech, Sweden

[2]MVD-Enferplex BCV/BRSV multiplex, Enfer Scientific, Naas, Ireland

Evaluation of the BCV/BRSV multiplex

In Paper III, we validated the new BCV/BRSV multiplex for BTM testing in the Norwegian dairy population. As described previously, we randomly selected samples from 360 herds from two counties. All 720 samples were analysed with the multiplex along with two commercially available ELISAs (SVANOVIR® BRSV-ab and BCV-ab, Svanova Biotech, Sweden). Diagnostic test evaluation was performed using latent class analysis in a two test, two populations scenario, assuming conditional independence between tests (Hui and Walter, 1980). The response of a positive reaction for each antigen included in the multiplex test is light emission, measured in relative light units, resulting in a continuous variable. Because the multiplex consisted of several antigens, each giving a separate response, a separate cut-off value was used for each antigen. Antigens were combined in a parallel reading, i.e. the test was considered positive when the relative light units of at least one antigen was above the applied cut-off. The cut-off values recommended by the manufacturer were used as starting points, and test parameters and subpopulation prevalences were estimated for several different cut-offs of the multiplex test. Because we did not have reliable prior information on test parameters or subpopulation prevalences, we could not estimate a possible correlation between tests in the chosen scenario. However, the effect of

relaxing the conditional independence assumption was assessed in a sensitivity analysis, exploring the effect of correlation, if present, on test parameters. For this part, correlation was included as a proportion of the maximum possible value in a conditional covariance model following the method of Vacek (1985). A detailed description of the models is included in Paper III, Appendix A.

Probability of freedom

In Paper IV, we developed a framework for a frequently updated estimate of probability of freedom from BRSV and BCV antibodies at the herd level, based on information from BTM testing, geographic location and animal movement data. We included all animal movements to study herds over a period of approximately 3 years, resulting in records of 45,208 movements to 1802 destination herds. By letting all animal purchases into a herd have an associated probability of introducing infection, we could update the herds' probability of freedom regularly, thus providing a more updated estimate than we could get from the previous herd test result. The probability of introducing infection (or antibodies) from purchase of animals depends on the within-herd prevalence in the source herd, and the probability that the source herd is positive at the herd level. The within-herd prevalence was set to 0.5 (50/50 probability of infection/freedom), and for the latter we used the prevalence in the county of the source herd based on the BTM screening in 2016. In addition to possible introduction through animal purchase, we also had to account for the possibility of introduction through other transmission routes. This included indirect transmission or direct transmission through routes other than officially recorded animal movements, such as use of shared pasture. Four different levels (categories) of local transmission rates were estimated based on herd size (small/large) and location (southern county/northern county), assumptions supported by the literature and the previous risk factor study (Paper I). For calculation of the posterior probability of freedom from antibodies (*PostPFree*) after each successive (three-month) time period, the prior probability was obtained by discounting the previous month's posterior probability by the probability of introduction during the time period.

In summary, the information used in estimating the change in probability of freedom over time was comprised of BTM test results, purchase of animals, location of the herd and herd size. The incorporation of both historical surveillance data and results from ongoing surveillance in temporal discounting of the probability of freedom was first described by

Martin et al. (Martin et al., 2007a; 2007b). The method was further adapted for use at the herd level for surveillance of Johne's disease in Ireland (More et al., 2013). Later, Frössling et al. (2014) developed a method to identify herds with an increased probability of disease due to animal trade. The developed framework was based on a combination of methods from these studies, and is described in detail in Paper IV.

Results and discussion

This section will provide a brief summary of the main results, together with a discussion including methodological considerations. First, a presentation and discussion of the main results from each of the four papers is given: the risk factor and spatial analysis, the consequences of winter dysentery, the diagnostic test evaluation and the estimation of herd-level probability of freedom. Then , sections discussing the use of anybody testing and the quality of the register data follow, before some overall considerations on validity and finally implications for control both in Norway and beyond.

Risk factor analysis

The risk factors identified in Paper I were purchase of animals, herd size, distance to other farms and geographical location. Each factor is discussed below.

Purchase of animals

In Paper I, the 5-year *in-degree* was a significant risk factor for BCV positivity. *In-degree* can be defined as the number of incoming animal movements from individual herds, through animal purchase, over a defined time period (Wasserman and Faust, 1994). The odds of antibody positivity for BCV increased with increasing *in-degree*. For *in-degree* higher than nine, the odds of antibody positivity were six times higher than for herds with zero or one purchased animal. *In-degree* was not significant in the BRSV model, in line with a previous study from Sweden (Frössling et al., 2012). A possible explanation could be that direct transmission routes between herds are more important for BCV than BRSV. However, buying an antibody-producing cow could suffice to switch the BTM test result to positive, and because the prevalence of BCV is high, so is the risk of buying an antibody-positive animal. Therefore, the coarse classification of herds, provided by BTM antibody testing, limits further inference. Classification based on a group of homebred animals would have been better suited for the risk factor analysis, to avoid the estimated effect of *in-degree* to be influenced by potential introduction of antibodies. In Paper IV, 18% (BCV) and 27% (BRSV) of closed herds (i.e. herds with no purchase of livestock) changed their BTM status from negative to positive in 3 years. In other words, the proportion of herds that changed to positive was higher for BRSV than BCV. In concordance with the findings from Paper I, this indicates that the importance of transmission via routes other than officially recorded movements was higher for BRSV than for BCV. Indications of the importance of indirect transmission can be found in historical data as well: During the first regional outbreak of

BRSV in Norway (in Hadeland/Oppland county), movement restrictions were implemented in outbreak herds; however, the virus continued to spread despite these restrictions (Norström et al., 1999).

Our findings indicate that virus can enter herds via officially recorded animal-movements as well as through other routes. However, many factors contribute to the complexity of assessing transmission routes: The relative importance of direct vs. indirect transmission is likely different during epidemics, compared to more endemic phases. The importance of different routes might also differ between regions. Furthermore, it seems likely that animal trade is important for long-distance transmission, whereas local transmission might also be effectively performed through fomites. Further research is needed to assess the importance of different between-herd transmission routes of BRSV and BCV across different regions. More knowledge about the contact network between cattle herds in Norway would therefore be highly beneficial.

Herd size

Results from the risk factor analysis in Paper I showed that an increase in herd size was associated with increased odds of antibody positivity for both viruses. The odds of positivity increased, with 72% for BRSV and 84% for BCV, with an increase in herd size across the interquartile range. An association with herd size has been shown in previous studies as well (Tråvén et al., 1999; Norström et al., 2000; Solís-Calderón et al., 2007; Ohlson et al., 2010b). In Paper II, there was a clear difference in herd size between herds that reported winter dysentery and herds that did not, with a mean herd size of 30 (SD 16.6) and 25 (SD 15.7) cows, respectively. This might suggest that the risk of having a winter dysentery outbreak was higher in larger herds, thus giving support to herd size as a risk factor for BCV introduction. However, it is worth noting that reporting was voluntary, and thus the difference could reflect that outbreaks were more likely to be reported from larger herds than smaller herds. A likely explanation for the association between herd size and the risk of BRSV and BCV positivity is that larger herds have more contacts. More visitors in large herds have been shown in Sweden (Nöremark et al., 2013). Furthermore, it is possible that large herds also have a more extensive animal trade network contributing to the effect of herd size, although in Paper I, this was to some extent accounted for by introducing *in-degree* in the BCV model. The interpretation of herd size as a proxy for the number of indirect contacts was suggested also by Ohlson et al (2010b). It could be argued that herd size can also be a risk factor in itself, as larger herds facilitates longer virus circulation.

However, even the large herds in this study were not very large, and thus virus circulation for prolonged periods of time was unlikely or at least of minor importance.

Farm proximity

In Paper I, we assessed the effect of farm proximity, i.e. the mean Euclidean distance to the five nearest herds, on the odds of antibody positivity. The impact was similar for both BRSV and BCV, with decreasing odds of positivity with increasing distance. Not surprisingly, being remotely located reduced the risk of virus introduction. Previously, bordering cattle herds gave increased risk of BRSV positivity in Ecuador (Saa et al., 2012); and in a Swedish study, increasing distance to neighbours reduced the odds of BCV positivity, but had no significant effect for BRSV (Ohlson et al., 2010b). The location of a farm, and the distance to its neighbours, is not something that is readily changed, and is therefore not a target for intervention. Nevertheless, knowledge about factors that differentiate herds in different risk categories is important for informed decision-making, both for the farmer, for instance when purchasing animals, and for systematic control work.

Spatial patterns

Prevalence

In the two counties in western Norway that constituted the study area in Paper I, the overall apparent prevalence of seropositive herds was 46% for BRSV and 72% for BCV. Furthermore, 41% of the herds were positive against both viruses and 22% were negative against both. The high coexistence of BRSV and BCV antibodies at the herd level was expected due to many common risk factors. The estimated true prevalence was 49% for BRSV and 85% for BCV. However, this calculation was based on the test parameters (Se and Sp) provided by the manufacturer, calculated for serum samples. We found a much lower Sp of the BRSV ELISA in Paper III, implying that the presented estimate of true BRSV prevalence in Paper I is likely biased upwards, i.e. an overestimation of the true prevalence. Vaccination is a potential source of misclassification when BTM is used; however, it had been of limited use in the study region. Note that although an imperfect test or the use of vaccination could potentially cause misclassification of herds, the most important aspect to be aware of is that the prevalence of antibody-positive herds reflects the proportion of herds that have been exposed to virus over the last few years. Thus, the proportion of herds with current viral circulation is likely much lower.

Maps

The point maps made in Paper I revealed negative and positive herds in close proximity, which has also been shown in previous studies from Norway and Sweden (Ohlson et al., 2010a; Klem et al., 2013). Results from the multivariable logistic regression models showed that first-order spatial effects were notable for both viruses, although the x-coordinate (longitude) was not significant in the BCV model. The overall trend was an increase in prevalence from south to north, and for BRSV from east to west as well. This was in concordance with the visual impression of the prevalence risk maps. However, trends at the national scale could well be different, and studies from Sweden found the opposite trend at a national level, namely a lower prevalence of BRSV and BCV in the north compared to the south (Elvander, 1996; Ohlson et al., 2010b). Generalizing the trend found in our study region to a national level is therefore not recommended.

Spatial cluster analysis

In Paper I, we detected several high-risk spatial clusters in the study area. Using the Spatial Scan Statistic under the Bernoulli model, we identified five spatial clusters of BRSV-positive herds, and four of BCV-positive herds ($p < 0.05$). In a Swedish study, Ohlson et al. (2010b) did not find clustering of positive herds; however, cluster analysis was only performed for one region. Another study from Sweden detected local clusters of positive herds for both BRSV and BCV, and analogous to our results, the high prevalence regions were similar for both viruses (Beaudeau et al., 2010). Transmission dynamics between herds have also been reported to vary between regions (Ohlson et al., 2013). In Norway, one former study detected spatio-temporal clustering of respiratory disease during the first large regional outbreak assumed to be caused by BRSV (Norström et al., 2000). Outside Scandinavia, few studies describing spatial analysis of BRSV and BCV exist. In general, spatial and/or temporal clustering of positive herds are not unexpected for infectious diseases. In Paper I, the temporal effect could not be entangled from the spatial effect, as time of sampling was strongly correlated with the geographic coordinates. A longitudinal study with repeated sampling would have been more suitable for assessing temporal trends.

Scanning for high values of residuals from the logistic regression models gave similar results to those obtained from the Bernoulli model. By comparing different models, we found that models including all significant risk factors reduced the number of spatial clusters. However, the remaining clusters of high values of residuals indicate that some unidentified risk factors are spatially dependent and/or demonstrate the importance of local

transmission. More research is needed to assess transmission routes; however, our results suggest that local transmission of virus is of importance, and that the risk of viral introduction to a herd is not only dependent on factors related to that herd, but also on its neighbours. This calls for joint efforts to prevent viral spread between herds.

The overall prevalence detected in the study area was relatively low, especially given that BTM testing was used for herd classification. However, large variations in herd prevalence were detected, as can be seen in the prevalence risk maps, indicating that careful evaluation of spatial patterns at a national scale should be made to inform optimal surveillance and control. The existence of high-risk as well as low-risk areas could imply that a regional approach to control might be useful.

Evaluating the consequences of winter dysentery

Effect on milk production

In Paper II, we quantified the effect of winter dysentery on milk production. We estimated that, on average, production was reduced from 23.0 L/cow per day 7 days before notification of outbreak, to 19.4 L/cow per day at the lowest level, 2 days after notification. This equals a drop of 3.6 L/cow, or 15%. In total, the estimated average loss across the modelled time period was 51 L/cow, from 7 days before to 19 days after notification of the outbreak. Descriptive statistics indicated a large spread in milk production loss ranging from a slight increase in production, for two herds, to a loss of more than 60% for some herds. A few previous studies have reported loss in milk production in herds with winter dysentery (Durham et al., 1989; Jactel et al., 1990; Tråvén et al., 1993). However, this is the first quantification from a large-scale study, and the first to document changes in milk composition. The effect on composition was only notable for free fatty acids, with an estimated increase of 11% for WD+ herds. Milk production loss has been shown for other infectious agents undergoing control, such as BVDV, bovine herpesvirus 1 and *Mycobacterium avium* subsp. *Paratuberculosis* (MAP). An outbreak with bovine herpesvirus 1 was reported to result in an estimated milk production loss of 0.92 kg/cow per day during a period of 9 weeks (van Schaik et al., 1999). For BVDV it is often the adverse effects on reproduction that is emphasized, but the virus is also associated with reduced milk production. A study from New Zealand reported that milk production was reduced by 0.99 kg/cow per day for cows in infected herds (i.e. herds with high level of antibodies in BTM, and thus most likely presence of persistently infected animals), which equals 5.8% of total

production (Heuer et al., 2007). For paratuberculosis, milk production loss at cow-level was estimated in a recent systematic review and meta-analysis to constitute 1.87 kg per day, equivalent to 5.9% of their yield, for cows that were test positive on fecal culture or PCR (McAloon et al., 2016).

In summary, our findings showed a marked effect of winter dysentery on milk production, and for some herds the disease had a detrimental impact. Other effects, such as effects on reproduction or effects on calves and young stock, were not assessed. As BCV gives rise to both respiratory and enteric disease, the total impact of a high BCV prevalence in a population is complex. The effect of outbreaks of winter dysentery is one part of the picture. Because of the high contagiousness, resulting in high morbidity (Boileau and Kapil, 2010), preventing introduction into the herd is the best measure to avoid the negative effects. The notable effect in the acute phase of an outbreak, the effect on milk quality and indications that the reduced production can be long-lasting, should be a wakeup call for stakeholders in the industry to prevent between-herd spread of BCV in areas where epidemics of winter dysentery are a recurrent problem.

Cost-benefit of control

Prior to launching the control programme, the industry performed a cost- benefit assessment (Olav Østerås, personal communication). Cost-effectiveness of systematic control was estimated through a simulation model comparable to the one described for the Norwegian BVDV programme (Valle et al., 2005). The work was performed by the industry, and has so far not been published.

The contribution of the work included in this thesis to the cost- benefit analysis of systematic control, was the estimation of milk loss associated with winter dysentery. In the period around outbreak the milk production loss was estimated to 51 L/cow for the average herd. However, descriptive statistics indicated that production could be reduced for a long time. At 150 days after the outbreak the average production was still less than before the outbreak, whereas WD- herds increased their production slightly during this period. Assuming that WD+ herds would have followed the same trend had they not had an outbreak, the total loss during first 150 days after reported outbreak amounts to 313 L/cow. With premium milk quality, and no adjustment for season, the milk price is 4.9 NOK/L (0.50 EURO, conversion rate as per March 2019). Consequently, the estimated production

loss across the first 150 days after a reported outbreak amounts to approximately 1500 NOK (147 EURO) per cow, or 46000 NOK (4500 EURO) for a herd with 30 cows.

Misclassification bias

Misclassification bias is bias resulting from errors in classification of categorical variables (Dohoo et al., 2009, p. 255). In Paper II, herds were classified based on a voluntary reporting system. Thus, it is likely that not all herds that had an outbreak of winter dysentery reported it, resulting in misclassification of exposure in the WD- group (non-exposed). The simulation of pseudo-outbreak dates was performed to minimize misclassification bias due to under-reporting. Because diagnosis was generally not confirmed by laboratory diagnostics, some misclassification of exposure in the WD+ group (exposed) was also expected. However, the list of differential diagnosis is limited, as the population is free of many infectious diseases with similar clinical presentation. Another limitation of the study was the inaccurate measure of the time of outbreak. The time of reporting was used as day zero, but for some herds, the reporting might have happened early in the course of an outbreak and some for some herds late, or even after the outbreak burned out. Consequently, we most likely failed to capture the maximum consequences for some herds. In total, the bias resulting from the misclassification of exposure and the inaccuracy of the time variable was expected to lead to an underestimation of the effect of WD on milk production. Communicating that the estimates are likely conservative is therefore important when mediating findings to farmers and stakeholders in the industry. Additionally, the large variation in the milk drop among study herds should be emphasized, as it means consequences of an outbreak are difficult to predict for individual herds.

Evaluation of the BCV/BRSV multiplex immunoassay

Test accuracy and prevalence

At the cut-off values recommended by the manufacturers, the estimated median Se for BRSV was 94.4 [89.8–98.7 95% Posterior Credibility Interval (PCI)] and the median Sp was 90.6 [85.5–94.4 95% PCI] for the multiplex. For the Svanovir® BRSV-Ab the Se was 99.8 [98.7–100 95% PCI], and Sp 57.4 [50.5–64.4 95% PCI]. The low Sp of the commercial ELISA improved with a higher cut-off without concurrent loss in Se. For BCV, the estimated median Se and Sp was 99.9 [99.4–100 95% PCI] and 77.3 [69.8–84.8 95% PCI] for the multiplex, and 99.0 [96.9–100 95% PCI] and 99.5 [97.1–100 95% PCI] for the Svanovir® BCV-Ab. The estimated true subpopulation prevalences for BRSV ranged from

84.5–87.3 in subpopulation one (Oppland) and from 29.9–30.5 in subpopulation two (Sogn og Fjordane) across the different cut-offs. The BCV prevalences in the two subpopulations were 91.5–94.0 and 51.5–61.5, respectively. The relatively narrow range of prevalence estimates indicated that the underlying disease definition seemed robust to changes in the antigen cut-off values.

Cut-off values

Investigation of different cut-offs was performed to learn more about test accuracy and assess the test's fitness for purpose in the targeted population. The accuracy parameters for different cut-offs can be found in Tables 3 and 5 in Paper III. The cut-offs recommended by the manufacturer were used as starting points. For BRSV, there was limited gain in increasing the cut-off for the BRSV-A antigen, as a small increase in Sp resulted in a relatively large reduction in Se. For BCV, Sp increased and Se decreased with increasing cut-off, as expected. However, a large gain in Sp was obtained by reducing the number of antigens from 3 to 1, resulting in a median Sp of 99.2 at the highest cut-off, and only a minor reduction in Se (97.1). Our interpretation was that two of the antigens might add false positive results. In general, setting the cut-off for tests with a continuous scale output is a trade-off between optimizing test Se and Sp. Which cut-off value to apply therefore depends on the purpose and context of the testing. It is important to consider the implications of false positive versus false negative classifications. This might include the associated cost of misclassification, implications for the tested subject and/or a need to ensure early detection. Furthermore, the prevalence in the population should be taken into account, as it affects the predictive values of the test. During the course of a control programme, a change in the choice of cut-off might be necessary, as the disease prevalence changes in the population. Early on, when prevalence is high, a less than optimal Sp might still have a reasonable positive predictive value, whereas a high Sp might be more important when prevalence starts to decrease to avoid the consequences of a high false positive rate.

Model assumptions

The model used relies on the following assumptions: there must be a difference in prevalence between populations, test parameters must be constant across populations and the tests must be conditionally independent, given disease status (Hui and Walter, 1980). A detailed discussion on the validity of these assumptions is provided in the discussion in Paper III, and will not be repeated here. However, we explored the consequences of relaxing

the assumption of conditional independence in a sensitivity analysis, and an elaboration on this issue is provided in the following section.

Conditional independence

The assumption of conditional independence implies that, given a true positive (or negative) herd, the probability of a positive (or negative) outcome of the first test is the same regardless of a known outcome of the second test. It can be argued that when two tests are measuring the same biological trait (e.g. antibodies), this assumption might not be met (Gardner et al., 2000). For instance, false positive results due to cross-reactions to antibodies other than BCV/BRSV could potentially be produced by both tests. Ignoring conditional dependence between tests can introduce bias in the estimation of accuracy parameters (Vacek, 1985). To assess the magnitude of this possible bias we performed a sensitivity analysis, where the dependence between tests was kept at a fixed proportion of the maximum possible value. For BCV, introducing covariance had little effect: less than 5% change in accuracy parameters for a covariance of 75% of the maximum possible value. For BRSV, the effect was small for a covariance of less than 25% of the maximum. A notable effect on Sp was observed for an Sp covariance of more than 25% of the maximum. In other words, if a positive covariance of the tests' Sp exists for BRSV, this would cause the estimated specificities to be biased upwards.

Interpretation of bulk tank milk test results

In Paper III, test accuracy was estimated by cross-classifying samples according to the diagnostic-test outcomes of two tests, both performed on BTM, using a latent class approach. Because we did not have individual samples, we could not assess the number (or proportion) of positive cows needed to produce a positive BTM test. Christensen and Gardener (2000) state that the factors affecting pooled Se (BTM being an extreme version of pooling) are probably complex, and mention how a dilution effect likely depends on the concentration of antibodies for the individual cow(s) and her (their) production level, i.e. the relative contribution to the bulk tank. These factors are especially important when within-herd prevalence is low. In the relatively small Norwegian herds, one or a few antibody-producing cows might be enough to alter the BTM test result. Furthermore, it is not known exactly how long the animals produce antibodies, although as previously mentioned, studies indicate several years after infection (Alenius et al., 1991; Klem et al., 2014b). Branscum et al. (2005) mention how the latent class (i.e. true disease status) can be unclear for such cases, where the antigen is rapidly cleared but antibodies persist for long, and suggest

evaluations consisting of antibody-antibody tests are best interpreted as previous exposure. For practical purposes, a positive BTM test result can be interpreted as the herd having at least one cow contributing to the bulk tank that has previously been exposed to the respective virus. A negative BTM result, on the other hand, is more informative, as it indicates the herd has not had a circulating virus for year, provided that one can trust a negative test result. At a herd prevalence of 50%, using the recommended cut-offs (and antigen-A only for BCV), the negative predictive value is 95% for BRSV and close to 99% for BCV, supporting reasonable trust in a negative test.

The continuous output (i.e. relative light units) of the multiplex test could potentially have more information than the dichotomized test result if it is associated with the proportion of antibody-producing cows in the herd (i.e. within-herd prevalence). An association between the optic density value of the BTM test and within-herd prevalence has been shown for other ELISA tests, e.g. for *Neospora caninum* (Frössling et al., 2006), *Mycobacterium avium* subsp. *Paratuberculosis* (Nielsen and Toft, 2014) and BVDV (Beaudeau et al., 2001). BRSV and BCV result in rapid seroconversion after exposure, similar to many other viral infections (Uttenthal et al., 2000; Oma et al., 2016). In combination with a high morbidity of disease, this leads to an expected high within-herd antibody prevalence shortly after introduction of virus into the herd. For practical purposes, it is therefore less of an issue if low-prevalence herds go undetected, as many of these likely have only a few old cows contributing antibodies to the bulk tank, and a low risk of virus circulation. An investigation of the relationship between the continuous output of the test and within-herd prevalence would be useful for making informed decisions concerning the choice of cut-off; it might also be useful for classification of herds to establish probabilities of the herd harbouring an active infection.

Probability of freedom

PostPFree – shades of green

In the control programme, a negative herd test results in a ‘green status’ which is valid for one year. But are all herds the same shade of green? In the previous section, the diagnostic test evaluation was discussed, briefly mentioning how test accuracy and prevalence affect the trust in a negative test. In Paper IV, we also accounted for the time elapsed since sampling, purchase of livestock, the location of the herd and herd size to estimate an updated posterior probability of freedom (*PostPFree*) for each herd in each three-month

time period. *PostPFree* from BRSV antibodies over the three-year study period is shown for three example herds in Figure 4.

The *PostPFree* started close to one immediately after a negative test for both BRSV and BCV, due to the high Se of the BTM tests. Purchase of livestock had a large effect on the decrease in *PostPFree* for both viruses, compared to the effect of local transmission. The median *PostPFree* in the 12th, i.e. the last, time period was 0.62 (range 0–0.91) for BRSV and 0.80 (range 0–0.95) for BCV. When comparing *PostPFree* for the final time period to subsequent BTM results, the Wilcoxon Rank Sum Test gave a significant ($p < 0.01$) difference in *PostPFree* between test-negative and test-positive herds for both viruses. Dividing herds into four groups based on quartiles of *PostPFree* also showed a large difference in the proportion of positive herds between the first and fourth quartile of *PostPFree* (see Figure 4 in Paper IV). In summary, *PostPFree* provided a more accurate measure of antibody status than the previous test result when validated against a new BTM test after three years. Because annual re-testing is recommended in the control programme, we would ideally have evaluated *PostPFree* after each year. Nevertheless, a notable proportion (29% for both viruses) of the BTM-negative herds changed to positive in three years, indicating that a categorization of test-negative herds, i.e. some 'shades of green', might be useful.

Results from the sensitivity analysis showed that changing the local transmission rates by 50% resulted in a moderate change in *PostPFree* for the final time period. It is therefore important to ensure that the parameter used for local transmission is as correct as possible, and assessment of dynamics on a larger scale and for other regions is recommended before the framework is applied nationally. Another important constraint is that all input variables relate to antibodies, and consequently the interpretation of *PostPFree* is the probability of freedom from antibodies. The probability of freedom from current infection is likely much higher. An extended discussion on this matter can be found in Paper IV.

