Capture ELISA systems for the detection of bovine coronavirus-specific IgA and IgM antibodies in milk and serum

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Abstract

Isotype-capture ELISAs for BCV-specific IgA and IgM were developed and tested on milk and serum samples from Swedish cattle. The capture ELISAs showed higher sensitivity than indirect ELISAs for detection of BCV-specific IgA and IgM. In the capture ELISAs the agreement between detection in milk and serum samples was 94% for IgA and 86% for IgM. The correlation between $\log_{10}$ titres in milk and serum was $r=0.82$ ($P<0.001$) for IgA and 0.84 ($P<0.001$) for IgM. Milk seemed a better target than serum for diagnosing specific IgA at low levels. There was no variation in the isotype-specific BCV antibody titres between healthy quarters of the same udder, but subclinical mastitis was associated with higher levels of IgA antibodies and weak false IgM positive reactions in undiluted milk. Bovine IgA and IgM antibodies in milk and serum showed high stability towards freezing and thawing and storage at room temperature.

The antibody responses to BCV were followed in milk and serum from six dairy cows and in serum from four calves for a period of 1 year after an outbreak of winter dysentery (WD). In this outbreak some animals became reinfected with BCV. The IgA and IgM capture ELISAs differentiated between primarily BCV infected and reinfected animals. In the primarily infected cattle, IgM antibodies were first detected in milk and serum four to nine days after the first WD

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symptoms observed, and were subsequently detected for at least 2–3 weeks. IgM was also detected in the reinfected cows, but mostly at lower levels and for a shorter period of time than in the primarily infected animals. In milk, however, the IgM response of the reinfected cows was detected for a longer period of time than in serum. Six months after the outbreak, IgA was still detected in both serum and milk of all six cows and also in serum of one calf. The reinfected cows showed higher and more long-lasting peak levels of IgA in milk and serum than the primarily infected cows, indicating boosting of the IgA response. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Bovine coronavirus (BCV) is a common pathogen in Swedish cattle herds (Tråvén et al., 1998) causing winter dysentery in adult cattle (Alenius et al., 1991), calf enteritis (Mebus et al., 1973) and respiratory infections (McNulty et al., 1984). Faecal detection of the virus in EM is time consuming and laborious and the enzyme-linked immunosorbent assay (ELISA) shows low detection rates in adult cattle (Smith et al., 1998) and in conventionally reared calves (personal observations). Attempts at diagnosing these infections serologically are often problematic because in adult cattle high BCV-specific serum IgG levels are often encountered in the acute samples, presumably due to reinfections with the virus, obscuring the detection of a possible titre increase in paired samples. Since adult cattle usually are seropositive (Alenius et al., 1991; Tråvén et al., 1998), maternal antibodies frequently obliterate the detection of infection in calves (Atterhem et al., 1996). There is also a demand for serologic tests that do not require the cumbersome and expensive paired samples necessary for an IgG-based diagnosis. In the light of these facts, diagnostics based on specific antibodies of the IgA and IgM isotypes would be highly desired improvements.

IgM seen 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 (Tizard, 1996), although at a lower level and the detection is hampered depending on the extent of competition for antigenic binding with IgG. Calves experimentally reinfected with bovine respiratory syncytial virus (BRSV) did not show detectable IgM in serum, but in respiratory and eye secretions (Kimman et al., 1987a).

IgA is produced by plasma cells mainly in the lamina propria beneath mucosal surfaces and skin, 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 protect from mucosal infection in vivo (Liew et al., 1984; Renegar and Small, 1991). 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 (Rosenthal and Gallichan, 1997). Serum IgA responses were detected after both the first and the second experimental infection with BRSV in the respiratory tract of

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 detectable after the first immunisation (Hu et al., 1998).

In the bovine, IgG1 and IgG2 are the dominant serum Ig subclasses (Butler, 1983), while in milk IgG1 is the major isotype, being selectively transported across the udder epithelium (Butler, 1983).

BCV-specific IgA and IgM were detected by indirect ELISAs in serum and mucosal secretions from calves (Saif, 1987; Heckert et al., 1991a, b, c). In an indirect system, however, where antigen is bound to the solid phase, detection and quantification of specific antibodies in classes 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 antiserum or monoclonal antibodies (mAbs) are bound to the solid phase, the BCV-specific IgA suffers only from the competition with IgA carrying other specificities. Intra-isotype competition is probably much less severe than that from antigen-specific IgG (Kimman et al., 1987b). Capture and indirect IgA and IgM ELISA results were compared for BRSV-infected calves in the studies of Zaane and Kimman, but only in a small number of samples.

In the present work, we evaluate the performance of capture versus indirect ELISAs for IgA and IgM on a larger material of samples from BCV-infected cattle. In the second step, the diagnostic potential of IgA and IgM to detect the early antibody response and to detect reinfection was investigated. The particular aim was to explore the diagnostic capacity of these tests on milk samples.

2. Materials and methods

2.1. Purification of IgA

Bovine IgA was purified as described by Fey et al. (1976). Approximately 1 l of bovine saliva was collected from two adult cows and pooled. The saliva was dialysed against 0.005 M sodium phosphate buffer (pH 7.0), clarified by centrifugation and purified on DEAE cellulose (DE 52, Whatman, Maidstone, Kent, UK) according to Fey et al. (1976). The fractions eluted from the column were tested with mouse monoclonal antibodies (mAbs) to bovine IgM, IgA, IgG1 and IgG2 (kindly provided by Dr. A.J. Guidry, USDA-ARS, Beltsville, MD, USA) in an indirect ELISA (described below). Fractions containing only IgA were pooled and purified by gel filtration on a Sephacryl S-300 column (Amersham Pharmacia Biotech), to remove high molecular contaminants. The IgA containing fractions were identified with the anti-IgA mAb in ELISA, pooled and used for immunisation of mice and to check the specificity of the hybridoma clones produced.

2.2. Purification of IgM

Approximately, 100 ml of bovine serum from an adult cow was saturated with 50% ammonium sulphate and dialysed against 0.5 M NaCl buffered with 0.05 M Tris–HCl, pH

8.0. The dialysate was clarified by centrifugation at $2000 \times g$ for 30 min and gel filtered on a Sephacryl S-300 column (Amersham Pharmacia Biotech). The eluted fractions were tested in ELISA with mAbs to bovine IgM, IgA, IgG1 and IgG2. The purified IgM was used to check the specificity of the hybridoma clones produced.

2.3. Preparation of BCV antigen

BCV strain Munich W 270/83 was prepared in primary bovine foetal turbinate (BT) cells. The BT cells were grown in EMEM (National Veterinary Institute, NVI, Uppsala) supplemented with 3 g/l tricine, 50 mg/l gentamicin, 2 mM L-glutamin and 10% FCS with extremely low Ig content (NVI), until confluence in 225 cm^2 cell culture flasks (Costar, Life Technologies, Täby, Sweden). The growth medium was removed and the cell monolayer rinsed with PBS (pH 7.4). 100 TCID$_{50}$ of the virus preparation in 10 ml of EMEM without FCS were added per flask. The cultures were incubated at 37°C for 1 h, then 65 ml of EMEM without FCS were added per flask and the cultures incubated for a further 5–7 days when 90–100% of the cells were destroyed. The virus was harvested by two cycles of freezing and thawing and the fluid was clarified by centrifugation for 30 min at 2000 rpm (Sorvall RC-3B). This crude BCV preparation was used as antigen in the Ig capture ELISAs for antibody detection. To concentrate the BCV for immunisation of mice and for use in the indirect ELISAs, the virus was pelletted by ultracentrifugation (Kontron, TFA 20 rotor) at 4°C and 20 000 rpm for 18 h and the virus pellet re-suspended in phosphate buffered saline (PBS, pH 7.4).

Negative control antigen used in the capture ELISAs for antibody detection was cell culture fluid from uninfected BT cells collected after freezing and thawing as above.

2.4. Production of mAbs to bovine IgA, IgM and to BCV

Female Balb/c mice, 4–6-weeks-old, were immunised subcutaneously with 40 μg of purified bovine IgA, 100 μg of bovine IgM (I-8183, Sigma–Aldrich, Tyresö, Sweden) or 10 μg of BCV, respectively, in 250 μl of PBS, pH 7.4, adjuvanted with 5 μg of ISCOM-Matrix (kindly supplied by Dr. K. Lövgren). A booster dose was given 14 days later and after 3 more days the mice were euthanised. The spleenocytes were fused to the myeloma cell line Sp2/0 according to the procedure described by Galfre and Milstein (1981), using 50% PEG (Hybri Max, Sigma–Aldrich). Following fusion, hybridoma cells in FDMEM (Full Dulbecco's modified Eagle's medium, NVI) supplemented with 10% FCS (NVI) were incubated at 37°C for 12 h. Cells were seeded onto microtitre plates and cultured in complete medium supplemented with HAT (Hybri Max, Sigma–Aldrich) for 14 days.

