JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1997, p. 33–40 Vol. 35, No. 1
0095-1137/97/$04.00+0


# Characterization of Monoclonal Antibodies to the Hemagglutinin-Esterase Glycoprotein of a Bovine Coronavirus Associated with Winter Dysentery and Cross-Reactivity to Field Isolates

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Received 8 July 1996/Returned for modification 20 August 1996/Accepted 27 September 1996

**Seven hybridoma cell lines producing monoclonal antibodies (MAbs) to the hemagglutinin-esterase (HE) glycoprotein of bovine coronavirus (BCV) were obtained from BALB/c mice that were immunized with an enriched peplomeric fraction of the winter dysentery (WD)-associated strain BCQ.2590. The specificities of these MAbs to either the dimeric (140-kDa) or the monomeric (65-kDa) form of the HE glycoprotein were determined by Western immunoblotting experiments with purified virus and immunoprecipitation tests with [$^{35}$S]methionine-labelled infected cell extracts. Four of these anti-HE MAbs inhibited the hemagglutinating activity of the virus and three weakly neutralized its infectivity in vitro. In addition, competition binding assays allowed for the definition of two independent antigenic domains (domains A and D) and two overlapping antigenic domains (domains B and C) for the HE glycoprotein of the WD-associated strain; epitopes located within antigenic domain A were not associated with hemagglutination inhibition (HAI) and virus neutralization activities. In HAI tests, the four anti-HA MAbs defined two distinct antigenic subgroups among 24 BCV field isolates that have been associated with either typical outbreaks of WD or neonatal calf diarrhea (NCD) in Quebec dairy herds from 1986 to 1996. The Quebec WD-associated strains of BCV, as well as some of the NCD-associated strains isolated since 1991, fell within a subgroup distinct from that of the prototype Mebus strain.**

Bovine coronavirus (BCV) is an enteropathogenic agent which replicates in the differentiated enterocytes of the small and large intestines, causing severe diarrhea in newborn calves (27, 28) and chronic shedding in adult cattle (4). BCV infection of the upper respiratory tract has also been described in growing calves (33, 39). More recently, several reports have suggested the association of BCV with acute enteric infection in adult cattle during the winter season (2, 17, 19, 34, 35). The disease, often referred to as winter dysentery (WD), is characterized by an acute onset of dark, bloody, liquid diarrhea accompanied by decreased milk production and variable depression and anorexia in adult cattle.

The BCV virion is mostly spherical and enveloped, with a diameter of about 120 nm, and displays two fringes of surface projections (10, 25, 28). The viral genome consists of a large single-stranded RNA with positive polarity, approximately 30 kb in length, complexed with a phosphorylated nucleocapsid (N) protein (52 kDa) in a helical configuration (38). The ribonucleoprotein is surrounded by a lipid envelope which contains three viral glycoproteins: the integral or matrix (M) glycoprotein (24 to 26 kDa), the spike (S) glycoprotein (180 to 200 kDa) which forms the long club-shaped surface projections, and the hemagglutinin-esterase (HE) glycoprotein (125 to 140 kDa) associated with shorter granular projections (12, 14, 25, 26). The S glycoprotein is often posttranslationally cleaved by host cell proteases into two 100-kDa fragments, fragments S1 and S2, corresponding to the N- and C-terminal subunits, respectively (1, 32). The HE glycoprotein is split by reducing agents into two 65-kDa subunits (15, 23, 25, 31). Both S and HE glycoproteins carry determinants for virus neutralization (14, 16, 29, 41, 43) and interact with receptors on the cell surface (36, 37). In addition to being the hemagglutinin, the HE glycoprotein exhibits an acetylesterase activity which inactivates receptors for BCV on susceptible cells by hydrolyzing an ester bond to release acetate from C-9 of sialic acid (36, 42). Other reports suggested that the HE glycoprotein also has a significant role in the initiation of BCV infection (14, 16).

Although the WD-associated BCV isolates can be distinguished from those responsible for neonatal calf diarrhea (NCD) by their clinical syndromes, they appear morphologically and antigenically similar (3, 4, 40). We have already demonstrated (9) that recent Quebec WD-associated isolates differ from the prototype Mebus strain (BCV.Meb) with respect to hemagglutination inhibition (HAI), their hemagglutination patterns at different temperatures, and their receptor-destroying enzyme activities with rat erythrocytes. Furthermore, analysis at the molecular level of one representative WD-associated strain (BCQ.2590) and two highly cytopathic Quebec NCD-associated strains isolated during the same period in comparison with the BCV.Meb strain revealed distinct amino acid substitutions within the signal peptide and near the sequences of the putative esterase domain of the HE glycoprotein of the WD-associated strain (9).

In this report, we describe the production and characterization of monoclonal antibodies (MAbs) to the HE glycoprotein of the BCQ.2590 strain. MAbs were raised against epitopes of at least four antigenic domains of the HE glycoprotein of this

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WD strain. A study of their cross-reactivities in HAI tests toward WD- and NCD-associated BCV isolates collected during a 10-year period revealed the presence of two distinct antigenic subgroups. The Quebec WD-associated strains of BCV, as well as some of the NCD-associated strains isolated since 1991, fell within a subgroup distinct from that of the prototype BCV.Meb strain.

## MATERIALS AND METHODS

**Cells and viruses.** The cell culture-adapted BCV.Meb strain (28) was obtained from the American Type Culture Collection (ATCC VR-874), Rockville, Md. This prototype BCV strain was originally isolated from a diarrheic newborn calf and was propagated for at least 30 consecutive passages in fetal bovine kidney cells (28). A total of 23 other BCV isolates were recovered from newborn calves with clinical cases of epidemic diarrhea (NCD), adult cattle with chronic diarrhea (AD), or adult cattle with acute hemorraghic enteritis (WD) in different Quebec dairy herds from January 1987 to April 1996 (see Table 3). The fecal samples were obtained from five regional provincial laboratories covering a territory of more than 300,000 $km^2$. No commercial BCV vaccine had been administered to these herds during the year preceding these outbreaks. For the present studies, the BCV strains were passaged not more than five times in human rectal tumor (HRT-18) cells in the presence of 10 U of bovine pancreatic trypsin per ml (8). The extracellular virions of the various BCV isolates were purified from clarified supernatants of infected cell cultures by differential and isopycnic ultracentrifugation on continuous sucrose gradients as described previously (8, 10).

