Epidemiology and Eradication of Bovine Viral Diarrhoea Virus Infections

Studies on transmission and prenatal diagnosis of persistent infection

Ann Lindberg

Department of Ruminant Medicine and Veterinary Epidemiology

Uppsala

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Abstract

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This thesis describes the principles for eradication of bovine viral diarrhoea (BVDV) from cattle populations without the use of vaccines, as they have been applied in the Scandinavian countries since 1993-94. It also presents five studies concerning transmission of BVDV and prenatal diagnosis of persistent BVDV infection. The studies relate to large-scale eradication by addressing issues of importance for elimination of virus in infected herds, for management of recently infected herds and for prevention of BVDV transmission through livestock trade.

Transmission of primary type I BVDV infections in the absence of persistently infected (PI) animals was studied. Calves undergoing acute infection with BVDV were brought in contact with healthy, non-immune calves. Also, calves inoculated with BVDV were housed with non-immune calves, while they all underwent a concurrent infection with bovine coronavirus. In both studies, none of the in-contact calves seroconverted. This suggests that primary infections are of low infectivity and that virus circulation will cease after PI animals have been removed.

The ability to identify dams pregnant with PI foetuses (PI carriers) before parturition was investigated. Using an indirect antibody ELISA on samples taken in late gestation, it was possible to discriminate PI carriers from other antibody positive pregnant cows in herds with ongoing infection. It was also possible to detect viral antigen in foetal fluids from PI carriers by an RT-PCR assay and by virus isolation (VI). RT-PCR was superior to VI in this respect. Serology can be used to prevent PI carriers from being traded, and both methods can be used to delimit outbreaks of BVDV in infected herds.

The infectivity associated with foetal fluids and uterine lochia was tested by exposing non-immune calves to such samples. It was shown that foetal fluids can be infectious, but to a low extent. Uterine lochias are not likely to be infectious. Also, the infectivity associated with dams delivering PI calves, and their calving environment, was studied by putting susceptible calves in repeated contact with such dams after removal of the PI offspring. It was shown that rapid removal of newborn PI calves does not prevent further spread of BVDV infection to susceptible animals.

Keywords: cattle, pestivirus, BVDV, experimental study, test validation, foetal fluid, indirect transmission, control, risk factor.

Author's address: Ann Lindberg, Swedish Dairy Association, P.O. Box 7019, SE-750 07 UPPSALA, Sweden.

On BVDV eradication…

The ubiquity, ease of transmission, frequent inapparent infection, and presence of nonbovine hosts make eradication of BVD an unreasonable consideration.
– Robert. F. Kahrs

It's kind of fun to do the impossible.
– Walt Disney

On PhD studies….

My goal is simple. It is complete understanding of the universe, why it is as it is and why it exists at all.
– Stephen Hawking

It is more important to know where you are going than to get there quickly.
– Mabel Newcomber

To all other fools, sane or not

Contents

Appendix

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

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

II. Niskanen, R., **Lindberg, A**. and Tråvén, M. Failure to spread bovine virus diarrhoea virus infection from primarily infected calves despite concurrent infection with bovine coronavirus. Veterinary Journal (In press).

III. **Lindberg, A.**, Groenendaal, H., Alenius, S. and Emanuelson, U. Validation of a test for dams carrying foetuses persistently infected with bovine viral diarrhoea virus based on determination of antibody levels in late pregnancy. Preventive Veterinary Medicine (2001) 51: 199-214.

IV. **Lindberg, A**., Niskanen, R., Gustafsson, H., Bengtsson, B., Baule, C., Belák, S. and Alenius, S. Prenatal diagnosis of persistent bovine viral diarrhoea virus (BVDV) infection by detection of viral RNA in foetal fluids. Veterinary Journal (In press).

V. **Lindberg, A**., Stokstad, M., Løken, T., Alenius, S. and Niskanen, R. Indirect transmission of bovine viral diarrhoea virus at calving and during the postparturient period. (Submitted manuscript).

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

Abbreviations

BCV	bovine corona virus
BHV-1	bovine herpes virus type 1
BVD	bovine viral diarrhoea
BVDV	bovine viral diarrhoea virus
cp	cytopathogenic
EBL	enzootic bovine leucosis
EDQM	European Directorate for the Quality of Medicines
ELISA	enzyme-linked immunosorbent assay
IBR	infectious bovine rhinotracheitis
MD	mucosal disease
ncp	non-cytopathogenic
OD	optical density
PI	persistently infected
PI carrier	cow pregnant with a persistently infected foetus
R	reproductive rate
R_0	basic reproductive rate
RNA	ribonucleic acid
ROC	receiver-operating characteristics
RT-PCR	reverse transcriptase-polymerase chain reaction
Se	sensitivity
Sp	specificity
VI	virus isolation

Background

The clinical manifestations of infection with bovine viral diarrhoea virus (BVDV) was first described by Olafson and colleagues (1946) who reported about an apparently new disease in cattle characterised by acute gastroenteritis and erosions in the digestive tract. During the 55 years that have followed, the virus has been identified and its epidemiology successively better understood.

Today the causal association between infection in the first trimester of pregnancy, establishment of persistent infection (Malmquist, 1968, Coria & McClurkin, 1978b) and the subsequent death from mucosal disease in immunotolerant animals (Brownlie et al., 1984, Roeder & Drew, 1984) is clear. The wish to control the negative effects of the virus has lead to the development of numerous vaccines, but also of eradication schemes. These schemes are based on identification of infected herds and subsequent removal of persistently infected (PI) animals (Bitsch & Rønsholt, 1995). In connection with improved methods for herd and individual diagnosis (Niskanen et al., 1991, Houe, 1992, Niskanen, 1993), the test and cull approach has proven successful, whereas non-systematic approaches involving vaccination are still struggling with safety and efficiency issues (Levings & Wessman, 1991, Thomson & Vickers, 1991, van Campen & Woodard, 1997, van Campen et al., 2000, Barkema et al., 2001).

In Sweden, a scheme with the objective to eradicate BVDV without vaccination was launched in April 1993, close in time to the implementation of similar measures in the other Scandinavian countries (Husu & Kulkas, 1993, Olsson et al., 1993, Bitsch et al., 1994, Waage et al., 1994). The scheme has lead to a reduction in the prevalence of BVDV infected herds in Sweden from approximately 50% in 1993 to 3% today (May 2002). The present study is aimed at answering some of the questions that have arisen during the implementation of the scheme.

Introduction

Structure and management of cattle herds in Sweden

In June 2000, there were over 77,000 agricultural holdings with over 2 acres of land. Cattle, the target species for eradication of BVDV, were present on 42% of these holdings (SBA, 2002).

Dairy herds

Like in many other countries, the structural trend in the Swedish dairy industry is towards fewer, but larger units. In 1993, when the national BVDV scheme was launched, there were approximately 18,500 dairy herds. Today, the corresponding figure is 10,800. Approximately 75% of the dairy herds are affiliated to the milk recording scheme and 95% use artificial insemination. The number of cattle has also decreased, but not at the same pace. Consequently, the average dairy herd size has increased from 32 to 41 during this period (for herds affiliated to the milk recording scheme). There is a distinct regional trend in herd size, currently ranging from 31 cows in the north to 51 in the south. In 1993/94, the average Swedish dairy cow produced 8,000 kg of milk per year. Today the average annual milk yield is 8,800 kg (SHS, 1994, SDA, 2002b). Dairy cattle are housed during the winter. In the southern parts of the country, the housing season lasts from October-November to April-May whereas in the north, cattle are housed from August to June.

Beef herds

The trend within the beef industry is the same as for dairy. Between 1993 and 2001, the number of beef breeding herds has decreased from 17,800 to 13,500. During the same period, the average herd size increased from 6 to 12 (SBA, 2002). In addition to the beef breeding herds, there are also approximately 5,500 rearing enterprises employing market purchased calves of 6-8 weeks age. Approximately 50% of the animals slaughtered are bull calves that originate from dairy herds. A majority of the cattle slaughtered for beef production are 16-18 months of age, at a mean weight of 322 kg (L. Lindell, personal communication). Like dairy cattle, beef cattle are also housed during the winter, but the housing season often starts later.

National disease control

In addition to the BVDV scheme, national eradication schemes have been launched in Sweden for enzootic bovine leucosis (EBL) and infectious bovine rhinotracheitis (IBR), in 1990 and 1994 respectively. IBR was present only to a low degree, but for EBL, the situation was different. In 1990, the prevalence of infection in dairy herds was estimated to be 25-30%, which was high in an international perspective. However, after 10 years, Sweden was officially declared

to be free from the infection by the European Union in December 2000 (SDA, 2002a). To a large extent, the organisation of the BVDV scheme has built on the practical experiences gained from the EBL scheme. Vaccination against viral infections is not performed in Swedish cattle herds.

