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Antigenic and biological comparisons of bovine coronaviruses derived from neonatal calf diarrhea and winter dysentery of adult cattle

Brief Report

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Summary. The antigenic and biological properties of 6 strains of bovine coronavirus (BCV) derived from neonatal calf diarrhea (CD) and 8 strains of BCV from winter dysentery (WD) of adult cattle, propagated in HRT-18 cells, were compared to determine if CD and WD strains belong to distinct serotypes or subtypes of BCV. All strains hemagglutinated both mouse and chicken erythrocytes at 4 °C, but the ratios of hemagglutination titers with mouse erythrocytes compared to chicken erythrocytes showed diversity for both CD and WD strains. Some CD and WD strains did not hemagglutinate chicken erythrocytes at 37 °C and showed receptor-destroying enzyme activity against chicken erythrocytes. Hyperimmune antisera were produced in guinea pigs against 3 and 7 strains of BCV from CD and WD, respectively. No significant differences in antibody titers against these strains were observed by indirect immunofluorescence tests. However, in virus neutralization tests, antisera to 1 CD and 2 WD strains had 16-fold or lower antibody titers against 3 WD and 1 CD strains than against the homologous strains, and this variation reflected low antigenic relatedness values (R=13–25%), suggesting the presence of different subtypes among BCV. In hemagglutination inhibition tests, some one-way antigenic variations among strains were also observed. These results suggest that some antigenic and biological diversity exists among BCV strains, but these variations were unrelated to the clinical source of the strains; i.e. CD or WD.

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Bovine coronavirus (BCV) is a primary cause of neonatal calf diarrhea (CD) worldwide [4, 13]. Bovine coronavirus is also associated with acute diarrhea in adult cattle during the winter season referred to as winter dysentery (WD) [4, 17, 18, 24, 26]. Besides infecting the small and large intestines of calves, BCV also possesses a tissue tropism for the upper respiratory tract [15].

Bovine coronavirus has four major structural proteins: the nucleocapsid protein (N), the transmembrane protein (M), the spike protein (S) and the hemagglutinin-esterase protein (HE) [21]. The S and HE proteins form fringes of longer and shorter surface projections, respectively [4]. Both proteins can cause hemagglutination (HA) and contain neutralizing epitopes [7, 20]. The HE protein also possesses receptor-destroying enzyme (RDE) which inactivates cellular receptors for BCV by hydrolyzing an ester bond to release acetate from C-9 of sialic acid [4, 27].

Because the primary isolation of BCV remains difficult, there have been only a few investigations on antigenic comparisons of BCV strains [6, 8, 11, 14]. Specifically, whether antigenic or biological differences exist between strains derived from CD and WD remains obscure [2]. The purpose of this study was to isolate BCV strains from feces derived from CD and WD in human rectal tumor (HRT-18) cells and to compare the antigenic and biological properties of these strains.

The Mebus strain of CD BCV [13] was provided by Dr. K. W. Theil of our institute (originally supplied by Dr. D. A. Brian, The University of Tennessee, Knoxville, Tennessee, U.S.A.), and had been passaged at least 40 times in fetal bovine kidney (FBK) cells and 20 times in embryonic bovine kidney (BEK) cells. It was propagated 8 times in bovine turbinate (BT) cells, 6 times in bovine kidney (MDBK) cells and 3 times in HRT-18 cells in our laboratory. The 216XF strain isolated from a newborn beef calf with diarrhea in Japan was propagated 10 times in HRT-18 cells [25]. The DBA strain of WD BCV was propagated 8 times in HRT-18 cells in our laboratory as previously described [2]. The SD strain of WD BCV was kindly supplied by Dr. D. A. Benfield, The South Dakota University, Brookings, South Dakota, U.S.A., and was propagated 8 times in HRT-18 cells. Six WD isolates of BCV designated as the TS, CN, BE, BM, AW and BW strains, were isolated from fecal samples of gnotobiotic calves which had been inoculated with fecal filtrates from affected adult dairy cows from 6 herds in the U.S.A. including Ohio (3 herds) and Wisconsin (3 herds), which were experiencing typical outbreaks of WD during the winters of 1987 to 1993. Three CD isolates of BCV designated as the DB2, OHC and SDC strains, were isolated from fecal samples of neonatal calves with acute diarrhea from 3 herds in the U.S.A. including Ohio (2 herds) and South Dakota (1 herd). A fourth CD isolate (JAZ) was isolated from a neonatal calf with acute diarrhea in Hokkaido, Japan. Virus isolation on HRT-18 cells was done as previously described [2, 25] except that 10 μg/ml of pancreatin (Gibco Laboratories, Grand Island, N.Y.) was added to the maintenance medium. Confirmation of the isolates as BCV was done by using immune electron microscopy [16] and immunofluorescence (IF) [2] with hyperimmune antiserum prepared against the Mebus strain of CD BCV. The isolated viruses, which were passaged a total of 5 to 10 times in HRT-18 cells were plaque-purified at least once before use.

