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Winter Dysentery Caused by Bovine Coronavirus: No Rule Without an Exception

Diagnostics, clinical picture, epidemiology and herd immunity

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SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

Winter dysentery caused by bovine coronavirus: No rule without an exception. Diagnostics, clinical picture, epidemiology and herd immunity.

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Akademisk avhandling som för vinnande av veterinärmedicine doktorsexamen kommer att offentligen försvaras i "Ettan", Klinikcentrum, Uppsala, fredagen den 26 maj 2000, kl 13.00.

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

Abstract

This study examines the strength of association between winter dysentery (WD) and bovine coronavirus (BCV), describes the clinical, epidemiological and serological features of the disease in Swedish dairy herds and investigates herd level immunity.

The significance of WD was determined by an interview study comprising 256 dairy herds in central Sweden. The prevalence and distribution of BCV antibodies in dairy herd bulk milk was assessed by a nation-wide survey in 2236 herds.

Isotype-capture ELISAs for BCV-specific IgM and IgA and an ELISA for BCV antigen were developed. Winter dysentery was experimentally reproduced in lactating, BCV seronegative cows with a virulent field strain of BCV. BCV seronegative, colostrum-fed calves infected with the same BCV strain developed similar diarrhoea. However, the general condition was clearly less affected in the calves. The kinetics of the isotype-specific nasal and systemic antibody responses were followed for 6 to 22 months and very long-lasting IgA responses were documented in milk, serum and nasal secretions. Also in naturally infected cows and calves, long-lasting IgA levels were recorded in milk and serum. Cows showed a longer duration of the systemic IgA response than calves.

Use of the BCV-specific IgM and IgA capture ELISAs in addition to the conventional IgG1 antibody indirect ELISA detected significant milk or serum antibody responses in 90% of 236 cattle sampled in 38 WD outbreaks. IgM detection was particularly useful for diagnosing primary BCV infections in cattle where the acute samples were taken late. IgM detection can be used

diagnostically in samples obtained on only one occasion, due to the comparatively short duration of high IgM levels after infection. An IgA titre increase in paired samples was particularly useful for diagnosing BCV reinfections. Both IgM and IgA detection diagnosed BCV in a higher proportion of the paired bulk milk samples (n=28) than IgG1.

The overall severity score of the WD outbreak and the decrease in milk yield were significantly associated with the herd BCV immunity level, expressed as time since the previous BCV infection.

One typical WD outbreak in south-eastern Sweden was not associated with BCV infection.

Key words: Cattle, winter dysentery, bovine coronavirus, isotype-specific antibody response, isotype-capture ELISA, antibody survey, milk, bulk milk, experimental infection, herd immunity.

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Winter Dysentery Caused by Bovine Coronavirus: No Rule Without an Exception

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The secret of good writing is rewriting
Mario Puzo
...and rewriting and rewriting
Bengt Larsson
...and cutting a lot
Bror Morein

Feces are often passed with little warning and considerable velocity
OM Radostits et al.
(Winter dysentery in cattle.
In: Veterinary Medicine, 1999)

Duties of the PhD student: To remember that the supervisor mostly is right
(In: Handbook for PhD students, SLU)

All you have is time
Bodil Jönsson

Abstract

Tråvén, M. 2000. Winter dysentery caused by bovine coronavirus: No rule without an exception. Diagnostics, clinical picture, epidemiology and herd immunity.
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This study examines the strength of association between winter dysentery (WD) and bovine coronavirus (BCV), it describes the clinical, epidemiological and serological features of the disease in Swedish dairy herds, and investigates herd level immunity to BCV.

The WD incidence is high, implicating economic significance due to the decrease in milk yield. Also, the BCV antibody prevalence is high in dairy herd bulk milk throughout Sweden.

Isotype-capture ELISAs for BCV-specific IgM and IgA and an ELISA for BCV antigen detection were developed. Winter dysentery was experimentally reproduced in lactating, BCV seronegative cows with a virulent field strain of BCV. BCV seronegative, colostrum-fed calves infected with the same BCV strain developed similar diarrhoea. However, the general condition was clearly less affected in the calves. The kinetics of the isotype-specific local and systemic antibody responses were followed for 6 to 22 months and very long-lasting IgA responses were documented in milk, serum and nasal secretions. Also in naturally infected cows and calves, long-lasting IgA levels were recorded in milk and serum. Cows showed a longer duration of the systemic IgA response than calves.

Use of the BCV-specific IgM and IgA capture ELISAs in addition to the conventional IgG1 antibody indirect ELISA detected significant milk or serum antibody responses in 90% of 236 cattle sampled in 38 WD outbreaks. IgM detection can be used diagnostically in samples obtained on only one occasion, due to the comparatively short duration of high IgM levels after infection. Further, IgM detection was particularly useful for diagnosing primary BCV infections when the acute samples were taken late. An IgA titre increase in paired samples was particularly useful for diagnosing reinfections. Both IgM and IgA detection diagnosed BCV in a higher proportion of the paired bulk milk samples than IgG1.

The overall severity score of the WD outbreak and the decrease in milk yield were significantly associated with the herd BCV immunity level, expressed as time since the previous BCV infection.

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Contents

Appendix

Papers I-V

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

I **Tråvén, M.**, Sundberg, J., Larsson, B. & Niskanen, R., 1993. Winter dysentery diagnosed by farmers in dairy herds in central Sweden: incidence, clinical signs and protective immunity. *Vet. Rec.* 133, 315-318.

II **Tråvén, M.**, Björnerot, L. & Larsson, B., 1998. Nation-wide survey of antibodies to bovine coronavirus in Swedish dairy herd bulk milk. *Vet. Rec.* 144, 527-529.

III Näslund, K., **Tråvén, M.**, Larsson, B., Silván, A. & Linde, N., 2000. Capture ELISA systems for the detection of bovine coronavirus-specific IgA and IgM antibodies in milk and serum. *Vet. Microbiol.* 72, 3-4, 183-206.

IV **Tråvén, M.**, Näslund, K., Linde, N., Linde, B., Silván, A., Fossum, C. & Larsson, B., 2000. Experimental reproduction of winter dysentery in lactating cows using BCV - comparison with BCV infection in milk-fed calves. *Submitted.*

V **Tråvén, M.**, 2000. Winter dysentery serologically linked to bovine coronavirus in 38 out of 39 outbreaks, symptom patterns in relation to herd BCV immunity. *Manuscript.*

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Abbreviations

BCV	bovine coronavirus
BHV1	bovine herpesvirus 1
BRSV	bovine respiratory syncytial virus
BToV	bovine torovirus
BVDV	bovine viral diarrhoea virus
CD	colostrum deprived
dpi	days post infection
dpo	days post outbreak start
ELISA	enzyme-linked immuno-sorbent assay
EM	electron microscopy
gp	glycoprotein
HA	haemagglutination
HCV	human coronavirus
HE	haemagglutinin esterase protein
HI	haemagglutination inhibition
HRSV	human respiratory syncytial virus
HRT-18	human rectal tumour-18
IEM	immuno electron microscopy
Ig	immunoglobulin
IgA, IgG1, IgG2, IgM	immunoglobulin A, G1, G2, M
ISCOM	immuno-stimulating complex
mAb	monoclonal antibody
MHV	mouse hepatitis virus
NCD	neonatal calf diarrhoea
OD	optical density
PAG-IEM	protein A-gold immuno electron microscopy
PIV3	parainfluenza virus 3
rt-PCR	reverse transcriptase polymerase chain reaction
S	spike protein
TGEV	transmissible gastroenteritis virus
VN	virus neutralisation
WD	winter dysentery

Introduction

Background

In the winter of 1986-87 there was a WD epizootic in several areas in central Sweden. Seroconversions to BCV were detected in all 9 outbreaks investigated (Alenius et al., 1991). However, the difficulty to detect significant titre increases to BCV in seropositive cows was experienced, and the need for improved diagnostic methods to examine the association of WD with BCV was identified. Many questions also were raised concerning the epidemiological peculiarities of the disease, e.g. why calves seemed to be refractory to WD, while BCV is an established calf diarrhoea pathogen. Further, the influence and duration of immunity was not clear. In face of the economic losses incurred by the pronounced decrease in milk yield in affected dairy herds, there was a demand for more knowledge about the disease in Swedish cattle. This study was initialised in 1991.

