Epidemiology and Molecular Tracing of Bovine Coronavirus and Bovine Respiratory Syncytial Virus Infections in Cattle Herds

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Cover: A cute French Charolais heifer in a pretty pasture located in Härkeberga, Uppland county of Sweden, is curiously looking at the author while taking photo of her. The Author took this photo while visiting Härkeberga landscape and church with his nice SLU friends on 2012-Oct-06. The author's shadow with an Indiana Jones hat (a sweet American present from Erin & Kevin) is also present in the photo. Schematic shapes of BCoV and BRSV drawn by the author are then added to the cover photo. All right and permission are reserved by the author and his friends.

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Abstract

Bovine coronavirus (BCoV) and bovine respiratory syncytial virus (BRSV) are highly contagious pathogens that cause respiratory disease in cattle worldwide. BCoV is also associated with enteric disease in cattle of all ages. The aim of this thesis was to gain serological and molecular knowledge of BCoV and BRSV infections to help establish efficient future control in Sweden.

In the first study, the prevalence of antibodies to BCoV and BRSV infections was studied in 20 conventional and 20 organic dairy herds in south-east Sweden. On two occasions, with a 1-year interval, 699 serum samples from 624 cows were tested by ELISA. The antibody prevalence to BCoV and BRSV was high (> 80%) at both sampling times. Conventional herds had a significantly higher seroprevalence than the organic herds ($P < 0.01$). There was a significant association ($p < 0.001$) of antibody prevalence and age of the cow, where titres were higher in older individuals. The findings in this study suggest that organic farm management routines may be effective in reducing the seroprevalence of these viruses.

The second study was conducted on the molecular tracing of BCoV and BRSV throughout Sweden and also evolution of *Betacoronavirus1*. To investigate the molecular epidemiology of BCoV, the spike (S) gene of 27 PCR- positive samples from 2005 to 2009 were sequenced from 25 cattle herds. For BRSV, the glycoprotein (G) gene of PCR- positive samples during four years (2007 to 2011) were sequenced from 30 cattle herds. Sequence analysis revealed a high degree of identity among Swedish strains (> 97.7% for BCoV, > 94.5% for BRSV) regardless of clinical signs or animal age. Circulation of BCoV and BRSV between herds was found to occur throughout the year most often during the winter period. Identical sequences found in herds sampled within a few months' time suggested that these herds were part of a common transmission chain. Evolution analysis of the S gene of *Betacoronavirus1* obtained since 1965 implies that BCoV strains are evolving genetically close to their human and canine counterparts. This study suggests that molecular analysis of strains can be a useful tool to support or rule out suspected transmission routes. Such knowledge is essential for the control of the spread of BCoV and BRSV between herds, regions and even countries.

Keywords: bovine coronavirus, spike gene, bovine respiratory syncytial virus, glycoprotein gene, epidemiology, organic, cattle herd, risk factors, molecular tracing, evolution.

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Dedication

To my Family with Best Love Ever

And with special loyalty to Zakariya Razi
(Physician and philosopher, 26August 865 – 925)

"His writings on smallpox and measles show originality and accuracy, and his essay on infectious diseases was the first scientific treatise on the subject."
WHO bulletin, May 1970 about Zakariya Razi

It is not the strongest of the species that survives, nor the most intelligent that survives. It is the one that is the most adaptable to change.
Charles Darwin

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List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Bidokhti, M.R., Tråvén, M., Fall, N., Emanuelson, U. & Alenius, S. (2009). Reduced likelihood of bovine coronavirus and bovine respiratory syncytial virus infection on organic compared to conventional dairy farms. *Veterinary Journal* 182(3), 436-40.

II Bidokhti, M.R., Tråvén, M., Ohlson, A., Baule, C., Hakhverdyan, M., Belák, S., Liu, L. & Alenius, S. (2012). Tracing the transmission of bovine coronavirus infections in cattle herds based on S gene diversity. *Veterinary Journal* 193(2), 386-90.

III Bidokhti, M.R., Tråvén, M., Ohlson, A., Zarnegar, B., Baule, C., Belák, S., Alenius, S. & Liu, L. (2012). Phylogenetic analysis of bovine respiratory syncytial viruses from recent outbreaks in feedlot and dairy cattle herds. *Archives of Virology* 157(4), 601-7.

IV Bidokhti, M.R., Tråvén, M., Krishna N. K., Munir, M., Belák, S., Alenius, S., Cortey, M. Evolutionary Dynamics of Bovine Coronaviruses: Natural Selection Pattern of the Spike Gene Implies Adaptive Evolution of the Strains. (manuscript).

Abbreviations

aa	Amino acid
BCoV	Bovine coronavirus
BRD	Bovine respiratory disease
BRSV	Bovine respiratory syncytial virus
BToV	Bovine torovirus
BVDV	Bovine viral diarrhoea virus
CD	Calf diarrhoea
CoVs	Coronaviruses
CRCoV	Canine respiratory coronavirus
E	Envelope protein gene
ECoV	Equine coronavirus
ELISA	Enzyme-linked immunosorbant assay
EM	Electron microscopy
F	Fusion protein
G	Glycoprotein
HCoV-OC43	Human coronavirus OC43
HE	Hemagglutinin-esterase
HECV-4408/US/94	BCoV-like human enteric coronavirus – 4408/US/94
HI	Haemagglutination inhibition
HRSV	Human respiratory syncytial virus
HRT-18	Human rectal tumor type 18 cell line
ICTV	International Committee for Taxonomy of Viruses
IFA	Immunofluorescent antibody
ISCOM	immunestimulating complex
kb	Kilobase
L	Large protein
M	Membrane glycoprotein gene
M2	Matrix gene
MAbs	Monoclonal antibodies
MDBK	Madin-Darby bovine kidney
MHC	Major histocompatibility complex
mRNA	Messenger RNA

N	Nucleocapsid phosphoprotein gene
N- PCR	Nested - PCR
NADRS	National animal disease recording system
Neu5,9Ac2	5-N-acetyl-9-O-acetylneuraminic acid
NGS	Next generation sequencing
NS1 and NS2	Nonstructural proteins
nt	Nucleotide
P	Phosphoprotein
PHEV	Porcine hemagglutinating encephalomyelitis virus
RdRp	RNA-dependent RNA polymerase
RSV	Respiratory syncytial virus
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase – polymerase chain reaction
S	Spike glycoprotein gene
SARS-CoV	Severe acute respiratory syndrome CoV
SH	Small hydrophobic protein
SN- PCR	Semi nested - PCR
SOMRS	Swedish official milk recording scheme
tMRCA	The most recent common ancestor
VNT	Virus neutralization test
WD	Winter dysentery

1 Introduction

Bovine coronavirus (BCoV) and Bovine respiratory syncytial virus (BRSV) have emerged as worldwide viral pathogens that annually cause enormous economic losses in dairy and beef herds. BCoV infection manifests as diarrhoea and respiratory tract symptoms with varying degrees of severity, while infection with BRSV demonstrates as respiratory disorders. Both infections in severe cases can cause death (Saif, 2010; Larsen, 2000; Baker *et al.*, 1997; Clark, 1993). These viruses are of major causative agents of the bovine respiratory disease (BRD) complex. BRD is a major welfare and economic burden affecting both beef and dairy cattle, and costing around a billion dollars per annum in the United States (Brodersen, 2010; Smith, 2009; Snowder *et al.*, 2006). Similar to Sweden, recent studies have proposed these two viral pathogens as the most costly infections in Norwegian cattle (Stokstad, 2013; Gulliksen *et al.*, 2009a) after successfully eradication of several other infectious diseases from the cattle population (Norwegian Veterinary Institute, 2011). Vaccines against BCoV and BRSV are used around the world, but their efficacy has been questioned (Fulton *et al.*, 2013; Windeyer *et al.*, 2012; Saif, 2010; Larsen, 2000).

Like most other countries, Swedish cattle farms are immensely infected with these two highly contagious pathogens, leading to the thought that control of these infections among most of the herds is almost impossible. However, evidence coming from recent studies shows the possibility to have cattle herds free of BCoV and BRSV (Ohlson *et al.*, 2013; Bidokhti, 2008). Biosecurity, herd management and rapid diagnosis of these infections during outbreaks are thought to play essential roles in improving the control strategies. The key to understanding the evolution and epidemiology of BCoV and BRSV, and hence central to our efforts to control these viruses, is determining the role played by antigenic variation. Such studies would provide worthwhile information about genetic diversity of the viral strains and their circulation pattern among the herds.

The overall aim of this doctoral research was to obtain serological and molecular knowledge about BCoV and BRSV infections, in order to help the establishment of efficient control measures in the future. In the first study, we assessed individual and herd risk factors which may affect seroprevalence of BCoV and BRSV in a south-eastern region of Sweden (paper I). In the second study, we studied molecular tracing of BCoV (paper II) and BRSV (paper III) in the herds throughout Sweden during recent years. Additionally, we studied evolution of BCoV strains and their relationship with other virus species in *Betacoronavirus1* obtained since 1965 (manuscript IV).

2 Background

2.1 Cattle rearing in Sweden

The number of dairy cows in Sweden is continuing to decrease, whereas herd size is increasing. The number of beef cows has stabilized after a large increase during the first half of the 1990s (Swedish National Veterinary Institute, 2012). In 2011 there were roughly 1.5 million cattle in Sweden, 347000 of which were dairy cows and 196000 beef cows (Swedish board of Agriculture, 2012). Approximately, 5300 dairy herds, with average herd size of 65 cows, and 12000 herds, with average herd size of 17 beef cows, were distributed throughout the country. There is a stable season and an outdoor season during a year; all adult cattle should be kept on pasture during summer (2–4 months, depending on geographical location). Herd density varies a lot in the country; it is higher in the southern regions of Sweden and lower in the north. The main counties for cattle farming are in southern Sweden; including Västra Götaland, Kalmar, Skåne, Jönköping, Halland and Östergötland.

Farm data including individual treatments, vaccinations and diagnosis for all production animals are compulsorily reported by veterinarians to the Swedish board of agriculture through the national animal disease recording system (NADRS) (Emanuelson, 1988). Over 90% of Swedish dairy cows are also registered in the Swedish official milk recording scheme (SOMRS) where individual data on production and reproduction are constantly recorded (Wolff *et al.*, 2012; Mörk *et al.*, 2010).

Scandinavia has chosen an approach that is both unusual and successful to deal with infections through control programs in closed herds as an alternative to comprehensive use of antibiotics and mass vaccinations of cattle. This has resulted in infectious bovine rhinotracheitis virus and bovine leukaemia virus being cleared from the Scandinavian herds and currently more than 99.8% of the cattle herds in Sweden are free from bovine viral diarrhoea virus (BVDV)

(Swedish National Veterinary Institute, 2012). The BVDV control has been successfully performed according to the rules of the national eradication program (Lindberg & Alenius, 1999).

