

**Antigenic variation among bovine enteric coronaviruses (BECV)
and bovine respiratory coronaviruses (BRCV) detected
using monoclonal antibodies**

Brief Report

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Summary. Bovine coronavirus (BCV) causes neonatal calf diarrhea (CD) and is associated with winter dysentery (WD) in adult dairy cattle. It can also be isolated from the respiratory tracts of cattle entering feedlots. Monoclonal antibodies (MAbs) specific for the hemagglutinin esterase (HE) and spike (S) surface proteins of 2 bovine enteric coronavirus (BECV) strains and two bovine respiratory coronavirus (BRCV) strains were tested against 6 BECV strains and 6 recently isolated BRCV strains, in order to characterize the antigenicity of BCV strains with varied tissue tropisms. All MAbs had high immunofluorescence (IF) titers against BECV and BRCV strains, indicative of conserved cross-reactive epitopes. In hemagglutination inhibition (HI) tests, the S-MAbs were more broadly reactive than HE-MAbs. The BRCV and CD MAbs were more broadly reactive in HI than the WD MAbs. The HA activity of the Mebus vaccine CD strain was not inhibited by any of the MAbs tested. The HI activity of BRCV strain R6 was unique among the 6 BRCV isolates. In virus neutralization assays, MAbs to the BRCV strain R4 neutralized all 6 BECV strains tested. Antigenic variation exists among both BECV and BRCV strains, but it cannot be attributed solely to the clinical origin of the strain.

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Table 1. Hemagglutination inhibition (HI) reactivity of selected monoclonal antibodies with BECV and BRCV strains

MAb	VP ^a	Isotype	Calf diarrhea BECV			Winter dysentery BECV			Feedlot BRCV					
			DB2	Mebus	SDC	DBA	BM	BE	R1	R2	R3	R4	R5	R6
DB-2 MAbs (CD)														
CD-1	ND ^b	IgG2b	+ ^c	- ^c	-	-	+	-	+	+	+	-	-	-
CD-2	S	IgG2a	+	-	-	-	+	-	+	+	-	-	-	-
DBA MAbs (WD)														
WD-1	S	IgG2a	+	-	-	+	-	-	+	+	+	-	-	-
WD-2	HE	IgG1	-	-	-	+	-	-	-	-	-	-	-	-
WD-3	HE	IgA	-	-	-	+	-	-	-	-	-	-	-	-
R4 MAbs (BRCV)														
BRCV-1	ND	IgG1	+	-	-	-	-	+	+	+	+	+	+	-
BRCV-2	ND	IgG1	+	-	-	-	-	-	+	+	-	+	-	-
BRCV-3	ND	IgG1	+	-	-	-	-	+	+	+	-	+	-	-

^aViral protein^bNot determined^c“+” = HI titer \geq 40; “-” = HI titer < 40

Bovine coronavirus (BCV) causes calf diarrhea (CD), is associated with winter dysentery (WD) in adult dairy cattle, and may contribute to respiratory disease in feedlot cattle [9, 14]. There are several studies of the antigenicity of BECV isolates using polyclonal or monoclonal antibody [5, 6, 12, 17], but there are no studies of BRCV isolates using MAbs. Although some biologic and antigenic differences between BECV strains have been detected, it is unclear if these differences are distinctive between BECV (CD, WD) and BRCV strains, and if such differences can be used to differentiate and identify the sources of individual strains.

In this investigation, 3 MAbs to BRCV strain R4, 2 MAbs to the CD strain DB2, and 3 MAbs to the WD strain DBA were tested by IF, HI and VN to determine the antigenic relatedness of 6 BECV and 6 BRCV strains. The BECV strains (Tables 1, 2) were plaque isolated or cloned by limiting dilution as described previously [1, 5]. The respiratory strains (Tables 1, 2) were isolated in HRT-18 cells from cattle in Ohio feedlots from 1995–1996 and were cloned by limiting dilution [5, 8].

Monoclonal antibodies to CD strain DB2, WD strain DBA, and BRCV strain 76 were produced as described previously [7, 16, 19]. Briefly, female Balb/c mice were given an intraperitoneal (IP) injection of 0.1 ml of inactivated and purified BCV strains (80 μ g protein/mouse). Mice with IF titers to BCV ranging from 51,200–102,400 and HI titers of \geq 640 were used for Mab production. The hybridoma supernatants were screened by IF, then by fluorescent focus neutralization (FFN) and HI tests against homologous virus strains. The IF and FFN tests were performed using HRT-18 cell cultures grown in 96 well microplates as

Table 2. Virus neutralization reactivity of monoclonal antibodies with BECV and BRCV strains