The anti-Ig hybridoma supernatants were assayed against purified bovine IgA, IgM, IgG1 and IgG2 using an indirect ELISA to exclude cross-reacting hybridomas. The IgG1 and IgG2 used were purified from bovine colostrum or bovine serum, respectively, on DE 52 (Whatman) as described by Fey et al. (1976) and the fractions were selected by aid of isotype-specific mAbs as described for IgA.

The anti-BCV hybridoma supernatants were first screened against BCV in an indirect ELISA and positive clones were re-tested in a blocking ELISA to confirm the specificity. The selected hybridomas were cloned by limiting dilution and the isotype determined

using Western blot (Inno-Lia, Innogenetics, Ghent, Belgium). The hybridomas were cultured in FDMEM supplemented with 2% FCS and the supernatants harvested. After concentration of the supernatants through precipitation with 33% ammonium sulphate and dialysis of the precipitate against PBS (pH 7.4), the mAbs were further purified on a Protein A sepharose column (Amersham Pharmacia Biotech) according to the manufacturer's description. The Ig-containing fractions were identified in indirect ELISA and SDS–PAGE and pooled. Purified mAbs were conjugated to horseradish peroxidase (HRP) according to Nakane and Kawaoi (1974).

2.5. Virus neutralisation

The ability of the anti-BCV mAbs to neutralise virus infectivity was determined in a virus neutralisation (VN) test. mAbs were titrated by doubling dilution from 1:2 to 1:256 in complete EMEM. To each antibody dilution 100 $TCID_{50}$ of BCV were added. Virus controls containing 1000, 100, 10, and 1 $TCID_{50}$ were incubated simultaneously with the virus/mAb mixtures for 1 h at 37°C. 10^5 TB cells were added per well and the plates incubated at 37°C in a CO_2 environment until all virus control wells showed 100% CPE. The titre was determined as the highest mAb dilution completely preventing CPE.

2.6. Haemagglutination inhibition

The ability of the anti-BCV mAbs to prevent the virus from agglutinating mouse erythrocytes was evaluated in a haemagglutination inhibition (HI) test using U bottomed microtitre plates (MicroWell, Nunc, Roskilde, Denmark). The anti-BCV mAb supernatants were titrated by doubling dilution in saline (0.15 M, 0.01% BSA), 50 μl per well. Fifty μl saline containing 4 HA units of the crude BCV preparation were added to each well and the plates were incubated for 30 min at room temperature. Virus control without mAb was included on each plate and a mAb control without virus was included for every mAb tested to check for spontaneous agglutination. Fifty μl of a 1% erythrocyte suspension in saline were added to each well and the plates were incubated for 1 h. The HI titre was determined as the highest mAb dilution completely preventing HA.

2.7. IgA and IgM capture ELISA

2.7.1. Coating and samples
Polysorp microtitre plates (Nunc) were coated with the purified mAbs to IgA or IgM at a concentration of 5 μg/ml in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (coating buffer), and incubated over-night at +4°C. After washing, bovine sera were added in 3-fold dilutions, from 1:10 to 1:7290, and for milk samples from undiluted to 1:2430. The lowest dilution of each sample was added to duplicate wells. Reagents of 100 μl each were used per well, except for the substrate solution and the H_2SO_4 used to stop the reaction. Milk samples were defatted through centrifugation at $2000 \times g$ for 10 min, unless otherwise stated.

2.7.2. Washing, dilution and incubation

PBS with 0.05% Tween-20 (PBST, pH 7.4) was used as dilution and washing buffer. All incubations were made at $+37^\circ C$ for 1 h, unless otherwise stated, followed by washing three times in PBS-T.

2.7.3. BCV antigen

After incubation of the coated wells with the test samples, the standard positive and negative samples, the plates were washed and 100 µl of the BCV preparation added to each test well. Negative control antigen was added to one of the duplicate wells of the lowest dilution used for each sample to check for non-specific binding.

2.7.4. Conjugate

After incubation and washing, the HRP-conjugated mAb to BCV (15:11, selected as described in Section 3), diluted in PBST, was added and the plates were incubated.

2.7.5. Substrate

After washing, 200 µl of the substrate buffer (0.1 mg tetramethyl benzidine/ml with 0.05% H_2O_2) were added to each well. The reaction was stopped after 10 min at room temperature through adding 50 µl of 1 M H_2SO_4 and the optical density (OD) at 450 nm was measured in an ELISA reader (Titertek Multiscan, Labsystems, Helsinki, Finland).

2.8. Indirect ELISA for purity control of the bovine Ig

For coating, the collected peak fractions of isotype-purified bovine Ig were diluted 1:50 in coating buffer and each fraction was dispensed into four wells. Mouse mAbs specific for bovine IgA, IgM, IgG1 and IgG2 (see above) were used as detection antibody. Bound mAbs were detected with a HRP conjugated rabbit anti-mouse Ig (P 0260, Dakopatts, Älvsjö, Sweden) diluted 1:1000 in PBST with 2% horse serum. The procedure was continued as above.

2.9. Indirect ELISA for control of the mAbs to bovine Ig and to BCV

Purified bovine IgA and IgM for coating were diluted 5 µg/ml in coating buffer. The BCV antigen preparation for coating (described above) was mixed with Triton X-100 (0.01%) and sonicated for 3×5 s before diluted 1:2000 in coating buffer. The optimal coating dilutions were determined by checkerboard titration with positive and negative bovine sera. After incubation and washing the hybridoma culture supernatants, diluted 1:10, were added and the plates incubated. The procedure with conjugate (anti-mouse Ig-HRP) and substrate was continued as above.

2.10. Blocking ELISA for control of the mAbs to BCV

BCV-coated ELISA plates were prepared as described above. BCV antibody positive or negative bovine sera, diluted 1:50 in PBST, were dispensed into wells and the plates

incubated. The hybridoma culture supernatants to be tested were diluted 1:10 in PBST and added to one well prepared with positive and one with negative serum, and incubated. The ELISA procedure was continued with conjugate and substrate as above.

2.11. Confirmation of the Ig-specificity of the mAbs

Six mAbs with high anti-IgA activity in the indirect ELISA and one each of the anti-IgM, anti-IgG1 and anti IgG2 mAbs were further tested for specificity and cross-reactivity in a checkerboard analysis scheme. The cross-testing was performed using a modified capture ELISA where plates were coated with predetermined optimal dilutions of each mAb. After incubation over-night at +4°C and washing, bovine serum, milk, saliva or PBS were added in 10-fold dilution steps. After incubation and washing, the HRP conjugated mAbs were added. This design was employed on every possible pair of coating and conjugated mAbs.

2.12. Western blot

Specific binding of the anti-Ig mAbs to Ig heavy chain was tested in western blot (WB). Purified IgA or IgG1 were analysed in SDS–PAGE (Bio–Rad Laboratories, Sundbyberg, Sweden), 12% gel with LMW standard (Amersham Pharmacia Biotech). Separated polypeptides were transferred to nitrocellulose membranes (Schleicher and Shüell, Germany) according to Towbin et al. (1979) and incubated with the mAbs for 2 h at room temperature. An alkaline phosphatase-conjugated rabbit antiserum to mouse Ig (Dakopatts) was used as secondary antibody and bound enzyme was detected with BCIP/NBT. The anti-Ig mAbs and the conjugate were diluted in a blocking solution of PBST with 5% non-fat dry milk.

Binding of the anti-BCV mAb BCV15:11 to separated BCV proteins was tested in WB. The concentrated BCV preparation was analysed in SDS–PAGE using a 10–15% gel and transferred as above. Nitrocellulose strips were incubated with the mAb or anti-BCV reference mAbs directed towards the S or HE proteins (kindly provided by Dr. L.A. Babiuk, University of Saskatchewan, Canada). HRP-conjugated rabbit anti-mouse Ig (P 0260, Dakopatts) was used as secondary antibody and visualised with DAB.