Winter dysentery

History

Bovine winter dysentery (WD) is a clinically defined disease that has been around for a long time. It was first described in USA by Steffen in 1915 (cited by Jones and Little, 1931). Less well-defined descriptions can also be found in European veterinary literature (Brieg, 1917). The disease has subsequently been described under names like bovine winter diarrhoea, winter scours, infectious diarrhoea, virus enteritis, epidemic enteritis or epizootic diarrhoea of adult cattle from many countries: Great Britain (Rollinson, 1948), Canada (Macpherson, 1957), Israel (Komarov et al., 1959), Australia (Edwards and Sier, 1960), France (Charton et al., 1963), New Zealand (Horner et al., 1975), Japan (Takahashi et al., 1980), Belgium (Broes et al., 1984) and from other parts of the USA (Merriman, 1953, Roberts, 1957). The first report of a similar disease in Sweden was published in 1951 (Hedström and Isaksson, 1951), but the disease was probably not a new entity in the country.

Most of these reports come from countries within the temperate climate zone, together with a few descriptions from subtropical areas with winter rain climate, except for the Israeli report. In warmer regions, the disease seems to be practically unknown. However, studies show a high BCV seroprevalence in Mozambique calves (Baule, 1993) and high detection rates of BCV antigen in faeces of Indian calves (Vijayakumar and Dhinakaran, 1996) and Ethiopian calves (Abraham et al., 1992), indicating that BCV infections occur also in those areas, although the epidemiology may be different.

In early reports, WD was associated with *Campylobacter spp* (*Vibrio jejuni*) (Jones and Little, 1931, Rolle et al., 1955), but these findings could not be reproduced. The disease showed the basic characteristics of a viral infection (Macpherson, 1957), and several viruses were proposed and discarded as etiologic agents (reviewed by Campbell and Cookingham, 1978). Most WD

work since then has focused on BCV as the causative agent (see below). Also, a serological association of WD with bovine torovirus (BToV) (Koopmans et al., 1991, Kruiningen, 1992) has been reported, while others detected seroresponses to BVDV in cows with WD (Smith et al., 1998a). Further, group B and C rotaviruses have in a few instances been detected or isolated from faeces of cows with WD (Saif et al., 1991, Tsunemitsu et al., 1991a, 1999a, Parwani et al., 1996, Chang et al., 1997).

Clinical picture

WD outbreaks usually occur during the winter season. The disease is highly contagious, spreading through susceptible herds in less than a week, mainly affecting the adult cattle. The dominating symptom is diarrhoea, often with an admixture of blood in the faeces of some cows, and with a characteristic smell of the faeces. Diarrhoea attack rates usually are high (up to 100%) in adult cattle. Affected herds experience a severely decreased milk yield (25 to 90% reported), implicating the economic importance of the disease, although mortality usually is low. Respiratory symptoms are seen in a variable proportion of the affected herds, but fever is frequently absent. Young calves mostly are reported to be unaffected (Jones and Little, 1931, Rollinson, 1948, Hedström and Isaksson, 1951, Roberts, 1957), although diarrhoea attack rates similar in both calves and older cattle occasionally have been described (Kahrs et al., 1973).

Bovine coronavirus

Structure and properties

Bovine coronavirus is an enveloped, single-stranded RNA virus with a mean size of 100-120 nm. In EM, the virus has a characteristic morphology with a double fringe of surface projections, the spike (S) glycoprotein (gp) making up the large (20 nm) club-shaped peplomers, and the hemagglutinin esterase (HE) gp constituting the shorter projections (Fig. 1). Further, the virion possesses the integral membrane (M) gp and the small-membrane (sM) protein completing the envelope. The phosphorylated nucleocapsid (N) protein, is closely associated with the viral RNA, forming a helical nucleocapsid (Clark, 1993). The BCV genome also encodes a number of proteins which are present in infected cells, but not incorporated into the virion. Some of these non-structural proteins take part in the regulation of viral replication, while the function of others is unknown (Brown and Brierley, 1995). The major neutralising epitopes are located on the S and HE gps, both proteins giving rise to separately neutralising antibodies (Deregt and Babiuk, 1987). Both the S and HE gps require N-acetyl-9-O-acetylneuraminic (or sialic) acid ($Neu5,9Ac_2$) as a determinant in their not fully characterised receptors. This carbohydrate is present on glycoproteins and glycolipids (Vlasak et al., 1988, Schultze et al., 1991, Schulze and Herrler, 1992) of many cell types (Holmes and Compton, 1995). Both the S and HE gps mediate agglutination of mouse

and rat erythrocytes (Schultze et al., 1991, Schulze et al., 1991), a property used diagnostically in HA/HI tests (Sato et al., 1977, Akashi et al., 1980). The S gp initiates membrane fusion with the host cell or between infected and non-infected cells, by a still unidentified process. The HE protein has acetylesterase activity, disconnecting an acetyl group from the $Neu5,9Ac_2$ under certain conditions, abrogating BCV binding. This esterase activity may prevent virus from becoming trapped on mucin carbohydrates (Holmes and Compton, 1995). Antigenic variation between BCV strains has been documented in a number of studies (Reynolds et al., 1985, El-Ghorr et al., 1989, Zhang et al., 1994, Tsunemitsu and Saif, 1995, Dea et al., 1995, El-Kanawati et al., 1996, Milane et al., 1997, Chouljenko et al., 1998, Fukutomi et al., 1999, Hasoksuz et al., 1999a, b). This variation, however, has not been large enough to designate more than one serotype. Also, no consistent relationships between antigenic variants and clinical outcome or source of the isolates were demonstrated. As viral infectivity is completely lost with disruption of the lipid envelope, BCV is sensitive to detergents, organic solvents and commonly used disinfectants (Mebus, 1990) (Maris, 1990).

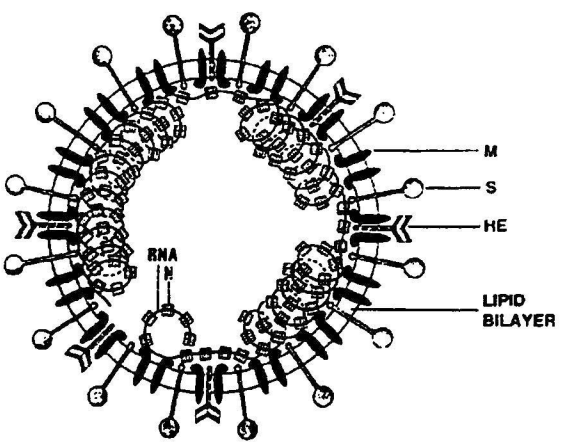

Fig. 1. BCV proteins and particle structure. The sM protein is not shown in the model. From: KV Holmes, MC Lai, Coronaviridae. In: Fields' Virology, 3rd Ed. BN Fields, DN Knipe, PM Howley (Eds.), Lippincott Raven Publishers, 1996. Reprinted with kind permission of the authors and the publisher.

Disease associated with BCV infection

BCV was initially detected and described as a neonatal calf diarrhoea (NCD) agent (Stair et al., 1972, Mebus et al., 1973), causing disease mainly in calves 5 days to 3 weeks old, usually with a high morbidity, but with mortality varying widely, depending on the calf management and the presence of co-infections (Mebus et al., 1972).

