Epidemiology, Detection and Prevention of Respiratory Virus Infections in Swedish Cattle

with Special Reference to Bovine Respiratory Syncytial Virus

Sara Hägglund
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

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Abstract

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This thesis deals with the dynamics of bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (PIV-3) and bovine coronavirus (BCoV) infections in Swedish cattle herds. It also describes the development of a fluorogenic reverse transcription (fRT)-PCR for BRSV diagnosis. Furthermore, BRSV immunostimulating complexes (ISCOMs) and a commercial inactivated BRSV vaccine were evaluated *in vivo* in calves.

By serology, high incidences of PIV-3 and BCoV, and a lower incidence of BRSV, were demonstrated in 118 herds in one area of Sweden and at a Swedish bull testing station for beef breeds. In the dairy herds, absence of these infections and bovine viral diarrhea virus (BVDV) was correlated to absence of respiratory disease, confirming the clinical importance of the viruses. There appeared to be a geographical clustering of BRSV and BCoV infected herds and infections were as common in herds that did or did not introduce new animals, indicating indirect transmission between herds. At the bull testing station, an effective spread of virus within the herd was demonstrated. BRSV was involved in a disease outbreak, emphasizing the need for effective means to prevent this infection. By testing animals prior to admission, the bull testing station was kept free from BVDV during a six-year study period.

A single tube BRSV fRT-PCR was shown to be a sensitive, specific and rapid tool to detect BRSV in both naturally and experimentally infected animals. The assay required less laboratory time, had a lower risk of contamination and allowed higher sample throughput than a nested PCR with a comparable sensitivity. Accordingly, the fRT-PCR is suitable for BRSV research as well as for routine diagnostic purposes and was used in the vaccine evaluation.

The immunogenicity and protection induced by BRSV-ISCOMs were evaluated and compared to those induced by a commercial vaccine (CV) in calves. An experimental BRSV challenge was used for this purpose. The ISCOMs induced rapid humoral immune responses and strong clinical and virological protection despite maternal antibodies in calves at vaccination. In contrast, calves vaccinated with the CV and control calves all shed virus and developed moderate to severe respiratory disease after challenge. Thus, BRSV-ISCOMs are promising candidates for future industrial production after further evaluation and may also be used to investigate protective immunological parameters against BRSV. The CV needs to be further evaluated in controlled field trials.

Keywords: cattle, BRSV, PIV-3, BCoV, BCV, BVDV, respiratory disease, PCR, ISCOM, vaccine.

Authors address: Sara Hägglund, Swedish University of Agricultural Sciences, Department of Clinical Sciences, Division of Ruminant Medicine and Veterinary Epidemiology, P.O Box 7054, SE-750 07 UPPSALA, Sweden. E-mail: sara.hagglund@kv.slu.se

Everything is possible

- *H. Hägglund*

To my family

Contents

Appendix

Paper I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Hägglund, S., Svensson, C., Emanuelson, U., Valarcher, J.F. & Alenius, S. 2005. Dynamics of virus infections involved in the bovine respiratory disease complex in Swedish dairy herds. *The Veterinary Journal*. In press.

II. Hägglund, S., Hjort, M., Öhagen, P., Törnqvist, M. & Alenius, S. A six-year study on respiratory viral infections in beef calves. Submitted.

III. Hakhverdyan, M., Hägglund, S., Larsen, L.E. & Belák, S. 2005. Evaluation of a single-tube fluorogenic RT-PCR assay for detection of bovine respiratory syncytial virus in clinical samples. *Journal of Virological Methods* 123, 195-202.

IV. Hägglund, S., Hu, K.F., Larsen, L.E., Hakhverdyan, M., Valarcher, J.F., Taylor, G., Morein, B., Belák, S. & Alenius, S. 2004. Bovine respiratory syncytial virus ISCOMs--protection in the presence of maternal antibodies. *Vaccine* 23, 646-655.

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Abbreviations

ag	antigen
APC	antigen presenting cells
BCoV	bovine coronavirus
BHV-1	bovine herpes virus 1
bp	basepairs
BRD	bovine respiratory disease
BRSV	bovine respiratory syncytial virus
BRV	bovine rotavirus
BVDV	bovine viral diarrhea virus
cDNA	complementary DNA
COD	corrected optical density
CPE	cytopatic effect
CV	commercial vaccine
dNTPs	deoxynucleotide triphosphates
ELISA	enzyme-linked immunosorbant assay
F	fusion
fRT-PCR	fluorogenic RT-PCR
G	glyco
HE	hemagglutinin
HN	haemagglutinin-neuraminidase
HRP	horseradish peroxidase
HRSV	human respiratory syncytial virus
HRT-18	human rectal tumour cells
Ig	immunoglobulins
ISCOMs	immunostimulating complexes
MAbs	monoclonal antibodies
NSAID	non-steroid inflammatory drug
ORSV	ovine respiratory syncytial virus
PCR	polymerase chain reaction
PFU	plaque forming units
PI	persistently infected
PIV-3	bovine parainfluenza virus 3
ps	pig serum
RT	reverse transcription
S	spike
s.c.	subcutaneously

Introduction

Respiratory disease in cattle, general background

Nomenclature

The bovine respiratory disease (BRD) complex includes several syndromes that have been defined according to different epidemiological conditions. Since many of the disease-causing pathogens are ubiquitous and adult animals are often immune, BRD occurs most frequently in young calves, during the period between passive and active immunity. The disease is then called enzootic pneumonia (Bryson *et al.*, 1978a; Radostitis *et al.*, 2000). When, in contrast, whole cattle populations are naïve to specific infections, BRD epizootics occur that may severely affect both calves and adults (Inaba *et al.*, 1972; Elvander, 1996).

The term shipping fever is used when BRD appears after long, stressful transports and mixing of animals from different origin, as performed in feedlot rearing. Pneumonic pasteurellosis is also often used in these cases although *Pasteurella* or *Mannheimia spp.* are probably seldom the only aetiological agents (Yates, 1982; Storz *et al.*, 2000a). Some forms of interstitial pneumonia, such as 'fog fever', 'bovine farmer's lung', or hypersensitivity reactions against massive *Dictyocaulus viviparus* infections, have previously been classified as atypical interstitial pneumonia. However, Radostitis *et al.* (2000) recommend that the word 'atypical' be avoided since it might lead to confusion. In a review Yates (1982) stated that there is a lack of uniformity in the use of the BRD terminology and no clear definitions have been made by scientific commitees. Clinically, the disease often appears as pneumoenteritis, since many agents have tropism for epithelial tissue both in the respiratory and digestive tract.

Viral and bacterial aetiologies

Viral infections are detected in most BRD cases that are investigated early after the onset of clinical signs (Bryson *et al.*, 1978a). Different viruses are involved, such as bovine respiratory syncytial virus (BRSV), bovine herpes virus 1 (BHV-1), bovine parainfluenza virus 3 (PIV-3), bovine adenoviruses, bovine coronavirus (BCoV) and bovine viral diarrhea virus (BVDV) (Stott *et al.*, 1980; Kapil & Basaraba, 1997). They sometimes occur in combination and they frequently precede bacterial invasion of the lung (*e.g.* with *Mannheimia hemolytica, Pasteurella multocida, Haemophilus somnus, Salmonella dublin* and *Arcanobacterium pyogenes*) (Babiuk, Lawman & Ohmann, 1988). Bacteria and viruses may also interact with *Mycoplasma spp.* to induce chronic or more severe disease in a synergistic manner (*e.g. M. bovis, M. dispar, M. bovirhinis* and *M. canis*) (Thomas *et al.*, 2002). Bovine rhinovirus and bovine reovirus have low pathogenicity as single agens (Stott *et al.*, 1980), whereas the clinical effects of bovine influenzavirus and ovine RSV (ORSV) infections in cattle are unclear (Graham, Calvert & McLaren, 2002).

Predisposing factors

Several management factors affect the animals' immune responses against respiratory infections, and thus the development of clinical disease: for instance low colostrum intake, no previous exposure to the pathogen (by natural infection or by vaccination with effective vaccines) and immunosuppressive stress (caused by *e.g.,* poor nutrition, dehydration, low or high temperatures, little rest and transportation.) (Virtala *et al.*, 1999). Ammonia and hydrogen sulphide gases from manure, as well as dust particles, may additionally serve as irritants and predispose for BRD (Callan & Garry, 2002).

The infection dose is also probably important in the clinical outcome of infections, because high doses may overcome the host immune responses (Yates, 1982). Crowding, large animal groups, poor hygiene, poor ventilation and high humidity increase the number of infectious particles in the environment and in aerosols (Wathes, Jones & Webster, 1983; Stärk, 1999). Isolation of sick individuals is probably an important measure to lower the infection pressure in herds.

A range of factors influence the animals' risk for exposure to new respiratory pathogens, for example:

i) direct contact between animals from different sources (*e.g.* by introduction of animals in herds, by mixing of animals from several herds, or by contact over fences at pasture).
ii) introduction of new pathogens into herds by indirect contact (*e.g.* by vectors or contaminated material).
iii) housing of young calves with older animals that may serve as carriers or that shed pathogens during transient infections.
iv) large animal groups (increases contact between infectious and susceptible animals and might favour virulent subpopulations of pathogens through passages between many individuals).

Economical impact and incidence

BRD is economically important. Costs are due to losses of animals, decreased weight gains, increased work loads and a considerable consumption of therapeutic drugs. Moreover, much is spent on vaccines and prophylactic arrival medication in fattening herds, especially in North America. In 1991 the economic impact of BRD in the United States was estimated to more than 600 million US dollars (Smith, 2000).

European investigators have reported BRD incidences (or BRD treatment rates) of 22-38% and BRD mortalities of 2-9% in beef herds where animals were observed between a few weeks of age to slaughter (Stott *et al.*, 1980; Howard *et al.*, 1987; Frankena *et al.*, 1994). Similarly, BRD treatment rates were on average 20% in 30 Swedish fattening herds during 5-12 weeks of study (19-216 days of age) (Bengtsson & Viring, 2000) and BRD incidences were about 7% in Swedish dairy calves between birth and three months of age (Svensson *et al.*, 2003; Ortman & Svensson, 2004). Svensson *et al.* (2003) found highest odds ratios for calves housed in large-group pens and fed with automatic milk-feeding systems.

Cattle rearing in Sweden

Herd data

The total number of cattle in Sweden has decreased during recent years and was about 1,600,000 heads in 2003 (Swedish Board of Agriculture, 2004). As in other parts of Europe (Clausen, 2005), there is a continuous restructuring of the industry towards larger and fewer herds. In 2003, the Swedish dairy production was concentrated to 9700 holdings (~73% tie stalls and ~27% free stalls) with a mean herd size of 41 cows. The housing season lasts from October to May and even longer in northern Sweden. Artificial inseminations are performed on about 85% of animals and most calvings occur during autumn. The two main dairy breeds are Swedish Red and White Breed and Swedish Friesian and the mean annual yield was 8073 kg per cow in 2003 (Swedish Dairy Association, 2005a).