2.1.1 Organic farming

The first organic farms in the Nordic countries were established during the 1930s and practiced biodynamic methods (Lund & Algers, 2003). During the 1960's and 70's, when the ideas of organic agriculture were highlighted, the organic movement provided ideas of more natural and animal friendly ways of livestock production (Padel *et al.*, 2004). In 1999, the first EU- regulations on organic livestock production were agreed upon, where the principle of prevention rather than treatment is emphasized (The Council Of The European Communities, 1999). The aims of organic dairy farming are to produce milk and meat based on good animal health and welfare and also reducing the use of antibiotics. Major approaches to fulfil these aims are to improve immunity and resistance to disease through appropriate nutrition, breeding and reduction of stress by allowing the animals to express a natural behaviour. Likewise, the organic farming aims at reducing disease prevalence by limiting mixing of animals from different herds, reducing animal density, implementing good hygiene and biosecurity to prevent infections (KRAV, 2012). In 2007, 7.5 % of the Swedish dairy cows were organically managed and this number has increased reaching 14% in 2011 (KRAV, 2012; Svensk Mjölk, 2012). In several previous studies animal health has been assessed in Swedish organic dairy herds, measured as udder health, reproductive performance, metabolic profiles, and longevity of dairy cows, not demonstrating any important differences compared to conventional farming (Blanco-Penedo *et al.*, 2012; Fall & Emanuelson, 2009; Fall *et al.*, 2008a). However, no previous studies focused on whether organic management (with less animal movements) would reduce the prevalence of viral infections such as BCoV and BRSV.

2.2 Bovine coronavirus

2.2.1 Virus

BCoV is an enveloped virus belonging to the *Coronaviridae* family, order *Nidovirales* (de Groot *et al.*, 2012; Cavanagh, 1997). Like other coronaviruses (CoVs), BCoV possesses the largest contagious ribonucleic acid (RNA) genome among RNA viruses, with an approximate size of 31 kilobase (kb). The viral genome includes 13 open reading frames. Its positive-sense, single-stranded, non-segmented RNA acts as a messenger RNA (mRNA) in infected cells and directly exploits the cell protein machinery to produce viral proteins

(Saif, 2004; Clark, 1993; Almeida *et al.*, 1968). The coronavirus particles are helical in symmetry, about 120 nm in diameter. Their pleomorphic to spherical shape with prominent surface projections is just like the corona of the sun (Lai, 1990). A schematic illustration of the BCoV particle and its components are presented in Figure 1 A. The envelope is derived from the plasma membrane of the host cell and it is essential for infectivity of the virus. The genome contains five major structural protein genes including a nucleocapsid (N) phosphoprotein gene, a transmembrane (M) glycoprotein gene, a spike (S) glycoprotein gene, an envelope (E) protein gene, and an envelope-associated hemagglutinin-esterase (HE) glycoprotein gene (Figure 1 B) (Lai & Cavanagh, 1997; Saif, 1993; King & Brian, 1982).

The classification of CoVs is being modified. The latest proposal of the International Committee for Taxonomy of Viruses (ICTV) has described two sub-families for *Coronaviridae*; *Coronavirinae* and *Torovirinae*, the former comprising three genera named as *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus* (de Groot *et al.*, 2012) and with a novel (but yet to be approved) genus, provisionally named *Deltacoronavirus* (Woo *et al.*, 2012). Four separate lineages (A through D), some of them encompassing multiple virus species, are commonly recognized within the genus *Betacoronavirus*. BCoV, together with human coronavirus OC43 (HCoV-OC43), equine coronavirus (ECoV) and porcine hemagglutinating encephalomyelitis virus (PHEV) belongs to the virus species *Betacoronavirus1* of the lineage A of the genus *Betacoronavirus* (de Groot *et al.*, 2012).

2.2.2 Spike protein

The large petal-shaped surface S protein of BCoV is a type 1 membrane glycoprotein of 1363 amino acids that comprises two hydrophobic regions: the N-terminus is a signal sequence and the C-terminus acts as a membrane anchor (Figure 1 C) (Parker *et al.*, 1990; St Cyr-Coats *et al.*, 1988; Stair *et al.*, 1972). For BCoV, the S protein is cleaved by an intracellular protease at a cleavage site spanning amino acid (aa) residues 763-768 (motif RRSRR). The cleavage occurs between amino acids 768 and 769 to form two functionally distinct subunit domains, S1 (N-terminal domain) and S2 (C-terminal domain) (Figure 1 C) (Abraham *et al.*, 1990). The S1 subunit is a peripheral protein which has several crucial roles: mediating virus binding to host-cell receptors (Saeki *et al.*, 1997; Kubo *et al.*, 1994), induction of neutralizing antibodies (Yoo & Deregt, 2001; Yoo *et al.*, 1991b; Deregt & Babiuk, 1987), and haemagglutinating activity (Schultze *et al.*, 1991). S2 subunit is a transmembrane protein and its main function is to fuse viral and cellular membranes (Yoo *et al.*, 1991a; Sturman *et al.*, 1985). S1 subunit sequence is

more variable compared to S2 subunit and mutations in S1 sequence often changes its antigenicity (Yoo & Deregt, 2001; Vautherot *et al.*, 1992) and possibly virus pathogenicity (Hingley *et al.*, 1994; Fazakerley *et al.*, 1992).

The receptor-binding subunit S1 of coronaviruses uses a variety of cellular receptors including proteins and sugars. BCoV and HCoV-OC43 recognize a sugar moiety, 5-N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2), on cell-surface glycoproteins or glycolipids (Kunkel & Herrler, 1993; Schultze *et al.*, 1991). The regions spanning aa residues 146-179 and 458– 531 of the S1 subunit of BCoV have been identified as hyper variable regions (Hasoksuz *et al.*, 2002; Rekik & Dea, 1994). The hypervariable nature of the surface S protein formed the basis for targeting this gene to study the epidemiology and evolution of the virus.

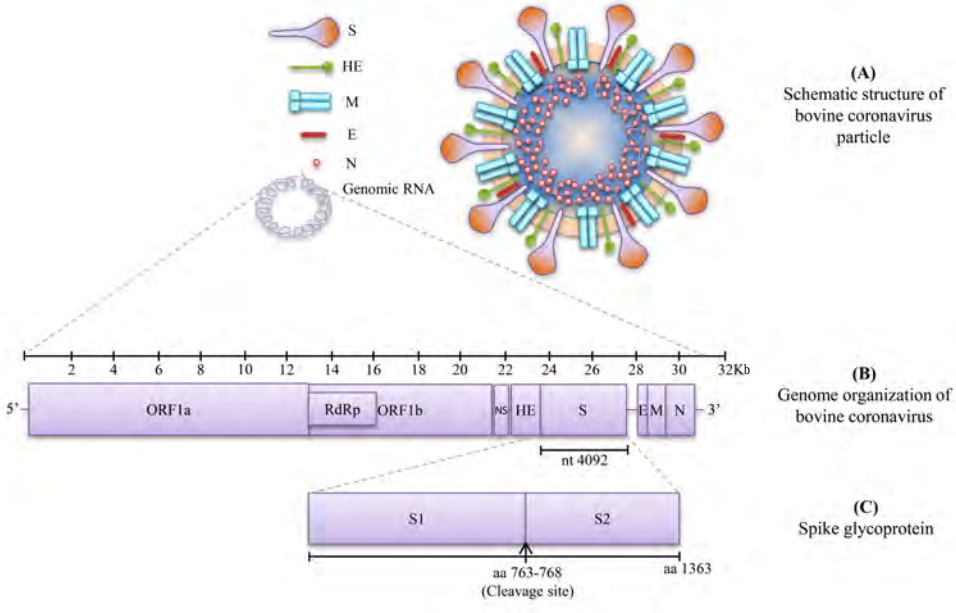

Figure 1. Schematic diagram of bovine coronavirus (BCoV) particles and its components: **(A)** A BCoV particle carrying all the structural components. **(B)** Expanded genomic RNA of BCoV illustrating characteristic of viral protein genes. Lengths are simulated based on the size of the corresponding gene. **(C)** Subunits of spike (S) glycoprotein; S1 and S2.

2.2.3 Pathogenesis

Bovine coronavirus is transmitted via the faecal-oral route through ingestion of contaminated feed or water and to a lesser extent, respiratory (aerosol) route (Thomas *et al.*, 2006; Heckert *et al.*, 1991b; Saif *et al.*, 1986). Respiratory secretions of infected animals may further enhance transmission. BCoV is shed

in mucosal secretions from the upper respiratory tract and excretions from the gastrointestinal tract (Heckert *et al.*, 1991b; Collins *et al.*, 1987).

BCoV is a pneumoenteric virus that replicates in the epithelium of the upper respiratory tract and the enterocytes of the intestinal tract (Boileau & Kapil, 2010; Park *et al.*, 2007). Researchers have proposed that replication and shedding of BCoV in nasal secretions is first initiated through the respiratory tract (oropharynx) then spreads to the gastrointestinal tract through the swallowing of large quantities of virus with subsequent shedding in the faeces (Saif, 2010; Thomas *et al.*, 2006). Inflammatory mediators cause hypersecretion in these organs. Damaged epithelial cells are replaced by immature cells, resulting in osmotic imbalance and finally malabsorptive diarrhoea (Boileau & Kapil, 2010). The haemorrhagic nature of the diarrhoea in some cases results from excess destruction of colonic cells and then transudation of extracellular fluid and blood (Tråvén *et al.*, 2001; Clark, 1993; Saif *et al.*, 1986). The infection is normally self-limiting and epithelial cells of the crypts can renew the damaged surfaces.

2.2.4 Clinical signs

BCoV infections are associated with respiratory and also enteric disorders. Replication and shedding of BCoV may start in the upper respiratory tract and spread to the gastrointestinal tract (Thomas *et al.*, 2006). However, it is still unclear whether respiratory and enteric BCoV isolates are distinctive in biological, antigenic and genetic characteristics and whether these isolates differ in their virulence and tropism for the respiratory and digestive tracts (Saif, 2010; Hasoksuz *et al.*, 2002).

The respiratory tract infections caused by BCoV occur in cattle of all ages (Cho *et al.*, 2001b; Lathrop *et al.*, 2000; Reynolds *et al.*, 1985; McNulty *et al.*, 1984). Clinical signs of respiratory disease are usually mild. Nasolacrimal discharge, rhinitis and cough are often present. These signs may accompany the diarrhoea in both calves and adults, manifesting mild respiratory symptoms (Saif, 2010). A typical respiratory outbreak of BCoV infection may last for 1-2 weeks (Saif, 2010). BCoV strains have been isolated from nasal swab samples or lungs of feedlot cattle with respiratory tract disease after shipping (Cho *et al.*, 2001b; Lathrop *et al.*, 2000).

BCoV is also a causative agent of calf diarrhoea (CD) in neonatal calves and winter dysentery (WD) in adult cattle (Alenius *et al.*, 1991; Saif *et al.*, 1988; Mebus *et al.*, 1973). The enteric infection is clinically manifested as an acute onset of fluid diarrhoea, possibly mixed with blood and unpleasant odor (Clark, 1993; Alenius *et al.*, 1991; Saif *et al.*, 1988). The incubation period is 2–7 days, and the diarrhoea commonly lasts for 3–6 days or even longer.

Calves with CD are usually aged from 1 day to 3 months (Tråvén *et al.*, 2001). Adult lactating dairy cows with WD show a marked drop in milk yield which usually lasts for up to 2 weeks (Takiuchi *et al.*, 2008; Saif *et al.*, 1988) or even months (Østerås, 2012). In general, calves show more severe symptoms than dairy cows. Other signs include mild colic, dehydration, depression, moderate fever, anorexia, and loss of body condition.

BCoV infection has a high morbidity but usually a low mortality (Saif, 2010; Alenius *et al.*, 1991); it is usually spread to all susceptible animals of a herd within a short time period (Hägglund *et al.*, 2006; Niskanen *et al.*, 2002). Treatment is usually not needed unless in severe cases to rehydrate and rebalance electrolytes (Tråvén *et al.*, 2001; Clark, 1993).