**Production of MAbs.** The sucrose-purified BCQ.2590 virus was treated with 1% *n*-octyl-β-D-glucopyranoside (OPG; Sigma) as described previously (36). The enriched peplomeric fraction was used to immunize female BALB/c mice by the immunization protocol described elsewhere (7). The fusion protocol for sensitized splenocytes with P3x63Ag8.653 myeloma cells was essentially similar to that described previously (7). Hybrid cells were cultured in hypoxanthine-aminopterine-thymidine medium containing 10% fetal calf serum and 10% Boehringer Mannheim-condimed H1 medium (supernatant of a mouse lymphoma cell line supplemented with 1 mmol of sodium pyruvate per liter, 0.2 μg of insulin per ml, and 10 ng of phorbol myristate acetate [pH 7.6] per ml) as a replacement for the feeder layers of mouse macrophages.

The supernatant from growing mouse-mouse hybridoma cells was screened for anti-BCV antibody production by indirect immunofluorescence (IIF) (7). Immunoglobulin isotyping was done by an enzyme immunoassay (Boehringer Mannheim, Laval, Quebec, Canada). Ascitic fluids containing MAbs were obtained by intraperitoneally injecting $10^6$ cloned hybrid cells into 16-week-old BALB/c mice that had been primed 14 days before with 0.5 ml of pristane (2,6,10,14-tetramethyl pentadecane; Sigma).

**Purification and conjugation of MAbs.** The antibodies were purified from ascitic fluids by affinity chromatography with the Affi-Gel protein A MAPS II kit (Bio-Rad). The protein concentration in the immunoglobulin G (IgG) fractions was determined by measuring the $A_{280}$ by using an extinction coefficient of 1.4 $cm^2$/mg. The purified immunoglobulins were conjugated to *N*-hydroxysuccinimido biotin (molecular ratio, 90:1; Sigma), according to the procedure described by Kendall et al. (22). The integrity of the labelled antibodies was assessed by comparing their reactivities with that of a purified preparation of BCQ.2590, before and after conjugation, by an indirect enzyme-linked immunosorbent assay (ELISA) (7).

**Antibody-binding and competitive binding assays.** An indirect ELISA for determination of antibody-binding curves and titration of ascitic fluids was performed as described previously, with minor modifications (7). Wells in microtitration plates (Linbro/Titertek; ICN Biomedicals Inc., Horsham, Calif.) were coated with 100 μl (approximately 0.5 μg of protein) of a purified preparation of BCQ.2590 in 0.05 M sodium carbonate buffer (pH 9.6). After an overnight incubation at 4°C, antigen-coated plates were washed twice in 0.05 M Tris-buffered saline (TBS; pH 8.0) containing 0.05% Tween 20 (TBS-T), and unreacted sites were blocked by a 1-h incubation at room temperature in a 1% solution of bovine serum albumin in TBS.

After washing in TBS-T, 100 μl of antibodies (purified labelled or unlabelled MAbs; clarified ascitic fluid) serially diluted in TBS-T containing 0.1% bovine serum albumin was added to the wells, and the plates were incubated for 1 h at 37°C. Thereafter, unlabelled MAbs were detected following a further 1-h incubation at 37°C in the presence of a 1/5,000 dilution of goat anti-mouse IgG (heavy and light chains; Boehringer Mannheim) conjugated to horseradish peroxidase and biotinylated MAbs with horseradish peroxidase-labelled streptavidin (Sigma) diluted 1/1,000 in TBS-T. After a final washing step, peroxidase activity was revealed with the TMB microwell peroxidase system (Kirkegaard & Perry Laboratories). The $A_{459}$s were read on a multichannel spectrophotometer (Titertek Multiskan MCC/340; Flow). Endpoint titers were determined from the point at which the antibody-binding curve crossed the average absorbance attributed to nonspecific binding of the conjugated antibody for each plate.

For competitive binding assays, 10-fold dilutions ($10^{-1}$ to $10^{-8}$) of competitor MAb (unlabelled) were incubated in the BCQ.2590-coated plates for 1 h at 37°C before adding the biotinylated MAbs at concentrations adjusted to give a net absorbance of 1.0 to 1.5 (60 to 80% saturation levels) in the direct binding assay (14, 30). The competition curves were calculated as described by Kimura-Kuroda and Yasui (24) by using the formula $100(A - n)/(A - B)$, where $A$ is the $A_{459}$ in the absence of competing antibody, $B$ is the $A_{459}$ in the presence of homologous antibody at a 1:10 (or 1:100) dilution, and $n$ is the $A_{459}$ in the presence of competitor at $1{:}10^1$ to $1{:}10^7$ dilutions.

**Labelling of viral proteins and RIPA.** Confluent monolayers of HRT-18 cells in 75-$cm^2$ tissue culture flasks (Falcon) were infected with BCQ.2590 at a multiplicity of infection of 10 50% tissue culture infective doses ($TCID_{50}$s) of virus per cell as described previously (8). At 6 h postinfection, infected cultures were rinsed and incubated in methionine-free RPMI medium (approximately 10 ml per flask) for 30 min, after which 50 μCi of [$^{35}$S]methionine (specific activity 1,200 Ci/mmol; Amersham) per ml was added. The cultures were reincubated at 37°C until 48 h postinfection and were then harvested by three freeze-thaw cycles. Labelled extracellular virions in clarified supernatants were purified as described above.