Bovine viral diarrhoea virus

BVDV is a relatively small (40-60 nm) enveloped, spherical virus (Thiel et al., 1993, Donis, 1995). The genome is a single-stranded, positive sense ribonucleic acid (RNA) molecule, consisting of approximately 12,500 base pairs. It has a single open reading frame that is translated to a polyprotein of about 4000 amino acids, which is then further processed by viral and cellular enzymes into the final components (Grassmann et al., 2001). Extensive taxonomy studies based on the conserved 5' region has resulted in the (current) classification which places the virus among the pestiviruses within the family *Flaviviridae*, which also contains the genera Flavivirus and Hepacivirus (Shukla et al., 1995, Neyts et al., 1999). Among the pestiviruses, two other important animal pathogens can be found; classical swine fever virus and border disease virus in sheep (Neyts et al., 1999). BVDV is able to infect a wide range of ungulate species, both domesticated and wild (Paton et al., 1992, Soine et al., 1992, Løken, 1995, Taylor et al., 1997b, Anderson & Rowe, 1998, Sausker & Dyer, 2002). Although cases of spread from sheep to cattle have been described , the most common direction of transmission is believed to be from cattle to other species (Vilcek et al., 2000, Graham et al., 2001a). It is not known if the virus can persist in wildlife populations.

BVDV can exhibit two different biotypes; non-cytopathogenic (ncp) and cytopathogenic (cp) (Corapi et al., 1988). Despite their denomination, the name of the biotypes does not correspond to the pathogenicity of the virus in the field, but rather to the effect the virus has when grown in cell culture. Ncp strains are adapted to persist. By avoiding the induction of a type I interferon response in the foetus, they can establish persistent infections, whereas cp strains can not (Brownlie et al., 1989, Charleston et al., 2001). Cytopathogenicity occurs as a result of genetic alterations (insertions, duplications and/or rearrangements), within the region encoding the non-structural NS2/3 protein (Kummerer et al., 2000). Such mutations are associated with the development of mucosal disease, a terminal condition which is further described below.

The virus can also be classified according to genotype. Two distinct types, type I and II, have been identified (Pellerin et al., 1994, Ridpath et al., 1994). In general, the two genotypes exhibit the same range of pathogenicity (Ridpath et al., 2000). However, type II strains have also been associated with severe outbreaks of acute BVDV with high morbidity and mortality (Ellis et al., 1998, Odeon et al., 1999). In Sweden, only type I has been detected (Vilcek et al., 1997, Vilcek et al., 1999, Vilcek et al., 2001).

Despite a high degree of heterogeneity within genotype, it has been shown that BVDV strains are, in general, herd-specific (Paton et al., 1995, Hamers et al., 1998, Vilcek et al., 1999, Luzzago et al., 2001). This means that any viruses isolated within a herd will be more or less identical unless new virus strains are actively introduced. This could be by vaccination with modified live vaccines, with any vaccine containing adventitious virus, or by introduction of infected animals from other herds.

Clinical manifestation

The outcome of an infection with BVDV depends mainly on whether the infected animal is pregnant, or not, and if it has been previously infected with the virus. Generally speaking, previous infection in cattle with a normal immune response results in life long immunity and foetal protection during future pregnancies (Moerman et al., 1993, Fredriksen et al., 1999b). However, the degree of foetal protection against heterologous challenge may be genotype-dependent. In ewes, natural immunity to type I strains can provide a good cross protection whereas immunity raised against type II strains may not (Paton et al., 1999).

Infection in non-pregnant non-immune animals

In susceptible, non-pregnant animals the infection is in most cases subclinical (Houe, 1995) but can, depending on genotype and strain also produce severe disease where animals succumb to the infection (David et al., 1994, Pellerin et al., 1994, Hamers et al., 1999). The virus causes leukopenia and thrombocytopenia and the degree and persistence of these haematological findings are also associated with the severity of the infection (Corapi et al., 1989, Bolin & Ridpath, 1992, Ridpath et al., 2000), as is the degree of viremia (Walz et al., 2001b). Typically, virus can be detected in most secretions for 4 to 10 days post infection, but intermittently and at low levels (Brownlie et al., 1987). Clinical symptoms frequently seen are fever around day 6-9 post infection, inappetence and mucosal lesions. In calves, the infection is often associated with respiratory and gastrointestinal symptoms such as coughing and diarrhoea (Tråven et al., 1991, Baker, 1995, Potgieter, 1997). Such symptoms can also be a result of secondary, or concurrent infections (Brodersen & Kelling, 1998, Elvander et al., 1998, de Verdier Klingenberg et al., 1999, de Verdier Klingenberg, 2000, Fulton et al., 2000a) since BVDV acts as an immunosuppressive agent by impairing immune functions mainly associated with the cellular response (Potgieter, 1995, Adler et al., 1996, Bruschke et al., 1997).

In adult bulls, an acute infection may be associated with a transient impairment of semen quality (Paton et al., 1989, Kommisrud et al., 1996, Kirkland et al., 1997). There are indications that virus may persist and replicate in testicular tissue for more than 6 months although it can not be isolated from semen (Givens et al.,

2002). One case has also been reported where virus was constantly shed in semen from a bull with otherwise normal immunity (Voges et al., 1998).

Infection in pregnant non-immune animals

The virus shows affinity to rapidly dividing cells and a growing foetus is therefore a favoured site of replication. In non-immune pregnant animals, the virus infects the conceptus, irrespective of the time of gestation, with effectively 100% probability (Duffell & Harkness, 1985). However, the exact route by which the virus reaches the foetus is unclear, as is the time sequence for infection of different tissues. Using intramuscularly inoculated heifers, Fredriksen and colleagues (1999a) noted that the earliest stage of infection at which BVDV antigen could be detected in the foetuses was 14 days post infection and 4 days later in the intercotyledonary foetal membranes. Swasdipan and colleagues (2002) detected virus in the allantoic and amniotic membranes already 72 h post infection, in the foetus 4 days later and in the endometrium first at 10 days after infection in intranasally challenged ewes. The specific outcome of the foetal infection depends on the stage of gestation and therefore a wide range of reproductive failures can be seen in infected herds (Roeder et al., 1986). They include *e.g.* failure to conceive, the birth of immunotolerant, persistently infected (PI) calves, malformations, foetal death and abortion or mummification, intrauterine growth retardation and weak or stillborn calves (Carlsson et al., 1989, Oberst, 1993, McGowan & Kirkland, 1995, Fray et al., 2000). Abortions may appear at any time during pregnancy and are not necessarily associated with the time of infection.

Infection in the first trimester, before the foetus becomes immunocompetent, can result in a persistent infection in the foetus (Done et al., 1980). PI animals are the key transmitters of the infection since they shed virus continuously, in large amounts and in all bodily fluids (Coria & McClurkin, 1978b, Meyling & Jensen, 1988, Brock et al., 1991, Kirkland et al., 1991). Typically, they do not develop a detectable antibody response to the persisting virus but if exposed to heterologous strains of BVDV they will produce neutralising antibodies (Bolin et al., 1985, Bruschke et al., 1998). The presence of specific neutralising antibodies may affect the ability to isolate virus from such animals (Brock et al., 1998). PI animals have impaired immune functions and they tend to be more susceptible to other infections (Potgieter, 1995). It is therefore quite common that they die or are culled before they reach adult age (Barber et al., 1985, Taylor et al., 1997a, Houe, 1999). However, they may also be clinically healthy. PI cows that reach adult age can conceive (McClurkin et al., 1979). If so, the infection will be transmitted to the foetus, and thus, the offspring will always be PI (Baker, 1987).

If the foetus is infected after it has become immunocompetent, it will develop antibodies (Howard, 1990). However, despite the ability to mount an immune response, the growing foetus is negatively affected and these animals can be weak

at birth, ill-thrifty and therefore more susceptible to other infections (Larsson et al., 1994, Moennig & Liess, 1995).

The pregnant dam, if non-PI, develops antibodies in response to the infection. If a persistent infection is established in the foetus, her immune response will be further triggered and antibody levels will continue to rise until the production of colostrum starts, shortly before parturition (Meyling & Jensen, 1988, Brownlie et al., 1998).

Infection in animals with passive immunity

Calves that receive colostrum containing antibodies to BVDV achieve a passive immunity that protects them from infection during their first months in life (Howard et al., 1989, Bolin & Ridpath, 1995). Usually, maternal antibodies will be detectable a few hours after the first meal and decline at a rate of one half their remaining antibody titre every 21 days (Brar et al., 1978). The duration of this protection depends on the concentration of neutralising antibodies in colostrum, the amount ingested and possibly also of the challenge experienced by the calf. In general, passive antibodies are detectable for 4-6 months (Coria & McClurkin, 1978a). In PI animals, maternal antibodies will decline at a higher rate (Palfi et al., 1993, Brock et al., 1998). Passive immunity interferes with vaccination. Ellis and colleagues (2001) showed that vaccination before sufficient decline of maternal antibodies did not protect against infection with a virulent type II BVDV strain. In another study, calves did not respond serologically to BVD vaccine until their maternal antibody titres were below 1:96 to 1:20 (Brar et al., 1978).

Mucosal disease

A sequel to persistent infection is mucosal disease (MD) which is a lethal condition that usually involves cattle aged 6 months to 2 years of age. The course of the disease can be either acute, with a duration of 2 days to 3 weeks, or chronic with animals surviving up to 18 months of age. Typically, cases exhibit fever, anorexia, massive mucosal erosions throughout the gastrointestinal canal and profuse diarrhoea leading to progressive wastage and death (Baker, 1995). In chronic cases, the animals show similar symptoms, but in a more protracted form. Also, apart from gastrointestinal symptoms like intermittent diarrhoea and chronic bloat, dermatological symptoms like erosive lesions on the skin and laminitis may develop.