Hyperimmune antisera were prepared in guinea pigs against 3 CD strains (Mebus, DB2, 216XF) and 7 WD strains (SD, BM, CN, AW, DBA, TS, BE) of BCV that had been propagated in HRT-18 cells for 6 to 11 passages and then purified on sucrose gradients [5]. These antisera were used for serological tests.

Hemagglutination (HA) tests were conducted by the microtiter method [19]. Bovine coronavirus strains were purified from infected-cell culture supernatants,

which were concentrated approximately 100-to 200-fold. Serial 2-fold dilutions of BCV were prepared in 0.05 ml of veronal buffered saline containing 0.1% bovine serum albumin and 0.001% gelatin and mixed with 0.05 ml of 0.8 and 0.4% suspensions of mouse and pooled adult chicken erythrocytes, respectively, in the same buffer. The mixtures were then incubated for 1h at either 4 or 37 °C and the HA titers were determined. The plates incubated at 4 °C were moved to 37 °C for 2 h to measure inactivation of receptors reflected by the disaggregation of the BCV-erythrocyte complexes mediated by the receptor destroying enzyme (RDE) activity [22].

Antigenic comparisons of BCV strains were done by indirect IF, virus neutralization (VN) and HA inhibition (HI) tests. The indirect IF tests were performed as previously described [8]. Virus titration and VN tests were conducted with HRT-18 cells in microplates as previously described [25]. Virus infectivity titers were expressed as median tissue culture infective doses ($TCID_{50}$)/ml. The VN antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited cytopathic effects (CPE). The antigenic relatedness (R) between the strains was calculated using the formula [1, 8]:

$$R = 100\sqrt{r1 \times r2}\,\%$$

in which $r1$ is heterologous titer (strain 2)/homologous titer (strain 1), and $r2$ is heterologous titer (strain 1)/homologous titer (strain 2). The HI test was done using standard techniques with mouse erythrocytes [19] and sera treated with kaolin and mouse erythrocytes. The antibody titers were expressed as the reciprocal of the highest serum dilutions producing complete HI.

Cytopathic effects were evident in HRT-18 cells inoculated with each of the strains of BCV. Cytopathic effects were characterized by enlarged, rounded, and densely granular cells that occurred in clusters at 2 to 3 postinoculation days [2], and no differences were observed in CPE among these strains. Syncytia were also clearly observed in HRT-18 cells at 2 days after inoculation with these strains following staining with fluorescein isothiocyanate-conjugated anti-BCV serum. Infectivity titers of BCV reached $10^{7.0}$ to $10^{8.7}$ $TCID_{50}$/ml at the 5th to 10th passages on HRT-18 cells.

The HA and RDE titers of purified BCV strains are summarized in Table 1. All strains agglutinated mouse erythrocytes and no differences were observed in HA titers against mouse erythrocytes at 4° and 37 °C. All strains also agglutinated chicken erythrocytes at 4 °C, but the HA titers varied among the BCV strains. This diversity was reflected in variations of the ratios of HA titer with mouse erythrocytes to HA titer with chicken erythrocytes (M/C HA titer ratio) at 4 °C. However there was no relation between M/C HA titer ratio and the clinical source (CD or WD) of the strains. At 37 °C, the Mebus and DB2 strains of CD BCV and the DBA and SD strains of WD BCV agglutinated chicken erythrocytes with the same HA titers as at 4 °C. However, the other strains of BCV did not agglutinate chicken erythrocytes at 37 °C, and showed RDE activity against chicken erythrocytes. Receptor-destroying enzyme activity with mouse erythrocytes was not

Table 1. Hemagglutination (HA) and receptor-destroying enzyme (RDE) activities of purified BCV strains

