

Comparison of bovine coronavirus isolates associated with neonatal calf diarrhoea and winter dysentery in adult dairy cattle in Québec

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Cytopathic coronaviruses were isolated in HRT-18 cells from bloody faecal samples collected from cows in Québec dairy herds having experienced typical outbreaks of winter dysentery (WD). The formation of polykaryons in the infected cell cultures was found to be dependent on the presence of trypsin in the medium. The WD isolates differed from the prototype Mebus strain of bovine enteropathogenic coronavirus (BCV.Meb) in respect to haemagglutination inhibition (HI), haemagglutination patterns at 4 °C and 37 °C, and receptor destroying enzyme activity with rat erythrocytes. Other field strains of BCV associated with outbreaks of neonatal calf diarrhoea (NCD) also differed from the BCV.Meb strain by demonstrating differences in HI. In all cases, no differences were detected by virus

neutralization and Western immunoblotting. Analysis and comparison of the nucleotide and deduced amino acid sequences of the PCR-amplified haemagglutinin esterase (HE) genes of one representative WD strain (BCQ.2590) and two highly cytopathic NCD strains (BCQ.3 and BCQ.571) revealed high degrees of similarities (nt and aa sequence homologies > 98%) with the BCV.Meb strain. The putative esterase active site FGDS was conserved among these four BCV strains, indicating that this domain is probably not a determinant for BCV virulence. Six amino acid substitutions occurred between the HE glycoproteins of BCV.Meb and BCQ.2590 strains; two proline substitutions occurred respectively in the signal peptide (at aa 5) and near the sequences of the putative esterase domain (at aa 53).

Outbreaks of acute diarrhoea in adult cattle during the winter season have been reported from a number of countries (Campbell & Cookingham, 1978; Durham *et al.*, 1989; Espinasse *et al.*, 1982; Saif *et al.*, 1988; Takahashi *et al.*, 1980), the disease often being referred to as winter dysentery. Classically, the clinical syndrome is characterized by an acute onset of dark, bloody, liquid diarrhoea in adult cows, accompanied by decreased milk production and variable depression and anorexia. Early investigations attributed the disease to *Campylobacter fetus* subspecies *jejuni* (Campbell & Cookingham, 1978); more recently, there have been several reports associating the disease with infection by coronaviruses (Durham *et al.*, 1989; Saif *et al.*, 1988). In several studies the coronaviruses identified were isolated in cell cultures and shown to be serologically related to the Mebus strain of calf coronavirus (Benfield & Saif, 1990; Saif *et al.*, 1991).

Bovine coronavirus (BCV) represents one of the better

characterized haemagglutinating coronaviruses. The viral particle is mostly spherical, enveloped with a diameter of around 100 nm, and displays two fringes of surface projections (Dea *et al.*, 1980; King *et al.*, 1985; Mebus *et al.*, 1973). The viral genome consists of a large ssRNA with positive polarity, approximately 30 kb in length, and encodes four major structural proteins, two of which are the phosphonucleoprotein N (M_r of 52000) and the matrix glycoprotein M (M_r of 24000 to 26000) (Spana *et al.*, 1988). The longer surface projections characteristic of the coronavirus are formed by the spike glycoprotein S (M_r of 180000 to 200000) which often is post-translationally cleaved by host-cell proteases into two M_r 100000 fragments, S1 and S2, respectively corresponding to the N- and C-terminal subunits (Abraham *et al.*, 1991; Parker *et al.*, 1990). A third envelope glycoprotein, the haemagglutinin, is a disulphide-linked dimer (M_r of subunits 62000) and is associated with the short granular projections located at the base of the typical large bulbous peplomers (Dea & Tijssen, 1989; Dea & Garzon, 1991; Deregt *et al.*, 1988; King *et al.*, 1985). Acetylcysteine activity (AE) is associated with this additional glycoprotein, which is referred to as haemagglutinin esterase (HE) (Parker *et al.*, 1989; Vlasak *et al.*, 1988). The enzyme is able to

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The nucleotide sequence data reported here will appear in the EMBL and GenBank nucleotide databases under accession numbers L38962 for BCQ.2590, L38963 for BCQ.3 and L38964 for BCQ.571.