MD develops as a result of a mutation in the non-structural part of the genome (Tautz et al., 1998, Kummerer et al., 2000), resulting in a change in biotype from ncp to cp. If the cp strain is homologous to the ncp strain, the PI animal does not produce neutralising antibodies to it. Consequently, where a herd-specific strain is established, the cp strain can spread from the index case to other PI animals in the herd, resulting in an outbreak-type appearance of the condition. In these situations, homologous ncp and cp strains can be isolated from clinical cases

(McClurkin et al., 1985). MD can also develop as a result of recombination between the persisting ncp strain and an exogenous cp strain. This can occur, for example, if vaccines containing cp strains are used (Baker, 1995). Recombinations with heterologous cp strains and/or mutations in the exogenous cp strain have been suggested as possible reasons for development of late-onset type of MD (Ridpath & Bolin, 1995, Fritzemeier et al., 1997, Sentsui et al., 2001). However, the degree of homology between the persistent and superinfecting strain does not seem to be the only predictor of the outcome of the disease (Bruschke et al., 1998, Loehr et al., 1998).

Herd level effects of BVDV infection

At the herd level, BVDV infections typically results in an increased incidence of reproductive disorders and in impaired calf health (Houe & Meyling, 1991a, Larsson et al., 1994, Moerman et al., 1994, Fray et al., 2000). Examples of reproduction parameters in which the effect of BVDV infection has been shown are conception rates, pregnancy rates, abortion rates and time to first calving (Houe et al., 1993a, McGowan et al., 1993, Rufenacht et al., 2001, Valle et al., 2001). Increases in the incidence of treatments of retained placenta and silent heat have also been reported, as well as an increase in the risk of infectious diseases in adult animals, such as masitis (Niskanen et al., 1995). Also, a negative association between milk yield and BVDV infection has been reported (Barber et al., 1985, Moerman et al., 1994, Lindberg & Emanuelson, 1997). In herds relying on the production of animals for slaughter, important causes of production losses are *e.g.* immunosuppression with subsequent increase in the prevalence of infectious diseases and growth retardation in sick non-PI and PI animals (Kelling et al., 1990, de Verdier Klingenberg et al., 1999, Taylor & Rodwell, 2001).

Prevalence and incidence of BVDV infection

Antibodies to BVDV have been detected in non-vaccinated cattle in all countries where prevalence studies have been made (Littlejohns & Horner, 1990, Rweyemamu et al., 1990, Shimizu, 1990, Zhidkov & Khalenev, 1990). Assuming that the presence of antibodies reflects exposure to the virus it can be concluded that it is present worldwide. However, the prevalence of herds with signs of recent exposure shows a wide variation between countries and between regions within countries (Alenius et al., 1986, Bitsch & Rønsholt, 1995, Paton et al., 1998, Rossmanith & Deinhofer, 1998, Nuotio et al., 1999, Graham et al., 2001b, Mainar-Jaime et al., 2001). Within herds, the prevalence of antibody positive animals will usually be high if there are PI animals present. (See Houe, 1999 for a review). In non-vaccinated herds that do not have the infection, the prevalence and age distribution of seropositive animals will reflect the time that has passed since the last PI animal left the herd (Houe, 1992). Animals born “post-PI” will be seronegative (unless they still have maternal antibodies) whereas those exposed and infected prior to this date will usually remain seropositive for the rest of their

life. The prevalence of seropositive animals will decrease with the replacement rate. This picture will be distorted to some degree if seropositive animals are purchased and to a high degree if vaccines are used (Houe et al., 1995a, Houe et al., 1995b, van Campen et al., 1998).

In most surveys performed, the prevalence of PI animals within the entire cattle population (including both infected and non-infected herds) seems to be consistently around 1-2% under endemic conditions (Depner et al., 1991, Houe & Meyling, 1991b, Braun et al., 1997, Schreiber et al., 1999). Considering the fact that a certain proportion of PI animals die prior to the time when they can be tested, the prevalence of PI animals *born* is likely to be higher. Consequently, differences in PI mortality rates may be a source of bias in prevalence studies as a higher rate will result in a lower apparent prevalence. This may be one reason as to why lower prevalences have been reported in some regions/countries with endemic BVDV infection (Houe et al., 1995b, Taylor et al., 1995). The estimated incidence of infection in the first trimester has been estimated to be 3.3 % in the entire population (Houe & Meyling, 1991b). In paper **III**, the prevalence of PI carriers among antibody positive cows and pregnant heifers in Swedish dairy herds subjected to elimination of virus was 13%.

At the herd level, the annual incidence risk will also vary between countries and regions, and has consequently been estimated to range from 0.08 and 0.48 under endemic conditions (Niskanen et al., 1995, Houe, 1999, Graham et al., 2001b). In areas with systematic control of the infection, reports show a decreasing risk trend and levels around 0.02-0.03 after 4-5 years implementation (Alenius et al., 1997, Valle et al., 2000a, Alban et al., 2001).

Transmission of BVDV

BVDV can be transmitted both by direct contact between an infected and a susceptible animal, and indirectly through different types of vehicles. Direct contact with a PI animal is by far the most efficient route of transmission. PI animals can also transmit the infection efficiently to other animals within the same housing unit without being in direct contact with them (Houe & Meyling, 1991b, Wentink et al., 1991). Indirect transmission has only been shown experimentally, and only where the initial contact was PI (Gunn, 1993, Lang-Ree et al., 1994, Mars et al., 1999, Niskanen & Lindberg, 2002). The probability of transmission by indirect means is dependent on time, temperature and dose. If the virus is preserved within the vehicle, *e.g.* in semen from acutely or PI bulls, contaminated embryos or contaminated injectables, the potential for spread is increased (Schlafer et al., 1990, Kirkland et al., 1991, Givens et al., 2000, Niskanen & Lindberg, 2002).

Different routes of transmission, their relative importance for within- and between herd spread and how they can be controlled are further elaborated on in paper **VI**.

Diagnosis of BVDV infection

Assays for BVDV are either aimed at detecting the virus itself (including traces of viral RNA), or antibodies to it. The diagnostic tools mentioned below are all developed for individual diagnosis, but some of them have also been validated for use at the herd level.

Diagnostic methods

Virus detection

The presence of BVDV in a sample can be demonstrated by isolation and detection in cell culture, by detection of viral antigens, or by detection of viral nucleic acid (See Sandvik, 1999 for a review).

Virus isolation (VI) is made by incubating samples on low-passage cultures of primary bovine kidney, turbinate or testis cells after which fluorochrome or enzyme labelled BVDV-specific antibodies are used to detect the presence of virus. VI is regarded as the reference test for virological diagnosis (Brock, 1995) and is a good indicator of the presence of live (and infectious) virus. However, the presence of toxic substances and/or antibodies in a sample can yield a false negative test result (Palfi et al., 1993, Brinkhof et al., 1996).

Several methods for detection of viral antigen by enzyme-linked immunosorbent assays (ELISAs) have been published (Fenton et al., 1991, Mignon et al., 1992, Crevat et al., 1993, Entrican et al., 1995, Foucras et al., 1996, Graham et al., 1998, Kramps et al., 1999). Such tests have the advantage of being rapid, sensitive and independent of cell culture facilities, and have therefore become widely popular. Most of them are of the sandwich type, with a capture antibody bound to the solid phase, and a detector antibody conjugated to a signal system, such as peroxidase. Some rely on extraction of viral antigen from the buffy coat of whole blood samples and small sample volumes can therefore be a practical problem. However, new antigen ELISAs are being developed that do not rely on extraction of antigen (Holmquist et al., 2002).
Immunohistochemistry is a method for detection of intracellular viral antigen and is the test of choice for demonstration of virus in tissues (Hewicker et al., 1990, Haines et al., 1992). Using ear notches as specimen, this method can be used to screen for PI animals (Njaa et al., 2000).

For detection of viral RNA, reverse transcriptase-polymerase chain reaction (RT-PCR) techniques are employed. They have the advantage of being insensitive to toxic substances in the specimen and to the presence of interfering antibodies, but are extremely sensitive to sample contamination which can lead to false positive results (Belák & Ballagi-Pordány, 1993). The development of closed analysis systems, where BVDV nucleic acid amplification and detection is made in the

same tube, has reduced this problem (Mahlum et al., 2002). Today, the development is towards quantitative and multiplex assays where virus can be quantified and where both genotypes, or additional viral agents can be assayed within the same sample (Gilbert et al., 1999, Onodera et al., 2002).

Antibody detection

ELISAs are also used for BVDV antibody detection. There are two principal types; indirect and competitive. In indirect ELISAs, antibodies are trapped by immobilised antigen, and detected using enzyme-conjugated species-specific antiglobulins and a chromogenic substrate. The optical density (OD) is then measured, which will be higher in a positive sample than in a negative. In competitive ELISAs (also called blocking ELISAs), virus-specific antibodies in the sample block the binding of conjugated virus-specific antibodies to fixed viral antigen. In contrast to the indirect ELISA, a positive sample in a blocking ELISA will yield a weaker signal than a negative sample (See Sandvik, 1997 for a review).

The gold standard for antibody detection is the virus neutralisation test (Edwards, 1990). It is sensitive and specific but cell culture dependent and labour demanding in relation to the ELISAs. Therefore, the latter are regularly used when a large sample throughput is required.

Objective of BVDV diagnosis

From a BVDV control perspective, the objective of BVDV diagnosis should be to differentiate between infected and non-infected herds respectively, and within infected herds, to differentiate between PI animals, immune animals and animals that are susceptible to infection. PI animals also have to be differentiated from acutely infected animals.