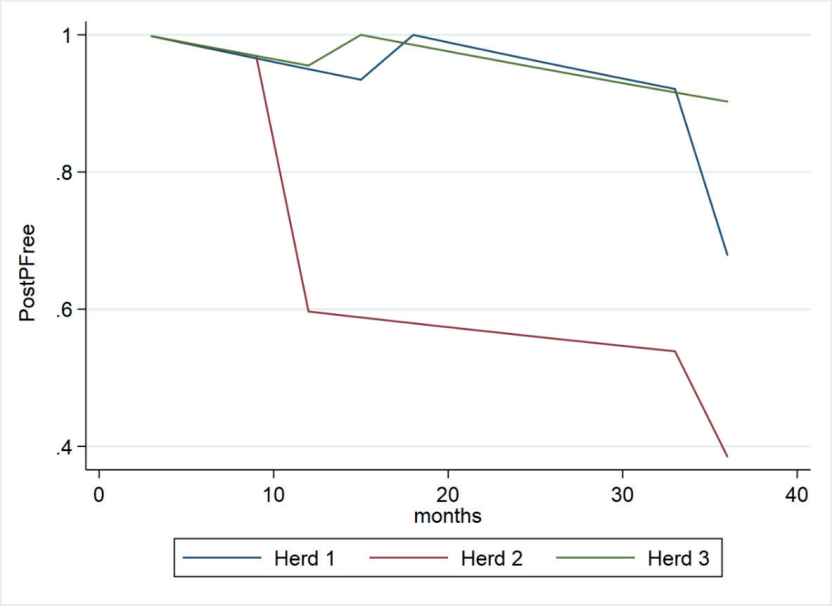

Figure 4. Herd-level probability of freedom (*PostPFree*) from BRSV antibodies over 36 months for 3 example herds all starting with a negative test. Herds 3 and 1 have a second BTM test with a negative result. Herd 2 has purchased livestock in two of the three-month time periods and Herd 1 has purchased livestock in the last time period.

Currently, the control programme uses pooled samples of serum from young stock and milk from primiparous cows to classify herds (Harald Holm, personal communication, January 24, 2019). The framework presented in Paper IV could be extended to include these other sampling methods, provided that test accuracy parameters are obtained for these applications of the test. A relevant example can be found in a recent Swedish study, where estimation of herd-level probability of freedom from *Salmonella* accounted for differences in HSe and HSp between sampling strategies. The study also included different prior probabilities based on location in a high vs. low prevalence region and if there was a prior suspicion of *Salmonella* in the herd (Ågren et al., 2018). As previously mentioned, a BTM-negative test result is, for BRSV and BCV, indicative of no virus having circulated in the herd for years. Consequently, it seems likely that the risk of introduction is lower in BTM-negative herds than for other herds, and the study sample in Paper IV likely represents a low risk stratum of the population. Herds classified as negative based on samples from young stock or primiparous cows might belong to a different risk stratum, as the infectious history reflected by the test is much shorter. Therefore, including other types of herd testing likely

entails a larger diversity among herds in terms of risk, and hence further increases the need for some 'shades of green'.

Application in systematic control work

The *PostPFree* can be used to separate herds in different risk categories, for which one possible application is as a decision support tool in a risk-based approach to sampling. Assessing the probability of infection (*PostPInf*), which is the complement to *PostPFree*, as a diagnostic test can illustrate this: At a cut-off of *PostPInf* > 0.25, the Se of *PostPInf* was 0.76 (95% CI:0.68–0.82) for BRSV and 0.55 (95% CI 0.42–0.68) for BCV. This means that if re-testing had been performed at this cut-off value, we would have captured an estimated 76% and 55% of false negative herds (i.e. antibody positive) for BRSV and BCV, respectively. The example is based on a small sample of herds using an arbitrary cut-off, and with the additional limitation that we did not have annual samples. It is therefore meant as an illustration of a possible application.

In the control programme, 'green' herds are advised to purchase livestock only from other 'green' herds. As *PostPFree* provides an updated measure of a herd's probability of freedom, it could be useful in the decision-making process behind livestock trade. In summary, Paper IV outlines a framework for how herds could be classified in different risk categories, with possible applications for surveillance and control of BRSV and BCV.

Antibody testing of herds

Three of four papers in this thesis are based on classifying herds by detection of antibodies. As described in the introduction, infection with BRSV and BCV generally results in a short viral shedding period (days–weeks), followed by a long lasting antibody response (years). This means that when a positive classification of animals or herds are based on detection of antibodies, positivity reflects previous exposure to virus, and hence the results of the studies must be interpreted accordingly. The risk factors reported in Paper I refers to an increased risk of previous virus exposure to lactating animals in the herd. The spatial clusters represents clusters of herds that have been exposed to virus sometime during the previous years. The test parameters presented in Paper III answers the tests ability to correctly classify herds with respect to antibodies in BTM. The probability of freedom calculated in Paper IV reflects the probability of freedom from antibodies. Among herds with antibodies, there will be a substantial number of false positives with respect to the proportion actually harboring the infectious agent. A quantification of this 'false positive rate' with respect to

actual infection is not described in the literature. It likely depends on sampling strategy, occurrence and transmission dynamics between herds. Ideally, classification of herds should reflect infective status, and thus be based on detection of antigen. However, there are several reasons for why antibody testing was used in this PhD-project as well as in the control programme, as opposed to detection of the actual antigen: The relatively short shedding period means that timing is essential to capture the antigen. There is no viremic phase, which excludes blood samples as a diagnostic option for antigen detection. Thus, samples must be taken from the respiratory tract (e.g. nasal swabs), or faeces for enteric BCV infection. Both viruses commonly cause subclinical infection, or disease with mild clinical signs, and consequently the presence of virus in a herd can easily go undetected if sampling is performed only when animals show signs of infection. Altogether, these factors make detection of virus suitable for diagnostic purposes during outbreaks, but unfeasible for surveillance, control and large scale studies. Despite its limitations, the feasibility and low cost of antibody testing was the reason for why this was used in much of the work included in this PhD-thesis as well as in the control programme.

Regardless of sampling method, a BRSV/BCV herd status is short-lived. Even if virus is detected in a herd (for instance by PCR), the virus is likely eliminated from the typical Norwegian herd within a few weeks. Moreover, a negative herd might be re-infected. Thus, there is (shortly after testing) an inevitable uncertainty regarding the probability of the herd harboring virus. The control programme relies on self-clearance, and no interventions are recommended for test-positive herds. The core principle is to find and protect the negative herds, and ideally, predicting which herds are likely to stay negative would benefit the control work, as discussed in paper IV. This differs somewhat from the control and eradication of more persistent infectious diseases, where locating positive herds is important to implement interventions. For example, identification and removal of persistently infected (PI) animals is a central element in the control of BVDV (Løken and Nyberg, 2013). Another example is the control of salmonella in Swedish cattle, where a list of hygiene measures are imposed on salmonella positive herds (Ågren et al., 2015).

Based on the known high morbidity of BRSV and BCV infections, one might assume that once virus enters a herd, close to all animals are exposed and subsequently seroconvert. The overall within-herd antibody prevalence is thus likely high immediately after infection but decreases as new animals are born. Limited knowledge exists of within-herd prevalence for herds with production systems comparable to Norway. In a Swedish study, Hägglund et al.

(2006) found that 13 of 118 (11%) herds had one or two calves seropositive to BRSV when three calves ~7 months of age was sampled from each herd. The remaining herds had either no positive calves or all three tested positive. An increase in seroprevalence with age has previously been shown (Bidokhti et al., 2009). Years after infection the seroprevalence could still be high among older animals, but low (zero) among young animals that have not been exposed to virus. The assumption of a seroprevalence of either zero or close to one within a group of animals of similar age is the background for why sampling of only 2 to 4 young stock is assumed to have adequate HSe, and is an important assumption for the design of the control programme. It should be noted that the HSe in this case is with respect to virus circulation in the herd within the previous year, approximately. However, scientific studies describing within-herd prevalences across age groups are lacking. If it turns out that a prevalence in the middle range is common within a group of animals of similar age, a sample of only two animals will result in low HSe. On the other hand, increasing the number of animals in the pool will generally decrease HSp (Christensen and Gardner, 2000). It is therefore wise to optimize the number of animals included for a herd diagnosis. It would thus be highly preferable to conduct a field study to investigate the within-herd prevalences in relevant age-groups.

Data quality of register data

All four papers in the present thesis utilized data from central registries. This information was collected primarily for purposes other than research, and such data are often called secondary data. Advantages such as availability and the possibility to save costs, because data collection has already been performed, are attractive features of secondary data. However, there are also potential disadvantages, such as not having the information one needs (or in the required format), and potentially unknown accuracy and completeness of the data.

The NDHRS database has been validated with respect to completeness and correctness of health events by evaluating agreement between NDHRS records and on-farm recordings (Espetvedt et al., 2013). The authors found full agreement or only minor discrepancies in 87.5% of the entries and thus concluded that the quality was generally good, although with room for improvement of completeness. The major source of missing health data was failure of reporting from CHC to NDHRS. Studies have also looked at the completeness in the NDHRS and other Nordic cattle databases for specific diseases, such as clinical mastitis,

locomotor disorders and metabolic disorders (Espetvedt et al., 2012; Lind et al., 2012; Wolff et al., 2012). These studies showed considerable variation in the completeness of reporting of different diseases. Most variables used in the studies included in this thesis were herd-level variables, and not health data retrieved from CHC.

The Norwegian recordings of milk shipment have not been validated for research purposes. The volume and composition of milk shipped to the dairy plant form the basis for the farmers' payment, and are therefore measured with high precision. Nevertheless, a recent study explored the potential use of milk shipment data for research and disease monitoring purposes, and found that the Swedish milk shipment data were 'noisy' and therefore challenging to use (Fall et al., 2018). Even though we did not perceive this as a big problem in Paper II, Norwegian milk shipment data should undergo validation to assess correctness and further elucidate the potential use of these data for research and surveillance.

The Norwegian livestock registry (in which animal movements are recorded) has not been previously used for research purposes. During the work with Paper IV, data cleaning revealed many duplicate records and also a few inconsistencies, such as the same animal moving back and forth from the same herd in the same day. The accuracy of the time of movement could not be assessed through data cleaning. Likely sources of incompleteness are temporary movements, for instance movements to pastures and temporary housing of animals on other farms. Such movements might go unrecorded. A validation of the Norwegian livestock registry would be beneficial both with regards to potential use for research and other purposes of this registry, such as ensuring traceability in the case of disease outbreaks.

Validity

Much of the previous discussion included methodological considerations with implications for validity. This section provides a brief overall assessment of validity.

A study is said to be internally valid if one can make unbiased inferences about the associations of interest in the source population, and externally valid if one can make correct inferences to populations beyond the source population (Dohoo et al., 2009, p. 244). Bias is a term used for systematic (as opposed to random) error in the design, conduct or analysis that renders results invalid (Thrusfield and Christley, 2018). Bias is often divided into three major types: selection bias, bias due to confounding and information bias. Kleinbaum et al.

(1982, pp. 190-191) uses the following definitions: Selection bias refers to a distortion in the estimate of effect resulting from the manner in which subjects are selected for the study population. Confounding is bias that results when the study factor effect is mixed in the data, with the effects of extraneous variables. Information bias refers to a distortion in estimation of the effect of interest that results when measurement of either the exposure condition or the outcome is systematically inaccurate.

Internal validity

In all analytical studies, groups that are compared should be selected from the same source population to avoid selection bias (Dohoo et al., 2009, p. 249). In Paper I, BTM samples were collected from 1347 out of 1854 of all dairy herds in the study area, i.e. 73% of all eligible dairy herds. Collection was performed by the milk truck driver at ordinary milk shipment, and sampling can be regarded as convenience sampling. We do not have reason to believe sampling was associated with the BTM status of herds. However, as we did not formally assess this, the risk of selection bias cannot be excluded. Furthermore, 153 herds had to be excluded from the risk factor analysis due to incomplete NDHRS records. These herds had a BTM result, and the proportion of positive herds were slightly higher in this group compared to the herds with complete records, indicating there could be differences of importance to the analysis. However, with only a moderate number of exclusions and a small difference in test results, the potential bias was believed to be minor.

Paper II was a cohort study for which selection from the same source population implies that subjects in the non-exposed group should be comparable to the exposed group with respect to all factors that might bias the measure of association. All NDHRS herds in one region were selected and then divided in WD+ (exposed) and WD- (non-exposed), depending on whether or not they reported winter dysentery. Even though groups were selected from the same source population, the WD+ herds differed from the WD- herds in having an overall higher yield. Thus, it was necessary to analytically adjust for milk production prior to the outbreak when assessing the effect of winter dysentery, to avoid confounding. For the analysis on milk composition, only a subset of herds had available records, potentially compromising the internal validity of these analyses. Several other factors could affect the internal validity in this study, of which important ones were the uncertainty of the time of outbreak, likely under-reporting of disease and lack of confirmative laboratory diagnostics. These potential sources of misclassification bias were discussed under consequences of winter dysentery.

In Paper III, the source population consisted of herds that delivered milk to the largest dairy company in Norway (TINE SA), and provided a BTM sample during the study period (March 2016). A random selection of herds from this source population was performed, thus preventing selection bias. The most important threat to internal validity in this study was therefore possible violation of the model assumptions. As previously described, a sensitivity analysis was performed to assess the potential impact of a violation of the conditional independence assumption, and the conclusion was that a positive covariance could, if present, bias the estimated Sp for BRSV upwards.

In Paper IV, the study sample was a subset of the herds included in Paper I. The total population of dairy herds in the region was not known throughout the study period. However, the majority of dairy herds in the region were included, and it seems likely that transmission dynamics are similar for herds located in the same region within the same time period. The internal validity is, however, also affected by the correctness of the input parameters, and as support from literature regarding the within-herd prevalence was scarce, this constitutes a potential weakness. Overall the internal validity was deemed acceptable, but applying the method beyond the study region might require modifications, discussed in the following section.

External validity

The target population for the work included in this thesis was the Norwegian population of dairy herds. In the following, external validity will refer to the ability to make correct inferences for this target population. Because Papers I, II and IV relied on information from the NDHRS, the source population was limited to NDHRS members, possibly affecting external validity. However, in 2017 the NDHRS contained records of 96% of all Norwegian dairy herds (TINE Advisory Service, 2018). Because of the high proportion, it seems likely that NDHRS members are largely representative of the Norwegian dairy population. In Paper I and Paper II, the study area was limited to one region of Norway, located in western and eastern Norway, respectively. Paper III was conducted with herds from both of these regions. Because the population is relatively homogenous, with similar production systems and management across the country, results from the risk factor analysis, the consequences of WD and the diagnostic test evaluation are likely generalizable to the national dairy herd.

In Paper IV, we estimated the probability of freedom for each herd. Only herds that started with a negative BTM test could be used for calculations of probability of freedom, and of

these, only herds with a second BTM test in 2016 could be used for validation. The inference for herds classified as negative based on other sampling regimes is therefore questionable. The local transmission rate could also be different for other regions and/or other time periods, and should be estimated for different regions, and preferably also over time. All in all, the small sample size and restricted study area is a limitation to the generalizability of the results of Paper IV. Nevertheless, the flexible framework allows for adjustments, and achieving fitness for purpose is likely also possible at a national scale.

Implications for control

In Norway

The present work has impacted how BRSV and BCV are managed in Norway. However, in the attempt to address the contribution of this work, it is important to emphasize that decisions related to the control of these viruses are made by the industry partners. Their decisions are rarely triggered solely by the work included in this PhD-thesis, but are grounded on the total body of knowledge along with other relevant considerations, such as cost and compliance. That said, the work of the project group, including the work presented here, has significantly added to the knowledge of BRSV and BCV in Norway, and a discussion on the implications for control follows.

Local transmission

The risk factor analysis in Paper I showed that distance to neighbors was of importance for herd antibody status for both BRSV and BCV, supporting that people traveling between farms could carry these viruses from one farm to the next. Farms with many other farms in close proximity were at higher risk of being positive, which could indicate that travelling time between farms matters. Other work from the project group should be mentioned in this regard; Oma et al. (2018) showed how BRSV and BCV loose infectivity outside the host, and that after 24 h a high load of both viruses could be detected on fomites, but remaining infectivity was only found for BCV. This also points to the importance of external biosecurity measures, and a possible effect of traveling time. Reducing the viral load on clothing and equipment to a minimum makes it less likely that enough infective virions reach the next farm. Biosecurity measures should be in place both when leaving a farm, to reduce the amount of virus brought out of the farm, and when entering the next farm to avoid introduction of any virus that might be left on clothing, hands or equipment from the previous farm. The current advice in the control programme is for all farmers to have an

infection sluice room that includes: a clear barricade (line or obstacle) to distinguish the outside zone from the inside of the barn, clothing and footwear available for all visitors and facilities for cleaning of hands and equipment.

Livestock trade

The importance of livestock trade as a transmission route between farms shown in the present work has attained attention. Live-animal trade in Norway is organized by the producer organizations, both for replacement animals and for animals shipped to slaughter. Considerable efforts are made to find measures to facilitate safe trade, and to increase the awareness among farmers on how infectious agents can enter herds. The industry is currently working on common guidelines (to be implemented 2019) for livestock trade, where one of the key features is for all farms to document a BRSV/BCV herd test < 12 months old, to be allowed sell animals (Harald Holm, personal communication, June 3, 2019). Since the launch of the control programme, separate transport vehicles have been used for animals from test-negative and -positive (or non-tested) herds. Purchased animals must have a certificate of their health status. Owner-declaration is sufficient for calves, but veterinary approved health declarations are required for pregnant heifers/cows and bulls. The health declaration has two parts: one certificate on animal level, and one on herd level. The herd level part was recently made electronically available online, and the BRSV and BCV status of the herd is now automatically included in the herd health declaration. The company organizing livestock trade has access to the status of all selling herds, and the request for animals from green herds is high (Harald Holm, personal communication, June 3, 2019). Risk communication to farmers has high priority, and information on biosecurity and disease control is available on the web pages of the producer organizations, who also arrange frequent meetings with producers all over Norway. These meetings are important for effective dissemination of information to farmers and veterinarians, and are also important to encourage local joint efforts. The latter being motivated by the strong local dependence found in Paper I, which indicates that local dedication is likely effective and necessary for successful control.

Consequences of winter dysentery

The quantification of milk loss described in paper II has provided important input to the cost-benefit analysis performed by the industry during the planning of the control programme, addressed in a previous section. Furthermore, documenting the impact has been essential to inform farmers of the potential consequences of BCV introduction into the herd,

which is important to gain motivation and compliance with the control programme. The evident drop in herd-level milk production quantified in paper II has lead the industry to plan a pilot project on syndromic surveillance, using real-time production data from automatic milking systems. This project will be initiated in the autumn of 2019 (Gunnar Dahlen, personal communication, May 12, 2019).

Herd classification

The results from the diagnostic test evaluation showed that both the commercial ELISAs and the BCV/BRSV multiplex can, with some adjustments, show similar performance for BTM classification. All tests can be used to classify herds with respect to antibodies in BTM. The multiplex test is today used in the Norwegian control programme. Its' advantage is that a single test can be used for both BRSV and BCV, thus potentially reducing costs. Based on the results from paper III, our advice was to use only one of three BCV antigens for BTM testing in the control programme.

It is important to note that the test accuracy parameters presented in Paper III are valid for BTM testing only, as only BTM samples were used in the analysis. The HSe and HSp can differ substantially between different sampling strategies, i.e. bulk milk samples are different from (smaller) pooled samples, which again differ from individual samples (Christensen and Gardner, 2000). The test is currently in use for pooled samples of serum and milk, and consequently further validation for these applications is needed. Our current recommendation to the industry partners is for this to be done. The framework for probability of freedom calculations presented in paper IV has so far not been used by the industry. This is partly because individual milk and serum samples are currently used for follow-up classification of herd status instead of BTM. It is possible to include other sampling strategies in the probability of freedom calculations, but this requires validation of the test to obtain the accuracy parameters for the chosen strategy.

By-products of control

The work conducted in this thesis has involved two common infectious agents, and provided knowledge that can be used to inform preventive efforts. However, many infectious diseases follow the same transmission routes i.e. live animal contact and/or indirectly through farm visitors or equipment. Thus, measures aiming at limiting the spread of BRSV and BCV are likely also effective in terms of preventing transmission of other infectious diseases. A positive by-product of increased awareness and external biosecurity could therefore be a

reduction in other endemic pathogens as well as future emerging pathogens. In particular there is ongoing work on preventing the spread of digital dermatitis, and these efforts are to some extent coordinated with BRSV/BCV control.

Beyond Norway

Infectious diseases cause substantial problems in the cattle industry worldwide. For many countries BRSV and BCV will be far down on the list of present concerns, as other infections are considered to have a larger impact. Nevertheless, the presented work has shown that winter dysentery is associated with substantial production losses in affected herds, and should be a reminder not to forget these common infections, and motivate initiatives to prevent between-herd transmission. In the presented work BTM negative herds were found even in areas where there has been known recent circulation of virus and there was a substantial variations in herd prevalence. These are strong indications that it is possible to stop these viruses at the farm gate. One could argue that the Norwegian herds are small and dispersed, therefore conditions are not comparable to other countries. However, regardless of the country a herd is located in, the virus enters through the farm gate, either via contaminated fomites or livestock. If it is possible to stay negative in Norway it could be possible elsewhere. The importance of livestock trade is of value to anyone interested in trying to limit the spread of these diseases, and even if no systematic control work is performed or planned, farmers should be informed that animal purchase is associated with a risk of introducing virus that might result in disease outbreaks.

The validation of the multiplex test for BTM is at present mostly of national interest, however, if the test is made commercially available it is of interest to anyone planning to use the test on BTM. The SVANOVIR® BRSV-ab and BCV-ab, are widely used, and as test parameters for the BTM application has previously not been published, they might be useful for anyone using the tests for this application. BTM testing of BRSV and BCV will not be meaningful in many areas due to the high occurrence of virus which means that all herds will have antibodies in the bulk tank. Validation for other strategies is thus likely of more interest. The framework for calculation of probability of freedom presented in paper IV is by no means a description of the control programme, but nevertheless one of the first publications outlining the potential for systematic control of BRSV and BCV without the use of vaccination or antimicrobials. Murray et al. (Murray et al., 2016a) concluded in a review that mass medication with antimicrobials provides inconsistent control of BRD, and posed a serious concern regarding emergence of antimicrobial resistance. Despite this,

antimicrobial mass-medication is frequently used to control BRD worldwide. Publications that draw attention to alternative, sustainable strategies to combat these diseases are therefore in demand.

Future perspectives

The research conducted in this PhD-project has provided some answers, but further issues remain to be addressed.

In Paper II, we assessed the consequences of winter dysentery on milk production. However, as outlined under knowledge gaps, this only constitutes a portion of the total impact of BRSV and BCV associated disease. There is a need for further studies identifying consequences on other production parameters under relevant conditions. This information can be used to motivate farmers and hence increase compliance to the control programme.

The new multiplex immunoassay has been validated for BTM, but test performance for other applications remains unknown. At present, the test is used for herd diagnosis based on pooled samples of milk from primiparous cows and serum from young stock, necessitating estimation of test accuracy parameters both at the individual level, and as a herd test for different sampling strategies (individual samples and different pooling schemes). In Paper IV, we presented a framework for calculating herd-level probability of freedom. As pooled samples are used for classification of herds in the control programme, it follows that an extension of the framework to include these sampling strategies could be useful. This requires HSe for these applications as input in the model, and is yet another reason to recommend evaluating the diagnostic test for individual and pooled samples. For another input parameter; the within-herd prevalence, support from the literature was scarce. Better knowledge of the range of within-herd prevalence in Norwegian herds and differences between groups (e.g. calves, young stock and adults) would be valuable knowledge, both to ensure correct input in the presented model, and when planning sampling strategies in the control programme. As previously discussed the current sampling strategy of 2 to 4 animals included in a pooled sample is based on an assumption of either very high or very low (zero) within-herd prevalences among animals of similar age. However, the assumption lacks scientific support, especially for BCV where transmission dynamics and the link to different clinical manifestations is still not fully understood. Thus, within-herd prevalences across age-groups should be investigated to assess the currently used sampling scheme, and enable assessment of different sampling scenarios.

Results from Paper I and Paper IV indicate that cattle trade is an important transmission pathway for BRSV and BCV between herds. However, the relative importance of different transmission routes is still not known. One possibility for future research is to conduct

studies implementing whole genome sequencing of virus from disease outbreaks. Combining genetic information with known contact patterns; animal movements, and preferably also other modes of contact, could be used to identify transmission routes. In the present work we explored the network parameter *in-degree* as a risk factor for BTM positivity. However, the cattle trade network in Norway is still not described. Social network analysis has been an increasingly popular technique used to describe livestock trade networks over the past few decades (Ortiz-Pelaez et al., 2006; Lentz et al., 2016; Kim et al., 2018). Investigating the intensity of movements, their range, essential nodes and the geographical and temporal trends of the cattle trade network in Norway is important to understand the potential for spread of other infectious diseases in addition to BRSV and BCV. An investigation of network properties could provide key information for surveillance, outbreak control, targeted interventions and preparedness against exotic diseases. Furthermore, data-driven models using real animal movement data can been used to simulate disease spread as recently shown for *E.coli* in Sweden (Widgren et al., 2018).

The following questions are therefore suggested as directions for future research:

- What are the herd level consequences of winter dysentery outbreaks on production parameters such as reproductive performance of cows, and how do outbreaks affect calves and young stock?
- What is the diagnostic test accuracy of the BCV/BRSV multiplex for individual samples (serum and milk)?
- What is the range of within-herd prevalences of BRSV and BCV in herds and in different age-groups?
- What is the relative importance of different routes of BRSV and BCV transmission between herds?
- What are central features of the livestock trade network in Norway? What are the network properties relevant for disease spread, and are there temporal or regional trends?

Concluding remarks

- Herd-level risk factors for BRSV and BCV antibody positivity in BTM were large herd size, close proximity to neighbours and geographic location. Antibody positivity for BRSV was associated with increased odds of antibody positivity for BCV.
- Purchase of livestock was an additional risk factor for BCV positivity at the herd level.
- In two counties in western Norway there were large differences in prevalence of BRSV- and BCV-positive herds across the study area. Both large geographic trends and spatial clustering were detected.
- The effect of winter dysentery on herd-level milk production is considerable, with an estimated reduction of 15% at the maximum compared to pre-outbreak production. There was also a significant effect on free fatty acids in milk.
- The MVD-Enferplex BCV/BRSV multiplex was validated for bulk tank milk testing of herds. For BCV, the estimated median Se was 99.9 [99.4–100 95% PCI] and median was Sp 77.3 [69.8–84.8 95% PCI]. For BRSV, the estimated median Se was 94.4 [89.8–98.7 95%] and median Sp was 90.6 [85.5–94.4 95% PCI], using the configuration and cut-off values recommended by the manufacturer.
- A framework for a frequently updated herd probability of freedom (*PostPFree*) was developed based on bulk tank milk testing, herd location and purchase of livestock. *PostPFree* can provide a better estimate of a herd's antibody status for both BRSV and BCV than what can be achieved by relying solely on the previous BTM test result.
- Unanswered questions still remain regarding the epidemiology of BRSV and BCV in Norway. Important aspects include transmission routes, within-herd prevalence and test-performance.

