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Bovine coronaviruses associated with enteric and respiratory diseases in Canadian dairy cattle display different reactivities to *anti*-HE monoclonal antibodies and distinct amino acid changes in their HE, S and ns4.9 protein

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Abstract

Bovine coronavirus isolates associated with recent outbreaks of respiratory disease in Ontario and Quebec dairy farms were compared to reference strains known to be responsible for neonatal calf diarrhea (NCD) or winter dysentery (WD) of adult cattle. In respect to their hemagglutinating properties and their higher RDE activities with rat erythrocytes, WDBCoV strains differed from NCDBCoV strains and respiratory bovine coronaviruses RBCoV strains. Serologically, three MAbs directed to the HE glycoprotein of the WDBCoV strain BCQ.2590 recognized two serogroups amongst NCDBCoV strains by hemagglutination inhibition, whereas only one of the MAbs failed to react toward three of the four RBCoV isolates tested. Sequencing analysis of the S (S1 portion), HE, ORF4 and ORF5 genes of BCoV isolates associated with different clinical syndromes indicated that neither insertions or deletions could explain their distinct tropism. For the HE glycoprotein, a total of 15 amino acids (aa) substitutions were identified by comparing field isolates to the prototype Mebus strain. Two specific proline substitutions were identified for virulent strains being located in the signal peptides (aa 5) and aa position 367; one specific aa change was revealed at position 66 for RBCoV field isolates. Analysis of the S1 portion of the S glycoprotein revealed a total of eight aa changes specific to enteropathogenic (EBCoV) strains and eight aa changes specific to RBCoV strains. For all BCoV isolates studied, the region located between the S and M genes (ORF4) apparently encodes for two non-structural (ns) proteins of 4.9 and 4.8 kDa. A specific non-sense mutation was identified for the nucleotide at position 88 of the putative 4.9 kDa protein gene of RBCoV isolates resulting in 29 rather that 43 aa residues. The ORF5, which encodes a 12.7 ns protein and the 9.5 kDa E protein, was highly conserved amongst the BCoV field isolates. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Bovine enteropathogenic and respiratory coronaviruses; Winter dysentery; Antigenic and genomic variability; Esterase; Hemagglutinin; Monoclonal antibodies; Structural proteins

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Bovine coronavirus (BCoV) is a member of the family Coronaviridae, order Nidovirales (De Vries et al., 1997). The genome of coronaviruses consists in a single stranded, capped, and polyadenylated positive-sense RNA molecule of approximately 26 to 30 kb in length, that yields a 3′-coterminal nested set of subgenomic mRNAs in the infected cells (Spaan et al., 1981; Lai, 1986). Coronaviruses are mostly spherical in shape, enveloped with a diameter ranging between 120 and 150 nm, and possess an helical nucleocapsid. The virion contains four structural proteins: the spike (S) glycoprotein of 160–200 kDa which forms the long club-shaped surface projections, the matrix glycoprotein (M) of 20–38 kDa, the phosphorylated nucleocapsid protein (N) of 50–60 kDa and the small membrane protein (E) of about 9.5 kDa (Spaan et al., 1988 (a review)). Based on serological cross-reactivities and genomic relatedness, the coronaviruses have been separated into three distinct subgroups (Schockley et al., 1987; Dea et al., 1990). The BCoV belongs to the second subgroup, with hemagglutinating encephalomyelitis virus of swine (HEV), human respiratory coronavirus (HCoV-OC43), turkey enteric coronavirus (TCoV) and murine hepatitis coronaviruses (MHVs). Members of this subgroup possess an additional 140 kDa envelope-associated glycoprotein, the hemagglutinin–esterase (HE), corresponding to a second fringe of granular surface projections (King et al., 1985; Hogue and Brian, 1986; Dea et al., 1990). Both the S and HE glycoproteins are able to hemagglutinate red blood cells by binding to *N*-acetyl-9-*O* neuraminic acid (Vlasak et al., 1988; Schultze et al., 1991b), elicit the production of virus neutralizing antibodies (Dea et al., 1990; Vautherot et al., 1992), and are probably involved in determining the viral tissue and host tropism (Spaan et al., 1988). The HE is further associated to acetyl esterase (AE) and receptor destroying enzyme (RDE) activities (Vlasak et al., 1988; Parker et al., 1990). Few investigators have also postulated that the regions situated between the S and M genes (ORF4 and ORF5), potentially encoding non-structural (ns) proteins of 4.9, 4.8 and 12.7 kDa, and the small 9.5 kDa membrane protein (E), could be involved in the presumed preferential respiratory tropism of HCoV-OC43 and HEV (Abraham et al., 1990b; Mounir and Talbot, 1993; Vieler et al., 1996).