Table 1. Propagation of Québec bovine coronaviruses in HRT-18 cells: cytopathogenicity, infectivity, haemagglutination titres and serological relatedness to the Mebus strain

Viral isolate	Disease	Passage no.	Type of CPE*	Infectivity titres (log ₁₀ TCID ₅₀ /ml)†	HA titres‡		Reactivity to anti-BCV.Meb§	
					4 °C	37 °C	VN	HI
BCQ.2439	WD	3	C	7.00	256	32	2560	80
BCQ.2508	WD	3	C	6.50	1024	64	1280	80
BCQ.2590	WD	5	C	6.42	512	< 16	2560	80
BCQ.7373	WD	5	C	4.80	512	< 16	NT	160
BCQ.3	NCD	3	B	6.20	512	512	1280	1280
BCQ.9	NCD	3	B	6.00	256	256	2560	1280
BCQ.189	NCD	3	A	6.25	512	512	1280	1280
BCQ.571	NCD	3	C	6.0	256	256	2560	80
BCQ.20	NCD	5	A	6.25	256	256	1280	40
BCQ.2070	NCD	4	C	5.75	128	128	2560	1280
BCV.Meb	NCD	7	A	7.00	1024	1024	2560	1280

* On the basis of cytopathic changes induced in HRT-18 cells, Québec BCV isolates could be classified into weak fusogenic (A), highly fusogenic (C) and non-fusogenic but highly cytolytic (B) strains.

† TCID₅₀ values evaluated using clarified infected cell culture fluids.

‡ Reciprocal value of highest dilution of infected cell culture fluids producing complete HA of rat erythrocytes after 1 h incubation at 4 °C or 37 °C (averaged values for four tests).

§ Reciprocal of highest dilution of polyclonal anti-BCV serum (Mebus strain) that inhibited 100 TCID₅₀ or four HA units of virus.

NT, Non tested.

inactivate receptors for BCV of susceptible cells by hydrolysing an ester bond to release acetate from the C-9 of sialic acid (Vlasak *et al.*, 1988). Both S protein and HE interact with receptors on the cell surface (Schultze *et al.*, 1991 *a, b*) and trigger the immune system eliciting the production of neutralizing antibodies (Dea & Tijssen, 1989; Michaud & Dea, 1993; Vautherot *et al.*, 1992).

Although BCV strains exist that can be distinguished by their pathogenicity, there is still some controversy as to the existence of distinct BCV serotypes (Reynolds *et al.*, 1985; Saif *et al.*, 1991; Takahashi *et al.*, 1980). Recent comparison of BCV isolates associated with outbreaks of neonatal calf diarrhoea in Québec, using polyclonal antisera and MAbs raised against the S glycoprotein, confirmed their close antigenic relationship, but also revealed that they can be assigned to at least three distinct antigenic subgroups (Michaud & Dea, 1993).

The present study was conducted in order to further characterize coronaviruses which have been associated with classical outbreaks of winter dysentery (WD) in adult dairy cattle in Québec (Athanasios *et al.*, 1994). The *in vitro* cultivation properties, enzymatic activities and serological cross-reactivities of Québec WD isolates as compared with the Mebus strain of neonatal calf diarrhoea coronavirus (BCV-Meb) have been investigated. We also reported the nucleotide sequences of the HE gene of a representative Québec WD isolate.

The cell culture-adapted BCV-Meb strain (Mebus *et al.*, 1973) was obtained from the ATCC (ATCC VR-874). The prototype BCV strain was originally isolated in fetal bovine kidney (FBK) cells from diarrhoea fluid of a

calf, and was attenuated by at least 30 consecutive passages in the FBK cells. It was propagated in our laboratory five to ten times in human rectal tumour (HRT-18) cells (Dea & Garzon, 1991). Coronaviral isolates BCQ.3, BCQ.9, BCQ.189, BCQ.571, BCQ.20 and BCQ.2070 were recovered during the winter of 1989 from clinical cases of epidemic diarrhoea in newborn calves (NCD) affecting Québec dairy herds located in four different geographic regions (Michaud & Dea, 1993). Four other isolates were recovered during the winters of 1992 and 1993 from bloody faecal samples collected from adult dairy cows from herds which were experiencing typical WD outbreaks (Table 1). No commercial BCV vaccine had been applied in these herds during the year preceding the emergence of clinical cases. The field strains of BCV were passaged not more than five times in HRT-18 cells in the presence of 10 U/ml of bovine pancreatic trypsin (Dea *et al.*, 1980, 1989). Infectivity titres were determined by titration of clarified tissue culture medium using an end-point dilution procedure and calculation of TCID₅₀/ml (Dea *et al.*, 1989). Haemagglutination (HA) tests with rat and chicken erythrocytes were also performed as previously described (Dea *et al.*, 1989).

Identification of the various Québec BCV isolates from faecal samples was confirmed by negative stain electron microscopy and by indirect ELISA (Athanasios *et al.*, 1994). Protein A-immunogold labelling of aggregated coronaviral particles, following incubation with rabbit hyperimmune serum raised against the BCV-Meb strain (Dea & Garzon, 1991), further confirmed the identification of the virus (data not shown).