2.2.5 Epidemiology

BCoV is highly contagious and easily introduced to herds by visitors, carrier animals, and fomites. CoVs remain infectious at low temperatures and at low ultraviolet light intensities, which can shield the virus in the environment during the colder seasons. Stress factors such as changes in diet, parturition, close confinement, lactation, cold weather, shipping, presence of other microorganisms, and wide fluctuations in temperature are defined as risk factors in initiating the disease. Large herds with a history of an outbreak are at high risk for reinfection (Smith *et al.*, 1998; Clark, 1993; Saif *et al.*, 1986). Therefore, mentioned factors lead BCoV infection to be widespread with high seroprevalence among dairy and beef herds. Viral shedding in clinically healthy cows was also shown in some studies (Collins *et al.*, 1988; Crouch *et al.*, 1985). But new introduction of the virus more likely contributes to the infection pattern rather than latency or carrier animals (Liu *et al.*, 2006).

Bovine-like CoVs have also been isolated from several domestic and wild ruminants thus far including buffalo (Decaro *et al.*, 2008c), elk (Majhdi *et al.*, 1997), giraffe (Hasoksuz *et al.*, 2007), alpaca (Jin *et al.*, 2007), deer, antelope, waterbuck (Alekseev *et al.*, 2008) and recently zoo animals including wisent, sitatunga, tahr and nyala (Chung *et al.*, 2011). Their ability to infect gnotobiotic calves (Tsunemitsu *et al.*, 1995) and their close genetic relationship to BCoVs (Alekseev *et al.*, 2008) have suggested potential reservoirs for the cattle infection.

After the 1970s, when BCoV was recognized as a causative agent for CD (Stair *et al.*, 1972), studies were done during the 1980s to find out its association with WD (Espinasse *et al.*, 1982; Takahashi *et al.*, 1980). Serological evidence confirmed these results and it emerged later that this virus is the causative agent of WD (Alenius *et al.*, 1991; Emanuelson *et al.*, 1989).

The first report of WD came from the USA in 1915 (Jones & Little, 1931), and was then followed by reports from many countries. In Sweden, the first outbreak was reported in 1946 and then by March 1948 WD was recognized as endemic disease spread overall the country (Hedström & Isaksson, 1951).

Today BCoV infection seems to be endemically present in cattle on all continents (Hick *et al.*, 2012; Brandao *et al.*, 2006; Khalili & Morshedi, 2006; Valle *et al.*, 2006; Hasoksuz *et al.*, 2005; Jeong *et al.*, 2005; Paton *et al.*, 1998; White *et al.*, 1989), as well as in the Scandinavian countries (Ohlson *et al.*, 2010a; Gulliksen *et al.*, 2009a; Hägglund *et al.*, 2006; Härtel *et al.*, 2004; Tegtmeier *et al.*, 1999). Swedish studies have shown high prevalence of this endemic infection among beef and dairy herds, causing annual outbreaks (Beaudeau *et al.*, 2010a; Ohlson, 2010; Bidokhti, 2008; Hägglund *et al.*, 2007; Tråvén *et al.*, 1999).

2.2.6 Immunology

The HE and S glycoproteins are recognized first by the bovine immune system while the N protein induces antibody responses during the later stage of initial infection and the early stage of reinfection. The M glycoprotein is the least immunogenic of the major viral structural proteins (Lin *et al.*, 2000).

BCoV-specific IgM increases from day 2-7 and remains detectable during 3-6 weeks, as measured by isotype capture ELISA after an experimental infection of seronegative animals. IgA can be detected from day 7-9 and during several months. BCoV-specific IgG is also detectable from day 9-11 in serum and milk (Tråvén *et al.*, 2001). The IgG may remain detectable for at least one year after the infection even without reinfection (Alenius *et al.*, 1991).

The efficiency and duration of a naturally-acquired immunity is unknown (Saif, 2010). This immunity is inefficient and the infection is still recurrent (Saif, 2010; Clark, 1993). Antibodies are passed to the offspring via the colostrum; these are detectable in the sera until approximately 5–6 months of age. The passively derived antibodies decrease or delay the active antibody response to infection in calves (Heckert *et al.*, 1991a).

2.2.7 Detection

BCoV, as a major causative agent of cattle diarrhoea should be distinguished from other enteric viruses, including its counterpart bovine torovirus (BToV) (Aita *et al.*, 2012). BCoV is difficult to isolate in the cell culture; however, the human rectal tumor type 18 (HRT-18) has been the most commonly used cell line for such purpose (Tsunemitsu *et al.*, 1991; Benfield & Saif, 1990). The viral particles can also be detected in field samples using direct or immune electron microscopy (EM) (Heckert *et al.*, 1989; Saif *et al.*, 1986).

Several nucleic acid based detection methods have also been routinely used to detect viral genes in nasal or bronchoalveolar lavage and faecal specimens. These methods also make it possible to study molecular epidemiology of viral infections. For such reasons different kinds of reverse transcriptase – polymerase chain reaction (RT-PCR); including one step-, nested (N-) or semi nested (SN-) PCR have been developed (Cho *et al.*, 2001a). These can target either hypervariable regions of BCoV genome for phylogenetic analysis (Martinez *et al.*, 2012; Liu *et al.*, 2006; Park *et al.*, 2006) or conserved regions for detection purpose (Jeong *et al.*, 2005; Hasoksuz *et al.*, 2002). Real-time RT-PCR technique could also provide a fast, sensitive and less laborious detection method for BCoV infection in field and cell culture samples (Decaro *et al.*, 2008a; Escutenaire *et al.*, 2007).

Immunological methods for detecting the antibodies to BCoV in serum samples have also been developed. The virus neutralization test (VNT) and Haemagglutination inhibition (HI) have been used to quantitate antibodies to BCoV and to compare antigenic variation between strains (Fulton *et al.*, 2013; Decaro *et al.*, 2008b). Likewise, an enzyme-linked immunosorbant assay (ELISA) has been developed with reliable results for mass monitoring of cattle population in serological surveys. It can detect the antibodies against BCoV in milk and serum samples (Ohlson *et al.*, 2013; Näslund *et al.*, 2000; Alenius *et al.*, 1991).

2.2.8 Evolution

Coronaviruses lack proof-reading activity of the RNA-dependent RNA polymerase (RdRp) which causes low fidelity of RNA replication machinery. This results in a high mutation rate of about one mutation per genome per replication round (Moya *et al.*, 2004; Drake & Holland, 1999). In addition, a genetic recombination mechanism (Woo *et al.*, 2009; Zhang *et al.*, 2005; Keck *et al.*, 1987) provides broad genomic diversity among CoVs (Smith & Denison, 2012; Herrewegh *et al.*, 1998; Chang *et al.*, 1996). Thus, over a short time period, a new variant may replace the old one and become the dominant virus that better adapts to its environment and even host (Woo *et al.*, 2009).

Studies are on-going to understand the genetic diversity of CoVs. Recently, bats were recognized as ideal hosts for the *Betacoronavirus* gene source (Woo *et al.*, 2012). The mean evolutionary rate estimate of the RdRp gene in CoVs is 1.3×10^{-4} nucleotide (nt) substitutions per site per year (Woo *et al.*, 2012). Molecular clock analysis using RdRp gene showed that the most recent common ancestor (tMRCA) of all CoVs was estimated at ~8100 BC, and that of *Betacoronavirus* at ~3300 BC (Woo *et al.*, 2012). The mean evolutionary rate of PHEV, BCoV, and HCoV-OC43 was estimated at 6.1×10^{-4} nucleotide

substitutions per site per year for S gene (Vijgen *et al.*, 2006). Molecular clock analysis using S gene showed that tMRCA of PHEV, BCoV, and HCoV-OC43 was estimated at 1777 (Vijgen *et al.*, 2006). This study also estimated the mean evolutionary rate of N gene in PHEV, BCoV, and HCoV-OC43 to be 3.6×10^{-4} nucleotide substitutions per site per year. Molecular clock analysis using N gene in PHEV, BCoV, and HCoV-OC43 estimated tMRCA at 1878 (Vijgen *et al.*, 2006). Molecular evolutionary analysis on HCoV-OC43 isolates suggested BCoV as their genetically closest counterpart compared to the other species (Vijgen *et al.*, 2006). A recently isolated canine respiratory coronavirus (CRCoV) has also shown a high genetic similarity to *Betacoronavirus1* (Erles et al., 2007).

BCoV-like CoVs transmissible to gnotobiotic calves have been found among various wild ruminants (Alekseev *et al.*, 2008; Tsunemitsu *et al.*, 1995). The public health impact of BCoVs has also been raised due to the isolation of a BCoV-like human enteric coronavirus – 4408/US/94 (HECV-4408/US/94) from a child with acute diarrhoea (Zhang *et al.*, 1994), and also the outbreaks of severe acute respiratory syndrome CoV (SARS-CoV) (Zhong & Wong, 2004; Groneberg *et al.*, 2003). Indeed, a novel coronavirus, HCoV-EMC, was recently found which has been circulating in the Middle East and caused death with similar clinical signs to SARS-CoV (Al-Ahdal *et al.*, 2012; Zaki *et al.*, 2012). Such veterinary and public health impacts thus reinforce studying evolution of BCoV strains circulating in cattle populations.

The possible mechanisms by which BCoV can evade the immune response and adapt itself to new host environments are still not completely known. A detailed understanding of the evolutionary mechanisms exploited by BCoV to mediate such evasion and adaptation will be useful in predicting possible changes in virulence. This will thus be crucial in vaccine development and control programs.

2.3 Bovine respiratory syncytial virus

2.3.1 Virus

BRSV, together with human respiratory syncytial virus (HRSV) and murine pneumonia virus is an enveloped RNA virus classified in the *Pneumovirus* genus of the *Paramyxoviridae* family, subfamily *Pneumovirinae*, order *Mononegavirales* (Wang *et al.*, 2012; Van der Poel *et al.*, 1994). An illustration of the BRSV particle and its components is presented in Figure 2 A. The viral particles possess a non-segmented, single-stranded, negative-sense RNA genome of around 15 kb in length (Figure 2 B). The genome encodes 11 viral proteins which result from translation of 10 mRNAs. EM of BRSV

demonstrates a pleomorphic, spherical particle, 150 to 200 nm in diameter, although filamentous particles of up to 400 nm in length have been observed (Belanger *et al.*, 1988). The envelope is derived from the plasma membrane of the host cell. The name refers to the characteristic syncytia formation in cell culture, a hallmark cytopathic effect (Chanock *et al.*, 1957). Syncytia are large multi-nucleated cells resulted from fusion of respiratory syncytial virus (RSV) - infected cells with neighboring cells. The helical capsid of the viral particles contains three transmembrane glycoproteins: the large glycoprotein (G), the fusion (F) protein, and the small hydrophobic (SH) protein (Figure 2 A). These are arranged separately into spikes on the surface of the virion. The F and the G proteins play main roles in attachment and entry of host cells and syncytia formation. Other encoded proteins include two nonstructural proteins (NS1 and NS2); four RNA-associated proteins to form the ribonucleoprotein (RNP) complex, namely, the nucleoprotein (N), the phosphoprotein (P), the large (L) catalytic subunit of the viral RNA-dependent polymerase protein, the membrane (M) protein, and a transcription elongation factor (M2-1) is encoded by the first of two overlapping open reading frames of the matrix (M2) gene. The second frame of the M2 gene also encodes an RNA regulatory protein (M2-2) (Figure 2 B) (Samal *et al.*, 1993; Mallipeddi *et al.*, 1990).