Althought BCoV isolates are generally recognized as being associated with enteric diseases of newborn calves (NCD: neonatal calf diarrhea) (Mebus et al., 1973) and winter dysentery (WD) or chronic diarrhea in adult cattle (Crouch et al., 1985; Benfield and Saif, 1990), there exist much evidences that other isolates may be responsible or contribute to acute respiratory problems in feedlot cattle (Reynolds et al., 1985; Storz et al., 1992). A recent survey conducted in feedlots or livestock shows in several states in the USA, emphasized the role of RBCoV in the bovine shipping fever syndrome pneumonia (Storz et al., 1996, 2000). Despite their association to different clinical pathologies, BCoV isolates apparently belong to a single serotype on the basis of virus neutralisation (VN) and hemagglutination inhibition (HAI) tests using polyclonal hyperimmune sera. However, data obtained from recent serological findings using *anti*-HE and *anti*-S MAbs suggest the existence of different subgroups of BCoV, each containing EBCoV and WDBCoV strains responsible for either NCD, WD or chronic shedding in adult cattle (El Ghorr et al., 1989; Tsunemitsu and Saif, 1995; Milane et al., 1997; Fukutomi et al., 1999). Antigenic and genomic variations have been also recently reported among EBCoV and RBCoV strains (Chouljenko et al., 1998; Hasoksuz et al., 1999). The significance of such variability in the virulence and tropism of field isolates of BCoV is still unknown.

The purpose of the present study was to analyse the biological, serological and genomic properties of cytopathogenic RBCoV strains recently isolated in Quebec and Ontario, Canada, in comparison to the reference Mebus strain. We further compared the RBCoV strains to previously characterized Quebec EBCoV strains isolated in newborn calves with diarrhea and to Quebec WDBCoV strains in order to identify specific strain markers that should be considered for diagnosis and development of vaccines.

The cell culture-adapted and attenuated Mebus strain of BCoV was obtained from the American Type Culture Collection (ATCC VR 874,

Rockville, MD). This prototype NCD strain was originally isolated from diarrhea fluid of a 2-week-old calf and attenuated following more than 30 successive passages in fetal bovine kidney (FBK) cells (Mebus et al., 1973). The 67N strain of porcine hemagglutinating encephalomyelitis virus (VR 741) was also obtained from the ATCC, whereas the HCoV-OC43 virus was kindly provided to us by PJ Talbot, INRS-Institut Armand-Frappier, Laval, Quebec, Canada. The RBCoV reference strain OK-0514, isolated from the nasal swab of a calf with clinical respiratory tract disease subsequent to shipping from Oklahoma to a feedlot in Kansas, was kindly provided to us by J. Storz, School of Veterinary Medicine of Louisiana (Storz et al., 1996). Field strains of EBCoV associated with either diarrhea in newborn calves or WD in adult cattle were obtained from affected dairy herds from different geographical areas in Quebec, Canada (Dea et al., 1995; Milane et al., 1997). The RBCoV strain BCQ.3994 was isolated from the lungs of pneumonic growing calves from a dairy farm in Southern Quebec that developed signs of respiratory tract disease during 1998, whereas two other Canadian RBCoV strains (BCO.43277 and BCO.44175) were isolated from pneumonic growing calves with pneumonia from two different Ontario dairy farms during 1997 (kindly provided to us by S. Carman, Ontario Ministry of Agriculture and Fisheries, Ontario Veterinary College, Guelph, Ontario, Canada). All field isolates of BCoV were propagated for less than five passages in the G clone of humal rectal tumor cells (HRT-18G) developed through selection and medium modulation (Storz et al., 1996). Trypsin-treatment of field isolates, as well as routine addition of 10 U/ml of bovine pancreatic trypsin in the maintenance medium of infected cultures, were done as described elsewhere (Dea et al., 1990). All the BCoV isolates propagated in the HRT-G cells behaved as highly fusogenic (induced production of small syncytia with less than 10 nuclei) and cytolytic (complete destruction of the monolayers within 4–5 days after infection) strains (data not shown).

Hemagglutination (HA) of rat erythrocytes at 4 and 37°C, and hemagglutination inhibition (HAI) tests were performed, as previously described (Dea et al., 1980). The receptor destroying (RDE) activities of the various BCoV isolates were determined according to Storz et al. (1992), and expressed as the ratio between HA titers of infected supernatant fluids determined after incubation at 1 h at 4°C and HA titers determined after shifting to 37°C for 1 h, leading to activation of the enzyme activity and elution of the viral particles from the surface of the erythrocytes (Vlasak et al., 1988). Following three freeze and thaw cycles of the infected cultures, the extracellular virions of the various BCoV isolates were purified by isopycnic ultracentrifugation on sucrose gradients (Dea et al., 1989). To determine the esterase activities associated to the HE glycoprotein of the various BCoV isolates, aliquots (15 μl) of purified viral preparations, adjusted to contain approximately the same amounts of viral particles as determined by negative stained electron microscopy (Dea et al., 1989), were added to 1 ml of PBS containing 1 mM *p*-nitrophenyl acetate (PNPA). Hydrolysis of the substrate following an incubation of 5 min at room $T°$ was monitored by determining the optical density at a wavelength of 405 nm (A_{405}) (Parker et al., 1990).