For immunoprecipitation experiments, MAbs within the ascitic fluids (50 μl) were first incubated in the presence of a 10% suspension of formaldehyde-fixed and heat-inactivated *Staphylococcus aureus* cells (Pansorbin; Calbiochem, La Jolla, Calif.) for 2 h at 4°C in radioimmunoprecipitation assay (RIPA) buffer (final volume, 100 μl) under gentle agitation. The RIPA buffer consists of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% phenylmethylsulfonyl fluoride, and $10^3$ U of aprotinin per ml (Boehringer Mannheim). Antibody-coated bacteria were washed twice, resuspended in 100 μl of RIPA buffer, and incubated in the presence of diluted radiolabelled virus (500,000 cpm) overnight at 4°C. The immune complexes were then collected by centrifugation and washed four times in RIPA buffer. The resulting pellets were dissolved in 50 μl of electrophoresis sample buffer in the presence or absence of 5% β-mercaptoethanol. After boiling for 3 min, the specimens were clarified at 10,000 × *g* for 15 min, and analyzed by electrophoresis (SDS-polyacrylamide gel electrophoresis [PAGE]) on SDS–12.5% polyacrylamide slab gels as described previously (6). For fluorography, the gels were soaked in Amplify (Amersham, Oakville, Ontario, Canada) for 1 h, dried, and autoradiographed with Kodak X-OMAT RP film at −70°C.

**Western immunoblotting.** Viral proteins separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (pore size, 0.45 μm; Schleicher & Schuell) as described previously (6). After saturation with 3% BLOTTO (skim milk powder) in TBS-T, the blots were cut into strips that were incubated for 2 h at room temperature in mouse ascitic fluid (1/200 dilution) or rabbit anti-BCV polyclonal serum (1/1,000 dilution) diluted in TBS-T containing 1% BLOTTO. After washing in TBS-T, the nitrocellulose strips were incubated with a 1/1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG or sheep anti-rabbit IgG (Bio-Rad) for 1 h at room temperature. The immune complexes were revealed with a commercial alkaline phosphatase conjugate substrate kit (Bio-Rad) containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in TBS buffer.

**VN and HAI.** Hyperimmune rabbit sera, mouse ascitic fluids, and purified preparations of MAbs were tested for their virus neutralization (VN) and HAI activities as described elsewhere (8, 10). The VN titers were expressed as the reciprocal of the highest serum or ascitic fluid dilution neutralizing 100 $TCID_{50}$s of virus. The HAI titers were expressed as the reciprocal of the highest serum or ascitic fluid dilution inhibiting agglutination of rat erythrocytes by BCV (8 hemagglutination [HA] units) after 2 h at 4°C.

**PAG immunoelectron microscopy.** The protein A-gold (PAG) immunoelectron microscopy technique was done as described previously, with minor modifications (5, 7). Briefly, aliquots (25 μl) of purified preparation of the BCQ.2590 strain were mixed with an equal volume of rabbit anti-BCV.Meb hyperimmune serum (diluted 1:250) or clarified ascitic fluid (diluted 1:100) in phosphate-buffered saline (PBS) containing 0.05% Tween 20. After incubation for 10 min at room temperature, droplets of these mixtures were deposited on paraffin strips, and carbon-stabilized 400-mesh naked nickel grids were floated for 30 min on the droplets. The grids were then washed for 5 min on several drops of PBS, and incubated in diluted PAG complex (15-nm diameter granules) for 30 min. The grids were finally washed with PBS, rinsed three times in distilled water, and counterstained with 3% sodium phosphotungstate (pH 6.0). The grids were examined on a Philips EM 300 electron microscope at a potential of 80 kV. The specificity of the labelling was demonstrated with controls including an irrelevant MAb and incubation with the PAG complex alone.

## RESULTS

**Production and characterization of anti-HE MAbs.** An enriched peplomeric fraction was obtained following OPG treatment of the purified BCV.2590 virion to solubilize the components of the lipid envelope. After differential ultracentrifugation of the treated virus, the two envelope glycoproteins S (gp200/100) and HE (gp140/gp65) remained in the supernatant (Fig. 1, lane C), whereas most of the N protein (p52) was

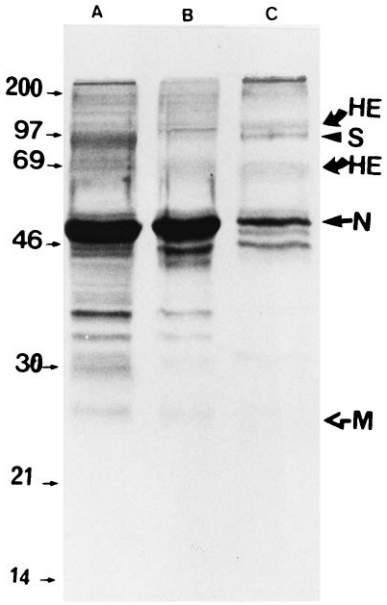


FIG. 1. Polypeptide profiles of the WD-associated BCV strain BCQ.2590. The sucrose gradient-purified virions were analyzed by SDS-PAGE under reducing conditions before (lane A) or after (lanes B and C) treatment with *N*-octylglucoside. Lane B, viral pellet following the detergent treatment; lane C, supernatant containing the enriched peplomeric fraction. The acrylamide concentration in the gel was 12.5%. The viral proteins were revealed after staining with Coomassie blue. The positions of the molecular mass markers are indicated on the left (in kilodaltons), and the positions of the viral structural proteins are indicated on the right.

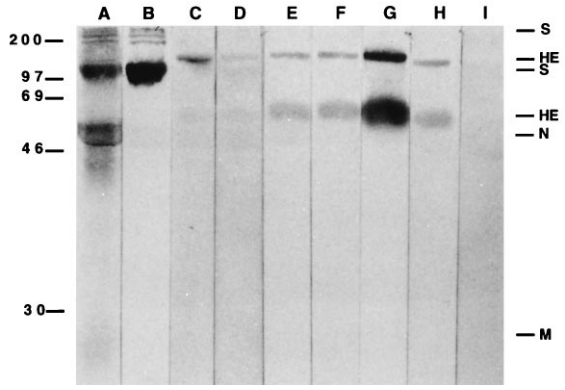


FIG. 2. Protein specificity of anti-BCV MAbs determined by Western immunoblotting of sucrose gradient-purified WD-associated BCV strain BCQ.2590. Viral proteins separated by SDS-PAGE under reducing conditions were electrophoretically transferred to a nitrocellulose membrane. After saturation, individual nitrocellulose strips were incubated with rabbit hyperimmune serum to the BCV.Meb strain (lane A), a pool of anti-S glycoprotein (gp100) MAbs to the BCV.Meb strain (lane B), and MAbs to the BCQ.2590 strain. Blots obtained with anti-HE glycoprotein MAbs are represented in lanes C (9F2-1), D (7B11-19), E (6E7-26), F (6E7-11), G (2G1-20), H (1D6-2), and I (1D6-3). Immune reactions were revealed as described in the text. The positions of the molecular mass markers are indicated on the left (in kilodaltons), and the positions of the viral structural proteins are indicated on the right.

found in the pellet (Fig. 1, lane B). The glycosylated M protein was also absent from the enriched peplomeric fraction.