2.13. Binding of the anti-Ig mAbs to sera from various animal species

The binding of mAbs to Igs of other species was investigated using a competitive ELISA. Micro-titre plates were coated with purified IgA or IgM. Then serum samples from several animal species in 10-fold dilution steps were added simultaneously with the HRP-conjugated mAbs, except for the unconjugated M70:15 that was detected with anti-mouse-Ig HRP (P 0260, Dakopatts).

2.14. Evaluation on bovine milk and serum samples

All bovine samples used for the evaluation of the isotype-capture ELISAs originated from Swedish cattle.

2.14.1. BCV antibody negative milk and sera

Forty-one sera and 21 milk samples were collected from dairy Herd T that had not experienced winter dysentery (WD) for more than 10 years. Herd T consisted of 28 cows, eight heifers and five calves that were all IgG1 antibody negative to BCV. Twenty-nine sera and 17 milk samples were collected from dairy Herd R that had not experienced WD for more than 4 years. The cows born before the last WD outbreak in Herd R (investigated by us, Alenius et al., 1991) were IgG1 antibody positive to BCV. Only the IgG1 antibody negative cattle (nine cows, seven heifers and two calves) were included in the negative reference population. Sera were analysed in 1:10 dilution and milk samples undiluted. Each sample was analysed simultaneously in two wells with BCV antigen, and for the capture ELISAs also in two wells with control antigen.

2.14.2. IgM and IgA isotype-capture versus indirect ELISA

Winter dysentery convalescent serum samples from 29 cattle and milk samples from 31 cows in 12 herds were used to compare cIgA and iIgA ELISA performances. These cattle were sampled 15–23 days post outbreak start (DPO).

The cIgM and iIgM tests were compared using a series of milk and serum samples from two cows in Herd AN (2 and 45, see below) sampled eight times during 2 months after WD.

2.14.3. IgM and IgA detection in milk versus serum

Milk and serum antibody levels were compared in cIgA and iIgG1 ELISAs using samples from nine BCV negative cows in Herd R, eight WD convalescent cows in five herds sampled 19–23 DPO, and 31 cows that were patients with various diagnoses at the University ruminant clinic in Uppsala. These cows originated from 23 different farms with unknown WD history. For each cow the milk and serum were analysed on the same micro-titre plate.

Milk and serum IgM levels were compared in the cIgM ELISA using 72 milk and serum samples from the cows in Herd AN (described below) after a WD outbreak.

2.14.4. Antibody response after primary infection and reinfection

Bulk milk and individual serum and milk samples from six cows and four calves (Table 2) were collected in dairy Herd AN 4, 6, 9, 12, 18, 24, 38 DPO and 2, 4, 6, 9 and 12 months after the start of a WD outbreak. The herd comprised 19 milking cows with young stock and calves. The previous WD outbreak in this herd had occurred more than 12 years ago but BCV seropositive cows had been incorporated into the herd on several occasions. BCV-specific cIgA, cIgM and iIgG1 were analysed in 2-fold dilution steps. The first sampling was done 4 days after the first diarrhoeic animal was noticed.

2.14.5. Milk antibody level comparison between udder quarters

Serum and milk from all four quarters were analysed in 3-fold dilution steps for BCV-specific antibodies using the cIgA, cIgM and iIgG1 tests. Cell count level for each quarter was estimated by the California mastitis test (CMT) on a scale from 1 to 5 (1=no visible gel, 5=heavy gel formation). Samples were obtained from four cows in Herd TR, a BCV antibody negative experimental herd, and from five WD convalescent cows from Herds

SK and KA, both sampled 10 days after the respective outbreak started. Milk from all quarters of one cow was analysed on the same plate.

Milk samples from all quarters of 10 cows in Herds SA and RU were analysed for BCV-specific IgA and IgG1 together with bacteriological examination. Herd SA was sampled 2 months after a WD outbreak and Herd RU was sampled during a subclinical BCV infection indicated by rising titres or seroconversion in paired samples (data not shown).

2.14.6. IgA and IgM antibody level in centrifuged versus uncentrifuged milk

Binding of IgA to fat globules in milk has been reported (Honkanen-Buzalski and Sandholm, 1981; Pivont et al., 1984), possibly leading to a significant proportion of the IgA being discarded with the fat fraction when centrifuging milk samples before the analysis. To examine this question, eight milk samples from four experimentally BCV infected cows described elsewhere (Tråvén, 2000) were analysed to test the effect of centrifugation on IgA and IgM levels. Samples were titrated in 3-fold dilution steps, and all samples from one animal were run on the same plate.

2.14.7. Stability of bovine Ig isotypes

To check the stability of the antibodies towards sample handling procedures, milk from four BCV antibody negative cows in Herd TR and sera and milk from five WD convalescent cows in Herds SK and KA were given the following treatments. Aliquots of serum and milk from one udder quarter per cow were stored at room temperature or at 4°C for 0, 3, 5 or 7 days before centrifugation (0, 3, 7 and 10 days for the BCV negative milk). Aliquots of the same samples were frozen at −20°C for 24 h and thawed at 4°C for 24 h one, three or five times, and stored at −20° until analysed. Samples were analysed in 3-fold dilution steps for BCV-specific IgM, IgA and IgG1 and for each treatment series, all samples from one animal were analysed on the same plate.

Milk and serum samples from five cows in Herd RU (complete treatment) and from five cows in Herd SA (only freeze/thaw treatment) were analysed for BCV-specific IgA and IgG1.

2.15. Statistical analyses

The analytical error of the capture IgA ELISA was determined using 200 bulk milk samples and 116 sera analysed in duplicate and calculated from the formula $S = \left(\sum d^2 / 2n \right)^{0.5}$ where n is the number of duplicates and d is the difference in a certain pair. The bulk milk samples were obtained during a BCV antibody survey in an area of southwestern Sweden. Fourty-eight sera originated from cows and 37 from calves sampled 5 weeks to 9 months after an experimental BCV infection described elsewhere (Tråvén, 2000) and 37 sera came from newborn to 6-months-old calves sampled during a 2-year study of calf diseases in a large dairy herd in southwestern Sweden (Klingenberg et al., 1999). For the capture IgM ELISA, 21 individual milk samples and 37 sera were analysed. The milk samples and nine sera came from experimentally BCV infected cows and calves and 28 sera came from the 2-year calf study. Bulk milk samples were analysed undiluted and individual milk samples in 1:2 dilution. Sera from the experimental

infection were analysed in 1:25 dilution and sera from the 2-year calf study in 1:100 dilution.

The inter-assay coefficient of variation was calculated from the formula $CV = 100\% \times SD/mean$. In the IgA test, one each moderately strongly positive sample of milk and serum (S187) was analysed on 65 plates, and one each weakly positive sample of milk and serum (K5008) was analysed on 48 plates. In the IgM test, one each moderately strongly positive sample of milk and serum (SK250) were analysed on 59 plates.

3. Results

3.1. Selection of mAbs to bovine Ig

Several mAb clones showed strong binding to bovine Ig of one subclass and low or undetectable binding to the other three in the indirect ELISA. Cross-testing of the mAbs for confirmation of the Ig specificity showed that mAbs anti-IgM M69:2, anti-IgA A12:2 and A39:6, anti-IgG1 G1/42:40 and anti-IgG2 G2/98:40 did not cross-react with other Ig classes or subclasses. All mAbs showed good HRP conjugation ability. mAbs A12:2, A39:6 and M69:2 showed strong binding when the same mAb was used both for coating and as conjugate. This property of the mAbs indicated that the recognized epitope appeared at least twice on each Ig molecule, that the mAb had bound to the plate in a functional manner and had reasonably high affinity. The mAbs G1/42:40 and G2/98:40 showed no signal when they were used both for coating and as a conjugate, indicating that the recognised epitope appeared only once on the respective Ig molecule.

The five mAbs above were run in WB against bovine IgA. Only mAbs A12:2 and A39:6 showed detectable binding to the α-chain band at approximately 59 kd. mAb G1/42:40 showed strong binding to the γ1-chain band at 55 kd (Butler, 1983). The mAb subclass determinations are shown in Table 1.

The binding test with sera from other species revealed that mAbs A12:2 and A39:6 bound to serum Ig from several domestic and wild ruminants comparable with binding to

Table 1
Specificity, subclass and species cross-reactivity in mouse anti-bovine Ig mAbs

mAb	Specificity, bovine Ig	Subclass	Cross-reacting with serum from
A12:2	IgA	IgG2b	sheep, goat, deer, reindeer, water buffalo[a]
A39:6	IgA	IgG1	sheep, goat, deer, reindeer[b]
M69:2	IgM	IgG2a	bovine only[c]
M70:15	IgM	IgG1	sheep
G1/42:40	IgG1	IgG1	n.d.[d]
G2/98:40	IgG2	IgG1	n.d.