2.3.2 G protein

The G protein of BRSV mediates attachment of the virus to cells. It is a major protective antigen of BRSV. The G protein is structurally different from its counterparts in other viruses of the *Paramyxoviridae* (Levine *et al.*, 1987). Its amino acid composition is not homologous to any other paramyxovirus proteins. It is a type II G protein with a signaling domain between residues 38 and 66. The G protein varies in length from 257 to 299 amino acids (Figure 2 C). It is synthesized as a 32-kDa polypeptide precursor which is extensively glycosylated with both *N*- and *O*-linked oligosaccharides and a high proportion of proline residues. These modifications result in a mature G protein of 80 to 90 kDa (Ishiguro *et al.*, 2004; Collins & Mottet, 1992; Wertz *et al.*, 1989; Lambert, 1988; Gruber & Levine, 1985). This gives the protein features similar to those of the mucinous proteins of the respiratory tract. Likewise, modulation of glycosylation may contribute to immune evasion by either abolishing G protein recognition by carbohydrate specific antibodies or by masking antigenic sites (Palomo *et al.*, 2000; Melero *et al.*, 1997). The G protein can be divided into an intracellular domain, a transmembrane domain, and a large ectodomain; the latter is comprised of two highly glycosylated mucin-like variable regions that sandwich a central conserved hydrophobic region between aa 154 to 192 (Figure 2 C) (Langedijk *et al.*, 1997; Langedijk *et al.*, 1996).

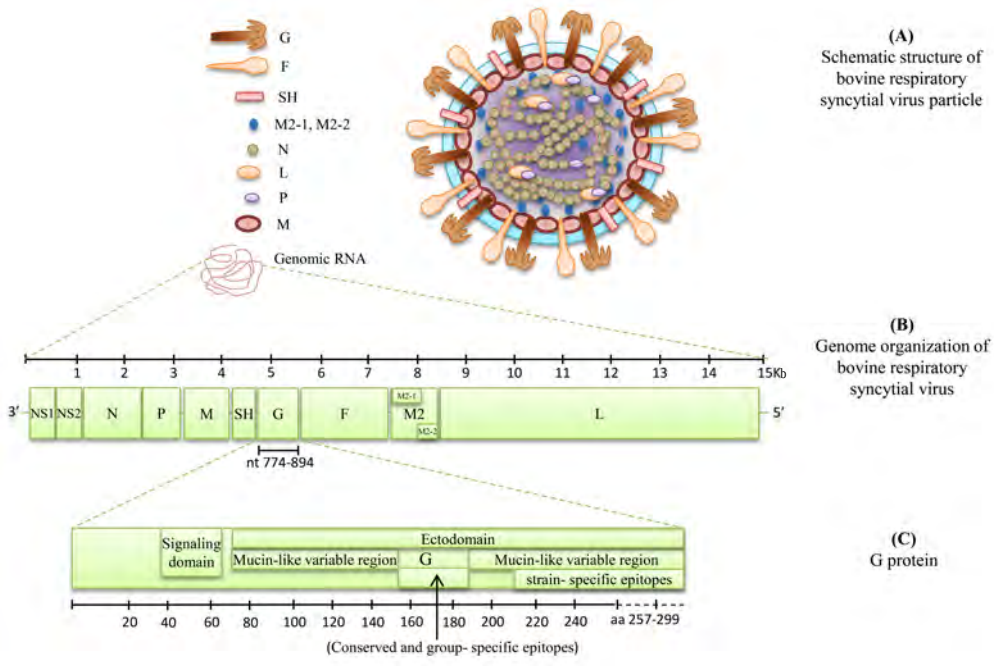

Figure 2. Schematic diagram of bovine respiratory syncytial virus (BRSV) particles and its components: **(A)** A BRSV particle carrying all the structural components. **(B)** Expanded genomic RNA of BRSV showing features of viral protein genes. Lengths are simulated based on the size of the corresponding gene. **(C)** Characteristics of G protein.

Peptides based on this region located between two polymeric mucin-like regions were found to be immunodominant in the G proteins of both HRSV (Akerlind-Stopner *et al.*, 1990) and BRSV (Langedijk *et al.*, 1996).

Antigenic variations in the major surface ectodomain of G protein may play a role in pathogenesis of BRSV infections. G protein-specific monoclonal antibodies (MAbs) (Prozzi *et al.*, 1997) and the inherent G protein genetic diversity (Elvander *et al.*, 1998) have been helpful in grouping BRSV strains. Six genetic subgroups (I-VI) have been arbitrarily assigned to represent the temporal and geographical distribution of BRSV strains (Valarcher *et al.*, 2000). Similarly, antibody reaction pattern (Furze *et al.*, 1994; Mufson *et al.*, 1985) and genetic variety (Zlateva *et al.*, 2005; Cane & Pringle, 1995; Sullender *et al.*, 1991) of the G protein have been practically exploited to group the HRSV strains. Hypervariability of surface ectodomain of the G protein makes this gene a logical choice for investigating the epidemiology and evolution of the virus strains.

2.3.3 Pathogenesis

BRSV is mainly transmitted via direct contact with respiratory secretions (Easton *et al.*, 2004; Van der Poel *et al.*, 1994). However, its airborne transmission has been experimentally confirmed (Mars et al., 1999). The viral particles of BRSV have been detected in nasal discharge and droplets up to 12 days after experimental infection (Castleman *et al.*, 1985; McNulty *et al.*, 1983; Elazhary *et al.*, 1980; Jacobs & Edington, 1975). After entry, BRSV replicates in the epithelial cells of the respiratory tract (Viuff *et al.*, 2002). Infected epithelial cells, without demonstrating cytopathic effects, mediate secretion of chemokines and cytokines, attracting inflammatory cells including neutrophils, macrophages, and lymphocytes to the airways. These immune-mediated mechanisms seem to play the major role in pathogenesis of BRSV in respiratory tract (Larsen, 2000; Kimman *et al.*, 1989). As a result, interstitial pneumonia with mucopurulent exudate and haemorrhage are usually observed in the bronchi and bronchioles particularly involving the cranio-ventral parts of the lungs combined with widespread emphysema and edema throughout the lungs (Baker *et al.*, 1997; Collins *et al.*, 1988; Bryson *et al.*, 1983). In the alveoli, the viral infection causes necrosis of type I pneumocytes and hypertrophy of type II pneumocytes (Tjornehoj *et al.*, 2003). The infection is normally self-limiting and epithelial cells can regenerate the damaged surfaces.

2.3.4 Clinical signs

BRSV is known as a major causative agent of BRD complex in calves under six months of age and dairy cattle (Larsen, 2000; Baker *et al.*, 1997; Elvander, 1996). BRSV infection, after a short incubation period of 2-5 days, causes a fever of around 40°C, inappetence, and clinical signs of upper and lower respiratory disease. These signs include coughing, abdominal breathing, nasal discharge, lung crackling sounds, and high respiratory rate (Elvander, 1996; Harrison & Pursell, 1985; Verhoeff & van Nieuwstadt, 1984). Adult dairy cows with BRSV infection experience a drop in milk production which lasts 3-5 days or even longer (Beaudeau *et al.*, 2010b). In severe cases, BRSV-infected animals can develop polypnea, dyspnea, subcutaneous emphysema, mouth-breathing, anorexia and emaciation (Elvander, 1996). However, BRSV infection often occurs in subclinical or asymptomatic form. Interestingly, and unlike HRSV infection, natural BRSV infection is often accompanied by concomitant bacterial infection (*Mannheimia haemolytica, Pasteurella multocida, and Haemophilus somnus*), resulting in what has been defined as BRD complex (Easton *et al.*, 2004). The morbidity rate in herd with clinical BRSV disease has been estimated at 30 to 50% (Larsen, 2000; Baker *et al.*,

1997). This rate could reach up to 100% in severe infections. The case mortality rate is usually as low as 3 to 5% (Baker *et al.*, 1997).

2.3.5 Epidemiology

Primary BRSV infections are highly contagious and almost exclusively observed in cows under 2 years of age. BRSV outbreaks usually occur in autumn and winter (Hägglund *et al.*, 2006; Van der Poel *et al.*, 1993). Like most enveloped viruses, BRSV is sensitive to inactivation (Larsen, 2000; Baker *et al.*, 1997). Studies on HRSV have shown that detergents for washing hands and equipment can effectively inactivate the virus and control its transmission (Hall, 2000; Contreras *et al.*, 1999). However, no study has been specifically performed for its bovine counterpart. Outbreaks have been associated with several risk factors such as high density of cattle population, cold weather, housing conditions, lactation, conception and age distribution in herds (Norström *et al.*, 2000; Baker *et al.*, 1986). Close proximity between non-infected and infected herds have suggested that airborne transmission between herds might not be of great importance and herds can still stay free from the infection despite virus circulation in the area (Ohlson *et al.*, 2010a).

BRSV was first isolated from cattle with respiratory disease in Switzerland and Japan (Inaba *et al.*, 1970; Paccaud & Jacquier, 1970). In Scandinavia, the first epizootic outbreak of respiratory disease associated with this infection was reported from Norway in the summer of 1976 and BRSV was isolated (Odegaard & Krogsrud, 1977). In Sweden, in the winter of 1988/89, BRSV was isolated during epizootics of respiratory disease in dairy herds (Elvander *et al.*, 1991). Since then, several outbreaks have been reported from not only Norway (Norström *et al.*, 2000) and Sweden (Hägglund *et al.*, 2007; Elvander, 1996), but also Finland (Autio *et al.*, 2007; Härtel *et al.*, 2004), and Denmark (Larsen *et al.*, 2000; Uttenthal *et al.*, 1996), suggesting that BRSV infection has become endemic in Scandinavian countries. Today BRSV seems to be spread worldwide and highly prevalent suggesting that the infection is endemic in most areas (Almeida *et al.*, 2005; Van der Poel *et al.*, 1994; Baker *et al.*, 1986).

2.3.6 Immunology

Colostrum-deprived calves showed BRSV-specific IgM and IgA detectable in serum and nasal secretions as early as 8 days after experimental BRSV infection, and remain detectable during 12-29 days (Kimman *et al.*, 1987). Serum IgG1 becomes apparent from day 13-17 (Schrijver *et al.*, 1996; Kimman *et al.*, 1987) and is detectable for more than 30 days (Uttenthal *et al.*, 2000) and probably for several years after a primary infection. IgG2 is detectable from

day 30-90 (Kimman *et al.*, 1987) and probably lasts at least eight months (Larsen, 2000). Maternally-derived antibodies could be detectable up to 6 months of age; these can suppress the humoral immune response (Kimman *et al.*, 1987; Baker *et al.*, 1986). Passive immunity derived from colostrum feeding decreased the severity of BRSV infections in calves (Belknap *et al.*, 1991). BRSV-specific IgE can rise up in some infected animals; this can be correlated with increased clinical symptoms (Gershwin *et al.*, 2011).

Reinfection is common since BRSV does not evoke a strong immunological memory response (Gershwin, 2012). Little or no clinical signs, however, are observed in re-infected animals (Kimman *et al.*, 1987). Reinfection associated with increased antibody levels is usually observed in younger cows, and it has thus been hypothesised that immunity increases with re-exposure to BRSV (Van der Poel *et al.*, 1995).

BRSV infection induces depletion of T cells in the respiratory tract which leads to delayed clearance of BRSV in calves (Taylor *et al.*, 1995). Thus cell-mediated immunity also plays an important role in response to BRSV (Gershwin, 2012). Polymorphisms in the genes of Th2 response in adaptive immunity influence the pathology observed in young cattle infected with BRSV (Leach *et al.*, 2012). Host genetics may thus play a role in determining the immune response of cattle to BRSV infections (Glass *et al.*, 2012).

