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Neonatal Calf Diarrhoea with Special Reference to Rotavirus Infections

Significance, epidemiology and aspects of prevention

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

Neonatal calf diarrhoea with special reference to rotavirus infections. Significance, epidemiology and aspects of prevention.

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i "Ettan", Klinikcentrum, Uppsala, fredagen den 1 oktober 1999, kl 13.00.

Av fakultetsnämnden utsedd opponent: Professor Linda J. Saif, Food Animal Health Research Program, OARCD/The Ohio State University, Wooster, Ohio 44691, USA

Abstract

This thesis deals with neonatal calf diarrhoea (NCD) in Sweden, with special reference to bovine rotavirus (BRV) infections. Clinical significance, molecular epidemiology and aspects of prevention were investigated.

The prevalence, incidence and role of BRV were investigated in outbreaks and endemic situations, and by an experimental study in a calf model. Group A BRV was found to be the most commonly occurring causative agent in NCD. The clinical signs in calves were aggravated after experimentally induced dual infection of BRV and bovine viral diarrhoea virus (BVDV). Otherwise, dual infections were rarely detected in the herds, and *Escherichia coli K99+* was a rare finding, suggesting that the indications for antimicrobial drugs in NCD therapy are few. Poor growth in diarrhoeic and recovered calves was demonstrated, which implies financial loss and stresses the need for continued milk feeding to diarrhoeic calves.

A one-step on-site BRV test was developed and evaluated. When compared to the reference ELISA, the sensitivity of this test was 89% and the specificity 99%. The simplicity and rapidity of the test procedure make it suitable for use in practice.

Four BRV G-types (G3, G6, G8, G10) were detected in faecal samples, of which G10 and G6 were predominant (91%). G10 was the major G-type in samples from dairy calves and G6 the sole type in samples from beef suckler calves. One major G-type persisted in a herd for a period of 4 years, probably due to the stable character of BRV and to restricted calf trading.

Aspects of prevention and control of BRV infection were investigated in a cohort study. A dramatic decline in the incidence of NCD was demonstrated after strict closure and eradication of BVDV in a herd. This was considered to be an effect of the overall reduction of the herd's exposure to pathogenic agents. An association between low serum

IgG and increased incidence of diarrhoea highlights the importance of colostral feeding to newborn calves.
Attention is called to the claim that control and prevention of NCD is possible without vaccination programmes and antimicrobial therapy, by reduction of the exposure of calves to pathogenic agents through control of direct contact between cattle, and by optimal colostral feeding.

Key words: neonatal calf diarrhoea, rotavirus, bovine viral diarrhoea virus, closed herd, prevention, immunoglobulin, risk factor, G-type.

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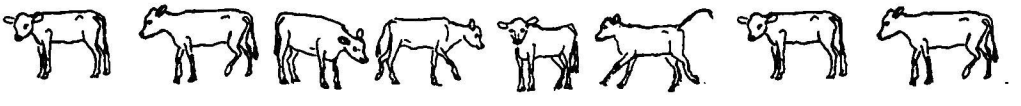
Que s çay-je?
Michel de Montaigne



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Contents

Appendix

Papers I-V

This thesis is based on the following papers, which will be referred to in the text by their corresponding Roman numerals:

I. Group A rotavirus as a cause of neonatal calf enteritis in Sweden. K.deVerdier Klingenberg & L. Svensson. Acta vet scand 1998, 39, 195-199.

II. Evaluation of a one-step test for rapid, in practice detection of rotavirus in farm animals. K. de Verdier Klingenberg & J. Esfandiari. Vet Rec 1996, 138, 393-395.

III. Rotavirus G-type restriction, persistence and herd type specificity in Swedish cattle herds. K. de Verdier Klingenberg, M. Nilsson, L. Svensson. Clinical and Diagnostic Laboratory Immunology 1999, 6, 181-185.

IV. Incidence of diarrhea among calves after strict closure and eradication of bovine viral diarrhea virus in a dairy herd. K. de Verdier Klingenberg, I. Vågsholm, S. Alenius. JAVMA 1999, 214, 1824-1828.

V. Bovine viral diarrhoea virus aggravates clinical signs in experimentally rotavirus infected calves. K. de Verdier Klingenberg. (Submitted).

Abbreviations

BCV	bovine coronavirus
BHV-1	bovine herpesvirus 1
BLV	bovine leukaemia virus
BRSV	bovine respiratory syncytial virus
BRV	bovine rotavirus
BVDV	bovine viral diarrhoea virus
dsRNA	double stranded ribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
Ig	immunoglobulin
Mabs	monoclonal antibodies
NCD	neonatal calf diarrhoea
NSP	nonstructural protein
RNA-PAGE	ribonucleic acid - polyacrylamide gel electrophoresis
RT-PCR	reverse transcription - polymerase chain reaction
spp	species
VP	viral protein

Introduction

Diarrhoea has been part of the disease panorama of the young throughout the ages, and is still a major cause of morbidity and mortality throughout the world (Saif 1990a, Bern & Glass 1994, Glass et al 1996). The neonatal diarrhoea complex affects both man and animals and demonstrates many similarities between species in the etiology and epidemiology of the disease. Morbidity and mortality differs depending on the pathogenic agents involved, the infectious dose, the host resistance, and—especially—on the immidiate environment of the neonate, i.e., management as well as physical environmental factors. Much research has been done on this topic and a large body of knowledge accumulated, but successful control of diarrhoeal disease in man and animals is highly dependent on practical concerns—how the knowledge is put into practice. This poses a great challenge for researchers as well as a great opportunity for collaboration between researchers, physicians and veterinarians.

This thesis deals with neonatal calf diarrhoea with special reference to bovine rotavirus (BRV) infections in Swedish calves.

General Background

Rotaviruses

Rotaviruses are a major cause of diarrhoea in the young of many mammalian species, including humans, cattle, pigs and horses (Flewett & Woode 1978). Such disease in an animal is primarily caused by rotavirus originating from the same animal species. The extent to which transmission of rotaviruses between species occurs is unclear. Interspecies infection can occur under experimental conditions, but the significance under natural conditions seems to be of little importance (Kapikian & Chanock 1996).

Structure and function

Rotaviruses are classified as a genus within the family *Reoviridae* (Matthews 1979) and was first described in diarrhoeic calves in 1969 (Mebus et al. 1969), when Mebus and collaborators detected an agent, later referred to as the reference rotavirus strain NCDV, Nebraska calf diarrhea virus. Rotaviruses have a distinct morphology (Figure 1), which has been elucidated in detail (Estes & Cohen 1989, Mattion et al. 1994), and is identical for all species evaluated to date.