[a] Moose, pig, horse, human, mouse, rabbit and guinea-pig sera were tested.

[b] Moose, pig, horse, human, mouse and guinea-pig sera were tested. Weaker binding was recorded to goat serum than for A12:2.

[c] Sheep, goat, water buffalo, moose, reindeer, horse, human, mouse and guinea-pig sera were tested.

[d] n.d.: not determined.

bovine Ig (Table 1). mAb M69:2 bound only to bovine Ig but mAb M70:15 showed binding to sheep serum Ig comparable with that to the bovine. mAbs A12:2 and M69:2 were selected for work on bovine samples.

3.2. Evaluation of mAbs to BCV in functional tests

Four anti-BCV mAbs with high binding and blocking activity in the ELISAs were selected for further characterisation.

Virus neutralising (VN) activity was demonstrated for all four mAbs, with VN titres of 16, 16, 32 and 32 for mAbs 15:11, 41, 59 and 63, respectively. This indicated that all mAbs were directed towards the S or the HE protein since neutralising epitopes have been demonstrated on the envelope proteins S and HE of BCV (Deregt and Babiuk, 1987).

HI activity, expressed as reciprocal HI titres, was eight for mAbs 41 and 59, whereas mAbs 15:11 and 63 showed no HI activity. Both the HE and the S proteins of BCV have been shown to possess epitopes with HA activity (Schultze et al., 1991).

mAb BCV15:11 with VN but without HI activity was analysed in WB together with anti-BCV reference mAbs to determine the epitope specificity. The BCV15:11, however, did not bind to SDS-denatured BCV proteins, indicating that the mAb is directed against a conformational epitope as is often the case with VN mAbs. mAb BCV15:11 was selected for use in the Ig capture ELISAs for bovine milk and sera. The subclass was IgG2a.

3.3. Capture ELISA for BCV-specific IgA and IgM show higher sensitivity than indirect ELISA in milk and serum samples

The sensitivity and performance of the capture and indirect IgA and IgM ELISAs were tested on sera and defatted milk samples. The antibody results were related to the well-established BCV IgG indirect ELISA (Alenius et al., 1991), using a bovine IgG1-specific mouse mAb (2:2, NVI) as conjugate.

3.3.1. Evaluation on negative sera and milk

All samples fell below $2\times$ the negative bovine control samples (adult cow serum and bulk milk from Herd T) in the cIgA (capture) ELISA. In the iIgA (indirect) ELISA, however, one cow in each herd showed a 'positive' reaction in milk only (OD 0.57 and 0.16). Two cows in Herd T showed cIgM 'positive' reactions in serum only (OD 0.30 and 1.02). The iIgM test showed an unacceptably high background level, 0.25–0.50 for milk and 0.70–0.80 for serum. The samples from Herd R were not analysed using the iIgM test.

Cut-off value (CO) calculations were performed on the Herd T and R samples, omitting the false positives. Mean+2SD gave a CO of 0.15 on serum (n=59) and 0.17 on milk (n=30) for cIgA and 0.13 on serum (n=48) and 0.11 on milk (n=30) for cIgM. However, due to the variation between plates it was considered more reliable to use $2\times$ the negative reference sample as CO limit.
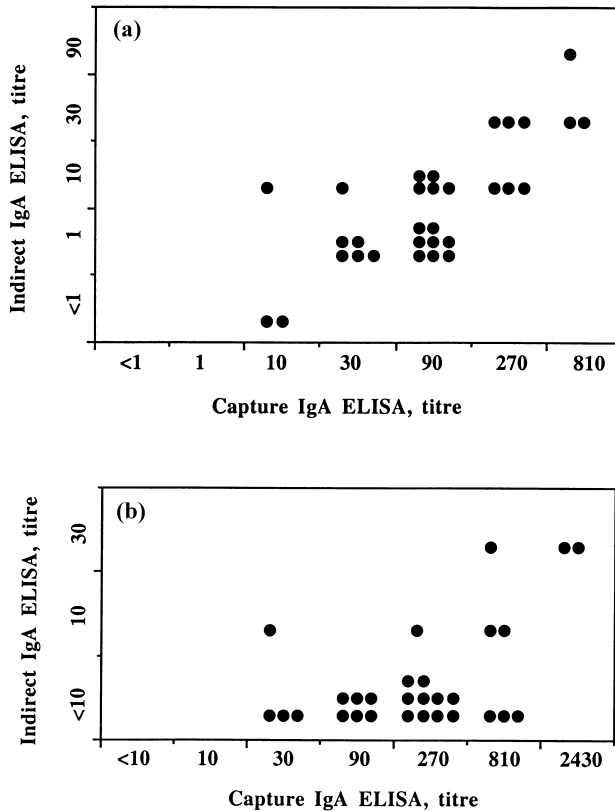
Fig. 1. Comparison of capture versus indirect ELISA for BCV-specific IgA in winter dysentery convalescent milk (a) and serum (b) samples.

3.3.2. Evaluation of cIgA versus iIgA on positive sera and milk

All WD convalescent samples were positive for IgG1 antibodies to BCV. The titres ranged from 270 to 2430 in sera and from 10 to 810 in milk samples. The difference in sensitivity between the cIgA and the iIgA ELISAs was dramatic. In the cIgA ELISA milk titres ranged from 30 to 810 (Fig. 1a) and serum titres from 30 to 2430 (Fig. 1b), all samples being positive. iIgA titres ranged from <1 to 90 in milk and from <10 to 30 in sera. As many as 22 sera (76%) and two milk samples (6%) tested negative in the iIgA ELISA, all of which were positive in the cIgA ELISA.

3.3.3. Evaluation of cIgM versus iIgM on positive sera and milk

The indirect system showed a background level of at least 0.50 for the sera, compared with 0.10 for cIgM. The amplitude of the signal was about the same in the capture and the indirect system. Therefore, only the cIgM ELISA was used subsequently.
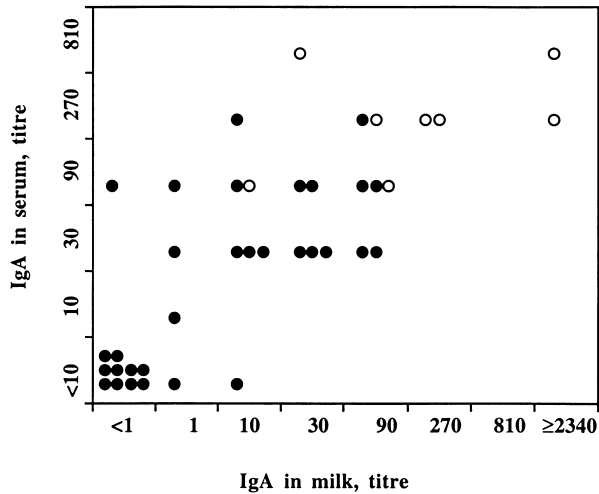
Fig. 2. BCV-specific IgA levels in milk versus serum measured in isotype capture ELISA. The samples originated from WD convalescent cows (o) and from patients at the University ruminant clinic, Uppsala (●).

3.4. Capture IgA ELISA show good agreement between detection in milk and serum

The milk and sera from Herd R fell below a CO of $2\times$ the negative reference sample for all three Ig isotypes tested. Among the WD convalescent cows all serum and milk samples were positive for BCV-specific IgG1 and IgA. Seven cows showed higher IgG1 titres in serum than in milk (9–81-fold) and one cow showed equal titres (810). Two cows showed higher IgA titres in milk than in serum (3–9-fold) and three cows showed higher IgA titres in serum than in milk (3–27-fold, Fig. 2). Four cows were IgM negative in both serum and milk, two cows showed weak IgM reactions in undiluted milk only and two cows showed higher IgM levels in serum than in milk. None of the cows, however, showed a high IgM response.