Herd level tests are aimed at differentiating between herds with ongoing infection and herds likely to be free. This can be done either by antibody tests, or by virus detection. Antibody tests indirectly detect the recent or current presence of PI animals through the serological response in surrounding animals. Antibody tests are performed either on pooled milk (*e.g.* bulk tank milk), or on spot samples from expectedly seronegative animals (Niskanen et al., 1991, Houe, 1992, Beaudeau et al., 2001). Bulk milk tests can not be used in herds that vaccinate, but spot tests seem to work well (Pillars & Grooms, 2002). Virus can be detected either in pooled serum or in pooled milk (usually bulk milk). However, pooling means that it is highly likely that any virus positive samples are mixed with samples from antibody positive animals. Therefore VI can be difficult and RT-PCR is often the method of choice (Radwan et al., 1995, Drew et al., 1999), or a combination of the two (Renshaw et al., 2000). Because PI animals are in general more prevalent among young animals, bulk milk virus tests are principally used to *exclude* the presence of PI animals among lactating cows. To find all PI animals, those not included in the bulk also have to be tested (Drew et al., 1999).

At the individual level, BVDV infection can be diagnosed either by detection of the virus itself, or by demonstration of seroconversion in paired samples. A PI animal is typically virus positive and antibody negative unless it has been exposed to and infected with a heterologous strain. Persistent infection is then confirmed by repeated detection of virus in samples taken 2-3 weeks apart. Virus can sometimes be isolated also from acutely infected animals but such animals will be virus negative and antibody positive if they are retested (Sandvik, 1999).

Economic importance of BVDV infections

As indicated above, BVDV has got the potential to cause considerable damage in infected herds due to its broad effect on health and production, including reduced milk production, reduced reproductive performance, growth retardation, increased occurrence of other diseases, unthriftiness, early culling and increased mortality especially among young stock. In conjunction with the high prevalence and incidence of new infections it is obvious that this is a source of substantial economic losses to the cattle industry.

Important factors that affect the magnitude of the losses in any single case are the initial herd immunity, the number of animals in different stages of gestation at the time of the infection and the virulence of the virus. Consequently, the calculated losses in individual herd outbreaks have varied from a few thousand up to US$ 100,000 (Duffell et al., 1986, Wentink & Dijkhuizen, 1990, Alves et al., 1996, Stelwagen & Dijkhuizen, 1998). Since losses at the herd level show such a wide range of variation, the losses for the industry as a whole is perhaps better reflected in calculations of national losses. These have been estimated to lie within the range of US$ 10-40 million per million calvings (Bennett & Done, 1986, Harkness, 1987, Houe et al., 1993b).

New infections in naïve herds can be associated with extreme, but transient, reproductive losses. As a large proportion of the adult animals become immunised the losses will change in nature, from mainly reproductive losses to losses due to impaired calf health. To the author's knowledge, the long-term losses associated with BVDV infection, *e.g.* loss of genetic material and effect on the longevity of cattle infected as calves, have not been characterised, nor quantified.

In a French study, it was estimated that an eradication program would not be cost efficient until after approximately 15 years following implementation (Dufour et al., 1999). However, cost-benefit assessments of implemented control schemes have shown that control of the virus without vaccination can give a good pay-off. For example, the net benefit of the Danish eradication programme has been estimated to be over US$ 24 million after 4 years (V. Bitsch, personal communication). A similar study of the Norwegian control and eradication program from 1993 to 1997 showed that the program was already cost-effective

from the second year (Valle et al., 2000b). A major contributor to the cost-efficiency of the Scandinavian schemes is the use of cheap methods for initial screening and subsequent monitoring, *e.g.* by testing bulk milk for antibodies. Another fact is the instant reduction in the risk of new infections, which is achieved as soon as PI animals are prevented from being marketed.

Vaccination and BVDV control

In many countries, vaccines are used to control BVDV. Classical BVDV vaccines are of two different types; modified live and inactivated (killed). Modified live vaccines contain a live but attenuated strain of the virus, and generally give a better immunological response than killed vaccines. The latter consist of virus that has been inactivated, together with an immuno-stimulating additive (See van Oirschot, 2001 for a review). There is a tendency to move towards the development of non-replicating vaccines (similar to classical killed vaccines), because of safety issues. New types of non-replicating vaccines are *e.g.* subunit vaccines, recombinant subunit vaccines, peptide vaccines, DNA vaccines and some vector vaccines.

The main objective with vaccination against BVDV is to prevent transplacental infection and thus the establishment of new persistent infections (van Oirschot, 2001). In countries where virulent type II strains are present, prevention of postnatal infections is also a concern as the clinical manifestations may be severe. However, there is clear evidence that all products on the market do not sufficiently fulfil those objectives (Kelling et al., 1990, Holland et al., 1993, van Campen & Woodard, 1997, Cortese et al., 1998, van Campen et al., 2000, Thurmond et al., 2001, Wittum et al., 2001). The problem with current BVDV vaccines and vaccination schemes is complex, and includes both epidemiological, technical, sociological, political and economic considerations that will be discussed later in this thesis. Currently, the only approaches that have been successful in reducing the impact of BVDV infections are those that put emphasis on biosecurity in general, and control of direct animal contacts in particular – with or without the complementary use of vaccines. In paper **VI**, a review of current control options is given, including the principles for eradication of BVDV that have been applied in Scandinavia and now also in Austria (Rossmanith et al., 2001).

Epidemiological aspects on transmission and eradication

The potential for an infectious disease to spread between units (animals, pens, herds, regions, countries) within a population can be expressed in terms of its reproductive rate, **R**. The reproductive rate describes the average number of secondary cases an infected individual gives raise to during its infectious period. It is a function of the risk of transmission per contact, the number of infectious

contacts per time unit, the duration of the infection and the proportion immune (or infectious) already in the population. A special case is the *basic* reproductive rate (**R_0**) which corresponds to the case when one infected individual enters a totally susceptible population. R_0 has to exceed 1 in order for an infection to be able to cause any major outbreaks. If R_0 is less than 1, the infection will, theoretically, fade out. (For an extensive reference, see Anderson & May, 1991, for an applied text book , Giesecke, 1994).

The general formula for calculating R_0 is

$\beta * k * D$

where

β = the probability of transmission per contact type
k = frequency of (different types of) potentially infectious contacts per time unit
D = duration of infectious period

The value of these parameters, and thus R_0, will differ depending on contact type. Theoretically, for BVDV the contact types are direct or indirect contact with a PI, and direct or indirect contact with an acutely infected animal. In addition, indirect contacts can be further divided upon type of vehicle. However, what we can observe in the field is the net force of infection, that is, the average R_0 for all routes by which the infection can be transmitted.

For a situation when the population is no longer fully susceptible (*e.g.* during an epidemic or under endemic conditions) it is more correct to consider the *net* reproductive rate**.** If the number of susceptible individuals changes as a result of immunisation, R_0 will be reduced. This is particularly obvious if the population is small. For an immunogenic infection with a high R_0, like postnatal BVDV infection transmitted from a PI animal, the net reproductive rate R within a herd will rapidly decrease to <1. This is why so many small herds spontaneously eliminate BVDV infection without intervention. The concept of R_0 can be applied both to transmission between individuals and to transmission between herds. To clear a herd from BVDV infection $R_{0(\text{within herd})}$ has to be below 1; for eradication at a larger scale $R_{0(\text{between herds})}$ will have to be <1.

Putting this into the BVD context and looking at transmission between herds, the probability of transmission (β in the formula above) will be higher if PI animals or PI carriers are moved from infected to susceptible herds than if the animals introduced are only transiently infected. β is believed to be even less for a contact involving a commodity or a person carrying BVDV. For each contact type, however, a low probability of transmission can be counter-weighed by a high frequency of contacts.

In the BVDV scheme, there are a number of measures in place to control different routes of transmission and consequently reduce $R_{0(\text{between herds})}$. Control of the livestock trade, only allowing free herds to have pasture contacts and recommending double fences towards neighbouring herds are all aimed at changing the contact structure and reduce the frequency of contacts (κ) that are with PI animals, PI carriers and acutely infected animals. Other biosecurity recommendations, like the use of protective clothing and not allowing transportation staff to enter cow houses, are aimed at reducing the probability of transmission associated with that particular type of contact (β). Finally, the scheme is aimed at shortening the duration of the infectious period for any herd (D) by monitoring for rapid detection of new infections, and by using a robust and efficient protocol for clearing herds from the infection (further described in paper **VI**).

The theory behind vaccination is to reduce the average number of susceptible individuals that an infectious individual meets during its infectious period, and thereby reduce R. As described above, if R becomes less than 1, the infection should fade out, or at least – there should be no major outbreaks. The higher R is, then the higher will be the proportion that has to be immunised. In this context, immunisation (*i.e.* "true" protection) is a function of the efficacy of the vaccine and the extent to which it is used in the population. The phenomenon that immunity in a proportion of individuals can be protective for others is often referred to as *herd immunity*. Vaccines give raise both to individual immunity and herd immunity, and the combined effect is referred to as vaccine *efficiency* (Anderson & May, 1991).