For serological studies, monoclonal antibodies 1D6-2, 1D6-3 and 9F2-1R directed to the HE glycoprotein of the Quebec WDBCoV reference strain BCQ.2590 were used (Milane et al., 1997), as well as rabbit hyperimmune sera produced to the concentrated and purified Mebus and BCQ.2590 strains (Dea et al., 1995). The *anti*-HE MAbs were obtained following fusion experiments with splenocytes from Balb/c mice which have been immunized with an enriched peplomeric fraction of the WDBCoV BCQ.2590 strain (Milane et al., 1997). The specificities of the MAbs toward 65 and/or 140 kDa polypeptides, corresponding to the monomeric and dimeric forms of the HE glycoproteins, have been determined by Western blotting and radioimmunoprecipitation experiments. No reactivity was observed toward the S (100 or 200 kDa) glycoprotein, and none of these *anti*-HE MAbs had neutralizing activity against the homologous and Mebus BCoV strains (Milane et al., 1997).

The capacity of BCoV field isolates to attach to 9-*O*-acetylated sialic residues present at the surface of rat erythrocytes at different incubation *T*° is a biological property which has been previously reported for distinguishing between NCD- and WD-associated BCoV strains (Dea et al., 1995; Milane et al., 1997). In agreement with previous findings, HA titers of all BCoV strains studied at 4°C varied between 640 and 5120, and only HA titers of WDBCoV strains diminished significantly when switching the incubation *T*° from 4 to 37°C, due to the activation of the higher RDE activity of the WDBCoV strains (Table 1). For RBCoV and NCDBCoV strains, the capacity to destroy the host cell receptors was not influenced by variation of the incubation *T*°, except for BCQ.1523 strain for which a minimal RDE activity of 2 was determined, meaning that its HA titers at 4°C was at least twice the value obtained at 37°C. The esterase activities of the WDBCoV and RBCoV strains were comparable and appeared superior to that of NCD strains. Study of the cross-reactivity of the *anti*-HE MAbs revealed a lack of reactivity (HAI titers < 20) of MAbs 1D6-2, 1D6-3 and 9F2-1R toward NCD-associated Mebus and BCQ.571 strains, whereas HAI titers of at least 2560 were obtained when testing MAbs 1D6-2 and 1D6-3 against all other EBCoV and RBCoV strains studied. However, MAb 9F2-1R reacted only weakly (HAI titers of 40–80) to both Ontario and Quebec RBCoV strains tested, a titer of 2560 being obtained when tested against the reference OK-0514 RBCoV strain. Despite the lack of reactivity to the *anti*-HE MAbs tested, all BCoV strains tested reacted positively by HAI against both rabbit hyperimmune sera (*anti*-Mebus and *anti*-WD.2590).

For the preparation of genomic RNA, aliquots (50 μl) of purified virus or infected cell lysates were supplemented with 1 μl of RNA guard (Pharmacia) and extraction was performed by one-step guanidinium isothiocyanate-acid phenol method (Chomczynski and Sacchi, 1987). The approach of reverse transcription (RT) followed by the polymerase chain reaction (PCR) (Rekik and Dea, 1994) was used to amplify the S1 region of the S genes, the entire HE genes, and entire ORF4 and ORF5 regions of the BCoV isolates studied. To access the error rate of the RT and *Taq* polymerase, clones from different RT-PCR events were sequenced. The primers were choosen according to the cDNA sequence of the Mebus strain of BCoV (GenBank accession numbers M31053, M30612 and M76372) (Abraham et al., 1990a,b; Parker et al., 1990). The nt and deduced aa sequences were computer analysed with the aid of the GeneWorks 2,4 (IntelliGenetics Inc., Mountain View, Calif.) program. The nucleotide sequence accession numbers (EMBL/GenBank/DDBJ) that have been assigned to the HE, S1, ORF4 and ORF5 genomic regions of the EBCoV and RBCoV strains studied are: AF239307, and AF239316 for BCQ.1523; V06093 and AF239315 for BCQ.571; AF239306, AH010061 and AF239314 for BCQ.7373; L38962, AF239313 and AF239317 for BCQ.2590; AF239308 and AF239310 for BCO.43277; AF239309 and AF239311 for BCO.44175; AF339836 and AF239312 for BCQ.3994. To eliminate nt and aa changes that could be attributed to adaptation in HRT-G cells, comparison study was made with sequences of the HE and S genes of the avirulent Mebus strain initially propagated either in bovine fetal kidney cells or MDBK cells (GenBank accession numbers M30612, D00662, S50936) (Abraham et al., 1990a,b; Kienzle et al., 1990). Comparison was also made with the sequences of the BCoV.Que strain, another attenuated NCD-BCoV strain isolated in 1972 from diarrheic calves in Quebec, Canada, which has been propagated for at least 41 passages in Vero, followed by 10–15 passages in MDBK cells (Dea et al., 1980; Parker et al., 1990) prior sequencing studies (Rekik et al., 1994) (GenBank accession numbers D00662, M31053).