Mice that were immunized with the enriched peplomeric fraction of the BCQ.2590 strain developed a high specific antibody response to the homologous virus. Antibody titers in serum from mice providing the immune cells ranged from 12,800 to 25,600 by IIF, whereas titers of 128 to 256 and 1,280 to 5,120 were obtained by VN and HAI, respectively. From a total of 40 hybridoma cell lines initially secreting anti-BCV MAbs, as determined by IIF, seven were found to produce MAbs directed against the HE glycoprotein. Both IgA and IgG (IgG1 and IgG2b subisotypes) anti-HE glycoprotein MAbs were obtained; their polypeptide specificities were confirmed by Western immunoblotting and RIPA (Table 1).

By Western immunoblotting, five of these MAbs (MAbs 1D6-2, 2G1-20, 6E7-11, 6E7-26, and 7B11-19) reacted to both the monomeric (65-kDa) and dimeric (140-kDa) forms of the HE glycoprotein (Fig. 2). On the other hand, MAb 9F2-1 revealed only the dimeric form (Fig. 2, lane C), whereas MAb 1D6-3 appeared to be directed to a conformationally dependent epitope, with no reactivity to the viral structural protein being observed by immunoblotting (Fig. 2, lane I). In the RIPAs, five of the MAbs reacted toward the monomeric (gp65) and dimeric (gp140) forms of the HE glycoprotein, whereas two others (MAbs 1D6-2 and 1D6-3) immunoprecipitated only the dimeric form from BCV-infected cell lysates (Fig. 3, lanes H and I, respectively). Anti-HE glycoprotein MAbs also appeared to coprecipitate the M glycoprotein, in

TABLE 1. Characterization of MAbs to the HE glycoprotein of BCV strain BCQ.2590 associated with WD in adult cattle

| MAb identification | Immunoglobulin isotype | IIF titer[a] | HAI titer[b] | VN titer[b] | Polypeptide specificity[c] | |
|---|---|---|---|---|---|---|
| | | | | | WB | RIPA |
| 1D6-2 | IgG2b | 10,240 | 2,560 | 80 | HE (65, 140)[d] | HE (140) |
| 1D6-3 | IgG2b | 10,240 | 5,120 | 80 | 0[e] | HE (140) |
| 2G1-20 | IgG2b | 20,480 | <20 | <20 | HE (65, 140) | HE (65, 140) |
| 6E7-11 | IgA | 10,240 | <20 | <20 | HE (65, 140) | ND[f] |
| 6E7-26 | IgA | 10,240 | <20 | <20 | HE (65, 140) | HE (65, 140) |
| 7B11-19 | IgG1 | 5,120 | 1,280 | <20 | HE (65, 140) | HE (65, 140) |
| 9F2-1 | IgG1 | 20,480 | 5,120 | 80 | HE (140) | HE (65, 140) |
| MAb-S[g] | ND | 1,280 | <20 | 5,120 | S (100, 200) | S (100, 200) |
| P-5801 | ND | 20,480 | 160 | 1,280 | S, HE, N, M | S, HE, N, M |

[a] Reciprocal values of the highest dilution of ascitic fluids detecting cytoplasmic fluorescence in BCQ.2590-infected HRT-18 cells.
[b] HAI and VN titers of ascitic fluids are expressed as described in Materials and Methods.
[c] Polypeptide specificities of hyperimmune serum (P-5801) to the BCV.Meb strain and MAbs to the BCQ.2590 strain were determined by Western immunoblotting (WB) and RIPA experiments.
[d] The molecular masses of the viral proteins are indicated in kilodaltons.
[e] 0, absence of reactivity.
[f] ND, not determined.
[g] MAb-S corresponds to a pool of MAbs directed to the S glycoprotein of the BCV.Meb strain (10).

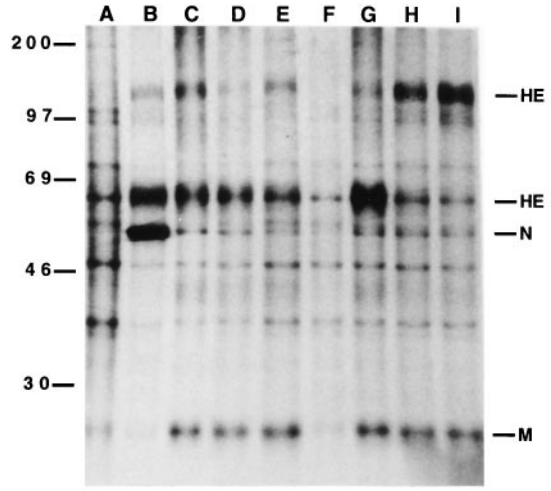


FIG. 3. RIPA of viral proteins from WD-associated BCV strain BCQ.2590. Viral proteins were precipitated from lysates of [$^{35}$S]methionine-labelled infected cell extracts following incubation in the presence of rabbit hyperimmune serum to the BCV.Meb strain (lane B), an MAb to an irrelevant virus (porcine reproductive and respiratory syndrome virus; lane F), or an MAb to the BCQ.2590 strain. The immunoprecipitation profiles obtained with anti-HE MAbs glycoprotein are represented in lanes C (9F2-1), D (7B11-19), E (6E7-26), G (2G1-20), H (1D6-2), and I (1D6-3). Lane A, noninfected cell lysate. The positions of the molecular mass markers are indicated on the left (in kilodaltons), and the positions of the viral structural proteins are indicated on the right.