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Bovine respiratory syncytial virus and bovine coronavirus antibodies in bulk tank milk – risk factors and spatial analysis

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ABSTRACT

Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCoV) are considered widespread among cattle in Norway and worldwide. This cross-sectional study was conducted based on antibody-ELISA of bulk tank milk (BTM) from 1347 herds in two neighboring counties in western Norway. The study aims were to determine the seroprevalence at herd level, to evaluate risk factors for BRSV and BCoV seropositivity, and to assess how these factors were associated with the spatial distribution of positive herds. The overall prevalence of BRSV and BCoV positive herds in the region was 46.2% and 72.2%, respectively. Isopleth maps of the prevalence risk distribution showed large differences in prevalence risk across the study area, with the highest prevalence in the northern region. Common risk factors of importance for both viruses were herd size, geographic location, and proximity to neighbors. Seropositivity for one virus was associated with increased odds of seropositivity for the other virus. Purchase of livestock was an additional risk factor for BCoV seropositivity, included in the model as *in-degree*, which was defined as the number of incoming movements from individual herds, through animal purchase, over a period of five years. Local dependence and the contribution of risk factors to this effect were assessed using the residuals from two logistic regression models for each virus. One model contained only the x- and y- coordinates as predictors, the other had all significant predictors included. Spatial clusters of high values of residuals were detected using the normal model of the spatial scan statistic and visualized on maps. Adjusting for the risk factors in the final models had different impact on the spatial clusters for the two viruses: For BRSV the number of clusters was reduced from six to four, for BCoV the number of clusters remained the same, however the log-likelihood ratios changed notably. This indicates that geographical differences in proximity to neighbors, herd size and animal movements explain some of the spatial clusters of BRSV- and BCoV seropositivity, but far from all. The remaining local dependence in the residuals show that the antibody status of one herd is influenced by the antibody status of its neighbors, indicating the importance of indirect transmission and that increased biosecurity routines might be an important preventive strategy.

1. Introduction

The overall health among Norwegian dairy cattle is good with few endemic infectious diseases present. Several infections, such as bovine tuberculosis, bovine brucellosis and bovine viral diarrhea (BVD), have been eliminated through successful control programs (Sviland et al., 2015a, 2015b; Åkerstedt et al., 2015). However, bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCoV) are endemic and prevalent in the national herd (Gulliksen et al., 2009). The prevalence of these infections is considered high in most parts of the world, and they cause disease problems leading to reduced animal welfare, increased use of antibiotics and financial loss for the farmer (Valarcher and Taylor, 2007; Boileau and Kapil, 2010; Sacco et al., 2014). BRSV causes respiratory disease, most often in young animals, and bronchopneumonia due to secondary bacterial infection is common (Larsen, 2000). BRSV was the most commonly isolated agent in respiratory outbreaks in cattle herds in a recent Norwegian study (Klem et al., 2014a). BCoV is the cause of calf diarrhea, respiratory disease and winter dysentery (contagious diarrhea in adult cattle) (Boileau and Kapil, 2010). Studies

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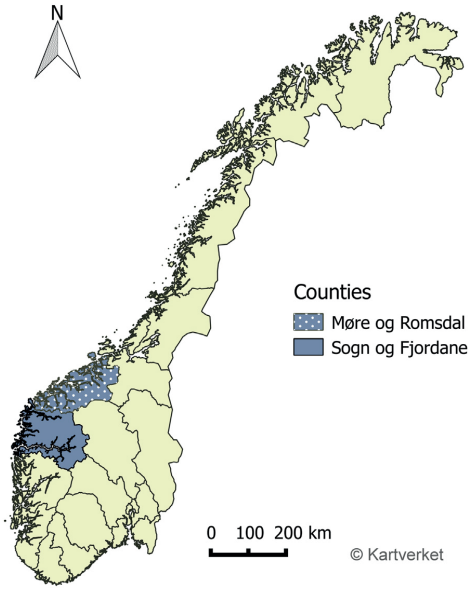

Fig. 1. Study area: Møre og Romsdal and Sogn og Fjordane county located at the northwest coast of Norway.

have shown significant effects of BCoV infection on production in terms of decreased milk yield and poor growth rate (Tråvén et al., 2001; Beaudeau et al., 2010b) which both result in economic loss.

Bulk tank milk (BTM) serology is a cheap and effective method used to screen herds for infectious diseases. However, due to long lasting seropositivity after infection, a herd will stay test-positive for many years after circulation of virus in the herd (Alenius et al., 1991; Tråvén et al., 2001; Klem et al., 2014b). Likewise, test-negative herds might have been virus free for years and serology on bulk tank milk is therefore an indicator of herd status with an inherent time-lag.

Herd level risk factors previously found to be of importance for BCoV status in Swedish dairy herds are herd size, not providing boots for visitors and geographic location (Tråvén et al., 1999; Ohlson et al., 2010). For BRSV seropositivity, herd level risk factors found to be of importance both in Scandinavia and beyond are herd size, age profile of the herd, type of production and existence of bordering cattle herds (Norström et al., 2000; Solís-Calderón et al., 2007; Ohlson et al., 2010; Saa et al., 2012).

Previous studies in Scandinavia have indicated large variations in prevalence of BRSV and BCoV between regions (Elvander, 1996; Tråvén et al., 1999; Beaudeau et al., 2010a; Klem et al., 2013), but spatial analyses involving BRSV and BCoV infections are infrequently reported. For control- and eradication purposes, locating high and low risk areas is important in order to know which control strategies should be applied to different regions. Risk factors like herd size, animal movement between herds, and proximity to neighbors are likely to vary geographically. However, it is currently not known how geographical differences in risk factors are associated with the spatial variation in prevalence of positive herds for these two viruses. Because the spatial pattern of antibody-positive herds may be largely driven by the spatial patterns of herd characteristics, such as herd size and distance to neighbors, spatial clusters of positive herds might only be reflecting the geographical distribution of known characteristics. Hence, it is of major interest to determine if adjusting for these factors changes the appearance of the spatial clusters.

BRSV and BCoV can be spread between herds by direct animal contact and indirect transmission. Direct contact includes physical contact between animals from different herds, for instance through shared pasture, or by live animal trade. Indirect transmission happens through passive transfer of animal secretions and excretions between herds by fomites like clothing or equipment.

The topography in western Norway, where the area under investigation is located, is characterized by mountains and fjords separating the herds and limiting direct contact. However, animal movements between holdings might provide an important route of transmission. *In-degree* is a measurement of animal contact which is defined as the number of incoming animal movements from individual herds, through animal purchase, over a defined time period (Nöremark and Widgren, 2014). Livestock movements are often registered in central databases which allows for calculation of *in-degree*, but factors affecting indirect transmission can be more difficult to assess because information on movement of people and biosecurity routines are not readily available in central registries. Nevertheless movement of people is associated with herd size, because larger herds have more visitors (Nöremark et al., 2013).

The aim of this study was to determine the spatial variation in herd-level prevalence of BRSV and BCoV, as measured by BTM-antibodies, across the study region in western Norway. Furthermore, the effect of the risk factors herd size, location, animal movement, and proximity to neighbors were evaluated and the effect of these risk factors on the spatial distribution of positive herds was assessed.

2. Materials and methods

2.1. Study population

This cross-sectional study was performed in "Sogn og Fjordane" and "Møre og Romsdal" counties on the west coast of Norway (Fig. 1). The region was thought to be a suitable study area because of an expected mix of BTM-positive and negative herds. One BTM sample from each of 1347 herds was collected by the dairy company (Tine, Norwegian Dairies SA), between December 2012 and June 2013. In 2013, 1854 herds delivered milk in the two counties, which means samples were collected from 73% of all eligible dairy herds. Milk samples were treated and analyzed as described in Section 2.2, and each herd was categorized as either positive or negative based on the BRSV and BCoV antibody test results, respectively. If a herd contributed more than one sample during the study period, only the result from the first sample was included. Prevalence estimates were calculated for the region as a whole and for each county separately. True prevalence was calculated using the Rogan-Gladen-estimator based on the sensitivity and specificity of the tests as specified by the manufacturer (Greiner and Gardner, 2000).

During the study period, 98% of all dairy herds were members of the Norwegian Dairy Herd Recording System (NDHRS) which provides reliable records on herd characteristics, production parameters and disease occurrence (Espetvedt et al., 2013). The medical company distributing the only registered BCoV vaccine in Norway was contacted to get information regarding the number of units sold. The use of the only registered BRSV vaccine was recorded by contacting the veterinary practitioners by phone. Veterinarians in all municipalities of the study area with more than 10 herds were contacted, covering 1295 of 1347 herds.

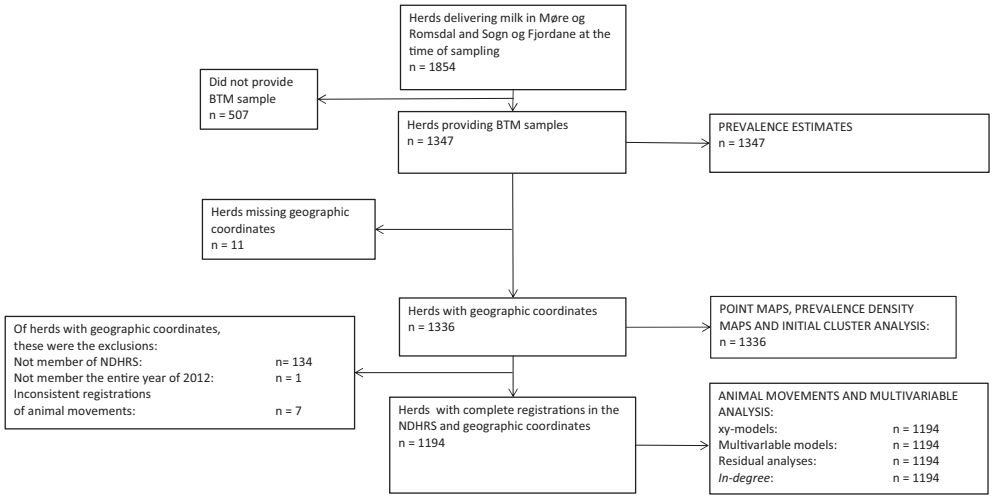

Fig. 2. Flow diagram of eligible, sampled and analyzed herds.

2.2. Laboratory analysis/outcome variable

BTM samples were collected by the milk truck drivers and transported at a temperature of 4 °C to the dairy plant were they were frozen at between −18 and −20 °C, and were kept at this temperature until thawing at the time of laboratory analysis (Tine mastittlaboratoriet in Molde). All BTM samples were analyzed using indirect ELISA (SVANOVIR® BRSV-ab and BCoV-ab, Svanova Biotech, Uppsala, Sweden). The optical density (OD) reading of 450 nm was corrected by the subtraction of OD for the negative control antigen, and per cent positivity (PP-value) (Takiuchi et al., 2009) was calculated as (corrected OD/positive control corrected OD) × 100. The cut-off for a positive result was set at a PP-value of 10 for both tests (Anon., 2016a,b) and the dichotomized results (BRSV +−/and/or BCoV +−) were used as the outcome in all analyses. The sensitivity and specificity of the ELISAs provided by the manufacturer were 94% and 100% for BRSV, and 84.6% and 100% for BCoV respectively (Alenius et al., 1991; Elvander et al., 1995).

2.3. Explanatory variables

Test results were combined with production data and health recordings from the NDHRS. All statistical analyses were done using Stata/SE (StataCorp. 2011. *Stata Statistical Software: Release 12*. College Station, TX: StataCorp LP) unless otherwise specified. Permission to use the database was given by the owner, Tine, Norwegian Dairies SA. All recordings were from the year of 2012. To describe the general characteristics of the herds in the area the mean, standard deviation and range were calculated for the following herd parameters retrieved from tables of annual summary data in the NDHRS: herd size, milk production, somatic cell count (SCC), overall herd disease incidence, and replacement rate. Herd size was defined as the herds' mean number of cow-years in 2012 (one cow-year = 365 days for a cow in a herd, calculated for each cow from date of first calving). SCC was measured as mean somatic cell count in BMT and milk production was measured as kg milk produced per cow-year. Herd disease incidence was the combined incidence rate for all recorded diseases per 100 cow-years in 2012, where recorded diseases include all cases treated by a veterinarian as this is reported in on-herd health recordings. Replacement rate was the number of cows in first lactation divided by the herds' number of cow-years, multiplied by one hundred. Reports of respiratory disease was available at the individual level, and this information was dichotomized to whether or not the herd had one or more animals with reported respiratory disease during the year of 2012. Herds that were not NDHRS members, or had incomplete registrations during this time, had to be excluded from the risk factor analysis, but were still included in prevalence estimates, point maps and isopleth maps. For an overview of eligible, sampled and analyzed herds see Fig. 2.

Data on animal movements between holdings were provided by the Norwegian Food Safety Authority, and *in-degree* was calculated as a measure of animal purchase as described in Section 2.4. Access to recordings on the location of each herd, given by geographic coordinates (latitude, and longitude, projection: EPSG: 4326-WGS 84), was provided by Tine, Norwegian Dairies SA. No information on the location of non-dairy cattle holdings was available. As a measure of proximity to neighbors, the mean Euclidian distance to the five closest dairy herds was calculated. This calculation also included herds outside the study area to avoid biased values for herds close to the county borders. The date at which the sample was collected by the tank milk driver, was divided into two categories: winter; December 1st–March 31th vs. summer; April 1st–June 30th.

2.4. Animal movements

In this study, the term 'animal movement' refers to change in ownership of an animal. Registration of cattle purchases is mandatory in Norway. *In-degree* was used as a measure of livestock movement, and is the number of direct ingoing contacts, from individual herds, through animal purchase (Nöremark and Widgren, 2014). *In-degree* was calculated as a sum of purchases from individual herds for a period of almost five years; January 1st 2008–December 5th 2012. I.e. an in-degree of five for a given herd in this study indicates that the herd has purchased live animals from five different holdings during the five year period described. Purchases reported after December 5th were excluded from the *in-degree* calculations because this was the date of collection of the first BTM sample. A total of 347 holdings had not registered purchases during this time period. This was assumed to be true

and no herds were excluded due to missing values, but herds with inconsistent duplicate registrations were omitted (Fig. 2).

2.5. Risk factor analysis

2.5.1. Univariable analyses

A total of 1194 out of the 1347 sampled herds, or 89%, had complete records, and were included in the risk factor analysis. To assess the probability of selection bias the proportion of positive herds was calculated for the 153 herds (11%) that did not have complete NDHRS records as well as all sampled herds. Herds lacking geographic coordinates were excluded from the maps and spatial analyses (Fig. 2).

Univariable analyses for a set of 11 predictors were performed in order to select which variables to include in the multivariable models. These predictors were chosen from the available data based on a causal diagram and biological plausibility of an association with the dichotomized test result of BRSV and BCoV antibodies in BTM. The same variables were evaluated for both viruses. Continuous variables assessed for an effect on the outcome were: herd size, herd disease incidence, average milk production per cow-year, replacement rate, mean SCC in BTM, geographic coordinates and average distance to the five nearest herds. The continuous variables were included as such in the analyses unless otherwise mentioned. Dichotomous variables were: time of sampling (winter; December 1st–March 31th vs. summer; April 1st–June 30th) and whether or not the herd had reported respiratory disease the year before sampling. The association between *in-degree* and the outcome was assessed treating *in-degree* both as a continuous- and as a categorical variable. For analytical and interpretational reasons *in-degree* was eventually included as a categorical variable with three categories: category 1 for 0–1 direct ingoing contacts, category 2 for 2–9 direct ingoing contacts and category 3 for more than 9 direct ingoing contacts.

For all variables the association with the outcome was evaluated by simple logistic regression (Wald-test), and the predictor was included in the subsequent model-building process if the p-value < 0.2.

Linearity of continuous predictors was assessed by grouping observations in groups of equal size, and making plots of the group means against the log odds of the outcome. In case of non-linearity, different transformations were evaluated. To avoid multicollinearity in the model, correlation coefficients between all pairs of two predictors were calculated before the multivariable analysis was performed (Dohoo et al., 2003).

2.5.2. Multivariable analyses

Based upon the significant associations from the univariable analyses, two logistic regression models with different outcomes were built: one with the BRSV antibody status of the herd as the outcome and the second with BCoV antibody status as the outcome. Large scale trends, also called first-order spatial effects, relate to variation in the mean value of a spatial process (Dohoo et al., 2003), and to control for possible first-order effects the x-coordinate (longitude) and y-coordinate (latitude) were added in the model as continuous variables. Biologically plausible pairwise interactions between significant variables from the final models were assessed by adding their cross-product in the model and then determining if the coefficient for the term was statistically significant. For interactions, a more stringent criterion was used for model inclusion ($p < 0.02$) in order to choose the most parsimonious model. Possible confounding factors were identified through a causal diagram and monitored by calculating the changes in other covariates when one factor was added and withdrawn from the model. The final models were fitted using a manual backward stepwise procedure, with a selection threshold of $p < 0.05$. The area under the curve (AUC) of the receiver operating characteristic (ROC) was used to evaluate overall model performance, and the Hosmer-Lemeshow test was used as a test for the modelís goodness of fit, with data grouped in ten groups on the basis of percentiles of estimated probability.

Pearson and standardized deviance residuals were calculated for both models. To detect possible influential observations, Q–Q plots of Pearson residuals were made, and the delta beta statistics were calculated. Observations with high residual values or delta beta value above 0.2 were omitted, and the analyses were rerun to evaluate their impact on the estimates.

2.6. Spatial patterns

2.6.1. Point maps and maps of the prevalence risk distribution

All maps were created using QGIS 2.4.0 (QGIS Development Team, 2014). Point maps were created to show the point location of all study herds with respect to their antibody status for the two viruses. Kernel density estimation was used for both BRSV and BCoV positive herds in addition to all herds, using the isotropic Gaussian kernel function implemented in the "spatstat" library in R (Baddeley and Turner, 2005). Kernel density estimation is a weighted moving average method that can be used to estimate the intensity, or mean function, for point processes (Berke, 2005). The resulting values can be presented as a raster map with one density value for each grid cell. A common fixed bandwidth determined from the coordinate ranges from the study herds ($(1/8) \times \min(x_{range}, y_{range})$) was used. Dividing the range distance by eight was done to avoid over smoothing the intensity function, as reported elsewhere (Vanderstichel et al., 2015). Generating a risk map with spatial point data (locations of cases and non-cases) is based on the ratio of two intensities as described by Berke (2005). Thus, the isopleth map showing prevalence risk on a smoothed color scale was made by dividing the Kernel density raster layer for the cases by the Kernel density raster layer for the background population.

2.6.2. Local clusters

The spatial scan statistic test was applied to explore spatial clusters of positive herds by using the software SaTScan version 8.1.1 (Kulldorff, 2009). The spatial scan statistic can analyze spatial point data (Kulldorff and Nagarwalla, 1995; Kulldorff, 1997), and cluster detection is done by gradually scanning a window across space, noting the number of observed and expected observations inside the window at each location (Kulldorff, 2015). Clusters of positive herds were detected using the Bernoulli model with analysis settings as purely spatial, scanning for areas with high rates and maximum spatial cluster size 20% of population at risk. No overlap of clusters was allowed. Results from the analyses includes location of clusters, the value of observed/expected cases, the relative prevalence (not shown) and a p-value for each cluster obtained by the Monte Carlo method (999 iterations).

To evaluate spatial clusters of positive herds first after correcting for first order effects and then after adjusting for other herd level risk factors, two sets of logistic regression models were built using BRSV- and BCoV-test results (0/1) as the outcome. One set included only the x- (longitude) and y- (latitude) coordinates (called the xy-models). The other set also included the predictors of interest that remained in the model as described in section 2.5.2. After model diagnostics and evaluation of model fit, the deviance residuals from all four models were obtained and analyzed using the spatial scan test under the normal probability model with analysis settings as purely spatial, scanning for areas with high values of residuals, and maximum spatial cluster size 20% of population at risk. No overlap of clusters was allowed. The output reports key statistics, including the location, the number of herds, the log-likelihood ratio and a p-value for each cluster obtained by the

Table 1
Mean, standard deviation (STD) and range for herd parameters obtained from NDHRS from the year 2012 in 1194 dairy herds, included in a study of BRSV and BCoV as measured by bulk tank milk antibodies in the study area of Sogn og Fjordane and Møre og Romsdal county on the northwest coast of Norway.

Variable	Mean				Overall mean	STD	Range
	BRSV+	BRSV−	BCoV+	BCoV−			
Herd size	25.8	16.8	23.3	14.9	20.9	14.7	3.3–123.6
Average milk production per cow-year, in kg	7295	7161	7306	7012	7222	1123	2984–13682
Mean somatic cell count in BTM	122.8	116.0	121.3	113.0	119.1	40.4	24–273
Mean distance to the 5 nearest herds, in km	1.8	2.3	1.9	2.6	2.1	1.7	0.19–18.0
Replacement rate	41.9	42.2	42.9	40.0	42.0	16.9	0–128(IQR: 31–51)
Herd disease incidence*	94.0	91.4	95.2	86.0	93	66.9	0–500
*In-degree***	2	2	2	1	2	9.7	0–181

* Herd disease incidence per 100 cow-years (year 2012).
** *In-degree*: median number of direct ingoing contacts through animal purchase over a period of almost five years.

Monte Carlo method (999 iterations). Because the same modelling approach was used for the spatial assessment of the residuals from both the xy- and the final model it was possible to compare the spatial clusters of residuals before and after correcting for the risk factors.

3. Results

3.1. Study population

Mean values, standard deviations and ranges of descriptive parameters for the study population are presented in Table 1. The overall apparent prevalence of seropositive herds in the study area was 46.2% for BRSV, and 72.2% for BCoV. 40.7% of all herds were positive for both viruses and 22.3% were negative for both viruses on BTM. This means that a herd which is antibody positive for one virus had a 5.3 times increased odds of positivity for the other virus. The prevalence of positive herds was higher in the northern county (Møre og Romsdal), 54.4% for BRSV and 79.8% for BCoV, compared to the southern county (Sogn og Fjordane), where the prevalence of BTM positive herds was 36.7% for BRSV and 63.4% for BCoV.

Based on the sensitivity and specificity of the ELISA tests given by the manufacturer, the calculated true prevalence was 49.1% for BRSV and 85.3% for BCoV. For the 153 herds (11%) that provided milk samples but were not part of the multivariable analyses (see Fig. 2), the prevalence was 50.3% and 77.1% for BRSV and BCoV, respectively.

Vaccination against BRSV was known to have been used in a total of six herds before the time of sampling. Five of these where in the northern county (Møre og Romsdal) and one in the southern county (Sogn og Fjordane). It was decided not to exclude any herds due to vaccination because vaccination was so rarely used and because the herds that had used it reported a prolonged history of respiratory disease and were likely to be antibody positive on BTM sampling regardless of the use of vaccine. Regarding the BCoV vaccine, no units of the vaccine were sold to pharmacies in the study area during 2012. This is not a guarantee that it is not used, but strongly implies limited use, and thus the risk that use of vaccine would influence the results was considered negligible.

3.2. Animal movements

Incoming animal movements were registered from most parts of the country, but the majority of purchases were across short distances within the study region. For the 1194 herds that had complete records, the median *in-degree* over the period of almost five years (January 1st 2008–December 5th2012) was 2 – with a range of 0–181.

3.3. Multivariable model

Time of year for sampling was excluded from the model due to collinearity with the geographic coordinates ($r = -0.79$ for x and $r = -0.81$ for y). The x- and y-coordinates were also correlated ($r = 0.71$), which was expected due to the north-east slope of the coastline. Because no herds are located off-shore, an increase in y will tend to entail an increase in x. The stability of the models was tested by removing the coordinates one at a time, fitting the model with only the x-coordinate, only the y-coordinate and both. No substantial changes were observed in the estimates for the other covariates in the model, and it was decided to keep both coordinates despite the correlation in order to correct for large geographic trends (first order effects) so that any remaining geographic variation in the residuals could be attributed to local dependence. The distance to the five closest dairy herds showed lack of linearity with the log odds of the outcome, and was therefore log transformed (natural logarithm).

3.3.1. BRSV-model

Variables included in the final BRSV logistic regression model were: herd size, x- and y-coordinates and log of mean distance to the five closest dairy herds (in km). Results from the BRSV logistic regression model are shown in Table 2. The area under the ROC curve was 0.73, and the p-value of the Hosmer-Lemeshow goodness of fit test with ten groups was 0.91 indicating acceptable overall fit of the model. Calculation of the delta beta statistics revealed no obvious outliers and no observations had delta beta >0.2.

3.3.2. BCoV-model

Variables included in the final BCoV logistic regression model were: herd size, herd disease incidence, x- and y-coordinates, log

Table 2
Estimated odds ratios with 95% CI and coefficients with standard errors, along with p-values based on a logistic regression model on factors associated with herd level BRSV-status as measured by antibodies in bulk tank milk in 1194 dairy herds in two counties on the west-coast of Norway.

Variable	OR	95% CI	Coefficient	Std. Error	P-value
Herd size	1.05	(1.04–1.06)	0.046	0.006	<0.01
x-coordinate (longitude)	0.60	(0.49–0.72)	−0.53	0.01	<0.01
y-coordinate (latitude)	3.55	(2.58–4.90)	1.27	0.16	<0.01
Log of mean distance to 5 nearest herds, in km	0.53	(0.44–0.64)	−0.63	0.09	<0.01
Constant	–		−76.08	9.66	<0.01

Table 3
Estimated odds ratios with 95% CI and coefficients with standard errors, along with p-values based on a logistic regression model on factors associated with herd level BCoV-status as measured by antibodies in bulk tank milk in 1194 dairy herds in two counties on the west-coast of Norway.

Variable	OR	95% CI	Coefficient	Std. Error	p-value
Herd size	1.05	(1.03–1.07)	0.052	0.009	<0.01
Herd disease incidence*	1.003	(1.001–1.005)	0.003	0.001	<0.01
x-coordinate (longitude)	0.78	(0.63–0.95)	−0.25	0.11	0.017
y-coordinate (latitude)	3.54	(2.53–4.95)	1.26	0.17	<0.01
Log of mean distance to 5 nearest herds, in km	0.46	(0.37–0.56)	−0.78	−0.78	<0.01
*In degree*** , category 1	1, reference				
In degree, category 2	1.73	(1.28–2.34)	0.53	0.15	<0.01
In degree, category 3	5.97	(2.94–12.10)	1.80	0.36	<0.01
Constant	–		−77.02	10.12	<0.01

* Herd disease incidence per 100 cow-years (year 2012).
** The number of a herd's direct ingoing contacts through animal purchase from unique herds over a period for almost five years. Category 1 includes herds with *in-degree* 0–1, category 2 for *in-degree* 2–9 and category 3 for *in-degree* more than 9.