Among the patients at the ruminant clinic, five cows were BCV antibody negative in all three isotypes. IgG1 titres among the other 26 ranged from 90 to 2430. Eighteen cows showed detectable IgA titres in both milk and serum, milk titres ranging from 1 (undiluted) to 90 and serum titres ranging from 10 to 270 (Fig. 2). Two cows were IgA positive in milk only (Titres 1 and 10) and one cow in serum only (Titre 90). Three cows showed higher IgA titres in milk than in serum and 12 cows showed higher IgA titres in serum. Two cows showed detectable IgM levels, one in serum only (Titre 90) and the other in milk only (Titre 10).

For IgG1 antibodies there was a 100% agreement between detection in serum and milk. For IgA, milk and serum detection agreed to 94%. The correlation coefficient of $\log_{10}$ IgA titres in milk versus serum was $r=0.82$ ($P<0.001$) and that for log IgG1 titres was $r=0.97$ ($P<0.001$). Too few IgM positive samples were obtained in this material from WD convalescent cows and patients at the clinic to enable us to determine detection agreement or titre correlation. Comparing WD convalescent milk and serum responses of the cows in Herd AN, however, gives an estimate of both agreement and titre correlation,

Table 2

IgM antibody responses to BCV in individual milk and serum samples from six cows and four calves and in bulk milk after a winter dysentery outbreak in Herd AN. The first samples were taken at 4 days post outbreak start (DPO)[a]

ID No.	Age, sex	Sample	1st IgM (DPO)	IgM detected (days)	IgM peak	
					Titre	At DPO
2	3y	serum	6	19	320	6–12
	F	milk	6	54[b](54)[c]	640	9
22	5–6y	serum	n.d.	n.d.	n.d.	
	F	milk	9	19[b](4)	10	9
45	5–6y	serum	6	13	2560	9
	F	milk	6	33 (13)	1280	6
67	3y	serum	4	21	10240	6
	F	milk	4	6m (19)	160	6–9
329	4y	serum	9	4	640	9
	F	milk	6	33 (10)	160	12
432	3y	serum	6	33	2560	9
	F	milk	9	4 then dry[b,d]	5120	9–12
7	4–5w	serum	6	54+at 9m[e]	5120	9–12
	F					
A	3w	serum	6	19	≥20480	9
	M					
B	3m	serum	6	19	10240	9–12
	M					
C	3m	serum	6	19[b]	10240	9
	M					
–		bulk milk	6	19[b]	320	9

[a] y: years, m: months, w: weeks, MPO: months post outbreak start, F: female and M: male, n.d.: not detected.

[b] Continuous period of detection displayed. Low levels of IgM were detected in some of the samples after this period.

[c] No. of days that IgM was detected at a level above 1:1 indicated in parenthesis.

[d] Cow 432 was dried off before calving, milk not sampled at 18 and 24 DPO.

[e] Calf 7 showed a high IgM titre (1280) at 9 MPO indicating reinfection.

although milk and sera were not analysed on the same plate. Agreement between IgM detection in milk and serum was 59% including the results from undiluted milk, but could be improved to 86% if the results from undiluted milk samples were excluded. The correlation coefficient for IgM $\log_{10}$ titres in milk versus serum was $r=0.81$ ($P<0.001$) including and $r=0.84$ ($P<0.001$) excluding the results from undiluted milk samples. Thus, exclusion did not have the same impact on the correlation coefficient as on the agreement.

3.5. Capture IgA and IgM ELISAs differentiate between primary infection and reinfection

Details of the individual serum and milk antibody responses are given in Tables 2 and 3. It is evident that all animals that were BCV IgG1-antibody negative in the first serum and milk sample showed primary responses to BCV, characterised by strong IgM responses

Table 3

IgA and IgG1 antibody responses to BCV in individual milk and serum samples from six cows and four calves and in bulk milk after a winter dysentery outbreak in Herd AN. The first samples were taken at 4 days post outbreak start (DPO) start[a]

ID No.	Sample	1st IgA (DPO)	IgA peak		1st IgG1 (DPO)	IgG1 peak		Comments
			Titre	At DPO		Titre	At DPO	
2	serum	6	640	9–12	9	1280	18–4 m	
	milk	9	320	9–12	9	40	24–38	
22	serum	4	10240	9–12	4	≥10240	9–12	reinfection response,
	milk	4	1280	9–12	4	≥320	9–18	introduced to herd as adult
45	serum	6	1280	9–12	6	640	12–59	
	milk	6	2560	9	9	40	24–59	
67	serum	4	2560	4–6	6	≥1280	12–38	first cow ill
	milk	4	320	9	6	80	18–24	
329	serum	6	10240	9	4	40960	9–18	reinfection response,
	milk	6	10240	12–18	4	5120	9–12	introduced as adult?
432	serum	6	20480	6–12	4	10240	9	reinfection response,
	milk	6	655360	9–12	4	≥2560	6–12	introduced as adult
7	serum	6	2560	9–12+59	9	≥1280	18–12 m	obvious reinfection
			5120	9 m				at 9 MPO
A	serum	9	640	9	9	640	38	removed after 38 DPO
B	serum	9	2560	12	9	≥1280	24–4 m	died after 6 MPO
C	serum	9	2560	9–12	9	1280	24–6 m	
–	bulk milk	4	1280	9	4	320	9–12	

[a] y: years, m: months, w: weeks, MPO: months post outbreak start.

and the IgA and IgG1 antibodies first detected simultaneously with the first detection of IgM or a few days later. IgM antibodies were first detected 6 or 9 days post outbreak start (DPO). In serum IgM was detectable for 2–3 weeks, whereas in milk IgM was often detectable at low levels for longer periods after the peak than in serum. Cow 67, being the first animal to show WD symptoms in the herd, had IgM in serum and milk already at the first sampling (4 DPO). IgM was also detected in bulk milk from Day 6 to 24.

Three cows (22, 329 and 432) showed the characteristic features of a reinfection antibody response to BCV: IgG1 antibodies being detectable in the first samples, with or without IgA but at least 2 days before IgM, with a high peak titre of IgG1 and the IgM response being short, of low level or absent (except in 432). The reinfected cows also showed higher IgA peak titres than the primarily infected cows. The extremely high milk IgA peak of cow 432 was recorded just before drying-off for calving 4 weeks later. Cow 22 showed the most pronounced reinfection response, without detectable IgM in serum and a weak IgM response in milk, showing a titre above 1 for 4 days only. Cow 329 had a detectable IgM response in serum for only 4 days, but a longer response in milk. Cow 432, however, showed a strong IgM response in serum and milk comparable to the primarily infected cows.

3.6. Duration of the IgA and IgG1 responses to BCV

Differences in the duration of the IgA and IgG responses between primarily infected and reinfected animals could not be properly evaluated in Herd AN, since there was a subclinical reinfection with BCV some time between 6 and 12 months after the WD outbreak, indicated by most of the animals showing a $\geq$4-fold IgG1 and/or IgA titre increase in the 9-months sample. In Calf 7 and in all six cows IgA was still detectable 6 months after the start of the outbreak, with titres ranging from 20 to 320 in serum and from 80 to $\geq$640 in milk. The highest IgA titres at 6 months were detected in the three cows that showed antibody responses indicating reinfection at the beginning of this study. Calf B and C became IgA negative to BCV before the subclinical reinfection, i.e. no IgA antibodies were detected at the 6 and 4 months sampling, respectively. Calf A was removed from the herd after the 38 DPO sampling and was lost to follow-up. No animal became IgG1 seronegative to BCV before the reinfection. Examples of the isotype-specific primary infection and reinfection antibody response kinetics are shown in Fig. 3.

3.7. Subclinical mastitis influences milk antibody levels

The four cows from Herd TR were BCV antibody negative in all isotypes tested in serum and milk from all quarters. Cell count level did not influence the antibody negative test results in these cows (see below). CMT values for these cows were 4/2/2/3, 2/1/2/5, 2/2/1/5 and 2/3/3/2. Thus, both milk samples with low and high cell count levels were tested.

The five WD convalescent cows from Herds SK and KA were IgA and IgM positive in milk (IgA titres 10–810, IgM titres 90–810) with little variation between quarters. However, only one cow showed high cell count levels (Table 4).

Nine of the 10 cows in Herds SA and RU had IgG1 and IgA antibodies to BCV in milk and one had only IgG1. These cows had little or no BCV-specific IgM. Six cows showed no or little variation in IgA and IgG1 antibody level between quarters (OD differences less than one titration step), all except one having low CMT values (1 and 2). The other four cows (Table 4) showed 3- to -30-fold IgA titre variations between quarters, and higher cell counts were associated with higher titres. One of these cows also showed a 3-fold titre variation in IgG1. Subclinical infections with coagulase negative Staphylococci were detected in the quarters with the highest IgA titres in three cows. Bacteriological infection of the udder was not detected in any of the other seven cows studied. In contrast to the Herd TR samples, three Herd SA undiluted milk samples with elevated cell counts turned out weakly positive (OD 0.14–0.25) in otherwise IgM negative cows. This false positivity is probably connected with the high cell count. Two of these IgM false positive quarters were infected with coagulase negative Staphylococci.