BCV strain (Derivation[c])	HA titer[a]					RDE titer[b]	
	4 °C			37 °C			
	Mouse	Chicken	M/C[d]	Mouse	Chicken	Mouse	Chicken
Mebus (CD)	102400	25600	4	102400	25600	<12.5	<12.5
DB2 (CD)	102400	12800	8	102400	12800	<12.5	<12.5
DBA (WD)	51200	3200	16	51200	3200	<12.5	<12.5
SD (WD)	51200	3200	16	51200	3200	<12.5	<12.5
216XF (CD)	25600	1600	16	25600	<12.5	<12.5	≥1600
CN (WD)	6400	1600	4	6400	<12.5	<12.5	≥1600
BE (WD)	51200	1600	32	51200	<12.5	<12.5	≥1600
AW (WD)	51200	1600	32	51200	<12.5	<12.5	≥1600
OHC (CD)	51200	200	256	51200	<12.5	<12.5	≥200
SDC (CD)	51200	200	256	51200	<12.5	<12.5	≥200
JAZ (CD)	51200	100	512	51200	<12.5	<12.5	≥100
TS (WD)	51200	100	512	51200	<12.5	<12.5	≥100
BM (WD)	25600	100	256	25600	<12.5	<12.5	≥100
BW (WD)	25600	100	256	25600	<12.5	<12.5	≥100

[a] Expressed as the reciprocal of the highest dilution of virus showing complete HA of 0.4 and 0.2% suspensions of mouse and chicken erythrocytes, respectively, after 1 h incubation at 4 °C or 37 °C

[b] Expressed as the reciprocal of the highest dilution of virus causing complete disappearance of HA patterns at 4 °C after 2 h incubation at 37 °C

[c] *CD* Calf diarrhea, *WD* winter dysentery of adult cattle

[d] *M/C* Ratio of HA titer with mouse erythrocytes to HA titer with chicken erythrocytes

detected for any strain of BCV. According to these results, BCV strains were classified into 3 groups. The first group (CD isolates, Mebus, DB2; and WD isolates, DBA, SD) showed low M/C HA titer ratios(≤16), no differences in HA titers against chicken erythrocytes at 4 and 37 °C and no RDE activity against chicken erythrocytes. The second group (CD isolate, 216XF; and WD isolates, CN, BE, AW)showed low M/C HA titer ratios (≤32), no HA against chicken erythrocytes at 37 °C and RDE activity with chicken erythrocytes. The third group (CD isolates, OHC, SDC, JAZ; and WD isolates, TS, BM, BW) showed high M/C HA titer ratios (≥256), no HA against chicken erythrocytes at 37 °C and RDE activity with chicken erythrocytes. These variations in HA and RDE activities were unrelated to the clinical source of the isolates (CD or WD).

In indirect IF tests, all of the antisera reacted to each virus strain with high titer (102 400 to 409 600), and each antiserum showed no significant differences in reactivity with the homologous and heterologous strains (not greater than twofold differences).

The results of VN tests are shown in Table 2. All of the antisera neutralized the heterologous strains, showing that the strains were closely related antigenically.

Table 2. Virus neutralization (VN) antibody titers of hyperimmune guinea pig sera against BCV strains