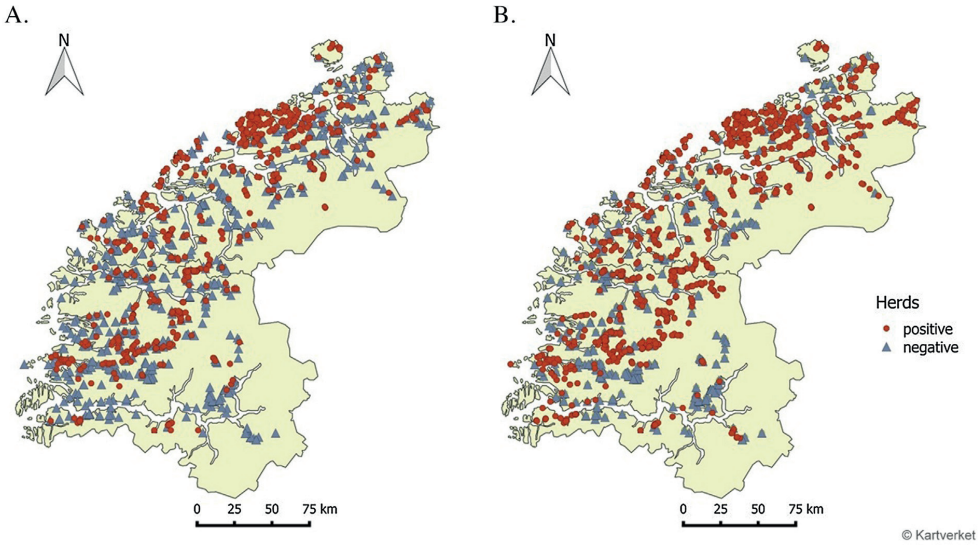

Fig. 3. Point map showing the location of 1336 dairy herds in the study area at the northwest coast of Norway. Herds were classified based on antibody-ELISA of one bulk tank milk sample collected during the period December 2012 to June 2013, and positive herds are marked as red dots whereas negative herds are marked as blue triangles. Map A shows BRSV antibody status and Map B shows BCoV antibody status.

of the distance to the five closest dairy herds and *in-degree*. After the introduction of *in-degree* the variables "replacement rate" and "reported respiratory disease" were no longer positively associated with BTM positivity. Results from the logistic regression model are shown in Table 3. The BCoV model had an area under the ROC curve of 0.81, and a Hosmer-Lemeshow goodness of fit test with ten groups gave a p-value of 0.63, indicating good overall fit of the model. Calculation of the delta beta statistic detected five possible influential observations (delta beta >0.2). However, omitting these did not substantially influence the model estimates. The model had lowest predictive ability for large BCoV negative herds, with a relatively short distance to the five nearest dairy herds, located in the northern county. These herds were BCoV-negative despite the high probability of a positive outcome predicted by the model.

3.4. Spatial patterns

3.4.1. Point maps and maps of prevalence risk distribution

The point location of all study herds are shown in Fig. 3. Kernel density estimation was used to make smoothed maps of the prevalence risk distribution for evaluation of large trends regarding spatial variation of positive herds for the two viruses. These maps show the density of positive herds over and above the density of the background population, and are shown in Figs. 4 and 5. The spatial distribution of risk is similar for the two viruses with the highest prevalence risk in the northwestern region, and the lowest prevalence risk in the south.

3.4.2. Local clusters

Application of the spatial scan test under the Bernoulli model identified five spatial clusters of BRSV-positive, and four of BCoV-positive herds ($p < 0.05$). The BRSV-positive clusters included from 15 to 182 herds and the ratio of observed/expected cases ranged from 1.91 to 2.17. For clusters of BCoV-positive herds the number of herds in a cluster ranged from 30 to 160 and the ratio of observed/expected cases ranged from 1.23–1.39.

The location of spatial clusters of high values of deviance residuals from the xy-models and the final models are shown in Fig. 6. Key statistics from the analyses are shown in Table 4. A spatial cluster of high values of residuals is an area with an excess of cases based on what is expected under the current model. For the xy-models cluster analysis using the spatial scan test identified several areas with

Table 4
Key statistics from the cluster analyses of residuals from the logistic regression model with x- and y-coordinates as the only predictors, and the final logistic regression model with all risk factors included, for BRSV and BCoV antibodies in bulk tank milk in 1194 dairy herds in two counties on the northwest coast of Norway. "Mean inside" and "Mean outside" refers to the mean value of deviance residuals inside and outside the cluster, respectively.

	Number of cases	Mean inside	Mean outside	Standard dev.	Log-likelihood ratio	p-value
BRSV xy-model:						
1. cluster	180	0.66	−0.15	1.11	39.92	0.001
2. cluster	41	1.21	−0.072	1.12	25.08	0.001
3. cluster	31	1.08	−0.057	1.13	15.13	0.001
4. cluster	16	1.38	−0.047	1.14	12.30	0.005
5. cluster	10	1.55	−0.041	1.14	9.64	0.040
6. cluster	30	0.86	−0.051	1.14	9.38	0.044
BRSV final model:						
1. cluster	180	0.58	−0.13	1.06	33.32	0.001
2. cluster	41	0.90	−0.060	1.08	15.51	0.001
3. cluster	31	0.96	−0.053	1.17	13.22	0.003
4. cluster	16	1.25	−0.044	1.08	11.27	0.009
BCoV xy-model:						
1. cluster	160	0.64	0.047	1.03	22.15	0.001
2. cluster	52	0.86	0.092	1.04	13.52	0.001
3. cluster	233	0.43	0.052	1.04	12.47	0.001
4. cluster	72	0.69	0.090	1.04	11.31	0.003
BCoV final model*:						
1. cluster	72	0.69	0.067	0.96	13.86	0.001
2. cluster	37	0.88	0.080	0.96	12.46	0.001
3. cluster	122	0.50	0.060	0.96	11.44	0.003
4. cluster	233	0.34	0.048	0.96	8.32	0.027

* Note that for BCoV the order of the clusters are not the same from the two models because of change in log-likelihood ratio. The 1. cluster from the final model is equivalent (regarding location) to the 4. cluster from the xy-model.

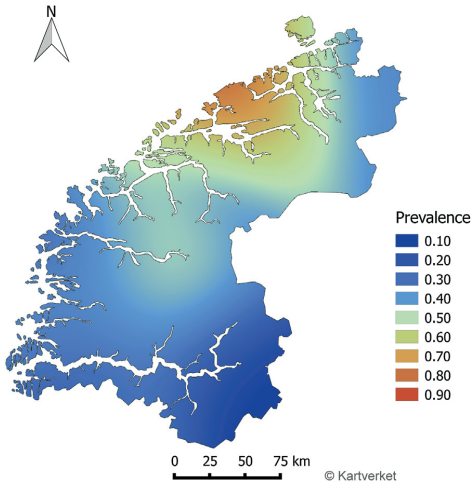

Fig. 4. Isopleth map of the prevalence risk distribution of BRSV-positivity based on classification of herds by antibody ELISA on bulk tank milk. Samples were collected during the period December 2012–June 2013, and 551 out of 1336 dairy herds were BRSV antibody positive.

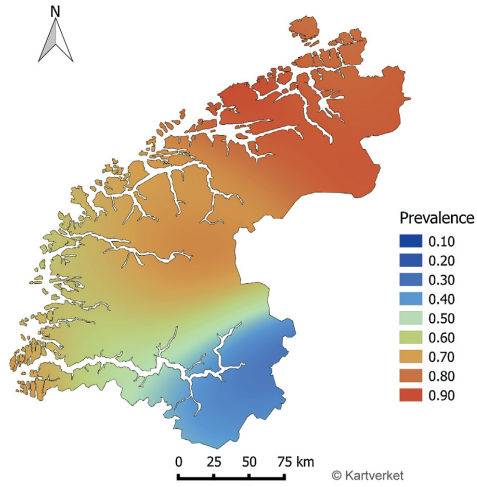

Fig. 5. Isopleth map of the prevalence risk distribution of BCoV-positivity based on classification of herds by antibody ELISA on bulk tank milk. Samples were collected during the period December 2012–June 2013, and 863 out of 1336 dairy herds were BCoV antibody positive.

high values of deviance residuals. These clusters consist of positive herds with a low probability of positivity predicted by the model, i.e. the herds were expected to be negative when correcting for large (first order) geographic trends. Clusters with a p-value > 0.05 were excluded. The cluster analyses of model residuals detected six spatial clusters of BRSV-positive, and four of BCoV-positive herds. The BRSV-positive clusters included from 10 to 180 herds, two clusters were located in Møre og Romsdal, one on the border between the two counties, and three were located in Sogn og Fjordane. For clusters of BCoV-positive herds the number of herds in a cluster ranged from 52 to 233, one cluster was located in Møre og Romsdal and the other three in Sogn og Fjordane. The most northern cluster had approximately the same geographic location for both viruses, a peninsula in the northwest of Møre og Romsdal (Romsdal-shalvøya). For the final models the deviance residuals were spatially clustered in four locations for both viruses ($p < 0.05$, Fig. 6). For the BRSV-model the number of clusters was reduced, but the changes in log-likelihood ratio of the remaining clusters were small. For BCoV the number of clusters remained the same, but there were substantial changes in the log-likelihood ratio, see Table 4.

The spatial scan statistic will search for clusters with high values of residuals, and what is considered high values is relative to the

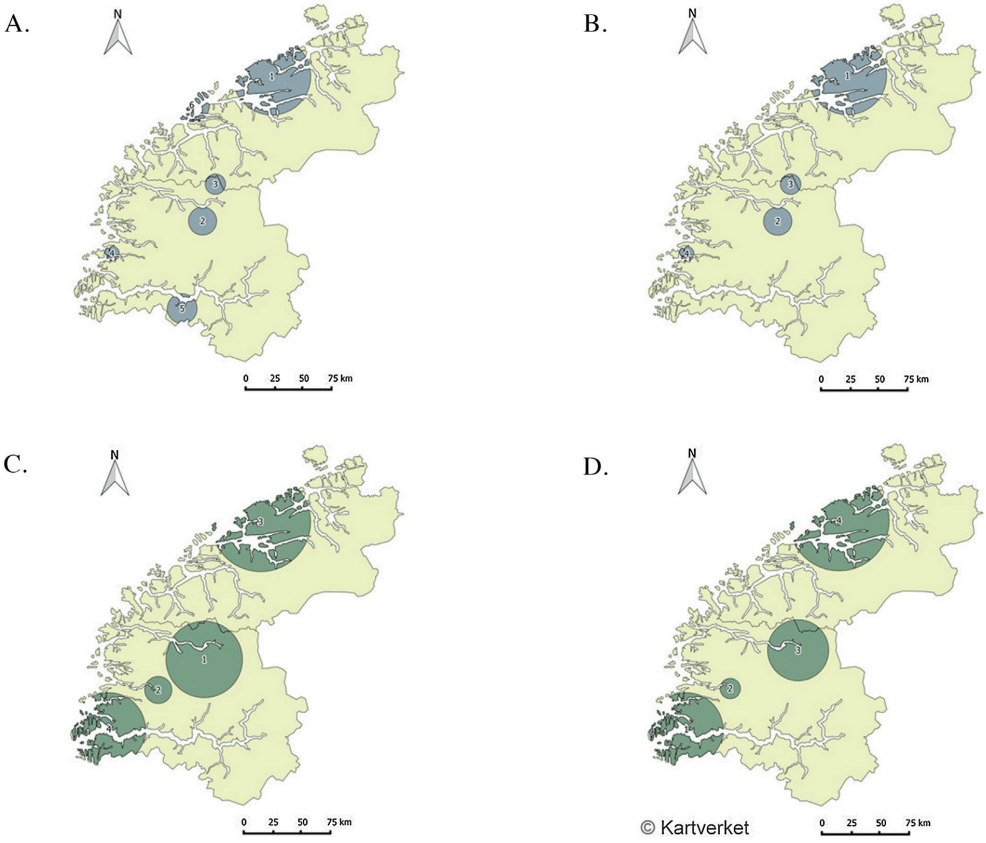

Fig. 6. Geographic map of the study area indicating the location of clusters of high values of deviance residuals from the BRSV (A and B) and BCoV (C and D) logistic regression models. Clusters from xy-models are shown in A and C, and spatial clusters of high values of deviance residuals after correcting for all the risk factors in the final logistic regression models are depicted in B and D. Analyses were performed for n = 1194 herds in the study area situated in the northwest part of Norway. Clusters were detected using the normal probability model of the spatial scan statistic, and all clusters have a p-value < 0.05. Clusters are sorted according to likelihood ratio, with the most likely cluster as number one (number displayed in the center).

rest. The reference values was different for the BRSV and the BCoV models because the BRSV models had higher values of residuals on average both for the xy-model and for the final model. This means that the evaluation of spatial clusters must be interpreted as clusters of unexplained variation in the outcome for that model, and comparison of the spatial clusters of BRSV positive herds and BCoV positive herds must be done with caution.

4. Discussion

The overall apparent prevalence of seropositive herds in the study area was 46.2% for BRSV and 72.2% for BCoV, which is low compared to reports worldwide (Paton et al., 1998; Uttenthal et al., 2000; Ohlson et al., 2010). This is also lower than estimates from previous studies in Norway using serologic methods (Gulliksen et al., 2009; Klem et al., 2013). The present study classified herds according to detection of antibodies measured in a milk sample taken from the BTM. This methodology generally increases the prevalence of a disease when compared to individual sampling of a group of young animals – the method used by the previous Norwegian studies. Hence, it makes the discrepancy between the present study and the previous ones even larger, and is most likely due to differences between study regions. The study region was selected as it was believed it would contain a mix of BTM positive and negative herds. The large variation in prevalence across regions is in agreement with a study performed by Klem et al. (2013).

For both models the odds of being BTM positive increased from south to north (latitude) and for BRSV from east to west (longitude). These large trends can be interpreted as first order effects, but because the time of sample collection was correlated with the geographic coordinates, and had to be omitted from the model, the observed geographic trends cannot with complete certainty be separated from a possible temporal effect. About 40% of the herds were positive against both BRSV and BCoV, and the odds of being positive for one virus were approximately five times larger if a herd was positive for the other virus. The large proportion herds with antibodies against both viruses was not surprising given known common risk factors.

Herd size was positively associated with seropositivity for both BRSV and BCoV. Increasing the herd size by one cow-year increased the estimated odds of being antibody positive by 5% for both viruses. This corresponds to a 72% and 84% increase in the odds of BTM positivity when increasing the herd size across the interquartile range for BRSV and BCoV, respectively. The association between herd size

and BRSV and BCoV positivity is well documented (Tråvén et al., 1999; Norström et al., 2000; Solís-Calderón et al., 2007; Ohlson et al., 2010). The dairy production in Norway is typically organized in small units with a mean of 24.2 cow-years per herd in 2013 (Anon., 2015). Even though this is smaller than in most developed countries this association holds true. The reason for the association remains unclear; however, it may be linked to larger herds having more indirect contact for instance via visits from veterinarians, AI technicians, advisory personnel or others (Norström, 2001). Furthermore, herd size might be associated with differences in management, and larger herds might provide better conditions for intra-herd virus circulation.

It is interesting that *in-degree* was only a significant predictor in the BCoV model, and not for BRSV. For the study population, the majority of purchased livestock came from within the region, and the fairly low herd level prevalence of BRSV in this region could explain why the number of ingoing contacts (*in-degree*) was not associated with BRSV positivity. The most commonly purchased animals are calves and young-stock, and because the prevalence on calf level is lower than on herd level, the risk of buying a young animal with either current viral infection or antibodies might not be high enough to show an association with the outcome. The prevalence of BCoV is higher, and thus the risk of buying antibody positive or infected animals is also higher, and more likely to affect the BTM result. A biological explanation behind differences in the likelihood of direct transmission between the two viruses should also be considered. An important difference in the pathogenesis of the two viruses is that BCoV replicates both in cells in the respiratory tract and intestinal epithelial cells, leading to shedding of virus in nasal secretions as well as in feces (Boileau and Kapil, 2010). On the other hand, BRSV only replicates in cells of the respiratory tract (Valarcher and Taylor, 2007). However, several important aspects of the pathogenesis are common for the two viruses: Shedding of virus is highest in the acute stage of the infection and disease can vary from subclinical to severe (Larsen, 2000; Cho et al., 2001; Boileau and Kapil, 2010).

Increasing mean distance to the 5 nearest dairy herds was associated with a significant decrease in odds of BTM positivity for both viruses. Association between existence of bordering herds and BRSV was also found by Saa et al. (2012). Another study found that the odds of BCoV positivity decreased as the distance to the nearest cattle herd increased, but no association was found for BRSV (Ohlson et al., 2010). Norström (2001) found an increased risk of outbreak of BRSV if at least one positive herd was within a radius of 500 m of a herd. BRSV and BCoV are enveloped viruses, with relatively short survival time outside the host, depending on environmental factors such as temperature, humidity and light (Hall et al., 1980; Larsen, 2000; Wolff et al., 2005; Casanova et al., 2010). As the number of infective virions on equipment decreases over time, the likelihood of indirect transmission by fomites decreases with increasing travelling time and therefore distance. Distance to neighbors will also influence on the number of possible indirect contacts for a herd, and thus the likelihood of exposure. This effect might be more evident during periods of high infectious pressure (epidemics). It is also possible that the distance between herds is associated with the risk of direct transmission, if animals in herd dense areas have more contact during pasture time in the summer. However, we did not have any information on the location of pastures.

The results of this study show that the geographic distribution of BRSV and BCoV in the study area are far from uniform, and that there are both local high risk clusters (Fig. 6), and large geographic trends (Figs. 4 and 5). The cluster analyses on the residuals showed that some of the local dependence changed when correcting for other risk factors. In other words, local dependence seems to be partially explained by spatial variation in the distribution of risk factors included in the logistic regression model, such as proximity to neighbors, herd size and large geographic trends (x- and y-coordinates). However, spatial clusters of high residual values from the final models indicates that there are still spatially dependent unmeasured risk factors. No information on biosecurity was available and good hygiene and husbandry practices could be an unmeasured preventive factor. A study by Ohlson et al. (2010) showed a preventive effect of using boot covers on BCoV positivity. Other non- measured potential risk factors that could be spatially dependent include the use of common grazing, and historical data on previous disease outbreaks. Both winter dysentery and respiratory disease typically occur as epidemics with years between (Boileau and Kapil, 2010). An epidemic spread of these viral infections might cause all, or the majority of, herds in an area to be antibody positive, and thus affect the spatial distribution of positive herds for years.

For BCoV the number of clusters remained the same after correcting for the risk factors in the final model, however with large changes in the likelihood ratio. These changes in the log-likelihood ratio mean that adjusting for geographic differences in herd size, proximity to neighbors and *in-degree* results in a more random distribution of the residuals. However, the effect was not uniform for all clusters, indicating that the effect of the risk factors might not be the same in all areas. Compared to the BRSV-model, the moderate values of observed/expected for the BCoV clusters from the Bernoulli model also support that local dependence might be more important for BRSV than for BCoV. The spatial clusters of BRSV antibody positive herds had high values of observed/expected from the Bernoulli model, and there were relatively small changes in log-likelihood ratio of the clusters between the xy- and the final model, which might indicate strong local dependence. This also agrees with the lower predictive ability of this model compared to the BCoV model (AUC values 0.73 and 0.81, respectively). For BRSV the results imply the existence of spatially dependent unmeasured risk factors and that each herd relies strongly on the status of its neighbors, thus indicating the importance of indirect transmission routes. The implementation of a high level of biosecurity could, therefore, be important to prevent virus introduction. The higher overall predictive ability of the BCoV model compared to the BRSV model means that despite a higher overall prevalence of BCoV it is easier to predict the serologic status of a herd, or to locate "high risk herds", for BCoV than for BRSV, based on the number of animals purchased and relatively constant factors like herd size, proximity to neighbors and location. The difficulty in finding strong associations between the investigated risk factors and BRSV positivity, and the strong local dependence, could mean that the spread of BRSV in this region has been of a more epidemic character, involving more stochasticity than what has been the case for BCoV.

Classification of herds in this study was based on a single BTM sample. The use of BTM serology cannot be relied on to give an updated picture of the infection status of a herd because animals shed antibodies for years after infection (Alenius et al., 1991; Tråvén et al., 2001; Klem et al., 2014b). The proportion of herds with ongoing or recent infection is therefore likely to be much lower than the prevalence of BTM-positive herds. Several other diagnostic options for classification of herds with respect to BRSV and BCoV status exists. Serology of individual animals, either using milk or blood samples, can give a more recent picture of the herds' infection history than BTM samples, depending on the age and number of animals sampled. (Ohlson et al., 2009; Klem et al., 2013). The ideal method for classifying herds with respect to detecting virus circulation would be to detect the virus; however, this is demanding on a larger scale (Klem et al., 2013).

The antibody ELISA tests used to classify herds as either positive or negative for either virus are imperfect. This means that there will be some misclassification of outcome, which could lead to an

underestimation of prevalence. (Estimates of true prevalence are shown in Section 3.1). However, as previously mentioned, prevalence estimates based on bulk tank milk serology will be much higher than the proportion of herds that have circulating virus, so compared to this the inaccuracy introduced by an imperfect test is negligible. When evaluating the effect of the risk factors, misclassification of the outcome was considered non-differential because the performance of the tests was not believed to be associated with any of the risk factors. Hence, this is not likely to have influenced the results.

The internal validity of this study was deemed high as all dairy herds in the study area were equally likely to be sampled and included in the study. The high proportion of sampled herds (73% of all eligible herds) also minimizes the risk of severe selection bias that could have affected the validity of the study. However, the prevalence estimates in the 153 herds that were excluded from the multivariable analysis due to incomplete NDHRS registrations, were slightly higher than for the entire population of sampled herds, which could indicate differences in, for example, management. But because the excluded herds represented only 11% of the sampled herds, and the difference in prevalence was modest, the introduced bias is likely to be small. The unknown location and BRSV-/BCoV status of beef herds in the area could potentially bias the results if the proximity to neighbors variable is incorrectly specified. However, the authors believe it is unlikely that their geographic distribution differs substantially from the distribution of dairy herds. The x- and y-coordinates were included in the models to reduce spatial heterogeneity. However, spatial correlation structures in the data may be more complex than a simple latitudinal/longitudinal gradient. In case of overdispersion due to spatial autocorrelation, this could alter the effective sample size, leading to increased chance of Type I error. However, given the low p-values and the inclusion of the x- and y-coordinates in the models, it is unlikely that the significance or direction of the effect estimates would change. The results of this study are believed to be representative for the Norwegian dairy herd as a whole, because the management systems for dairy production are comparable across the country. The external validity is therefore deemed good, and the results might also be valid for other temperate areas of smaller-scale dairy production.

The study demonstrates that the herd level prevalence of BRSV and BCoV as measured by antibodies in bulk tank milk varied considerably in the region investigated. Of all the herds, about 40% were positive for both viruses. Several herd level risk factors were of importance for both BCoV and BRSV, such as herd size, geographic location and distance to neighboring herds, and for BCoV also *in-degree*. Adjusting for these risk factors explains some of the spatial clusters of positive herds, but spatial clusters of unexplained variation in the outcome was also detected. The remaining local dependence indicates that the antibody status of one herd is influenced by the antibody status of its neighbors and that indirect transmission is likely to be important. This means that a joint effort in terms of implementing preventive measures in an area could be an effective way to lower the prevalence of these infections. Measures should involve caution when purchasing livestock, implementing a high level of biosecurity and increased awareness among farmers and other people travelling between herds in order to prevent between-herd transmission of virus.

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A cohort study of the effect of winter dysentery on herd-level milk production

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ABSTRACT

Winter dysentery (WD) is a contagious disease caused by bovine coronavirus. It is characterized by acute onset of diarrhea, fever, depression, and reduced milk yield in adult cattle. Although production loss is a well-known consequence of WD, large-scale studies estimating the effect on milk production are lacking. The objective of this study was to estimate the effect of farmer-reported WD on herd-level milk production and milk composition. A cohort study was performed based on reports of herd outbreaks of winter dysentery during a regional epidemic in Norway during the winter of 2011–2012. Reports were made by farmers, and diagnosis was based on a herd outbreak of acute diarrhea in adults. Milk shipment data were retrieved from the dairy company, and information on herd size and milking system were retrieved from the Norwegian Dairy Herd Recording System. We compared milk production in herds with reported outbreaks of WD (n = 224) with all herds in the same area without a reported outbreak (n = 2,093) during the same period. The outcome variable in the analysis was milk volume per cow per day, and the main predictor was whether the herd had a reported outbreak of WD or not. We assessed the effect of WD on milk production by fitting a linear mixed model, adjusting for milk production in the herd before the outbreak. Similarly, we assessed the effect of WD on milk composition using linear regression, adjusting for the levels of milk components before the outbreak. This study estimated a total loss of 51 L/cow during the study period, from 7 d before to 19 d after a reported outbreak. The lowest estimated production was 2 d after the outbreak was reported, when the average milk yield was 19.4 L/cow per day, compared with 23.0 L/cow per day 7 days before notification (i.e., a difference of 3.6 L/cow, or 15%). The effect gradually declined with time. The estimated effect on milk composition was modest, but an increase of 11% in free fatty acids and a small increase in fat/protein ratio indicated that WD might put cows into negative energy balance. Descriptive analysis indicated that herd milk yield was still reduced 4 mo after an outbreak. This cohort study showed that WD causes considerable decreases in milk production, and it alters milk composition. These findings highlight the important negative consequences of WD, and should motivate actions to prevent between-herd spread of bovine coronavirus.

Key words: dairy, bovine coronavirus, milk composition, milk yield

INTRODUCTION

Winter dysentery (**WD**) in dairy herds is characterized by the sudden onset of diarrhea in several adult cattle (Clark, 1993). It typically occurs as epidemics during the winter, and is caused by bovine coronavirus, which is endemic in cattle populations worldwide (Saif, 1990; Alenius et al., 1991; Paton et al., 1998; Boileau and Kapil, 2010). Previous studies have shown high prevalence in the Norwegian national dairy herd as well. Gulliksen et al. (2009) found that 39% of examined calves were antibody positive, and Toftaker et al. (2016) found antibodies in bulk tank milk in 72% of all study herds. Bovine coronavirus also causes calf diarrhea and respiratory disease in both calves and adult animals (Boileau and Kapil, 2010). The clinical signs of WD include watery diarrhea with or without blood in the feces, fever, depression, decreased milk production, anorexia, and sometimes cough or nasal discharge (Boileau and Kapil, 2010). Mortality is low, but morbidity in affected herds is high, and outbreaks can result in poor herd health and reduced animal performance (Clark, 1993; Tråvén et al., 2001; Boileau and Kapil, 2010). Reduced milk production is an important consequence for the farmer, because of associated economic losses. The acute drop in milk yield associated with WD is well known, but estimates of the magnitude of this drop are often based on a few animals or on outbreaks in only a few herds. Furthermore, the reported magnitude of this drop varies widely (Durham et al., 1989; Fleetwood et al., 1989; Tråvén et al., 2001).