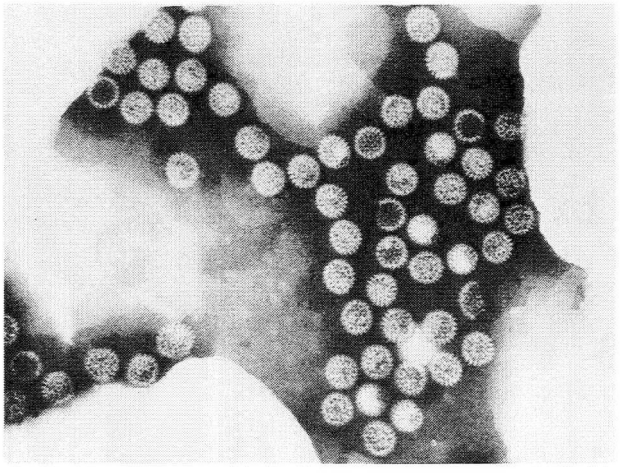

Figure 1: Rotavirus particles by electron microscopy. The term *rotavirus* is derived from the Latin word "rota" meaning "wheel". Photo by L Svensson.

The overall diameter of the virus is about 65-75 nm, and the rotavirus particle consists of a non-enveloped, triple-layered icosahedral protein capsid, that encloses the segmented dsRNA genome. Each of the 11 dsRNA segments

encodes one protein, i.e., 6 structural and 5 nonstructural proteins. The structural proteins build up the concentric triple-layered viral capsid (Figure 2). The outer protein layer is composed of the viral proteins VP7 and VP4. VP4 spikes penetrate the VP7 layer to interact with the middle protein layer (Shaw et al. 1996), composed of the major structural protein VP6. The inner, VP2, layer encloses VP1, VP3, and the viral genome. Electron cryomicroscopy and computer image analysis have enabled visualization of each one of the three protein layers, thus enhancing the understanding of the relation between structure and function (Shaw et al. 1996).

Only two viral proteins, VP4 and VP7, are known to induce neutralizing antibodies (Offit & Blavat 1986). The genes that code for VP3, VP4 and VP7, and NSP1, NSP2 and NSP4, have been associated with pathogenicity, but no single gene has been shown to determinate rotavirus pathogenicity under all circumstances, in all strains and in all hosts (Desselberger 1997). The diversity of pathogenicity is illustrated in the recent identification of NSP4 as a viral enterotoxin (Ball et al. 1996). NSP4 is a transmembrane glycoprotein of the endoplasmic reticulum, associated with budding through the membranes of the endoplasmatic reticulum during viral morphogenesis (Mattion et al. 1994). This new mechanism of rotavirus pathogenesis has been suggested to be associated with mutations in the NSP4 gene (Zhang et al. 1998).

Group and type

Although rotaviruses are morphologically indistinguishable, they are classified into different electropherotypes, serogroups, subgroups and serotypes/genotypes. Electropherotypes represent different patterns due to migration of the dsRNA-genome segments in polyacrylamide gels. Different migration patterns do not necessary indicate differences in the size, but in the flexibility of the molecules, due to nucleotide sequence differences and thus local changes in binding between the complementary RNA strands (Holmes 1996).

Serogroups and subgroups are based on antigenic specificities, where VP6 plays an important role. Seven different serogroups (A-G) have been reported, determined by epitopes on rotaviral proteins (Kapikian & Chanock 1996). Two distinct subgroups of group A rotaviruses, I and II, are described (Greenberg et al. 1983, Theil & McCloskey 1989), while group B-G rotaviruses have not been classified further.

The specificities of the two outer capsid proteins, VP7 and VP4, are used for serotype/genotype classification of group A rotaviruses. The VP7-specificity is referred to as G-type (*g*lycoprotein) and the VP4-specificity as P-type (*p*rotease-sensitive protein). The G-types have been closely studied and 14 G-types have been identified in animals and humans (data reviewed in Kapikian & Chanock

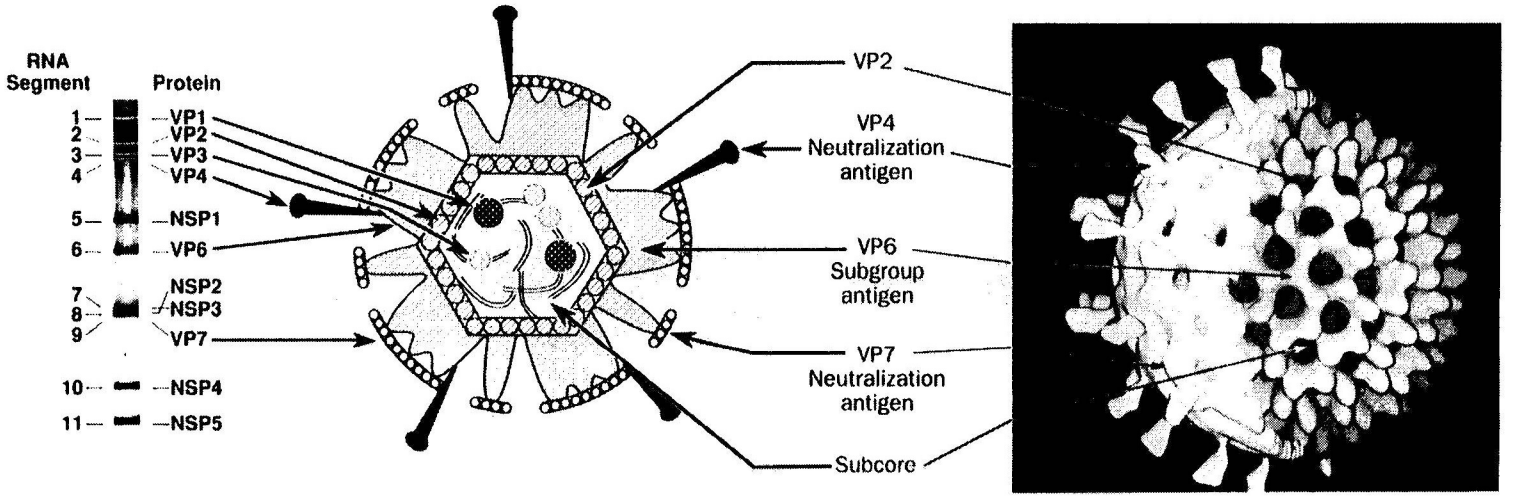

Figure 2: Rotavirus genes, proteins, and particle structure. Photo reprinted with the kind permission of Mattion, Cohen & Estes (1994).

1996). The classification based on P-specificity has been more complicated. Currently, 11 distinct serotypes and 19 genotypes according to P-specificity have been described, although there is an agreement that only VP4 serotypes as determined by neutralization assay are classified as P-types (Kapikian & Chanock 1996). Various combinations of P- and G-types have been found in reference strains and field isolates.