In 2003, cows for beef production were dispersed on 13,000 holdings with an average herd size of 13 cows. The largest beef breed is Charolais (~6000 cows registred in a national beef recording programme), followed by Hereford, Simmental, Highland Cattle, Limousin, Aberdeen Angus and Blonde d'Aquitaine (Swedish Dairy Association, 2005a). There are additionally about 8500 beef rearing enterprises, 200 of which purchase >150 calves/year, generally at 6-10 weeks of age (M. Törnqvist, Swedish Animal Health Service, personal communication). Most beef cattle are slaughtered at a mean carcass weight of 140 kg (34,000 calves in 2004) or at about 320 kg (241,000 bulls/bullocks in 2004) (Swedish Official Statistics, 2005a).

Disease control

Growth-promoting antibiotics have been banned in Sweden since 1986 (Casewell *et al.*, 2003) and antibiotics or vaccines for animal use can only be obtained by veterinary prescription. Efforts are made on disease prevention by management and most farmers are registered in organisations that are specialised in preventive medicine and that provide consultation (Swedish Dairy Association and Swedish Animal Health Service). Between 100 and 200 animals are imported and exported each year (Swedish Official Statistics, 2005b) and disease surveillance during import is carefully regulated by a national organisation, which is supported by both the beef and dairy industry (Swedish Farmers' Disease Control Programme).

As a result of national eradication programmes, Sweden has been declared free from BHV-1 since 1998 and from bovine leucosis since 2001. To continually monitor these disease free statuses, analyses are performed annually from all dairy herds and from a selection of beef herds. A national control programme against BVDV is undergoing and in August 2005, 98.5% and 97.2% of the dairy and beef herds, respectively, were declared free of this infection (Swedish Dairy Association, 2005b). This programme is based on i) serological identification of infected and non-infected herds, ii) certification and monitoring of non-infected herds and iii) virus clearance of infected herds by removal of persistently infected (PI) animals and, most importantly, by protection of non-immune pregnant animals from acquiring the infection (Lindberg & Alenius, 1999).

Because Sweden is declared free from BHV-1, and most dairy and beef herds are free from BVDV, this thesis focuses on three other important viruses in the BRD complex: BRSV, PIV-3 and BCoV. Special emphasis is given to BRSV because this virus causes severe annual outbreaks of BRD in both young and adult Swedish cattle.

Bovine respiratory syncytial virus (BRSV)

Viral characteristics

BRSV is an enveloped RNA virus classified as a *Pneumovirus* in the Paramyxoviridae family, order Mononegavirales. Its genomic RNA is single-stranded, non-segmented, negative sense and is incorporated in helical nucleocapsides. The fusion (F) and glyco (G) surface proteins have important functions in virus attachment and in cell membrane fusion of host cells. Viral polymerases are incorporated in the nucleocapsides and transcription as well as replication takes place in the cytoplasm of the cell. Virus assembly and spread occur by budding from the cell membrane, by fusion and by lysis of cells (Kingsbury, 1990). The major target cells for replication are epithelial cells in the respiratory tract and pneumocytes (Viuff *et al.*, 1996; Viuff *et al.*, 2002).

BRSV exists as a single serotype and antibody cross reactivity occurs between bovine, human, caprine and ovine RS viruses (Stott & Taylor, 1985). According to reactions with panels of monoclonal antibodies (MAbs), BRSV can be divided into three antigenic subgroups (A, AB and B) (Furze *et al.*, 1994; Grubbs, Kania & Potgieter, 2001) and sequence data reveal six distinct phylogenetic branches with isolates from different dates and geographical origin (Valarcher, Schelcher & Bourhy, 2000). The average nucleotide homology between BRSV strains is 92% and 98% for the G and F protein, respectively (Valarcher, Schelcher & Bourhy, 2000), as compared with 53% and 89% amino acid identity in G and F between human RSV (HRSV) strains of different subtypes (A and B) (McIntosh & Chanock, 1990). Bovine and human RSV share 30% and 81% amino acid identity in G and F, respectively (McIntosh & Chanock, 1990), and infections have been reproduced experimentally with HRSV in cattle (Thomas *et al.*, 1984). BRSV is most closely related to the caprine RSV (Trudel *et al.*, 1989), and a high seroprevalence to ORSV has been found in cattle, indicating inter-species transmission (Grubbs, Kania & Potgieter, 2001).

History

HRSV was initially isolated from a laboratory chimpanzee (Blount, Morris & Savage, 1956) and shortly thereafter from children with respiratory disease (Chanock, Roizman & Myers, 1957). After its discovery, this virus became rapidly recognised as a major cause of common cold, croup, bronchiolitis and pneumonia (Chanock *et al.*, 1961). In 1968, Doggett and co-workers found antibodies to HRSV in bovine sera (Doggett, Taylor-Robinson & Gallop, 1968) and in 1970, Paccaud and Jacquier isolated a distinct species of RSV from respiratory samples from cattle (Paccaud & Jacquier, 1970). Since then, the importance of BRSV in enzootic pneumonia of calves and as a cause of outbreaks of respiratory disease

also in adult cattle has been clearly established (Stott & Taylor, 1985; Wellemans, 1990; Van der Poel *et al.*, 1994; Elvander, 1996; Larsen, 2000).

Clinical signs

Infections with BRSV are often subclinical, but may cause clinical signs ranging between transient fever and dyspnoea (Van der Poel *et al.*, 1993; Larsen, 2000). In a study by Verhoeff and van Nieuwstadt (1984) 13 groups of 1- to 15- month-old calves with serologically confirmed BRSV infection were investigated for clinical signs of disease. Animals in three of these groups showed only mild clinical signs of BRD (increased coughing and nasal discharge), whereas 47% of animals in ten groups showed abdominal respiration, 50% abnormal breathing sounds on auscultation, 59% cough and 62% nasal discharge (Verhoeff & van Nieuwstadt, 1984a). Occasionally, BRSV infections lead to grave dyspnoea and sometimes also subcutaneous emphysema. In these severe cases, the destruction of lung parenchyma may lead to anoxia, which is clinically observed as cyanosis followed by death (Wellemans, 1990).

Secondary opportunistic bacterial or mycoplasma infections frequently aggravate the disease (Bryson *et al.*, 1978b) and although BRSV is sometimes detected as the only agent in fatal disease, necropsy in later stages often reveal bacterial infections when BRSV is no longer detectable (Verhoeff & van Nieuwstadt, 1984a; Larsen *et al.*, 1999). This illustrates the importance of early diagnosis in BRSV outbreaks.

In contrast to HRSV, which may cause disease during repeated infections (Hall, 2001), BRSV seems primarily to affect naive animals. Although virus shedding occasionally has been detected upon experimental BRSV re-infection, clinical disease is seldom observed (Kimman *et al.*, 1987; Tjornehoj, 2000; Taylor *et al.*, 2005). In agreement with this, i) natural disease is most often seen in calves younger than six months in areas where the infection is endemic, probably because older animals are immune (Van der Poel *et al.*, 1993) and ii) in areas where BRSV is not endemic and where adult cattle remain seronegative to BRSV, severe disease may be observed in animals of all ages when the infections are introduced (Elvander, 1996).

Maternally derived antibodies, which are predominantly of the IgG1-isotype (Uttenthal *et al.*, 2000), provide at least partial protection against clinical signs after natural and experimental BRSV infection (Kimman *et al.*, 1987, 1988; Belknap *et al.*, 1991). Thus, young animals are also often spared from severe disease in outbreaks in areas where BRSV is endemic (Wellemans, 1990).

Epidemiology

BRSV appears to be spread throughout the world (Inaba *et al.*, 1972; Baker, Ames & Markham, 1986; Motha & Hansen, 1998; Almeida *et al.*, 2005). Studies on bulk tank milk showed that 41-89% of herds were antibody positive in 1990 in Sweden (n=2237) (Elvander, 1996) and 100% of herds were antibody positive in 1996 in UK (n=341) (Paton *et al.*, 1998).

Clinical disease caused by BRSV is mainly diagnosed in autumn and winter in temperate climate zones (Stott *et al.*, 1980). It is unclear which factors favour disease during these periods and whether the viruses continue to circulate between animals and herds also when they are not frequently detected. It has been proposed that BRSV stays latent in animals during summer and is re-activated the following winter season, but this has been difficult to prove. In a longitudinal survey, including six closed dairy herds with annual BRSV infections in winter, primary serological responses were only detected in one herd in summer (June-August), indicating that the virus circulates at low levels or not at all during summer (Van der Poel *et al.*, 1993). Antibody titre-rises in seropositive cattle were recorded during summer in five of six herds, but were not associated with virus transmission to sentinel animals. These serological responses were interpreted as activation of latent infections that did not lead to virus shedding at levels high enough for successful transmission. Provided that the virus was not re-introduced by indirect contact, BRSV persisted within these herds and was possibly latent in seropositive cattle between high incidence winter seasons.

Attempts to reactivate virus have been made in animals previously infected with BRSV. Despite corticosteroid treatment, stress and BVDV or BHV-1 co-infections, BRSV re-excretion has not been detected, by either virus isolation, reverse transcription (RT)-PCR or by sentinels. BRSV specific antibody titre rises after provocation have nevertheless been interpreted as an indication of virus latency and reactivation (Van der Poel *et al.*, 1993, 1997). Viral messenger RNA has also been detected by RT-PCR in lymphnodes of animals 71 days after BRSV infection (Valarcher *et al.*, 2001) and, given that the virus persists on farms, individual latency rather than continuous circulation between animals is supported by mathematic modelling (De Jong *et al.*, 1996). Sequence data from recurrent outbreaks in the same herds in different years have revealed considerable nucleotide variation, implying that new viral strains are introduced or that latent strains evolve dramatically (Larsen, Tjornehoj & Viuff, 2000). Consensus sequences of BRSV strains obtained in a herd during one outbreak are identical, even if obtained 2-3 weeks apart (Larsen *et al.*, 1998; Larsen, Tjornehoj & Viuff, 2000; Valarcher, Schelcher & Bourhy, 2000).

Humoral immune responses

Detection of immunoglobulins (Ig) in body fluids is a specific measure of previous infection (or passive immunisation) and isotype kinetics in non-immunised animals may be used to estimate the infection date. The kinetics varies according to the sensitivity of the assay used for detection and is described below for virus-specific antibody detection by enzyme-linked immunosorbant assays (ELISAs).

Antibody responses to primary and secondary infections of colostrum-deprived, seronegative animals

Naïve, colostrum-deprived calves show IgM and IgA serum responses from day 8-14 after experimental BRSV infection, in titres that remain detectable during 12-29 days (Kimman *et al.*, 1987). Serum IgG1 becomes apparent from day 13-17 (Kimman *et al.*, 1987; Schrijver *et al.*, 1996) and is detectable for more than 30 days (Uttenthal *et al.*, 2000). IgG2 is detectable from day 30-90 (Kimman *et al.*,

1987) and probably lasts at least eight months (Larsen, 2000). In lung, nasal and eye secretions, IgA stays detectable during several months.

No serum IgM, but rapid secondary serum IgA responses (from day 6-8) and increase in serum IgG1 and IgG2 are observed after BRSV re-infection of colostrum-deprived calves (four months after primary infection). Strong, rapid secondary IgA responses (from day 4-11) and IgM resembling primary responses are detected in the respiratory tract (Kimman *et al.*, 1987).