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Diseases associated with reduced general condition often result in reduced milk production. A rapid decrease in milk yield has been described for several viral diseases in cattle, including foot and mouth disease, bovine herpesvirus 1 infection, and bovine leukemia virus infection (Lyons et al., 2015; Statham et al., 2015; Yang et al., 2016). Studies have also shown that viral infections can affect milk quality (Rola et al., 2015; Yang et al., 2016). However, this has not previously been shown for WD on a larger scale. Possible effects on milk composition are important because altered composition could adversely affect milk quality, which in turn has economic consequences for the farmer and the processing industry.

The Norwegian dairy herd is a suitable study population for quantifying the effects of WD, because the presence of other endemic diseases that could confound results is low. This also means that the list for differential diagnosis of WD is limited. Norwegian cattle are free of many infectious agents such as bovine viral diarrhea virus, bovine herpes virus 1, *Mycobacterium avium* ssp. *paratuberculosis,* and *Brucella abortus,* and they are virtually free of *Salmonella* spp. (prevalence <0.5% in farmed species; Sviland et al., 2015; Åkerstedt et al., 2016a; Åkerstedt et al., 2016b; Heier et al., 2016).

The endemic occurrence of bovine coronavirus regularly causes respiratory disease and diarrhea, and is a concern for animal health and economic sustainability (Gulliksen et al., 2009; Klem et al., 2014). Large-scale observational studies estimating the effect of WD on milk production under field conditions are lacking, and further knowledge in this area is in demand. Reliable estimates of both the magnitude and duration of effect of WD on milk production are important for motivating famers and others to prevent the spread of bovine coronavirus between herds. Furthermore, quantifying the effects of WD on milk composition would add valuable input to the overall picture of the economic consequences of this disease. The objectives of this study were to estimate the effect of an outbreak of farmer-reported WD on (1) herd-level milk production, as measured by volume of milk per cow per day at the time of outbreak, and (2) herd-level milk composition. A secondary objective was to explore the duration of the effect on milk production.

MATERIALS AND METHODS

Background

During the winter of 2011–2012, a seasonal epidemic of WD took place in Norway. It started in the eastern part of the country in the autumn, and spread in an epidemic pattern throughout most parts of the country. Initially, bovine coronavirus was confirmed as the causative agent in a limited number of herds by antigen (PCR) or antibody detection (seroconversion), or both. Salmonellosis, bovine viral diarrhea, and Schmallenberg virus infection were ruled out. Later in the outbreak, laboratory confirmation of the diagnosis was usually not performed. During this epidemic, the advisory service of the largest dairy company (TINE SA) developed a voluntary surveillance system in the eastern part of Norway, where farmers and veterinarians were encouraged to report outbreaks of contagious diarrhea, so that herd-level biosecurity measures could be implemented. The farmers were advised to report outbreaks of acute diarrhea affecting several adult cattle. These reports were the basis for the present study.

Study Population

In total, 241 cases of farmer-reported WD in dairy herds were made from November 4, 2011, to March 13, 2012. These reports were from 7 counties in eastern Norway: Østfold, Akershus, Oslo, Hedmark, Oppland, Buskerud, and Vestfold, which constituted the study area and defined the source population. We performed a cohort study, the study unit being the herd. Herds from which outbreaks were reported by the producer were considered exposed (**WD+**). All other dairy herds in the same area were considered non-exposed (**WD−**). Inclusion criteria were member of the Norwegian Dairy Herd Recording System; milk shipment data available for the time of the outbreak (at least 21 d before and 19 d after the day of notification); and location within the study area. In this study, the day the farmer notified the advisory service of a current outbreak was day 0, and all references to time were relative to this. Because all included herds had milk shipments throughout the study period, study groups were considered closed, and a risk-based design was applied (Dohoo et al., 2009). For a visual overview of all study herds with respect to exposure status, a point map was made.

Data

Access to milk shipment data on volume and composition was provided by the dairy company (TINE SA). The total volume of milk was recorded for each shipment (i.e., every time the milk truck collected milk from the farm bulk milk tank). Milk quality was evaluated at the dairy plant by analyses of milk composition approximately twice per month. The number of cows contributing monthly test day samples was retrieved from the Norwegian Dairy Herd Recording System, along with data on the average annual herd size, milking system, and production type (freestall/tiestall).

Variables

Outcome Variables. The continuous outcome variable was herd-level milk production, measured by volume of milk per cow per day. This was calculated as follows. The volume of milk shipped on each shipment date was divided by the number of days since the last shipment to obtain the herd's daily milk production. The number of cows contributing to the bulk tank each day was estimated from the number of cows contributing on 2 consecutive monthly test days, calculating the average change in herd size per day, allowing imputation of the average herd size per day for all days between test days. The volume of milk per day was then divided by the estimated number of cows on that day to obtain herd-level milk production. Available data on milk composition consisted of records for fat, urea, protein, lactose, free fatty acids, and SCC in the analysis description. Fat, protein, urea, and lactose were measured in %, free fatty acids was measured in millimoles per liter and SCC was measured in 1,000 cells/mL of milk.

Explanatory Variables. The main predictor (i.e., exposure of interest), was the binary variable of whether or not a herd had a reported an outbreak of WD (WD+/−). To obtain comparable time at risk for the exposed and non-exposed herds, we simulated a set of pseudo-notification dates for the non-exposed herds using frequency distribution of the actual notification dates. A pseudo-notification date was randomly assigned to each of the non-exposed herds using a list of computer-generated random numbers. In this way, milk production for the period around the outbreak for WD+ herds and around the pseudo-outbreak for WD− herds could be compared.

To account for any initial differences in milk production between WD+ and WD− herds, we calculated pre-outbreak milk production as average production (L/cow per day) for the time period from 21 to 8 d before the day of notification for each herd. We estimated the number of cows contributing to the bulk tank each day as described for herd-level milk production. Records from the last week before the notification date were omitted to avoid overlap with the disease period, because the accuracy of the reported time of outbreak was unknown. In a similar way, we calculated the average values of the different milk components for the period from 21 to 8 d before the outbreak. The time variable was the time relative to the notification date. The average annual herd size was retrieved from the annual summary tables of the Norwegian Dairy Herd Recording System, using the number of cow-years in 2012 (or 2011 for the herds that lacked a record for 2012). One "cow-year" equaled 365 cow days at risk. Milking system was divided into 3 categories: automatic milking system, pipeline milking, and milking parlor. Information on whether the herd had tiestalls or freestalls was also included.

Descriptive Statistics

We assessed the distribution of milk production and herd size among WD+ and WD− herds using histograms (results not shown). We calculated mean values and spread for herd characteristics and main variables with respect to the herd's exposure status (WD+/−), along with descriptive statistics for milk composition before and after the outbreak/pseudo-outbreak in WD+ and WD− herds, respectively. We explored the duration of effect beyond the modeled time period by calculating the average production in 20 d intervals (i.e., the first interval was d 10–29, the next d 30–49, and so on). We did this for all herds with available records, up to d 150. To assess the spread of the drop in milk production, we calculated the maximum difference between pre-outbreak milk production and herd-level milk production for each herd, and visualized it in a histogram.

Multivariable Models

Milk Production. We identified possible confounders for the effect of WD on herd-level milk production through a causal diagram, and evaluated their effects by closely monitoring the other estimates as a potential confounder was included and removed from the multivariable model. Variables assessed as possible confounders were herd size, milking system, and milk production in the period before the outbreak. The distribution of DIM was also calculated in both groups of herds. Pairwise correlations between all predictors were assessed. To allow for a different effect of time since outbreak/pseudo-outbreak for WD+ and WD− herds, an interaction term between WD and number of days since outbreak was included in the model. To assess linearity for the continuous predictor days after outbreak, smoothed line plots were drawn visualizing their relationship with the outcome. For the WD+ herds, the relationship was clearly nonlinear, so different transformations were tried. Models were compared based on Akaike's information criteria, and the best fit was accomplished by modeling the interaction term as a cubic spline. Knots were chosen a priori based on biological considerations, and knots at d −3, 2, 7, and 14 were used (Vittinghoff et al., 2012). Finally, assessment of the effect of WD on milk production was

carried out by fitting a linear mixed model with a herd random effect to account for dependence between repeated milk shipments from each herd. The model was fit using a manual backward stepwise procedure, with a selection threshold of $P < 0.05$. We used the restricted maximum likelihood approach. Ten different correlation structures were explored for the random effect: autoregressive (AR1 and AR2), moving average (MA1) and Toeplitz 1 to 7. The different correlation structures were evaluated by comparing log-likelihood and Akaike's information criteria. Residuals on herd and shipment level were assessed for possible outliers and normality plots, and plots of residuals against predicted values were made. Predicted values were calculated, and average herds in terms of milk production before outbreak were visualized through a line plot. To obtain confidence intervals for selected predicted values, the variance was calculated as suggested by Kleinbaum et al. (1982). The total loss per cow over the study period was calculated as the difference in estimated milk production per day between WD+ and WD− herds plus the initial difference at the start of the study period (d −7).

Milk Composition. We assessed the effect of WD on 6 different milk components: fat, urea, protein, fat/protein ratio, lactose, free fatty acids, and SCC. Free fatty acids and SCC were log-transformed. Records for milk composition were available for a subset of the study population, consisting of 1,539 farms: 167 WD+ and 1,372 WD−. We used the first available milk composition analysis before d 20 for this part of the analysis. Assessment of the effect of WD on the different outcomes of milk composition were performed by linear regression, adjusting for the level of the outcome measure before the outbreak, as described in the milk production section. Possible confounding factors were identified using a causal diagram and monitored by calculating the changes in other covariates when one factor was added and withdrawn from the model. For all outcomes, final models were fitted using a manual backward stepwise procedure with a selection threshold of $P < 0.05$.

Software

Data set assembly was done in SAS (version 9.3; SAS Institute Inc., Cary, NC) and further processing and data analyses were performed using Stata (Stata SE/14; Stata Corp., College Station, TX). Maps of the study area and study population were created in QGIS 2.12.2 (QGIS Development Team, QGIS Geographic Information System, Open Source Geospatial Foundation, http://qgis.osgeo.org).

RESULTS

Descriptive Statistics

The final study population consisted of 2,317 herds: 224 WD+ and 2,093 WD−. For an overview of eligible and analyzed herds, see Figure 1. An overview of the location of all study herds, together with their exposure status, is presented in Figure 2. Descriptive statistics of daily milk production 21 to 8 d before notification, daily milk production 0 to 19 d after notification, herd size, milking system, and production type (freestall/tiestall) in WD+ and WD− herds are shown in Table 1. The study herds had milk shipments 1 to 16 times, with a mean of 11 shipments from 7 d before to 19 d after the day of notification. Milk shipments happened at uneven intervals, typically with either 2 or 3 d intervals [mean 2.5, SD 1.4]. The distribution of milk components in the WD+ and WD− groups is shown in Table 2. The average herd size was 25.7 cow-years (SD 16.0), and the overall average milk production from 21 to 8 d before the outbreak was 22.2 L/cow/day (SD 6.0). Smoothed line plots of milk production in WD+ and WD− herds are shown in Figure 3. The spread in maximum herd-level milk drop (herd-level milk production – pre-outbreak milk production) was illustrated by the interquartile range: 13 to 29%. The distribution is visualized in Figure 4. For 2 WD+ herds we found no drop in milk production. For 212 of the 224 WD+ herds and 1,977 of the 2,317 WD− herds, we were able to follow milk production up to 150 d after the outbreak. The average milk production between 130 and 150 d after reporting was 22.4 L/cow per day (SD 5.9) for WD+ herds and 22.7 L/cow per day (SD 5.8) for WD− herds. For these herds, milk production before the outbreak was 23.8 L/cow per day (SD 4.9) for WD+ herds and 22.1 L/cow per day (SD 6.0) for WD− herds, suggesting that more than 4 mo later, the WD+ herds still had not regained the production they had before the outbreak. The WD− herds had a slight increase in milk production during the same period.

Statistical Analysis

Milk Production. We detected no indications of multicollinearity for the factors in the model. Of the variables considered as possible confounders, only pre-outbreak milk production was kept in the final model: introducing pre-outbreak milk production led to a large change in the estimated effect of the primary predictor WD+/−. Including milking system produced only negligible changes in the estimate of the main predictor (3%), and because this variable had a considerable number of missing observations (13% of herds), it was

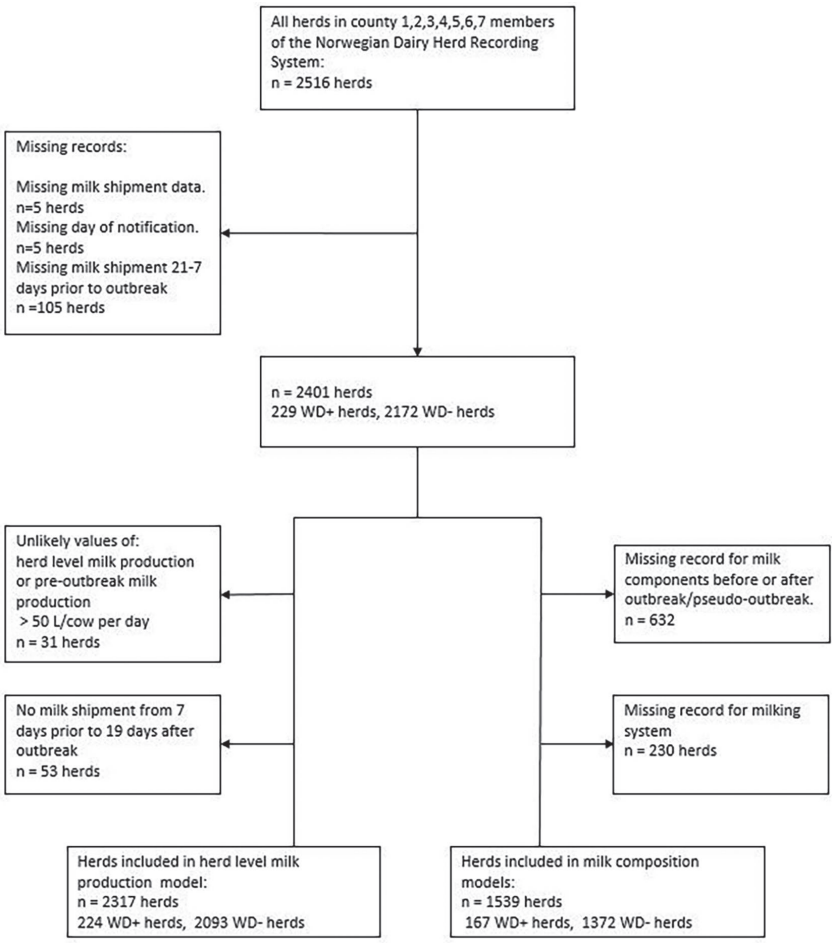

Figure 1. Flowchart of eligible and analyzed herds. Herds that reported an outbreak of winter dysentery were WD+ herds, and herds that did not make a report were WD− herds.

not included in the final model. Herd size was not significant ($P > 0.05$) in the model, and its effect on the coefficient of the main predictor was negligible, so it was also omitted. The distribution of DIM was virtually identical in the 2 groups and was not included in the model. The best model fit was achieved by applying a Toeplitz 6 correlation structure. Residual plots revealed no major shortcomings.

Estimates from the linear mixed model are presented in Table 3, and predicted milk production values for the average herd (milk production before outbreak) in the period around outbreak are shown in Figure 5. The predicted maximum difference in milk production between WD+ and WD− herds occurred at d 2. Furthermore, the model predicted that a herd with average milk production before an outbreak would fall from 23.0 L/cow per day (95% CI: 22.6–23.4 L/cow per day) 7 d before an outbreak to 19.4 L/cow per day (95% CI: 19.1–19.8) 2 d after an outbreak, whereas for a WD− herd we estimated a slight increase (<0.1 L) in milk yield during the same period. This equaled an estimated maximum herd-level drop in milk yield of 15% for a WD+ herd. The effect gradually declined over time and, around d 10 the slope for milk produc-

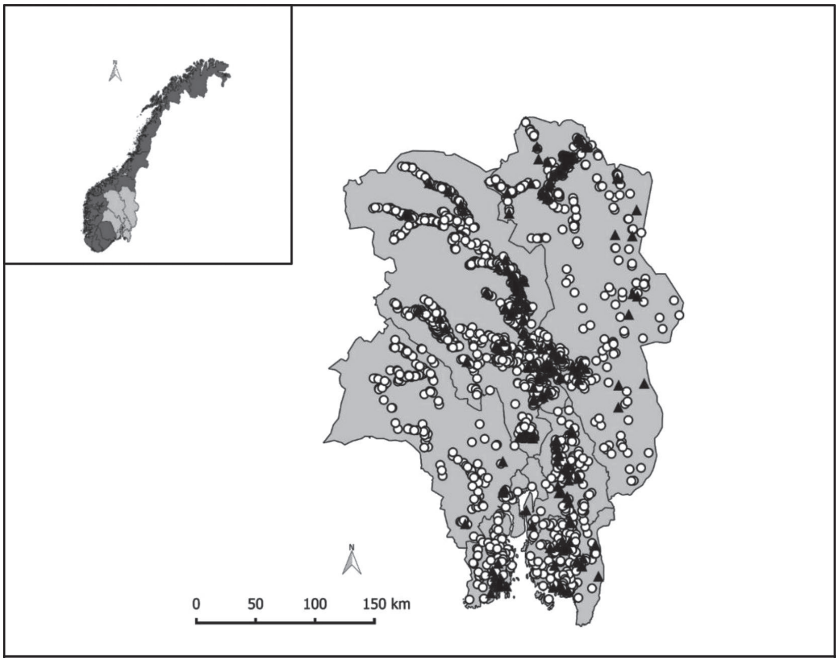

Figure 2. The study area consisting of 7 counties in eastern Norway. The black triangles (n = 224) were herds that reported an outbreak of winter dysentery, and the white dots (n = 2,093) were herds that did not make a report during the study period (November 4, 2011, to March 13, 2012).

tion flattened out for the WD+ herds. However, they did not completely regain the milk production they had before the outbreak within the modeled time period (Figure 5). For an average herd, the total estimated loss was 51 L/cow over the study period, from 7 d before to 19 d after the day of notification.

Milk Composition. Records on milk composition from d 0 to 19 were available for 1,539 herds: 167 WD+

Table 1. Descriptive statistics for the main variables and herd characteristics among herds that reported an outbreak of winter dysentery (WD+), and herds that did not report an outbreak (WD−)

	WD+ (n = 224)		WD− (n = 2,093)	
Variable	Mean	SD	Mean	SD
Average herd-level milk production d −21 to −8 (L/d)	570	341	399	308
Average herd-level milk production per cow d −21 to −8 (L/cow per d)	23.8	4.9	22.1	6.13
Average herd-level milk production d 0 to 19 (L/d)	558	334	409	312
Average herd-level milk production per cow d 0 to 19 (L/cow per d)	22.4	4.13	22.2	5.44
Average number of cow-years at risk per herd[1]	30	16.6	25	15.7
Milking system (n = 1,844; no. of herds)				
Pipeline	106		1,090	
Milking parlor	44		277	
Automatic milking system	57		270	
Barn type (n = 1,815; no. of herds)				
Freestall	98		546	
Tiestall	107		1,064	

[1]Number of cow-years at risk in 2012 or 2011 for all herds that had missing record for 2012, in total this includes n = 2,069, 223 WD+ and 1,846 WD− herds.

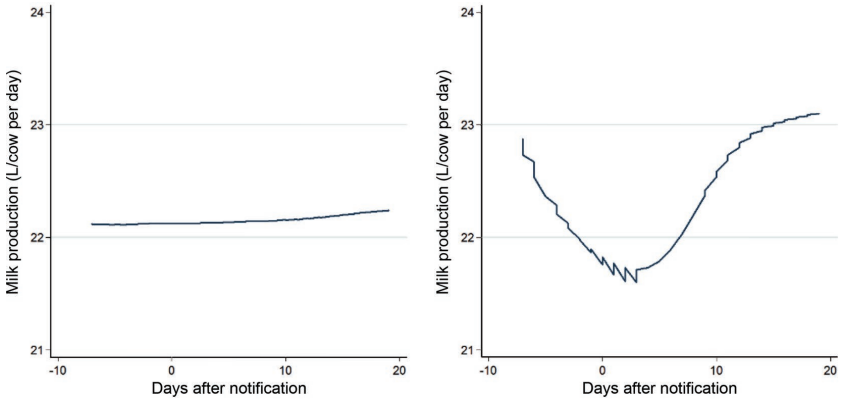

Figure 3. Smoothed line plots of the relationship between the number of days after notification and herd-level milk production (in L/cow per day) in herds that did not report winter dysentery (WD−; left) and herds that reported an outbreak of winter dysentery (WD+; right). Color version available online.

and 1,372 WD−. The median time of first available composition analysis after outbreak was at d 8. Herd size and milking system were tested in the models as possible confounders, and after a backward stepwise elimination procedure, milking system was kept in the model for lactose, protein, and free fatty acids. Herd size was not significant in any of the models, and was therefore omitted. The effect of WD on milk composition was statistically significant ($P < 0.05$) for fat, protein, fat/protein ratio, lactose, and free fatty acids. We were unable to detect any significant effect on urea or SCC. For fat, protein, and lactose, the estimated effect of WD was small (i.e., <1% difference between WD+ and WD−, given equal values before outbreak/pseudo-outbreak, results not shown). The observed effect of WD on fat/protein ratio was also small, in the direction of increased fat/protein ratio for WD+ herds: the estimated coefficient of WD was 0.018 (95% CI: 0.009–0.027). For free fatty acids, the estimated coefficient of WD was 0.010 (95% CI: 0.027–0.18), meaning the estimated average content of free fatty acids on the original scale was 0.029 mmol/L higher for a WD+

Table 2. Descriptive statistics for milk composition before and after outbreak/pseudo-outbreak in herds that reported an outbreak of winter dysentery (WD+), and herds that did not report an outbreak (WD−)

	WD+ (n = 167)		WD− (n = 1,372)	
Milk composition	Mean	SD	Mean	SD
Before outbreak[1]				
Fat (%)	4.08	0.23	4.14	0.29
Protein (%)	3.34	0.14	3.38	0.19
Fat/protein ratio	1.22	0.069	1.23	0.071
Urea (%)	5.54	0.96	5.59	0.99
Lactose (%)	4.61	0.08	4.58	0.16
Free fatty acids (mmol/L)	0.28	0.20	0.33	0.23
SCC ($\times 10^3$ cells/mL)	138	79.2	141	83.2
After outbreak[2]				
Fat (%)	4.16	0.28	4.12	0.30
Protein (%)	3.30	0.14	3.37	0.19
Fat/protein ratio	1.25	0.098	1.23	0.074
Urea (%)	5.50	1.06	5.53	0.99
Lactose (%)	4.60	0.09	4.59	0.15
Free fatty acids (mmol/L)	0.32	0.20	0.33	0.21
SCC ($\times 10^3$ cells/mL)	147	83	147	94

[1]Average level of milk component in the period from 21 to 8 d before outbreak/pseudo-outbreak.

[2]Average level of milk component in the period from 0 to 19 d after outbreak/pseudo-outbreak.

Table 3. Results from a linear mixed model with a herd random effect and a Toeplitz 6 correlation structure estimating the effect of winter dysentery (WD) on herd-level milk production (L/cow per day) in 2,317 herds (224 WD+ and 2,093 WD−)

	Coefficient	SE	$P > \|z\|$	95% Lower	95% Upper
Intercept	4.93	0.20	<0.01	4.53	5.32
WD[1]	−2.52	0.21	<0.01	−2.93	−2.12
Pre-outbreak milk production[2]	0.77	0.009	<0.01	0.75	0.79
Days[3]	0.0065	0.003	0.023	0.0007	0.012
Slope 1[4]	−0.50	0.024	<0.01	−0.55	−0.45
Slope 2[4]	2.05	0.10	<0.01	1.85	2.26
Slope 3[4]	−5.01	0.30	<0.01	−5.56	−4.46

[1]Winter dysentery outbreak versus no outbreak.

[2]Average milk production (L/cow per day) for 21 to 8 days before the day of notification.

[3]Number of days after notification of outbreak.

[4]Slopes 1–3 were the coefficients generated from a cubic spline of the interaction of WD[1] × days[3].

than for a WD− herd, given equal values before the outbreak. This was equivalent to an estimated average difference in free fatty acids between WD+ and WD− herds of 11%.

DISCUSSION

This study estimated the loss in milk production associated with a farmer-reported outbreak of WD to be 15% (2 d after notification) at the herd level. For an average herd, the total estimated loss per cow was 51 L for the entire study period, from 7 d before 19 d after notification. Former studies vary widely with respect to the magnitude of drop in milk yield. A few studies have explored the effect of WD on milk production at the herd level. One study reported that 90% of farmers had observed a decrease in milk yield after WD, but farmers were not asked to quantify the loss (Tråvén et al., 1993). A study describing an outbreak of WD in 2 herds in Canada reported that herd-level milk production dropped to less than half of normal production (Durham et al., 1989), and Jactel et al. (1990) estimated a drop of 6 to 30%, based on outbreaks in 7 herds. The latter study emphasized the large variation in severity of disease among the study herds, which was in line with the large spread in drop in milk production we found in the present study (Figure 4). Studies including only a few herds generally have limited generalizability, and that, combined with large variations in severity at both the cow and herd level, likely contributes to variations in estimates of the effect of WD on milk production between studies. An observational study

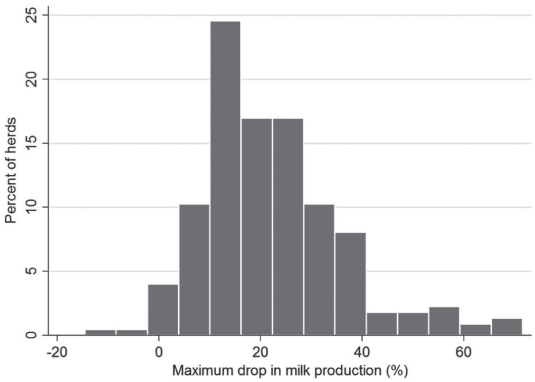

Figure 4. Histogram showing the distribution of maximum drop in milk production for herds that reported outbreak of winter dysentery (WD+). Maximum drop was calculated as the difference between the minimum daily production from 0 to 19 d after the day of notification and the average production before the outbreak (from 21 to 8 d before the day of notification).