Characterization of rotaviruses by determination of serogroup, subgroup, electropherotype and/or serotypes/genotypes enables the study of rotavirus molecular epidemiology and enhances the understanding of how rotaviruses appear in populations. In addition, it offers an elegant system for surveillance of rotavirus strains, e.g., in the national rotavirus vaccination program for children started in the US in August 1998 (Offit 1998, Ramachandran et al. 1998).

Bovine rotavirus infections

Clinical manifestations

Since 1969, bovine group A rotavirus (BRV) infections have been studied under both natural and experimental conditions, and in conventional as well as in gnotobiotic calves (Woode & Crouch 1978). A striking feature of BRV in calves all over the world, is the potential for diarrhoea outbreaks with a sudden onset and a rapidly spreading infection. Several characteristic properties of rotavirus contributes to this. BRV is physicochemically stable (Theil 1990) and ubiquitous in the environment of cattle, which allows persistence in the environment and retainment of the infectiousness for months. In addition, BRV is highly contagious, the infectious dose required is low (Kapikian & Chanock 1996), the incubation period is short, 1-2 days (Mebus et al. 1969), and BRV is shed in large quantities in diarrhoeic faeces.

The clinical signs are variable (Mebus et al. 1969, McNulty et al. 1976, Woode & Crouch 1978, de Leeuw et al. 1980a, McNulty & Logan 1983) and subclinical BRV infections are common (Myers et al. 1984, Snodgrass et al. 1986, de Rycke et al. 1986, Waltner-Toews et al. 1986). The diarrhoeal disease caused by BRV is usually mild, but may vary clinically from mild to severe. The clinical picture comprises depression, anorexia, mild fever and diarrhoea, and the consistency of the diarrhoeic faeces varies from liquid to pasty, often containing mucus and occasionally blood. The colour of the faeces is commonly yellowish if the calf is on a milk diet, but variation in colour is considerable.

Pathogenesis

BRV infections are localized to the small intestine. BRV is transmitted by the faecal-oral route, possibly aided by inhalation of aerosolized BRV (Ijaz et al. 1994). Via the luminal surface, BRV infects the mature, absorptive epithelial cells of the apical half of the villi in the small intestine (Mebus et al. 1971, Reynolds et al. 1985, Hall et al. 1988). The virus multiplies within the cells, normally causing rapid death by lysis (Altenburg et al. 1980, Estes 1996). Destruction of the villous enterocytes results in villous atrophy, stunting and fusion of villi, resulting in a reduced absorptive capacity of the villous enterocytes due to morphologic and functional impairment (Saif 1990b). The damaged enterocytes are replaced by proliferating and migrating crypt cells, which are refractory to further infection (Mebus & Newman 1977). The reduction of absorptive intestinal surface leads to malabsorption and maldigestion. Nevertheless, the mechanisms of production of diarrhoea are not fully understood, and still new mechanisms of viral pathogenesis are discovered, as e.g., the recently described rotaviral enterotoxin (Ball et al. 1996).

Mixed infections e.g., with coronavirus, *Cryptosporidium spp* or enterotoxigenic *Escherichia coli,* are considered common (Acres et al. 1977, Moon et al. 1978, Reynolds et al. 1986, Hall et al. 1988) and able to increase the severity of the BRV infection, although the pathogenic mechanisms for this remain unclear (Snodgrass 1990a). Besides co-infections, the course of the disease may also be complicated by dehydration and electrolyte imbalance, acidosis, and intestinal damage. The impact of rotaviral virulence on the course of the disease has been discussed, and BRV virulence has been associated with the site of infection (Bridger et al. 1992) and with calf age (Bridger 1994a, Varshney et al. 1995). A number of immunologic and nonimmunologic host-related factors also affect the course of a rotavirus infection (Offit 1996), and in calves, the protection from clinical illness provided by maternally aquired specific IgG has been well established (McNulty et al. 1976, Snodgrass et al. 1980, Snodgrass et al. 1982, Saif et al. 1983, Saif & Smith 1985).

Clearance of the infection probably occurs by several mechanisms: immature and refractory enterocytes, gut peristalsis, interferon production, and virus-specific humoral and cellmediated interactions (Offit 1996). For the most part, natural rotavirus infection induces protection against disease by reinfection, probably by immunologic intestinal mucosal defense (Offit 1996).

Diagnosis

The clinical signs alone are not sufficient for establishing a rotavirus diagnosis. Detection of seroconversion in young calves is rarely an appropriate diagnostic

method for BRV infection, because of the masking of passive immunity, although detection of specific IgM and IgA antibodies may be relevant.
However, the diagnosis of BRV infection is most commonly based on the detection of the virus, viral antigen or viral nucleic acid in faecal samples. Diarrhoea usually coincides with the duration of rotaviral shedding, and the optimal faecal sample should be collected during the first four days of illness (Kapikian & Chanock 1996). Various methods for detection of BRV has been used, and the diagnostic methods applied are, with few exceptions, similar for rotaviruses of different species origin (Yolken & Wilde 1994).

BRV was successfully cultivated in cell culture early (Mebus et al. 1971), however rotaviruses are not routinely isolated on cell cultures. Electron microscopy (EM) and RNA polyacrylamide gel electrophoresis (RNA-PAGE) remain mainstays in rotavirus diagnosis, permit visualization of the viral particle or the viral nucleic acid, and are able to detect all groups and all sero/genotypes of rotaviruses (Saif et al. 1977, Theil et al. 1981, Herring et al. 1982). RNA-PAGE also provides a simple method for electropherotypic characterization of rotaviruses (Fijtman et al. 1987, Theil & McCloskey 1989, Bellinzoni et al. 1987, Holmes 1996). Several different immunoassays for detection of BRV are available, of which group A rotavirus antigen enzyme-linked immunosorbent assay (ELISA) represents a commonly used, fast and simple diagnostic method, suitable for routine diagnosis (Ellens & de Leeuw 1977, Yolken & Leggiadro 1986, Yolken & Wilde 1994). These rotavirus ELISAs, that utilize antibodies directed against VP6, detect all group A rotavirus strains comprising all serotypes, and thus is appropriate for group A rotaviruses from all mammalian species. Non-group A rotaviruses, however, remain undetected. Besides detecting group A rotaviruses, ELISAs based on monoclonal antibodies (Mabs) can be used in more specific ways for sub- and serotyping. If the number of samples is limited, latex agglutination tests are often faster and simpler than ELISAs, but false positive reactions may occur (Sanekata et al. 1981, Sukura & Neuvonen 1990, Bendall et al. 1991, Brink et al. 1992).
The development of the reverse transcriptase-polymerase chain reaction (RT-PCR) (Gouvea et al. 1990, Wilde et al. 1991, Taniguchi et al. 1992, Isegawa et al. 1993, Chinsangaram et al. 1993) and gene probes (Parwani et al. 1992, Hussein et al. 1993, Muñoz et al. 1995) allows sero/genotyping of rotaviruses and thus has enhanced the possibility for studies on molecular epidemiology.