Antibody responses to primary and secondary infections of colostrum-fed, seropositive animals

During primary BRSV infections in presence of maternally derived serum IgG1 (titres of 1280-2560, 3-week-old calves), both mucosal and serum IgG1, IgG2 and IgA responses are completely suppressed (Kimman *et al.*, 1987). A weak serum IgM response is sometimes observed and the grade of inhibition tends to be related to the level of passively derived serum IgG1 pre-inoculation (Kimman *et al.*, 1987; Uttenthal *et al.*, 2000). Nevertheless, after re-infection at a low IgG1 titre (80), these animals show rapid serum IgA, IgG1 and IgG2 rises (from day 6-8) and IgA in the respiratory tract, indicating that an immunological memory is induced during the primary infection.

Experimental models

Experimental BRSV infections have in most cases resulted in no or very mild clinical signs of disease and many vaccines have therefore been evaluated mainly for reduction of virus shedding after infection (Belknap, Ciszewski & Baker, 1995; Larsen, 2000). Different strategies have been applied, such as for route, mode and frequency of inoculation, type of inoculum and type of animals. In most experiments, the usage of colostrum-deprived calves or calves given BRSV-specific antibody-negative colostrum has been necessary for reproduction of some clinical disease. It is difficult to extrapolate results from such studies to a natural situation, where market-purchased young calves, some of which have maternal antibodies, are likely candidates for vaccination and where the infection sometimes causes severe clinical signs. Recently, however, with the aerosol inoculation technique and with a virulent low passage Danish isolate, even conventional colostrum-fed calves were severely affected after experimental infection (Tjornehoj *et al.*, 2003). This has opened up new possibilities for vaccine evaluation in this important animal group.

Bovine parainfluenza virus 3 (PIV-3)

Viral characteristics

PIV-3 is an enveloped, negative sense, non-segmented, single-stranded RNA virus that belongs to the genus *Respirovirus* in the Paramyxoviridae family. Its major site of replication is epithelial cells in the respiratory tract (Tsai & Thomson, 1975), but viremia may occur with replication in monocytes (Adair *et al.*, 2000). The surface haemagglutinin-neuraminidase (HN) protein and fusion (F) protein are important for mucus penetration, attachment and fusion with the host cell

membranes. PIV-3 replicates in the cytoplasm of cells and transcription of its RNA is made by a viral transcriptase. Virus assembly occurs through budding from the host cell membrane and infected cells sometimes form syncytia or lyse (Kingsbury, 1990). Variation in strains can be detected by MAbs and sequence analysis, but only one serotype occurs in cattle (Bryson, 1990).

Bovine PIV-3 is closely related to ovine and human PIV type 3, which causes respiratory disease in lambs and children. There is 77 and 80% amino acid homology in the HN and F proteins, respectively, between bovine and human strains (Chanock & McIntosh, 1990). Although not regarded as a zoonosis, infections have been reproduced experimentally with bovine PIV-3 in primates (Pennathur *et al.*, 2003) and one report exists about natural infection with the bovine virus in man (Ben-Ishai *et al.*, 1980). Cross-infection between sheep and cattle has also been reproduced experimentally (Woods, Sibinovic & Marquis, 1965).

Clinical signs

PIV-3 infections cause less serious disease than BRSV (Verhoeff & van Nieuwstadt, 1984a), but are nevertheless significantly correlated with BRD (Stott *et al.*, 1980). Mild clinical signs such as slight fever, coughing and nasal discharge are observed after pure PIV-3 infections in young animals (Bryson *et al.*, 1978a., 1979; Verhoeff & van Nieuwstadt, 1984a). When animal groups are of uniform age (and have uniform susceptibility), many or all individuals in young groups are simultaneously affected, whereas older animals generally are immune and do not show clinical signs of disease (Verhoeff & van Nieuwstadt, 1984a).

The virus is thought to have a predisposing role in shipping fever and enzootic pneumonia; it may be isolated from severe or fatal BRD cases together with bacteria and *Mycoplasma spp.* (Bryson *et al.*, 1978b; Storz *et al.*, 2000a). The BRD predisposing function of PIV-3 is probably correlated to its immunosuppressive effects on leucocytes (*e.g.* decreased phagocytosis by alveolar macrophages and decreased lymphocyte proliferation) and destruction of the mucociliary system (Hesse & Toth, 1983; Babiuk, Lawman & Ohmann, 1988; Basaraba *et al.*, 1994; Adair *et al.*, 2000). A decrease in surfactant production by alveolar type II cells and impairment of the basement membrane of epithelial cells in bronchioli have also been suggested (Tsai & Thomson, 1975).

Epidemiology

Bovine PIV-3 was first isolated in the 1950s from cattle with clinical signs of BRD in the USA (Reisinger, Heddleston & Manthei, 1959). It has subsequently been found to have a worldwide distribution with high serum antibody prevalences in adult animals (Bryson, 1990). PIV-3 was diagnosed in 11 of 47 and 15 of 17 BRD outbreaks in Northern Ireland in 1978 and 1998, respectively (Bryson *et al.*, 1978a; Graham *et al.*, 1999), and PIV-3 infections occurred in replacement animals in 19 of 19 Dutch dairy herds during the summer and autumn of 1979 (Verhoeff & van Nieuwstadt, 1984a).

Humoral immune responses

PIV-3 specific serum IgG is detected about day 8-9 and IgA about day 12 after experimental PIV-3 infection. Serum IgM can be detected from day 7 and is also detectable after experimental re-infections with short intervals (Graham *et al.*, 1999). A four-fold increase in serum IgG or a long-lasting IgG antibody titre is not always seen after infection in calves with maternal antibodies and this phenomenon impairs the serological diagnosis of PIV-3, as for BRSV (Bryson *et al.*, 1978a; Graham *et al.*, 1999).

Bovine coronavirus (BCoV)

Viral characteristics

BCoV is a group II member of the *Coronavirus* genus in the Coronaviridae family, order Nidovirales. Its virions are enveloped, containing non-segmented, positive-sense, single-stranded RNA genomes incorporated in helical nucleocapsides. The spike (S) and hemagglutinin (HE) surface proteins are important for attachment and entry into cells and the genomic RNA is directly translated into an RNA polymerase. This then synthesises a full genome-length negative-sense strand, from which sets of mRNA are transcribed with different length and a common 3'end. Replication occurs in the cytoplasm of the cell and assembly occurs by budding at the membranes of the endoplasmatic reticulum and the Golgi apparatus. Virus is released by secretory mechanisms of the cell, by cell-to-cell fusion and by lysis of cells (Clark, 1993). The primary site of replication is epithelial cells in the distal small intestine, in colon and in the respiratory tract (Reynolds *et al.*, 1985; Saif *et al.*, 1986).

Clinical signs

BCoV has been associated with calf diarrhea since the early 1970s (Stair *et al.*, 1972) and is now also generally accepted as a major causative agent of winter dysentery in adult cows (Saif, 1990). Infections in both calves and adults implicate to varying degrees respiratory clinical signs in addition to diarrhea (Alenius *et al.*, 1991; Tråvén *et al.*, 2001). The faeces, and sometimes also the respiratory secretions (unpublished observations) may contain blood (Espinasse, Savey & Viso, 1990; Kapil & Basaraba, 1997).

BCoV is involved in shipping fever and has been significantly associated with BRD (Storz *et al.*, 1996; Kapil & Basaraba, 1997; Storz *et al.*, 2000a; Plummer *et al.*, 2004). Fatal respiratory disease with large amounts of virus in lungs have been reported from calves in feedlots (Storz *et al.*, 2000b). Although most BCoV isolates produce only enteric or combined enteric and respiratory signs when inoculated experimentally (Reynolds *et al.*, 1985; Kapil *et al.*, 1991), it has been suggested that BCoV strains vary in tissue tropism (Lin *et al.*, 2000). However, results from animal experiments with strains obtained from animals with respiratory or enteric clinical signs do not consistently support this theory (Cho *et al.*, 2001). Host factors, such as age, production state or diet, may contribute to the fact that respiratory disease is more pronounced in certain animals. The same

strain may cause disease in both seronegative calves and adults (Tråvén *et al.*, 2001) and cows are probably immune to the virus in herds with problems only in young individuals (Tråvén, 2000).

Epidemiology

BCoV is widespread in many countries with high seroprevalences in adult cattle (Paton *et al.*, 1998; Tråvén, Bjornerot & Larsson, 1999; Hasoksuz *et al.*, 2005; Jeong *et al.*, 2005). Winter dysentery is mainly seen during the winter in temperate climate zones and the virus has been suggested to persist between seasons in healthy carrier animals (Crouch *et al.*, 1985; Collins *et al.*, 1987). However, the diagnostic assays in these studies have been questioned and the results need to be confirmed (Saif, 1990). Supporting the theory about latent infections, BCoV inoculated experimentally has been detected in intestinal crypts for 21 days (Kapil, Trent & Goyal, 1990). In other experiments, shedding was terminated by the latest 19 days after experimental infections (as determined by PCR) suggesting that the infection is self limiting (Cho *et al.*, 2001).

Humoral immune responses

BCoV-specific serum and milk IgG is detectable from day 9-11 after experimental BCoV infection of seronegative animals (Tråvén *et al.*, 2001) and remain so at least one year after natural infections without reinfections (Alenius *et al.*, 1991). BCoV-specific IgM increases from day 2-7 and remains detectable during 3-6 weeks, whereas IgA can be detected from day 7-9 and during several months, as determined by isotype capture ELISAs (Tråvén *et al.*, 2001). Passively derived antibodies decrease or delay the active antibody response to infection in calves (Heckert *et al.*, 1991).

Vaccination

Live vaccines

Live vaccines against respiratory infections in cattle are based on virus strains that have been attenuated by extensive passages in cell culture (Wellemans, 1990). When administered intramuscularly, these viruses replicate only to a very limited extent at the site of injection and probably never reach the respiratory tract (Stott *et al.*, 1984). Intranasal administration on the other hand, lead to replication, but possibly also to spread to unvaccinated controls (Bryson *et al.*, 1999).

It is recommended to administer live vaccines only to healthy individuals in order not to exaggerate the clinical signs of an eventual concurrent infection (Kimman *et al.*, 1989). Moreover, the storage is delicate because the vaccine strains may be inactivated by heat, freezing or needle disinfectants and freeze-dried products have a short life-span once a dissolvent has been added. Maternal antibodies suppress the humoral response to vaccination and immunization during this period gives a poorer response (Stott, Thomas & Taylor, 1986; Kimman, Westenbrink & Straver, 1989). Other shortcomings are the risk of reversion to pathogen forms and spread

as well as spread of adventitious contaminating pathogens, such as BVDV (Stott *et al.*, 1984; Barkema *et al.*, 2001).

Most field studies on the efficacy of vaccines lack good experimental design and formal analysis of data (Martin, 1983; Perino & Hunsaker, 1997), but some live vaccines against BRSV and BRSV/BHV-1/BVDV (Rispoval and Rispoval 3, SmithKline Beecham Animal Health) have shown significant protection in blind field trials (Verhoeff & van Nieuwstadt, 1984b; Frankena *et al.*, 1994). Likewise, a live BCoV/bovine rotavirus (BRV) vaccine (Calf-guard, Pfizer) induced a significant reduction of BRD in calves seronegative for BCoV in a randomized blind study with natural BCoV infections (Plummer *et al.*, 2004).