The amplification of entire HE genes of RBCoV strains OK-0514, BCO.43277, BCO.44175 and BCQ.3994 were obtained using primers HE.Ad5 sens 5′ GGATCC GGATCC GCC GCC GCC ATG TTT TTG CTT CTT AGA TTT GTT C 3′ and HE.Ad5 antisens 5′GGATCC GGATCC TCA CTA AGC ATC ATG CAG CCT AGT ACC 3′, and comparison were made with the HE sequences of two Quebec WDBCoV strains (BCQ.2590 and BCQ.7373) and two Quebec NCDBCoV (BCQ.571 and BCQ.1523) previously

Table 1
The hemagglutinating (HA), esterase and RDE activities of selected Quebec field isolates of RBCoV and EBCoV, and their cross-reactivities in HAI tests to *anti*-HE MAbs directed against the WD-strain BCQ.2590[a]

Strains	Year isolation	Disease	HA titers[b]	Esterase[c] activities (per 15 μl)	RDE[d]	HAI titers of *anti*-HE Mabs or rabbit antiserum[e]			
						1D6-2	1D6-3	9F2-1R	*anti*-Mebus
BCoV.Meb	1972	NCD	2560	1.481	1	<20	<20	<20	1280
BCQ.1523	1994	NCD	640	1.950	2	10 240	10 240	1280	640
BCQ.571	1989	NCD	640	1.782	1	<20	<20	<20	160
BCQ.2590	1991	WD	2560	3.122	128	2560	2560	1280	160
BCQ.7373	1992	WD	1280	2.181	256	10 240	5120	2560	640
OK-0514-3	1996	Pn	2560	2.202	1	5120	5120	5120	320
BCO.43277	1997	Pn	1280	2.178	1	5120	5120	40	320
BCO.44175	1997	Pn	2560	2.170	1	5120	5120	80	320
BCQ.3994	1998	Pn	5120	2.110	1	1280	1280	<20	320

[a] BCoV, Bovine coronavirus; BCQ, Bovine coronavirus Quebec; BCO, Bovine coronavirus Ontario; Meb, reference avirulent Mebus strain; WD, Winter dysentery; NCD, Neonatal Calf Diarrhea; RBCoV, Respiratory bovine coronavirus; Pn, Pneumonitis.

[b] Reciprocal of the highest dilution of infected cell culture fluids producing complete HA of rat erythrocytes after 1 h incubation at 4°C (averaged values for four tests after three to five passages on HRT-18 cells).

[c] Absorbance at 405 nm after 5 min of reaction with 1 mM-*p*-nitrophenyl acetate: the extracellular virions of each BCoV isolates was purified from 100 ml of infected cell culture fluids following isopycnic ultracentrifugation on sucrose gradients.

[d] Reciprocal value of highest dilution producing complete disaggregation of BCoV-erythrocyte complexes after 1 h at 37°C.

[e] Reciprocal values of highest MAb dilution inhibiting HA of rat erythrocytes by the various BCoV isolates (8 HA units) after 2 h at 4°C.

characterized in our laboratory (Dea et al., 1995; Kourtesis et al., 2001). Their nt and deduced aa sequences were then compared to those of the cell culture-attenuated reference NCD-associated Mebus strain of BCoV which has been resequenced in our laboratory following passaged in HRT-G cells. For this reference strain, data were similar to those previously reported by other authors following propagation on the same cell line (Zhang et al., 1991b) or on FBK cells (Abraham et al., 1990a). Thus, HE gene sequences of the reference Mebus strain remained unchanged following adaptation in HRT-G cells (Table 2). No nt substitution were also demonstrated when comparing the HE genes of the attenuated Mebus and BCoV.Que strains. For all BCoV strains studied, the HE genes contained a large ORF of 1272 nts encoding a predicted protein of 424 amino acids (aa). Table 2 presents the aa changes observed for all strains analysed when compared to the reference Mebus strain; the majority of the nucleotidic variations identified were silent mutations. The mutations of a leucine (L) by a proline (P) residue at aa position 5 and the substitution of a serine (S) by a proline (P) at aa position 367 determined for virulent BCoV isolates had been already reported (Zhang et al., 1991a; Dea et al., 1995). The mutation of an aspartic acid (D) by a glycine (G) at aa position 66 seems to be specific for all RBCoV strains.