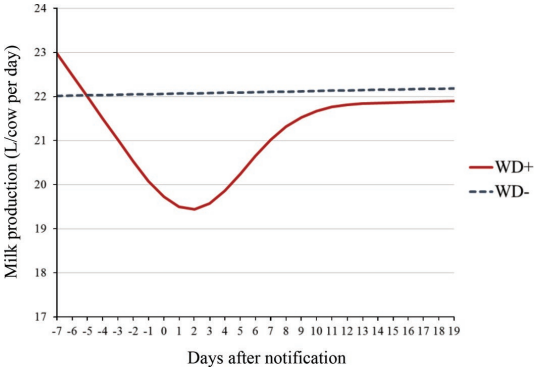

Figure 5. Predicted values of herd-level milk production (measured in L/cow per day) for herds that reported outbreak of winter dysentery (WD+) and herds that did not report winter dysentery (WD−) estimated from a linear mixed model, adjusting for milk production before the outbreak. Milk production before the outbreak was set to the study sample mean of 22.2 L/cow per day for 21 to 8 d before the day of notification. Color version available online.

by Beaudeau et al. (2010) did not reveal any effect of seroconversion for bovine coronavirus in pooled milk samples of primiparous cows on test-day milk yield. However, seroconversion for bovine coronavirus does not necessarily mean that the herd had a WD outbreak. Furthermore, samples were taken 6 mo apart, making the exact time of virus introduction unclear, and the use of test day records for milk yield provides an additional limitation, because they are typically taken 1 month apart. Tråvén et al. (2001) found that milk production was reduced by 19 to 56% at the cow level in an experiment with 5 naturally infected cows. Comparing herd-level evaluations to cow-level studies is problematic, because a herd might consist of a mixture of naïve, immune, noninfected and infected animals, and the infection might vary from subclinical to severe. For the same reason, the results of the present study are not generalizable to individual cows. The relatively large sample size, the cohort of WD− herds from the same area for comparison, and access to milk shipment data for calculations of daily milk production were major strengths of the present study.

Milk production in the WD+ herds was still reduced 150 d after outbreak, compared with a slight increase in production for the WD− herds. However, uncertainty about causal inference increases with time since an outbreak. Furthermore, the long-term effect likely depends on the number of cows infected, the duration of clinical signs in the herd, and the number of new cows calving in the period after outbreak. Hence, the factors affecting long-term effects at the herd level are likely complex. We had no information on the factors mentioned in the current study. Virtually no other studies exist describing the long-term effects of WD, although Jactel et al. (1990) described reduced production up to 28 d for 1 study cow, and Clark (1993) stated that decreased production might last several months.

This is the first large-scale study to show altered milk composition as an effect of WD. The change in composition was small except for the increase in free fatty acids of about 11% for WD+ herds. Jactel et al. (1990) reported a decline in fat and protein content in 2 herds with WD outbreaks, but did not measure free fatty acids. However, an increase in free fatty acids has been described for bovine herpes virus 1 (Rola et al., 2015), and might be associated with negative energy balance due to anorexia or increased energy demand in diseased cows. The fat/protein ratio has been used as an indicator of lipo-mobilization (Toni et al., 2011), and the observed increase in this parameter gives additional support to the theory that negative energy balance is a consequence of WD. Increased free fatty acids in milk can be associated with reduced quality and cause off-flavor (Santos et al., 2003), and are therefore important for farmers for economic reasons, because they might affect the price of the milk.

In the present study, we found that the maximum difference in milk production between the WD+ and WD− groups was at d 2 after the notification date. However, we did not know the exact date of peak outbreak in terms of severity of clinical signs or maximum morbidity, because the farmer might have made the report before or after this peak. Figure 4 shows that a few herds did not experience a drop in milk production within the study period. It seems likely that we failed to capture the maximum effect of WD within the investigated time period for some WD+ herds, contributing to underestimation of milk loss.

The diagnosis in this study was based on clinical signs without laboratory confirmation for the majority of herds. However, the rapid spread of disease strongly indicated a contagious disease, and bovine coronavirus was confirmed as causative agent in some herds where diagnostics was performed. The Norwegian dairy herd is free of many infectious diseases that could be mistaken for WD. Feed-related diarrhea is also a differential diagnosis for WD, and because farmers' ability to distinguish WD from other causes of diarrhea probably differs, some exposure was likely misclassified in the WD+ group. Reporting of WD to the advisory service of the dairy company was done voluntarily, and because this might be associated with underreporting, misclassification of exposure was also likely present in the WD− group. Assigning pseudo-notification dates was done to minimize this problem: although many herds recorded as WD− probably had an outbreak during the winter season of 2011 to 2012 but failed to report it, it was less likely that such an outbreak would fall within the 27 d that represented the time at risk for this analysis. Altogether, the uncertainty regarding time of outbreak, the likely underreporting of WD, and the fact that the diagnosis was done by farmers means that the drop in milk production found in this study should be considered a conservative estimate (bias toward the null). As well, the negative effect of WD on milk production likely represents only a part of the total economic loss associated with a WD outbreak. Other effects of WD, such as adverse effects on reproduction, effects on calves and young stock, and treatment costs were not investigated in this study.

The initial differences between WD+ and WD− herds with respect to milk production (before the outbreak) were considerable (see Table 1). Hence, it was necessary to adjust for the difference in milk production before exposure to draw inferences from comparisons of the 2 groups. We did this analytically by including prior milk production in the model. The WD+ herds were also larger than the WD− herds on average (see

Table 1), indicating that large herds were more likely to make a report, or that large herds had increased risk of clinical outbreak of WD. The latter has been described by White et al. (1989). The milk production values in Table 1 were averaged over the number of days in the table range, and are not comparable to the maximum drop estimated by the model.

The internal validity of this study was deemed acceptable after bias was minimized as described above. However, the milk composition analysis data were available for a smaller subset of the study herds, so selection bias cannot be ruled out for this part of the analysis. The external validity was considered good for the Norwegian population of dairy herds, because 98% of all dairy herds were members of the Norwegian Dairy Herd Recording System in 2013 (Espetvedt et al., 2013), and the herds in the study area were not likely to differ significantly from Norwegian dairy herds across the country with respect to management systems and breeds. The results are likely also valid for other populations of smaller-scale dairy herds in temperate areas.

Our results indicate that the effect of WD on milk production at the herd level is considerable. We also found an increase in free fatty acids and fat/protein ratio, indicating that WD can induce negative energy balance and adversely affect milk quality. The findings of this study emphasize the importance of preventive measures and should encourage farmers, veterinarians, and others to avoid between-herd spread of bovine coronavirus.

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III

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Evaluation of a multiplex immunoassay for bovine respiratory syncytial virus and bovine coronavirus antibodies in bulk tank milk against two indirect ELISAs using latent class analysis

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ABSTRACT

Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) are responsible for respiratory disease and diarrhea in cattle worldwide. The Norwegian control program against these infections is based on herd-level diagnosis using a new multiplex immunoassay. The objective of this study was to estimate sensitivity and specificity across different cut-off values for the MVD-Enferplex BCV/BRSV multiplex, by comparing them to a commercially available ELISA, the SVANOVIR® BCV-Ab and SVANOVIR® BRSV-Ab, respectively. We analyzed bulk tank milk samples from 360 herds in a low- and 360 herds in a high-prevalence area. As none of the tests were considered perfect, estimation of test characteristics was performed using Bayesian latent class models. At the manufacturers' recommended cut-off values, the median sensitivity for the BRSV multiplex and the BRSV ELISA was 94.4 [89.8–98.7 95% Posterior Credibility Interval (PCI)] and 99.8 [98.7–100 95% PCI], respectively. The median specificity for the BRSV multiplex was 90.6 [85.5–94.4 95% PCI], but only 57.4 [50.5–64.4 95% PCI] for the BRSV ELISA. However, increasing the cut-off of the BRSV ELISA increased specificity without compromising sensitivity. For the BCV multiplex we found that by using only one of the three antigens included in the test, the specificity increased, without concurrent loss in sensitivity. At the recommended cut-off this resulted in a sensitivity of 99.9 [99.3–100 95% PCI] and specificity of 93.7 [88.8–97.8 95% PCI] for the multiplex and a sensitivity of 99.5 [98.1–100 95% PCI] and a specificity of 99.6 [97.6–100 95% PCI] for the BCV ELISA.

1. Introduction

Bovine coronavirus (BCV) and bovine respiratory syncytial virus (BRSV) are commonly occurring agents among cattle worldwide (Valarcher and Taylor, 2007; Boileau and Kapil, 2010). They are endemic and prevalent also in the Norwegian dairy herd (Gulliksen et al., 2009; Klem et al., 2014a). BCV causes respiratory disease, calf diarrhea and winter dysentery (contagious diarrhea in adult cattle) (Boileau and Kapil, 2010). BRSV causes respiratory disease mostly in young animals but can affect animals of all ages, and is a common cause of respiratory outbreaks in Norway (Larsen, 2000; Klem et al., 2014a). Consequences of these infections are herd health problems, reduced animal welfare and increased use of antibiotics due to secondary bacterial infections (Larsen, 2000; Valarcher and Taylor, 2007; Boileau and Kapil, 2010). Therapy costs and reduced production entails considerable financial loss for the farmer, and contributes to a present focus in Nordic countries on how to limit the spread of these viruses in the cattle population.

In 2016, a national control program against BRSV and BCV infections was launched in Norway as a joint initiative between the producer organizations. This prompted the need for an easy and cost-effective way to screen dairy herds for a herd level diagnosis for BRSV and BCV. The initial screening in the control program was conducted using bulk tank milk samples (BTM). There are already commercially available indirect enzyme-linked immunosorbent assays (ELISAs) widely used in routine diagnostics and research in the Nordic countries (SVANOVIR® BRSV-Ab and SVANOVIR® BCV-Ab) (Tråvén et al., 1999; Klem et al.,

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2014b; Toftaker et al., 2016). However, in order to optimize cost-effectiveness of the control program, the development of a new multiplex antibody ELISA was initiated (MVD-Enferplex BCV/BRSV multiplex). The new test allowed screening for both viruses by the use of a single test.

The performance of a diagnostic test is characterized by the test's sensitivity (Se) and specificity (Sp), where Se is the proportion of true positives correctly classified as positive by the test, and the Sp is the proportion of true negative subjects correctly classified as negative. The true antibody status of each test subject can be determined in two ways: By use of a perfect reference test, or based on populations with known status. However, a perfect reference test (often termed a "gold standard") is rarely available and for endemic diseases, which is the case for BRSV and BCV in Norway, no reference population with complete certainty regarding disease or disease freedom exists. Consequently, the underlying true infection status for test subjects remains unknown. Test validation studies (erroneously) assuming perfect reference tests are common, even though this has been shown to introduce bias in the estimation of accuracy parameters (Valenstein, 1990; Lijmer et al., 1999). Latent class analysis (LCA) allows for the estimation of test parameters in populations where the underlying true infection status cannot be determined (Hui and Walter, 1980). In LCA the true infection status is treated as an existing, but unknown (latent), variable and test accuracy and prevalence are parameterized according to this latent variable.

As the BRSV/BCV multiplex is a new test, it needs to be validated. Test characteristics are different when a test is used as a herd test, compared to when it is used on individual samples (Christensen and Gardner, 2000) and validation for the relevant application is therefore important. BTM testing is a key component of the Norwegian BRSV/BCV control-program, it is therefore of interest to estimate test accuracy, at different cut-off values, for this application.

The aim of this study was to estimate the test sensitivity and specificity of the newly developed MVD-Enferplex BCV/BRSV multiplex across different cut-off values, for detection of antibodies in BTM. The BCV part of the multiplex was compared to the commercially available SVANOVIR® BCV-Ab, and the BRSV part of the multiplex was compared to the SVANOVIR® BRSV-Ab. As neither test could be considered perfect, the evaluation was done using LCA.

2. Materials and methods

2.1. Study population and sample material

A cross-sectional sampling design was used for the present study. Herds were eligible for inclusion if they delivered milk to the largest dairy company in Norway (TINE SA), and provided a BTM sample during the study period (March 2016). Herds from two counties with an expected difference in true prevalence (TP) were selected in order to meet the model assumptions, described in the LCA section. Using a random numbers generator, 360 samples were randomly chosen from herds in "Oppland" (Pop 1) and 360 from herds in "Sogn og Fjordane" (Pop 2) counties. "Sogn og Fjordane" is located in western Norway, and was assumed to have a relatively low prevalence, based on results from a previous study (Toftaker et al., 2016). Oppland county, located in eastern Norway, was thought to have higher prevalence based on known patterns of animal movements and a history of previous outbreaks of disease (Toftaker et al., 2017).

BTM samples were collected from nearly all Norwegian dairy herds delivering milk to the largest dairy cooperation (TINE SA) during March 2016. The samples were collected as part of the national control program against BRSV and BCV. The milk truck driver collected samples at ordinary milk shipment using standard procedures for BTM sampling. The milk was then stored at 4 °C until received at the laboratory (TINE Mastitis Laboratory, Molde, Norway) where samples were frozen and shipped over-night to the Enfer laboratory in Ireland (Enfer Scientific, Naas, Ireland). Samples were kept frozen until the time of laboratory analysis.

2.2. Diagnostic tests

2.2.1. ELISA

The SVANOVIR® BRSV-Ab, hereafter designated the BRSV ELISA, and SVANOVIR® BCV-Ab, hereafter designated the BCV ELISA, were used on all 720 samples, following the manufacturer's instructions. The optical density (OD) reading of 450 nm was corrected by the subtraction of OD for the negative control antigen, and percent positivity (PP-value) was calculated as (corrected OD/positive control corrected OD) × 100. According to the test manuals, the recommended cut-off values of sample positive > 10 PP for both tests were used as a starting point for these tests (Svanova; Svanova). For the BRSV ELISA the Se and Sp provided by the manufacturer were 94% and 100%, respectively. These parameters are calculated from serum samples, and parameters specific for BTM samples have not been reported (Elvander et al., 1995). For the BCV ELISA the test parameters provided by the manufacturer were Se of 84.6% and Sp of 100%, and as for BRSV the calculations are based on serum samples (Alenius et al., 1991).

2.2.2. Multiplex

All 720 samples were analyzed using the MVD-Enferplex BCV/BRSV multiplex, hereafter referred to as the BRSV/BCV multiplex (Enfer Scientific, Naas, Ireland). A panel of three BCV recombinant proteins (BCV A-C), along with a panel of two recombinant proteins and two synthetic peptides for BRSV (BRSV A-D) were used as antigens. Briefly, the antigens were deposited in a multiplex planar array as individual spots into wells of 96 well microtiter plates to produce arrays of antigens. Samples were diluted 1:3 into sample dilution buffer and mixed before added to the well and incubated at 37 °C for 60 min with agitation. After washing procedures, the detection antibody diluted in conjugate buffer was added and plates were incubated (37 °C for 60 min with agitation) before new washing. Finally, the chemiluminescent substrate was added. Relative light units (RLU) were captured (45 s exposure) immediately, using Quansys biosciences imaging system, and data was extracted using Quansys Q view software (v 1.5.4.7). Antigens were combined in a parallel reading, i.e. the test was considered positive when the RLU-value of at least one antigen was above the applied cut-off. Laboratory personnel were not formally blinded to test results, but due to the large volume of samples they were considered blinded for any practical purposes.

2.3. Data management and descriptive statistics

Because the multiplex consisted of several antigens each giving a separate response, a separate cut-off value was needed for each antigen. We calculated the proportion of herds that had a positive response to each of the individual antigens within the test-positive group (at manufacturers recommended cut-off values), and defined the antigen with the highest proportion of positive responses as the most influential. This was done for both viruses. When later choosing which cut-off values to assess, changing the cut-off for the most influential antigen for each virus was prioritized. We used an explorative approach to selecting cut-off values, and several different cut-off values were tried for the most influential antigen (Fig. 1). Furthermore, we evaluated test performance when including only the single most important antigen. Data preparation and descriptive analysis were performed in Stata (Stata SE/14; Stata Corp., College Station, TX).

2.4. Latent class analysis

In the present study, we used guidelines for reporting of diagnostic accuracy in studies that use Bayesian LCA (Kostoulas et al., 2017).

The target condition was herds with one or more animals producing

Cut-off alternative	BRSV-A	BRSV-B	BRSV-C	BRSV-D
1	1000	4000	7000	1700
2	2000	4000	7000	1700
3	3000	4000	7000	1700
4	4000	4000	7000	1700
5	4000	6000	9000	2000
6	8000	4000	7000	1700
7	2000	-	-	-
8	4000	-	-	-
9	2000	4000	7000	1700

Cut-off alternative	BCV-A	BCV-B	BCV-C
1	10000	11000	30000
2	15000	15000	35000
3	20000	15000	35000
4	25000	15000	35000
5	30000	15000	35000
6	35000	15000	35000
7	40000	15000	35000
8	10000	-	-
9	35000	-	-

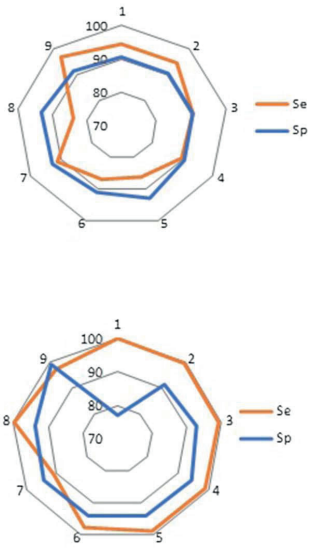

Fig. 1. To the left are the different cut-off values (relative light units) for the BRSV antigens (top) and BCV antigens (bottom) included in the BCV/BRSV multiplex. To the right are spider plots of median Se and Sp for the different cut-off alternatives. The BRSV ELISA cut-off was fixed at sample positive > 10 PP, except for alternative 9 where sample positive > 50 PP was used. For the BCV ELISA the cut-off was fixed at sample positive > 10 PP. Test parameters are estimated from a Bayesian LCM analysis.

BCV/BRSV-antibodies while contributing to the bulk tank. The underlying latent state could be considered as previous exposure, leading to antibodies in BTM.

The use of LCA methodology for diagnostic test evaluation requires a set of assumptions of the tests and test populations to be fulfilled. (1) two or more populations with different prevalence are included, (2) the Se and Sp of the diagnostic tests are the same across the populations, and (3) the tests are conditionally independent (CID) given disease status (Hui and Walter, 1980). We ran the analyses assuming CID between tests; however, we also explored the consequences of relaxing this assumption as explained below. For the CID-models, parameters were estimated for several cut-off values (Fig. 1). Models were fit using Bayesian LCA in the OpenBugs version 3.2.1 rev 781 software. We used non-informative priors in the shape of uniform distributions on the interval between zero and one, modelled using the beta (1, 1) distribution for test properties and sub-population prevalence in all analyses. Models were run with 20,000 iterations, of which 10,000 were used as burn in and discarded. Convergence of the Markov Chain Monte Carlo (MCMC) chains were assessed by visual inspection of history plots, time-series plots and Gelman-Rubin diagnostic plots using three sample chains with different initial values, as suggested by Toft et al. (2007). Posterior inference was done by calculating medians and 95% posterior credibility intervals (PCI) for Se, Sp and true prevalence. The model description is included in Appendix A.

2.5. Sensitivity analysis

A correlation between tests, if present, is not possible to estimate in a two tests scenario without including informative priors. We did not have any reliable prior information on test performance or prevalences in the present study. However, the consequences of relaxing the assumption of conditional independence given disease status was first explored by Vacek (1985), who examined the impact of conditional dependence by assuming a fixed proportion of the maximum possible covariance between tests. Following this approach we explored the consequences of conditional dependence between tests for the cut-off values with the preferred test characteristics. (Fig. 1: alternative 2 for BRSV, alternative 8 for BCV.) See Appendix A for details.

We compared the results of the conditional independence model to models allowing 25, 50, 75 and 90% of the maximum possible positive covariance, as well as a negative covariance of −25%.

3. Results

3.1. Descriptive statistics

A combination of different cut-off values for the included antigens, (cut-off alternatives 1–9) are presented in Fig. 1 for the BRSV- and BCV multiplex. For the BRSV multiplex, the BRSV-A antigen was responsible for detecting the majority of the positive samples. For the BCV multiplex the antigen detecting the majority of positive samples was the BCV-A. Counts of test outcomes for the tests are presented in Tables 1 and 2 for the BRSV and BCV tests, respectively.

Table 1
Counts of paired test outcomes in the two sub-populations for the BRSV-antibody tests (BRSV multiplex/BRSV ELISA). For the BRSV multiplex varying cut-off values for the included antigens were used (shown in Fig. 1). The BRSV ELISA cut-off was fixed at sample positive > 10 PP, except for alternative 9 where sample positive > 50 PP was used.

	BRSV multiplex/BRSV ELISA							
	Pop 1				Pop 2			
Cut-off alternative	+/+	+/−	−/+	−/−	+/+	+/−	−/+	−/−
1	299	0	35	26	111	16	103	130
2	299	0	35	26	111	16	103	130
3	287	0	47	26	105	16	109	130
4	283	0	51	26	102	16	112	130
5	272	0	62	26	94	12	120	134
6	274	0	60	26	93	15	121	131
7	289	0	45	26	107	12	107	134
8	264	0	70	26	84	11	130	135
9[a]	295	4	12	49	105	22	18	215

[a] BRSV ELISA cut-off: sample positive > 50 PP.

Table 2
Counts of paired test outcomes in the two sub-populations for the BCV-antibody tests (BCV multiplex/BCV ELISA). For the BCV multiplex varying cut-off values for the included antigens were used (shown in Fig. 1). The BCV ELISA cut-off was fixed at sample positive > 10 PP.

	BCV multiplex/BCV ELISA							
	Pop 1				Pop 2			
Cut-off alternative	+/+	+/−	−/+	−/−	+/+	+/−	−/+	−/−
1	336	7	0	17	219	34	0	107
2	335	3	1	21	215	14	4	127
3	334	2	2	22	207	11	12	130
4	330	2	6	22	198	9	21	132
5	329	2	7	22	187	9	32	132
6	324	2	12	22	182	9	37	132
7	301	2	35	22	174	9	45	132
8	336	2	0	22	219	10	0	131
9	321	0	15	24	180	1	39	140

3.2. Latent class analysis

3.2.1. BRSV

Estimates of median Se and Sp and true prevalence in the two sub-populations for the BRSV-multiplex and BRSV ELISA when applying different cut-off values are presented in Table 3. As a starting point the recommended cut-off values from the test manufacturers were applied (alternative 2 in Fig. 1), resulting in median Se of 94.4 and Sp of 90.6 for the BRSV multiplex, and Se 99.8 and Sp 57.4 for the ELISA. The Sp of the ELISA increased to 99.4 (Se 93.4) when a cut-off of sample positive > 50 PP was used. For the multiplex, increasing the cut-off value for the BRSV-A antigen generally resulted in lower Se and higher Sp estimates as could be expected. Discarding all antigens except the BRSV-A resulted in increased specificity, however, with the cost of significantly reduced sensitivity, as can be seen from comparing cut-off alternative 2 and 7 in Table 3. Point estimates (median) of true BRSV antibody prevalence ranged from 84.5 to 87.3 for pop 1, and from 25.2 to 30.5 for pop 2. Results from the COC-models with fixed covariance, showed that allowing for covariance altered specificity estimates for both the ELISA and the multiplex. The change was small for a covariance of 0.25 or less of the maximum possible covariance. The Se estimates were not noticeably affected by allowing for covariance. Results from the sensitivity analysis are presented in Table 4.

3.2.2. BCV

Estimates of test parameters and true prevalence in the two sub-populations across different cut-off values for the BCV multiplex and the BCV ELISA are presented in Table 5. When we applied the cut-off values currently recommended by the test manufacturers (alternative 1 in Fig. 1), the estimated median Se and Sp was 99.9 [99.4–100 95% PCI] and 77.3 [69.8–84.8 95% PCI], for the BCV multiplex, and 99.0 [96.9–100 95% PCI] and 99.5 [97.1–100 95% PCI] for the BCV ELISA, respectively. Similar to what we observed for BRSV, increasing the cut-off for the most important antigen (BCV-A) resulted in a lower Se and a higher Sp for the BCV multiplex. When we used the BCV-A as the sole antigen (cut-off alternative 8, Table 5) the median Sp increased to 93.7 while the median Se remained unchanged (99.9). Point estimates (median) of true BCV antibody prevalence ranged from 91.5 to 94.0 for pop 1, and from 52.4 to 61.5 for pop 2. Results from the sensitivity analysis, i.e. allowing for covariance between tests, showed negligible effect on the estimated test-parameters; less than 5% change in parameters for covariance at 75% of maximum possible (results not shown).

4. Discussion

We estimated the sensitivity and specificity of a new multiplex and two commercial ELISAs for detection of BRSV and BCV antibodies in BTM using LCA. This is the first study evaluating the MVD-Enferplex BRSV/BCV multiplex. The present study is also the first to present test parameters for the SVANOVIR® BRSV-Ab and SVANOVIR® BCV-Ab on BTM. The BRSV multiplex showed a somewhat lower Se, but a much higher Sp than the BRSV ELISA at the recommended cut-off values. However, when we increased the cut-off of the BRSV ELISA to sample positive > 50 PP, this resulted in a large increase in Sp without a notable decrease in Se, as shown in Table 3. Our results therefore suggest that a higher cut-off than recommended by the manufacturer might be appropriate when using the SVANOVIR® BRSV-Ab on BTM. For BCV, the specificity of the multiplex was notably lower than the BCV ELISA at the recommended cut-off when using all three antigens. However, when using the BCV-A antigen only, the Sp improved without the cost of reduced Se, and the test performance was then similar to the BCV ELISA. This implies that the extra antigens are adding false positive samples, hence reducing Sp. Overall; the two tests in this study both showed good performance for detection of both BRSV and BCV antibodies. A possible benefit of choosing the multiplex therefore lies in enabling screening for both agents simultaneously as this will reduce screening costs. As the multiplex evaluated in the present study is a new

Table 3
Test parameter estimates for the BRSV multiplex and BRSV ELISA: Sensitivity, specificity, and estimates of true prevalence (TP) in the two sub-populations. Cut-off alternative 1- 9 represents different cut-off alternatives for the BRSV multiplex (presented in Table 1). The BRSV ELISA cut-off was fixed at sample positive > 10 PP for all alternatives except for alternative 9, where the BRSV ELISA cut-off was increased to sample positive > 50 PP.