MAb	VP ^a	Isotype	Calf diarrhea BECV			Winter dysentery BECV			Feedlot BRCV					
			DB2	Mebus	SDC	DBA	BM	BE	R1	R2	R3	R4	R5	R6
DB-2 MAbs (CD)														
CD-1	ND ^b	IgG2b	+ ^c	+	+	+	- ^c	+	+	+	+	+	+	-
CD-2	S	IgG2a	+	+	+	+	-	+	+	+	+	+	+	-
DBA MAbs (WD)														
WD-1	S	IgG2a	+	+	-	+	+	+	+	-	+	-	+	-
WD-2	HE	IgG1	+	+	-	+	-	-	-	-	-	-	-	-
WD-3	HE	IgA	-	+	+	+	-	-	+	+	+	-	+	-
R4 MAbs (BRCV)														
BRCV-1	ND	IgG1	+	+	+	+	+	+	+	+	+	+	+	+
BRCV-2	ND	IgG1	+	+	+	+	+	+	+	+	+	+	+	+

^aViral protein^bNot determined^c“+” = CPE reduction VN titer of ≥ 40 ; “-” = VN titer of < 40

described previously [7]. The CD, WD and BRCV hybridomas which were positive for FFN and HI antibodies to BCV were used to produce ascites by injection of the cloned (2–3 times) hybridoma cells into pristane-primed mice [7, 16, 19]. The mice were injected with 0.2 ml of hybridoma cells (1×10^6 cells/ml) and 5–10 days later ascites were collected. The isotypes and subspecies of the MAbs were determined by immunodiffusion assay of hybridoma supernatant fluids with monospecific anti-mouse immunoglobulin sera (ICN Biomedicals, Aurora, OH) and are summarized in Tables 1 and 2.

The hemagglutination inhibition (HI) test was performed using 96-well U-bottom plates [17]. Monoclonal antibodies to BRCV and BECV were serially diluted 2-fold with veronal buffered saline (VBS) and mixed with the same volume of 8 HA units of purified BCV [17] and incubated at 22 °C for 1 h. After incubation, a 1% mouse erythrocyte suspension was added and incubated for 2 h at 22 °C. HI titers were expressed as the reciprocal of the highest dilution of MAb which completely inhibited HA (pellet formation). HI titers of ≥ 40 are indicated by a plus (+) in Table 1, while a lack of HI (titer of < 40) is indicated by a dash (-).

Two of the CD strains of BECV, including the Mebus vaccine strain, failed to react with any of the MAbs tested by HI (Table 1). The third CD strain, DB2, was the most broadly reactive of the BECV strains tested, as its hemagglutination was inhibited by 6 of the 8 MAbs tested. Among the WD strains, each strain reacted with only one classification of MAb (CD, WD or BRCV). Among the DBA MAbs, WD-1, directed against the S protein was more reactive than either of the MAbs directed against the HE protein. BRCV strains R1 and R2 shared similar HI patterns when tested with all 8 of the MAbs. BRCV strains R4 and R5

reacted similarly with 6 of the 8 MAbs, but differed when tested against 2 of the BRCV MAbs. All of the MAbs, from both enteric and respiratory strains, failed to inhibit the hemagglutination caused by BRCV strain R6.

The HI results showed that for the DBA MAbs, the S-MAbs were more broadly reactive in HI than the HE-MAbs (Table 1). Because the S protein is more efficient than the HE protein in hemagglutination [23], it follows that the S-MAbs would be more efficient in inhibiting hemagglutination than the HE-MAbs. The BRCV and CD MAbs were more broadly reactive in HI than the WD MAbs.

A CPE reduction virus neutralization (VN) test was performed using HRT-18 cell cultures grown in 96-well microplates as described previously [18]. Briefly, serial 2-fold dilutions of MAbs were mixed with the same volume of virus suspensions containing 100 TCID₅₀/0.1 ml and then incubated at 37 °C for 90 min. Four wells of HRT-18 cells were inoculated with 0.1 ml of each virus-serum mixture and incubated for 5–7 days at 37 °C. Neutralizing antibody titers of MAbs were expressed as the reciprocal of the highest dilution that inhibited 50% of the CPE. VN titers of ≥ 40 are indicated by a plus (+) in Table 2, while a lack of VN titer (< 40) is indicated by a dash (–).