For the S1 subunits of the S genes, a total of 2304 nts were obtained for all BCoV strains studied using primer pair S1.Ad5 sens 5′GGATCC GGATCC GCC GCC GCC ATG TTT TTG ATA CTT TTA ATT TCC 3′ and S1.Ad5 antisens AS 5′GGATCC GGATCC TCA TCT ACG ACT TCG TCT TTT TG 3′, corresponding to 768 predicted aa residues. As previously reported by other investigators (Zhang et al., 1991a), sequences of the Mebus and the BCoV.Que attenuated strains propagated either on FBK or MDBK cells were identical (data not shown). Most of the 58 aa changes identified were strain-specific, but aa changes observed at position 11 (M changed for T) of the signal peptide and those observed at positions 40 (I for T), 169 (N for H), 173 (N for H), 248 (M for L), 253 (S for N), 465 (A for V), and 470 (D for H) could have resulted from the propagation on HRT-18 cells since they were also identified for the Mebus strain following propagation on these cells (data not shown). The aa changes at positions 115 (D for K) and 173 (N for H) were observed for all virulent BCoV isolates. Eight aa substitutions, corresponding to aa residues 154, 182, 196, 215, 273, 320, 509 and 759, appeared to be specific to the EBCoV strains (Table 3). Only one of these variations, an asparagine (N) residue replaced by a threonine (T) at aa position 509, was situated in the hypervariable region of S1 (aa 452–593) (Rekik and Dea, 1994). Another aa substitution at position 759, where an aspartic acid (D) was replaced by a valine (V) residue, appeared to be specific for virulent NCDBCoV strains. The inclusion of an asparagin (N) residue at aa position 320 specific to EBCoV strains did not result in the appearance of an additional putative glycosylation site, and the same situation was noted in the case of both WDBCoV strains for which a specific inclusion of an asparagin (N) residue at aa position 196 was revealed.

Interestingly, eight aa substitutions in the S1 subunit of the S gene also appeared to be more specific to RBCoV strains, corresponding to aa residues 118, 147, 256, 362, 510, 531, 578, and 691 (Table 4). Three of these aa substitutions appeared to be situated in the hypervariable region of S1. Three of these specific changes located at aa positions 510, 531 and 578 had been already reported by other investigators (Chouljenko et al., 1998) and were present in all RBCoV strains studied, except the Quebec RBCoV strain BCQ.3994 for which only the aa change at position 510 (S for T) was demonstrated. The aa substitution at positon 118 which was also previously reported for the reference RBCoV strain OK-0514 was only demonstrated in the Ontario BCO.43277 strain. Three other aa changes identified at aa positions 256 (M for L), 362 (S for T) and 691 (A for T) were observed only in the sequences of both Ontario RBCoV strains; the aa change at position 256 being also present in the Quebec RBCoV strain BCQ.3994. Chouljenko et al. (1998) had identified five other variations specific to RBCoV strains situated at the N-terminus of the S1 portion of the S gene at aa positions

Table 2
Variations observed in the aa sequence of the HE glycoproteins of BCoV field strains compared to that of the reference Mebus strain[a]

Amino acid	Pathology and strains										
	EBCoV strains							RBCoV strains			
	NCD					WD					
	Mebus (HRT)	Mebus (FBK)	BCoV.Que (MDBK)	BCQ.571 (HRT)	BCQ.1523 (HRT)	BCQ.2590 (HRT)	BCQ.7373 (HRT)	OK-0514 (HRT)	BCO 43277 (HRT)	BCO 44175 (HRT)	BCQ 3994 (HRT)
5	L	*	*	P	P	P	p	p	p	p	p
8	V	*	*	*	*	A	*	*	*	*	*
11	S	*	*	*	C	*	*	*	*	*	*
49	N	*	*	*	T	*	T	T	T	T	T
53	R	*	*	*	*	P	*	*	*	*	*
66	D	*	*	*	*	*	*	G	G	G	G
139	V	*	*	*	*	*	*	G	*	*	*
182	G	*	*	*	*	*	R	*	*	*	*
245	F	*	*	*	*	*	L	*	*	*	*
282	D	*	*	*	*	*	G	*	*	*	*
344	D	*	*	*	*	A	*	*	*	*	*
350	Q	*	*	*	*	R	*	*	*	*	*
367	S	*	*	P	P	P	P	P	P	P	P
392	L	*	*	I	I	*	I	I	I	I	I
394	L	*	*	*	*	*	*	*	*	F	*
400	G	*	*	*	*	*	*	S	*	*	*

[a] An asterisk (*) indicates a consensus with the reference Mebus strain.