3.8. Comparison of IgA and IgM in centrifuged and uncentrifuged milk

The cows showed milk IgA titres ranging from 30 to 810 and milk IgM titres ranging from 30 to 2430. All pairs of centrifuged and uncentrifuged samples showed differences less than one titration step in both the IgA and the IgM analysis. For the majority of

sample pairs, the OD value was slightly lower in the uncentrifuged sample in both analyses, probably due to simple dilution by the fat.

3.9. Bovine IgA and IgM antibodies show high stability towards sample handling procedures

All milk samples from the Herd TR cows were IgM, IgA and IgG1 antibody negative to BCV both before and after all treatments.
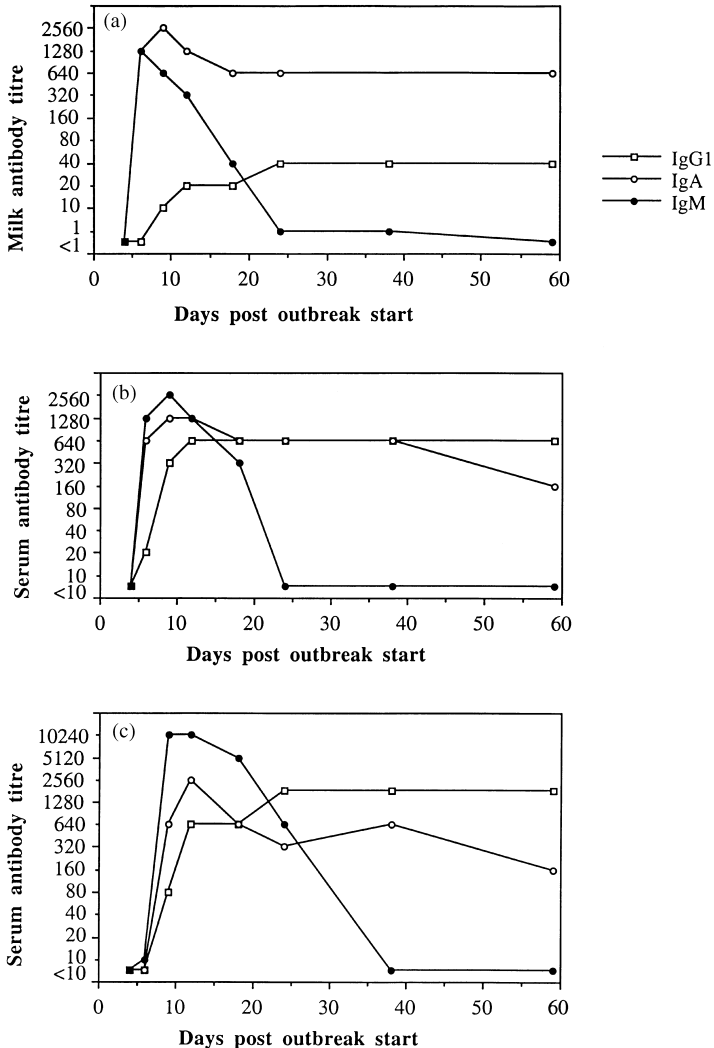

Fig. 3. Isotype-specific antibody responses to BCV in milk and serum after winter dysentery in Herd AN. IgA and IgM antibodies were measured in capture ELISAs and IgG1 in an indirect ELISA. Responses of primarily BCV infected Cow 45 (a,b) and Calf B (c) and BCV reinfected Cow 329 (d,e) as well as bulk milk (f) responses are shown.
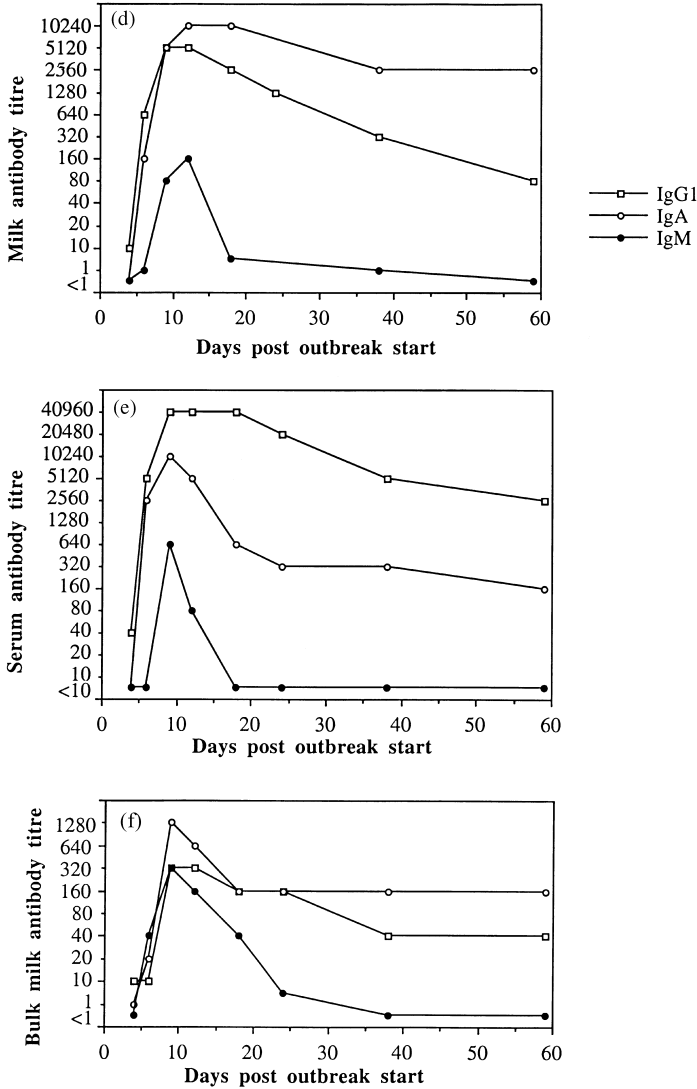
Fig. 3. (*Continued*)

The untreated milk and sera from the WD convalescent cows in Herds SK and KA showed high levels of BCV-specific IgA and IgM (see above). The untreated Herd SA and RU samples showed more varying antibody levels, milk IgA titres ranging from <1 to 810 and IgG1 from 10 to 30. Serum IgA titres ranged from <10 to 2430 and IgG1 from 270 to 810. Antibody titres at all levels were little affected by any of the treatment series. Most often no change or a slight OD decrease was seen with increasing treatment, but no result changed more than one titration step. There was no difference in the stability of Ig towards storage at room temperature versus at 4°C.

Table 4
BCV-specific Ig titres in serum and milk from all four quarters in relation to cell count level (CMT) and bacteriological findings. Results from 10 cows with identical titres in milk from all quarters are not shown

Herd	ID	Analysis	Udder quarter				Serum	Bacteriological findings
			RF	RH	LH	LF		
SA	206	CMT	1	3	1	1		negative
		IgG1	10	10	10	10	810	
		IgA	30	90	30	30	810	
		IgM	<1	1	<1	<1	<10	
	215	CMT	5	4	1	3		RF & RH
		IgG1	30	30	10	30	810	moderate coagulase
		IgA	270	810	30	270	810	negative Staphylococci[a]
		IgM	1	1	<1	<1	<10	
	216	CMT	2	1	1	1		RF moderate
		IgG1	10	10	10	10	810	coagulase negative
		IgA	30	10	1	10	30	Staphylococci
		IgM	<1	<1	<1	<1	<10	
RU	17	CMT	1	4	1	1		RH moderate
		IgG1	10	10	10	10	270	coagulase negative
		IgA	90	810	90	90	30	Staphylococci
		IgM	1	1	1	1	10	
SK	249	CMT	4	1	5	4		not tested
		IgG1	not tested					
		IgA	270	90	270	270	270	
		IgM	270	270	270	270	2430	

[a] Moderate growth of coagulase negative Staphylococci.