The 7 MAbs tested were more broadly reactive in VN tests (Table 2) than in HI tests (Table 1). Whereas the Mebus vaccine and SDC calf diarrhea strains failed to react with any of the MAbs in HI testing, both were neutralized by MAbs directed against both DB2 CD and R4 BRCV strains. Additionally, the Mebus CD strain was also neutralized by MAbs directed against the DBA WD BECV strain. The VN reactivity patterns of the Mebus CD strain and the DBA WD strain were identical to one another. The CD strain DB2 had a very similar pattern, varying only in regard to its lack of neutralization by the WD-3 MAb. The 2 BRCV MAbs tested in VN successfully neutralized all BCV strains, both enteric and respiratory. Five of the 6 BRCV strains were neutralized by MAbs directed against the DB2 CD strain, and all 3 CD strains were neutralized by both BRCV MAbs. BRCV strain R6 is unique, as it was in HI testing, failing to react with any of the CD or WD MAbs.

Based on HI and VN testing with MAbs, we found that antigenic and biologic variation exists among BCV strains, but that this variation was not always related to the clinical source of the isolates. This is in agreement with previous studies using polyclonal antisera and BECV isolates [5, 11, 13, 17, 18, 20]. Tsunemitsu et al. [18] tested BCV strains isolated from nasal swabs and feces of calves and found them to be indistinguishable when tested by IEM, HA, IF, and VN, using hyperimmune serum. Tsunemitsu et al. [17] determined the HI and VN titers of polyclonal antibodies against various BECV strains and found that the reactivity patterns for these strains could not be grouped according to their clinical origin (CD or WD). Using the same isolates, but testing them with the MAbs produced in this study, rather than polyclonal hyperimmune antiserum [5], similar HI patterns were found, which could not be predicted based on clinical origin.

Reynolds et al. [12] found that hyperimmune antiserum to three different BCV isolates (2 respiratory, 1 enteric) showed VN with the homologous and heterologous isolates and significant cross-reactions with eight other isolates,

which were of intestinal and respiratory origin. Using polyclonal antiserum and 14 MAbs directed against the S protein of the Mebus CD strain, Michaud and Dea [10] found that 9 WD isolates from Quebec were all closely related, using polyclonal antibodies, but could be classified into 3 distinct antigenic subgroups based on their reactivity with the S-MAbs of the Mebus strain in VN and HI tests. This finding is in agreement with the results of the present and our previous study [5]: BECV isolates were closely related using polyclonal antiserum, but could be classified into 2 to 3 antigenic subgroup based on VN and HI test results. With MAbs, Milane et al. [11] indicated that variations (unrelated to clinical source) affecting the antigenic determinant of the HE protein occurred among the BECV strains isolated in Quebec and that these strains were grouped differently by their MAb HI titers from the prototype Mebus strain. Similarly, in the present study, the HA activity of the Mebus strain was not inhibited by any of the MAbs tested. The BRCV isolate R6 was unique among the respiratory isolates when tested against the BECV MAbs, a finding consistent between HI and VN tests and in testing with both MAbs and hyperimmune sera [5]. However, using the BRCV-MAbs, this strain was antigenically related to the other BRCV strains when tested by HI and VN.

Differences in VN and HI test results are to be expected, as there was no correlation between these activities [11]. Disparate VN and HI results have been reported for MAbs to the S and HE proteins for the Quebec BCV isolates [3], MAbs to the S protein for the Mebus strain [10] and MAbs to the HE protein for the WD strain (BCQ.2590) [11]. Moreover, MAbs were reactive with separate epitopes on the BCV HE or S protein. The HE protein contains three antigenic regions, A, B, and C. Domain A is further subdivided into two epitopes, A1 and A2, which react with neutralizing antibodies [2]. The S protein has three antigenic regions, two (A, B) or 3 (A, B, C) or which react with neutralizing antibodies [2, 10]. In the present study, the DB2 MAbs, CD-1 and CD-2 reacted with a hemagglutinating epitope, and a third DB2 MAb (data not shown) reacted with a non-hemagglutinating epitope, although both epitopes were separately involved in VN activity. This was also true for most of the MAbs, as they demonstrated VN activity with strains for which they had failed to inhibit hemagglutination. This finding is in agreement with E1-Ghorr, et al. [4] that their MAbs against the HE protein were both neutralizing, but only one had HI activity. In addition, the R4 MAb BRCV-3 inhibited HA activity, but it had no neutralizing activity. This is in agreement with Michaud and Dea [10] who reported that HE-MAbs to the Mebus strain had HI activity, but no neutralizing activity.

The results of this study indicate antigenic and biologic variation, as detected by MAbs, exists among BCV isolates, but that this variability is not always related to the clinical source of the isolate. One BRCV strain, R6, and to some extent the Mebus vaccine strain, represent unique BCV isolates, distinct from the other enteric and respiratory strains, based at least on one-way HI and VN reactivity patterns using MAbs.

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