The first report on electron microscopic (EM) detection of coronavirus-like particles in faeces of cows with winter dysentery was published in 1975 (Horner et al., 1975), and followed by many more (Takahashi et al., 1980, Espinasse et al., 1982, Broes et al., 1984, Kruiningen et al., 1987, Saif et al., 1988, Durham et al., 1989a, Fleetwood et al., 1989). Serological responses to BCV in WD cows have also been reported in several studies (Takahashi et al., 1980, Alenius et al., 1991, Saif et al., 1991, Kruiningen et al., 1991). The bulk of evidence for an association between BCV and WD was reviewed by Saif (1990) using Koch's postulates as criteria. Despite the large number of studies, there has been doubt concerning the etiology of WD because of the low detection frequency of faecal BCV in WD cows (Espinasse et al., 1982, Fleetwood et al., 1989), while other studies reported high frequencies of BCV shedding among clinically healthy cows (Crouch and Acres, 1984, Crouch et al., 1985, Collins et al., 1987, Bulgin et al., 1989). Further, the frequency of serological responses to BCV was low in some WD studies (Koopmans et al., 1991, Smith et al., 1998a). Above all, experimental evidence, reproducing WD by the use of BCV, has been lacking.

BCV antigen detection/isolation or serological responses frequently are associated with respiratory symptoms or lung lesions in calves (Thomas et al., 1982, Möstl and Bürki, 1988, Herbst et al., 1989, Heckert et al., 1990a, Appel et al., 1992, Storz et al., 1996) and an association of BCV with respiratory disease in older cattle has also been reported (Wellemans et al., 1985, Bosgiraud and Nicolas, 1986, Läuchli et al., 1990). However, in many of these cases mixed infections were detected, and only in a few trials (McNulty et al., 1984, Kapil et al., 1991) have respiratory symptoms been experimentally produced with BCV. The vast majority of experimental BCV infections have yielded no such symptoms (Reynolds et al., 1985, Saif et al., 1986, Saif, 1987, El-Kanawati et al., 1996, Tsunemitsu et al., 1999b). Presently, the specific contribution of BCV to bovine respiratory disease is not clear.

Epidemiology

Winter dysentery displays the classical features of a highly contagious disease, appearing in smaller or larger epizootics (Hedström and Isaksson, 1951, Edwards and Sier, 1960, Takahashi et al., 1980) with a cyclic variation in an area (Roberts, 1957) depending on the immunity level in the population. Most of the transmission between herds is considered to occur by indirect contacts via people and equipment (Roberts, 1957). Most outbreaks occur during the winter season (Merriman, 1953, Roberts, 1957, Macpherson, 1957, Jactel et al., 1990). This pattern has been attributed to:

1) Viral survival outside the host being higher when temperature is lower, humidity is higher and particularly when UV radiation is reduced, increasing the likelihood that the virus will survive indirect transmission between herds (Clark, 1993, Saif, 1990).

2) Increased host contacts during stable conditions.

3) Increased host susceptibility to infection due to overcrowding and other stress factors, like sudden temperature drops (Campbell and Cookingham, 1978, Jactel et al., 1990).
4) Possibly also increased viral shedding from carriers around parturition (Collins et al., 1987, Bulgin et al., 1989).
The WD frequency during summer is low, disease seldom being seen on pasture (Merriman, 1953, Roberts, 1957). Serum antibody responses to (Elezhary et al., 1981) or faecal shedding of coronaviruses have been documented in several species of wild ruminants (Chasey et al., 1984, Tsunemitsu et al., 1995, Majhdi et al., 1997). These viruses were highly similar to BCV in genetical (Majhdi et al., 1997) or antigenic and biological comparisons (Tsunemitsu et al., 1995). These observations led to the proposition that wild ruminants may act as a reservoir, transmitting BCV to cattle (Tsunemitsu et al., 1995).

BCV survival outside the host has not been directly evaluated, but BCV is considered rather fragile and sensitive to inactivation (Clark, 1993, Saif, 1990) in contrast to the rotaviruses, which may survive for many months in faeces (Ramos et al., 2000).

Diagnostic methods

In the early studies, BCV was detected by EM (Horner et al., 1975, Takahashi et al., 1980, Espinasse et al., 1982, Broes et al., 1984, Kruiningen et al., 1987, Durham et al., 1989a), which, apart from being laborious and time-consuming, is a method with limited sensitivity (Flewett, 1978). Also, the projections are frequently lost during sample preparation, rendering morphological identification difficult (Bridger et al., 1978). The sensitivity of EM detection was improved by aggregating the virus with specific antibodies in immuno EM, IEM (Langpap et al., 1979, Saif et al., 1988, 1991), and identification of virus-antibody complexes with colloidal gold in protein A-gold IEM, PAG-IEM (Saif et al., 1988, El-Ghorr et al., 1988, Heckert et al., 1989, Athanassious et al., 1994). Immuno-assays have the advantage of being practical methods for routine use, facilitating the analysis of large numbers of samples. ELISA tests have been used for BCV antigen detection in cow faeces (Crouch and Acres, 1984, Fleetwood et al., 1989, Smith et al., 1998a, b). Detection with reverse transcriptase polymerase chain reaction (rt-PCR) has been described in recent studies (Tsunemitsu et al., 1999b, Fukutomi et al., 1999), but the analytical sensitivity of the rt-PCR in those studies was not significantly higher than that of IEM and ELISA.

Primary BCV isolates from calves have been described in a large number of studies, using many different cell types and organ cultures (reviewed by Saif and Heckert, 1990). However, the isolation of BCV from patient samples is insidious (Saif, 1990, Clark, 1993) and only a low number of isolates from adult cows have been described (Takahashi et al., 1980, Benfield and Saif, 1990, Dea et al., 1995, Chung et al., 1997). Major break-throughs were the discovery of trypsin enhancement of viral in vitro proliferation (Dea et al.,

1980, Storz et al., 1981, St.Cyr-Coats et al., 1988) and the identification and use of the HRT-18 cell line. These cells have retained important features of highly differentiated brush border cells and are particularly sensitive to BCV (Tompkins et al., 1974, Laporte et al., 1980). Further, the heterogeneous HRT-18 has been sub-cloned, and the clone most sensitive to BCV, the HRT-18G, has been identified (Storz et al., 1996). Still, using the HRT-18 or HRT-18G, some BCV strains could not be isolated (Kapil et al., 1996, Silva et al., 1999). Therefore, viral isolation presently is not a suitable gold standard for identifying BCV infected cattle.

Serological identification of BCV infected cattle in WD outbreaks has been used in a number of studies. The proportion of cattle showing a significant response to infection has been highly variable: 59% (Takahashi et al., 1980) 73% (Saif et al., 1991), 63% (Kruiningen et al., 1991), 61% (Kruiningen, 1992), 95% (Alenius et al., 1991), 9% (Koopmans et al., 1991), and 19% (Smith et al., 1998a). These studies comprised 10, 6, 8, 9, 19 and 12 WD herds, respectively. In the earlier studies, HI (Takahashi et al., 1980) and VN tests were used (Alenius et al., 1991, Saif et al., 1991, Kruiningen, 1992). VN tests are specific, but the sensitivity varies and these methods are laborious. The use of ELISA reduces the laboratory work and facilitates analysis of large numbers of samples. The ELISA used in the study of Alenius et al. (1991) showed 85% sensitivity and 100% specificity compared with the VN test as gold standard. However, the ELISA sensitivity is discredited by this comparison, since it measures only antibodies of the IgG class, while early IgM responses to infection can give VN positive results. ELISA was further used in the studies by Koopmans et al. (1991), Kruiningen et al. (1991) and Smith et al. (1998).