Table 2. Infectivity, AE, HA and RDE activities of purified preparations of BCV strains associated with NCD and WD in adult cattle

Isolate*	Disease	Infectivity titre† (log ₁₀ TCID ₅₀ /50 µl)	Acetylerase‡ (per 15 µl)	Haemagglutination§		RDE titre	
				Chicken	Rat	Chicken	Rat
BCQ.2439	WD	7.2	3.292	16	8192	16	8
BCQ.2508	WD	8.7	3.089	32	8192	4	64
BCQ.2590	WD	9.4	0.764	64	4096	64	128
BCQ.7373	WD	8.7	2.151	64	4096	64	256
BCQ.3	NCD	8.4	0.492	64	4096	32	< 2
BCQ.9	NCD	9.7	1.826	16	8192	16	2
BCQ.189	NCD	8.9	3.388	128	8192	64	< 2
BCQ.2070	NCD	7.7	3.210	8	4096	8	< 2
BCQ.571	NCD	7.5	1.782	32	8192	2	< 2
BCQ.20	NCD	7.9	1.877	64	4096	32	< 2
BCV.MEB	NCD	9.4	3.155	128	16384	8	2

* Field strains of BCV were propagated not more than five passages in HRT-18 cells in the presence of bovine trypsin.

† Extracellular virions were concentrated and purified by isopycnic ultracentrifugation on continuous 20 to 50% (v/v) sucrose gradients.

‡ Absorbance at 405 nm after 5 min of reaction with 1 mM-*p*-nitrophenyl acetate.

§ Reciprocal value of highest dilution producing complete haemagglutination after 1 h incubation at 4 °C.

|| Reciprocal value of highest dilution producing complete disaggregation of BCV-erythrocyte complexes after 1 h at 37 °C.

On the basis of cytopathic changes induced upon the third to fifth passages in HRT-18 cells, the Québec BCV isolates could be classified into weak fusogenic (induced production of small discrete syncytia, containing less than 10 nuclei), highly fusogenic [syncytia progressively increased in number and size, containing up to 40 nuclei, leading to complete destruction of the cell sheets within 72 to 96 h post-infection (p.i.)], and non-fusogenic but highly cytolytic (no syncytia produced but intense degeneration of the monolayers obtained within 96 h p.i.) strains (Table 1). The four WD isolates behaved as highly fusogenic strains, provided trypsin was present in the maintenance medium. Under standard conditions, the virus yield after three to five successive passages was similar for the different BCV isolates, as revealed by indirect immunofluorescence, indirect immunoperoxidase (Michaud & Dea, 1993) and the calculation of infectivity titres. The extracellular virions of the various BCV isolates were purified from clarified infected cell culture fluids by differential and isopycnic ultracentrifugation on continuous 20 to 55% (v/v) sucrose gradients (Dea *et al.*, 1989). Electron microscopic examination of purified viral preparations revealed that most viral particles possessed the double fringe of surface projections (Dea & Garzon, 1991).

The HA patterns of the NCD and WD isolates differed. From supernatant fluids of infected HRT-18 cells, comparable HA titres with rat erythrocytes were obtained for both WD and NCD isolates after 1 h incubation at 4 °C. Interestingly, a drastic drop in the HA titres of WD isolates was noticed if the test was conducted at 37 °C, whereas the incubation temperature

did not seem to affect the HA activity of NCD isolates when tested with rat erythrocytes (Table 1). On the other hand, the approximately 100-fold concentrated and purified preparations of all BCV strains tested agglutinated chicken erythrocytes at very low titres at 4 °C (Table 2).

To determine the antigenic relatedness of the WD and NCD isolates, virus neutralization (VN) and haemagglutination-inhibition (HI) tests were performed as previously described using rabbit hyperimmune serum raised against the BCV.Meb strain (Dea *et al.*, 1980; Dea & Tijssen, 1989). Although no differences were detected by VN, a 16- to 32-fold difference in HI titres was obtained between the WD coronaviruses and the reference BCV.Meb strain (Table 1). Except for BCQ.20 and BCQ.571, other NCD isolates reacted to HI titres similar to those of BCV.Meb strain.

The structural polypeptide profiles of sucrose gradient-purified WD isolates were compared to that of BCV.Meb by SDS-PAGE and by Western immunoblotting (Dea & Tijssen, 1989). Under non-reducing conditions, similar polypeptide profiles were identified for all BCV isolates analysed. The four major proteins described previously for reference NCD coronavirus strains (King *et al.*, 1985; Deregt *et al.*, 1988; Dea & Tijssen, 1989) were identified for all WD isolates and reacted similarly to the BCV.Meb strain in Western immunoblotting analyses using homologous rabbit hyperimmune serum (data not shown).

To further investigate the biological properties of WD isolates compared with NCD isolates, different viral functions were assessed with purified BCV isolates containing infectivities of 10^{7.2} to 10^{9.4} TCID₅₀/ml.