Epidemiology

For three decades, studies on the BRV epidemiology have been carried out in a variety of countries, all over the world (White et al. 1970, McNulty et al. 1976, de Leeuw et al. 1980a, Schusser et al. 1982, Reynolds et al. 1985, Bellinzoni et al. 1989, Lucchelli et al. 1992, Huang et al. 1992). Seroprevalence in the cattle population is high and most adult cattle are seropositive to BRV (Schlafer &

Scott 1979, Schwers et al. 1984, Saif & Fernandez 1996) The prevalence of BRV shedding in calf faeces is high (Reynolds et al. 1986, Chinsangaram et al. 1995, Lucchelli et al. 1992, de Rycke et al. 1986, Bellinzoni et al. 1987), and calves are commonly infected during the first weeks of life (de Leeuw et al. 1980a, McNulty & Logan 1983).

The BRV serotypes G6 and G10 are the predominating G-types in cattle populations (Snodgrass et al. 1990b), however G1 (Blackhall et al. 1992), G2 and G3 (Hussein et al. 1993), G7 (Brüssow et al. 1992), and G8 (Snodgrass et al. 1990b) have also been reported. Association of G-type distribution, with herd type, region, management conditions, clinical symptoms and calf age, has been suggested (Bellinzoni et al. 1989, Clark et al. 1996, Hussein et al. 1995, Lucchelli et al. 1994, Muñoz et al. 1993). The effect of BRV vaccination on the G-type distribution has not been investigated, but in US, G6 remains the most common G-type detected despite the widespread use of a G6 vaccine (Saif & Fernandez 1996). Four BRV P-types have been reported, namely P6[1], P7[5], P8[11], P[12] (Snodgrass et al. 1992). Certain rotavirus types, e.g., G8 (Kelkar et al. 1996) and P8[11] (Gulati et al. 1999), are present in human and bovine populations and have been suggested to represent natural reassortants between human and bovine rotaviruses.

The data on the seroprevalence of bovine non-group A rotaviruses is limited, and comparatively few reports on the clinical importance of non-group A rotaviruses in cattle have been published (Saif 1990c, Tsunemitsu et al. 1992, Bridger 1994b, Chinsangaram et al. 1995). Group B rotaviruses have been detected in the faeces from diarrhoeic calves (Mebus et al. 1978, Snodgrass et al. 1984a, Chinsangaram et al. 1994, Chang et al. 1997). Group B (Parwani et al. 1996, Chang et al. 1997, Tsunemitsu et al. 1999) and C rotaviruses (Tsunemitsu et al. 1991) have been isolated and characterized in adult cows.

Therapy, prevention and control

There is no specific therapy for BRV-induced neonatal diarrhoea, but antimicrobial drugs are used to treat possible secondary bacterial infections. Fluid therapy is essential in neonatal scours. The withholding of milk to dairy calves has been considered advantageous, but modern studies have shown beneficial effects of continued milk feeding during diarrhoea (Bywater 1980, Heath et al. 1989, McGuirk 1998).

Since BRV is highly stable and shed in enormous amounts from infected calves, it is not possible to eradicate the virus from infected cattle herds and to date, no fully effective methods of prevention and control of BRV infections are avilable. The major strategies currently used for prevention and control are boosting the immunity of susceptible calves and reducing their exposure to BRV. This is

achieved in practice by adoption of vaccination programmes and of good management practices (housing, feeding, hygiene), where the importance of adequate colostral feeding can not be over-emphasized.

Since BRV infections are endemic and the infection is restricted to the small intestine, current vaccination strategies focus on maternal passive immunization (Saif & Fernandez 1996), to boost colostral and milk BRV antibodies titers, in combination with management practices to ensure adequate colostral feeding. Immunization of cows against BRV gives rise to a heterotypic BRV antibody response to the vaccination strain and boost preexisting BRV antibodies as well (Snodgrass et al. 1984b, Brüssow et al. 1987, Saif & Jackwood 1990, Conner et al. 1994). The immunologic basis of heterotypic protection, however, is not clear (Offit 1996). The aim of maternal immunization is to provide protection to the neonatal calf against diarrhoeal disease. Thus a natural BRV infection has a subclinical course, induces active immunity and thereby prevention of subsequent disease. The impact of maternal vaccination, on reduction of calf diarrhoea and BRV shedding, has been studied in several field trials, but the effects have been difficult to evaluate (Saif & Fernandez 1996). BRV live and killed vaccines for maternal immunization are commercially available in many countries.

Oral vaccination with live, attenuated vaccine to induce active immunization in calves was tried in the early history of rotaviruses (Mebus et al. 1973, de Leeuw et al. 1980b, Thurber et al. 1977, Saif & Fernandez 1996), before detailed knowledge of the antigenic complexity of rotaviruses and the induction of passive immunity was aquired. Poor results in field trials, due to interference by colostral antibodies, rotavirus exposure prior to protection and disadvantages in farm handling practices, led to this strategy soon being replaced by maternal vaccination (Saif & Fernandez 1996). However, a commercial rota-corona-vaccine for oral administration to calves is presently available in the US, but is frequently not effective in protecting the young calves and outbreaks of enteric disease associated with BRV continue in vaccinated herds (Saif & Jackwood 1990, Lucchelli et al. 1992, Saif et al. 1994).

In the 90s, the development of rotavirus subunit vaccines composed of VLP (virus-like particles) and CLP (core-like particles) represent a promising new approach to inducing maternal immunity. This new generation of vaccines are noninfectious, stable, antigenically authentic, highly immunogenic, and can be modified according to serotypic changes in the field (Labbé et al. 1991, Crawford et al. 1994, Saif et al. 1994, Saif & Fernandez 1996).

Neonatal calf diarrhoea

A multifactorial disease

The term *neonatal calf diarrhoea* generally refers to a disease complex characterized by acute, undifferentiated diarrhoea in young calves. Economic losses in neonatal calf diarrhoea have been estimated (House 1978, Gunn & Stott 1996) and neonatal scours constitute substantial cost in terms of calf mortality, opportunity costs for labour and capital, veterinary costs and loss in calf value.