Epidemiological aspects on test validation

The epidemiological performance of a test, *i.e.* its ability to discriminate between truly diseased and non-diseased individuals can be expressed in terms of two operational parameters, namely sensitivity (Se) and specificity (Sp). Se is the proportion of test positives among those truly diseased, and Sp corresponds to the proportion of test negatives among those truly healthy. In any given situation, an increase in Se will produce fewer false negative test results, and an increase in Sp leads to fewer false positive results (Martin et al., 1987). Note that Se and Sp is a different concept than the analytical sensitivity and specificity, which concerns the ability of a test to detect small amounts and specific substances.

Strictly speaking, Se and Sp should be regarded as inherent characteristics of a test, given that the test is used in a population that is comparable to the one in which it has been evaluated. However, also within a given population, Se and Sp may differ depending on the "strength" of the biological signal. One example is the increased sensitivity of antigen tests for canine heartworm with increasing worm burdens (Courtney & Cornell, 1990). Ideally, test validation studies should

identify variables that interact with test performance and provide estimates of Se and Sp for each level of those variables (Greiner & Gardner, 2000).

Typically, the performance of a test should be validated against the true disease status, a gold standard. Unfortunately, the ”true” disease status often has to be assessed by another test, which by definition is not perfect. However, today there are methods available that provide a way to make simultaneous inferences about Se and Sp without imposing constraints on any of the parameters (*e.g*. assuming one of the tests have perfect Se and/or Sp) (Hui & Walter, 1980, Joseph et al., 1995). The idea is to allocate individuals from each population into a truly diseased or a truly non-diseased, but unobservable (latent) class. In general, these methods require that two or more populations with different apparent prevalence are sampled, or that the data can be subdivided in such a way. The two tests compared should have the same Se and Sp in both populations and be conditionally independent. This implies that given an animal is diseased (or not), the probability of positive/negative outcomes for one test is the same, regardless of a known outcome of the other test. This assumption is met to some extent if it can be shown that the tests measure different aspects of the same condition (Enoe et al., 2000).

Receiver-operating characteristic (ROC) analysis can be used to assess and visualise test performance (Greiner et al., 2000). Typically, in the ROC analysis curves will be constructed with the true positive fraction (Se) on the Y axis, and the false positive fraction (1-Sp) on the X axis, giving an image of test performance over the whole range of possible cut-off levels. ROC analysis can be used *e.g*. to evaluate the discriminatory power of a test, to compare different tests and to select an optimal cut-off value (Greiner et al., 2000). For tests measured on a continuous scale, the choice of the cut-off level is a trade-off between sensitivity and specificity. In general, this choice will depend on the cost of the error, from an economic and/or ethical point of view (Vizard et al., 1990, Hilden, 1991).

Aims of the study

The overall aim of this work has been to address questions arising in connection with the ongoing Swedish BVDV eradication scheme. These are related to the preconditions for successful elimination of virus in infected herds, to the efficient management of recently infected herds and to prevention of transmission of the infection through the livestock trade.

More specifically, the following aims were set:

1) To investigate whether acute type I BVDV infection can be perpetuated to susceptible animals in the absence of persistently infected animals, with and without a co-infection with bovine corona virus (**I** and **II**),

2) To evaluate serology and foetal fluid sampling as methods for prenatal diagnosis of persistent infection (**III** and **IV**),

3) To investigate whether BVDV infection can be perpetuated by indirect means despite rapid removal of suspected PI calves directly after they are born (**V**), and

4) To describe the epidemiological principles for large-scale eradication of BVDV without the use of vaccines (**VI**).

Materials and methods

In this section, the materials and methods applied in studies related to aim 1-3 are summarised. A more detailed description is given in each paper. Also, some methodological considerations are given.

Transmission of primary BVDV infections in the absence of persistently infected animals (I, II)

In paper **I**, secondary transmission of acute BVDV infection was studied. Primary BVDV infection was initiated in a group of 5 calves by exposing them to direct contact for 6 hours with a calf that was PI with a type I BVDV strain. After the contact they were put in a 5.7 m^2 pen. On days 4, 7, 14, 21, 28, 35 and 42 after this initial infectious contact, new calves seronegative to BVDV, were introduced (2 per occasion) and kept with the group of 5 for 48 hours. The stocking density during this period was 1.2 calves/ m^2. At every introduction and removal, direct nose-to-nose contact between the calves was ascertained. The 5 primarily infected calves were followed serologically and virologically for 6 weeks post infection, and the in-contact calves were followed in the same manner for 4 weeks after they were removed from the group.

In paper **II**, a co-infection with bovine corona virus (BCV) was added to the picture. Ten calves were infected by intranasal inoculation with type I BVDV on day 0, and were thereafter randomly allocated to one of two groups – A and B – into which they were introduced on day 1. In addition to the BVDV infected calves, the two groups consisted of four calves susceptible to BVDV so that each group consisted of 9 calves – 5 infectious and 4 susceptible. Also on day 1, BCV infection was introduced into group A by letting a BCV infected calf stay with the group for 2 hours. A control group (group C) with two susceptible calves was also kept on the premises, in a pen adjacent to group B but separated by a floor-to-roof solid wall. The stocking density in pens A, B and C was 0.75, 1.6 and 0.33 m^2, respectively. BCV was rapidly transmitted to all groups despite the biosecurity routines in place. Thus, the study came to be on transmission of BVDV from calves with concurrent BCV infection to other calves infected with BCV. From day –1 up to and including day 35 post infection the calves were clinically examined and blood and faecal samples as well as nasal swabs were collected for analysis of the presence of BVDV, BCV and antibodies to the viruses.

Evaluation of two different methods for prenatal diagnosis of persistent infection (III-IV)

Validation of the performance of an indirect ELISA when used for prenatal diagnosis

The performance of an antibody test used in routine diagnostics, namely an indirect ELISA, was evaluated with respect to its ability to detect cattle pregnant with PI foetuses (**III**). This was done by analysing a data set including records on 2,162 cow-calf pairs where the cow had been tested antibody positive to BVDV during pregnancy (while clearing the herd from the infection) and where also her calf had been tested for antibodies and virus. The sensitivity and specificity of the test was modelled at 12 different decision thresholds (corresponding to OD values from 0.5 to 1.6 with increments of 0.1) using a generalised linear mixed models approach (binomial error, logit link). The dependent variable was the test result (+/-) at each decision threshold and the gold standard (the calf's BVDV status) was included in the model as one of the covariates. Other covariates included in the models were month of gestation, specimen (blood/milk) and lactation number. To account for dependence between observations (within herds), a random effect of herd was included.

Evaluation of the ability to use foetal fluid as a specimen for prenatal diagnosis

The study described in paper **IV** was aimed at investigating whether foetal fluid sampling could be used as a complement to the serological test evaluated in paper **III**. Foetal fluid samples were obtained in late gestation (244-267 days) from 9 heifers that were pregnant with PI foetuses as a result of an experimental infection in early pregnancy. The PI status of the offspring was confirmed after birth. In addition, a sample from a non-infected cow was obtained and used as a negative control. After appropriate sedation and local anaesthesia, the dams were sampled by making a "blind" perpendicular puncture through the abdominal and uterine walls and into the foetal compartments. The target area is located approximately 10 cm cranial of the udder and 10 cm medial of the flank. The sampling was successful in all cases and the heifers were followed clinically after the sampling, during calving and for 30 days afterwards. The control cow was followed for 10 days after sampling. Samples were assayed using VI, by nested RT-PCR and in an indirect ELISA for detection of the presence of antibodies.

Indirect transmission of BVDV in connection with the birth of PI calves, despite rapid removal of the newborn (V)

The potential for transmission of BVDV to susceptible cattle via foetal fluids, uterine lochia and the environment in which the PI calf is born was investigated in four different trials. The objective was to mimic a situation when a PI calf is removed, or dies, shortly after birth and thus ceases to constitute a source of contagion. In the first three trials, calves that were negative to BVD virus and antibodies were exposed to samples of foetal fluid, or uterine lochia that had been collected after calving from the heifers used in study **IV**. In trial I, the calves were exposed to pooled samples, collected from different cows but on the same day *post partum* (0, 2, 4, 7, 10 and 14). In the second trial, the samples came from days 0, 2 and 4, were not pooled and originated from dams where it had been possible to isolate virus from their foetal fluids *pre partum* (**IV**). The samples used in trial III had been collected in a Norwegian study on days 0 and 1 and were screened for antibodies prior to selection. The samples with the lowest antibody levels were selected for exposure. In total there were 20 exposures, of which 8 were done with samples from day 0. VI was later attempted on all samples from days 0 and 1.

Trial IV was executed in Norway where 4 susceptible calves were penned for 8 hours with cows that had delivered PI calves within the preceding 24-48 hours, after the PI offspring had been removed. The procedure was repeated twice, except for one calf that was exposed four times to such dams within 24 hours after they had calved. In addition, 2 calves were kept within the same housing unit without having contact with the cows or the other calves. All calves were tested for seroconversion after the trials.

Methodological considerations

Although the relative insignificance of acute infections in relation to persistent infections is acknowledged, the perception of their ability to maintain a herd infection after the removal of PI animals is still a matter of discussion. However, to the author's knowledge, studies specifically aimed at qualitative or quantitative assessment of the transmission associated with acutely infected animals have not been performed. Numerous experimental and observational studies have been carried out, both at the individual and herd level. However, most of them either do not address transmission at all, or they do not differentiate between acute and persistent infections.

Transmission of an infection can be studied by mathematical modelling, in experimental studies and by observational studies in the field. Below are some considerations regarding these different approaches in relation to our studies and to the study of BVDV transmission in general.