2.3.7 Detection

BRSV should be distinguished from other respiratory viruses, including infectious bovine herpes virus 1, BVDV, parainfluenza virus 3 and BCoV. The isolation of BRSV in the cell culture is rarely performed. However, primary bovine embryonic turbinate, lung cells, hamster kidney and Madin-Darby bovine kidney (MDBK) cell lines have been used for its propagation (Spilki *et al.*, 2006; Arns *et al.*, 2003; West *et al.*, 1998). The viral particles can be detected in cell culture or field specimens using direct or immune EM (Larsen, 2000).

The most routinely used methods for BRSV-specific antibody detection in milk and serum are the virus neutralisation test and ELISA (Uttenthal *et al.*, 2000; West *et al.*, 1998; Ellis *et al.*, 1995; Elvander *et al.*, 1995). The immunofluorescent antibody (IFA) assay is also a rapid, reliable, and sensitive test for BRSV antigen detection in clinical samples (Larsen, 2000).

Several nucleic acid based methods have also been commonly used to detect BRSV genes in samples (Socha & Rola, 2011; Larsen *et al.*, 1999; Vilcek *et al.*, 1994). These methods including conventional RT-PCR also make it possible to study molecular epidemiology of BRSV strains (Socha *et al.*, 2009; Yaegashi *et al.*, 2005; Kovarcik & Valentova, 2004; Stine *et al.*, 1997).

Molecular beacon oligonucleotide probes with a reporter fluorophore have also been developed to reliably detect the BRSV genome in infected living cells (Santangelo *et al.*, 2006). From other genomic detection methods, real-time RT-PCR assays are known as the fastest and least laborious methods for BRSV gene detection in the field and in cell culture samples (Thonur *et al.*, 2012; Boxus *et al.*, 2005; Hakhverdyan *et al.*, 2005; Achenbach *et al.*, 2004).

2.3.8 Evolution

Genetic variability between RSV strains is a signature feature that may change the pathogenicity and fitness of the virus, and contribute to the viral ability to cause repeated infections and yearly outbreaks by immune system evasion (Eshaghi *et al.*, 2012; Parveen *et al.*, 2006; Larsen *et al.*, 2000; Hall *et al.*, 1991). The presence of positively selection sites, either in the entire G gene or mostly in its ectodomain, has suggested that immunological pressures may differ among RSV strains, resulting in the induction of different immune responses (Larsen *et al.*, 2000; Uttenthal *et al.*, 2000; Melero *et al.*, 1997; Stine *et al.*, 1997; Cane & Pringle, 1995; Garcia *et al.*, 1994; Sullender *et al.*, 1991). The receptor binding domain, however, may lack variability or have a very limited number of changes. This is either because of its structural–functional constraints or because it is not immunodominant (Walsh *et al.*, 1987).

Studies are on-going to analyse the genetic diversity of paramyxoviruses. Bats were recently recognized as tentative hosts at ancestral nodes to both of the major *Paramyxoviridae* subfamilies (*Paramyxovirinae* and *Pneumovirinae*) (Drexler *et al.*, 2012). The rates of evolution of the synonymous and nonsynonymous substitutions along the G protein gene were 5.2×10^{-4} and 6.4×10^{-4} nucleotide changes per year per site, respectively. For the N protein gene, the rates were 1.6×10^{-3} and 5.4×10^{-5} nucleotide changes per year per site, respectively. These rates for the F protein gene were 6.3×10^{-4} and 1.9×10^{-4}, respectively. These may indicate a continuous evolution of BRSV genes particularly in regions where vaccination is broadly used (Valarcher *et al.*, 2000). However, other risk factors such as herd size and animal breeds may also affect BRSV evolution in cattle populations. The high rate of evolutionary change in BRSV may confer selective advantage and facilitate its reinfections. It has been hypothesized that RSV genotypes that accumulate several mutations could be the precursors of new lineages due to antigenic drift resulting from selective pressure from the host immune response (Botosso *et al.*, 2009; Cane & Pringle, 1995). Continued genotyping and molecular epidemiological surveillance of BRSV is essential to further understand BRSV evolution, transmission in communities, herd health settings, changes in viral phenotype and immunogenicity.

3 Aims

The overall aim of this doctoral research was to get serological and molecular knowledge about BCoV and BRSV infections to help establish efficient control of these viruses in Sweden in the future.

The specific aims were:

- to compare the serological prevalence of BCoV and BRSV infections in dairy cows managed under organic and conventional management systems (paper I).

- to investigate potential risk factors associated with these infections at individual and herd levels (paper I).

- to study the molecular epidemiology of BCoV and BRSV infections and possibility of tracing their transmission in cattle herds (papers II and III).

- to describe the circulation pattern of BCoV and BRSV infections in Sweden annually and longitudinally (papers II, III & manuscript IV).

- to analyse the evolution of BCoV strains and determine potential receptor binding domains and selection pattern along the S gene (manuscript IV).

4 Materials and Methods

This section briefly describes sampling, data collection, and research methods used in this thesis. More detailed descriptions are provided in each individual paper (I-IV).

4.1 Risk assessment study

4.1.1 Study herds

In the first study, to investigate herd and individual risk factors that may affect the seroprevalence of BCoV and BRSV infections, 40 herds were analysed (20 organic and 20 conventional). Selected herds had more than 40 cows, were enrolled in the SOMRS and were located in the Uppland (n=15), Södermanland (n=8), Östergötland (n=14) and Småland (n=3) counties of south-east Sweden. Selected organically managed herds had produced milk in accordance with organic standards for at least 2 years previous to the study, to avoid confounding from herds in transition to organic standards. Of the 52 eligible organic farms, 24 were willing to participate and, of these, 20 were randomly selected. Of the 156 eligible conventionally managed farms, 32 agreed to participate and 20 were randomly selected. All 40 study herds were free of BVDV as defined by The National Control Program (Lindberg & Alenius, 1999). The herds were all visited on two occasions; in the spring of 2005 and 2006. All herd visits were performed by Dr. Nils Fall to study metabolic parameters in peri-parturient cows (Fall *et al.*, 2008b). Data on herds and individuals were also retrieved via the SOMRS and from farmer questionnaires at each sampling time.

4.1.2 Sampling

On each occasion sampled cows were between 7 days before their predicted calving date and 42 days after calving. If the number of such animals in a herd

was less than 12, all were sampled. If the herd contained more than 12 eligible animals, 12 were randomly selected to be included in this study using a random number table. Blood samples were drawn from the tail vein of the selected cows using 10ml Vacutainers without anticoagulant. Sample tubes were transported at room temperature within four hours after sampling to a laboratory where they were centrifuged at 2000g for 10 min. The separated sera were then stored at - 20°C until analysed.

A total of 699 serum samples were taken from 624 cows in the 40 herds. At the 2005 sampling, 169 cows were sampled on conventional farms and 169 on the organic farms. At the 2006 sampling, 189 animals were sampled in the conventional herds and 172 in the organic herds. By chance 75 cows were sampled at both sampling times to study seroconversion.

4.1.3 Antibody testing

All sera were tested using commercially available indirect ELISAs SVANOVA (Biotech) to detect BCoV- and BRSV-specific IgG. Sera were diluted 1:25 and analysis performed according to the manufacturer's instructions. Serum samples that generated a corrected optical density (COD) value of ≥ 0.2 at 450nm were regarded as positive in both BCoV and BRSV tests, whereas samples generating a value below this cut-off point were regarded as negative (Elvander *et al.*, 1995; Alenius *et al.*, 1991). Both positive and negative control sera were included in each assay. Seroconversion was defined as a negative COD value converting to a positive in paired sera.

A sensitivity of 84.6% and 94.6% was estimated for BCoV and BRSV, respectively, while a specificity of 100% was estimated for both of them, according to the manufacturer's manual. The estimates for BRSV are based on a parallel analysis with the ELISA used at the National Veterinary Institute, Sweden and the ELISA used at the Central Veterinary Laboratory, England (Elvander *et al.*, 1995). For BCoV ELISA, the estimates were obtained in comparison with a virus neutralisation test (Alenius *et al.*, 1991).

4.1.4 Statistical analysis

The Fisher's exact and Wilcoxon Rank Sum tests were used to investigate possible associations between serological status and animal and farm management details. The association between potential risk factors and seroprevalence to BCoV or BRSV was analysed using multivariable logistic regression models, with each herd sampling considered the unit of analysis. The regression parameters and their variances were estimated using the generalised estimation equation, which adjusts for clustering of the data at group level (Liang & Zeger, 1986), assuming a compound symmetry

correlation structure. The model was validated using the Pearson v2 statistic. All statistical analyses were carried out using SAS (Release 9.1, SAS Institute Inc.).

4.2 Molecular tracing and evolution study

4.2.1 Study herds

In the second study, to investigate molecular epidemiology of BCoV based on partial-length (1038 nt) S gene, herds experiencing enteric and/or respiratory case reports during outbreaks 2005 to 2009 were investigated for this infection. BCoV infection was diagnosed in 23 dairy herds and two feedlot herds located in different parts of Sweden namely Västerbotten (n=2), Uppland (n=6), Gotland (n=4), Skåne (n=2; one herd sampled two times), Halland (n=4; one herd sampled two times), Västergötland (n=1), Västmanland (n=1), Ångermanland (n=1) and Jämtland (n=6). Samples were obtained from October 2005 to May 2006 (n=5), October 2006 to May 2007 (n=5), October 2007 to May 2008 (n=11), and October 2008 to July 2009 (n=5) (Table 1 of paper II).

To study the evolution of BCoV based on full-length (4092 nt) S gene, samples from 29 herds mainly in Sweden and few in Denmark were included. This study was performed on enteric and/or respiratory case reports during outbreaks 2002 to 2010 (Table 1 of manuscript IV). The old Swedish BCoV strain which was detected in 1992 from a WD outbreak in Uppland and previously used to experimentally reproduce disease (Niskanen *et al.*, 2002; Tråvén *et al.*, 2001), was also included in this study.

To investigate molecular epidemiology of BRSV, on the other hand, herds experiencing outbreaks of respiratory disease during the time period 2007 to 2011 were screened for this infection. The BRSV infection was diagnosed in 19 dairy herds and 11 feedlot herds located in several counties of Sweden namely Västerbotten (n=4), Uppland (n=5), Södermanland (n=1), Östergötland (n=5), Jönköping (n=1), Småland (n=3), Öland (n=1), Skåne (n=1), Västergötland (n=3), Värmland (n=1), Västmanland (n=2) and Dalarna (n=3). Samples were obtained from February 2007 (n=1), January – July 2008 (n=9), February – August 2009 (n=7), December 2009 to February 2010 (n=4), and January – March 2011 (n=9) (Table 1 of paper III).

4.2.2 Sampling

Faecal and respiratory samples were collected either by local veterinarians or visiting farms. Dr. Madeleine Tråvén and Dr. Anna Ohlson collected several of the samples upon visiting farms with relevant disorders. Sampled cattle in all

herds were clinically showing respiratory and/or enteric signs. Fresh samples were then mailed or transported to a laboratory and stored at -70°C until analysed.

Two herds, N1 and M1, were sampled two times with some months apart to study intra-herd circulation pattern of the BCoV strains (Table 1 of paper II). Samples SWE/I/07-3 and SWE/I/07-4 were obtained from one herd and SWE/N/05-1 and SWE/N/05-2 from another large dairy herd (Table 1 of manuscript IV). 14 samples; 3 nasal and 11 faecal, of sequenced BCoV strains were included in both paper II and manuscript IV.