Bovine Ig isotypes and their use in diagnostics

IgM detected in serum and milk is the major Ig class of the primary immune response, disappearing after the acute phase. IgM is also produced during a reinfection, with strong responses at sites of mucosal infection like nasal and eye secretions (Kimman et al., 1987a), but at a lower or undetectable level in serum depending on the competition for antigenic binding with IgG (Zaane et al., 1986, Kimman et al., 1987a). Recently, specific IgM serum antibody responses to parainfluenza virus 3 (PIV3) were reported in experimentally PIV3 reinfected calves that had maternal antibodies at the time of the first infection (Graham et al., 1999).

IgA is produced by plasma cells mainly in the lamina propria beneath mucosal surfaces and skin (Jonard et al., 1984), most of it being secreted onto these surfaces. The level of total IgA in bovine serum is usually lower than that of total IgM (Butler, 1983). IgA does not show obvious virus neutralising activity measured by in vitro assays (Kimman et al., 1987b). However, local IgA antibodies have been shown to provide protection from mucosal infection in vivo (Liew et al., 1984, Renegar and Small, 1991) and the important role of secretory IgA for mucosal immunity in all mucosal organs is now well

established (Rosenthal and Gallichan, 1997). Earlier, IgA protection was thought to be conveyed only by preventing the adherence of micro-organisms to mucosal surfaces. Recent work has indicated that other possible modes of action are trapping of intracellular viral precursors and submucosally invading virus, followed by excretion into the lumen of the mucosal organ (reviewed by Rosenthal and Gallichan, 1997). Earlier, serum IgA was considered a leakage from mucosal sites (Vaerman et al., 1973), but later work has indicated that serum IgA is mainly produced at systemic sites (bone marrow, spleen, lymph nodes) by B-cells derived from mucosal induction sites (Brown et al., 1987, Murphy, 1994, Pinxteren et al., 1998). Serum IgA responses were detected after both primary infection and reinfection with BRSV in colostrum-deprived calves (Kimman et al., 1987a, b). Mice immunised intranasally with human RSV ISCOMs also showed IgA responses in serum after both the first and second inoculation in contrast to the IgM response that was only detected after the first immunisation (Hu et al., 1998). BCV-specific IgA and IgM response in serum and mucosal secretions from calves have previously been studied using indirect ELISAs (Saif, 1987, Heckert et al., 1991a, b, d).

In the bovine, IgG1 and IgG2 are the dominant serum Ig subclasses (Butler, 1983), while in milk IgG1 is the major isotype, in contrast to non-ruminant mammals where IgA is the dominant milk isotype. The bovine IgG1 is selectively transported into milk across the udder epithelium (Butler, 1983). The milk Igs serve the dual purpose to protect the udder from infection, as in other mucosal secretions although in ruminants this function is comparatively inefficient (Lascelles et al., 1981), and to provide the offspring with passive mucosal protection, and in ruminants also systemic immunity (Goldblum and Goldman, 1994).

Dairy herd management in Sweden

In an international perspective, Swedish dairy herds are comparatively small. In 1991, when the bulk milk BCV antibody survey was carried out, there were 505,000 dairy cows in a total of 24,000 herds (mean 21 cows/herd). The mean milk production was 6,900 kg/year (Statistics 1991/1992, SHS). However, the dairy industry is consistently subject to rationalisation. In 1998, there were only 14.000 dairy herds left, but the total number of dairy cows was 442,000 (mean 32 cows/herd). The milk production was mean 8,000 kg/year (Cattle statistics 1997/1998). In 1991, the number of beef herds was about 12,000, but increased considerably during the following years, to about 15,500 in 1998. Most of the beef herds are small (mean cows 1991: 8, 1998: 11) (Yearbook of agricultural statistics 1991, 1998).

National eradication programmes for BLV, BVDV and BHV-1 started in 1990, 1993 and 1994, respectively. The BHV-1 prevalence was very low at the start of the compulsory program, only 19 infected herds have been identified in 1990-1997 by extensive sampling of both dairy and beef herds (Björnerot et al., 1997) and in 1998 Sweden was declared free from BHV-1. Gradually, all dairy and most beef herds were affiliated with the voluntary BLV and

BVDV programs. BLV infected cattle were present in 25-30% of the dairy herds (Klintevall, 1995), and the BVDV seroprevalence in dairy cattle was about 51% (Alenius et al., 1997) at the start of the respective program. In 1998, BLV infected cattle remained in only 42 herds and more than 70% of the dairy herds have been certified free from BVDV (Cattle statistics, 1999). Vaccination for viral infections are not performed in Swedish cattle.

Another important factor when studying infectious diseases is that Swedish dairy herds by tradition have, in an international perspective, comparatively low frequencies of cattle introductions or other direct contacts with cattle from other farms.

Aims of the study

The over-all aim of the study was to explore the strength of the association between winter dysentery and bovine coronavirus. The specific aspects of this work were:

* To describe the clinical and epidemiological features of winter dysentery in Swedish dairy herds, in relation to BCV infection.

* To develop sensitive and reliable isotype-capture ELISAs for BCV-specific IgM and IgA antibodies to make feasible the detection of antibody responses in a large proportion of infected cattle.

* To study the isotype-specific systemic and mucosal antibody responses to BCV infection, in order to establish reference data for diagnostic use of the IgA and IgM tests, with particular emphasis on milk.

* To develop a BCV antigen ELISA to study the viral excretion of infected cattle.

* To develop a BCV infection model for experimental reproduction of winter dysentery in adult cattle and diarrhoea in young calves.

* To explore, by use of the infection model, if cows and calves could become infected and develop disease with the same BCV strain.

* To study the differences in BCV infection and disease between cows and calves, both after natural and experimental infection, and to generate hypotheses as to explain the seemingly different epidemiology of winter dysentery in adult cattle and BCV calf diarrhoea.

Comments on methods

Isotype-capture ELISA vs. indirect ELISA for detecting virus-specific IgA and IgM

The analytical sensitivity and performance of the isotype-capture ELISAs were compared with indirect ELISAs using the same mAbs (**III**). The sensitivity of both the IgA and the IgM capture ELISAs were higher than the corresponding indirect tests, the difference being dramatic for IgA detection. The results of this study confirmed previous findings on a more limited number of samples (Zaane and Ijzerman, 1984, Kimman et al., 1987b).

The major reasons for these differences are that:

1) In an indirect system, where antigen is bound to the solid phase, detection and quantification of specific antibodies in isotypes that occur at low levels, like IgA in serum, will be hampered by inter-isotype competition for binding sites mainly with IgG (Chantler and Diment, 1981, Zaane and Ijzerman, 1984). In the capture system, where isotype-specific mAbs (or antiserum) are bound to the solid phase, the BCV-specific IgA suffers only from the competition with IgA carrying other specificities. This intra-isotype competition is probably much less severe than the competition from antigen-specific IgG (Kimman et al., 1987b).

2) Furthermore, the basic lay-out of the isotype-capture tests contains an internal amplification step, since the virus with many identical epitopes is added after the sample antibodies. This was also reflected in earlier studies, where a capture IgA ELISA produced considerably higher titres than an indirect IgA ELISA in samples not containing detectable IgG (Zaane and Ijzerman, 1984, Kimman et al., 1987b). The very extended BCV-specific IgA responses detected in serum, milk (**III, IV**) and nasal secretions (**IV**) using the IgA capture ELISA compared with the short IgA responses of primarily infected colostrum deprived (CD) calves detected by an indirect ELISA (Saif, 1987) underlines the effect of inter-isotype competition. However, CD calves experimentally infected with BRSV (Kimman et al., 1987a) and rotavirus (Zaane et al., 1986) and seronegative calves naturally infected with BRSV (Kimman et al., 1987b) also showed much shorter IgA serum responses than in our studies (**III, IV**), although IgA capture ELISAs were employed, suggesting differences in sensitivity of the tests. However, differences in the biology of the infections cannot be excluded, since the nasal IgA in BRSV infected calves was more extended than the serum response (Kimman et al., 1987a).