BCV. Meb	CTAAACTCAGTGA AAAATGTTTTTCTTCTAGATTGTTCTAGTTAGCTGCATAAATGGTAGCC TAGGTTTTGATAACCCCTCTACCAATGTTGTTTCGC	100
BCQ. 3AA.....C.....TT.....C.....	100
BCQ. 571AT.....T.....TC.....T.....	100
BCQ. 2590GA.....C.....CT.....C.....	100
BCV. Meb	ATTTAAATGGAGATTGGTTTTTATTGGTGACAGTCGTTTCAGATTGTAATCATGTTGTTAATACCAACCCCGTAATTATCTTATATGGACCTTAATCC	200
BCQ. 3G.....T.....	200
BCQ. 571G.....T.....	200
BCQ. 2590C.....C.....	200
BCV. Meb	TGCCCTGTGATCTCGTAAATAATCATCTAAAGCTGGCAACTCCATTTT TAGGAGTTTTCACTTTACCGATTTTTATAATTACACAGGCGAAGGTCAA	300
BCQ. 3C.....	300
BCQ. 571T.....	300
BCQ. 2590C.....	300
BCV. Meb	CAAATTATTTTTATGAGGGTGTAAATTTACGCCTTATCATGCCCTTAAATGCACCACTTCGTTAGTAGTAATGATATTTGGATGCAGAATAAAGCCTTGT	400
BCQ. 3C.....G.....C.....	400
BCQ. 571T.....C.....T.....	400
BCQ. 2590C.....G.....T.....	400
BCV. Meb	TTTACACTCAGGTTTATAAGAATATGGCTGTGTATCGCAGCCCTTACTTTTGTAAATGTACCATATGTTTATAATGGCTCTGCACAATCTACAGCTCTTTG	500
BCQ. 3	500
BCQ. 571	500
BCQ. 2590	500
BCV. Meb	TAAATCTGGTAGTTAGTTCTTAATAACCCCTGCATATATAGCTCGTGAAGCTAATTTGGGGATTATTATAAGGTTGAAGCTGACTTTTATTGTC	600
BCQ. 3T.....G.....T.....C.....	600
BCQ. 571T.....T.....A.....C.....	600
BCQ. 2590C.....T.....T.....T.....	600
BCV. Meb	GGTTGTGACGAGTATATCGTACCCTTTGTATTTTAAACGGCAAGTTTTTGTGCGAATACAAAGTATTATGATGATAGTCAATATTATTTTATAAAGACA	700
BCQ. 3	700
BCQ. 571	700
BCQ. 2590	700
BCV. Meb	CTGGTGTATTATTTATGGTCTCAATCTACTGAAACCATTACCCTGGTTTTGATTTTAAATGTCAATTATTTAGTTTTACCCCTCTGGTAATTATTTAGCCAT	800
BCQ. 3	800
BCQ. 571	800
BCQ. 2590	800
BCV. Meb	TTCAAATGAGCTATTGTTAACTGTTCTACGAAAGCAATCTGTCTTAAACAAGCGTAAGGATTTTACGCCCTGTACAGGTTGTTGATTACGGTGAACAAT	900
BCQ. 3T.....C.....T.....A.....	900
BCQ. 571G.....T.....C.....G.....	900
BCQ. 2590G.....T.....C.....G.....	900
BCV. Meb	GCCAGGCAGTCTGATAACATGACGGCGTTGCTTGTCAACCCCGTACTGTTATTTTCGTAATCTACTACCAACTATGTTGGTGTTTATGATATCAATC	1000
BCQ. 3G.....T.....	1000
BCQ. 571A.....T.....	1000
BCQ. 2590A.....T.....	1000
BCV. Meb	ATGGGGATGCTGGTTTTACTAGCATACTCAGTGGTTTGTATATGATTCACCTTGTTTTTCGCAGCAAGGTGTTTTTAGGTATGATAATGTTAGCAGTGT	1100
BCQ. 3AC.....A.....	1100
BCQ. 571AC.....A.....	1100
BCQ. 2590CC.....G.....	1100
BCV. Meb	CTGGCCTCTCTATTCCCTATGGCAGATGCCCTACTGCTGCTGATATTAATACCCCTGATGTACCTATTTGTGTGTATGATCCCGCTACCCTTATTTGCTT	1200
BCQ. 3TT.....A.....C.....A.....	1200
BCQ. 571TT.....A.....C.....A.....	1200
BCQ. 2590CC.....A.....T.....C.....	1200
BCV. Meb	GGCATCCTTTTGGGTGTTGCGGCATAAATTATTGAGTTTTTGTGTTATATTTATGGTGATAATGGTACTAGGCTGCATGATGCTTAGACCATAAATC	1300
BCQ. 3T.....	1300
BCQ. 571T.....	1300
BCQ. 2590C.....	1300