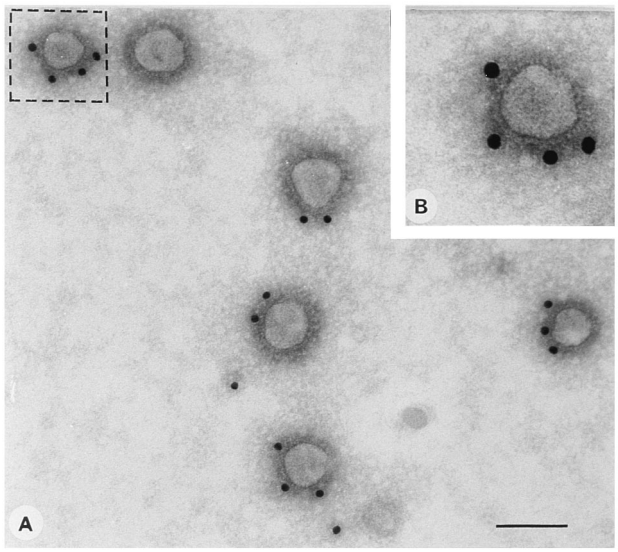


FIG. 4. Protein A-immunogold labelling of BCQ.2590 virions after incubation with anti-HE glycoprotein MAb 2G1-20. (A) The gold granules are specifically associated with the surface projections of the virus. (B) Higher magnification of the viral particle outlined with dashed lines in panel A showing the specific location of the gold granules on or near the shorter surface projections of the BCV virion. Bar, 150 nm.

agreement with findings which showed that the envelope and M glycoproteins of coronaviruses are closely associated during the intracellular maturation process (20).

Four of the anti-HE glycoprotein MAbs (MAbs 1D6-2, 1D6-3, 7B11-19, and 9F2-1) inhibited the HA activity of the homologous virus and three MAbs (MAbs 1D6-2, 1D6-3, and 9F2-1) weakly neutralized (VN titers, 80) its infectivity in vitro (Table 1). The indirect PAG electron microscopy technique was used in an attempt to determine the location on the virion of the protein recognized by the MAbs directed against the BCQ.2590 strain. As indicated in Fig. 4A, following incubation with MAb 2G1-20, most of the gold granules appeared to be closely associated to the viral envelope, with minimal gold background staining. At a higher magnification, gold labelling was more apparent near or on the short granular projections of damaged viral particles (Fig. 4B), whereas such particles were not labelled by anti-S glycoprotein MAbs, as previously reported elsewhere (5, 7). Control experiments, where MAb IAFK8 directed to a porcine arterivirus (13) was used or where the anti-BCV MAb was omitted, showed that the viral particles in the test were specifically labelled.

**MAb reactivity in competitive binding assays.** Competitive antibody-binding assays were conducted in order to determine the topography of epitopes on the HE glycoprotein recognized by the various MAbs raised against the BCQ.2590 strain. Each of the anti-HE MAbs described in Table 1 (with the exception of MAb 6E7-11, which induced an insufficient amount of ascitic fluid) was used both as a competitor and a probe (conjugated to D-biotin *N*-succinimide ester) and tested for its ability to compete for the binding of the five other MAbs. Conjugation had no apparent adverse effect on the binding properties of these MAbs, and endpoint titers ranged from approximately $10^{3.5}$ to $10^5$.

The results of typical competition binding experiments are presented in Fig. 5 and are summarized in Table 2. Competition was considered to be positive only if it occurred reciprocally and over a range of several $\log_{10}$ dilutions. Binding of biotinylated MAb was inhibited from approximately 50 to greater than 99% (>80% for all MAbs) in the presence of the homologous competitor ascitic fluid diluted between $10^2$ and $10^5$. The six MAbs tested appeared to delineate two independent antigenic domains (domains A and D) and two overlapping antigenic domains (domains B and C) for the HE glycoprotein of the WD-associated strain; epitopes located within antigenic domain A were not associated with the HAI and VN activities. MAbs 2G1-20 and 6E7-26 reciprocally competed with each other (Fig. 5C and D) and defined antigenic domain A. The other antigenic domains, domains B, C, and D, were defined by MAbs 1D6-3, 9F2-1, and 1D6-2, respectively. MAb 7B11-19, which also competed with MAb 1D6-3 (Fig. 5B), but only partially with MAb 9F2-1 (Fig. 3, lane F), delineated an overlapping region between antigenic domains B and C (Fig. 5A and E). Other partial competitions were observed between MAb 9F2-1 and MAbs 1D6-2 (Fig. 5A) and 1D6-3 (Fig. 5B). However, MAb 1D6-2 did not compete with any of the other MAbs (Fig. 5B to F). These partial competitions suggest spatially closed, but distinct epitopes.

**Cross-reactivities of anti-HE glycoprotein MAbs toward BCV field isolates.** The MAbs (1D6-2, 1D6-3, 7B11-19, and 9F2-1) that inhibited HA of the homologous strain were also evaluated for their ability to inhibit the HA activity of other BCV isolates which have been associated either with typical outbreaks of NCD, chronic shedding (AD), or WD in adult cattle of different Quebec dairy herds (Table 3). Fecal specimens were collected from diarrheic animals from January 1987 to April 1996 and were collected from five different geographical regions of the province. Diagnosis of a coronaviral infection was obtained by negative-stain electron microscopy and by indirect ELISA of clarified fecal samples (2). A total of 23 BCV isolates could be cultivated in HRT-18 cells, provided that trypsin was added to the culture medium. Upon their first two or three passages in these cells, BCV isolates behaved as weakly fusogenic strains (they induced the production of small