A further advantage of isotype-capture IgM ELISAs, although not examined in these studies, is that this test design largely avoids interference with IgM rheumatoid factors (RF) in serum (Meurmann, 1983, Tuokko, 1984, Osorio et al., 1989), which cause false positive results in indirect IgM ELISAs unless sera are depleted of either the RF or IgG before testing (Ungar-Waron and Abraham, 1991, Graham et al., 1998).

Infection model for the reproduction of winter dysentery

An infection model was developed (**IV**), using 1) a faecal sample as source of virus, and 2) a BCV seronegative calf, orally and intranasally inoculated with the faecal sample, as viral transmitter to the experimental cattle. An infected bovine as transmitter mimics, as closely as possible under experimental conditions, the route(s) and dose of a natural infection, bearing in mind that transmission routes and their relative importance in the natural infection are incompletely known. The virulence of the virus was retained by avoiding cell culture adaptation and attenuation before infection. The importance of the virulence aspect is exemplified by the difficulties to experimentally reproduce severe BRSV disease using cell cultured virus in a number of experiments (Kimman et al., 1987a, Elvander et al., 1998). The advantages of the 'natural transmission' model overshadows the disadvantage that the infectious dose is unknown and varying between individuals due to differences in contact intensity. The risk of introducing other infections into the experimental situation, by using conventionally reared cattle, is difficult to completely preclude even if cattle are tested and carefully observed before the experiment. Other infections need to be monitored, but reliable diagnostic tests are lacking, particularly for a number of potential respiratory pathogens.

Herd immunity determined from isotype-specific antibody responses in relation to age of sampled cattle

A model for assessing herd immunity was developed (**V**). This method makes it possible to determine the herd immunity level prior to the outbreak after the outbreak has actually started, which is a major advantage. The method was useful in the population of Swedish dairy cattle where a majority of herds contract BCV infections with an interval of several years. In populations with a higher frequency of infections, other approaches to herd immunity assessment may be required. Determining herd immunity from the distribution of primary infection vs. reinfection responses in relation to the age of sampled cattle requires that:

1) All seronegative cattle become infected (clinically or subclinically) once a BCV infection enters the herd, as evidenced by the 100% seroconversion in seronegative cattle sampled in all age groups (**V**).

2) The ability to mount clear-cut systemic anamnestic responses (from memory cells) is very long-lasting, as indicated by the distinct reinfection responses of 6-year-old cows in herds where 5.5-year-old cows were clearly primarily infected, and the IgG1 titres being clearly detectable in all cows sampled 4.5 years after a WD outbreak (**V**).

3) The number of introduced cows is low, since their immune status is ignored using this approach.

Results and discussion

Significance of winter dysentery in Swedish dairy herds

The clinical syndrome WD was determined, by farmer enquiries, to be a common disease in Swedish dairy herds (28.5% one-year incidence, **I**) implicating a significant economic impact by up to 56% reduction in milk yield (**V**), which is evaluated in a forthcoming paper. The enquiry results were confirmed by the high BCV antibody prevalence in dairy herd bulk milk (89%) throughout Sweden (**II**). In the latter study, larger herd size and location of the herd in areas more densely populated with cattle, were identified as significant risk factors for a high BCV antibody prevalence and level indicating a higher incidence of infection and probably also a higher incidence of clinical WD. This assumption is supported by a higher incidence of clinical WD associated with large herds reported previously (White et al., 1989). These results are consistent with the view that most BCV infections are indirectly transmitted between herds (Roberts, 1957), since large herds have a higher frequency of indirect contacts with, for example, AI assistants, slaughter transports and veterinarians. Shorter distances between herds increase the likelihood that the virus will survive the transport, with the vectors mentioned above and with local contacts between neighbours or mediated by pets and wild-life, and possibly also air-borne transmission (White et al., 1989).

WD strongly associated with BCV infection

WD was strongly linked to BCV infection by the significant antibody responses to infection detected in 90% of sampled cattle in 38 outbreaks (**V**), using the IgM and IgA isotype-capture ELISAs developed in this study (**III**) in addition to the conventional IgG1-specific indirect ELISA. Most of the 23 cows not showing significant antibody responses to infection in any of the isotypes tested were older cows with high BCV-specific IgA and IgG1 levels already in the acute samples, indicating a rapid anamnestic response.

As BCV is a common infection, and infections without obvious clinical symptoms have been documented in this work (**III, V**), there is a risk that acute BCV antibody responses are picked up by chance in cases where BCV infection is not associated with the disease. Preliminary results from an IgA antibody bulk milk survey indicate an incidence of subclinical or 'sub-WD' infections of about 5% during one year (data not shown). This would implicate a random BCV incidence of less than 1% in the material of paper **V**, assuming (from the WD occurrence data, **I, V**) that BCV infections occur during a period of at least 6 months and that each herd in study **V** is monitored for BCV infection occurrence during 1 month.

The etiologic relationship between BCV and WD was strongly supported by the experimental reproduction of the disease in BCV seronegative lactating cows (**IV**).

The exception

One herd outbreak clearly fulfilling the clinical definition of WD (V) was not associated with BCV infection. The levels of BCV-specific IgG1 and IgA antibodies were low to moderate in the acute samples and increases were completely absent in the convalescent samples. Neither was BCV IgM detected in any sample from this herd. This herd had the unusual anamnesis of having experienced 2 typical WD outbreaks only 6 weeks apart, the one sampled being the second. Since all BCV strains so far examined (see Introduction) belong to a single serotype, it is unlikely that an overt clinical WD outbreak caused by BCV should induce such poor protective immunity that a new outbreak would be elicited by another strain of the same virus only six weeks later. Herds not included in these studies also have reported the occurrence of 2 or even 3 WD-like outbreaks during the same stable season. However, samples have not been available. These findings strongly indicate that other etiologic agents may cause WD-like outbreaks in Sweden, possibly the bovine torovirus (BToV), as has been reported from the Netherlands (Koopmans et al., 1991) and USA (Kruiningen, 1992). Also, group B rotavirus has been isolated from cows with diarrhoea in USA (Saif et al., 1991, Parwani et al., 1996, Chang et al., 1997) and group B and C rotaviruses are reported from Japan (Tsunemitsu et al., 1991a, 1999a). The causation in these cases, however, is unclear because BCV infections co-occurred in at least 7 of the 9 outbreaks where torovirus seroconversions were detected (Koopmans et al., 1991, Kruiningen, 1992). BCV also was detected in one of the 2 herds with group B rotavirus (Saif et al., 1991, Chang et al., 1997) where BCV occurrence was examined. Excretion of group A rotaviruses in the faeces of healthy adult cows has been reported (Crouch and Acres, 1984, Bulgin et al., 1989), and may also occur with other rotavirus types. Seroconversions to BVDV associated with WD in the absence of seroconversions to BCV have been reported in some herds (Koopmans et al., 1991, Smith et al., 1998a). There was no response to BVDV infection in the non-BCV herd of paper V. However, dually infected individual cows (BCV and BVDV) showed severe disease, both in study V and in the previous Swedish WD study (Alenius et al., 1991). Further, the possibility that feed-stuffs with poor hygienic quality may cause WD-like outbreaks cannot be completely excluded.