Table 3
Variations observed in the aa sequence of the S1 portion of the S glycoproteins of BCoV field strains associated to WD, NCD, or pneumonia, specific to virulent EBCoV strains when compared to the reference Mebus strain[a]

Amino acid	Pathology and strains										
	EBCoV strains							RBCoV strains			
	NCD					WD					
	Mebus (HRT)	Mebus (FBK)	BCoV.Que (MDBK)	BCQ.571 (HRT)	BCQ.1523 (HRT)	BCQ.7373 (HRT)	BCQ.2590 (HRT)	OK-0514 (HRT)	BCO 43277 (HRT)	BCO 44175 (HRT)	BCQ 3994 (HRT)
154	L	*	*	*	F	F	F	*	*	*	*
182	E	*	*	K	K	K	K	*	*	*	*
196	K	*	*	*	*	N	N	*	*	*	*
215	E	*	*	K	K	K	K	*	*	*	*
273	A	*	*	P	P	P	P	*	*	*	*
320	D	*	*	N	N	N	N	*	*	*	*
509	N	*	*	*	T	T	T	*	*	*	*
759	D	*	*	V	V	*	*	*	*	*	*

[a] An asterisk (*) indicates a consensus with the reference Mebus strain.

Table 4
Variations observed in the aa sequence of the S1 portion of the S glycoproteins of BCoV field strains associated to WD, NCD, or pneumonia, specific to virulent RBCoV strains when compared to the reference Mebus strain[a]

Amino acid	Pathology and strains										
	EBCoV strains							RBCoV strains			
	NCD					WD					
	Mebus (HRT)	Mebus (FBK)	BCoV.Que (MDBK)	BCQ.571 (HRT)	BCQ.1523 (HRT)	BCQ.2590 (HRT)	BCQ.7373 (HRT)	OK-0514 (HRT)	BCO 43277 (HRT)	BCO 44175 (HRT)	BCQ 3994 (HRT)
118	M	*	*	*	*	K	*	K	K	M	M
147	L	*	*	*	F	*	*	*	F	F	*
256	M	*	*	*	*	*	*	*	L	L	L
362	S	*	*	*	*	*	*	*	T	T	T
510	S	*	*	*	*	*	*	T	T	T	T
531	N	*	*	*	G	*	*	G	G	G	*
578	T	*	*	*	*	*	*	S	S	S	*
691	A	*	*	*	*	*	*	*	T	T	*

[a] An asterisk (*) indicates a consensus with the reference Mebus strain.

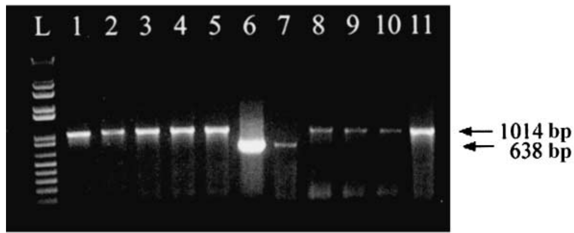

Fig. 1. RT-PCR amplification of the genomic region between the spike (S) and matrix (M) protein genes of BCoV isolates associated to different clinical syndromes as compared to the corresponding region of the porcine HEV and human HCoV-OC43 viruses. As expected, the use of primer pairs BCoV.5327S and BCoV.ORF4AS yielded DNA amplicons of 1014 bp for BCoV isolates associated to either enteric or respiratory diseases, whereas smaller DNA amplicons of 638 bp were obtained for both HEV-67N and HCoV-OC43 viruses. L, 1 kb DNA ladder; 1, Mebus strain (NCD); 2, BCQ. 2590 (WD); 3, BCQ.7373 (WD); 4, BCQ.571 (NCD); 5, BCQ.1523 (NCD); 6, HEV-67N; 7, HCoV-OC43; 8, OK-0514 (RBCoV); 9, BCO.43277 (RBCoV); 10, BCO.44175 (RBCoV); 11, BCQ.3994 (RBCoV).

11, 115, 118, 173 and 179. In the present study, these subtitutions were also present in the S1 sequence of EBCoV strains.