4.2.3 Sequencing and phylogenetic analysis

From clinical samples, RNA extraction using TRIzol LS reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis with random priming were performed as described previously (Liu *et al.*, 2006). PCR was then performed as described in detail in each of the papers II, III and IV (manuscript) using Taq DNA polymerase (AmpliTaq; Perkin–Elmer), Pfu Ultra DNA polymerase (Stratagene) and corresponding primer pairs. Additionally, N- and SN-PCR assays were developed in this study to increase the sensitivity of the PCR method for clinical detection. For paper II, the targeted amplicon was an initial 1038-nt region of the S protein gene of BCoV strains. For paper III, the targeted amplicon was a 603-nt gene region encoding the extracellular part of the G protein of BRSV strains. Likewise, for manuscript IV, the full-length S gene of BCoV strains with a length of 4092 nt was the target amplicon. The amplicons were then purified, further sequenced. The sequence alignment was performed with software BioEdit version 7.0.9.0 (Hall, 1999) with an engine based on the ClustalW algorithm.

Maximum likelihood analysis of aligned sequences of BCoV and BRSV strains was performed with various softwares; PHYML (Guindon & Gascuel, 2003), PAUP* ver 4.0b10 (Swofford, 2003) and MEGA 5 (Tamura *et al.*, 2011), to find the ‘‘best’’ tree by a heuristic search using the likelihood criterion. Bootstrapping of 1000 replicates was performed using neighbour-joining method. Rates of evolution and divergence times were estimated using the BEAST v.1.6.2 package (Drummond & Rambaut, 2007). The prediction of the receptor binding domain of the S protein was performed using InterProScan (Apweiler *et al.*, 2001). The existence of selective pressures along the S gene sequences was assessed using the SNAP server (Korber, 2000) and also HYPHY package (Pond *et al.*, 2005) through the Datamonkey facility (Pond & Frost, 2005).

5 Results & discussion

5.1 Risk assessment study

The seroprevalence to BCoV and BRSV at an individual level for all sampled cows was 86% (292/338) and 79% (267/338) in 2005, and 84% (304/361) and 82% (297/361) in 2006; no significant difference was observed between the two samplings (Table 2 of paper I). The results confirm endemic status of BCoV and BRSV in dairy cattle in southern Sweden (Ohlson *et al.*, 2010a; Hägglund *et al.*, 2006; Tråvén *et al.*, 1999; Elvander, 1996), as it is in most countries with intensive milk production systems (Boileau & Kapil, 2010; Larsen, 2000; Paton *et al.*, 1998).

5.1.1 Herd management related seroprevalence

The conventional herds studied in paper I had a significantly higher mean seroprevalence to BCoV and BRSV than the organic herds in both 2005 and 2006. The mean prevalence of cows positive to both BCoV and BRSV was 85% in 2005 and 89% in 2006 for the cows in conventional herds, and 54% in 2005 and 59% in 2006 for cows in the organic herds ($P < 0.05$) (Table 2 of paper I). However, in two organic herds all sampled animals were seronegative to BRSV. In organic herds 32% of the youngest cows (1–3 years old) were seropositive to both BCoV and BRSV, whereas this figure was 70% in conventional herds ($P < 0.001$) (Figure 1 of paper I). Although the conventional and organic herds included in this study were similar in many aspects, organic herds had a significantly lower seroprevalence to BCoV and BRSV. The fact that in five of the organic herds, only cows over fourth lactation time were seropositive to BRSV, suggests such herds have remained free from this infection for several years. Several risk factors related to farm, animal and environment have been proposed for BCoV and BRSV infection such as herd size, stall type, dense farming, animal purchasing and infection

experience (Frössling *et al.*, 2012; Gulliksen *et al.*, 2009a; Gulliksen *et al.*, 2009b; Larsen, 2000; Smith *et al.*, 1998; Clark, 1993; Baker *et al.*, 1986). Our results support the hypothesis that the stricter regulations on animal trade and quarantine of purchased individuals seen in organic production (KRAV, 2012) reduce the risk of direct transmission (Saif, 2010; Larsen, 2000). However, since indirect contact is considered more important in transmission of both BCoV and BRSV (Norström *et al.*, 2000; Tråvén, 2000), we concluded that further epidemiologic studies are required to test this hypothesis.

5.1.2 Age related seroprevalence

The seroprevalence to both BCoV and BRSV increased with increasing animal age (Figure 1 of paper I). The youngest age group (1–3 years old) had a significantly lower seroprevalence to both viruses than did the older cows (>3–5 years old) ($P < 0.001$). The mean in each of the four age clusters was 28, 46, 69, and 98 months, respectively. The finding indicates an age-dependent seroprevalence to these viruses. The likelihood of animal seropositivity to both these viruses in either conventional or organic herds gradually increases with age until all cows in the oldest cluster (>7 years old) were seropositive. Indeed, the mean antibody levels against BCoV and BRSV in the oldest cluster were also the highest relative to the younger clusters, suggesting that the oldest cows may have the highest levels of antibodies in their colostrum against BCoV and BRSV. This most probably increases the protective value of colostrum from the old cows.

5.1.3 Seroprevalence related to stall type but not to visitors

Results of logistic regression modeling of seroprevalence and potential risk factors are presented in tables 3 and 4 of paper I. The odds of animals being seropositive to BCoV or BRSV or both was significantly lower in organic compared to conventional herds. The odds of animals being seropositive to both BCoV and BRSV was significantly higher on farms using tie-stalls and own AI facilities (AIF). The increased risk of BCoV and BRSV infection on farms practicing tie-stalls for the cows has previously been reported for BCoV infection (Smith *et al.*, 1998). In such a system animals are more confined and the stocking is denser, which may facilitate indirect inter-herd transmission. Farm visitors such as veterinarians and AI technicians have been proposed to play a role in the transmission of BCoV and BRSV, especially in large herds with frequent visitors (Larsen, 2000; Norström *et al.*, 2000; Tråvén *et al.*, 1993). However, we didn't observe such association and, in contrast, herds using AIF had a higher seroprevalence than herds using professional AI technicians, as also reported in another study (Ohlson *et al.*, 2010a). It is likely

that biosecurity measures undertaken by visiting AI technicians and farmer education on the importance of biosecurity all helped to avoid infections despite regular visitors in the herds. In the same vein, one studied organic herd with a high number of public visitors, such as school children, had only one BRSV positive 5-year-old cow out of 15 animals sampled. Thus contacts unrelated to the cattle farming were not a transmission risk.

5.1.4 Long-lasting seropositivity and highly contagious features

Seventy-five cows in 15 of the conventional and 17 of the organic herds were sampled in both 2005 and 2006. Of the samples taken in 2005, 92% (69/75) were seropositive to BCoV and 79% (59/75) to BRSV. Seropositive animals stayed positive on the second sampling occasion, indicating that antibodies remain detectable for several years after infection with BCoV and BRSV (Tråvén, 2000; Elvander, 1996). Seroconversion to BRSV was found in some herds, where all animals sampled on the second occasion were seropositive. This finding is in the line with previous observations that following introduction of BCoV and BRSV to a herd, most animals seroconvert within a short time period (Tråvén, 2000; Alenius *et al.*, 1991).

5.2 Molecular tracing and evolution study

5.2.1 BCoV circulation pattern in Sweden

An initial 1038-nt fragment of the S glycoprotein gene was amplified by PCR from all 27 field samples and sequenced directly. BCoV was detected in outbreaks or case reports showing signs of either respiratory or enteric disease in both dairy and feedlot herds most often during the winter period but also during the summer months (May to August, Table 1 of paper II). We concluded that BCoV strains have circulated between cattle herds all the year round. Additionally, the full-length (4092 nt) S gene sequence of 33 field samples didn't show any consistent differences between BCoV strains obtained from respiratory and enteric disease (manuscript IV). The results imply that the same strains of BCoV cause respiratory disease, CD and WD, which is in agreement with previous studies (Saif, 2010; Niskanen *et al.*, 2002; Tråvén *et al.*, 2001).

Genetic variation of the S gene of BCoV strains

Comparative genetic analysis of the 1038-nt sequence showed that all the 27 Swedish strains obtained from 25 herds in 2005–2009 shared a high identity both at the nt level (>97.7%) and at the deduced aa level (>96.7%) (Paper II). The analysis of the full-length S gene of all 33 Swedish and Danish strains also

showed a high degree of identity both at nt level (>97.8%) and deduced aa level (>97.4%) (manuscript IV).

More genetic variation was observed within southern strains than within northern and central strains. In fact all strains obtained from northern and central Sweden during the third season (October 2007 to May 2008) of paper II were highly similar (nt identity >99.7%). These strains were collected from regional outbreaks of WD. These regions were shown to have a low herd immunity to BCoV; 70% of the herds had BCoV seronegative primiparous cows in the northern regions compared to 10% in the southern parts in 2007 (Ohlson, 2010). This may be related to a lower density of herds in northern regions, less transportation of animals and/or a difference in biosecurity between the regions (Ohlson *et al.*, 2010a). The low herd immunity suggests that BCoV is not continuously circulating in the northern parts of Sweden, and that virus causing regional epizootics is introduced from other regions. Such a transmission is also suggested by the finding of strain N3-0605 in southern Sweden prior to and highly identical (nt identity >99.8%) to the strain causing the Jämtland outbreaks of WD (Z1– Z5).

In this study, we reported a close genetic relationship (98.9% nt identity, 98.6% aa identity) and high simulated structural similarity of the S protein of SWE/C/92 with a human enteric CoV isolate, HECV-4408/US/94 (Figure 1 and Figure 3A, 3B and 3C of manuscript IV). This was also in accordance with nested positioning of the oldest Swedish strain in the phylogenetic trees (Figure 1 of paper II, Figure 1 of manuscript IV). Thus, the similarity between SWE/C/92 and HECV-4408/US/94 S protein conformation further supports the hypothesis of interspecies transmission of these viruses. Future studies to find novel strains in humans and animals of *Betacoronavirus1* and determination of the structure of the S protein would greatly assist in determining how such interspecies transmissions occur.

Phylogenetic analysis of BCoV strains

The Swedish BCoV strains fell into four clusters in the consensus tree based on maximum likelihood analysis of the 1038-nt fragment of the S gene (Figure 1 of Paper II). Cluster I and II included strains obtained from all over Sweden. Cluster II also included the Italian strain Bubalus/Italy/179/07, a BCoV strain detected in buffalo calves with severe enteritis (Decaro *et al.*, 2008c). Cluster III contained the two Swedish strains I2-0702 and I4-0801 from Gotland and the strains Italy/438/06 and Italy/339/06 that were isolated from an outbreak of enteric and respiratory disease in dairy cattle (Decaro *et al.*, 2008b). Cluster IV contained the strain C1-9202 together with the Swedish strain Rc1N-99 and the Danish strain HcF-01 that were collected in 1999 and 2001, respectively. The

grouping of these three sequences was supported by a bootstrapping value of 62% (Figure 1 of Paper II). Analysis of full-length S gene sequences of all 33 Swedish and Danish BCoV strains also showed low genetic diversity that resulted in their clustering as a unique clade with Italian strains in the phylogenetic tree (Figure 1 of manuscript IV). A high similarity was observed between Italian and Swedish strains. This molecular pattern suggests that the BCoV strains circulating in Swedish herds are closely related to strains present in other parts of Europe. While it is easy to understand the clustering of strains from the central and southern parts of Sweden with strains from Denmark, which borders with Sweden, grouping of Swedish strains with Italian strains was surprising. As there were no records of animals purchased from Italy, it is unlikely that the virus was introduced to Sweden by transportation of animals directly from Italy. There have, however, been animals exported from Sweden to central and southern Europe in recent years. Samples sequenced from other European countries are needed in order to trace the origin of Swedish strains.