BCV infection induces high IgM and long-lasting IgA responses systemically and at mucosal sites

BCV-specific IgM was detected for 13 to 54 days in serum and for 1 up to several months in milk of primarily infected cattle (**III, IV**). The long-lasting weak IgM responses, particularly in milk, are probably not of interest when using the IgM test as a single sample diagnostic method. High titres (≥320 in serum and ≥50 in milk) were detected for a more limited period, 2 to 4 weeks, in both serum and milk and should be more suitable detection levels for single sample testing. Lower IgM response levels may, however, be used for detecting

BCV-specific IgM responses in reinfected cows (**III, V**), and to monitor sequential viral infections in disease outbreaks of, for example, respiratory disease and neonatal calf diarrhoea, where several infectious agents are likely to contribute, although not necessarily present at exactly the same time. Sequential infections are difficult to diagnose with antigen detection or isolation methods and with IgG seroconversion tests, due to the narrow time frame of these methods. A local IgM response was also detected in nasal secretions for at least a week (**IV**). IgM nasal responses in reinfected calves of the same magnitude and duration as those in primarily infected ones (Kimman et al., 1987a) suggest that nasal IgM may have diagnostic potentials. However, the tampon method for sampling of nasal secretions, although producing undiluted samples of high quality, is too laborious and time consuming for field sampling.

Very long-lasting IgA responses were detected in milk, where many cows tested positive 2 years after the BCV infection (**V**), and in serum (1 to 2 years, **IV, V**). Also, the local IgA response in nasal secretions was very extended (>6 months when the follow-up was terminated, **IV**). High mucosal IgA levels are important for protective immunity to mucosal infections (Rosenthal and Gallichan, 1997), and mucosal or systemic IgA may serve as a marker for protection. The nasal IgA is more easily detected and evaluated than faecal IgA (not determined in this study), which showed low or undetectable levels, particularly in older calves (Zaane et al., 1986, Kimman et al., 1987a, Heckert et al., 1991c, d). The serum IgA responses of primarily BCV infected cattle in our studies (**III, IV**) were considerably more extended than reported in other studies of primary viral infections in bovines (Zaane et al., 1986, Kimman et al., 1987a, b, Saif, 1987), while the nasal IgA responses in the study of Kimman et al. (Kimman et al., 1987a) were detected throughout the 4 months follow-up. However, our results are in line with the serum IgA responses in rotavirus infected children, exceeding in duration the follow-up periods of 4 (Grimwood et al., 1988) and 6 months (Hjelt et al., 1986).

Antibody responses in milk - diagnostic utility

For diagnosing infections in dairy cows, individual milk and bulk milk are easily accessible samples that can be drawn and sent to the laboratory by the farmer. Extensive work has been published on IgG antibody level surveillance in bulk milk, and detection of seroconversions in individual paired milk samples concerning BVDV (Niskanen et al., 1989, 1995, Niskanen, 1993, Larsson et al., 1994, Lindberg and Alenius, 1999) and BLV (Klintevall et al., 1991). Individual milk and bulk milk were also shown to be suitable for analysing diagnostic responses in the IgM and IgA isotypes (**III, IV, V**). Milk was more reliable than serum for measuring low IgA levels, which may be of interest for monitoring the duration of protective immunity to infection. Bovine IgA and IgM antibodies showed stability to freezing, thawing and storage similar to that of IgG in both milk and serum (**III**). Long-term storage

of milk and serum samples at -20°C affected the specific antibody levels marginally compared with storage at -70°C (Table 1).

Table 1. Comparison of BCV-specific IgM and IgA optical density (OD) values for aliquots of milk samples stored 7 to 8 years at -20°C or -70°C. Samples were diluted 1:2, 10, 50 and 250 and tested in isotype-capture ELISAs. In no case was the end-point titre changed. OD values are shown for a dilution on the linear part of the titration curve

Cow No.		201	206	214	34	169	174	178
IgA	-20°	1.38	0.91	0.91	0.97	0.86	0.97	0.88
	-70°	1.17	0.77	0.88	0.99	0.78	0.92	0.99
IgM	-20°	0.60	0.75	0.67	0.85	0.84	0.84	0.73
	-70°	0.59	0.66	0.68	0.94	0.69	0.95	0.71

A disadvantage of milk compared with serum samples, is that with long-term storage (years) a proportion of the milk samples develop a gelatinous consistency, making them more difficult to pipette. The antibody levels, however, seem to be well retained (data not shown).

The IgA antibodies, and to a lesser extent IgM, in milk showed a higher variation between quarters of the same udder depending on the presence of subclinical mastitis than did IgG1 antibodies (**III**). For field diagnostic purposes, however, IgA antibody detection in paired samples produced consistent results, diagnosing BCV infection in 62% of the cows in 38 WD outbreaks (**V**). The lower diagnostic rate for IgA than for IgG1 (73%, **V**) may in part be due to IgA level variations, since milk somatic cell count levels were not measured in study **V**. However, the fact that IgA antibodies become detectable, and also peak before IgG1 antibodies after an infection, thereby making an IgA increase in paired samples more easily obscured by delayed acute sampling than IgG1, is more influential on the diagnostic rate. The diagnostic rate of IgA in paired serum samples (≥4-fold increase) from rotavirus infected children was also lower than that of IgG (Grimwood et al., 1988), the frequencies being similar to the results in this study.

Diagnostic value of the BCV-specific IgA and IgM isotype-capture ELISAs

The IgA and IgM ELISAs proved useful for increasing the frequency of individual BCV diagnoses in sampled cattle, increasing the frequency of positive herd diagnoses, as well as the confidence in the herd diagnosis (**V**). Residual IgG1 titres in previously BCV infected cattle obscured the IgG1 increase, supporting previous findings (Alenius et al., 1991) and resulted in a

substantially lower IgG1 diagnostic rate (41%) than for IgA (70%) in paired samples, making the IgA test particularly suitable for detecting reinfection responses. In primarily infected cattle, however, the IgG1 test showed a higher diagnostic rate (93%) than IgA (57%), mainly because the IgA antibodies become detectable in serum and milk a couple of days before the IgG1 antibodies (**III, IV**), rendering an IgA increase more easily missed by delayed acute sampling (>3-4 dpo) (**V**).

In two herds with delayed acute sampling (6 and 11 dpo), the BCV diagnosis was made possible only through specific IgM detection in the acute samples. The IgM test produced the highest diagnostic rate (95%), based on detection in either of the acute and convalescent samples, in the primarily infected cattle (**V**), while the diagnostic rate was low in reinfected cattle (23%). However, an IgM detection rate of 23% is high in reinfected cattle, compared with earlier results from experimentally reinfected cattle (Kimman et al., 1987a, Heckert et al., 1991a, b), confirming the results obtained in the earlier study (**III**), where all 3 reinfected cows showed detectable IgM responses, but varying widely in magnitude. The IgM test also produced the highest frequency of diagnostic results in calves with maternal antibodies (**V**), supporting previous reports (Kimman et al., 1987b, Kimman et al., 1988, Graham et al., 1998).

In the majority of cattle studied, the IgM response at high levels was sufficiently short (2-4 weeks, **III, IV**) to render the IgM isotype-capture ELISA suitable for use on single samples taken close to the IgM peak response (approximately 6 to 14 dpo). The IgM test may also be used on a single sample of bulk milk taken approximately 7 to 12 dpo, to enable detection even when the number of primarily infected cows is low. The diagnostic potential of the IgM test for bulk milk (79%) was, however, evaluated on paired samples (**V**). The IgA test also showed a higher diagnostic frequency (57%) than IgG1 (29%) on bulk milk. The combined use of IgA and IgM detection diagnosed BCV infection in 96% of the paired bulk milk samples (**V**). Using bulk milk for diagnostic purposes in herd outbreaks would reduce the cost, compared with analysing individual samples from a number of animals, and may potentially increase the demand for diagnostic tests.