BCV. Meb	AAAC	1304
BCQ. 3	1304
BCQ. 571	1304
BCQ. 2590	1304

Fig. 1. Nucleotide sequence comparisons of the HE genes of the Québec WD strain BCQ.2590, Québec NCD strains BCQ.3 and BCQ.571, and the reference BCV.Meb strain. cDNA synthesis and PCR amplification were performed as described previously (Rekik & Dea, 1994). The following primers were chosen in order to permit amplification of a product of 1387 bp in length spanning the entire HE gene: oligo HE.A, 5' TTTATGAATCTCCAGTTG 3', a sense primer corresponding to the sequence located -40 to -22 nt upstream of the 5' end of the HE gene, and oligo HE.B, 5' TTAATCTCCTATAACAGC 3', an antisense primer representing the sequence at position 40 to 58 of the S protein gene (Parker *et al.*, 1989, 1990). Sequencing of cDNA clones was performed on both

Acetylcholinesterase activity of purified BCV preparations was determined according to Vlasak *et al.* (1988) with modifications suggested by Storz *et al.* (1992). Aliquots (15 µl) of purified preparations of the different BCV strains were added to 1 ml of phosphate-buffered saline containing 1 mM-*p*-nitrophenyl acetate (PNPA). Hydrolysis of the substrate following an incubation period of 5 min was monitored at a wavelength of 405 nm in a spectrometer (Spectronic 2000, Milton Roy). The method described by Storz *et al.* (1991) was used to determine the titre of the receptor destroying enzyme (RDE) activity. Haemagglutination tests were first done as described above and HA titres were determined after an incubation period of 1 h at 4 °C. Thereafter, the microtitre plates were shifted to 37 °C for 1 h to monitor inactivation of receptors reflected by the breakdown of the BCV-erythrocyte complexes mediated by the AE in the RDE assay. The enzymatic activities of the different purified viral preparations are compared in Table 2.

Although high variability was observed amongst the infectivity titres of the BCV strains tested, they appeared to possess comparable AE activity in the PNPA test, with the exception of Québec isolates BCQ.3 and BCQ.2590 that showed a very weak AE activity. Purified NCD and WD isolates agglutinated rat erythrocytes with similar titres at an incubation temperature of 4 °C.

The RDE titres determined for WD isolates with rat erythrocytes varied from 8 to 256. In contrast, elution through RDE activity was minimal or not detectable in tests involving the NCD isolates since no reduction in the HA titres of the NCD strains with rat erythrocytes was noted following incubation at 37 °C. On the other hand, all BCV strains tested had HA titres 128- to 256-fold lower with chicken erythrocytes, elution through RDE activity being detected with titres of 16 to 64.

For the preparation of genomic RNA, aliquots (50 µl) of purified virus were supplemented with 1 µl of RNAGuard (Pharmacia) and RNA extraction was performed by the one-step guanidinium isothiocyanate-acid phenol method (Chomczynski & Sacchi, 1987). cDNA synthesis, PCR amplification and DNA sequencing were performed as reported previously (Rekik & Dea, 1994). To assess the error rate of the reverse transcriptase and *Taq* polymerase, clones from different RT-PCR events were sequenced. Errors generated by T7 polymerase were avoided by sequencing four randomly selected clones in both directions.

We sequenced the complete HE genes of one representative WD isolate (BCQ.2590) and two Québec NCD isolates (BCQ.3 and BCQ.571). The alignments of nucleotide and deduced amino acid sequences are presented in Figs 1 and 2, respectively. These sequences