3.10. Reproducibility of the capture ELISAs

The analytical error of the IgA test, expressed as a percentage of the mean OD value, was 6% for the undiluted bulk milk samples and 7% for the sera analysed in 1:25 or 1:100 dilution. The analytical error of the IgM test was 8% for the milk samples diluted 1:2 and 6% for the sera in 1:25 or 1:100 dilution. The inter-assay CV was 19 and 26% for the moderately strongly IgA positive milk and serum, respectively, and 25 and 29% for the weakly IgA positive milk and serum, respectively. The inter-assay CVs for the moderately strongly IgM positive milk and serum were 22 and 24%, respectively.

4. Discussion

The anti-bovine IgA (A12:2), the anti-bovine IgM (M69:2) and the anti-BCV (BCV15:11) mouse mAbs were selected for use in the capture ELISAs for BCV-specific bovine IgA and IgM because of high specificity, good binding and conjugation abilities. Work with the indirect IgA and IgM ELISAs was discontinued because of low sensitivity, and for the iIgM test also because of a high background level. The sensitivity and

specificity of the capture ELISAs could not be evaluated against reference methods for BCV-specific IgA and IgM since such methods are not available. Instead, the accuracy of the capture ELISAs was proven by the finding that the IgA and IgM results were in good agreement with the IgG1 level in all samples where it was possible to establish the timing of the BCV infection. The cIgA ELISA seems to be most promising since no false positives were recorded, while two cows in the otherwise BCV antibody negative reference herd showed false cIgM positive reactions in serum, but not in milk. These false positives could not be completely ruled out as unspecific reactions since they did not react with the control antigen. IgM rheumatoid factors (RF) are well known to cause false positive reactions in IgM analyses on human sera (Salonen et al., 1980; Meurmann, 1983) and have also been described in bovine sera (Ungar-Waron and Abraham, 1991; Graham et al., 1998). We have not further explored possible RF reactions in this study, but analysing IgM in milk might be a way to avoid RF interference. Reproducibility of the cIgA and cIgM ELISAs was high in the within-plate comparison. The between-plate variation was larger, but quite acceptable in view of the analyses being done during an evaluation period of 6 months.

Individual milk and bulk milk samples proved suitable for detection of both IgA and IgM BCV-specific antibodies. This is the first report, to our knowledge, of IgA and IgM detection in bovine milk for serologic diagnostic purposes. To fully quantify an IgA response in mucosal secretions in relation to that in serum, the level of specific IgA should be related to the level of total IgA, because total IgA varies with the amount of secretion produced (Hordnes et al., 1997; Hu et al., 1999). Milk is a specialised and easily accessible form of mucosal secretion and possibly a more stable source of IgA than for example respiratory secretions, indicated by the fact that there was such a high correlation ($r=0.82$) between IgA titres in milk and serum in this study. In contrast, anti-RSV IgA in respiratory, intestinal and genital secretions of mice showed no correlation to the specific IgA levels in serum (Hu et al., 1999). No obvious variations in milk level of BCV-specific IgA due to lactational stage were seen in Herd AN cows during the observation period of 1 year. In other samples, however, we noticed a small to moderate rise in milk IgA and IgG1 levels shortly before drying off (data not shown), most likely due to the higher total concentration of IgA (Guidry et al., 1980). No colostrum or dry period secretion IgA levels were tested in our study. For three cows in Herd RU serum IgA was on the border of the detection limit. Milk, as expected, gave more consistent results than serum when analysing weak IgA responses.

Differences in milk BCV-specific IgA levels between udder quarters were detected in some cows due to increased BCV-titres being associated with high cell count levels. This is not surprising, since most of the IgA and some of the IgM are locally produced in the udder (Lascelles et al., 1981) where local inflammation and cytokine activity are likely to affect the Ig production. It is also possible that differences in milk volume between quarters, not measured in this study, accounted for some of the titre differences. Serum IgG1 leaking into inflamed udder tissue may explain the IgG1 variation (though small) between quarters seen in one cow. Only one cow had both high cell counts and IgM levels (Table 4). This cow did not show any titre difference between quarters in IgM corresponding to the difference in IgA level. For routine diagnostic purpose differences between healthy quarters, i.e. with low cell counts, are sufficiently small to give reliable

results in the IgA and probably also in the IgM test. In milk with high cell counts weak false positive IgM reactions can be eliminated by using a higher CO for undiluted milk or by diluting the milk.

BCV-specific IgA and IgM showed stability comparable to that of IgG1 towards the storage and handling procedures used. The levels of IgA and IgM should be little affected by the handling normally encountered by samples sent for routine analysis. None of the milk samples in the treatment series coagulated in spite of 7–10 days in room temperature. However, analysis of other milk samples showed a substantial reduction in the level of BCV-specific IgG1 in the whey fraction after coagulation from microbial activity (data not shown). Probably IgA and IgM levels would be likewise affected by coagulation.

The IgA and IgM determinations in this study made it possible to differentiate between primarily BCV infected and reinfected cattle. IgM was detectable for at least 2–3 weeks after the outbreak in both serum and milk from all primarily infected animals and bulk milk. Surprisingly, IgM was detected in milk from all three and in serum from two of the reinfected cows. In the study of Kimman et al. (1987b) IgM was not detectable in serum after experimental BRSV reinfection 4 months after the primary infection. Our results, however, show that in field outbreaks with longer intervals between infections probably with different strains of the virus, specific IgM can be detected after the reinfection, especially in milk. Conclusions about the usefulness of reinfection IgM responses for diagnostic purposes are difficult to draw from the results of these few animals. Clearly, the short duration of the response in some animals is a limitation. Low levels of IgM were detected beyond 2 months after the outbreak started in milk from four cows and in serum from two calves. Such reactions were not present in the first samples of the study, but are of doubtful diagnostic value and might not be specific for BCV. The cows in Herd AN showing BCV reinfection responses probably had made their primary contacts with the virus on other farms. Two of them were introduced into the herd (purchased) as adults (Table 3) and the third reinfected cow was probably also introduced, since her ID number did not coincide with the ID series of the home-raised animals. All other cattle tested in the herd showed primary responses to BCV, which is in accordance with the approximate 12-year interval since the previous WD outbreak on the farm.

It is evident that the IgA response to BCV was detectable for a much longer period of time than the IgM response in all 10 cattle in this study. Serum IgA responses in calves experimentally infected with BCV (Saif, 1987) were much shorter than in our study, as expected from the detection in indirect ELISA. In our study, in contrast to other field studies (Heckert et al., 1991c), we were able to find seronegative cattle that became naturally infected, which facilitates the evaluation. Serum IgA responses detected using an isotype capture ELISA in seronegative calves naturally or experimentally infected with BRSV were also very short and mean peak levels were lower (Kimman et al., 1987a, b) compared with our results. Reasons for this discrepancy may be differences in sensitivity of the ELISAs or that BCV causes a more extended course of infection than BRSV. The levels and kinetics of the IgM response and the first detection of IgA in the naturally infected calves in the studies of Kimman et al. corresponded well with our results.

Appearance of detectable IgA before IgM in some of the early samples indicated the presence of IgA memory cells in the three originally reinfected cows. The higher peak levels of IgA in milk and sera shown by these cows compared with the primarily infected ones indicated that boosting of the IgA response occurs, as was also seen in the experimental calf studies with BCV (Heckert et al., 1991a) and BRSV infection (Kimman et al., 1987a). The three originally reinfected cows also displayed the highest IgA titres at the 6-months sampling, indicating that these cows probably would have retained detectable IgA levels longer than the originally primarily infected ones. Serum IgA levels were correlated with protection from respiratory challenge with influenzavirus in mice (Liew et al., 1984). Likewise, detection of, or a certain level of, systemic IgA might be used as a marker for protection in the bovine. The reinfection contracted by most animals in Herd AN before the 9-months sampling was subclinical to the farmer, indicating protection against disease. Symptoms were not even noted in the two bull calves that were IgA negative at the time of reinfection. Calves and young stock, however, are often asymptomatic in WD outbreaks among cows (Hedström and Isaksson, 1951; Roberts, 1957).

5. Conclusions

The capture ELISAs for BCV-specific IgA and IgM performed well in our test series of milk and sera, and should become valuable diagnostic tools capable of discriminating between primary infection and reinfection. Milk, besides being practical for sampling, proved suitable for detecting both IgA or IgM responses to infection in lactating cows. The IgM response after infection is sufficiently short to render a single-sample diagnostic procedure feasible. Further work is needed to examine the possibility to use certain levels of IgA for single-sample diagnostic purposes, probably in combination with IgM detection. Furthermore, the isotype capture ELISAs can be adapted for the diagnosis of other infections.