BCV strain (Derivation[b])	VN antibody titers[a] of hyperimmune guinea pig sera to									
	Mebus	SD	BM	DB2	CN	AW	DBA	TS	BE	216XF
Mebus (CD)	**10 240** (100)[c]	5 120	2 560	10 240	1 280	10 240	2 560	5 120	10 240	20 480
SD (WD)	5 120 (50)	**10 240** (100)	1 280	10 240	1 280	5 120	640	10 240	10 240	10 240
BM (WD)	10 240 (50)	10 240 (35)	**10 240** (100)	10 240	5 120	10 240	1 280	20 480	10 240	20 480
DB2 (CD)	5 120 (100)	5 120 (100)	1 280 (50)	**5 120** (100)	1 280	5 120	640	5 120	5 120	5 120
CN (WD)	5 120 (35)	5 120 (35)	10 240 (100)	5 120 (50)	**5 120** (100)	10 240	1 280	10 240	5 120	10 240
AW (WD)	2 560 (71)	2 560 (50)	1 280 (50)	5 120 (100)	1 280 (71)	**5 120** (100)	1 280	5 120	5 120	5 120
DBA (WD)	320 (18)	640 (13)	640 (18)	1 280 (25)	1 280 (35)	5 120 (71)	**2 560** (100)	20 480	10 240	20 480
TS (WD)	640 (13)	640 (18)	640 (25)	2 560 (35)	5 120 (71)	5 120 (50)	1 280 (71)	**20 480** (100)	10 240	20 480
BE (WD)	160 (13)	160 (13)	320 (18)	640 (25)	640 (25)	1 280 (35)	1 280 (71)	10 240 (71)	**10 240** (100)	20 480
216XF (CD)	640 (25)	320 (13)	320 (18)	2 560 (35)	1 280 (35)	1 280 (25)	1 280 (71)	10 240 (71)	10 240 (100)	**20 480** (100)
BW (WD)	320	640	640	2 560	1 280	2 560	640	10 240	10 240	10 240
JAZ (CD)	320	640	640	2 560	1 280	2 560	640	10 240	5 120	10 240
OHC (CD)	320	640	640	2 560	1 280	2 560	2 560	20 480	10 240	20 480
SDC (CD)	320	320	640	2 560	1 280	2 560	1 280	20 480	10 240	20 480

[a]Expressed as the reciprocal of the highest dilution of serum inhibiting cytopathic effects. Homologous titers are in bold. Titers which differed by 16-fold or greater with homologous titers are underlined

[b]*CD* Calf diarrhea, *WD* winter dysentery of adult cattle

[c]In brackets-*R*% values [1, 8]

However, antisera to the Mebus CD, and SD and BM WD strains showed 16-fold or lower VN antibody titers against the DBA, TS, BE and BW WD strains and the 216XF, JAZ, OHC and SDC CD strains than against the homologous strains. These differences were reflected in the *R*% values: the Mebus, SD and BM strains generated *R*% values of 13 to 25 against the DBA, TS, BE and 216XF strains.

The HI antibody titers are shown in Table 3. All of the strains showed cross-reactivity, but differences in antibody titers were observed. The DB2 strain of CD BCV and the SD strain of WD BCV were closely related in the HI tests, and antisera to these strains distinguished most other strains with 16-fold or greater differences in the HI antibody titers.

Bovine coronavirus causes neonatal CD [13] and is also associated with WD of adult cattle [17]. Based on epidemiological data, these disease syndromes often occur as separate and distinct outbreaks in herds [4, 12, 17]; hence antigenic or biological differences between CD and WD BCV might be expected. Calf diarrhea BCV isolates belong to a single serotype [4, 15, 25], but minor antigenic and biological variations among them have been revealed in limited studies [6, 8, 11, 14, 23]. In this study, we compared the antigenic and biological diversity of a variety of WD and CD BCV isolates.

Storz et al. [23] reported that variations in the ratios of HA titers with mouse erythrocytes to those with chicken erythrocytes (M/C HA titer ratio in this report)

Table 3. Hemagglutination inhibition (HI) antibody titers of hyperimmune guinea pig sera against BCV strains

BCV strain (Derivation[b])	HI antibody titers[a] of hyperimmune guinea pig sera to									
	DB2	SD	Mebus	216XF	DBA	CN	BM	AW	TS	BE
DB2 (CD)	≥**20480**	≥20480	320	1280	160	320	320	320	≥20480	5120
SD (WD)	≥20480	≥**20480**	80	2560	160	320	320	320	20480	5120
Mebus (CD)	2560	320	**160**	2560	640	160	320	320	5120	1280
216XF (CD)	1280	80	160	**2560**	80	640	160	320	10240	2560
DBA (WD)	1280	160	160	1280	**160**	640	320	320	≥20480	1280
CN (WD)	640	80	40	640	40	**640**	160	160	10240	640
BM (WD)	640	80	40	1280	80	160	**160**	80	5120	1280
AW (WD)	1280	80	40	2560	80	1280	160	**640**	20480	5120
TS (WD)	5120	160	80	1280	160	1280	320	640	**20480**	5120
BE (WD)	1280	160	160	1280	160	640	80	80	5120	**5120**
BW (WD)	640	80	40	1280	40	160	40	160	10240	2560
JAZ (CD)	1280	160	40	1280	40	320	40	160	10240	5120
OHC (CD)	1280	160	80	2560	80	320	80	320	5120	2560
SDC (CD)	1280	160	40	2560	40	320	80	160	5120	2560