Inactivated vaccines

Inactivated vaccines, which are virus strains treated with a virus-inactivating agent and mixed with an adjuvant, are safer with regard to innocuity and are less sensitive to storage. During the inactivation, however, the surface proteins may be deformed and may give rise to poor, non-protective and even allergic immune responses to a subsequent infection, with poor cytotoxic T-cell activation. This vaccine-induced phenomenon have been suggested as the cause of enhanced disease upon HRSV infection in children (Openshaw, Culley & Olszewska, 2001) and upon natural (Schreiber *et al.*, 2000) and experimental BRSV infection in calves (Gershwin *et al.*, 1998; Antonis *et al.*, 2003). The BRSV vaccine that was believed to cause enhanced disease upon natural infection also failed to protect calves against BRSV two months after vaccination in other herds and was consequently withdrawn from the market in 1998 (Larsen, Tegtmeier & Pedersen, 2001). It is noteworthy that inactivated BRSV vaccines are annually sold in a large number of doses and that disease enhancing effects have only been reported once from a field situation. Another, perhaps larger hinder to the success of the inactivated vaccines is that, like for the live vaccines, the presence of maternal antibodies may suppress the humoral response to vaccination. The short duration of immunity of some vaccines (Larsen, Tegtmeier & Pedersen, 2001) may lead to indefensibly expensive re-vaccination schedules for an optimal effect.

Nevertheless, significant protection was obtained in blind field trials on young calves immunised with a inactivated vaccine against BRSV, PIV-3 and *Mycoplasma spp.* (Torvac, C-Vet Veterinary Products) (Howard *et al.*, 1987; Stott *et al.*, 1987). The BRSV component in this vaccine is based on glutaraldehyde-fixed cells persistently infected with BRSV. This component also induced significant virological protection against a subclinial experimental infection in calves with maternal antibodies against BRSV (Stott, Thomas & Taylor, 1986). Another inactivated vaccine, against BCoV, BRV and *E. Coli* K99 (Lactovac, Hoechst), resulted in significantly higher antibody titres in calves during the first 3-4 weeks of life when administered to cows in the last trimester. The clinical effect on calf health, however, has not been thoroughly documented in peer-reviewed journals (Waltner-Toews *et al.*, 1985; Kohara *et al.*, 1997). No respiratory vaccine had been licensed in Sweden until 2004, when an inactivated vaccine against BRSV, PIV-3 and *M. haemolytica* was launched (Bovilis

Bovipast® vet., Intervet). To the authors' knowledge, the efficacy of this vaccine has not been evaluated in published field trials.

Immunostimulating complexes (ISCOMs)

Like Torvac in calves, subunit immunostimulating complexes (ISCOMs) have also shown promise in animals with maternal antibodies (Osterhaus, van Amerongen & van Binnendijk, 1998). This antigen-delivery system has been used experimentally in a variety of species and for a range of antigens (Sjolander *et al.*, 2001; Morein, Hu & Abusugra, 2004). Virus ISCOMs are built up of viral proteins, often surface proteins, that are incorporated in ISCOM particles of virus-like sizes (40-100nm), with inbuilt adjuvance. Spherical particles form spontaneously when cholesterol and phospholipids are added to viral antigens and Quil A adjuvant (Morein *et al.*, 1984). Quil A is a semi-purified preparation of *Quillaia saponins* from the bark of the tree *Quillaia saponaria* Molina. This adjuvant is commonly used in other inactivated vaccines for animals, but has fewer side-effects (local inflammation, hemolysis) and induces stronger T-cell responses when administered as ISCOMs (Sjolander *et al.*, 2001). Lower Quil A doses are required when using ISCOMs, compared to other systems where the adjuvant is not physically linked with the antigen (Barr & Mitchell, 1996).

The close association between purified antigens and Quil A in ISCOMs is thought to be important in the priming of immunity, possibly due to uptake of antigen and adjuvant by the same antigen presenting cells (APCs). ISCOMs enhance cytokine production and expression of MHC class II on APCs and stimulate the targeting of antigens to lymphoid organs. In addition, they induce strong humoral responses with low antigen doses (Sjolander, Cox & Barr, 1998). The adherence of ISCOMs to cell membranes is probably reflected by the high affinity of Quil A to cholesterol (Barr & Mitchell, 1996). Commercially available ISCOM vaccines exist against equine influenza, BVDV and equine gonadotropin-releasing hormone and several vaccines against human viruses are in phase I and II clinical trials (B. Morein, personal communication). Furthermore, experimental ISCOM vaccines against BVDV and BHV-1 have generated very good protection when evaluated in sheep and cattle (Trudel *et al.*, 1988; Carlsson, Alenius & Sundquist, 1991; Merza *et al.*, 1991). One obstacle to commercial virus-ISCOM production is the requirement of large amounts of virus of high titre for purification, if the proteins are not artificially produced.

Diagnosis

The laboratory diagnosis of viral diseases can be made directly, by detection of virus, or indirectly, by detection of virus-specific antibodies.

Direct laboratory diagnosis

Commonly used techniques in direct detection of BRSV, PIV-3 and BCoV are isolation of virus in tissue culture, immune based detection of antigens in histological sections or body secretions (immunohistochemistry or ELISAs) and detection of viral RNA by classic or real-time PCR. Whereas the window of

detection for the former techniques is only a few days after the onset of clinical signs, the PCR can detect viral RNA during two weeks in optimal cases (Larsen *et al.*, 1999; Valarcher *et al.*, 1999; Cho *et al.*, 2001). There are also other techniques, such as electron microscopy and haemadsorption, which are more used for research purposes than for routine laboratory diagnosis today.

Virus isolation in tissue culture

A classic method to directly detect BRSV, PIV-3 and BCoV is to isolate virus in tissue culture. Difficulties of this approach might contribute to an underestimation of the frequency of infections, in particular the type of cells chosen for culture. BRSV and PIV-3 are preferably propagated on primary bovine embryonic turbinate (Elvander *et al.*, 1998; Valarcher *et al.*, 1999), lung (Bryson *et al.*, 1999; Tjornehoj *et al.*, 2003) or kidney cells (Taylor *et al.*, 1995; Adair *et al.*, 1999), whereas a human cell-line (human rectal tumour cells, HRT-18) is often required for isolation of BCoV (Storz *et al.*, 2000b). The lability of RNA viruses is problematic because virus isolation depends on viable virus upon admission to the laboratory. Although the method is sensitive under suitable conditions, the number of days after infection when isolation is possible is limited. Detection of virus in cell culture is based on cytopathogenic effects, such as lysis of cells (BRSV, PIV-3 and BCoV), syncytia (BRSV, sporadically also for PIV-3 and BCoV); intracytoplasmatic inclusions (BRSV and PIV-3) and verification by immune based antigen detection (Wellemans, 1990; Viuff *et al.*, 1996). Virus isolation always implies a risk of amplification of co-infections that are better gained by the culture conditions than the actual pathogen. One main benefit, however, is the guarantee that the animal was infectious at the time of sampling. This cannot be assured with the techniques described below.

Immune based detection of antigens

Immune staining is widely used for antigen detection in tissue samples and body fluids. In these assays virus-specific antibodies (*e.g.* polyclonal antibodies from a hyper-immunised animal or MAbs) are allowed to attach to antigen in the bovine sample and are visualised by enzymatic reactions or by fluorescence (Viuff *et al.*, 1996). Virus-specific antigen (ag) ELISAs work in a similar way, except that virus in the sample attaches to wells coated with virus-specific MAbs before the antibodies are added. Rapid pen-side antigen test-kits are available for the detection of BRSV (Valarcher *et al.*, 1999).

PCR

The classical polymerase chain reaction (PCR), which amplifies DNA in a specific manner, has improved the sensitivity of the direct diagnosis of viruses dramatically (Belák & Ballagi-Pordány, 1993a). This technique relies on the design of nucleotide primers that flank a conserved region in the virus genome and its complementary sequence. After isolation of RNA and generation of complementary DNA (cDNA) by RT, the primers are allowed to hybridize to opposite strands in the cDNA. The cDNA denaturates and primers anneal and extend at different temperatures so that new templates are formed. By repetitive thermocycling, amplicons of similar size accumulate, which are visible by eye after processing in an agarose gel and staining. The high sensitivity of this assay is also a weakness because complicated laboratory routines are required to avoid

sample contamination and amplification of contaminating agens (Belák & Thorén, 2001). The classical gel-based PCR is labour intensive, which makes it expensive.

Real-time PCR is a refinement of the classical PCR method, in which the post PCR processing is omitted. The real-time PCR assay can provide a high sensitivity and requires therefore only one round of amplification, which further reduces working time and decreases the risk of contamination of samples. The real-time PCR is based on a more objective detection of amplicons than the classical PCR and it can be used to quantify viral RNA in the original sample (Boxus, Letellier & Kerkhofs, 2005). It also allows the simultaneous analyses of up to 96 samples. A range of different systems is available to detect amplicons in real-time (*e.g.* TaqMan®, molecular beacon, CYBR Green and Primer-Probe energy transfer), all of which generate a fluorogenic signal that can be measured in each cycle. Instead of gel-based visualisation of the end product, as in the classical PCR, amplicons are objectively quantified in "real-time" by a computer. In the TaqMan® system, a probe, labelled with a dye (emitting light) and a quencher (absorbing light), anneals between the primers on the cDNA strands. When a Taq polymerase elongates amplicons between the primers, the probe is released in fragments so that dye and quencher separate. This gives rise to the measurable fluorogenic light.

Indirect laboratory diagnosis

The indirect laboratory diagnosis is based on detection of virus-specific antibodies in body fluids. Although this approach does not precisely determine the time of infection, it is applicable for a long period (months to years) after the few days of viral shedding in the case of a transient infection. The diagnostic sensitivity is therefore greatly improved. Seroconversion between paired samples can be used to estimate when the infection occurred (see humoral immunity sections). Adult animals with high levels of virus-specific IgG and young animals with high levels of maternally derived IgG1 do not always show titre rises after infection, but a transient rise in IgM can sometimes be detected in the latter case (Kimman *et al.*, 1987). For IgG analysis, paired samples should be obtained during the first week after the onset of clinical signs and again three to four weeks later, preferably from 6- to 7-month-old calves (or older animals), which have lost their maternally derived antibodies. For IgM analyses, sera are where possible obtained from the same age group, within the first or second week after the onset of clinical signs. Three commonly used antibody assays are listed below (serum neutralization test, and indirect and capture ELISAs). Others are for example haemagglutination-inhibition assays and blocking ELISAs.

Serum neutralization tests

Neutralizing antibodies are detected by adding dilutions of sera to a viral suspension of a defined concentration (or vice versa, *i.e.,* varying virus concentrations mixed with a constant serum concentration) and by inoculating these mixtures onto susceptible cell cultures. The highest dilution of antibody that protects the cells against destruction from viral infection represents the titre (Murphy *et al.*, 1999). The virus growth can be quantified by several means, *e.g.* by observation of cytopathic effect (CPE) by microscopy, or by a plaque-forming assay, in which a solid or semi-solid overlay is added to define viral growth into a

number of plaques. In the plaque-forming assay, the titre is calculated based on a reduction of plaques in comparision with virus controls (Kennedy *et al.*, 1988).

Indirect and capture ELISAs

Virus-specific antibodies in the sample adhere to viral antigens coated in the plastic wells of indirect ELISAs. By adding antibodies directed to the Fc part of bovine immunoglobulins (*e.g.* monoclonal antibodies against bovine IgG) and conjugated with an enzyme (*e.g.* horseradish peroxidase), the presence of virus-specific antibodies in the original samples may be visualised by a substrate (*e.g.* tetramethylbenzidine in H_20_2) and the reaction stopped by sulphuric acid (Portelle *et al.*, 1984; Elvander *et al.*, 1995).