Modelling studies

Simulation modelling has the advantage that the behaviour of an infection can be investigated, assumptions about different routes of spread can be tested and the potential effect of different control measures can be estimated even though there is incomplete knowledge about the system. At the same time, it is a good way of identifying critical gaps in the current knowledge (Anderson & May, 1991, Dijkhuizen & Morris, 1995). However, modelling studies on BVDV transmission models *per se* are scarce. Those seen in the literature are mainly aimed at assessing the economic impact and to compare different control strategies (*e.g.* Pasman et al., 1994, Sørensen et al., 1995). Consequently, they do not explicitly address acute infections from a transmission point of view, but of course include assumptions about them in order to estimate the total effect on reproduction and production. However, Cherry, Reeves and Smith (1998) developed a deterministic model where they examined the test-and-cull strategy, assuming that the transmission rate for acute infections was zero. Their conclusion was that the strategy could only work if PI animals were removed before 11 days of age. In their own criticism of the model, they appreciate that stochasticity is probably an important element in the dynamics of the infection and that a model that incorporates this would be superior (G. Smith, personal communication). Consequently, the stochastic model presented by Innocent and colleagues (Innocent et al., 1997) seems able to produce results that are more consistent with what is seen in the field. For example, it showed that the presence of PI animals is sufficient to maintain infection in a herd and that the differential mortality in PI calves and healthy calves have a strong influence on the probability of spontaneous elimination.

Somebody wisely said that "All models are wrong, but some are useful". In order to be useful, a model should be validated, preferably against real data. Transmission studies like ours (**I**, **II, V**) can perhaps, despite their lo-tech appearance, contribute to the development of more realistic models, make them more valid and thus better decision tools.

Experimental studies

In general, results from experimental studies have limited validity in the field, because stress, stocking density, co-infections and other factors that may facilitate transmission are not present to the same extent. For the purpose of our studies, and in order to mimic a real life situation, we chose to use conventionally reared calves (**I, II, V**). In the literature, colostrum-deprived calves are often used (Stoffregen et al., 2000, Walz et al., 2001a, Hamers et al., 2002), possibly due to difficulties to obtain naturally seronegative calves. Colostrum is important for the development and maturation of the immune system in the newborn (Xu, 1996) and consequently for host susceptibility. Thus, the use of animals that are deprived of colostrum may further delimit the ability to extrapolate results from the experiment to the field.

Small scale experimental studies can be used to quantify transmission and estimate R_0. In their study on Aujeszky's disease, de Jong and Kimman (1994) introduced infectious animals in a group of susceptible peers in a manner similar to what we did in paper **II**, but with the objective to study differences in transmission between groups of vaccinated and unvaccinated pigs. The same, or similar design has later been used to estimate R_0 for bovine herpesvirus type 1 (BHV-1), classical swine fever virus, *Sarcoptes scabiei* and porcine respiratory and reproductive syndrome virus (Bosch et al., 1996, Moormann et al., 2000, Stegeman et al., 2000, Nodelijk et al., 2001). Some of the conditions that have to be met in order to estimate R_0 based on data from studies of this type are that all infected animals are equally infectious and that all susceptible animals are equally susceptible. In order to conclude on the former, a measure of the dose exerted by the infectious animals is needed. Dose assessment generally requires the use of a proxy. Examples of such proxies are period of viremia or period of virus shedding in different bodily excretions and fluids (Bosch et al., 1996, Nodelijk et al., 2001). Their validity depends on how the presence of virus is determined and how the test actually correlates with infectivity. For example, RT-PCR will probably overestimate infectivity as the presence of viral RNA does not necessarily correspond to the presence of infectious virus. To ascertain equal susceptibility is difficult - is there such a thing? Still, although equal susceptibility could be difficult to obtain, a basic requirement should be that study animals are randomly allocated to ensure that the study groups are representative with respect to individual variation in susceptibility.

Field studies

There are several field studies where acute infections are said to circulate for long periods of time, despite the absence of PI animals (Barber et al., 1985, Moerman et al., 1993, Edwards, 1997). In papers **I** and **II**, we did not observe any secondary infections when only transiently infected animals were present, even when the animals had a concurrent corona virus infection. In contrast, we saw that PI calves only had to be present for a few hours in order to cause seroconversions in susceptible animals even though there were no direct contacts between the infectious and susceptible animals (**V**). In addition, we found that infectious virus can be present in foetal fluids from dams giving birth to PI calves, at doses sufficient to lead to infection. This emphasises that in order to draw conclusions on the infectivity of acute infections from field studies, the absence of PI animals at any time must be reassured.

Results and discussion

A detailed discussion of the results achieved is given in each of the papers **I-V.** Here, they will be discussed in terms of how they relate to the eradication of BVDV on a larger scale. Also, some of the ideas put forward in paper **VI** are discussed further, together with ideas for future research.

The prospects for large-scale eradication of BVDV

The Scandinavian test-and-cull strategy relies heavily on the assumption that BVDV can be eradicated from a herd (and eventually from the population) as long as PI animals are identified and removed. This assumption is supported by our findings in papers **I** and **II**. Similar experiences are reported from Denmark (Bitsch et al., 2000) and by studies on indirect transmission where the absence of further transmission from acutely infected animals have been noted (Gunn, 1993, Lang-Ree et al., 1994). This clearly shows that the early perception of BVDV as an infection that spreads readily and can be controlled only by vaccination (Kahrs, 1981, Harkness, 1987) does not hold.

Prevent new herd infections and delimit outbreaks

Both from the economic and the ethical perspective, it is better to prevent than to treat. As indicated earlier, the major routes by which BVDV is transmitted between infected and susceptible herds under endemic and uncontrolled conditions, are by movement of PI animals and dams pregnant with PI foetuses. Surprisingly, there are very few studies, if any, that elaborate on what can be done to reduce the latter risk, although it is readily acknowledged (Meyling et al., 1990, Fray et al., 2000). In paper **III**, we show that PI carriers can be identified with a high probability using a standard serological assay, as long as the sample is taken in late pregnancy and the assay used is able to quantitatively reflect the antibody titre. Thus, by applying a test of this type in the livestock trade, the risk of transmission of BVDV infection between herds can be reduced, without completely blocking of with pregnant, seropositive cattle. Also, in paper **IV** it was shown that it is possible to detect viral RNA in foetal fluids by the use of PCR. Prenatal diagnosis of BVDV infection, if put into context within a protocol for virus elimination, can shorten the duration of herd infection. It may even prevent the outbreak of an infection that is still latent from a herd point of view, *i.e.*, where PI animals are present only *in utero*. To the author's knowledge, the methods described in papers **III** and **IV** are currently the only available pre-emptive methods for delimiting the consequences of BVDV infections.

Shorten the herd level duration of infection

The first step in eliminating BVDV from herds is to identify and remove PI animals and secondly to do follow-up on all calves born during the subsequent year. Normally, these would be tested around 12 weeks of age, and there is of

course a risk that the infection is perpetuated if a PI animal is born and a susceptible animal in early pregnancy becomes infected during this time period. The studies presented here support the claim that after removal of any PI animals initially present, the infection will cease to circulate (**I**, **II**) and that it is possible to actively delimit any further spread associated with the birth of additional PI animals (**IV**, **V**). The key is to know what animals are at risk of giving birth to PI calves (**III**, **IV**) and what animals are seronegative, pregnant and at risk of perpetuating the infection, and to separate these groups in time and place (**V**).

The results in paper **IV** showed that virus is present in foetal fluids in sufficient amounts to be isolated. It was therefore suggested that such fluids could be a significant source of virus spread within a herd, in connection with the birth of PI calves (**V**). However, only one of 20 exposures to foetal fluids collected at and after calving resulted in a seroconversion (**V,** *trials I-III*). This suggests that any dissemination of foetal fluids in connection with the birth of PI calves is of minor importance. So, from a control perspective, it is once again the PI calves themselves that are the problem. In paper **V**, *trial IV*, one calf seroconverted although it was not deliberately exposed to any potential source of infection. During the period when this calf was in the study, three PI calves were born and kept in the same stable unit, but only for a short period of time after birth. The PI calves were never given colostrum and it is therefore possible that their ability to transmit the virus by indirect means (via aerosol or personnel), was relatively higher than it would have been under natural conditions. Still, the results emphasise that potential PI carriers have to calve in an environment where their offspring can do no harm. This is in isolation, or in a unit where all other animals are immune. However, the risk of transmission from mothers of PI calves to susceptible animals seems to be negligible within a few days after calving (**V**).

Applicability to systems where vaccines are used, and type II strains are present

One argument raised as to why the test-and-cull approach could not work in countries where vaccines are available is that vaccination precludes the use of serology for herd diagnosis. Also, vaccines are perceived as necessary to prevent BVDV outbreaks in general and of type II BVDV in particular. There is also some confusion regarding how infectious acute BVDV is, which is of particular concern with respect to type II strains. However, although a lot of the recent work on BVDV infections have focussed on type II infections (Ellis et al., 1998, Odeon et al., 1999, Walz et al., 1999, Walz et al., 2001b), there appear to be no studies that have addressed transmission from animals primarily infected with BVDV type II and if this differs from type I strains. Also, to bear in mind is that

even though type II strains are prevalent in some parts of the world (Bolin & Ridpath, 1998, Fulton et al., 2000b) it is evident that a majority of the strains are just as avirulent as type I strains (Ridpath et al., 2000). Thus, type II strains essentially behave like type I strains and should also be able to control with the same approaches.