Herd immunity to BCV influences disease outcome

Except for a few detections of BCV in lung tissue (Saif et al., 1986, Kapil et al., 1991), BCV replication in experimentally infected cattle has been described only in the epithelium of the small and large intestines and the upper respiratory tract, i.e. nasal cavity and trachea (Mebus et al., 1973, Reynolds et al., 1985, Saif et al., 1986, Kruiningen et al., 1987, Kapil et al., 1990, Tsunemitsu et al., 1991b, 1999b). Thus, BCV infection is a typical mucosal infection, for which mucosal immunity is considered essential for protection (Rosenthal and Gallichan, 1997). Protective immunity from many mucosal infections is rather ineffective and short-lived (Ahmed and Gray, 1996), repeated infections associated with clinical symptoms being reported with

HRSV (Henderson et al., 1979, Collins et al., 1996) and rotavirus (Offit, 1996). Also in our studies a relatively short duration of complete protection, preventing diarrhoea, was indicated by the finding that 5 herds out of 75 in study I had WD outbreaks in two consecutive stable periods. In study V, 2 herds out of 38 possibly had an outbreak in the previous stable period. However, the cattle in one herd that were sampled repeatedly during one year after the WD (III) did not develop diarrhoea when reinfected in the following season, between 6 and 9 months after the WD, indicating protection.

A more extended partially protective immunity, mitigating the disease outcome for years after an outbreak was indicated in the early study (I). To further explore the BCV immunity on herd level and its influence on the clinical outcome (V), a method to classify herd immunity, applicable for BCV infection with its specific properties, was needed. In earlier epidemiological studies of WD, the influence of various BCV immunity levels on risks and symptoms was only examined by IgG seroresponses to BCV (Smith et al. 1998a, b). For systemic infections, the proportion of subjects being seropositive is an established measure of herd immunity (Majok et al., 1991, Rümke et al., 1995, Flugsrud et al., 1997). However, since protection from mucosal infections is less long-lasting, an approach was chosen where immunity classification was based on the time since the previous herd BCV infection. Although not directly evaluated in the study (V), this measure will be correlated to the proportion of seropositive cattle in the herds, due to the normal culling/replacement procedures and the low frequency of introduced cattle in the herds. Applying this method, a highly significant correlation ($p<0.001$) was obtained between herd immunity level and the severity of the disease outcome and the amplitude of the decrease in bulk milk yield during the WD (V). Further, severe bloody diarrhoea in adult cows and older heifers was seen almost exclusively in naive cattle (V), in contrast to the results of American studies, where bloody diarrhoea was commonly seen in BCV seropositive cows (Saif et al., 1991, Smith et al., 1998a, b, c).

Why do WD and BCV calf diarrhoea appear as two epidemiologically separate entities?

This question contains 3 sub-questions:

1. Why are cows not diseased in herds with neonatal calf diarrhoea (NCD) problems attributable to BCV?

Herds with severe NCD problems have not been studied in this work, and in the limited studies from Swedish herds BCV was only occasionally detected (by ELISA or EM) in NCD cases (Klingenberg, 1992, Viring et al., 1993, Klingenberg and Svensson, 1998) in contrast to findings in other countries (Snodgrass et al., 1986, Reynolds et al., 1986, Durham et al., 1989b, Heckert et al., 1990b, d, Lu et al., 1991, Steiner et al., 1997), a discrepancy possibly related to herd management and structure differences (Klingenberg, 1999). Generally, the serological status of the cows has not been examined in studies of neonatal diarrhoea. A few studies have, however, investigated whether

intermittently BCV shedding adult cows can be a source of endemic BCV neonatal diarrhoea (Crouch and Acres, 1984, Crouch et al., 1985, Collins et al., 1987, Bulgin et al., 1989). The cows investigated were seropositive to BCV, and even if some of the investigators concluded that these cows were chronically infected, a more likely explanation might be that most of them were repeatedly reinfected by BCV circulating between cows and calves, keeping the BCV immunity of the cows on a high level, thus preventing disease in the cows.

2. Why are calves often clinically unaffected in WD outbreaks although BCV is a well established primary calf enteritis pathogen?

In several studies BCV strains isolated from WD and from calf diarrhoea have been compared, in vivo and in vitro, in searching for differences indicating that separate strains cause disease among cows and calves (Reynolds et al., 1985, Tsunemitsu and Saif, 1995, Dea et al., 1995, El-Kanawati et al., 1996, Milane et al., 1997, Hasoksuz et al., 1999a). So far, however, no evidence of BCV strain differences linked to the clinical picture WD vs. NCD has been detected. In papers **IV** and **V** we show that conventionally reared calves become infected by BCV when the cows have WD, even when no calf diarrhoea is observed by the farmer. The 100% seroresponse to BCV in the calves sampled indicates that calves seldom escape infection. Both in the experimental study and in the field study, however, it was evident that even if the occurrence of calf diarrhoea may have been strongly underestimated by some farmers, the calves showed less depression of general condition than the cows, confirming early observations (Hedström and Isaksson, 1951, Roberts, 1957). The experimentally BCV infected calves (**IV**) developed diarrhoea of comparable severity and duration to that in the cows, indicating lack of an immediate link between the severity of diarrhoea and the degree that their general condition was affected. This finding is in line with recent reports of viral diarrhoea being mediated by stimulation of the enteric nervous system (Lundgren et al., 2000) rather than loss of absorptive epithelial cells and villous atrophy, as was earlier presumed (Bachmann, 1984, Torres-Medina et al., 1985).

The more severely affected general condition of BCV infected seronegative cows compared with calves observed in both experimentally (**IV**) and naturally (**V**) infected cattle, indicates a more severe gut damage in the former, possibly due to a higher viral dose contracted by the cows. In naturally infected young calves, the viral dose may be reduced by BCV-specific passive antibodies constantly supplied to the gut by whole-milk feeding or even by milk replacer (**IV**). Cows housed in tie-stall barns with their heads rather close together may receive a high viral dose by aerosol and by nasal contacts, as previously suggested (Smith et al., 1998a). None of these conditions, however, prevailed in the experiment (**IV**), since the calves were fed BCV antibody negative whole-milk during the entire clinical study and the cows were housed untied in individual pens of horse box type, within a common barn compartment.

A possible explanation is that intestinal mucosa in the adult cow supports the proliferation of BCV to a higher degree than that of the calf, either because of differences in distribution of the viral receptor or differences in the enzyme environment. Organ-specific protein receptors have been identified for the other coronaviruses MHV (mouse), HCV 229E (human) and TGEV (swine, Holmes and Compton, 1995). Except for an essential carbohydrate moiety, the BCV receptor has not been fully characterised, and information on age-related differences in receptor distribution is presently not available. However, BCV primary isolation and propagation in cell culture is greatly facilitated and enhanced by trypsin (Dea et al., 1980, Storz et al., 1981, St.Cyr-Coats et al., 1988, Kapil et al., 1996) or pancreatin, a mixed pancreatic enzyme preparation (Benfield and Saif, 1990), completing the proteolytic cleavage of the BCV S protein (into S1 and S2) necessary for induction of fusion with the cell membrane (Holmes and Compton, 1995). Trypsin facilitating the in vitro isolation and propagation was also determined for myxo-, paramyxo- and rotaviruses (Rott, 1979, Homma and Ohuchi, 1973, Theil et al., 1978). These observations led to the investigation of the importance of trypsin-like cleavage for infectivity in vivo. Recently, such in vivo trypsin-like cleavage by membrane-bound or excreted host cellular enzymes was demonstrated for Influenza A and Sendai virus in the bronchi and nasal cavity of humans and rats (Kido et al., 1996, 1999). Thus, enzymes with trypsin-like activity are present both in the upper respiratory tract and in the small intestine, and extracellular cleavage of the S protein is likely to facilitate BCV fusion with host cells also in vivo. Age-related differences in the proteolytic enzyme profile and levels in the intestines have been examined in pigs. The young animal, intended to live on milk for some time, has considerably lower levels of pancreatic enzymes than older pigs (Pond et al., 1971, Corring et al., 1978). Lower pancreatic trypsin levels, together with the presence of trypsin inhibitors found in colostrum and milk of many species, including bovines (Sandholm and Honkanen-Buzalski, 1979, McLean and Holmes, 1981), probably contributes to young calves being less sensitive to BCV infection than cows. Furthermore, the intestinal enzyme levels vary also in adult life, being up- and down-regulated according to the demands of the ration (Argenzio, 1993). The concept of high levels of intestinal proteolytic enzymes, leading to increased viral replication and increased intestinal damage, is supported by the findings (V) of high yielding cows being more severely affected than other cows in a proportion of the herds, a finding also reported by others (Smith et al., 1998b), and by higher diarrhoea frequencies in lactating than in dry cows (V). Trypsin influencing the pathogenicity of rotavirus in vivo has also been proposed, and supported by the increased severity and duration of diarrhoea in experimentally rotavirus-infected piglets fed pancreatic enzyme supplements (Steel and Torres-Medina, 1984). The lack of severe rotavirus infections in adult cattle compared with the situation in BCV infections, may be due to rotavirus immunity from repeated reinfections (Offit, 1996) and possibly developmental changes in the intestine (Bridger, 1994).