Neonatal calf diarrhoea is a multifactorial disease, where—besides the causative pathogenic agent—calf age, management and environmental factors, may influence the clinical outcome (Bruning-Fann & Kaneene 1992). The most commonly reported causative pathogens include rotavirus, coronavirus, *Cryptosporidium spp* and *Escherichia coli*. Other suggested pathogens in neonatal calf diarrhoea are *Salmonella spp*, bredavirus, calicivirus, astrovirus, parvovirus, *Campylobacter spp*, *Clostridium perfringens* (Tzipori 1981, 1985) and *Eimeria spp*. Many field studies have been conducted in several countries, to reveal the occurrence of enteropathogenic agents, and rotavirus is frequently the most commonly detected pathogen (Acres et al. 1977, Moon et al. 1978, Waltner-Toews et al. 1986, Snodgrass et al. 1986, de Rycke et al. 1986, Reynolds et al. 1986, de Visser et al. 1987, Hall et al. 1988, Bellinzoni et al. 1990, Viring et al. 1993, Bendali et al. 1999)

BVDV infections

Bovine viral diarrhoea virus (BVDV) belongs to the genus pestivirus of the family *Flaviviridae*, and is classified into BVDV type I and type II. Infections of BVDV type I are widespread all over the world and endemic in most cattle-rearing countries. Acute and persistent BVDV infections have been implicated in neonatal calf diarrhoea (Lambert et al. 1974), but the role of BVDV herein has been poorly defined and the pathogenesis is not clear (Baker 1995, Bielefeldt-Ohmann 1995). At present, probably more important than a direct cause of neonatal calf diarrhoea, is the ability of BVDV to cause immunosuppression and increase the susceptibility of the host to other pathogenic agents (Baker 1995, Potgieter 1995). This effect of acute and persistent BVDV infections may enhance the severity of calf diseases (Barber et al. 1985, Wray & Roeder 1987, Larsson et al. 1994, Moerman et al. 1994, Brodersen & Kelling 1998), including neonatal calf diarrhoea (Baker 1995), making knowledge about the BVDV status of a herd crucial, when investigating calf diarrhoea problems.

In the 1980s and 90s, several herd outbreaks of severe acute disease associated with BVDV type II, occurred in the United States and Canada (Pellerin et al.

1994). Recently, a severe and rapidly progressive disease with multisystemic distribution of BVDV in young calves, 35 days old, was demonstrated after experimental infection with BVDV type II (Ellis et al. 1998). This demonstrates a new clinical picture associated with acute BVDV infection in immunocompetent calves, indicating heterogeneity in the virulence in BVDV strains, which may also interfere with the neonatal calf diarrhoea syndrome.

Cattle management in Sweden

Cattle management practices vary greatly around the world. In Sweden, the dairy tradition of old has been strong, and the Swedish dairy cow of today is a thoroughbred high-producer, in one of 13,500 herds with an average size of 32 cows (Statistics Svensk Mjölk 1999). National control programmes (BVDV, BLV, BHV-1) comprise 100% of the dairy herds and, apart from ringworm and the occasional case of clostridiosis and babesiosis, no vaccination of cattle is performed. The neonatal calf mortality up to 30 days of age has been reported to be 1,5% (Olsson et al. 1993). Calves are rarely purchased externally and brought into a herd before the age of 6 weeks, and transportation of calves younger than 2 weeks is prohibited by law. By tradition, calves are separated from their dams at birth, and reared in individual pens by a twice-a-day bucket feeding system, although in recent years new management systems such as calving pens, group rearing, organic farming, etc., have gained ground. During the last decades, the number of beef suckler herds has increased to about 16,500, though the herds are small (on average 10 cows) and the management practices may be of an amateurish nature.

The Present Study

Aims

The first part in the present work (publications I, II, IV and V) deals with the *clinical significance* of BRV infections in Sweden. The objectives were to study the prevalence and incidence of BRV infections, and to investigate the role of BRV as a cause of diarrhoea in Swedish calves, the clinical epidemiology of BRV infections and some financial aspects on neonatal calf diarrhoea.

The second part deals with the *molecular epidemiology* of BRV in Sweden (III). The objectives were to study the distribution of BRV G-types in different regions of Sweden, over time, in different herd types and in clinically and sub-clinically BRV-infected calves.

Finally, *aspects of prevention* against BRV infection and neonatal calf diarrhoea were investigated (IV, V). The objectives were to identify and evaluate risk factors for diarrhoea, and to investigate the effect on diarrhoea of BVDV and of closure of the herd.

Definitions and comments on materials and methods

The *clinical significance* of BRV was investigated using faecal examination of BRV in naturally (I and IV) and experimentally (V) infected calves. Investigation of the clinical epidemiology of BRV was achieved in a two-year sudy of a large dairy herd (IV). To demonstrate the clinical significance of BRV for veterinarians and farmers, a one-step test using comparison with a reference ELISA was developed and evaluated for field diagnosis (II). In the BRV *molecular epidemiology* study (III), BRV in faecal samples were G-type characterized. The investigation of some *aspects of prevention* was achieved through a dairy herd cohort study (IV) and experimental study of BRV and BVDV infections in a neonatal calf model (V).

Several diagnostic methods were applied in the present work. A group A BRV antigen-ELISA was used throughout the study for detection of BRV in faecal samples, supplemented by RNA-PAGE (I, III), BRV G-typing RT-PCR (III) and by a one-step on-site rota test (II). Analyses for BVDV in serum were performed by an immunoperoxidase method (IV) or PCR (V) and for antibodies to BVDV by an indirect ELISA (IV, V). Serum samples were analysed by electrophoresis for protein fractions (IV) and the dry matter content in faecal samples was determined by weighing before and after evaporation (I, V). The presence of *Cryptosporidium spp* was determined by parasitological demonstrations of

oocysts in faecal smears and *Escherichia coli K99+* by slide agglutination test (I).

BRV infection was defined as detection of BRV in a faecal sample. This concept is commonly used in literature on BRV, although the definition has obvious limitations, a more correct definition being e.g., BRV replication in the enterocytes or BRV seroconversion. Nevertheless, the definition applied has practical advantages. The methods used for detection of BRV were ELISA (I-V), RNA-PAGE (I, III), RT-PCR (III) and a one-step on-site rota test (II). An indirect group A rotavirus antigen-ELISA, the reference method used for detection of human rotaviruses at the Swedish Institute for Infectious Disease Control, was used as overall diagnostic method, with the same cutoff applied for samples of bovine origin as well as for other species. The group A rotavirus ELISA as well as the RT-PCR have been described in several publications (Gouvea et al. 1990, Gouvea et al. 1993, Gouvea et al. 1994, Nakagomi et al. 1991, Santos et al. 1998, Svensson et al. 1983, Svensson et al. 1986). RT-PCR is primarily a typing method rather than a rotavirus detection method, since there is a significant concern of false negative results when the PCR method is used for diagnostic purposes. The RT-PCR method has a sensitivity comparable to RNA-PAGE (Gouvea et al. 1990), and RNA-PAGE and silver staining has a sensitivity of approximately 70% compared to EM and ELISA (Svensson et al. 1986).

Acute primary *infection of BVDV* in a neonatal calf was defined as seroconversion when paired serum samples were analyzed on the same ELISA plate. Seroconversion was considered to have occurred when the ELISA absorbance value for a calf's first serum sample was < 0.2 and a > 0.2 increase in absorbance value between the first and second serum samples was demonstrated. Confirmation of the BVDV infection status of the calves was obtained by analysing blood samples, by PCR, for BVDV (V), or by comparing test results with herd data from the official BVDV control (IV).
In the study presented in paper V, calves were experimentally infected with BVDV type I by inoculation of fetal calf serum containing BVDV into the nostril. The fetal calf serum originated from a single bovine fetus, and had a BVDV titer of 5×10^5 TCID50 per ml.