In capture ELISAs, wells are coated with isotype-specific monoclonal antibodies against the Fc parts of bovine immunoglobulins. Thereby all antibodies of a specific isotype in the sample adhere to the well, regardless of antigen specificity. By adding viral antigen and conjugated antibodies against the antigen, virus-specific antibodies of the addressed isotype are visualized in the sample, as in indirect ELISAs (Uttenthal *et al.*, 2000).

Aims of the study

The first part of this thesis deals with the incidences and infection dynamics of BRSV, PIV-3 and BCoV in Swedish cattle herds. The second part focuses on the development of a diagnostic method to detect BRSV and the evaluation of a candidate vaccine as well as a commercial vaccine against this virus *in vivo*. The specific aims were to:

- Investigate seroprevalences and incidences of BRSV, PIV-3 and BCoV a) in calves in 118 dairy herds in one area of Sweden and b) in calves arriving at a bull testing station from 99-124 Swedish beef-breeding herds, in each of six years.
- Describe BRD treatment rates in the bull testing station.
- Establish a fluorogenic RT-PCR (fRT-PCR) to detect BRSV in clinical samples.
- Evaluate the immunogenicity and protection induced by BRSV immunostimulating complexes (ISCOMs) and a commercial inactivated BRSV vaccine (CV) in calves, by using an experimental BRSV challenge.

Materials and methods

This section contains a brief explanation of materials and methods used in Paper I-IV and some methodological considerations. More attention is given to methods that are not described in detail in the papers.

Animals, herds and study design

Paper I

Paper I comprised 354 heifer calves in 118 dairy herds, selected as described previously (Svensson *et al.*, 2003). Briefly, herds in one area of south western Sweden were included in the study on the basis of size, rearing systems of heifer calves and the farmer's will and capability to participate in the investigation. The herds represented Swedish dairy herds affiliated to the official eradication programme for BVDV and that had between 28 and 94 (median 48) cows. Three heifer calves per herd were selected based on a recorded history of respiratory disease, as described in detail in Svensson *et al.* (2003). In herds where no calves fulfilled the criterion of disease (n=15), animals were randomly selected.

Blood samples were collected from the 354 calves when they were median 7 months old, during March to May 1999. Additional samples were obtained from 345 of these animals at median 15 months of age. The sera were analysed for IgG antibodies specific to BRSV, PIV-3, BCoV and BVDV. Information was collected through interviews with farmers on: i) estimated air-distance to the closest cattle-rearing herd, ii) purchase of animals during 1998-2000, iii) co-pasturing with animals from other herds between samplings and iv) estimated number of visitors during an average month.

Paper II

Animals at a Swedish bull testing station were monitored for viral infections and BRD antibiotic treatments during six years. Each year in August (1998-2003), 149-185 4- to 8-month-old calves arrived at the station from 99-124 different beef-breeding herds and stayed until March the following year (970 cattle in total). Only calves tested free from BVDV were allowed to enter the station and original animal groups were kept isolated from cattle three weeks before admission. Neither prophylactic antibiotic, nor BRD vaccines were used. The animals were kept in 10 semi-outdoor pens generously bedded with straw, in a barn with 3 walls. The same veterinarian started all treatments throughout the study.

Blood samples were collected on arrival at the station (August), in November and by the end of January. Additional sampling was performed in October 2002 and 2003 and also during outbreaks of respiratory disease or diarrhea. Analyses for BRSV, PIV-3, BCoV and BVDV IgG antibodies were performed on sera from all animals in 98/99 and 99/00, and on sera from a selection of animals from 00/01 and on (including animals from all pens).

Paper III

A BRSV fRT-PCR, based on the TaqMan® principle, was developed and evaluated on clinical samples. TaqMan® was chosen because this probe is less sensitive to point mutations than for example molecular beacons (M. Hakhverdyan, personal communication).

Samples for evaluation of the fRT-PCR originated from a number of sources. Twenty-eight lung samples derived from Danish BRD field cases and were previously tested with ag ELISAs. Moreover, 34 lung samples derived from Danish BRSV challenge experiments and were previously tested with ag ELISAs and BRSV conventional RT-PCR. Furthermore, 28 nasal swabs from the experimental study in Paper IV were used, which had previously been tested with BRSV nested RT-PCR and/or virus isolation. Eleven swabs (and paired sera) from three Swedish dairy herds with outbreaks of BRD, nine BRSV strains and eight symptomatically or genetically related virus species were also tested.

The oligonucleotide primers (19 and 25 nucleotides) and probe (34 nucleotides) against BRSV were designed from the published sequence of the F gene of isolate 394 (GenBank accession No. AF188554) and the predicted product size of the BRSV amplicon was 85 base pairs. Compatible primer sets were selected to ensure efficient amplification and detection of BRSV isolates from all six phylogenetic subgroups (Valarcher, Schelcher & Bourhy, 2000). The fRT-PCR was optimised by titration series of primers, probe and Mn^{2+}. Analyses were carried out on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

A ten-fold dilution series of a BRSV positive RNA template (three dilutions, five replicates of each dilution) were used to generate a standard curve and to calculate the reaction efficiency. In addition, the detection limit of the fRT-PCR was compared to that of nested RT-PCR, by testing nine steps of a ten-fold dilution series of a BRSV strain (No. 9402022) (Viuff *et al.*, 1996). The reproducibility of the fRT-PCR was assessed by testing 62 samples in duplicate.

Paper IV

For the challenge experiment in Paper IV, 14 conventionally reared Swedish Red and White breed male calves were obtained from a closed dairy herd, certified as free from BVDV infection under the Swedish control programme on BVDV. The calves were matched into three groups according to age and BRSV-specific serum IgG1 levels. Two groups of 5 calves each were immunised subcutaneously (s.c.), twice with three weeks interval, with either an experimental subunit BRSV vaccine (ISCOMs) or with a commercial inactivated BRSV vaccine (CV). Four calves were left as unvaccinated controls. The age of the calves at the day of first immunization ranged between 52 and 105 days and the mean age in each group was 78 (ISCOM), 85 (CV) and 86 days (controls). The mean maternally derived BRSV-specific serum IgG1 titres were 3.6 $\log_{10}$ for both vaccine groups and 3.7 $\log_{10}$ for the controls. After arrival at the Dept. of Ruminant Medicine and Veterinary Epidemiology, animals were kept group-wise in three pens divided by floor-to-roof walls.

Three weeks after the second immunization, all calves were challenged with 3 x $10^{4.5}$ $TCID_{50}$ of a seventh passage BRSV strain (no. 9492922, Denmark) (Viuff *et al.*, 1996). The inoculum was administered by aerosol inhalation, essentially as described earlier (Tjornehoj, 2000), except that no intra-tracheal injections were made. Virus inoculated foetal bovine lung cells were harvested with cell scrapers and treated with ultrasound. Visual examination in light microscope after treatment revealed many rounded, separated cells and that some cells were lysed. One aliquot were kept in the inoculation facilities during the inoculation and was thereafter titrated, in order to assure the virus stability throughout the inoculation.

A veterinarian, blinded as to treatment group, performed daily clinical examinations and nasal sampling for virus detection, from the day of challenge (day 0) to day 10. Sera were obtained on 14 occasions throughout the study and were analysed for BRSV-specific antibodies of several isotypes. Sera from day 30 was additionally analysed for BVDV-specific IgG antibodies.

Virus detection

Virus isolation

Isolation of BRSV from experimentally infected animals was attempted in Paper IV and these samples were also used as a part of the evaluation of the fRT-PCR in Paper III. Nasal cotton swabs in 1 ml F-DMEM, containing 20% gamma-irradiated foetal bovine serum were collected on day 3, 4 and 5 after infection and were immediately analysed, on a daily basis. Day 3, 4 and 5 were chosen because high levels of virus shedding were predicted on these days, thus increasing the probability of getting positive results from immunised animals. Because freezing might result in loss of virus infectivity (K Tjornehoj, doctoral thesis 2000), virus isolations were performed with fresh material filtered through a 45-µm filter onto a large cell surface (25cm^2). Virus isolations and PCR were performed on different swabs from the same animal (Virocult®, Medical Wire and Equipment Co Ltd. England, for PCR and cotton swab in medium and 20% FBS for virus isolation) and the swabs used for PCR were not filtered. It is possible that some infectious virus was lost during the filtration of sample material; however, it is more likely that results from samples negative in virus isolation and positive in PCR simply reflected a higher sensitivity of the PCR.

Antigen ELISA

A BRSV-specific ag-ELISA was used in Paper III, as previously described (Uttenthal, Jensen & Blom, 1996). Briefly, wells were coated with bovine anti-BRSV Ig extracted from hyperimmune sera or Ig from BRSV antibody negative sera (controls). After incubation of samples, biotinylated bovine hyperimmune anti-BRSV Ig, peroxidase-conjugated avidin and orthophenylenediamine substrate were added. The reaction was stopped with sulphuric acid. Plates were washed between each step.

RT-PCR

Precautions for contamination

Routine precautions and safety measures to avoid contamination of samples in our laboratory are described elsewhere (Belák & Ballagi-Pordány, 1993b). Briefly, five different rooms were used for i) handling of clean stocks ii) preparation of RNA and cDNA iii) performing PCR iv) preparation of nested PCR and v) gel-electrophoresis. Different coats, sample racks and pipettes were used in all rooms and gloves, racks and samples were never taken between rooms against the flow system. Disposable tubes were autoclaved before use and opened with opening tools, which were kept in 10% chloride between uses. Laminar flow cabinets were cleaned with chloride and exposed to UV radiation and aerosol-resistant tips were used at all steps.

RNA isolation for nested and fluorogenic RT-PCR

Total RNA was extracted from nasal swab media, faeces diluted in PBS or homogenated tissue. In brief, 250 µl sample material were mixed with 750 µl TRIzol LS Reagent (Invitrogen, USA; containing phenol and guanidine isothiocyanate) and incubated for 5 min at room temperature (to lyse cells and to disrupt nucleoprotein complexes, under inhibition of RNAse activity). Thereafter, 200 µl chloroform was added, mixed and centrifuged at 12 000 x g (Sigma, Germany) for 15 min (to separate nucleic acids from viral and cellular proteins). The aqueous phase was transferred to a new tube with 500 µl isopropanol and incubated overnight at -20°C (for RNA precipitation). After centrifugation at 12 000 x g for 20 min at 4°C and washing with 1 ml of -20°C 80% ethanol, the pellets were air dried, dissolved in 30 µl of dimethylpyrocabonate (DMPC) water and stored at -20°C.

Reverse transcription for nested PCR

Complementary DNA (cDNA) was constructed from 5 µl RNA in 5 µl DMPC water, by first denaturating RNA aggregates in the presence of 1 µl random hexamers (0.02 U), for 10 min at 65°C and cooling on ice. Thereafter, the hexamers were extended for 90 min at 37°C by 1 µl Moloney murine leukaemia virus reverse transcriptase (200U), in the presence of 2.5 µl deoxynucleotide triphosphates (dNTPs, 2mM), 5 µl 1st standard buffer and 1 µl RNAse inhibitor (RNAguard, Amersham Biosciences). The enzyme was then inactivated for 5 min at 95°C.