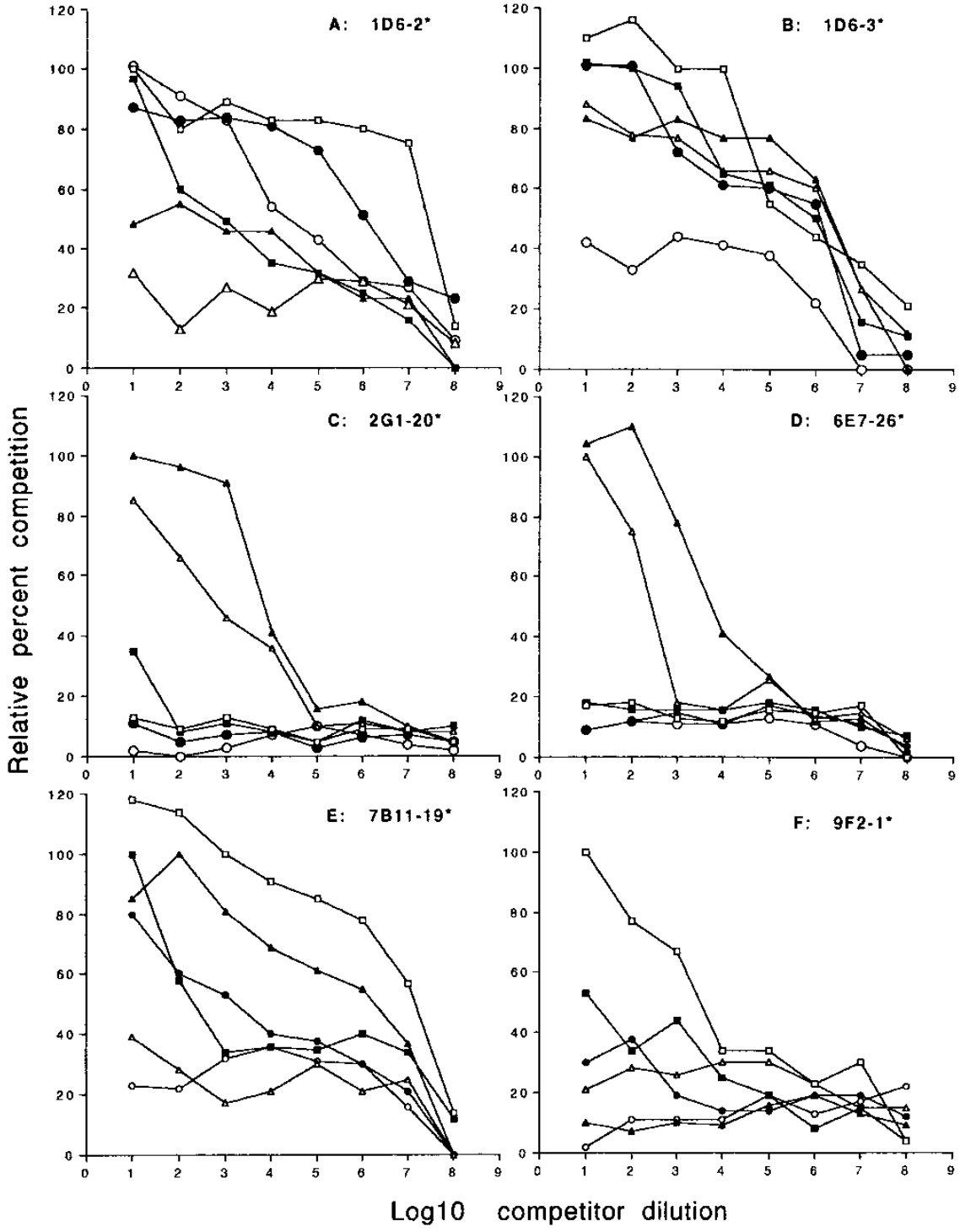


FIG. 5. Competition binding assays with biotinylated MAbs directed against the HE glycoprotein of the BCQ.2590 strain. Serial 10-fold dilutions of unconjugated antibodies (competitor) were allowed to react with coated antigen (purified BCQ.2590). After removal of the unbound antibodies, the binding of biotinylated MAb (probe) was determined. The extent of blocking by the competitor antibody of the conjugated probe was calculated and expressed as the relative percent competition, as described in the text. Biotinylated (*) antibodies were MAbs 1D6-2 (A), 1D6-3 (B), 2G1-20 (C), 6E7-26 (D), 7B11-19 (E), and 9F2-1 (F). The symbols used for competitor antibody correspond to MAbs 1D6-2 (○), 1D6-3 (●), 6E7-26 (△), 7B11-19 (■), 9F2-1 (□), and 2G11-20 (▲).

discrete syncytia containing less than 10 nuclei) or highly fusogenic strains (syncytia progressively increased in number and size, containing up to 40 nuclei, leading to the complete destruction of the cell sheets within 72 to 96 h postinfection). The virus yields after three to five passages ranged between $10^{5.75}$ and $10^{7.0}$ $TCID_{50}$s/ml. Final serological identification of the Quebec BCV strains was confirmed by IIF following incubation of the infected cell monolayers with rabbit hyperimmune serum raised against the reference BCV.Meb strain (8).

Five of the BCV isolates tested (isolates BCQ.2590,

TABLE 2. Summary of competition binding assays with MAbs to the HE glycoprotein of BCV strain BCQ.2590 associated with WD in adult cattle

| Competitor MAb | Competition with biotinylated probe MAb[a] | | | | | |
|---|---|---|---|---|---|---|
| | 6E7-26 | 2G1-20 | 1D6-3 | 7B11-19 | 9F2-1 | 1D6-2 |
| 6E7-26 | + | + | + | − | − | − |
| 2G1-20 | + | + | + | + | − | − |
| 1D6-3 | − | − | + | + | − | + |
| 7B11-19 | − | − | + | + | ± | + |
| 9F2-1 | − | − | + | + | + | + |
| 1D6-2 | − | − | − | − | − | + |
| | | A | B | | C | D |

[a] Results are expressed as relative percent competition: +, >70%; ±, 40 to 70%; −, <40%. By definition, binding of each conjugate to viral antigen in the absence of competitor represents 100% binding (0% competition), while the binding of the conjugate following prior incubation with excess homologous unlabeled MAb represents 0% binding (100% competition) (see Materials and Methods). The percent competition usually reached a plateau over a range of 2 or 3 10-fold dilutions. A, B, C, and D are putative antigenic domains of the HE glycoprotein, as defined by competition with the various anti-HE glycoprotein MAbs. The boxes indicate the reactivity of MAbs which defined the various antigenic domains.