An enteric strain Z2-0711 obtained from the northern region (Jämtland) also showed a high similarity (99.6%) to a respiratory strain U1-0907 obtained from a herd in the central region (Västmanland) (paper II). We also identified a high similarity (99.4%) between the strain SWE/M/06-3 and six other strains that circulated in 2007 to 2009 in distant regions of Sweden, implying that certain strains may have the potential to spread directly or indirectly to distant regions or to other countries. No identical strains obtained from different epidemic seasons have been identified, but some strains were highly similar. High stability of certain BCoV strains was shown by the finding of identical strains in Gotland island in 2007 (e.g. SWE/I/07-3) and a highly similar strain obtained from another region in 2009 (SWE/P/09-1). Highly similar strains were also found in different regions in 2010 (SWE/Y/10-3, SWE/P/10-4) (manuscript IV). These suggest that these BCoV strains were part of common transmission chains. These findings imply that sequence analysis of the S gene can support or rule out suspected transmission routes of BCoV between regions (paper II and manuscript IV).

The finding of identical 1038-nt sequences of two strains obtained 4 months apart from young calves in a large dairy herd (N1, >200 cows) indicates, for the first time, that a BCoV strain may circulate for an extended period of time within large herds (Paper II). In this large dairy herd we also found two slightly different (99.8%) CD strains, SWE/N/05-1 and SWE/N/05-2, which were circulating at the same time (manuscript IV). This finding indicates that strains with genetic diversity, though limited, can circulate in such herds. On the other hand, strains from another large herd (M1) obtained 3 months apart had several mutations, suggesting introduction of a new strain into that herd (Paper II).

Large dairy herds were previously found to have a higher incidence of BCoV infection (Ohlson *et al.*, 2010b; Smith *et al.*, 1998) which is consistent with the concept that large herds may foster a favourable environment for virus introduction and circulation of the strains.

Evolution analysis of BCoV strains

The evolutionary analysis of this study encompassed a large data set of *Betacoronavirus1* sequences of full-length S gene obtained over 45 years (1965-2010), including newly sequenced Swedish and Danish BCoV strains from the last decade and one strain from 1992 (Table 1 of manuscript IV). Sampling in time provides us heterochronous data to estimate the time of divergence of the recent BCoV sequences. The estimated rate of nt substitution in the S gene of BCoV (8.7×10^{-4} substitution /site /year) is comparable to that observed as standard range (orders of 10^{-3} to 10^{-5}) in other rapidly evolving RNA viruses, such as nonstructural protein NSP2 of rotavirus A (Donker & Kirkwood, 2012) and E gene of Dengue virus-3 (Sall *et al.*, 2010).

Molecular clock analysis of the spike gene of the recent BCoV strains and HCoV-OC43 strains estimated an evolutionary rate in the order of 4.1×10^{-4} substitution per site per year; similar to a previous estimate of 4.7×10^{-4} substitution per site per year (Vijgen *et al.*, 2005). Bayesian coalescent approach dated tMRCA around 1899, highly similar to the previous estimate of around 1890 (Vijgen *et al.*, 2005).

Evolutionary analysis of our BCoV strains along with other virus species in *Betacoronavirus1* demonstrated a closer relationship of BCoV to canine and human CoVs than to porcine and equine CoVs. TMRCA of CoVs is in accordance with their clustering in the phylogenetic tree (Table 3 and Figure 1 of manuscript IV). The time of divergence of BCoV and CRCoV strains was estimated to have occurred five decades after that of BCoV and HCoV-OC43 strains, suggesting a closer common ancestor of the former. The spike protein of CRCoV-4182/UK/03 has been shown to have a higher genetic similarity to BCoV/Mebus/US/72 and BCoV/LY138/US/65 than to HCoV-OC43/VR759/UK/6 (Erles *et al.*, 2007). In that study, the cross-reactivity of CRCoV-4182/UK/03 with polyclonal antisera against BCoV was also shown (Erles *et al.*, 2007). This corresponds to what is illustrated in the phylogenetic tree (Figure 1 of manuscript IV); the clade of ruminant CoVs is clustered closer to the clade of CRCoV strains than to the other virus species in *Betacoronavirus1*. At the tree level, CoVs from bovines and several wild ruminant species clustered closely together, implying that such interspecies transmission of CoVs may occur as suggested previously (Woo *et al.*, 2009; Alekseev *et al.*, 2008).

Positive selection on the S protein

The selection profiles of the aa sequence of all 33 Swedish and Danish BCoV strains showed two general patterns within the S protein. The cumulative differences between non-synonymous and synonymous (dN-dS) revealed several motifs under positive selection. Two regions of the S1 subunit (spanning aa residues 109-131 and 495-527) were under strong positive selection (Figure 2A of manuscript IV). In contrast, most of the motifs spanning between aa residues 36-97, 315-420, 498-713, 910-1032, 1059-1234 and 1245-1279 were under negative selection. They covered most of the S2 subunit, indicating S2 is relatively stable in BCoV (Figure 2A of manuscript IV). The SNAP analysis identified 133 positively selected sites. 89 of them are in S1 and 44 in S2 domain (Figure 2B of manuscript IV).

There is an association between positively selected sites along S1 subunit identified in this study and mapped neutralizing epitopes (Rekik & Dea, 1994; Yoo *et al.*, 1991b; Deregt *et al.*, 1989). It has been reported that mutations in the S1 and the N-terminus of the S2 sequence often result in changes in antigenicity (Kanno *et al.*, 2012; Yoo & Deregt, 2001; Vautherot *et al.*, 1992). Likewise, parts of the putative C-domain defined in this study; spanning aa residues 326-540, and the N-terminal domain (NTD) defined in details in a previous study (Peng *et al.*, 2012) were shown to be under strong positive selection in the BCoV strains (Figure 2A of manuscript IV). Taken together, the strong positively selected motifs among the S protein may thus be associated with the immune response and receptor-binding and would thus be important in future BCoV vaccine development.

The negative selection pattern of the S2 subunit is also reported (Figure 2 of manuscript IV). Negative selection is usually reported in genome fragments with essential functions in the viral lifecycle (Yang, 2005). For example, extensive syncytia formation was observed in cells infected with an S2 recombinant of BCoV (Yoo *et al.*, 1991a). Thus we speculate that the S2 subunit, except its N-terminus, would mostly interact with cellular compartments rather than immune system elements of the host.

5.2.2 BRSV circulation pattern in Sweden

A 603-nt fragment encoding the extracellular part of the G glycoprotein was amplified by PCR from all 30 field samples and sequenced directly. BRSV was detected in outbreaks of respiratory disease in both dairy and feedlot herds most often during the winter period but also during the summer months (May to August, Table 1 of paper III). We concluded that BRSV strains have circulated among herds throughout the year, causing annual outbreaks in Sweden.

Genetic variation of the G gene of BRSV strains

Comparative sequence analysis showed a high degree of sequence similarity (>97%) among 29 of the Swedish strains of BRSV. One strain from Skåne (M1/0908) was 99% similar to a Danish strain but only 94-96% similar to the other Swedish strains. Some strains from different herds were found to be identical, for instance E1, E2 and H2 sampled February-May 2008 in south-eastern Sweden. More than one BRSV strain was detected in the 2011 outbreak in Västerbotten county (AC, Figure 1 of paper III). The findings of this study show the BRSV strains that have been circulating among the Swedish herds during recent years are highly similar. This is in contrast to the observation of extensive sequence divergence (up to 11%) among BRSV strains detected in Danish herds (Larsen *et al.*, 2000). One explanation for the limited sequence diversity among Swedish strains may be very limited import of live animals to Sweden. These facts purpose the potential of inter-herd transmission within and between countries for BRSV strains mainly via animal trading. Certain outbreak strains of HRSV and BRSV have shown the potential to spread over different countries (Socha *et al.*, 2009; Kovarcik & Valentova, 2004; Zlateva *et al.*, 2004).

Phylogenetic analysis of BRSV strains

Phylogenetic analysis showed that all Swedish strains of BRSV belong to subgroup II as previously proposed (Valarcher *et al.*, 2000), where 29 out of 30 recent Swedish strains formed a clade with strong bootstrap support (Figure 1 of paper III). In general, the phylogeny suggested that the recently analysed Swedish BRSV strains are more closely related to the neighbouring Danish strains than to strains from other countries. In particular, a single novel strain M1/0908 was identified in Skåne and formed a well-supported cluster with a Danish strain (9314893/DK/93), indicating that both strains share a common ancestor. This cluster was only distantly related to the majority of the recent Swedish strains. There are two possible explanations for this unexpected event: either the virus had been present in Swedish herds for a long time but remained undetected or it was a new introduction into Swedish herds. Interestingly, nearly identical G gene sequences were found between strains M1/0908 and 9314893/DK/93 which were collected more than 16 years apart. This may suggest that some BRSV strains can remain stable for many years in the population, as it has been previously shown for HRSV strains (Ratiefard *et al.*, 2004; Zlateva *et al.*, 2004). More relevant sample sequences, however, are required to test such speculation.

During the study period, we investigated a major epizootic of BRSV in one area within the county of Västerbotten. This occurred among several herds

from November 2010 to February 2011. Cattle herds in this area were found to be sensitive to BRSV in a long-term serological study from 2006 to 2009 (Ohlson, 2010). Due to frequent trade of animals from the south of Sweden to the north, this epizootic in 2010/2011 was expectable. In this epizootic, BRSV affected young stock and cows in at least 30 dairy herds and caused three animals dead (low mortality). However, individual animals in more than 50% of the affected herds had severe signs of respiratory disease, forcing to be treated with antibiotics.

Outbreaks of BRSV mainly occur during autumn and winter when the animals are housed indoors in temperate climate areas (Van der Poel *et al.*, 1993). Interestingly, we found BRSV-positive samples during the summer in animals having respiratory disease. In the same line, serological studies have been previously confirmed seroconversion to BRSV in cattle herds during summer (Ohlson, 2010; Hägglund *et al.*, 2006). This indicates that BRSV is circulating between herds throughout the year. During this study, no report of BRSV outbreaks in Jämtland county was recorded. A long-term serological study also confirmed a BRSV-free status in herds within this county from 2006 to 2010 (Ohlson *et al.*, 2013). Also 10 out of 11 bulk milk samples from Jämtland collected in February 2013 tested negative for BRSV antibodies and only a low level of antibodies in the bulk milk sample of one of the herds was detected. This BRSV-free status at present of Jämtland is probably related to improved biosecurity, confined animal transportation and less herd density in this county. The knowledge obtained from phylogenetic analysis of strains in this thesis and from epidemiological studies (Ohlson *et al.*, 2013; Ohlson *et al.*, 2010b) helps to set up strategies to prevent transmission routes and the inter-herd spread of BRSV. Therefore it should be possible to establish effective control strategies for BRSV primarily based on biosecurity on a regional and even national level.

6 Conclusions

- Conventional herds had significantly higher mean seroprevalence to BCoV and BRSV compared to organically managed herds, suggesting that farm management methods play an important role in reducing the seroprevalence of these viruses through better biosecurity routines.

- Increased animal age was significantly associated with increased seroprevalence to BCoV and BRSV. The old cows were shown to have higher levels of antibodies in their serum against BCoV and BRSV and probably better protective value of their colostrum compared to younger cows.