Serum IgG was analysed with electrophoresis (IV, V), making a direct quantitative comparison with the results obtained by other IgG methods difficult. The electrophoresis used in this study correlated well to the total serum protein values obtained with the biuret method, and the mean serum IgG concentration 5.9 g/l corresponded to a total protein concentration of 53 g/l (de Verdier Klingenberg, unpublished data). The electrophoresis was previously applied in a study by Möllerberg and collaborators (1989), in which serum IgG was analysed in calves that received well-defined quantities of colostrum under experimental conditions.

The definition of *diarrhoea* in this study was based on the evaluation of faecal consistency, comprised of three steps: 1. Clinical observation of defecation (I-V), 2. Visual evaluation of faecal samples (I-V), 3. Determination of the dry matter content of faecal samples (I, V). The correlation between the steps was considered good (Figure 3).

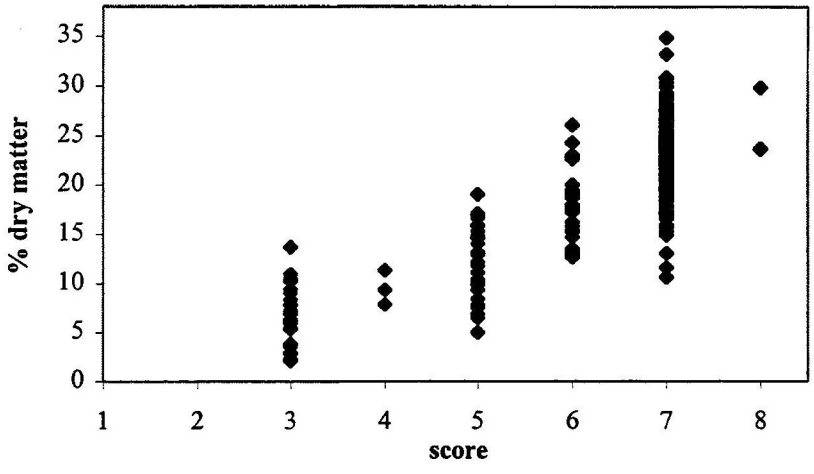

Figure 3: Dry matter content and visual evaluation of faecal consistency in 360 calf faeces samples. Visual evaluation expressed as a score of 1-8, where 7 represents normal consistency and < 7 diarrhoeic faeces.

The calves in this study were up to one month old and referred to as *neonatal*. They were born and reared in conventional cattle herds. In the analysis and evaluation of samples and data from animals, a thorough knowledge about the animals, the herds and their management is advantageous or even crucial. One way to obtain this knowledge is by way of a longterm study, as that described in paper IV, where a large dairy herd was monitored and recorded for two consecutive years. In the study described in paper V, the calves were experimentally infected with BRV and BVDV. To ensure that the calves had not been naturally infected with BRV before commencement, daily faeces samples from the calves were analysed for rotavirus, starting at birth and continuing throughout the study. The calves were born in a herd which had been declared free from BVDV for three years, a circumstance which, together with the negative analyses for BVD virus and antibodies in the calves, was a guarantee that the calves had not encountered BVDV before the experiment.

The herds described in paper IV and V were or became strictly closed and free from BVDV, i.e., according to Swedish BVDV control schemes (Lindberg & Alenius 1999), whose main principles are identification of non-infected and

infected herds by serological herd test, certification of non-infected herds by repeated sampling, and protection of non-infected herds on an advisory basis. Besides BVDV, the herds were also free from infections with BLV and BHV-1, and *Salmonella spp* was not detected in the herds. No vaccines or prophylactic antibiotics were used. Since many of the other risk factors can be held constant, studies of closed herds present a method to control multiple effects and interactions when the role of a certain risk factor in the development of neonatal calf diarrhoea is assessed. Nevertheless, the results are realistic and applicable to a farm situation.

Results and discussion

Clinical significance of BRV infections

In this study, BRV infections in Swedish calves were clearly associated with neonatal calf diarrhoea ($p<0.001$), in outbreaks (I) as well as in an endemic situation (IV). No association was found between the clinical outcome and the BRV G-type (III), though this does not exclude a genetic effect on BRV virulence variation. Compared to detection of other known enteropathogenic agents, BRV was the most commonly occurring infectious agent in neonatal diarrhoeic calves (I), where BRV was detected in 42.4% (14/33) of the faecal samples from diarrhoeic calves and 3.3% (1/30) from non-diarrhoeic calves. Although, study I was performed locally, and comprised only 63 calves in 14 herds, the results were confirmed in a larger study in another part of Sweden (Table 1).

Table 1. Detection of enteropathogenic agents in faecal samples from 135 diarrhoeic and 135 non-diarrhoeic calves (0-90 days) in a study comprising 123 dairy herds in southwest Sweden (Björkman C, de Verdier Klingenberg K, Svensson C. Forthcoming manuscript)

	Number of infected calves / total number of calves	Number of infected diarrhoeic calves / number of diarrhoeic calves	Number of infected non-diarrhoeic calves / number of non-diarrhoeic calves
Group A rotavirus[1]	45/270 (16.7%)	33/135 (24.4%)	12/135 (8.9%)
Cryptosporidium parvum[2]	21/270 (7.8%)	15/135 (11.1%)	6/135 (4.4%)
Bovine coronavirus	4/270 (1.5%)	4/135 (3.0%)	0/135 (0%)
Escherichia coli K99+	3/270 (1.1%)	1/135 (0.7%)	2/135 (1.5%)

[1]Association between BRV and diarrhoea: $p < 0.001$, [2]Association between *Cryptosporidium parvum* and diarrhoea: $p < 0.01$.

The results from these studies, as well as several other investigations of herds experiencing calf diarhoea problems, show that BRV is the most commonly occurring causative agent in neonatal calf diarrhoea in Swedish herds. In study IV, BRV infections in calves at 4 weeks of age were associated with diarrhoea ($p<0.001$), but not BRV infections in younger calves, suggesting that calves of the herd in this study were protected from BRV-induced diarrhoea by maternal immunity for more than 3 weeks after birth.

The generally considered endemic situation of BRV infection, with almost 100% seroprevalence of BRV in cattle, was supported by the results of serological screening of the calves in a rearing unit (de Verdier Klingenberg, unpublished data).