BCQ.2508, BCQ.2439, BCQ.2442, and BCQ.7373) that have been associated with typical WD outbreaks in 1991 and 1992 reacted with approximately similar titers to the four anti-HE glycoprotein MAbs; the HAI titers ranged from 1,280 to 10,240. On the other hand, the four MAbs were unable to inhibit the agglutination of rat erythrocytes by the reference BCV.Meb strain as well as the HA activities of NCD- and AD-associated BCV isolates recovered from Quebec dairy farms prior to 1992. On the basis of their reactivities to this library of four anti-HE glycoprotein MAbs, BCV strains isolated from animals with AD or WD in 1994 to 1996 fell within at least two distinct serogroups. The BCQ.1523 isolate showed reactivity to the four anti-HE MAbs similar to that of the homologous BCQ.2590 strain, whereas BCQ.376 and BCQ.3708 behaved like the BCV.Meb strain. Interestingly, the HA activities of the other isolates collected from 1994 to 1996 (isolates BCQ.1486, BCQ.2344, BCQ.701, BCQ.748 and BCQ.838) were inhibited by MAbs 1D6-2, 1D6-3, and 9F2-1R, but not by MAb 7B11-19.

## DISCUSSION

In a previous study, Quebec BCV isolates associated with WD in adult dairy cattle were found to differ from the prototype BCV.Meb strain with respect to HAI, HA patterns at 4 and 37°C, and receptor-destroying enzyme activity with rat erythrocytes (8). At the molecular level, two proline substitutions were identified in the signal peptide and near the putative esterase domain FGDS of the HE glycoprotein gene of one representative WD-associated strain, strain BCQ.2590. These findings are in agreement with earlier findings by others of antigenic differences between cell culture-adapted WD- and NCD-associated strains (3). In order to extend our studies of the antigenic diversity between the two types of BCV isolates, MAbs were raised against the HE glycoprotein of the WD-associated BCQ.2590 strain and were used in competitive antibody-binding assays for topographical analysis of the epitopes involved in VN and HAI. These MAbs were further analyzed with respect to their cross-reactivities in HAI tests toward NCD- and WD-associated Quebec BCV isolates collected during a 10-year period.

An enriched peplomeric fraction could be obtained following OPG treatment of the purified BCV.2590 virion. This detergent treatment has previously been described for the separation of the HE, S, and M glycoproteins of BCV and porcine hemagglutinating encephalomyelitis virus (36). Because OPG

TABLE 3. Cross-reactivity in HAI tests of anti-HE glycoprotein MAbs (BCQ-2590 strain) to Quebec field isolates of BCV collected over a 10-year period

| Strain | Yr of isolation | Disease | HAI titers of MAbs[a] or antiserum | | | | |
|---|---|---|---|---|---|---|---|
| | | | 1D6-2 | 1D6-3 | 7B11-19 | 9F2-1R | Rab. 5801 |
| BCV.Meb | 1972 | NCD | <20 | <20 | <20 | <20 | 320 |
| BCQ.3 | 1986 | NCD | <20 | <20 | <20 | <20 | 1,280 |
| BCQ.4 | 1986 | NCD | <20 | <20 | <20 | <20 | 640 |
| BCQ.7 | 1986 | NCD | <20 | <20 | <20 | <20 | 640 |
| BCQ.9 | 1986 | NCD | <20 | <20 | <20 | <20 | 1,280 |
| BCQ.189 | 1988 | NCD | <20 | <20 | <20 | <20 | 1,280 |
| BCQ.571 | 1989 | NCD | <20 | <20 | <20 | <20 | 160 |
| BCQ.718 | 1989 | NCD | <20 | <20 | <20 | <20 | 640 |
| BCQ.1750 | 1989 | NCD | <20 | <20 | <20 | <20 | 80 |
| BCQ.2590 | 1991 | WD | 2,560 | 5,120 | 1,280 | 5,120 | 160 |
| BCQ.2508 | 1991 | WD | 5,120 | 5,120 | 1,280 | 640 | 320 |
| BCQ.2439 | 1991 | WD | 5,120 | 5,120 | 1,280 | <20 | 160 |
| BCQ.2442 | 1991 | WD | 5,120 | 5,120 | 2,560 | 5,120 | 320 |
| BCQ.A130 | 1992 | AD | <20 | <20 | <20 | <20 | 640 |
| BCQ.A209 | 1992 | AD | <20 | <20 | <20 | <20 | 640 |
| BCQ.7373 | 1992 | WD | 10,240 | 5,120 | 2,560 | 5,120 | 320 |
| BCQ.1486 | 1994 | NCD | 10,240 | 10,240 | <20 | 2,560 | 320 |
| BCQ.1523 | 1994 | NCD | 10,240 | 10,240 | 1,280 | 5,120 | 640 |
| BCQ.3708 | 1994 | AD | <20 | <20 | <20 | <20 | 320 |
| BCQ.2344 | 1995 | NCD | 1,280 | 1,280 | <20 | 160 | 640 |
| BCQ.376 | 1996 | NCD | <20 | <20 | <20 | <20 | 80 |
| BCQ.701 | 1996 | NCD | 10,240 | 10,240 | <20 | 5,120 | 320 |
| BCQ.748 | 1996 | AD | 10,240 | 2,560 | <20 | 640 | 640 |
| BCQ.838 | 1996 | NCD | 10,240 | 2,560 | <20 | 160 | 320 |

[a] Reciprocal values of highest MAb dilution inhibiting agglutination of rat erythrocytes by BCV (8 HA) after 2 h at 4°C.

can be removed by dialysis, it is possible to obtain detergent-free glycoproteins, preserving their biological, enzymatic, and binding activities. We expected that such a detergent treatment would allow for the conservation of possible conformationally dependent epitopes which could be associated with VN and HAI activities. A total of seven hybridoma cell lines secreting MAbs directed to the HE glycoprotein of the BCQ.2590 strain could be established. Five of these anti-HE glycoprotein MAbs inhibited the HA activity of the virus and three weakly neutralized its infectivity in vitro. These results are in agreement with previous findings that the HE glycoprotein of hemagglutinating coronaviruses possesses antigenic determinants involved in HAI and VN and that no correlation exists between both activities of the anti-HE MAbs (7, 16, 29, 41).