Nested RT-PCR

Nested BRSV and BCoV RT-PCR were used in Paper II, III and IV. As previously described (Vilcek *et al.*, 1994), these assays amplified conserved regions of the F protein coding part of the BRSV genome (711 and 481 basepairs, bp) and of the HE protein coding part of the BCoV genome (560 and 407 bp).

Briefly, the PCR mix contained 24 µl DMPC water, 5 µl 10xPCR buffer (Perkin-Elmer), 1 µl dNTPs (10mM), 5 µl BSA, 1.5µl of each primer, 5 µl $MgCl_2$ (25mM), 2 µl Taq DNA polymerase (Ampli Taq, Perkin-Elmer, diluted 1:10) and 5 µl of cDNA. Two drops of mineral oil were added to overlay the reaction mix. The thermocyling profile included initial denaturation at 94°C for 2 min, followed by

35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 60 s, and extension at 72°C for 90 s, followed by a final extension at 72°C for 7 min. An internal primer set was added for a second PCR and each cDNA amplicon was further amplified as above. The PCR products were processed by gel electrophoresis (for verification of the size of amplicons) and visualised by ethidium bromide under UV light (Vilcek *et al.*, 1994). A conventional BRSV RT-PCR was used for comparative studies in Paper III, as previously described (Larsen *et al.*, 1999).

Antibody detection

Serum neutralisation tests

Neutralising antibodies were detected by a plaque reduction assay, in which dilutions of deconjugated sera were mixed with a viral suspension (50-100 plaque forming units, PFU, Snook strain) and incubated for 1 h at room temperature. The mixtures were thereafter inoculated onto foetal calf kidney cells and incubated during 1 h at 37°C (in 6-well plates, samples were run in duplicate). After removal of the virus/serum mixtures, the cells were overlayed with medium including 2% agarose. Viral growth was defined into a number of plaques after 7 days inoculation at 37°C, fixation and staining (Kennedy *et al.*, 1988). The endpoint titres were calculated by linear regression analyses and determined as the dilutions that produced a 50% reduction in the mean number of plaques compared with virus control wells.

Indirect IgG ELISAs

Commercially available indirect ELISAs (SVANOVA Biotech®, Sweden) were used to detect BRSV, PIV-3, BCoV and BVDV-specific IgG in Paper I-IV. A single dilution of samples were analysed (1:25, milk undiluted) and the cut-off set to a corrected optical density (COD) value of 0.2 (milk 0.05) at 450nm, as previously described (Niskanen *et al.*, 1989; Alenius *et al.*, 1991; Niskanen *et al.*, 1991). Seroconversions were defined as a conversion from a negative to a positive COD value in paired samples. When investigating serum antibodies in dairy calves in Paper I, an animal was considered infected if it was seropositive in a sample obtained at ~7 months of age and remained positive at ~15 months of age. This definition was set to exclude animals with remaining maternal antibodies at the first sampling, which then decreased to negative. Herds were considered infected if at least one sampled animal was infected according to the above definition.

Because the IgG subtype specificity of the commercial indirect ELISAs was unclear, additional indirect BRSV-specific IgG1 and IgG2 ELISAs were used for the vaccine evaluation in Paper IV. Briefly, the BRSV Snook strain (Thomas *et al.*, 1998) and control antigen (calf kidney cell lysate) were diluted with deionised water (1:50), were added to a 96-well immunoassay plate (50 µl/well) and were left to dry overnight at 37°C. After blocking of unspecific binding with 5% pig serum (ps) and between each incubation step, the wells were washed with PBS and 0.05% tween 20 (PBS/tween), four times. Sera were serial three-fold diluted with ps/PBS/tween (starting on 1:30), 50 µl of each dilution were added to control

and antigen wells and incubated at room temperature for 1h. One row was left as background control. After washing, MAbs against bovine IgG1 (B37) or IgG2 (B192), and thereafter horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma), were added and incubated at room temperature for 1 h each. Tetramethyl benzidine was used as substrate and the reaction stopped with 2M H_2SO_4. ELISA titres were calculated after subtraction of the OD with control antigen from OD with BRSV antigen and plotting of this COD against sample dilution. By regression analysis of the linear part of this curve, the end point was deduced at an OD that was 1.5 times the background.

IgA and IgM immunocapture ELISAs

For the vaccine evaluation in Paper IV, BRSV-specific IgA and IgM were analysed with immunocapture ELISAs. As previously described in detail (Uttenthal *et al.*, 2000), wells were coated with MAbs against bovine IgA and IgM (16-35 and 17-3) and sera added in single dilutions (1:20 in PBS/tween, 500µl/l). The BRSV strain 88Lu195 (Larsen *et al.*, 1998), biotin conjugated rabbit serum against HRSV, HRP conjugated avidin and ortho-phenylen diamine were further used for antibody detection. The immunocapture ELISAs were chosen because of a lowered risk of saturation of the antigen by the dominating antibody isotype (IgG), which is known to decrease the sensitivity of indirect ELISAs during detection of antibody isotypes in minority (*e.g.* IgM) (Graham *et al.*, 1999). The IgM assay has previously been used to detect BRSV serum antibodies from day 6-10 until day 16-17 after experimental infections and three weeks apart after natural infections, suggesting a high sensitivity (Uttenthal *et al.*, 2000). BRSV-specific serum IgA was only detected in one of three experimentally infected calves (day 9 to 26) and in two of six naturally infected calves, suggesting a lower sensitivity (Uttenthal *et al.*, 2000).

Data analysis

The χ^2 -test, a two-tailed Students *t*-test and the Fisher's exact test were used for statistical analysis.

Results

Virus epidemiology (Paper I and II)

Paper I

The seroprevalences of BRSV, PIV-3 and BCoV were investigated in young animals in 118 dairy herds in one area of Sweden. The observed data were generally homogenous in each herd, so that no or all sampled animals were seropositive to respective virus. In 70% (BRSV), 68% (PIV-3) and 82% (BCoV) of herds with one ~7-month-old calf seropositive for respective virus, all three sampled animals were positive. These figures rose to 80% (BRSV), 83% (PIV-3) and 97% (BCoV), when animals were ~15 months old.

The results showed a lowest seroprevalence for BRSV and highest for PIV-3. In total, 30% of the ~7-month-old calves were seropositive for BRSV, 48% for PIV-3 and 34% for BCoV. The incidence of infections between samplings was also lowest for BRSV, but highest for BCoV: seroconversions to BRSV, PIV-3 and BCoV occurred in 26%, 38% and 50% of seronegative animals (n=242, 177 and 228) and in 31%, 39% and 48% of negative herds (n=84, 56 and 77). Only six herds were classified as negative for all three infections and also BVDV in both samples. BCoV infections were statistically more common in BVDV positive than in BVDV-negative herds in the second sample (94% vs. 61%, P=0.01, Fisher's exact test). All herds had BRSV and BCoV antibody-positive bulk tank milk and 38% of herds had BVDV antibody-positive bulk tank milk in 2000.

No significant association was found between purchases of animals, co-pasture with animals from other herds or the estimated number of visitor per month. However, herds with an estimated air-distance of >1.0 km to the closest cattle-rearing herd were at significantly lower risk to be classified positive for BCoV at first sampling, compared to herds in more densely populated areas (P= 0.03, x^{2}-test). In addition, there appeared to be a geographical clustering of infected herds and of herds in which seroconversions occurred.

Of 18 herds, in which paired samples were obtained between March and August (outside the peak period when BRD is most often seen), seroconversions to BRSV, PIV-3 and BCoV were observed in 3 (17%), 4 (22%) and 1 (6%), respectively. A significantly higher proportion of herds in which no calves had a recorded history of respiratory disease (n=15) were classified as negative to the four infections at first sampling, when compared to herds in which disease was observed (60% *vs.* 14%, P=0.0002, Fisher's exact test).

Paper II

As observed in the dairy herds, the seroprevalence on arrival at the bull testing station from beef-breeding herds (*i.e.* reflecting infections in the original herds before sampling), was lowest for BRSV (37-46%), highest for PIV-3 (51-75%) and in-between for BCoV (39-65%). The seroprevalence for PIV-3 on arrival was significantly lower in 2002 than in 1998 ($P \leq 0.01$, x^{2}-test). All seronegative animals seemed to seroconvert concurrently when a virus was introduced at the

bull testing station. PIV-3 and BCoV infections occurred in 6 of 6 years whereas BRSV occurred only every second year (3 of 6 years). Thus, just as in the dairy herds, both the seroprevalence and incidence were relatively lowest for BRSV.

There was some evidence that the infections occurred outside winter period. PIV-3, and occasionally also BRSV and BCoV, occurred in the early autumn (between August and October or November) and some calves born in April were shown to be infected with BRSV and BCoV before arrival at the end of August.

BRSV was involved in an outbreak of BRD at the bull testing station during the winter 99/00, with high treatment rates and fatal disease. In contrast, when BRSV was introduced in August 2003 and between November and January 01/02, the treatment rates remained low. PIV-3 or BCoV was never associated with high BRD treatment rates, but seronegativity to either of these viruses was significantly correlated to BRD treatment in Charolais calves, during one year when BRSV was absent. Younger calves were at significantly higher risk of being treated during the BRSV outbreak (P=0.01, students t-test), but not throughout the whole study period. A difference in treatment rates for breeds was observed, with higher treatment rates for Aberdeen Angus and Hereford.

Fluorogenic RT-PCR to detect BRSV (Paper III)

The single tube BRSV fluorogenic RT-PCR (fRT-PCR) was shown to be a sensitive, specific and rapid tool to detect BRSV in both naturally and experimentally infected animals. Whereas all BRSV isolates and serologically confirmed field cases were tested positive, genetically or symptomatically related viruses, interspersed water controls and samples from healthy animals were all tested negative. Furthermore, samples that were tested in duplicate were in complete accordance, suggesting a good reproducibility.

The fRT-PCR detected four more positive samples than ag-ELISA, three more than conventional RT-PCR and two more than virus isolation. Only nested RT-PCR detected one more positive sample than fRT-PCR. When testing ten-fold dilution series of a BRSV cell culture isolate, however, the nested and the fRT-PCR had similar detection limits. By including rTth DNA polymerase, which functions both as a reverse transcriptase and a DNA polymerase, RT and PCR were performed in the same reaction tube in the fRT-PCR. The assay required less than 3 h to complete, compared to 7-8 h for the separate RT and nested PCR assays. In addition, no post PCR handling of products was required.

BRSV-ISCOMs as vaccine candidate (Paper IV)

Clinical protection

Whereas all four unvaccinated controls and all five calves immunised with the CV developed respiratory disease, none of the five calves immunised with ISCOMs were clinically affected after BRSV challenge.

The four unvaccinated controls with and without detectable maternal IgG1 antibody titres at the day of challenge (titres of 0, 2.5, 2.6 and 3.0 $\log_{10}$) developed severe disease, confirming the Danish infection model to be reproducible. Peak rectal temperatures and respiratory rates in these animals ranged between 39.5-41.2ºC and 80-120 min^{-1} (mean 40.3ºC and 97 min^{-1}), within six days after BRSV challenge. Two controls that showed pronounced abdominal breathing and that did not respond to antibiotic and anti-inflammatory treatments were euthanized on day 6 after challenge, for humane reasons, and BRSV was detected in their lungs (immunostaining and fRT-PCR). The two remaining controls developed marked lethargy along with respiratory signs that were still present by the end of the experiment (day 10). These animals showed BRSV-specific serum IgM and IgA antibodies from day 10 and 12.