To conclude question 2, the likely explanations are a) less intestinal trypsin activity in calves than in cows, b) reduction of viral dose by specific antibodies in milk or milk replacer, and possibly c) age-related differences in receptor expression, a field which is unexplored.

3. If calves are less sensitive to BCV, then why have a lot of experimentally infected calves developed severe diarrhoea?
Among these experiments (Stair et al., 1972, Mebus et al., 1973, Saif et al., 1986, Heckert et al., 1989, 1991a, El-Kanawati et al., 1996), the vast majority were carried out in gnotobiotic or colostrum deprived calves. None of these have a normal gut flora, which is an important non-specific resistance mechanism towards invading pathogens (Kato and Owen, 1994). Also, contact with antigen stimulates the development and maturation of the neonatal immune system (Adkins, 1999), and colostrum feeding has a strong influence on the development and function of the GI tract (reviewed by Xu, 1996). Further, none of these experimental infections were naturally transmitted, but large doses of virus mostly were given orally. High frequencies of BCV diarrhoea and mortality in calves have been described in dairy and beef herds (Collins et al., 1987, Bulgin et al., 1989). In those herds, a high faecal BCV contamination of the environment may have been allowed to build up, leading to viral transmission patterns (i.e. mainly faecal-oral) differing from the normal occurrence during WD outbreaks (see below). Also, NCD is a multifactorial disease, and other pathogens, although not detected in these particular herds, may contribute to the disease, as well as environmental factors and unsuitable management routines (Bruning-Fann and Kaneene, 1992).

Winter dysentery - a respiratory infection?

BCV transmission to a sensitive herd probably can occur by faecal contamination, but within-herd transmission of BCV in WD outbreaks is unlikely to occur mainly by the faecal-oral route. Development of the disease in two highly BCV susceptible herds investigated in paper V is shown in Table 2.

Table 2. Cumulative incidences of diarrhoea during 2 WD outbreaks

No. of cows	Cumulative incidence of diarrhoea (cows)				
in the herd	Day 1[a]	Day 2	Day 3	Day 4	Day 5
20	10	16	17	17	19
19	1	7	15	19	19

[a] The first day when diarrhoea was observed by the farmer.

Similar within-herd spreads have been reported by others (Roberts, 1957, Kahrs et al., 1973). The experimentally BCV infected seronegative adult cows in paper **IV** showed an incubation period of 4-5 days. Faecal shedding at levels detectable in ELISA did not occur before the diarrhoea started, except in one of four cows where shedding started 1 day before the diarrhoea. Even if shedding should start before the diarrhoea in some cows, non-diarrhoeic faeces is unlikely to spread BCV throughout the barn within a couple of days. Possibly, the incubation period may be somewhat shorter than in our study, but considering the 2- to 5-day incubation periods in experimentally BCV infected older calves (Mebus et al., 1973, Bridger et al., 1978, Saif et al., 1986, Saif, 1987, El-Kanawati et al., 1996), and 3-16 days in seropositive cows (Tsunemitsu et al., 1999b), an incubation period shorter than 3 days is unlikely in adult cattle. Many studies have documented BCV replication in the upper respiratory tract (Thomas et al., 1982, Reynolds et al., 1985, Saif et al., 1986, Saif, 1987, Kapil et al., 1991, El-Kanawati et al., 1996). The very rapid increase in the cumulative incidence of diarrhoea shown in Table 2, suggests that most cows were already infected by the time the first cow developed diarrhoea. Aerosol transmission being the major route of within-herd transmission is the most likely explanation for such an incidence pattern. BCV shedding in nasal secretions of seronegative calves was detected as early as 1 to 2 days after experimental oral and/or intranasal infection (Reynolds et al., 1985, Saif et al., 1986, Saif, 1987, El-Kanawati et al., 1996) and 2 days after transmission via direct contact with an experimentally inoculated calf (Niskanen et al., 2000), the latter study using 2 to 5 months-old conventional calves. Nasal shedding probably indicates concomitant aerosol shedding but, so far, aerosol shedding has not been directly examined in any study.

Conclusions

1. Causation

BCV is the major causative agent of WD, at least in Swedish cattle herds. However, all WD outbreaks are not caused by BCV.

WD can be reproduced with BCV in seronegative cows, using virulent wild-type virus and natural transmission by a carrier animal.

2. Diagnostics

Isotype-capture ELISAs show higher sensitivity than indirect ELISAs for detecting IgA and IgM in serum and milk where inter-isotype competition is larger than intra-isotype competition.

Milk and bulk milk are suitable for analysing IgA and IgM responses to infections. Both specific IgA and IgM responses showed longer duration in milk than in serum.

High IgM levels are present for a short period after BCV infection, making specific IgM detection suitable for a single sample diagnostic procedure.
BCV infected cows and calves show long-lasting IgA responses in serum, milk and nasal secretions.
BCV-specific IgG1 antibodies are detectable in milk and serum for several years after infection.

IgM detection and IgG1 titre increase in paired samples, are the most efficient diagnostic procedures in primarily BCV infected cattle (95 and 93% diagnostic rate, respectively).
IgA titre increase in paired samples, is the most efficient diagnostic procedure in reinfected cattle (70% diagnostic rate).
The diagnostic rate, using all 3 tests combined, is lower in reinfected cattle than in primarily infected. Therefore, strategic sampling of young cattle is advisory in WD outbreaks.

IgM antibody detection makes possible a BCV diagnosis in herds when the acute sampling is delayed.

The IgM and IgA tests are more efficient diagnostic tools on paired bulk milk samples than the IgG1 test. Combined use of the IgM and IgA tests diagnoses BCV in a high proportion (96%) of paired bulk milk samples, being a simple and economic diagnostic procedure compared with the analysis of several individual samples.

IgM detection is the most efficient diagnostic tool in calves with maternal antibodies.

BCV excretion in faeces, at levels clearly detectable by ELISA, is often very short, even in primarily infected cows and calves.

3. Herd immunity, epidemiology and clinical findings

WD is a common disease in Swedish dairy herds, and the BCV antibody prevalence is high throughout the country.

By measuring BCV-specific IgM, IgA and IgG1 in paired milk or serum samples primarily infected and reinfected cattle are distinguished in >99% of the cases.

Seronegative cattle, including calves, seroconvert to 100% when BCV infection enters a herd.

95% of the cattle that develop bloody diarrhoea during the WD outbreaks that are associated with BCV infection are primarily BCV infected.

Severity of the disease and the decrease in milk yield during WD are strongly correlated with the herd BCV immunity level.

The occurrence of calf diarrhoea during WD outbreaks is underestimated by farmers, but calves show a milder disease than cows.

Aerosol is probably the major route of within-herd spread of BCV preceding the WD outbreak, at least in herds with rapid spread.

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