[a]Expressed as the reciprocal of the highest dilution of serum inhibiting HA activity. Homologous titers are in bold. Titers which differed by 16-fold or greater with homologous titers are underlined

[b]*CD* Calf diarrhea, *WD* winter dysentery of adult cattle

were observed among CD BCV strains. The L9 strain of CD BCV at the high cell culture passage level (the 78th passage) showed a low M/C HA titer ratio (8) whereas the wild type CD BCV strains at low cell culture passage levels (the 3rd to 8th passages) showed high M/C HA titer ratios (128 to 256). In this study, the high cell culture-passaged Mebus strain of CD BCV (the 77th passage) showed a low M/C HA titer ratio (2), but some low cell culture-passaged CD strains (DB2, 216XF) and WD strains (CN, DBA, SD, BE, AW) of BCV (the 5th to 10th passages) also showed low M/C ratios (4 to 32). The differences in HA titers against chicken erythrocytes at 4° and 37 °C showed good agreement with the RDE titers for chicken erythrocytes. This suggests that comparison of HA titers obtained at 4° and 37 °C may provide an alternative method for evaluting RDE activity. On the basis of HA and RDE patterns, BCV strains were classified into 3 groups. However, each group contained both CD and WD BCV and no relationship between each group and the clinical (CD or WD) or geographic origin of the strains was observed.

All strains of both CD and WD BCV examined in this study were related antigenically. Specially, each antiserum showed no significant difference between the homologous and heterologous strains in indirect IF antibody titers. However, some antigenic diversity among BCV strains was observed by VN and HI tests. In our previous report [2], hyperimmune antiserum prepared in a gnotobiotic calf to the Mebus strain of CD BCV had an 8- to 32-fold lower VN antibody titer against the DBA strain of WD BCV than against the homologous strain. In this study, guinea pig hyperimmune antiserum to the Mebus strain showed similar VN antibody titer differences between homologous and the DBA strains. In addition, this serum also distinguished three other strains of WD BCV and four strains of CD BCV from the homologous virus by 16-to 64-fold differences. Moreover, hyperimmune antisera to the SD and BM strains of WD BCV also distinguished the same strains which were distinguished by the anti-Mebus serum, form homologous strains by 16- to 64-fold differences in antibody titers in VN tests. Although these variations were recognized only in one-way reactions and all strains examined could be classified into a single serotype, the strains showing these variations might be further divided into 2 subtypes. The Mebus, SD and BM strains which belong to the same potential subtype could be distinguished from the DBA, TS, BE and 216XF strains, which constitute another possible subtype (R% values of 13 to 25). The BW, JAZ, OHC and SDC strains also appeared to belong to the latter subtype. The DB2, CN and AW strains comprised an intermediate group that cross-reacted with both subtypes. Interestingly, antiserum to the Mebus strain of BCV which had been prepared in guinea pig in Japan showed only a 2-fold lower VN antibody titer against the 216XF strain than against the homologous strain [25]. In the present study, antiserum to the Mebus strain prepared in the U.S.A. had 16-fold VN antibody titer differences against the homologous and 216XF strains. The reason for this discrepancy is unknown, but differences in the passage level of the Mebus strain at the preparation of antiserum might affect the virus antigenicity [10]. Alternatively, contamination of cultures with other BCV might occur after import and propagation in Japan.

Bovine coronavirus strains examined in this study showed minor antigenic and biological variations, but this diversity was unrelated to the geographic origin or

affected animal age groups (WD and CD) from which these strains were recovered. Based on preliminary data, gnotobiotic and colostrum-deprived calves inoculated orally and nasally with WD BCV shed BCV rectally and nasally and developed diarrhea, which was indistinguishable from disease symptoms in calves inoculated with CD BCV [9]. Also, a cow inoculated via a duodenal cannula with CD BCV developed diarrhea and shed BCV (H. Tsunemitsu et al. 1994' unpublished data). These results suggest that the differences in these disease syndromes (WD and CD) are not related to viral factors, but to host and environmental factors; e.g. the immunological status of animals, environmental temperatures, secondary or coinfections with other pathogens, etc. [3, 12, 17]. Further studies are in progress to compare the antigenicity of BCV strains using monoclonal antibodies and in vivo cross-protection tests.

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