The severity of the challenge was emphasized by inducing moderate to severe respiratory disease also in all five calves vaccinated with the CV. The peak rectal temperatures and respiratory rates in this group ranged between 39.4-40.8ºC and 60-100 min^{-1} (mean 40.0ºC and 78 min^{-1}) after challenge and the severity of disease appeared to be directly related to levels of maternally derived BRSV-antibodies at vaccination.

In sharp contrast to controls and animals vaccinated with the CV, the five ISCOM vaccinated animals remained healthy after challenge. They had peak rectal temperatures and respiratory rates ranging between 39.3-39.7ºC and 52-56 min^{-1} (mean 39.5ºC and 54 min^{-1}). These results show a strong clinical protection induced by ISCOMs. All calves were seronegative to BVDV on day 30, inferring that the challenge isolate was BVDV free.

Rapid humoral responses

Two doses of ISCOMs induced a 10 to 1000-fold increase of serum IgG1 in all animals within 32 days after the first vaccination (by the day of challenge, day 0). In contrast, two doses of the CV induced a 100-fold increase only in the animal with the lowest levels of maternal antibodies at vaccination. Two animals immunised with the CV showed less than a 10-fold increase and the two with the highest levels of maternal antibodies at vaccination did not show an antibody increase before challenge. Both nasal IgG and serum IgG of both subtypes were significantly higher in animals vaccinated with ISCOMs *vs.* CV on day 0 and 5. Furthermore, only the ISCOMs seemed to prime a rapid serum IgA response after challenge.

Virological protection

After experimental challenge, BRSV was re-isolated from the nasopharynx of all controls and from all calves immunised with CV, but from none of the calves immunised with ISCOMs. BRSV-RNA was detected by fluorogenic and nested RT-PCR from a single animal immunised with ISCOMs and from all animals in the other two groups. As measured with virus isolation, the virus shedding appeared to be directly related to maternal antibodies at vaccination in animals vaccinated with the CV.

Discussion

The most critical aspects in the control of BRD are probably the assurance of adequate intake of high-quality colostrum and improvement of other management and environmental factors that may impair the animals' immune responses to infections (*e.g.* hygiene, ventilation and nutrition). Another crucial factor is the animals' exposure to pathogens. Entry of new pathogens into herds should be reduced and susceptible young animals should be protected from exposure to large doses of different pathogens within the herds. Extensive knowledge on pathogen transmission and epidemiology is required to intervene in the transmission by management. When this information is insufficient and when the management is optimized without satisfactory results, vaccination with efficient vaccines could be an alternative in herds with substantial losses.

Before initializing costly vaccination programmes against bovine respiratory virus infections, it is important to understand their prevalence in the cattle population. The incidence rates of infections as well as the impact of the diseases on farmers finances and on animal welfare should be considered to compare costs and benefits with vaccination. There is also a need to objectively investigate vaccine efficacy in the target animal group against natural virus challenge. In addition, for any control strategy that aims to reduce the infections on a longer perspective (*e.g.* reduction of virus circulation by vaccination of strategic animal groups or by prevention of virus transmission through management), knowledge on the following is important: i) modes of virus transmission by direct and indirect routes, ii) virus survival in the environment, iii) natural inter-species transmission, iv) the importance of reinfections, v) virus latency and re-activation and vi) chronic infections. The data accumulated in this thesis give some valuable information on the appearance of BRSV, PIV-3 and BCoV in Swedish cattle, on BRSV diagnosis and on the efficacy of BRSV vaccination.

Virus epidemiology

Virus dynamics

Seroprevalences of infections were assessed in young animals in middle-sized dairy herds in one area of southwestern Sweden. In agreement with previous studies (Verhoeff & van Nieuwstadt, 1984a; Houe, 1992), the serological results in herds were extensively homologous, indicating that the spread of viruses within herds is efficient. As also previously suggested (Houe, 1992), this feature allowed data from a small number of animals to be interpreted as an infection status of each herd. The serological data on BVDV differed from the other viruses as data were less homologus in BVDV-positive herds, probably due to a less efficient spread of BVDV within herds. The spread of BVDV within herds was possibly also affected by early removal of PI animals within the control programme.

It became obvious that PIV-3 and BCoV had circulated in a large number of the dairy herds before samplings. In contrast, BRSV infections appeared to be less common. Serum antibodies of sampled animals roughly reflected infections in

herds during the lifetime of the animals (5-11 months), although some antibodies might have been maternally derived and although some infections obtained at early age might have been missed due to the suppressive effect of maternal antibodies on humoral responses (Kimman, Westenbrink & Straver, 1989; Heckert *et al.*, 1991; Graham *et al.*, 1999; Uttenthal *et al.*, 2000). Maternal antibodies to BRSV and BCoV have been observed to remain until on average 3.3 and 5 (max 7 and 6) months of age in dairy calves (Baker, Ames & Markham, 1986; Alenius *et al.*, 1991) and to BRSV and PIV-3 until on average 6.2 and 6.3 (max 7.3 and 8.5) months of age in suckling beef cattle (Fulton *et al.*, 2004). Thus, most of the maternal antibodies were assumed to have declined by the first sampling. High incidences of the infections were confirmed by frequent seroconversions between paired samples.

Seroprevalences of the infections were also assessed in 4- to 8-month-old calves originating from a number of beef-breeding herds throughout Sweden, on arrival at a bull testing station. As interpreted from serum antibodies on arrival, PIV-3 seemed to be more prevalent than BCoV and BRSV in the original herds, supporting the data from the dairy herds. At the bull testing station, the number of calves with antibodies to PIV-3 on arrival was significantly lower in 2002 than in 1998, which might have been an effect of bio-security measures within the national control programme against BVDV. Only 25% of beef herds that were registered in the programme in July 1998 were declared free from BVDV, as compared with 90% in July 2002 (Data obtained from the Swedish Dairy Association). Both PIV-3 and BCoV circulated annually at the bull testing station, whereas BRSV showed the lowest incidence; it circulated only every second year. This information complements previous nationwide studies on bulk tank milk, which show that BRSV and BCoV are widely spread in Sweden (Elvander, 1996; Tråvén, Bjornerot & Larsson, 1999).

Because the cattle density is lower in Sweden than in many countries and because some bio-security measurements might be higher due to our BVDV control programme, the incidences can be expected to be even higher in other parts of the world. It is difficult to interpret serologic data from countries where vaccination is commonly used; however, annual BRSV and PIV-3 infections have been observed in Dutch dairy herds (Verhoeff & van Nieuwstadt, 1984a; Van der Poel *et al.*, 1993) and 62-100% of 6- to 9-month-old beef cattle were seropositive to BCoV on arrival at feedlots in Canada and USA (Martin *et al.*, 1998; Plummer *et al.*, 2004). In addition, 100% of sampled Brittish herds that did not use vaccination (n=341) were BRSV and BCoV antibody positive in bulk tank milk (Paton *et al.*, 1998), compared to 41-89% (BRSV) and 70-100% (BCoV) of herds in nationwide Swedish studies (northern and southern parts of the country) during the 1990s (Elvander, 1996; Tråvén, Bjornerot & Larsson, 1999).

Disease

BRSV was previously shown to cause severe clinical signs of respiratory disease in Swedish cattle (Elvander, 1996) and this virus was also involved in an outbreak of BRD at the bull testing station during the winter 99/00. At this time, most of the animals in the herd had become seronegative for BRSV. Therefore, many

individuals probably shed large amounts of virus, which increased the infection dose for other susceptible herd mates. Interestingly, when the infection was introduced in August 2003 and between November 2001 and January 2002, the treatment rates remained low, confirming earlier observations that many BRSV infections are asymptomatic (Verhoeff & van Nieuwstadt, 1984a; Van der Poel *et al.*, 1993). Possibly, strains with different virulence were circulating in the different years and the plausible higher herd immunity and the climate in August 2003 influenced the virus dose to naive individuals. A dairy herd (herd A, Paper III) with naïve adult cows suffered from a clinical BRSV outbreak in August, suggesting that the season does not play a definitive role on the clinical outcome of BRSV infections.

High BRD treatment rates were not observed during periods when PIV-3 and BCoV infections occurred at the bull testing station. However, Charolais calves that were seronegative to either of these viruses on arrival were at significantly higher risk to receive BRD treatment, compared to seropostitive calves, during one year when BRSV was absent. Both PIV-3 and BCoV have previously been associated to BRD treatments (Stott *et al.*, 1980; Storz *et al.*, 2000a; Plummer *et al.*, 2004), and also, a significant association was obtained between absence of BRSV, PIV-3, BCoV and BVDV with absence of BRD in calves in the dairy herds. The disease caused by these viruses is probably more severe during harder environmental conditions, larger infection pressure (including additional infections) and lower herd immunity than during those studied at the bull testing station. It is important to further investigate the clinical impact of these infections, especially in Swedish fattening herds, which introduce young animals in a continuous manner.

Transmission

Based on the geographical proximity of infected dairy herds and on numerous infections in dairy herds that did not introduce new animals, the results indicated that indirect spread is an important way of virus transmission. Moreover at the bull testing station, BRSV and BCoV infections occasionally occurred without introducing animals. BRSV was probably introduced from outside, since cattle on a neighbouring farm showed similar disease prior to the outbreak. Undoubtedly BRSV and BCoV may spread rapidly over large areas and into herds without livestock trade (unpublished observations of outbreaks in herds with naïve adult animals, Sweden); nevertheless, this does not rule out latency and re-activation of virus in certain previously infected herds. It should be emphasized that the latter phenomenon has never been demonstrated. A significant association was obtained between herd air-distances and BCoV in the dairy herds, which may have been due to increased direct or indirect (human) contacts between neighbouring herds, of spread by small vectors (rodents, birds) or even by air. BCoV differs from BRSV and PIV-3 in that it is also shed in faeces and this is probably an important factor for its survival and transmission. Airborne spread between buildings have been described for SARS-CoV (Yu *et al.*, 2004) and porcine respiratory CoV (Stärk, 1999).

No significant association was obtained between the estimated number of visitors per month and dairy herd infection status in Paper I. However, all herds were visited by milk tanker drivers and all had at least one additional visit per month. A control group without visitors was thus not included. It is likely that the infections spread by humans (Wellemans, 1990; Callan & Garry, 2002) and outbreaks in closed herds are often preceded by visits (unpublished observations). It is therefore crucial to perform studies on how to prevent transmission by man. Data on this topic would be useful, especially for the protection of naïve herds in isolated parts of Sweden.

In contrast to BRSV and BCoV, PIV-3 virus was introduced on all six occasions when animals were commingled at the bull testing station in August. Quarantine procedures and commingling during summertime did not prevent the transmission of this infection. It appears therefore that PIV-3 is more easily transmitted than BRSV and BCoV, or circulates to a higher extent all year around. This is also in accordance with the different infection pattern observed in the dairy herds, where PIV-3 infected herds were distributed throughout the area, whereas BRSV and BCoV did not reach certain sub-areas. Indeed, PIV-3 also showed a higher prevalence.