The genomic region between the S and M genes, corresponding to the ORF4 and ORF5 genes, was amplified by RT-PCR from NCD- and WD-associated BCoV, as well as from the four RBCoV strains studied, using primer pair BCoV.5327 sens 5′GGATCC GGATCC ATG TGG TGG TTG TTG TGA TGA 3′ and BCoV.ORF4 antisens 5′GATTC GAATTC AAC GTGC ATC CAC ATC AAG AAC 3′. For all BCoV isolates studied, DNA amplicons of 1014 base pairs were obtained, whereas smaller DNA amplicons of 638 base pairs were obtained for both HCoV-OC43 and HEV-67N strains (Fig. 1). For both types of EBCoV strains, the ORF4 region apparently encodes for two small non-structural proteins of 4.9 and 4.8 kDa and comprises a total of 302 nts. The alignments of the aa sequences of the ORF4 region encoding the 4.9 kDa protein of all the BCoV strains studied are presented in Fig. 2. These sequences were compared to that of the reference Mebus strain and to those of HEV-NT9, HEV 67N and HCoV-OC43 strains (GenBank accession numbers X89861, X89863 and M99576). As previously reported by other authors (Mounir and Talbot, 1993; Vieler et al., 1996), the HEV and HCoV-OC43 strains showed a major deletion in the genomic region corresponding to the ORF4, which was not the case for EBCoV and BCoV strains. However, a specific non-sense codon was identified at nt position 88 of the ORF4 of the four RBCoV isolates studied resulting in a premature termination of the protein which comprises 29 rather than 43 aa residues. In contrast to data obtained by previous

Mebus (ATCC)	MTTKFVFDLL	APDDILHPFN	HVKLIIRPIE	VEHIIIATTM	PAV	43
BCQ.2590	....	....	....	....	..F	43
BCQ.7373	....	....	....	....	..F	43
BCQ.571	....	....	....	....	..F	43
BCQ.1523	....	....	....	....	..F	43
OK-0514	...K......	........S.	......N..L			29
BCO.43277	....	........S.	......N...			29
BCO.44175	...K......	........S.	......N..L			29
BCQ.3994	...K......	........S.	......N..L			29
HEV-NT9	......I..V..	TL...H...VTLSI	CQSF			24
HEV-67N	......IN...---	-GFH...VTLSI	CQSF			20
HCoV-OC43	........S.	H				11

Fig. 2. Comparison of aa sequences of the ORF4 region encoding the 4.9 kDa protein. In comparison to BCoV enteropathogenic strains, the human coronavirus OC43 and the porcine HEV ORF 4 lack two open reading frames, whereas all RBCoV field isolates tested possess these two open reading frames but the 4.9 ns protein is apparently not expressed because of the introduction of an antisense codon at position 88. Sequences of strains HEV-NT9, HEV-67N and HCoV-OC43 correspond to GenBank accession numbers X89861, X89863, and M99576, respectively.

investigators (Chouljenko et al., 1998), all virulent field strains, and not only RBCoV strains, contained a deletion (TAAT) located 31–34 bp upstream from the AUG of the 12.7 kDa ORF in the genomic region between the 4.8 and 12.7 kDA ORFs (data not shown). Only a four strain-specific aa changes were observed in the ORF5 region encoding the 12.7 and 9.5 kDa (E) proteins. The only substitution determined within the E gene was located at aa position 53 where a valine was replaced by a glycine for all virulent strains, as previously described (Mounir and Talbot, 1993; Chouljenko et al., 1998).

The data obtained in the present study indicate that genomic, biologic and antigenic variations, as detected by MAbs directed to epitopes of two different antigenic domains of the HE glycoprotein of a reference WDBCoV strain (Milane et al., 1997) exist among EBCoV and RBCoV strains. However, the majority of aa changes detected seems to be scattered over different structural genes and appeared to be strain-specific, in agreement with previous findings on NCD- and WD-associated strains (Zhang et al., 1991a,b; Rekik and Dea, 1994; Chouljenko et al., 1998; Kourtesis et al., 2001). Sequencing analyses were restricted to genes encoding the envelope-associated HE, S (S1 portion) and E proteins of the BCoV virion, as well as putative ns proteins (4.9, 4.8 and 12.7 kDa) encoded between the spike and membrane protein genes, which have been previously incriminated in the tissue tropism (Mounir and Talbot, 1993; Vieler et al., 1996), pathogenesis, humoral and cellular immune responses (Spaan et al., 1988 (a review)), as well as to genomic and antigenic variabilities amongst BCoV field isolates (El Ghorr et al., 1989; Michaud and Dea, 1993; Rekik and Dea, 1994; Tsunemitsu and Saif, 1995; Milane et al., 1997; Chouljenko et al., 1998; Hasoksuz et al., 1999). The N and M protein genes were not analysed since previously serological and genomic comparison studies revealed that the latter two structural proteins are well conserved among field strains of BCoV associated to various clinical conditions, and amongst members of the hemagglutinating coronaviruses subgroup (Lapps et al., 1987; Schockley et al., 1987; Verbeek and Tijssen, 1991; Mounir and Talbot, 1992; Chouljenko et al., 1998). A recent study demonstrated that sequences of the N were more conserved among RBCoV strains than to EBCoV strains compared, although the few aa changes identified were strain-specific (Chouljenko et al., 1998).