As compared to BRV in the Swedish studies metioned above, *Cryptosporidium spp* seems to be more restricted to certain herds, possibly demonstrating an infectious pattern of greater complexity in these herds, where dual infections and managemental factors may be of greater importance. It would be interesting to compare the characteristic features of such herds with those in which only BRV is detected. BCV was occasionnally diagnosed but seems to cause more severe clinical symptoms than BRV. In the prevalence studies mentioned above, *Escherichia coli K99+* was rarely detected and no association with neonatal calf diarrhoea was demonstrated. This finding points out that the indications for antimicrobial drugs in the treatment of neonatal calf diarrhoea in Sweden are few. To obtain a good terapeutic effect and to avoid antibiotic resistance, antimicrobial drugs in calves should be reserved for prudent use.

A finding of both Swedish studies mentioned above, was the rare detection of dual infections (4/63 and 4/270, respectively) which may indicate a low infectious load in the herds. The national control programmes for BVDV and BLV appear to have had a significant effect on herd health. Closed herds have highly reduced their import of new infections, and spontaneosly cleared themselves from other infections, e.g., BRSV and even BCV. Herds with a large number of pathogenic agents circulating are most likely to have a more severe disease situation in relation to herds where fewer infectious agent species are present. If, e.g., BRV plus BVDV plus *Salmonella spp* are simultaneously present in a herd, calf morbidity and mortality are likely to be high. On the other hand, if BRV is the only infectious agent detected in a calf diarrhoea outbreak, the prognosis should be regarded as very favourable compared to situations when dual or mixed infection are prevalent.

Study IV demonstrated poor growth in diarrhoeic and recovered calves, representing one part of the actual financial losses by BRV infections. The diarrhoeic calves in the study gained less weight ($p<0.001$) than non-diarrhoeic calves, 0.24 and 0.33 kg/day, respectively. BRV infections may have an impact on a variety of economical factors, of which some are obvious, e.g., the cost of

care and treatment of sick calves and enhanced calf mortality, but actual losses may also include enhanced susceptibility to respiratory infections. Cost-benefit calculations in beef suckler herds in northern Scotland have shown neonatal calf diarrhoea to be a more costly disease than respiratory disease in young stock (Gunn & Stott 1996), thus serving as a basis of advice to farmers. The size, type and distribution of Scottish cattle herds differ from those of Sweden, but the need for advice to farmers, based on thorough knowledge, appropriate diagnostic methods and farm-tailored advice, exists in both countries. Besides the financial aspect, the association between diarrhoea and low weight gain also emphasizes the risk of milk withdrawal from diarrhoeic calves.

Since an etiological diagnosis in cases of neonatal calf diarrhoea cannot be made on a clinical basis, BRV diagnosis requires laboratory testing. The ELISA performed in the studies of this thesis (I-V), proved to be an exellent diagnostic test for BRV. The cutoff value used for the bovine samples was the same as that used for faecal samples of human origin, with close agreement between the results obtained by RNA-PAGE and by ELISA (I), with two different commercial ELISAs for bovine samples (Eli-vet Bovine Tetra-kit, Belgium, and Svanova Biotech, S-751 83 Uppsala, Sweden) as well (de Verdier Klingenberg, unpublished data). However, the significance of a virus amount below the cutoff level in rotavirus diagnosis can be questioned. A low amount certainly is epidemiologically significant, and in early and late infection low amounts can be expected.

The clinical advantage with rapid test procedures in a diarrhoea outbreak is obvious, thus establishing an etiological diagnosis that facilitates immediate performance of adequate measures, such as fluid therapy, continued milk feeding, colostrum supplementation, etc., and the adoption of protective measures to uninfected calves. However, a "cow-side-test" may also have an educational aspect. In this study (II), the one-step rotavirus test proved to be a suitable tool to show farmers that neonatal calf diarrhoea is commonly caused by an infectious agent and that this particular agent is not susceptible to antimicrobial therapy. Besides neonatal calf diarrhoea, the one-step test has additionnal fields of application for other animal species, e.g., in screening for rotavirus-excreting diarrhoeic foals to be isolated on arrival to a stationary clinic.

Molecular epidemiology of BRV in Swedish herds

The molecular epidemiology of BRV in this study (III) demonstrated a pronounced stability, as one major BRV G-type persisted in a large dairy herd during a 4 year period, which is consistent with a previous report (Ishizaki et al. 1995). Two major G-types were detected in > 90% of the typed samples. The two predominant G-types were G10 (60%) and G6 (31%), which is a variation of previously reported results where the reverse prevalence was commonly

demonstrated (Bellinzoni et al. 1989, Snodgrass et al. 1990b, Hussein et al. 1995, Lucchelli et al. 1994, Parwani et al. 1993). Since G10 was dominant (54%) in samples from dairy calves, and G6 the only serotype detected in beef suckler calves, the G-type distribution most likely reflected the herd type distribution where the samples were collected. The herd type distribution might also affect possible differences in geographic distribution of BRV serotype, although no influence of the geographic region on the G-type distribution was obvious in our study (III).

The persistence of a serotype in a herd is likely to depend on the stability of BRV in the environment. Adult cattle have been incriminated as BRV reservoirs (Crouch & Acres 1984, Kodituwakku & Harbour 1990), but the transmission environment-to-calf and calf-to-cow seems more likely. The selection and persistence of a particular BRV serotype in herds is also likely to be enhanced when trading of neonatal calves is a rare event.

Detection of seven different BRV G-types, of which most are probably present in Swedish cattle herds—four of which (G3, G6, G8, G10) were detected in this study (III), has been reported worldwide. Rotaviruses are considered to have the potential to be transmitted across species, and to provide a reservoir for reassortants between human and animal rotaviruses. The serotype G3 has been demonstrated in samples from a variety of animal species, including man (reviewed by Kapikian and Chanock 1996), and the finding of BRV G3 in this study may indicate a possible rotavirus relation between cattle and other species. In Swedish dairy herds, the clinical significance of future BRV transmission to other species appears unlikely. Close contact between neonatal calves and the young of other species is not anticipated to increase, due to species restriction in animal husbandry, a decreasing part of the population employed in agriculture, and an enhanced awareness of disease transmission. In beef suckler herds, interspecies transmission is also likely to be of minor importance, which is supported by the pronounced BRV G-type stability demonstrated in beef suckler herds in this study. Nevertheless, the ability of BRV to provide a reservoir for new rotavirus types can not be excluded.

Aspects of prevention of neonatal calf diarrhoea

An increased future risk for enteric viral infections in humans, such as rotavirus, has been feared (Bern & Glass 1994). When compared to the farm animal situation, crowded conditions, intensive animal trading and immunosuppressing co-infections might pose a hazard to the young stock. Prevention remains the major route of battle against enteric viruses. The principal strategy currently used and accepted for prevention and control of BRV infections, i.e., boosting the immunity of susceptible calves and reducing the exposure of these calves to BRV, allows a variety of alternatives. Prevention strategies in cattle herds do not

implicate the same measures in all herds in all countries. On the contrary, each farm should have its own tailored neonatal calf diarrhoea prevention and control programme, based on and adapted to the local conditions. And although, globally, large amounts of antimicrobial drugs to prevent and control neonatal scours in calves are in common use, they have no medical indication in BRV infections.