BCV.Meb	<u>M</u> <u>F</u> <u>L</u> <u>L</u> <u>L</u> <u>R</u> <u>F</u> <u>V</u> <u>L</u> <u>V</u> <u>S</u> <u>C</u> <u>I</u> <u>I</u> <u>G</u> <u>S</u> <u>L</u> <u>G</u> <u>P</u> <u>D</u> <u>N</u> <u>P</u> <u>P</u> <u>T</u> <u>N</u> <u>V</u> <u>S</u> <u>H</u> <u>L</u> <u>N</u> <u>G</u> <u>D</u> <u>W</u> <u>F</u> <u>L</u> <u>F</u> <u>G</u> <u>D</u> <u>S</u> <u>R</u> <u>S</u> <u>D</u> <u>C</u> <u>N</u> <u>H</u> <u>V</u> <u>V</u> <u>N</u> <u>T</u> <u>N</u> <u>P</u> <u>R</u> <u>N</u> <u>S</u> <u>Y</u> <u>M</u> <u>D</u> <u>L</u> <u>N</u> <u>P</u> <u>A</u> <u>L</u> <u>C</u> <u>D</u> <u>S</u> <u>G</u> <u>K</u> <u>I</u> <u>S</u> <u>S</u> <u>K</u> <u>A</u> <u>G</u> <u>N</u> <u>S</u> <u>I</u> <u>F</u> <u>R</u> <u>S</u> <u>F</u> <u>H</u> <u>T</u> <u>D</u> <u>F</u> <u>Y</u> <u>N</u> <u>T</u> <u>G</u> <u>E</u> <u>G</u> <u>Q</u> <u>Q</u> <u>I</u> <u>I</u> <u>F</u> <u>Y</u>	100
BCQ.3	..P..V.....R.....	100
BCQ.571	..L..V.....R.....	100
BCQ.2590	..P..A.....P.....	100
BCV.Meb	<u>E</u> <u>G</u> <u>L</u> <u>N</u> <u>F</u> <u>T</u> <u>P</u> <u>Y</u> <u>H</u> <u>A</u> <u>F</u> <u>K</u> <u>C</u> <u>T</u> <u>T</u> <u>S</u> <u>G</u> <u>S</u> <u>N</u> <u>D</u> <u>I</u> <u>W</u> <u>M</u> <u>Q</u> <u>N</u> <u>K</u> <u>G</u> <u>L</u> <u>F</u> <u>Y</u> <u>T</u> <u>Q</u> <u>V</u> <u>Y</u> <u>K</u> <u>N</u> <u>M</u> <u>A</u> <u>V</u> <u>Y</u> <u>R</u> <u>S</u> <u>L</u> <u>T</u> <u>F</u> <u>V</u> <u>N</u> <u>P</u> <u>Y</u> <u>V</u> <u>Y</u> <u>N</u> <u>G</u> <u>S</u> <u>A</u> <u>Q</u> <u>S</u> <u>T</u> <u>A</u> <u>L</u> <u>C</u> <u>K</u> <u>S</u> <u>G</u> <u>S</u> <u>V</u> <u>L</u> <u>N</u> <u>N</u> <u>P</u> <u>A</u> <u>I</u> <u>A</u> <u>R</u> <u>E</u> <u>A</u> <u>N</u> <u>F</u> <u>G</u> <u>D</u> <u>Y</u> <u>Y</u> <u>Y</u> <u>K</u> <u>V</u> <u>E</u> <u>A</u> <u>D</u> <u>F</u> <u>Y</u> <u>L</u> <u>S</u> <u>G</u> <u>C</u> <u>D</u> <u>E</u> <u>Y</u>	200
BCQ.3	..V.....	200
BCQ.571	..L.....	200
BCQ.2590	..V.....	200
BCV.Meb	<u>I</u> <u>V</u> <u>P</u> <u>L</u> <u>C</u> <u>I</u> <u>F</u> <u>N</u> <u>G</u> <u>K</u> <u>F</u> <u>L</u> <u>S</u> <u>N</u> <u>T</u> <u>R</u> <u>Y</u> <u>Y</u> <u>D</u> <u>S</u> <u>Q</u> <u>Y</u> <u>F</u> <u>N</u> <u>K</u> <u>D</u> <u>T</u> <u>G</u> <u>V</u> <u>I</u> <u>Y</u> <u>G</u> <u>L</u> <u>N</u> <u>S</u> <u>T</u> <u>E</u> <u>T</u> <u>I</u> <u>T</u> <u>T</u> <u>G</u> <u>F</u> <u>D</u> <u>F</u> <u>N</u> <u>C</u> <u>H</u> <u>Y</u> <u>L</u> <u>V</u> <u>L</u> <u>P</u> <u>S</u> <u>G</u> <u>N</u> <u>Y</u> <u>L</u> <u>A</u> <u>I</u> <u>S</u> <u>N</u> <u>E</u> <u>L</u> <u>L</u> <u>L</u> <u>T</u> <u>V</u> <u>P</u> <u>T</u> <u>K</u> <u>A</u> <u>I</u> <u>C</u> <u>L</u> <u>N</u> <u>K</u> <u>R</u> <u>K</u> <u>D</u> <u>F</u> <u>T</u> <u>V</u> <u>Q</u> <u>V</u> <u>D</u> <u>S</u> <u>R</u> <u>W</u> <u>N</u> <u>N</u> <u>A</u> <u>R</u> <u>Q</u> <u>S</u> <u>D</u>	300
BCQ.3	300
BCQ.571	300
BCQ.2590	300
BCV.Meb	<u>N</u> <u>M</u> <u>T</u> <u>A</u> <u>V</u> <u>A</u> <u>C</u> <u>Q</u> <u>P</u> <u>P</u> <u>Y</u> <u>C</u> <u>Y</u> <u>F</u> <u>R</u> <u>N</u> <u>S</u> <u>T</u> <u>T</u> <u>N</u> <u>Y</u> <u>V</u> <u>G</u> <u>V</u> <u>Y</u> <u>D</u> <u>I</u> <u>N</u> <u>H</u> <u>G</u> <u>D</u> <u>A</u> <u>G</u> <u>F</u> <u>T</u> <u>S</u> <u>I</u> <u>L</u> <u>S</u> <u>G</u> <u>L</u> <u>L</u> <u>Y</u> <u>D</u> <u>S</u> <u>P</u> <u>C</u> <u>F</u> <u>S</u> <u>Q</u> <u>G</u> <u>V</u> <u>F</u> <u>R</u> <u>Y</u> <u>D</u> <u>N</u> <u>V</u> <u>S</u> <u>S</u> <u>V</u> <u>W</u> <u>P</u> <u>L</u> <u>Y</u> <u>S</u> <u>G</u> <u>R</u> <u>C</u> <u>P</u> <u>T</u> <u>A</u> <u>A</u> <u>D</u> <u>I</u> <u>N</u> <u>T</u> <u>P</u> <u>D</u> <u>V</u> <u>P</u> <u>I</u> <u>C</u> <u>V</u> <u>Y</u> <u>D</u> <u>P</u> <u>L</u> <u>L</u> <u>I</u> <u>L</u> <u>G</u> <u>I</u> <u>L</u> <u>L</u> <u>G</u>	400
BCQ.3D.....Q.....S.....I.....	400
BCQ.571D.....Q.....S.....I.....	400
BCQ.2590A.....R.....P.....	400
BCV.Meb	<u>V</u> <u>A</u> <u>V</u> <u>I</u> <u>I</u> <u>V</u> <u>L</u> <u>L</u> <u>L</u> <u>L</u> <u>L</u> <u>F</u> <u>M</u> <u>V</u> <u>D</u> <u>N</u> <u>G</u> <u>T</u> <u>R</u> <u>L</u> <u>H</u> <u>D</u> A.....	424
BCQ.3	424
BCQ.571	424
BCQ.2590	424