Possible modes of prevention

Eradication

Several modes of prevention of viral infections in cattle are used in different parts of the world. The most drastic approach is eradication of pathogens, which currently is applied for BVDV in Scandinavia (Lindberg & Alenius, 1999). This method requires detailed information on virus transmission and epidemiology in the cattle population. Eradication of virus without knowledge on how to prevent transmission might be hazardous (if possible), considering the risk of re-introduction of virus. Keeping in mind that areas with cattle naïve to BRSV have suffered large losses from BRSV outbreaks (Inaba *et al.*, 1972; Elvander, 1996), eradication will not be an alternative for BRSV, PIV-3 or BCoV until extensive data on how to prevent their spread is produced. Such research would also be essential for the design of control strategies.

A key factor that distinguishes BRSV, PIV-3 and BCoV from BVDV, and that makes eradication programmes against these viruses less likely to become successful, is their efficient transmission (probably both directly and indirectly) by transiently infected animals. Hypothetically, the infection dose for BRSV, PIV-3 and BCoV is much lower than for BVDV since transiently BVDV infected animals do not appear to transmit the virus to susceptible pen mates. Moreover, airborne transmission of BVDV may be prevented by walls within a building (Niskanen *et al.*, 2000; Niskanen, Lindberg & Traven, 2002; Niskanen & Lindberg, 2003). BVDV is mainly spread by PI animals that acquire the infection *in utero* and that shed large amounts of virus throughout their life. As shown in the dairy herds in Paper I, the spread of BVDV between herds is low when these animals are identified and slaughtered; when pregnant animals are protected from contact with PI animals and when herds are prevented from introducing PI animals

or cows pregnant with PI animals (Lindberg & Alenius, 1999). Besides isolation of calves from new cattle, the application of virus and antibody detection before arrival at the bull testing station in Paper II was shown sufficient to prevent BVDV in this herd.

As an alternative to obligatory eradication programmes, attempts to prevent introduction of new infections can be made on a herd basis according to the wish of each farmer. When prevention is successful, established infections will most likely be self-cleared, as have been observed for BRSV, BCoV and BVDV (Alenius *et al.*, 1991; Elvander, 1996; Lindberg & Alenius, 1999). One important aspect, however, is how to protect these herds from new introduction of virus, since the herd immunity will decrease from the moment the infection is cleared. If costs do not exceed benefits, these animals should perhaps be protected by vaccination.

Vaccination

This section focuses only on vaccination against BRSV because it is largely involved in severe BRD in Swedish cattle. Until 2004, no BRSV vaccine had been licenced for use in Sweden, partly because published data on the protection induced by commercial vaccines against natural challenge was sometimes disappointing or uncertain (Martin, 1983; Perino & Hunsaker, 1997; Schreiber *et al.*, 2000; Larsen, Tegtmeier & Pedersen, 2001). Moreover, one Swedish field trial showed enhanced disease after vaccination with a commercial inactivated multivalent vaccine (PIV-3/ *M. Haemolytica*/ *P. Multocida*/ BVDV, S Alenius and M Törnquist unpublished observations).

The experimental BRSV model

By using an infection model that is previously evaluated in conventional calves with varying levels of maternal antibodies to BRSV (Tjornehoj, 2000), severe disease was reproduced in unvaccinated calves with and without maternal antibodies in Paper IV. A similar degree of disease has been published with other models, but only after challenge in seronegative calves (West *et al.*, 1999; Woolums *et al.*, 1999). Experimental challenge in calves with maternal antibodies has previously induced mild or no clinical signs (Belknap *et al.*, 1991; Patel & Didlick, 2004; Mawhinney *et al.*, 2005). To our knowledge, Paper IV is the first report of a BRSV vaccine evaluation against an experimentally induced severe disease in animals with maternal antibodies.

Protection against virus shedding and clinical signs

The most notable result in Paper IV was the absence of virus shedding after infection in 4 of 5 BRSV-ISCOM vaccinated calves as determined by PCR (5 of 5 as determined by virus isolation). Reduced virus shedding has been observed before for other vaccines administered to calves with maternal antibodies, but only after challenge causing mild or no disease and by virus isolation attempts (Stott, Thomas & Taylor, 1986; Patel & Didlick, 2004; Mawhinney *et al.*, 2005). Full protection against virus shedding determined by the more sensitive fluorogenic and nested RT-PCR, together with severe disease in controls, has, to the author's

knowledge, never before been demonstrated. This finding suggests that the ISCOMs can be used to stop virus circulation within and between herds. All calves vaccinated with the CV shed virus in the same experiment, as determined by both virus isolation and PCR.

Importantly, the clinical signs after challenge were also completely absent in calves vaccinated with BRSV-ISCOMs. Both virus shedding and the severity of signs in calves vaccinated with the CV appeared to be directly related to their maternal antibody status at vaccination and inversely related to antibodies at challenge. The two calves with highest levels of maternal antibodies at vaccination thus developed the most severe disease. Although the calf with lowest levels of maternal antibodies in the ISCOM group developed the strongest IgG and neutralizing antibody response, the ISCOMs overcame the suppressive effect of maternal antibodies on the response to vaccination in all animals. A disease enhancing effect was not observed.

The protection of single shots of live (Rispoval; Pfizer Animal Health) and inactivated (Bovipast; Intervet) vaccines in 4- to 5-week-old calves with maternal antibodies (lower levels than in Paper IV, as measured with the same ELISA), has been investigated by challenge three weeks after vaccination (Mawhinney *et al.*, 2005). A significant reduction of viral shedding was observed in calves immunised with the inactivated but not with the live vaccine. However, the clinical disease did not differ from controls (mild BRD). An experimental inactivated BRSV vaccine (Intervet UK Ltd), based on persistently BRSV infected bovine cells and administered at two and six weeks of age, also induced partial protection against virus shedding (as determined by virus isolation) after a challenge that induced mild BRD. When boostered again at 18 weeks of age, however, no virus was isolated after challenge at 43 weeks of age and clinical signs were significantly decreased (respiratory rates of 51-70 min^{-1} and dyspnea, but no fever, was reported in controls) (Patel & Didlick, 2004). This indicated a protective and rather durable response.

Practical implications for the commercial inactivated vaccine

Probably the CV investigated in this thesis induces partial protection against virus shedding and clinical signs in seronegative calves (at least shortly after vaccination). The effect in calves with maternal antibodies at vaccination is questionable, but since the vaccine is intended for use in calves from two weeks of age, controlled field studies should be performed to further evaluate its efficacy in this animal group. The challenge in Paper IV might be more severe than during natural infections, however, the challenge in some other published experimental trials is probably milder.

Practical implications for the experimental BRSV-ISCOMs

As an efficient inactivated subunit vaccine, the BRSV-ISCOMs would be of great use in Sweden and worldwide. The strong virological protection opens up possibilities not only to fully prevent clinical disease, but also to stop virus circulation within and between herds. For BRSV, this is probably unique. However, before coming forward as a candidate for large-scale production, further

evaluation needs to be done on younger calves, the duration of immunity and the safety of this vaccine. The results will be important for HRSV vaccine research. In addition, it is possible to use the ISCOMs as an experimental tool to find immunological markers involved in the protection against BRSV. This will affect the understanding of other viral infections in cattle.

Implications for future research

In parallel with an improved communication on calf management with our 'new', expanding farmers and their employees, I believe that it is important to further investigate the epidemiological patterns of BRSV, PIV-3 and BCoV. To prevent these infections without effective vaccines, we need to understand whether they are constantly introduced from outside the herds or whether they are able to remain in herds. Investigations on outbreaks where many or all animals are followed up serologically during several years after virus introduction are valuable and should be repeated in herds with different production systems. Such investigations in Swedish herds have previously indicated self-clearance of BRSV and BCoV (Alenius *et al.*, 1991; Elvander, 1996).

Virus sequencing data on repeated infections in the same herd should be obtained to elucidate whether new strains are introduced (even though sequence variation may be explained by virus evolution in latently infected animals). In such herds, it is interesting to repeatedly sample a selection of animals for virus detection to understand which age groups of animals are the main viral shedders and how long the virus can circulate in the herd. The fRT-PCR evaluated in this thesis could be used for this purpose since many samples may be analysed at low costs. These data would be precious so that practical advice could be given on how long herds should be kept under restrictions after the debut of disease, *e.g.* for sale of animals to other herds.

Moreover, sentinel animals could be used to understand how long the viruses can survive in the environment, in a similar manner that has been done for BVDV (Niskanen & Lindberg, 2003). Studies on HRSV have shown survival of this virus in the environment for about six hours, in quantities large enough for isolation *in vitro* (Hall, Douglas & Geiman, 1980). No such studies have been done for BRSV, but it can be assumed that the two viruses have a similar susceptibility. Perhaps the survival of BRSV will be shown to be much longer (days, weeks) if sentinel animals are used for its detection (*e.g.* detection of infections in sentinels by serology). To investigate indirect spread by man, different standardized cleaning procedures can be performed after visiting a donor herd with a BRSV or BCoV outbreak and before visiting sentinel animals in isolation facilities. Sweden is a suitable country to carry out such studies because several other infections are absent and infections can be studied with less risk of viral co-infections.

Because of the complex etiology of enzootic pneumonia, a simultaneous detection of several viruses and bacteria is desirable. Real-time PCR assays should be

designed and the protocols standardised so that several pathogens may be targed in a single PCR run. This will facilitate the diagnosis.

Other areas to which efforts should be concentrated are BRD vaccine development, as well as evaluation of treatments with non-steroid anti-inflammatory drugs (NSAIDs) and steroids. Commercially available vaccines, NSAIDs and steroids should be objectively evaluated in controlled field studies, in which the evaluators are blinded as to treatment group, animals are randomized to treatments and disease criteria are clearly defined. The BRSV-ISCOMs evaluated in this thesis should also be tested in the field after additional experimental studies on safety and duration of protection.

Concluding remarks

- BRSV, PIV-3 and BCoV were common infections in investigated Swedish beef and dairy cattle and were likely circulating throughout the year.

- Serological data in herds were homologous, indicating efficient spread of viruses within herds. This allowed retrospective investigations on virus presence in herds by sampling of a small number of animals.

- The geographical distribution of infected herds indicated transmission of virus between herds; moreover, infections in dairy herds that were closed with regard to animal introduction indicated possible indirect transmission of virus.

- A bull testing station was kept BVDV-free during six years, by testing animals before admission. Freedom of BVDV and BHV-1 probably reduced treatment rates after commingling of animals from a large number of herds. Differences in treatment rates were observed for different breeds.

- BRSV was involved in an outbreak of respiratory disease at the bull testing station, supporting earlier data on the clinical importance of this virus. Also, in the dairy herds, absence of BRSV, PIV-3, BCoV and BVDV was significantly correlated to absence of disease, enforcing the benefit of limiting these infections.

- A fluorogenic RT-PCR to detect BRSV had high relative sensitivity and high analytic specificity. The assay was more rapid and allowed higher sample throughput than classical gel-based nested PCR, showing its suitability for BRSV research as well as for diagnostic purposes.

- A Danish BRSV infection model was confirmed to be reproducible. Severe clinical signs of BRD were reproduced in conventional colostrum-fed calves with maternal antibodies to BRSV.

- In contrast to a commercial inactivated vaccine, an experimental BRSV-ISCOM vaccine induced rapid humoral immune responses and strong clinical and virological protection against experimental BRSV challenge despite maternal antibodies in calves at vaccination. The BRSV-ISCOMs are not only candidates for future industrial production after further evaluation, they may also serve as tools to elucidate protective immunological parameters against BRSV.

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