Acknowledgements

References

Alenius, S., Niskanen, R., Juntti, N., Larsson, B., 1991. Bovine coronavirus as the causative agent of winter dysentery: serological evidence. Acta Vet. Scand. 32(2), 163–170.

Atterhem, K., Fossum, C., Tråvén, M., Linde, N., Näslund, K., Larsson, B., 1996. Type 1 interferon and virus-specific IgA and IgM antibodies as an aid in the diagnosis of ongoing infections in calves. Swedish J. Agric. Res. 26, 101–104.

Butler, J., 1983. Bovine immunoglobulins: an augmented review. Vet. Immunol. Immunopath. 4, 43–152.

Chantler, S., Diment, J.A., 1981. Current status of specific IgM antibody assays. In: Voller, A. (Ed.), Immunoassays for the 80s. MTP Press, Lancaster, England, pp. 417–430.

Deregt, D., Babiuk, L.A., 1987. Monoclonal antibodies to bovine coronavirus: characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology 161, 410–420.

Fey, H., Phister, H., Messerli, J., Sturzenegger, N., Grolimund, F., 1976. Methods of isolation, purification and quantitation of bovine immunoglobulins: a technical review. Zbl. Vet. Med. B 23(4), 269–300.

Galfre, G., Milstein, C., 1981. Preparation of monoclonal antibodies: strategies and procedures. Methods Enzymol. 73, 3–46.

Guidry, A., Butler, J., Pearson, R., Weinland, B., 1980. IgA, IgG1, IgG2, IgM and BSA in serum and mammary secretions throughout lactation. Vet. Immunol. Immunopath. 1, 329–341.

Graham, D.A., Mawhinney, K.A., Elvander, M., Adair, B.M., Merza, M., 1998. Evaluation of an IgM-specific indirect enzyme-linked immunosorbent assay for serodiagnosis of bovine respiratory syncytial virus infection: influence of IgM rheumatoid factor on test results with field sera. J. Vet. Diagn. Invest. 10(4), 331–337.

Heckert, R., Saif, L., Mengel, J., 1991a. Isotype-specific antibody responses to bovine coronavirus structural proteins in serum, feces and mucosal secretions from experimentally challenge-exposed colostrum-deprived calves. Am. J. Vet. Res. 52(5), 692–699.

Heckert, R., Saif, L., Mengel, J., 1991b. Mucosal and systemic antibody responses to bovine coronavirus structural proteins in experimentally challenge-exposed calves fed low or high amount of colostral antibodies. Am. J. Vet. Res. 52(5), 700–708.

Heckert, R., Saif, L., Myers, G., Agnes, A., 1991c. Epidemiologic factors and isotype-specific antibody responses in serum and mucosal secretions or dairy calves with bovine coronavirus respiratory tract and enteric tract infections. Am. J. Vet. Res. 52(6), 845–851.

Hedström, H., Isaksson, A., 1951. Epizootic enteritis in cattle in Sweden. Cornell Vet. 42, 251–253.

Honkanen-Buzalski, T., Sandholm, M., 1981. Association of bovine secretory immunoglobulins with milk fat globule membranes. Comp. Immunol. Microbiol. Infect. Dis. 4, 329–342.

Hordnes, K., Tynning, T., Brown, T., Haneberg, B., Jonsson, R., 1997. Nasal immunization with group B Streptococci can induce high levels of specific IgA antibodies in cervicovaginal secretions of mice. Vaccine 15, 1244–1251.

Hu, K.-F., Ekström, J., Lövgren-Bengtsson, K., Morein, B., 1999. Induction of antibody responses in the common mucosal system by an RSV ISCOM and its applications in vaccine formulation. Med. Microbiol. Immunol. 187, 191–198.

Hu, K.F., Elvander, M., Merza, M., Åkerblom, L., Brandenburg, A., Morein, B., 1998. The immunostimulating complex (ISCOM) is an efficient mucosal delivery system for respiratory syncytial virus (RSV) envelope antigens inducing high local and systemic antibody responses. Clin. Exp. Immunol. 113, 235–243.

Kimman, T., Westenbrink, F., Schreuder, B., Straver, P., 1987a. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. J. Clin. Microbiol. 25(6), 1097–1106.

Kimman, T.G., Westenbrink, F., Straver, P.J., Zaane, D.v., Schreuder, B.E.C., 1987b. Isotype-specific ELISAs for the detection of antibodies to bovine respiratory syncytial virus. Res. Vet. Sci. 43, 180–187.

Klingenberg, K.d.V., Vågsholm, I., Alenius, S., 1999. Incidence of diarrhea after strict closure and eradication of bovine viral diarrhea virus infection in a dairy herd. J. Am. Vet. Med. Assoc. 214, 1824–1828.

Lascelles, A., Beh, K., Husband, A., 1981. Origin of antibody in mammary secretions with particular reference to the IgA system. In: Butler, J.E. (Ed), The Ruminant Immune System, Adv. Exp. Med. Biol. 137, 493–511.

Liew, F., Russell, S., Appleyard, G., Brand, C., Beale, J., 1984. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur. J. Immunol. 14, 350–356.

McNulty, M.S., Bryson, D.G., Allan, G.M., Logan, E.F., 1984. Coronavirus infection of the bovine respiratory tract. Vet. Microbiol. 9, 425–434.

Mebus, C.A., Stair, E.L., Rhodes, M.B., Twiehaus, M.J., 1973. Pathology of neonatal calf diarrhea induced by a coronavirus-like agent. Vet. Pathol. 10, 45–64.

Meurmann, O., 1983. Detection of antiviral IgM antibodies and its problems — a review. Curr. Topics Microbiol. Immunol. 104, 101–131.

Nakane, P.K., Kawaoi, A., 1974. A peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem. 22(12), 1084–1091.

Pivont, P., collard, A., Antoine, H., 1984. L′association des IgA sécrétoires avec les globules graisseux du colostrum bovin. Ann. Méd. Vét. 128, 149–152.

Renegar, K., Small, P., 1991. Passive transfer of local immunity to influenza virus infection by IgA antibody. J. Immunol. 146, 1972–1978.

Roberts, S.J., 1957. Winter dysentery in dairy cattle. Cornell Vet. 47, 372–388.

Rosenthal, K., Gallichan, W., 1997. Challenges for vaccination against sexually-transmitted diseases: induction and long-term maintenance of mucosal immune responses in the female genital tract. Semin. Immunol. 9, 303–314.

Saif, L., 1987. Development of nasal, fecal and serum isotype-specific antibodies in calves challenged with bovine coronavirus or rotavirus. Vet. Immunol. Immunopath. 17, 425–439.

Salonen, E.M., Vaheri, A., Suni, J., Wager, O., 1980. Rheumatoid factor in acute viral infections: interference with determination of IgM, IgG and IgA antibodies in an enzyme immunoassay. J. Infect. Dis. 142(2), 250–255.

Schultze, B., Gross, H., Brossmer, R., Herrler, G., 1991. The S protein of bovine coronavirus is a haemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. J. Virol. 65(11), 6232–6237.

Smith, D., Fedorka-Cray, P., Mohan, R., Brock, K., Wittum, T., Morley, P., Hoblet, K., Saif, L., 1998. Evaluation of cow-level risk factors for the development of winter dysentery in dairy cattle. Am. J. Vet. Res. 59(8), 986–993.

Tizard, I.R., 1996. Veterinary Immunology, An Introduction, 5th Ed. W.B. Saunders Company, Philadelphia, USA, pp. 153–164.

Towbin, H., Staehelin, T., Gordin, J., 1979. Electrophoretic transfer of proteins from polyacrylamid gels to nitrocellulose sheets; procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350–4354.

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.

Tråvén, M., 2000. Experimental reproduction of winter dysentery in lactating cows using BCV — comparison with BCV infection in milk-fed calves (in preparation).

Ungar-Waron, H., Abraham, A., 1991. Immunoglobulin M indirect enzyme-linked immunosorbent assay and the involvement of IgM-rheumatoid factor in the serodiagnosis of BHV-1 infection. Vet. Microbiol. 26, 53–63.

Zaane, D.V., Ijzerman, J., 1984. Monoclonal antibodies against bovine immunoglobulins and their use in isotype-specific ELISAs for rotavirus antibody. J. Immunol. Methods 72, 427–441.