	Test								Sub-population			
	BRSV multiplex				BRSV ELISA				Pop 1		Pop 2	
Parameter	Se		Sp		Se		Sp		TP		TP	
Cut-off alternative	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]
1	94.4	[89.8;98.7]	90.6	[85.5;94.4]	99.8	[98.7;100]	57.4	[50.5;64.4]	87.2	[81.7;91.5]	29.9	[24.1;35.9]
2	94.4	[89.8;98.7]	90.6	[85.5;94.4]	99.8	[98.7;100]	57.4	[50.5;64.4]	87.2	[81.7;91.5]	29.9	[24.1;35.9]
3	90.7	[85.6;96.0]	90.6	[85.6;94.4]	99.7	[98.6;100]	56.7	[49.7;63.9]	86.8	[83.5;92.8]	29.2	[25.6;39.4]
4	89.5	[84.3;95.0]	90.5	[85.5;94.4]	99.7	[98.6;100]	56.3	[49.2;63.4]	87.0	[80.8;91.4]	28.5	[22.5;34.9]
5	86.3	[80.8;92.0]	92.9	[88.3;96.2]	99.7	[98.5;100]	55.8	[48.8;62.9]	86.8	[80.6;91.4]	27.8	[21.8;34.4]
6	87.0	[80.3;91.2]	91.1	[86.3;94.8]	99.7	[98.6;100]	54.8	[47.7;62.0]	86.6	[80.3;91.2]	26.5	[20.4;33.0]
7[a]	91.2	[86.3;96.3]	92.9	[88.3;96.2]	99.7	[98.6;100]	57.9	[50.8;65.1]	87.3	[81.4;91.7]	30.5	[24.6;36.7]
8[a]	84.1	[78.4;90.2]	93.4	[89.1;96.6]	99.7	[98.5;100]	53.9	[47.1;60.8]	86.4	[80.0;91.1]	25.2	[19.3;31.7]
9[b]	97.0	[94.0;99.2]	91.5	[87.6;94.6]	99.4	[97.5;100]	93.4	[89.0;97.0]	84.5	[80.1;88.2]	30.1	[25.1;35.4]

[a] Only BRSV-A antigen included.
[b] BRSV ELISA cut-off: sample positive > 50 PP.

Table 4
Results from the sensitivity analysis (BRSV): Median estimates and 95% posterior credibility intervals (PCI) of the sensitivity (Se) and specificity (Sp) of bulk tank milk BRSV multiplex and BRSV ELISA at the manufacturers' recommended cut-off (alternative 2, Fig. 1), for the conditionally independent (CID) model and conditionally dependent (COC) models where the covariance is expressed as proportions of maximum possible value.

Conditional covariance[a]	BRSV multiplex				BRSV ELISA			
	Se		Sp		Se		Sp	
	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]
CID model								
0.00	94.4	[89.8;98.7]	90.6	[85.5;94.4]	99.8	[98.7;100]	57.4	[50.5;64.4]
$COC_{SeandSp}$								
0.25	94.1	[89.5;98.6]	87.3	[80.5;92.5]	99.6	[98.1;100]	55.2	[48.3;62.3]
0.50	93.6	[88.8;98.3]	80.7	[70.6;88.6]	99.4	[96.9;100]	50.7	[43.3;58.1]
0.75	92.1	[84.9;97.6]	69.3	[62.5;79.4]	98.5	[93.2;100]	42.7	[36.8;50.3]
0.9	89.8	[82.1;96.6]	67.8	[62.1;75.4]	97.1	[91.7;99.9]	40.6	[35.2;46.8]
−0.5	94.5	[89.9;99.0]	93.7	[90.4;96.3]	99.8	[99.1;100]	59.6	[52.5;66.9]
COC_{Se}								
0.25	94.1	[89,5;98.6]	90.5	[85.6;94.4]	99.7	[98.1;100]	57.4	[50.5;64,4]
0.50	93.6	[88,7;98.4]	90.5	[85.5;94.3]	99.4	[96.9;100]	57.4	[50.5;64.6]
0.75	91.8	[84.3;97.5]	90.2	[84.8;94.2]	98.3	[92.8;100]	57.3	[50.2;64.4]
0.9	87.4	[81.1;95.6]	89.4	[83.5;93.7]	95.4	[90.9;99.7]	57.0	[49.9;64.1]
−0.25	94.3	[89.8;98.7]	90.5	[85.5;94.4]	99.8	[98.7;100]	57.4	[50.5;64.5]
COC_{Sp}								
0.25	94.3	[89.7;98.8]	87.3	[80.6;92.5]	99.8	[98.7;100]	55.2	[48.3;62.3]
0.50	94.3	[89.8;98.7]	80.9	[70.6;88.6]	99.8	[98.6;100]	50.8	[43.3;58.3]
0.75	94.3	[89.7;98.8]	69.6	[62.7;79.5]	99.7	[98.6;100]	43.0	[37.0;50.4]
0.9	94.3	[89.6;98.7]	68.2	[62.3;77.0]	99.7	[98.3;100]	40.8	[35.4;47.7]
−0.25	94.4	[89.7;98.8]	92.0	[87.8;95.3]	99.7	[98.7;100]	58.4	[51.7;65.3]

[a] Proportion of upper limit of conditional covariance.

Table 5
Test parameters for the BCV multiplex and BCV ELISA: Sensitivity, specificity, and estimates of true prevalence (TP) in the two sub-populations. Cut-off alternative 1- 9 represents different cut-off alternatives for the BCV multiplex (presented in Table 2). The BCV ELISA cut-off was fixed at sample positive > 10 PP for all alternatives.

	Test								Sub-population			
	BCV multiplex				BCV ELISA				Pop 1		Pop 2	
Parameter	Se		Sp		Se		Sp		TP		TP	
Cut-off alternative	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]	Median	[95% PCI]
1	99.9	[99.4;100]	77.3	[69.8;84.8]	99.0	[96.9;100]	99.5	[97.1;100]	94.0	[91.0;96.3]	61.5	[56.2;66.7]
2	99.6	[98.6;100]	91.1	[85.4;96.0]	99.4	[97.8;100]	97.4	[93.4;99.7]	93.5	[90.5;95.8]	60.3	[55.1;65.5]
3	99.5	[98.1;100]	93.1	[88.0;97.2]	99.5	[98.1;100]	92.3	[87.3;96.7]	93.1	[90.1;95.5]	58.0	[52.7;63.2]
4	98.9	[96.8;100]	94.3	[89.5;98.1]	99.5	[98.0;100]	87.5	[81.1;93.3]	92.6	[89.4;95.2]	55.8	[50.3;61.2]
5	99.0	[96.8;100]	94.3	[89.5;98.1]	99.5	[98.0;100]	81.6	[75.0;87.8]	92.2	[88.9;94.9]	52.4	[47.0;58.0]
6	97.9	[95.1;99.7]	94.3	[89.5;98.1]	99.5	[98.0;100]	79.8	[72.6;86.8]	91.8	[88.2;94.8]	51.5	[45.9;57.3]
7	90.6	[86.6;94.2]	94.3	[89.5;98.1]	99.4	[97.9;100]	82.6	[73.5;92.3]	92.1	[88.3;95.0]	53.3	[46.8;60.1]
8[a]	99.9	[99.3;100]	93.7	[88.8;97.8]	99.5	[98.1;100]	99.6	[97.6;100]	93.5	[90.6;95.7]	61.2	[56.0;66.2]
9[a]	97.1	[94.0;99.4]	99.2	[96.9;100]	99.8	[99.1;100]	80.4	[73.1;87.5]	91.5	[87.8;94.5]	51.6	[46.1;57.1]

[a] Only BCV-A antigen included.

test, there were no relevant studies we could compare estimates to. However, the multiplex technology has been shown useful for bovine tuberculosis in cattle and goats (Clegg et al., 2011; O'Brien et al., 2017). The parameter estimates provided by the manufacturer for the SVANOVIR® BCV-Ab are based on data from a study in which 91 serum samples were analyzed using both the ELISA and a virus neutralization test (VNT) (Alenius et al., 1991). The estimates, Se of 84.6% and Sp of 100%, were calculated using VNT as gold standard. For the SVANOVIR® BRSV-Ab, the Se (94%) and Sp (100%) were calculated in a study comparing the test results to another ELISA in 151 serum samples. Thus, test estimates were relative to the other ELISA (Elvander et al., 1995). Results from the former studies are not comparable to the present study due to different sample material (serum vs. BTM). Even so, it is important to note that in studies assuming a perfect reference test the estimated Se and Sp of the index test will never exceed those of the gold standard, thus the higher Se of both the BRSV and BCV ELISA found in our study was not unexpected.

To explore the effect of different cut-off values on test characteristics we applied a range of cut-off values for the multiplex antigens. Whenever the cut-off is changed this could entail a change in the definition of the latent condition and change the number of true positive and true negative herds. There was relatively little variation in the Se and Sp estimates of the BRSV- and BCV ELISA across the different cut-off values explored, and the change in estimates of true prevalence was minor. The tests generally agreed on the proportion of positive herds indicating that tests had good agreement on the underlying target condition. The explorative approach to choosing cut-offs is a potential weakness of the current study; however, the different scenarios provide examples of expected performance for different cut-offs and do not represent an optimization of the diagnostic tests. The chosen cut-off will

likely affect the number of antibody producing animals needed for a positive BTM result, and a positive correlation between within-herd prevalence and OD-value has been shown for other diseases (Muskens et al., 2011; Nekouei et al., 2015). Because the typical Norwegian dairy herd is small (mean herd size 25.7) (Anon., 2015) compared to most other developed countries, this might influence the generalizability of our results: In larger herds antibodies might be diluted in the bulk tank, and hence cause the test Se to decrease. However, larger herds might also have more positive animals.

Careful evaluation of the model assumptions is crucial when performing LCA, as violation of assumptions might lead to biased results. The assumption of different prevalence between populations is central to LCA models, and Toft et al. (2005) showed that the precision of the accuracy parameters improved with increasing difference in prevalence among the populations studied. In the present study, the difference in prevalence between the two sub-populations was relatively large, which in addition to a sufficient sample size, leads to narrow posterior credibility intervals for the Se and Sp estimates.

The second assumption is that the test characteristics are constant in both populations. The Norwegian dairy herd is relatively homogeneous, and the two sub-populations in this study are likely similar in terms of breeds and production systems. A potential source of variation in test characteristics between sub-populations could be antigenic diversity within the Norwegian dairy herd. Findings of antigenic diversity of BCV are summarized by Saif (2010) who concludes that only a single serotype is known based on virus cross-neutralization tests, and that a high level of cross protection has been shown between respiratory and enteric isolates. For BRSV, a Norwegian study found that the current Norwegian strains of BRSV belonged to the same subgroup as other North European isolates, indicating that the within-country diversity is likely to be limited (Klem et al., 2014a). Additionally, cross-reaction is likely to be common, and has even been shown for isolates from different species (Oberst et al., 1993). Even though it seems unlikely that spatial antigenic diversity plays an important role as source of bias it cannot be excluded with complete certainty.

The final assumption to be met is conditional independence of tests given the disease status. Several papers argue that if tests have similar biological basis, this assumption is likely not met (Gardner et al., 2000; Branscum et al., 2005). Conditional independence between tests means that the probability of a positive (or negative) result from one test is the same regardless of the result of the other test, given the true disease state (Enøe et al., 2000; Toft et al., 2005). Conditional dependence would, in terms of false positives, mean that the second test is more likely to pick up a herd as a false positive if it already tested (false) positive on the first test, for instance due to cross-reactivity with other agents. To estimate covariance between tests (γ_{Se} and γ_{Sp}) two extra degrees of freedom are needed. In a two tests, two populations scenario this results in an unidentifiable model i.e. it is not possible to estimate covariance without including prior information. No reliable prior information could be obtained for test parameters or prevalences in the present study. Another approach potentially allowing for estimation of covariance would be to include a third test: either another antibody test, or a test detecting the virus itself (e.g. a qPCR). The first option would not necessarily allow for estimation of covariance unless the third test had some underlying properties substantially different from the two other tests. Adding an antigen test might ensure conditional independence, however, it would change the underlying disease status to involve not only serological response, but also a coherent shedding of virus. We explored the consequences of conditional dependence (sensitivity analysis) by including fixed covariances as proportions of the maximum possible covariance between tests. For the BCV estimates, allowing for covariance in the latent class models had negligible effect on parameter estimates of both tests. As the Se of the BCV multiplex and the Sp of the BCV ELISA is close to one, the small effect of covariance was expected. It can be shown mathematically that test Se (Sp) are conditionally independent whenever one test has Se (Sp) = 1, see Appendix A for details. This was also the situation for BRSV-Se where the Se of the ELISA is close to 1. However, the COC-models with fixed covariances did yield changes in the estimated specificity for BRSV of both tests. This became most notable when the covariance was assumed larger than 25% of maximum. In summary, the effect of covariance was small except for BRSV-Sp for high values of covariance. It is important to note that the sensitivity analysis gives an indication of the effect of covariance if present, but does not answer whether covariance exists. Even though both tests in this study are antibody tests, they differ in the way they are designed. First, the ELISAs uses crude whole virus in the ELISA well, whereas the BRSV/BCV multiplex uses peptides and recombinant proteins. Second, the tests use different techniques for detection. The ELISAs use a chromogenic substrate and results are based on a reading of optical density, whereas the BRSV/BCV multiplex uses a chemiluminescent substrate where results are based on a reading of light emission. These differences make a violation of the conditional independence assumption less likely.

In conclusion, the BRSV/BCV multiplex and the BRSV/BCV ELISA showed similar performance when applied on BTM samples. The Sp of the BCV multiplex can be improved by using the BCV-A antigen only, and the low Sp of the BRSV ELISA can be improved by increasing the cut-off when using this test on BTM.

Conflict of interest

T., N.T., M.S., L.S. and A.N. declare no conflict of interest. G.H. and N.W. are the founders of MV diagnostics and developers of the multiplex test. A.O'B. is also developer of the multiplex test, and an Enfer employee. However, neither MV diagnostics nor Enfer were involved in the data-analysis, and could not have inappropriately influenced this work.

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Appendix A. Model description

Model description

The assumption of conditional independence between tests given disease status implies that for the population with infection present (D^+), the probability of test 1 and 2 both being positive given the test subject is truly infected is:

$$\Pr(T_1^+T_2^+|D^+) = \Pr(T_1^+|D^+)\Pr(T_2^+|D^+)$$

Similarly, for the population of non-infected subjects (D^-), the probability of test 1 and 2 both being negative given the test subject is truly non-infected:

$$\Pr(T_1^-T_2^-|D^-) = \Pr(T_1^-|D^-)\Pr(T_2^-|D^-)$$

If we define

$$\gamma_{Se} = \Pr(T_1^+T_2^+|D^+) - \Pr(T_1^+|D^+)\Pr(T_2^+|D^+)$$

and

$$\gamma_{Sp} = \Pr(T_1^-T_2^-|D^-) - \Pr(T_1^-|D^-)\Pr(T_2^-|D^-),$$

then γ_{Se} and γ_{Sp} are the conditional covariances (COCs) among infected and non-infected test subjects, respectively, and presence of COC between tests given disease status implies that $\gamma_{Se} \neq 0$ and/or $\gamma_{Sp} \neq 0$.

The latent class model assumes that for the *i*th subpopulation the counts ($\mathbf{O_i}$) of the different combinations of test results, e.g. POS/POS, POS/NEG, etc. for the two tests follow a multinomial distribution

$$\mathbf{O_i}|\ Se_j, Sp_j, p_i \sim \text{Multinominal}(\mathbf{Prob_i},\ n_i) \text{ for } i = 1,2,\ldots,S \text{ and } j = 1,2.$$

where S is the number of subpopulations; j is the index for the test; and $\mathbf{Prob_i}$ is a vector of probabilities of observing the individual combinations of test results for the *i*th subpopulation (with true prevalence,TP_i):

$$\text{Prob}_i = \begin{pmatrix} \Pr(T_1^+T_2^+) = \Pr(T_1^+T_2^+|D^+) + \Pr(T_1^+T_2^+|D^-) \\ \Pr(T_1^+T_2^-) = \Pr(T_1^+T_2^-|D^+) + \Pr(T_1^+T_2^-|D^-) \\ \Pr(T_1^-T_2^+) = \Pr(T_1^-T_2^+|D^+) + \Pr(T_1^-T_2^+|D^-) \\ \Pr(T_1^-T_2^-) = \Pr(T_1^-T_2^-|D^+) + \Pr(T_1^-T_2^-|D^-) \end{pmatrix}$$

$$= \begin{pmatrix} (Se_1Se_2 + \gamma_{Se})TP_i + ((1 - Sp_1)(1 - Sp_2) + \gamma_{Sp})(1 - TP_i) \\ (Se_1(1\text{-}Se_2) - \gamma_{Se})TP_i + ((1 - Sp_1)Sp_2 - \gamma_{Sp})(1 - TP_i) \\ ((1\text{-}Se_1)Se_2 - \gamma_{Se})TP_i + (Sp_1(1 - Sp_2) - \gamma_{Sp})(1 - TP_i) \\ ((1\text{-}Se_1)(1\text{-}Se_2) + \gamma_{Se})TP_i + (Sp_1Sp_2 + \gamma_{Sp})(1 - TP_i) \end{pmatrix}$$

The model with CID between tests can be obtained by letting $\gamma_{Se} = \gamma_{Sp} = 0$ in the above expression.

From the expression for $\mathbf{Prob_i}$ it is possible to derive upper and lower limits for γ_{Se} and γ_{Sp}, since each of the elements of the probability vector must be between zero and one, thus:

$$\max[-(1 - Se_1)(1 - Se_2),\ -Se_1\ Se_2] \leq \gamma_{Se} \leq \min[Se_1(1 - Se_2),\ Se_2(1 - Se_1)]$$

$$\max[-(1 - Sp_1)(1 - Sp_2),\ -Sp_1Sp_2] \leq \gamma_{Sp} \leq \min[Sp_1\ (1 - Sp_2),\ Sp_2\ (1 - Sp_1)]$$

If we let the Se or Sp of either test be equal to 1 in the above equations, it follows that the associated conditional covariance is limited to zero from above and below. Thus implying conditional independence (with respect to Se and/or Sp) between the two tests given disease status. In frequentist statistics, a 95% confidence interval not including zero is evidence for statistical significance. If a similar approach is adopted in a Bayesian setting, then a 95% posterior credibility interval for the conditional dependence without zero indicates that the conditional dependence should be included in the model. This covariance can be expressed as either γ_{Se} (or γ_{Sp}) or as the proportion of covariance relative to its maximum value.

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Herd level estimation of probability of disease freedom applied on the Norwegian control program for bovine respiratory syncytial virus and bovine coronavirus

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ABSTRACT

A national control program against bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) was launched in Norway in 2016. A key strategy in the program is to test for presence of antibodies and protect test-negative herds from infection. Because these viruses are endemic, the rate of re-introduction can be high, and a disease-free status will become more uncertain as time from testing elapses. The aim of this study was to estimate the probability of freedom (*PostPFree*) from BRSV and BCV antibodies over time by use of bulk tank milk (BTM) antibody-testing, geographic information and animal movement data, and to validate the herd-level estimates against subsequent BTM testing.

BTM samples were collected from 1148 study herds in West Norway in 2013 and 2016, and these were analyzed for BRSV and BCV antibodies. *PostPFree* was calculated for herds that were negative in 2013/2014, and updated periodically with new probabilities every three months. Input variables were test sensitivity, the probability of introduction through animal purchase and local transmission. Probability of introduction through animal purchase was calculated by using real animal movement data and herd prevalence in the region of the source herd. The *PostPFree* from the final three months in 2015 was compared to BTM test results from March 2016 using a Wilcoxon Rank Sum Test.

The probability of freedom was generally high for test-negative herds immediately after testing, reflecting the high sensitivity of the tests. It did however, decrease with time since testing, and was greatly affected by purchase of livestock. When comparing the median *PostPFree* for the final three months to the test results in 2016, it was significantly lower ($p < 0.01$) for test positive herds. Furthermore, there was a large difference in the proportion of test positive herds between the first and fourth quartile of *PostPFree*. The results show that *PostPFree* provides a better estimate of herd-level BTM status for both BRSV and BCV than what can be achieved by relying solely on the previous test-result.

1. Introduction

Bovine respiratory syncytial virus (BRSV) and bovine coronavirus (BCV) are widespread infectious agents, present in cattle populations around the world, including the Norwegian dairy population (Valarcher and Taylor, 2007; Gulliksen et al., 2009; Boileau and Kapil, 2010). BRSV causes respiratory disease, mostly in young animals, but can affect cattle of all ages (Valarcher and Taylor, 2007). Clinical signs vary from none to severe (Valarcher and Taylor, 2007). BCV is responsible for diarrhea in calves, and for respiratory disease and contagious diarrhea in adult cattle (winter dysentery) (Boileau and Kapil, 2010). These infections lead to increased use of antibiotics due to common secondary bacterial infections, they reduce animal welfare and the associated economic losses can be considerable (Larsen, 2000; Boileau and Kapil, 2010). In 2016, a national control program against BRSV and BCV was launched in Norway, as the first country in the world. The program is conducted as a joint initiative amongst producer organizations, and participation is voluntary. In early 2016, bulk tank milk (BTM) was collected from the majority of Norwegian dairy herds and analyzed for BRSV and BCV antibodies. In a previous study, dairy herds in two counties on the west coast of Norway had also been sampled and tested three years earlier (Toftaker et al., 2016).

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A key strategy of the control program is to protect uninfected herds by imposing restrictions on livestock trade. A negative herd status based on BTM lasts for one year after testing, regardless of the degree of contact with other herds. In a previous Norwegian study, it was shown that spread of BRSV between herds was rapid i.e. the elimination rates and introduction rates were high (Klem et al., 2013). Transmission dynamics for BCV has not yet been investigated in Norway, although one study describes a regional outbreak of winter dysentery (Toftaker et al., 2017). Studies from Sweden have shown that recent BCV infection is common, indicating that the infection is easily transmitted (Beaudeau et al., 2010; Ohlson et al., 2013). Due to the constant risk of virus introduction, the assumption that a negative status is valid for a long time is questionable. Several factors can affect the risk of change in status. Purchase of livestock is a well-known route of introduction of infectious agents, and herds that frequently purchase animals are likely at a higher risk of seroconversion (Elvander, 1996; Frössling et al., 2012; Toftaker et al., 2016). In addition to purchase of animals, previous studies have shown that location and herd size are important risk factors for BRSV- and/or BCV antibody positivity (Ohlson et al., 2010b; Toftaker et al., 2016)

Demonstration of freedom from different diseases at the national level is important for international trade purposes, and the use of scenario-tree models has recently provided a more advanced and flexible approach to these calculations (Martin et al., 2007a). More et al. (2013) applied this methodology at herd level within the Irish control program for Johne's disease. They included information on livestock trade along with test results to calculate probability of freedom from Johne's disease in test-negative herds. In Norway, information on location of herds, herd size and livestock trade are available from central directories. It was hypothesized that this information could be used along with test results to provide updated estimates of herd probability of freedom from antibodies reflecting the status more accurately than previous BTM test results alone. Estimating a time-varying probability of freedom could potentially form a tool for risk assessment in livestock trade or provide the basis for a risk-based approach to sampling.

The aim of this study was to develop a method for a frequently updated estimate of probability of freedom (*PostPFree*) from BRSV- and BCV antibodies at the herd level, based on information from BTM testing, geographic location and animal movement data.

2. Materials and methods

2.1. Study area and study population

The study area was two neighboring counties on the west coast of Norway. The southern county; "Sogn og Fjordane" and the northern county; "Møre og Romsdal". Herds located in the study region were included if they had either at least one ingoing animal movement or contributed with at least one BTM sample during January 2013 to March 2016. We had no information on herds without movements or BTM samples; hence, the total cattle population in the study region was not known. A flowchart was made to describe the different subsets of herds used for the different analyses (Fig. 1).

2.2. Sampling and analysis of BTM

During December 2012 to June 2013, BTM samples were collected from 1347 herds (out of 1854 herds delivering milk in 2013) in the study area as part of a cross-sectional risk factor study (Toftaker et al., 2016). For the *PostPFree* calculations, BTM samples collected in December 2012 were assigned to the first time period i.e. the first three months of 2013. Some of the test-negative herds were resampled the following year (n = 275, February 2014–August 2014). Finally, 1148 herds also had a BTM sample collected in March 2016 as part of the national BRSV/BCV control program. All BTM samples were collected by the milk truck driver in conjunction with milk collection and cooled

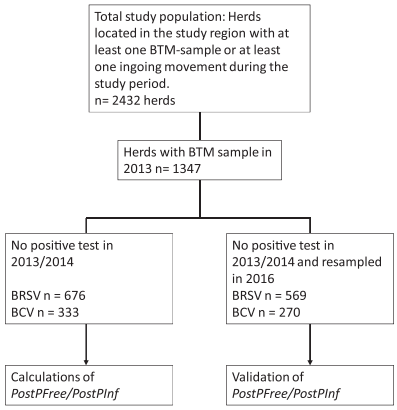

Fig. 1. Flow-chart outlining the study sample and subsets of herds included in different calculations in a study estimating the probability of freedom from BRSV- and BCV antibodies in dairy herds located in two counties in western Norway during the period January 2013–March 2016.

at a temperature of 2–4 °C until received at the laboratory (TINE Mastitis Laboratory, Molde, Norway) where samples were frozen between −18 and −20 °C until the time of analysis. The 2013 and 2014 samples were analyzed in the Norwegian laboratory, whereas the 2016 samples were shipped over-night to a laboratory in Ireland (Enfer Scientific, Naas, Ireland).

BTM samples collected in 2012–2014 were tested for antibodies against BRSV and BCV using the SVANOVIR® BRSV-Ab and SVANOVIR® BCV-Ab, respectively. Samples were analyzed following the manufacturer's instructions as described by Toftaker et al. (2016). A cut-off value of 10 percent positivity (PP) was used for both tests, according to the test manual (Svanova, 2018a,b). From 2016, all samples were analyzed with the new MVD-Enferplex BCV/BRSV multiplex, hereafter referred to as the multiplex. This test detects BRSV and BCV antibodies simultaneously using a panel of two recombinant proteins and two synthetic peptides for BRSV (BRSV-A -D) along with one recombinant protein (BCV-A) for BCV, as antigens. A positive test response results in chemiluminescence, captured by an imaging system, and measured in relative light units (RLU) by the Quansys Q view software (v 1.5.4.7). Antigens were combined in a parallel reading, i.e. the test was considered positive when the RLU-value of at least one antigen was above the cut-off. The applied cut-off values for the four different BRSV-antigens were: 2000 for BRSV-A, 4000 for BRSV-B, 7000 for BRSV-C and 1700 for BRSV-D. For BCV-A a cut-off value of 10,000 was used. The sensitivity (Se) of the multiplex was set to 0.94 for BRSV and 0.995 BCV. The Se was set to 0.998 for the SVANOVIR® BRSV-Ab and 0.999 for SVANOVIR® BCV-Ab. Test parameters at the applied cut-off values were based on a diagnostic test evaluation study, evaluating the multiplex along with the SVANOVIR® BRSV-Ab and SVANOVIR® BCV for BTM (Toftaker et al., 2018).