- Seropositive animals all remained positive on the second sampling occasion, indicating that antibodies remain detectable probably for several years after infection with BCoV and BRSV.

- Seroconversion was found in some herds, where all animals sampled on the second occasion were seropositive, indicating the highly contagious status of the infection. However, the finding of long-lasting seropositivity to these infections and herds in which only cows over fourth lactation were seropositive imply that herds could remain free from BCoV and BRSV for several years despite a high prevalence in the general cattle population.

- Comparative sequence analysis showed a high sequence identity among the Swedish BCoV strains, regardless of whether they were obtained from outbreaks of respiratory disease or diarrhoea or from calves or adults. Swedish BCoV strains unexpectedly showed a high homology with recently detected Italian strains. Circulation of a BCoV

strain during an extended period of time was demonstrated in calves in a large dairy herd. In northern Sweden, a regional outbreak of highly similar BCoV strains was detected, while in the southern and central regions more extensive transmission of the virus was indicated.

- Comparative sequence analysis showed a high sequence similarity among the Swedish BRSV strains and identified an unusual strain from a recent outbreak. Phylogenetic analysis indicated that this strain was closely related to a Danish strain with more than 16 years interval, implying that some BRSV strains remain stable for many years in the population.

- Evolutionary analysis of our BCoV strains along with other virus species in *Betacoronavirus1* demonstrated a closer relationship of BCoV to canine and human CoVs than to porcine and equine CoVs. At the tree level, the clade of ruminant CoVs is clustered closer to the clade of CRCoV strains than to the other virus species in *Betacoronavirus1*. Likewise, CoVs from bovines and several wild ruminant species clustered closely together, implying that such interspecies transmission of CoVs may occur.

- The finding of positively selected sites on the S protein was strongly associated with the mapped neutralizing epitopes and the putative receptor binding domains of the S protein. Therefore, the strong positively selected motifs among the S protein may be associated with the immune response and receptor-binding and would thus be considered in future BCoV vaccine development.

- Molecular genetic analysis of the S gene of BCoV and the G gene of BRSV is a useful tool for studying the epidemiology of these viruses. Such analysis provides data for tracing strains circulating among the herds. Identical sequences supported epidemiological data on inter-herd contact through purchased calves and that certain herds were part of a common transmission chain.

- The research performed during the last 6 years concerning the epidemiology of BRSV and BCoV infections and possibilities for control in Sweden, suggest that it will be possible to establish herds free from BRSV and also from BCoV on a regional and even national level.

7 Future research

- Further studies of the molecular epidemiology of BCoV and BRSV are warranted in order to clarify the indirect and direct transmission routes of these viral infections and to be able to effectively control the spread of the virus between herds, regions and even countries.

- Development of complex robust diagnostic panels, such as liquid-phase microarrays, for the, rapid, low-cost and high throughput simultaneous detection and identifications of the major pathogens in the BRD and enteric disease.

- Full genome sequences of BCoV and BRSV strains using next generation sequencing (NGS) would provide greater knowledge regarding the epidemiology and circulation pattern of these infections among herds.

- Development of efficient vaccines against BCoV and BRSV infections would be a great achievement. Such vaccines could be used along with biosecurity measures to improve herd immunity and control the infection, especially in regions with highly dense cattle populations. Molecular genetic analysis of BCoV and BRSV strains would also provide valuable knowledge regarding the selective pressure sites along antigenic proteins, which is important to consider in future vaccine development for these viruses. Such monitoring sequence changes in positive selection sites would provide also useful data for identifying future dominant epidemic strains.

- Isolation and full genome sequencing of BCoV and BRSV strains from Scandinavia and other countries would benefit the investigation

of genetic diversity of these infections among herds and also on their evolutionary aspects. Such characterized isolates could potentially be exploited as native candidates for vaccine development.

- It remains unclear how the genetic contribution of different bovine immunological components influences the level of protection and pathology in relation to BRSV (Meyer *et al.*, 2008) and most likely BCoV. An understanding of the genetics that control variation in immune responses and infectious disease may help to select for more resistant animals, as well as finding new approaches for improving vaccine efficacy (Leach *et al.*, 2012).

8 Populärvetenskaplig sammanfattning

Bovint coronavirus (BCoV) och bovint respiratoriskt syncytial virus (BRSV) är mycket smittsamma virus som orsakar respiratorisk sjukdom hos nötkreatur över hela världen. Dessa virus predisponerar för uppkomsten av sekundära bakteriella lunginflammationer hos framförallt kalvar men även kor och dessa lunginflammationer måste ofta behandlas med antibiotika för att kalvarna inte skall bli svårt sjuka eller dö. BCoV är även en vanlig orsak till diarrésjukdom hos nötkreatur i alla åldrar. Vacciner mot BCoV och BRSV används runt om i världen, men deras effektivitet har ifrågasatts. Syftet med denna avhandling var att få serologisk och molekylärbiologisk kunskap om BCoV- och BRSV-infektioner för att skapa en effektiv framtida kontroll i Sverige och resultaten av de genomförda studierna (3 publikationer och 1 manuskript) sammanfattas och diskuteras här kortfattat. I den första studien undersöktes förekomsten av antikroppar mot BCoV- och BRSV-infektioner i 20 konventionella och 20 ekologiska mjölkbesättningar i sydöstra Sverige Vid två tillfällen med ett års intervall, togs 699 serumprover från 624 kor och förekomsten av antikroppar undersöktes med ELISA-teknik. Antikroppsprevalensen för BCoV och BRSV var hög (> 80%) vid båda provtagningarna. Konventionella besättningar hade en signifikant högre seroprevalens än de ekologiska besättningarna ($P < 0,01$). Det fanns ett signifikant samband ($p < 0,001$) mellan antikroppsförekomst och kons ålder. Det var betydligt vanligare att äldre kor hade antikroppar i serum än yngre kor och de äldre korna hade även högre antikroppshalter i serum jämfört med yngre kor. Råmjölken från de äldre korna ger därför sannolikt kalvarna ett bättre passivt skydd mot både BCoV- och BRSV-infektioner jämfört med den från yngre kor. Serokonversioner mot BRSV hittades i vissa besättningar där samtliga provtagna kor vid det andra tillfället var seropositiva vilket tyder på att om denna virusinfektion introduceras till en besättning så sprids smittan snabbt till alla mottagliga individer i besättningen. Samtliga kor som var antikroppspositiva vid det första provtagningstillfället var även positiva vid det andra. Detta visar att antikroppar mot både BRSV och BCoV kvarstår under minst ett år efter en infektion och sannolikt i många fall livslångt. Slutsatsen grundas på att i vissa besättningar var det enbart de äldsta korna som var antikroppspositiva. I denna studie visade sig ekologiska

besättningar ha ett mer gynnsamt infektionsstatus jämfört med de konventionella sannolikt beroende på färre djurinköp och mindre antal indirekta kontakter med andra besättningar. Det kunde även noteras att i två ekologiska besättningar var samtliga kor negativa mot BRSV vilket visade att besättningar kan vara fria från BRSV under lång tid. Detta har visat sig stämma och i Jämtland har inga mjölkkobesättningar drabbats av BRSV från 2006 till februari 2013. Prevalensen av BRSV infektioner var mycket hög 2006 men nu måste Jämtland betraktas som en BRSV fri region i Sverige. Förhoppningsvis kommer det att förbli så men då enskilda djurägare utan några restriktioner kan köpa in djur från områden med en cirkulerande smitta finns det en uppenbar risk att smittan återinförs.

Med målet att få en ökad kunskap om de genotyper av BCoV och BRSV som finns i den svenska nötkreaturspopulationen genomfördes molekylärbiologiska studier av BCoV och BRSV positiva prover från nötkreatursbesättningar drabbade av dessa infektioner från olika delar av Sverige. De sekvenserade svenska genotyperna jämfördes även med genotyper från andra länder där sekvenserna hämtades från en internationell sekvensdatabas (GenBank).

För att undersöka den molekylära epidemiologin rörande BCoV sekvenserades 27 PCR-positiva prover från 25 besättningar från besättningar som drabbats av diarre och/eller luftvägssymptom under perioden 2005 till 2009. För BRSV sekvenserades PCR-positiva prover från 30 respiratoriska utbrott i nötkreatursbesättningar under perioden 2007 till 2011. Sekvensanalys avslöjade en hög grad av identitet bland svenska genotyperna, > 97,7% för BCoV och > 94,5% för BRSV tydande på att vi i Sverige har våra ”egna” BCoV och BRSV som cirkulerat bland svenska nötkreatur under lång tid. För BRSV påvisades dock en avvikande genotyp i Skåne som var mycket lik en som tidigare påvisats i Danmark. Mer förvånansvärt var dock att BCoV från Italien, både från vattenbufflar drabbade av diarré och från nötkreatur med lunginflammationer, var genetiskt mycket lika de svenska genotyperna. Det skall dock påpekas att hela genomet inte sekvenserats utan enbart en del av glykoproteinet (G) från BRSV och en del eller hela S proteinet från BCoV. De genomförda studierna i denna doktorsavhandling tyder dock på att molekylärbiologiska analyser kommer att vara ett mycket användbart verktyg för att undersöka misstänkta smittvägar för både BCoV och BRSV. Sådan kunskap är värdefull för kontroll av spridningen av BCoV och BRSV mellan besättningar, regioner och även länder. BCoV och BRSV diagnosticerades oftast som orsak till sjukdom under vintern, men även under sommaren förekom infektionerna. Detta tyder på att båda dessa infektioner cirkulerar och orsakar sjukdom bland nötkreatursbesättningar under hela året, något som varit ifrågasatt tidigare. För att studera den evolutionära utvecklingen av BCoV sekvenserades hela S genen från PCR positiva isolat från 30 besättningar. Den evolutionära analysen tyder på att BCoV utvecklats genetiskt nära andra coronavirus påvisade hos djur och människa. Coronavirus från nötkreatur visade ett närmare släktskap med coronavirus från hund och människa än med

coronavirus från svin och häst. Vi kunde inte hitta några genetiska skillnader i S proteinet hos BCoV från kalvar med diarré jämfört med virus från vuxna kor med diarré (s.k. vinterdysenteri) eller från kalvar och kor med luftvägssymptom. Detta tyder på att en genotyp kan ge upphov till samtliga dessa kliniska manifestationer vilket har ifrågasatts i den internationella litteraturen. Baserat på resultaten av den forskningen om BRSV- och BCoV-infektionernas epidemiologi och möjligheterna till profylax som genomförts under de senaste sex åren i Sverige är vi övertygade om att det går att etablera BRSV- och även BCoV-fria mjölkkobesättningar både på regional och nationell nivå. Detta genom ett frivilligt kontrollprogram baserat på ett stärkt smittskydd och regelbundna undersökningar av besättningarnas BRSV- och BCoV-status, genom analys av samlingsprover från förstakalvare och/eller tankmjölksprover. Detta skulle medföra en förbättrad djurhälsa och produktion samt ytterligare begränsa användningen av antibiotika i svenska nötkreatursbesättningar och därmed minska riskerna för uppkomsten av resistenta bakterier som skulle kunna drabba både djur och människor. I Norge kommer samtliga mjölkkobesättningar att undersökas med tankmjölksprover avseende både BCoV och BRSV i ett beviljat forskningsprojekt. Det är vår förhoppning att den redan nu i Sverige genomförda BRSV och BCoV forskningens resultat snart omsätts i praktiken, för att ytterligare förbättra både djurhälsan och produktionen i mjölk och köttproducerande besättningar i Sverige.

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