Epidemiological principles for large-scale eradication of BVDV without the use of vaccines (VI)

Since 1999, when paper **VI** was published, the Scandinavian countries have come further in their aims to eradicate BVDV infection using only zoo-sanitary measures. In Norway, the number of herds with restrictions has decreased from the top notation of 2,949 in July 1994 to 92 today (K. Plym-Forshell, personal communication). The corresponding figures for Denmark are approximately 6,000 in January 1997 to 350 today (PI status) (Bitsch et al., 2000, V. Bitsch, personal communication). In Sweden, the number of herds with confirmed or suspected infection peaked in July 1998 when 3,747 herds were under investigation. The current figure is 842 herds. The progress of the Swedish BVDV scheme is shown in figures 1-3. Figure 1 shows the number of herds declared free from infection after successful elimination of virus (n=3,272) and the number of new infections in herds previously certified as being free is shown in figure 2 (n=365). Figure 3 shows the successive decrease of herds with high antibody levels in bulk milk, indicating the decreasing prevalence of herds with active BVDV infection.

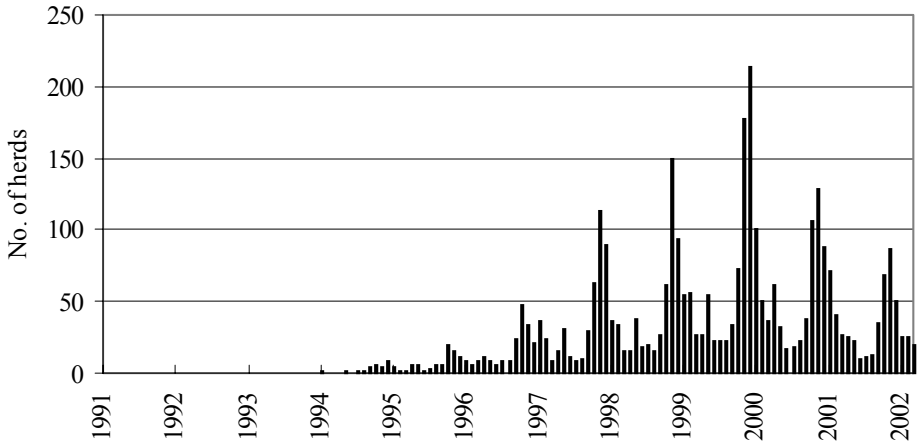

Fig. 1. Number of Swedish cattle herds certified as being free from the infection after successful elimination of BVDV within the voluntary national eradication scheme, per month from the start of scheme in September 1993 to April 2002.

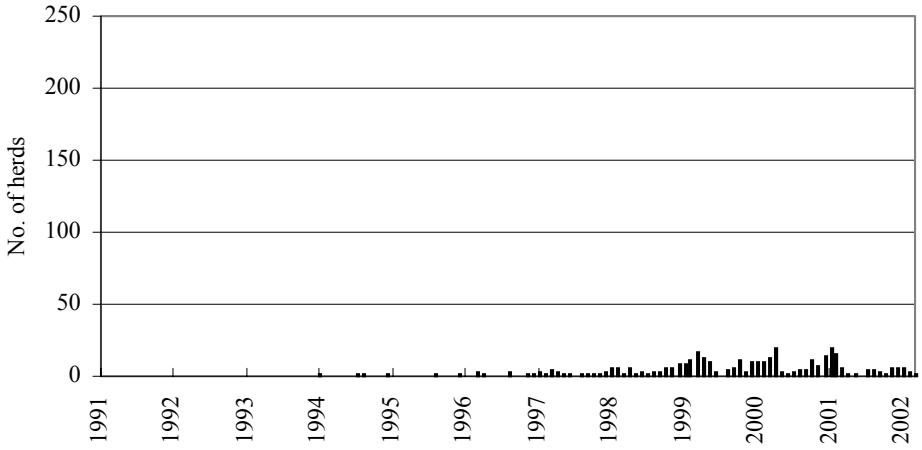

Fig. 2. Number of new cases of BVDV infection detected in Swedish cattle herds previously certified as being free from BVDV infection, per month from the start of the voluntary national eradication scheme in September 1993 to April 2002.

In contrast to what could be expected, the progress has been faster in high prevalence/high density areas like Denmark and South-East Sweden (Lindberg, 1996, Bitsch et al., 2000) than in low-prevalence areas like Finland and Northern Sweden. Thus, we do not have any reason to believe that the principles presented in paper **VI** will not hold also in other densely populated areas, as long as the known risk factors for transmission between herds are being managed. They are discussed in paper **VI** and some of them are further commented on below.

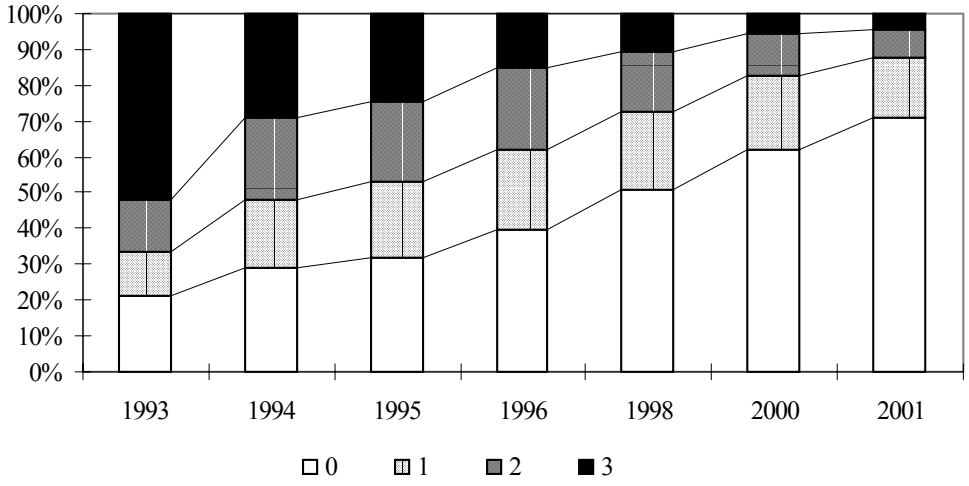

Fig. 3. Distribution of Swedish dairy herds over BVDV antibody classes in 7 national bulk milk surveys performed 1993-2001 (n=8,810 herds tested on all occasions). Class 0 and 1 are indicative of undetectable-low antibody levels. Class 2 is intermediate and class 3 reflects high levels of antibody to BVDV in bulk milk. Herds with recent or ongoing infection are usually found in class 3.

Wildlife reservoirs

The presence of non-bovine hosts of BVDV has been put forward as a reason why eradication could not be achieved (Kahrs, 1981). In Scandinavia, wild ungulates, mainly roe deer, can often be seen on cattle pastures. Consequently, any exposure to PI cattle could lead to the development of infected offspring. However, serological investigations made in roe deer populations do not suggest the presence of PI individuals (Nielsen et al., 2000), possibly because they are in early pregnancy at a time when cattle are not on pasture. Still, in areas where wild ungulates and cattle breed and graze synchronically, the situation could be different, as suggested by the results from Anderson and Rowe (1998). Persistent infection has been confirmed in eland (Vilcek et al., 2000). However, it is still unclear if the virus is able to persist in wildlife populations without being reintroduced, which would be required if it is to act as a long-term reservoir.

Semen and embryos

As discussed in paper **VI**, the main risks for reintroduction of BVDV after eradication are likely to be associated with the importation of livestock, semen and embryos and/or with the use of modified live vaccines. In Sweden, livestock and vaccines can more or less be disregarded, but semen and embryos are imported in significant quantities and often from countries with less control on BVDV infections. Both are regarded as safe means of introducing new genetic material, but recent studies have elicited that more knowledge is needed for proper risk management. It is becoming increasingly obvious that in-vitro fertilized embryos and contaminated biologicals are potential hazards in the use of embryo transfer (Trachte et al., 1998, Stringfellow et al., 2000, Vanroose et al., 2000). Also, the underlying biology of persistent testicular infection, described by Voges and colleagues (1998), is still unclear. For example, a recent study suggests that the timing of infection is irrelevant as virus could be isolated from testicular tissue 7 months after acute infection in post-pubertal bulls, although it could not be isolated from semen for more than 21 days (Givens et al., 2002).

In Sweden today, the import requirements for semen and embryos are regulated through the farmers' organisations. Essentially all imported semen is tested for the presence of viral RNA by RT-PCR. An exception is made for bulls that have been proven antibody negative after sampling, if they have been tested for virus at a previous occasion. Also, semen from antibody positive bulls from countries with a similar control system in place is excluded from testing. An alternative for countries that do not have control schemes would be to test seronegative bulls at AI stations on a monthly basis as suggested by Wentink and colleagues (2000). The risk of introduction of BVDV through embryo transfer is managed within the BVDV scheme. In affiliated herds, dams that receive imported embryos, or embryos from non-certified herds, have to be subjected to an antibody test 4-12 weeks after transfer to check for seroconversion.

Vaccination

The technical difficulties associated with BVDV vaccine production are acknowledged. BVDV is a virus that exhibits substantial variation and although the main antigenic epitopes are known, it is still difficult to produce vaccines that are able to prevent infection with heterologous genotypes and subtypes within these (Hamers et al., 2001). Also, it has not been possible to satisfy the needs for broad and high degree of protection with an ability to differentiate between natural infection and vaccination (van Oirschot, 1999).