Fig. 2. Alignment of the deduced amino acid sequences of the HE proteins of the Québec WD strain BCQ.2590, Québec NCD strains BCQ.3 and BCQ.571, and the reference BCV-Meb strain. The predicted signal peptide and intramembrane-anchoring sequences are underlined. The potential N-linked glycosylation sites are in bold and underlined. The putative esterase active site (FGDS) is marked by asterisks.

strands by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) using T7 DNA polymerase (Pharmacia). Sequences and additional primers used for sequencing HE gene were: oligo S418 (5' AAGAATATGGCTGTGTATCG 3'), oligo S881 (5' TTGATTCACGGTGAACAATG 3'), oligo S1083 (5' TGATAATGTTAGCAGTGTCTGG 3'), and the four corresponding reverse complementary primers. Sequences were analysed with the aid of the Mac Vector 3.5 (International Biotechnologies) and GeneWorks 2.2 (IntelliGenetics) sequence analysis programs. Asterisks indicate the conserved intergenic sequences and the start and stop codons are underlined.

were compared with those of the reference BCV.Meb strain (Parker *et al.*, 1989). All of the HE genes contained a large ORF of 1272 nt encoding a predicted protein of 424 amino acids. Frameshift, deletion or insertion, and nonsense mutations were not observed. Comparison of HE genes of the NCD isolates BCQ.3 and BCQ.571 did not demonstrate significant variations from that of the reference BCV.Meb strain. The only variations that were identified consisted of 10 to 12 nucleotide substitutions, which represented 1.2% of the HE gene sequence. In comparison, there were 19 nucleotide substitutions between the BCQ.2590 and BCV.Meb strains (nt sequence homologies of 98.5%), and 11 appeared to be specific to the WD isolate (Fig. 1). Despite these high degrees of similarity at the nucleotide level, the deduced amino acid sequences presented interesting features (Fig. 2). A proline substitution occurred in the signal peptide (at aa 5) between the avirulent BCV.Meb strain, the WD isolate BCQ.2590 and the NCD isolate BCQ.3; this proline substitution was also previously identified for the virulent BCV-LY138 strain (Zhang *et al.*, 1991). Whether this substitution could alter the maturation and intracellular transport of the HE glycoprotein remains to be elucidated. Five additional amino acid substitutions occurred between the HE proteins of the reference BCV-Meb strain and the WD isolate BCQ.2590: Val to Ala (aa 8), Arg to Pro (aa 53), Asp to Ala (aa 344), Gln to Arg (aa 350) and Ser to Pro (aa 367). The proline substitution at aa 367 was also previously reported for the virulent BCV-LY138 strain (Zhang *et al.*, 1991), whereas the proline substitution at aa 53 seems to be unique to the WD isolate. Interestingly, this substitution occurred in the vicinity of the sequence FGDS of the putative esterase active domain which was conserved in all BCV strains analysed.

Previous studies on the morphological and serological characteristics of coronaviruses associated with typical outbreaks of WD in adult cattle suggested a close resemblance with the reference BCV.Meb strain (Athanasious *et al.*, 1994; Saif *et al.*, 1991). However, in the first report by Benfield & Saif (1990) of the successful isolation in cell cultures of such WD strains of BCV, possible antigenic differences between the WD and the reference BCV strains were suggested. Our results are in agreement with these previous findings, but also provide additional information on the biological and molecular properties of these particular BCV strains.