The results of competitive antibody-binding assays suggested the presence of two distinct antigenic domains (domains A and D) and two overlapping antigenic domains (domains B and C) representing at least five distinct epitopes on the HE glycoprotein of the WD-associated strain. The antigenic site A was defined by two MAbs (MAbs 2G1-20 and 6E7-26), which recognized both the monomeric and dimeric forms of the HE glycoprotein by immunoblotting, but were not associated with HAI and VN activities. MAbs 1D6-3 and 9F2-1, which defined antigenic sites B and C, respectively, showed no reactivity or only weak reactivity toward the monomeric form of the HE glycoprotein by immunoblotting, and thus may be directed to distinct epitopes which are located close to each other following the assembly of the dimeric form of the HE glycoprotein. This possibility is substantiated by the fact that MAb 7B11-19 competed reciprocally with both MAbs 1D6-3 and 9F2-1, thus representing a putative overlapping region between antigenic sites B and C, which are both associated with HAI and VN activities. The reactivity patterns of different Quebec BCV isolates to these three anti-HE glycoprotein MAbs also suggested that they are directed against three independent epitopes of the HE glycoprotein. Finally, the antigenic site D was identified as an independent domain defined by MAb 1D6-2 which was also associated with VN and HAI activities.

In general, the data obtained from competitive antibody-binding assays are in agreement with previous findings on the topography of the HE glycoprotein of two reference NCD-associated strains. Indeed, identical experiments that have been performed with a set of 14 MAbs allowed for the definition of two independent antigenic domains on the HE glycoprotein of BCV strain G110 (41). All neutralizing anti-HE glycoprotein MAbs mapped within one major antigenic domain consisting of several, apparently three, overlapping epitopes. As demonstrated for the BCQ.2590 strain, antigenic domain A for this reference French strain of BCV was also defined by MAbs not associated with either VN or HAI activity. Three overlapping antigenic domains, representing four closely related neutralizing epitopes, were also identified on the HE glycoprotein of a Quebec reference NCD-associated strain (14). However, the data obtained in the case of these two NCD-associated strains differed from those obtained with the BCQ.2590 strain because the VN titers of the anti-HE glycoprotein MAbs were significantly higher (14, 16, 41).

Although BCV strains can be distinguished for their pathogenicities, there is still some controversy as to the existence of distinct BCV serotypes. Previous investigators detected only minor strain variations among cell culture-adapted BCV strains isolated from calves with NCD in Europe by both IIF and HAI tests (18), and similar findings were also reported for Quebec NCD-associated strains by counterimmunoelectrophoresis and immunodiffusion tests (11). More recently, anti-S glycoprotein MAbs were found to distinguish between vaccine (avirulent) and wild-type (virulent) BCV strains in mildly denaturating Western immunoblotting and neutralization assays (21). We also previously reported antigenic plurality among BCV isolates from calves with NCD in Quebec through the partial reaction in an indirect ELISA of MAbs against the S glycoprotein (29).

Therefore, the second objective of the present study was to determine if anti-HE glycoprotein MAbs raised against the BCQ.2590 strain could differentiate between NCD- and WD-associated Quebec BCV isolates. In preliminary comparative studies by IIF tests, no significant differences were demonstrated between the antibody titers of the various anti-HE glycoprotein MAbs when they were tested against cell culture-adapted NCD- or WD-associated strains. However, at least two distinct serogroups were identified among the 24 BCV isolates tested by the HAI test. Unexpectedly, the four anti-HE glycoprotein MAbs which inhibited the HA activity of the homologous BCV.2590 strain failed to react with the reference BCV.Meb strain, while they displayed strong HAI titers to the other WD-associated isolates tested. However, the reactivities of MAbs 1D6-1, 1D6-3, and 9F2-1R were not restricted to WD-associated strains because comparable HAI titers were obtained with some of the NCD- and AD-associated strains tested. Interestingly, none of these anti-HE glycoprotein MAbs reacted positively with BCV strains isolated in Quebec prior to 1991. These findings indicate that variations affecting antigenic determinants of the HE glycoprotein occurred among BCV strains isolated in Quebec within the last 10 years, and this diversity appears to be unrelated to the clinical source of the strains, in agreement with the recent findings of Tsunemitsu and Saif (40) in the case of BCV strains isolated in the United States. The nonreactivity in HAI tests of MAb 7B11-19 toward 18 of 19 NCD- or AD-associated BCV strains tested is of particular interest, since it implies that epitopes specific for the WD-associated BCV strains are present on the HE glycoprotein. The fact that all WD-associated isolates tested fell within the same antigenic subgroup further indicates that these strains possess common properties, some of which are shared by BCV strains associated with other clinical syndromes. Finally, the results derived from the cross-reactivity studies also suggest that epitopes of antigenic domains B, C, and D of the HE glycoprotein are involved in the antigenic diversity between NCD- and WD-associated BCV isolates.

In previous studies, differentiation between BCV strains associated with NCD and WD by VN tests has been also reported (3, 40). Although, it has been well demonstrated that the HE glycoprotein was also able to induce efficient neutralizing antibodies (14, 41), the S peplomeric protein of the BCV virion, particularly the S1 portion of the molecule, is known to carry the major and more powerful neutralizing epitopes (3, 7, 41). Studies are in progress to elucidate if the antigenic and genomic diversities identified for BCV strains isolated in Quebec from animals with typical cases of NCD and WD can also be related to changes in the S glycoprotein.

## ACKNOWLEDGMENTS

This research was supported by a grant from the National Sciences and Engineering Research Council of Canada (grant OGPOO46412).

We thank Nicole Sawyer for technical assistance. We are also grateful to C. Montpetit, G. Marsolais, and R. Assaf, Direction de la Santé Animale, Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec, for providing field strains of BCV.

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