All the tests detect antibodies, not the antigen itself, consequently we will in the present study use "positive" when referring to animals, herds or regions as having BRSV and/or BCV antibodies. Furthermore, all input variables in the probability model relates to antibodies, hence, the calculated probabilities relate to presences of antibodies, and not necessarily infection or presence of virus.

2.3. Data sources and software

The Norwegian food safety authority provided data on cattle movements (The Norwegian Livestock registry). In the current study, animal movements refer to movements where there is a change of

owner, for which reporting is mandatory. Information about herd size was retrieved from the Norwegian dairy herd recording system (NDHRS) which in 2011 included 98% of Norwegian dairy herds (Espetvedt et al., 2013). BTM test results were provided by the largest producer organisation, TINE SA, and information on location of herds (coordinates, EUREF89/WGS 1984 UTM-32) was provided by the Norwegian Agriculture Agency. All data management, calculations and analyses were performed using Stata (Stata SE/14; Stata Corp., College Station, TX).

2.4. Animal movements

All recorded animal movements where the destination herd was located in the study area were included. Duplicate records, i.e. movements where animal ID, source county, destination herd and movement date where identical, were reduced to single records (n = 8237). Records of movements where the same animal was moved back and forth between the same two herds, or to two different recipient herds, on the same day, were omitted (n = 179). Records where the source county or the source herd was missing, and could not be retrieved from other variables, were also omitted (n = 56). After editing, the dataset included records of 45,208 movements to 1802 destination herds located in the study region.

2.5. Probability of freedom

PFree was calculated for all herds starting the study period with negative BTM test results in 2013 (and, if tested, in 2014). This was done separately for each virus. The probability of freedom was updated periodically according to the chosen time period; every three months.

The framework presented here is based on a combination of concepts from the following studies; a) scenario-tree modelling of freedom from disease using multiple sources of data presented by Martin et al. (2007a, 2007b), b) calculations of probability of disease freedom on herd level in the Irish control program for Johne´s disease by More et al. (2013) and c) a novel method to identify herds with an increased probability of disease due to animal trade developed by Frössling et al. (2014). The probability of freedom was calculated for each herd using the following Eqs. (1)–(5):

First, the probability of introducing at least one positive animal, *PIntroTrade*, to the destination herd was calculated for each unique combination *sd* of source herd *s* and destination herd *d* for each time period:

$$\mathrm{PIntroTrade}_{sd}=1-(1-P(D+)_a)^n \quad (1)$$

where $P(D+)_a$ was the within-herd prevalence in the source herd, set to 0.5 (i.e. a 50–50 probability of infection/freedom) for all herds, and n was the number of animals purchased from the source herd.

The total probability of introduction from all animal purchases within each time period *t* was calculated for each destination herd:

$$\mathrm{PIntroTrade}_{all}=1-\prod(1-(PIntroTrade_{sd}\times P(D+)_h)) \quad (2)$$

where $P(D+)_h$ is the probability that the source herd is antibody positive at the herd level. As an estimate of $P(D+)_h$ the herd prevalence in the county of the source herd, based on the national BTM screening was used.

As virus can be introduced, not only through purchase of livestock, but also by indirect transfer, we included a factor for probability of indirect transmission; *PIntroLocal*. This factor was estimated using the proportion of herds that were negative at the first sampling (2013) and positive at the last sampling (2016), in the group that did not purchase animals, hereafter designated closed herds. This was done separately for the two viruses and for the two counties as we knew that the prevalence, and likely the infectious pressure, was higher in the northern county (Toftaker et al., 2016). In addition, herd size was taken into account as several studies have found an association between herd size and seropositivity (Norström et al., 2000; Solís-Calderón et al., 2007; Ohlson et al., 2010b; Toftaker et al., 2016). In the study by Toftaker et al. (2016) conducted in the same region, the odds of testing positive increased with 12% across the inter quartile range of herd size. The effect of herd size was the same for both viruses. Based on this, we divided the study herds into two groups with median herd size as cut-point and assigned a value of *PIntroLocal* 12% higher in the "large" compared to the small herds. In summary, this resulted in four categories of *PIntroLocal* for each virus based on herd size below or above median, and which county the herd was located in (north/south). The total probability of introduction through animal purchase and by indirect transmission for each time period *t* was then calculated:

$$\mathrm{PIntroTotal}_t=1-((1-PIntroTrade_t)\times(1-PIntroLocal)) \quad (3)$$

The prior probability of infection at time *t*, $PriorPInf_t$ was estimated as follows:

$$\mathrm{PriorPInf}_t=PIntroTotal_t+PostPInf_{t-1}-PIntroTotal_t\times PostPInf_{t-1} \quad (4)$$

For the first time period, the prior probability of infection (*PriorPInf*) was set to 0.5, resembling testing a herd with unknown status, i.e. no prior information on herd prevalence in the region available and an equal probability of being positive and negative. *PriorPInf* was then calculated for each time period by taking the posterior probability of infection from the previous time period ($PostPInf_{t-1}$) and adding the probability of introduction during time period *t* calculated from Eq. (3), and adjusting for the possibility that the herd might already have been antibody positive but undetected, at the end of the previous time period $(t-1)$.

After each three month period, an updated probability of freedom (*PostPFree*) was calculated using Bayes theorem as described by Martin et al. (2007b):

$$\mathrm{PostPFree}=\frac{(1-PriorPInf)}{(1-PriorPInf\times TotalSe)} \quad (5)$$

The probability of infection (*PostPInf*) was the complement to *PostPFree*. The change in *PostPFree* over time was visualized for two example herds in a line plot.

2.6. Sensitivity analysis

Due to the uncertainty of the local factor, a sensitivity analysis was performed, using 50% lower and 50% higher values of *PIntroLocal*, and assessing the effect on the outcome; *PostPFree*.

2.7. Model evaluation

To assess the usefulness of the developed method, the *PostPFree* calculations for the final three month period was compared to the results from BTM testing in 2016, using a Wilcoxon Rank Sum Test. Bar charts were made showing the proportion of test positive herds in each quartile of *PostPFree*. The accuracy of the *PostPInf* was explored by treating it as a diagnostic test, comparing the *PostPInf* results to the 2016 BTM test-results (used as gold standard). A smoothed line plot of Se and Sp versus probability cut-off of *PostPInf* was made, and the Se and Sp at different cut-offs of *PostPInf* were tabulated (results not shown).

3. Results

3.1. Study population

The dataset consisted of 2432 beef and dairy herds located in "Sogn og Fjordane" and "Møre og Romsdal" counties. A BTM result from 2013 was available for 1347 herds, of which 275 had a follow up sample in 2014. Of the 1347 herds, 676 and 333 did not have antibodies against

Table 1
Overview of BRSV- and BCV antibody test result for bulk tank milk samples in 2013 and 2016, in a study estimating the probability of freedom from BRSV- and BCV antibodies in dairy herds located in two counties in western Norway.

Year	BRSV+ n (%)		BRSV− n (%)		BCV+ n (%)		BCV− n (%)	
2013 n = 1347	622 (46.2)		725 (53.8)		973 (72.2)		374 (27.8)	
2016 n = 1565	688 (44.0)		877 (56.0)		1210 (77.3)		355 (22.7)	
2013/2016 n = 1148	+/+ 334 (29.1)	+/− 200 (17.4)	−/+ 178 (15.5)	−/− 436 (38.0)	+/+ 724 (63.0)	+/− 120 (10.5)	−/+ 89 (7.8)	−/− 215 (18.7)

BRSV and BCV (in 2013 or 2014), respectively, and were used for probability of freedom calculations. Of the 1347 herds sampled in 2013, 1148 also had a BTM sample in 2016 of which 569 and 270 were initially negative for BRSV and BCV, respectively, and were used for validation of *PostPFree/PostPInf*. For an overview of study sample and subset of herds used in different calculations, see Fig. 1.

3.2. BTM results

At the first sampling in 2013, 622 out of 1347 sampled herds were BRSV-antibody positive and 973 were BCV-antibody positive, i.e. a proportion of test positive of 46.2% for BRSV and 72.2% for BCV as previously reported (Toftaker et al., 2016). The national control program started in March 2016, resulting in BTM samples from 1565 herds in the study area. On this final screening, 688 herds (44.0%) were antibody positive for BRSV and 1210 herds (77.3%) were antibody positive for BCV. Of the initially negative herds that were also sampled in 2016, 178 (29%) had changed status for BRSV and 89 (29%) for BCV. An overview of counts and proportions of test outcomes are presented in Table 1.

3.3. Local transmission factor

3.3.1. BRSV

Of the closed herds (n = 384), 104 herds were initially test-negative for BRSV in each county. When retested in 2016, 21 (20%) of the initially negative herds had changed status in the southern county, and 36 (35%) in the northern county.

3.3.2. BCV

For BCV, 60 herds were initially test-negative in the northern county, and 66 in the southern county in the group that did not purchase animals. When retested in 2016, 16 (27%) and seven (11%) herds went from negative to positive in the northern- and southern county, respectively. The resulting local transmission rate, *PIntroLocal*, per three month time period for each virus is presented in Table 2.

Table 2
Local transmission rate, *PIntroLocal*, per three month time period in the four different categories of herds, in a study estimating the probability of freedom from BRSV- and BCV antibodies in dairy herds located in two counties in western Norway. *PIntroLocal* was estimated from the proportion of herds that went from antibody- negative to positive during the study period (2013–2016) and did not purchase livestock.

Herd size	*PIntroLocal*			
	BRSV		BCV	
	Northern county	Southern county	Northern county	Southern county
Small herds	0.025	0.015	0.019	0.0078
Large herds	0.028	0.016	0.022	0.0087

3.4. Probability of freedom

PostPFree was high after the initial negative tests for both viruses. The median *PostPFree* in the 12th, i.e. the last, time period was 0.62 (range 0–0.91) for BRSV and 0.80 (range 0–0.95) for BCV. The distribution of *PostPFree* in time period twelve is shown by county in Fig. 2. Purchase of animals greatly affected the *PostPFree* for both agents, resulting in different slopes for herds that purchased animals compared to closed herds, as illustrated by two example herds, in Fig. 3.

3.5. Sensitivity analysis

For BRSV, reducing the value of *PIntroLocal* by 50% gave a mean increase in *PostPFree* of 10.6% (SD 4.6%), and increasing the value of *PIntroLocal* gave a mean decrease in *PostPFree* of 9.6% (SD 3.8%). For BCV, reducing the value of *PIntroLocal* by 50% gave a mean increase in *PostPFree* of 5.4% (SD 3.3%), and increasing the value of *PIntroLocal* by 50% gave a mean decrease in *PostPFree* of 5.0% (SD 2.9%).

3.6. Model evaluation

The Wilcoxon Rank Sum test showed a significant ($p < 0.01$) difference in *PostPFree* between BTM positive and BTM negative herds in 2016. This was true for both BRSV and BCV.

3.6.1. BRSV

When assessing *PostPInf* as a diagnostic test, the Se decreased with increasing cut-off, and when 0.25 was used as cut-off, the Se for detecting herds that were BTM positive in 2016 was 0.76 (95% CI: 0.68–0.82). In a practical sense this means that a recommended retesting at this value would capture an estimated 76% of the positive herds i.e. herds that are misclassified as negative based solely on the previous BTM test. No herds had *PostPInf* < 0.05 (*PostPFree* > 0.95) at the end of the study period, but at the lowest estimated value, *PostPInf* < 0.086, two out of 15 herds (13%) were test positive. The proportion of test positive herds in each quartile of *PostPFree* is illustrated in Fig. 4, and the Se and Sp of *PostPInf* is illustrated in Fig. 5.

3.6.2. BCV

For BCV the Se decreased with increasing cut-off as for BRSV, however when using a cut-off value of 0.25 for *PostPInf* the Se for detecting BTM positive herds in 2016 was only 0.55 (95% CI 0.42–0.68). At a cut-off value of 0.1 the Se was 0.83 (95% CI 0.71-0.92). At *PostPInf* < 0.05, two out of 19 herds (10%) were test positive. The proportion of test positive herds in each quartile of *PostPFree* is illustrated in Fig. 4, and the Se and Sp of *PostPInf* is illustrated in Fig. 5.

4. Discussion

This study shows that the *PostPFree* of BRSV and BCV can be used as an updated measure of the probability of freedom from antibodies at the herd level. For both infections, *PostPFree* of a test-negative herd was high immediately after a negative test, reflecting the high sensitivity of

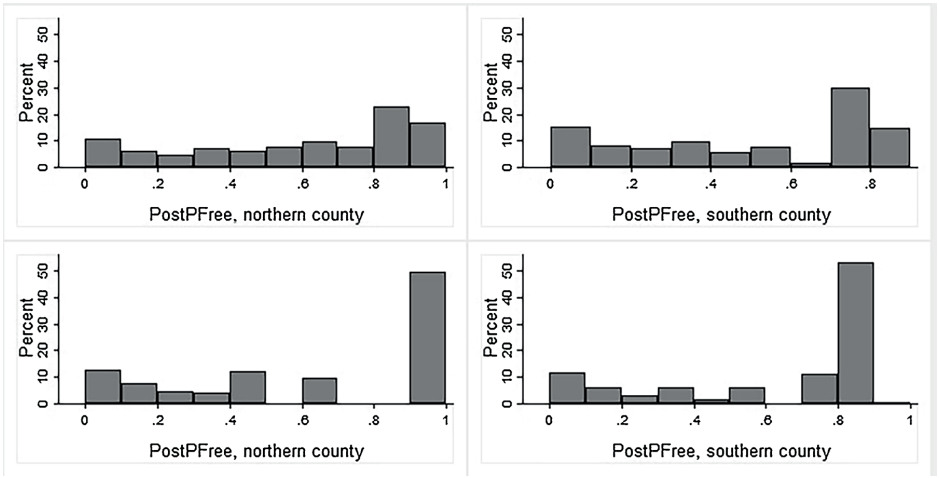

Fig. 2. The distribution of the estimated herd level probability of freedom (*PostPFree*) from BRSV antibodies (top panel) and BCV antibodies (bottom panel), by county, in the final three-month time period (time period 12) before subsequent testing. Calculations were based on BTM antibody testing, herd location and animal movement data during the period January 2013–December 2016, and were performed for n = 676 (BRSV) and n = 333 (BCV) dairy herds in two counties in western Norway during the period January 2013–December 2016. All herds had a negative test result at inclusion.

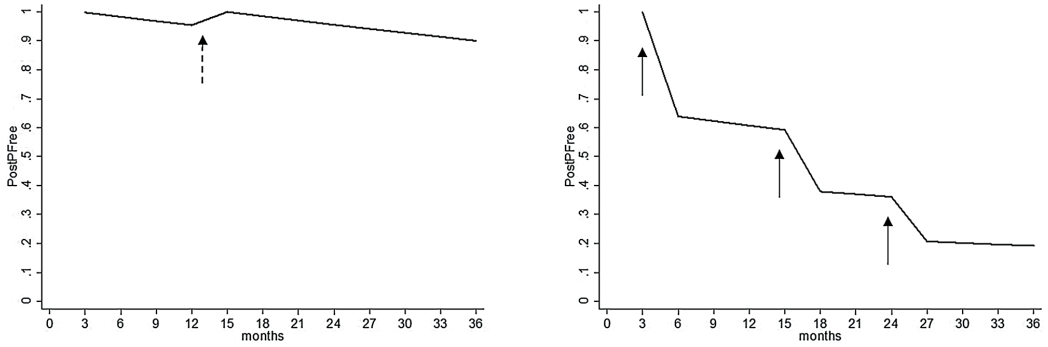

Fig. 3. Herd level probability of freedom (*PostPFree*) from BRSV antibodies over 36 months for two example herds both starting with a negative test. The herd to the left has no purchases, but a second bulk tank milk test indicated by a dashed arrow, whereas the herd to the right has purchased livestock on several occasions indicated by solid arrows. Calculations were based on BTM antibody testing, herd location and animal movement data during the period January 2013–December 2016. *PostPFree* was updated every three months.

the tests, but gradually decreased with time. It is intuitive that the confidence of freedom from infection will decrease with time since sampling, as long as there is a risk of introduction. The advantage of our approach is that it offers a quantification of this decrease in confidence, through the regularly updated *PostPFree* resulting in herd specific slopes over time based on purchase of livestock, location of the herd and herd size (Fig. 3). Based on our calculations, the effect of the local factor was small compared to the effect of purchasing livestock, which had a large impact on the probability of freedom. Large differences in *PostPFree* were observed in the study herds at the end of the study period, depending on to which extent the individual herd had purchased animals. When herds that were test negative in 2013 were retested in 2016, 29% had changed antibody status to positive, and even though this proportion was likely lower after only one year (when retesting is required), this indicated that, in many cases, inferring a herd's current status from an old BTM sample is problematic. Because most herds in the present study were not retested until 2016, a validation before this point was not possible. Consequently, we could only assess the overall performance of the method across three years, and not assess any variations between years. If implemented in the ongoing control program a continuous evaluation of the tool would be advisable so that adjustments can be made accordingly.

The estimated *PIntroLocal* was smaller for BCV than for BRSV. This was expected, as previous studies have indicated that the relative importance of purchase of livestock is higher for BCV than for BRSV (Frössling et al., 2012; Toftaker et al., 2016). The reason for the low estimated *PIntroLocal* was that few of the initially negative, closed herds seroconverted during the study period, 27% for BRSV and 18% for BCV. When herds purchasing animals also were included, 29% of the previously negative herds changed status to positive for each of the viruses. This is within the same range as in a Swedish study where between 11.1 and 66.7% of different categories of study herds went from BCV antibody negative to positive during a three-year period, when herd classification where based on pooled samples of primiparous cows (Ohlson et al., 2013). Only two herds had a negative BTM test. Even though the total study period was the same as in the current study, some herds did not become negative until after the study had started, thus the time at risk for each herd differed. Compared to our results for BRSV, Klem

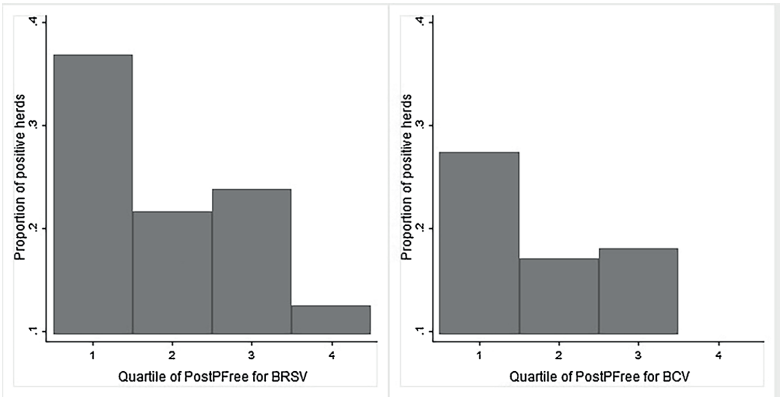

Fig. 4. Proportion of test positive herds in each quartile of the herd level probability of freedom (*PostPFree*) from BRSV- (left panel, n = 676) and BCV (right panel, n = 333) antibodies in the last of 12 (three month) time periods in dairy herds located in two counties in western Norway.

et al. (2013) found a considerably higher introduction rate (42%) over a period of only six months in a previous Norwegian study. However, the latter study differed from the present in two important aspects; it used a random sample of herds from the national dairy population, and herd classification was based on serum samples from a group of young stock. The difference in introduction rates could therefore be due to regional differences in disease occurrence and dynamics, and/or it might reflect that BTM negative herds represent a low risk stratum of the population. A negative BTM test means that the herd has likely been free from circulating virus for a long time, as animals continue to produce antibodies several years after infection (Alenius et al., 1991; Tråvén et al., 2001; Klem et al., 2014). If a herd has managed to stay free from infection for many years, it might have certain characteristics that makes it likely to remain free. *PIntroLocal* was used as a parameter for transmission through other routes than officially recorded animal movements. Indirect transfer via fomites is likely the most important factor, however, direct animal contact is possible e.g. on shared pastures, or if animals are temporarily moved (without change in ownership).

The estimation of *PIntroLocal* in the present study was based on a small sample size, and support from literature was scarce. The sensitivity analysis showed that the change in output (*PostPFree*) was moderate when *PIntroLocal* was increased or decreased with 50%, suggesting that if the true rate of local transmission is very different from the estimated local factor, this could affect the predictive ability of the model. It seems likely that local differences in prevalence and geographically dependent risk factors such as herd density might cause important differences in *PIntroLocal.* Differences in the importance of local transmission should therefore be investigated for different regions if the presented framework is to be applied at a national scale.

Currently, the control program is moving towards classification of herds based on pooled individual serum or milk samples, but these test results were not yet available for research purposes. The presented framework could be extended to encompass herd classification based on individual samples. This would include estimation of herd Se for the different types of sampling strategies, as described by More et al. (2013) for Johne's disease in Ireland and recently by Ågren (2017) for Salmonella surveillance in Sweden. When individual samples are used for herd classification the time span reflected by a positive test, in terms of time of exposure to virus, is shorter compared to using BTM. The length of the time span will depend on the age of the tested animals, i.e. young stock will reflect a shorter time period than primiparous cows. There might also be differences between categories of herds based on other factors, such as biosecurity level, production type, and herd size. The herd size in the study region is smaller than the national average (Anonymous, 2017), hence herds categorized as "large" in the present study, are small even in a Norwegian context. A different cut-off between large and small herds, or more categories of herd size might be appropriate for application at a larger scale.

As mentioned, *PostPFree* relates to presence of antibodies and not necessarily presence of virus. Ideally, one would prefer to use a test detecting the antigen itself in order to achieve a herd's true infection status; however, this is demanding to do on a large scale, and antibody

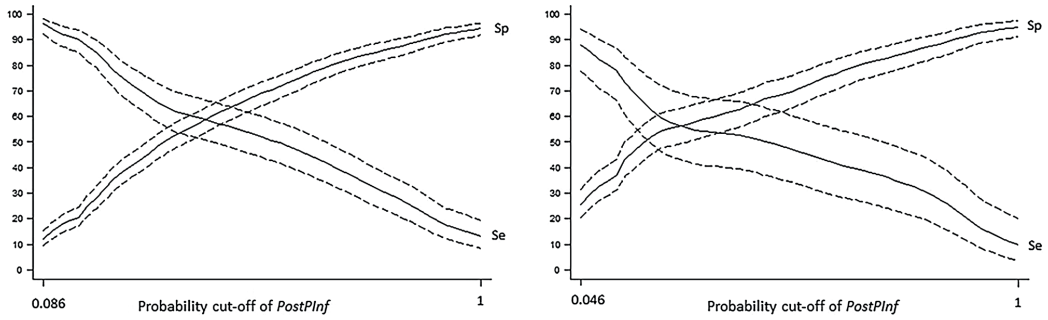

Fig. 5. Relative sensitivity and specificity of *PostPInf* (the probability of antibody positivity) for BRSV (left panel, n = 569) and BCV (right panel, n = 270)) in the last three month time period before subsequent bulk tank milk (BTM) testing versus cut-off value, when the subsequent BTM antibody-test was used as gold standard. Estimation of *PostPInf* was based on BTM antibody testing, herd location and animal movement data during the period January 2013–December 2016.

testing is commonly used (Hägglund et al., 2006; Beaudeau et al., 2010; Ohlson et al., 2010a). Animal purchase might mean introducing an antibody positive animal and not necessarily introducing virus. As Norwegian herds are small the purchase of a single antibody positive lactating cow will likely suffice to produce a positive BTM test. Because we used BTM testing as the "gold standard" the herd would be classified as a "true positive" in the validation. Altogether, it is important to keep in mind that serologic classification in general as well as the output of our model (*PostPFree*) likely overestimates the proportion of herds in which there is actual virus circulation. Therefore, the estimated *PostPFree* from antibodies is likely lower than the true probability of freedom from circulating virus. However, the consequences of a positive test result in the control program is the same, regardless of why there are test-positive animals in a herd.

In the present study, the within-herd prevalence was set to 0.5 (50%) for all source herds. There are likely variations in within-herd prevalences depending on time since outbreak, and an increase in seroprevalence with age has previously been shown (Bidokhti et al., 2009). In a previous Norwegian study, Klem et al. (2013) reported a mean within-herd prevalence for BRSV of 55% based on serology of young stock (> 6 months age), and Gulliksen et al. (2009) found a mean within-herd prevalence of 50% and 39% for BCV and BRSV, respectively, at calf level when calves with maternal antibodies were included. Studies sampling across age groups are lacking, hence the validity of the assumption of a 50% prevalence is hard to asses. Ideally, studies investigating the range of within-herd-prevalences should be performed.

The prior probability of infection, *PriorPInf,* was set to 0.5 for the first time period. This is a conservative estimate as it assumes no useful prior information about infection status (Martin et al., 2007b). However, the high Se of the BTM antibody tests will entail a high probability of freedom immediately after testing even if the prior probability is low. The model is therefore robust regarding choice of prior in this case.

Fixed values were used for all parameters in the present study. A stochastic approach is possible, and could potentially capture some of the uncertainty in the probability of disease. However, the aim of the present study was not to simulate disease spread, but to introduce a herd-specific measure as a decision support tool in the ongoing control program.

The model evaluation suggested that *PostPFree* is a useful tool for updated herd probability of freedom. When comparing *PostPFree* to the BTM result from 2016 (Wilcoxon Rank Sum Test), there was significant difference ($p < 0.01$) between groups for both models (BRSV and BCV), suggesting a benefit of using *PostPFree* instead of relying on the previous BTM result alone. Another indication of the usefulness of *PostPFree* was the clear differences in proportion of test positive herds between the first and fourth quartile of *PostPFree* as shown in Fig. 4. When assessing *PostPInf* as a diagnostic test we showed how many herds would be correctly classified at different cut-off values of *PostPInf*. In a practical setting, this is equal to the expected proportion of true positive herds that is detected if retesting is recommended at a certain value of *PostPInf* (*PostPFree*). If used on close to real time data, one could decide on a cut-off, and have an alarm when *PostPFree* drops below this value. This could enable timely intervention and a more risk-based approach to sampling and re-testing of herds. The relative Se (cut-off *PostPInf* > 0.25) was lower for BCV than for BRSV suggesting that a more stringent cut-off might be appropriate for BCV if used for targeted sampling. In addition to test strategy purposes, the *PostPFree* could be used to classify herds in more than two categories, thus providing a more updated input for risk assessment prior to livestock purchase.

In conclusion, estimation of the probability of freedom for individual herds over time, based on the framework presented in this study, gave considerable variation in values among study herds even when they had equal starting points, i.e. negative test results. Validation against subsequent BTM sampling indicated a benefit of using *PostPFree* for an updated probability of a herd's antibody status instead of relying solely on a previous BTM test result.

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