First, as previously reported by others (Benfield & Saif, 1990), the four WD isolates characterized in the present study behaved as highly fusogenic BCV isolates. The formation of polykaryons in the infected cell cultures appeared to be dependent on the presence of proteolytic enzymes in the medium, since a reduction in the visible CPE was noticed in the absence of trypsin. Although,

this could not be considered as a distinct feature of the WD isolates, it remains of interest since the CPE induced by the avirulent BCV.Meb strain was less severe. Recently, the NCD isolates BCQ.571 and BCQ.2070, which behaved also as highly fusogenic strains, could be differentiated from the prototype BCV.Meb strain by the amino acid sequences of their S glycoproteins proximal to the proteolytic cleavage site (Rekik & Dea, 1994). Whether such amino acid changes in the S glycoproteins might be related to cytopathogenicity of WD isolates remains to be elucidated. A second biological property that permitted us to differentiate WD from NCD isolates of BCV involves their interaction with different erythrocytes. Interestingly, such difference in the haemagglutinating properties between virulent (field strains) and avirulent (vaccine) BCV strains was also recently reported by Storz *et al.* (1992). In their study, these authors have demonstrated that all BCV strains tested agglutinated chicken erythrocytes and possessed RDE activity. On the other hand, as demonstrated in our study, both virulent (BCV.LY138) and avirulent (BCV.L9) BCV strains agglutinated mouse erythrocytes with higher titres, but elution through RDE activity was minimal. Thus, WD isolates are apparently distinct strains of BCV as the RDE activity of their HE glycoprotein is also very effective for inactivation of receptors on rat erythrocytes. This difference between WD and NCD isolates was further substantiated in HA tests with concentrated and purified viral preparations.

It has been postulated that difference in the HA pattern of avirulent and virulent BCV strains was probably determined by strain-dependent receptor binding properties, or possible differences between Neu5,9Ac₂-containing receptors on chicken and murine erythrocytes, or the greater abundance of receptors on murine erythrocytes (Schultze *et al.*, 1991*b*; Storz *et al.*, 1992). Results from comparative studies on the HA properties of WD and NCD isolates also suggest that the affinity for such receptors is probably dependent on the RDE activity of the HE protein. There exist several reports indicating that the AE of BCV is probably not directly involved in viral uptake during the infectious process (Storz *et al.*, 1991, 1992; Parker *et al.*, 1989; Vlazak *et al.*, 1988; Schultze *et al.*, 1991*a*). On the other hand, receptor binding and viral attachment to susceptible cells in infections of non-HA and HA coronaviruses are mediated by the S glycoprotein (Spaan *et al.*, 1988). The AE activity of the HE protein may play a role in facilitating virus release from infected cells and viral spread (Vlasak *et al.*, 1988). A higher RDE activity could partly explain the highly contagious nature of BCV strains associated with outbreaks of WD and the short duration of the syndrome within the affected herds (Durham *et al.*, 1989).

MAbs raised against the HE and S protein inhibited HA by BCV.L9 strain, and both proteins possess antigenic determinants that elicit the production of neutralizing antibodies (Dea & Tijssen, 1989; Michaud & Dea, 1993; Vautherot *et al.*, 1992). It has been also demonstrated that anti-HE MAbs are predominantly raised against the RDE activity (Storz *et al.*, 1991). The demonstration that purified HE of BCV.L9 had AE activity but agglutinated only mouse erythrocytes while purified S protein had a more powerful HA for chicken and mouse erythrocytes (Schultze *et al.*, 1991*a, b*) substantiates the need to investigate the genomic and antigenic variations between NCD and WD isolates at the S protein level. In the present study, no differences were detected between WD and NCD strains by seroneutralization, and sequencing analyses of their HE genes did not permit us to identify major differences that could explain the antigenic variability observed by HI. Nevertheless, it remains to be demonstrated whether the two proline substitutions that occurred in the signal peptide and in the vicinity of the sequences of the putative AE domain of the HE protein, respectively, could explain the weak reactivity of the BCQ.2590 isolate to the reference hyperimmune serum by HI, as well as enhancement of its RDE activity with rat erythrocytes. Inclusion of a proline residue in the sequence of a peptide or protein is expected to result in unique conformational preferences that may, or may not, be biologically significant (Yaron & Naider, 1993). Whether the proline substitutions in the HE protein of BCV isolates associated with WD could result in a conformational change facilitating elution of WD isolates from mouse or rat erythrocytes through RDE activity remains to be elucidated. Further antigenic and genetic characterization of additional WD isolates, from various geographical areas, is necessary to determine the extent of variation at the level of both HE and S protein genes. Such studies, as well as production of MAbs raised against purified HE protein of an antigenically distinct WD isolate are presently in progress.

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