This thesis, has identified and evaluated risk factors for neonatal calf diarrhoea in a dairy herd, particularly the effect of BVDV and of closure of the herd on neonatal calf diarrhoea (IV, V). The most striking result is the dramatic decline in the incidence of neonatal calf diarrhoea, from 70.6 to 19.4%, after strict closure and eradication of BVDV in a dairy herd (IV). Three years after the study, the herd remains closed and free from BVDV, and the incidence of neonatal calf diarrhoea is still low (personal communication).

These results were supported by the aggravation of the clinical signs in calves after dual infection of BVDV and BRV, compared to BRV infection alone (V). Although the prolonged duration of diarrhoea and aggravation of clinical signs in this study were rather modest, since neonatal diarrhoea affects a large proportion of calves worldwide, even a slight worsening is likely to have practical and financial bearing on the farmers. The effects of BVDV eradication and strict closure of herds on improvement of the calf health have also been observed in Scandinavian cattle practice and are likely a result of an overall reduction of the exposure of cattle to pathogenic agents.

Closure of herds also ensures that calves receive colostrum with adequate specificity of Ig. The importance of colostral feeding in prevention of neonatal calf diarrhoea was highlighted in this thesis by the association ($p<0.05$) of low serum IgG and increased incidence of neonatal calf diarrhoea (IV). Calves with high serum IgG level had less diarrhoea than calves with low serum IgG, though the number of calves with high serum IgG was low, so there was no statistically significant association. These results suggest that the incidence of neonatal calf diarrhoea could easily be reduced by ensuring that all calves receive an adequate amount of colostrum soon after birth, i.e., a calf should consume 2 litres of colostrum before it is two hours old and the first day of life the colostrum intake of a calf should amount to 15 % of its body weight. Maternal immunity passed on to calves can be additionally elevated as shown by the results from a Swedish study (Liberg et al. 1998), which suggest that elevation of serum IgG in calves can be achieved by control of the quality of colostrum before feeding it to calves. On a long-term basis, this immunity may be enhanced by breeding cows with high quality colostrum. The local maternal immunity in the gut of dairy calves can also be improved by feeding the calves fermented colostrum (Olsson 1981).

In this thesis, attention is called to the claim that control and prevention of neonatal calf diarrhoea is possible without vaccination programmes, through an

overall reduction of the exposure of cattle to pathogenic agents by control of direct contact between cattle, and by optimal colostral feeding to newborn calves. Globally, vaccination is the prevalent method used to control BRV infections in calves, despite the connected costs and risks (Babiuk et al. 1996, *Animal Pharm* 1999). With the development of safe and effective BRV vaccines, vaccination can be a useful tool in certain Swedish herds as well, e.g., to reduce BRV load and to minimizing BRV transmission in herds experiencing severe calf diarrhoea problems caused by BRV infection.

Conclusions

The significant decrease of neonatal calf diarrhoea observed in this study, after strict closure and eradication of BVDV in a herd, is suggested to be due to an overall reduction in the exposure of calves to pathogenic agents. It is also suggested that strict closure of herds increases the specificity of maternally transmitted IgG to calves. The conditions for prevention and control of neonatal calf diarrhoea in Swedish cattle herds are seen as good, without adoption of vaccination programmes, through improved management such as optimal colostral feeding, strict closure of herds and eradication of BVDV. This strategy is also suggested to hold other additional benefits for calf health, e.g., lower mortality and incidence of respiratory disease, and higher growth rates.

BRV infection remains a significant cause of neonatal calf diarrhoea. The clinical significance of BRV is reliant on its ability to persist on farms as an environmental contamination. This persistence was illustrated in the study by pronounced G-type stability, which it is suggested is due to BRV's stable character and to restricted calf trading.
Interspecies rotavirus transmission is likely to be of minor importance in Swedish cattle herds, but the ability of BRV to provide a reservoir for new rotavirus types can not be excluded. Continued investigation of BRV may reveal such strains of epidemiological significance.

The results of the study also suggest that dual infections, e.g., with BRV and BVDV, aggravate the clinical signs in calves, more than infections caused by one single agent. The outcome on farms that experienced calf diarrhoea outbreaks where BRV was identified as the only infectious agent was considered much more favourable than that of farms where dual or mixed infections prevailed. The rare incidence of naturally occurring dual infection in the herds in this study is seen as an indication of low infectious load.

The indications for antimicrobial drugs in neonatal calf diarrhoea therapy are few, which was demonstrated by the low incidence of *Escherichia coli K99+* in this study. To promote good weight gain, continued milk feeding to diarrhoeic calves is suggested to have beneficial effects.

Future implications of the study

In the cattle industry in Sweden and other countries, there is an increasing interest for new systems in calf rearing, e.g., organic farming. New management systems pose a challenge to veterinary practitioners and researchers to adapt individual advice to the current situation on each farm. There is a need for improvement of the knowledge of the factors that affect the health of neonatal calves. In addition to BRV, many pathogenic agents, e.g., adenovirus, coronavirus, and calicivirus, are likely to have impact on the health of neonatal calves, while the significance of others, e.g., circovirus and bovine immunodeficiency virus, on calf health is obscure. Future studies of interest comprise investigation of the interaction of calf pathogens on a herd level, increased detection of enteropathogenic agent species incriminated in diarrhoeic faeces, monitoring calf health in closed herds with low infectious load, and evaluating the effect of selection of cows with good colostral quality on calf health.

BRV will probably remain a major calf health pathogen in the future. Continued investigation of non-group A BRV in cattle herds and further characterization of group A BRV in faecal samples, by P-typing, sequencing of, e.g., the 7 untyped faecal samples described in study III etc., may reveal BRV strains of epidemiological significance. The development and evaluation of new generation BRV vaccines represent future studies of great interest.

Practical advice for prevention of calf scours in Swedish cattle herds

•Regard the neonatal calf as a vulnerable little creature. Good nursing care the first day of life as crucial.

•Provide the calving cow with a clean and dry environment, to protect the newborn calf from immediate infections.

•Promote good calf health by optimal colostral feeding, and by strict closure of herds and eradication of BVDV.

•Do not transport neonatal calves and be careful with grouping of calves during the first month of life.

•Consider BRV as the most commonly occurring infectious agent in neonatal diarrhoea in Swedish calves, and thus fluid therapy as the most important treatment.

•Consider *Escherichia coli* as a rare cause of neonatal calf diarrhoea, and thus antimicrobial therapy in this disease of little value. Reserve antimicrobial therapy for demonstrated complications, e.g., pneumonia.

•Prevent starvation and weight loss in diarrhoeic calves by continued milk feeding.

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