In a recent review, van Oirschot (2001) lists a number of characteristics of an ideal vaccine. It should:

- contain a variety of immunogens and thus be multivalent in a single stable formulation
- only need one or two non-invasive administrations
- induce broad humoral and cell-mediated immunity
- confer lifelong protection
- induce herd immunity
- induce correlates of protection (*i.e.* there should be a measurable parameter that corresponds well with true protection)
- not be inhibited by maternal immunity
- not compromise the ability to diagnose infection
- be safe
- be cheap

Being rather provocative, one could say that a vast majority of the BVDV vaccines currently on the market only fulfil the last criterion.

In the US, where vaccination is widely used, more than 140 different products are registered (Ridpath et al., 2000). The demands for registration are low (US Government, 1997) and this has lead to a plethora of vaccines with questionable efficacy. There is a problem with their ability to prevent postnatal infection (van Campen & Woodard, 1997, Rush et al., 2001, Thurmond et al., 2001, Wittum et al., 2001) and none of the products actually claim to prevent prenatal infection. Thus there is a general inability to actually target the critical control points in BVDV epidemiology[1].

In addition, live vaccines in general have a problem with safety issues related to pestivirus contamination. The problem of inactivation of any adventitious virus, as well as its deleterious consequences, are well documented (Wensvoort & Terpstra, 1988, Kreeft et al., 1990, Levings & Wessman, 1991, Løken et al., 1991, Yanagi et al., 1996, Falcone et al., 1999, Audet et al., 2000). Recently, the Dutch IBR scheme, in which a modified live vaccine was used, suffered from severe

[1] However, just recently, the first BVDV vaccine with the indication "prevention of transplacental infection" was registered (Bovilis BVD; Intervet).

outbreaks of BVDV after contaminated vaccine batches had reached the market (Barkema et al., 2001). As a result, the European Council/EDQM recently saw a need to revise its guidelines for the production of bovine serum and for products where pestivirus contamination is an issue (EDQM, 2001).

Another complicating factor is that currently there are no vaccines for BVDV available that allow differentiation between natural exposure and vaccination (van Oirschot, 1999). Consequently vaccination compromises the ability to use serology for diagnostic purposes, including the cheap and rapid herd level tests that are available. Thus, unfortunately, when the farmer decides to vaccinate he also reduces the veterinarian's ability to help him if complications arise. Interpreting serological patterns in vaccinated herds is difficult as they vary with the types of vaccines and immunisation programmes used (van Campen et al., 1998, S. Hietala, personal communication).

Yet another problem is the way in which BVDV vaccines are used in the field (Kelling, 1996). A survey performed in the US indicated that although a majority of the herds vaccinated, less than 30% were doing it correctly (Quaife, 1996). As indicated earlier, modified live vaccines are capable of producing transplacental infections in pregnant animals and MD in PI cattle, if they are used incorrectly. They have also been shown to have the same immunosuppressive properties as wild strains (Roth & Kaeberle, 1983). Killed vaccines are safer to use, but require that strict immunisation programmes are adhered to in order to provide adequate protection. Minor human mistakes, like failing to vaccinate one or two animals, are sufficient for new persistent infections to become established if a PI animal is introduced in the herd. Therefore, in order to control BVDV, the awareness that biosecurity is the top one priority must always be high, irrespective of whether or not vaccines are used. Several studies indicate how the use of vaccines can give a false sense of security and thus promote risky behaviour by livestock owners (Vannier et al., 1997, Engel & Wierup, 1999). A risky behaviour with respect to BVDV is, *e.g.*, to purchase untested stock, or to use common pastures without knowing the status of the other herds using the same pasture. Because of the flaws of current vaccines and vaccination schemes, this is a serious problem.

Vaccination and test-and-cull approaches for BVDV eradication are not mutually exclusive, as long as safe (killed) vaccines are used and it is ensured that the herd's BVDV status can be monitored. However, the message has to be recognised that biosecurity is the first line defense and that vaccination is back-up protection. Also in schemes based entirely on test-and-cull, vaccination could be a helpful tool to break the vicious circle in infected herds. However, it should be regarded as therapy – a time limited measure – and not as prophylaxis.

Biosecurity

Eradication at a national scale has been accomplished for other viral diseases in cattle, such as EBL and IBR. It has also been achieved in pig populations, where Aujeszky's disease is a good example of how a highly prevalent infection can be eradicated once the epidemiological understanding and good diagnostic tools are available (Andersen et al., 1989, Engel, 1999). The general experience with vaccines seems to be that they can be useful to prevent severe outbreaks, but also that they are not solely sufficient to prevent transmission between herds and achieve eradication (Stegeman, 1997, Vannier et al., 1997). Instead, the key factor to success seems to be biosecurity. How rigid the biosecurity barrier has to be will vary with type of infection, but for pseudorabiesvirus and BHV-1, the control of new introductions (by testing or by recruitment from certified free herds) seem to be a key issue, just like for BVDV (Stegeman et al., 1996, van Schaik et al., 1998).

However, despite massive information and education about biosecurity, it is unlikely that all farmers in an area will adopt perfect routines. Therefore, the single key measure for successful eradication of BVDV from cattle populations is to block PI animals from having access to 'hot-spots' like cattle auctions, common pastures and other places where animals from many different farms co-mingle. If animals in early pregnancy are present and become infected, they will efficiently introduce BVDV into their herd of destination. Thus, testing for BVDV *always* has to start with a test in the herd of origin.

Implications for future research

The purpose with research is not only to answer questions but also to identify new ones. I believe that it will be important to continue to clarify how acute type II infections differ from persistent infections with respect to infectivity, as this seems to be a paradigm that obstructs any initiatives to efficiently control BVDV infections in some countries.

Also, I think the prospects for prenatal diagnosis by using non-invasive methods like serology, should be further investigated, in particular to determine whether it is practicable in vaccinated herds. We made our study on antibody positive animals in herds with ongoing infection where the average antibody levels were generally high, and we were still able to get a good discrimination between antibody positive dams carrying healthy foetuses and those that were pregnant with PI calves. This implies that it could be possible to use this approach also in dams that are vaccinated.

The underlying immunological mechanisms responsible for the high antibody titres in PI carriers also warrants further study. We have been able to demonstrate the presence of virus in maternal circulation of PI carriers, in samples obtained

from the heifers used in study **IV** (data not shown). This indicates one means by which the maternal immune system could be continuously challenged, but the routes by which the virus travels do not yet seem to be understood.

We did not experience any negative side effects of the foetal fluid sampling but in the study by Callan and colleagues (2002), 14 of 169 animals aborted or delivered premature calves within 3 weeks of the procedure. It could not be determined if this was due to the procedure or a result of the infection. Still, this suggests that safety issues related to the methodology should be studied further.

Houe (2000) emphasizes that any choice of control approach should be based on thorough epidemiological investigations in the areas where the programme is going to be applied. However, to work with disease control in animals is nevertheless to work with people, and it is therefore important to have an understanding of how social factors contribute to positive progress. These are *e.g.* the infrastructure of the cattle industry, the educational level of farmers and other professionals involved in the eradication as well as the general attitude towards the scheme among stakeholders. The overall question is really how to communicate the right message at the right time to all concerned, not only in order to increase the general knowledge, but to actually change attitudes and behaviour. I believe that future cows and farmers would benefit from more research being done on the social epidemiology of farm animal disease control and eradication, alongside with the classical epidemiological questions still to be answered.

Concluding remarks

∴ Animals acutely infected with type I BVDV are highly inefficient in transmitting the infection to susceptible animals. The rate of transmission is not necessarily increased by the presence of a concurrent BCV infection, despite marked clinical disease in both infective and susceptible animals. Given these conditions, the probability that BVDV infection can be maintained in a herd after all PI animals have been removed is negligible.

∴ There are three ways in which diagnosis of persistent infection in the unborn calf can be made. The first is by serology, using an indirect ELISA able to quantitatively reflect the antibody titre on serum samples taken in the last trimester. The second is by analysing samples of foetal fluid obtained in late gestation using RT-PCR and the third is by isolating virus from the same specimen. Serology is highly sensitive, but the specificity is relatively low. RT-PCR is less sensitive, but performs better than VI. The specificity of the latter two tests has not been investigated.

∴ Antibodies can be present in foetal fluid, and this affects the sensitivity of VI and possibly also the RT-PCR when applied to this specimen. The presence of antibodies in foetal fluids is likely to reduce the infectivity associated with such fluids.

∴ Foetal fluids expelled in connection with the birth of PI calves can contain infectious doses of virus. If susceptible animals in early pregnancy are in contact with such fluids, a new persistent infection could be established, and BVDV infection could be perpetuated in the herd. However, the infectivity associated with foetal fluids is low and uterine lochias excreted after calving are not likely to be infectious.

∴ Rapid removal of newborn PI calves does not prevent further spread of BVDV infection if susceptible animals are kept within the same premises. Strategies aimed at delimiting further spread have to include separation of animals in early pregnancy from calving cows/heifers. If prenatal diagnosis of persistent infection can be achieved, separation can be restricted to dams identified as potential PI carriers.

∴ The principles for eradication of BVDV from cattle populations that have been applied in the Scandinavian countries and in Austria still hold.

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