Data further demonstrated that both envelope-associated glycoproteins, particularly the S1 subunit of the S peplomeric glycoprotein, appeared to contain aa changes specific to either RBCoV or EBCoV strains, in agreement with recent findings on RBCoV strains characterized in Louisiana (Chouljenko et al., 1998). Because the S protein is more efficient that the HE protein in hemagglutination (Vlasak et al., 1988; Schultze et al., 1991b), it is expected that antigenic variability would have been more evident using *anti*-S MAbs, rather than *anti*-HE MAbs. Interestingly, none of the *anti*-HE MAbs showed reactivity by HAI and indirect immunofluorescence (data not shown) to the reference Mebus strain, as well as two other NCDBCoV isolates, as previously reported (Milane et al., 1997). However, these *anti*-HE MAbs could not be used for specific identification of WDBCoV strains since they all reacted to the RBCoV field isolates. On the other hand, all BCoV strains tested reacted positively by HAI against both rabbit hyperimmune sera (*anti*-Mebus and *anti*-WD-2590). This may be explained by the fact that hemagglutination through S may still occur when the HE hemagglutinin is inhibited. However, epitopes of the S associated to virus neutralization are not necessarily associated to those associated to HAI, since both rabbit hyperimmune antisera were devoid of neutralization activities, in agreement with previous findings (Vautherot et al., 1992; Michaud and Dea, 1993).

One important finding which has been also observed previously for the reference WDBCoV strain BCQ.2590 (Milane et al., 1997) is the highest enzymatic activities (RDE and esterase activities) of WDBCoV field isolates in comparison to that of NCDBCoV and RBCoV isolates. Although earlier studies have demonstrated that BCoV esterase activity is probably not directly implicated in the processus of infection, particularly virus uptake (Vlasak et al., 1988; Schultze et al., 1991a), it could be related to the virulence by

facilating elution of the mature virions from the surface of infected cells, thus a possible higher potential for rapid dissemination (higher contagiosity) in the herds. Results from comparative studies of the HA properties of NCD- and WD-associated BCoV isolates also suggest that the affinity for *N*-acetyl-9-*O* neuraminic acid-containing receptors is probably dependent on the RDE activity of the HE protein (Dea et al., 1995). Accordingly, outbreaks of WD are usually of short duration and affected rapidly most of the animals within the herd, in contrast to NCD outbreaks that rather tend to evolve to a chronic status since newborn calves can shed the virus in their feces for long periods after recovery (Crouch et al., 1985; Dea et al., 1995; Fukutomi et al., 1999). It has been previously suggested that enhancement of the RDE activity of WDBCoV strains could be in part attributed to conformational changes of the HE protein due to inclusion of proline residues in the signal peptide and in the vicinity of the sequences of the putative AE domain (FGDS) of the HE protein. Data of the present study do not support this hypothesis since both proline substitutions were identified for all virulent BCoV strains studied, including those characterized by other investigators (Zhang et al., 1991b). The substitution of an aspartic acid (D) by a glycine (G) at aa position 66 of the HE glycoprotein was also previously identified as a specific marker for RBCoV strains (Chouljenko et al., 1998).

As also previously reported (Chouljenko et al., 1998), the region located between the S and M protein genes appeared of a particular interest when comparing EBCoV and RBCoV isolates. Indeed, this region contained four potential ORFs for all RBCoV isolates studied; thus BCoV isolates associated to different clinical symptoms did not have the 302 nt deletion previously identified for the human coronavirus HCoV-OC43 strain and HEV strains of swine that lack the two ORFs which potentially encode two non-structural (ns) proteins of 4.9 and 4.8 kDa (Mounir and Talbot, 1993; Vieler et al., 1996). Their absence in cases of both viruses indicates that they are most probably not essential for virus replication, at least in HRT-18 cells. Interestingly, an antisense mutation resulting in the truncation of the ns 4.9 protein was identified only for RBCoV field isolates, which may indicate that this protein could be involved, as for HEV and HCoV-OC43, in the preferential respiratory tropism. Further investigations are required to verify this hypothesis. This situation contrasts considerably with that of the porcine transmissible gastroenteritis virus (TGEV) for which deletions within the first 250 aa of S1 portion of S are associated with respiratory tropism and reduced enteropathogenicity (Wesley et al., 1991). On the other hand, the genomic regions encoding for the putative 12.7 kDa ns protein and the 9.5 kDa membranous protein E are probably essential for virus replication and infection process since both proteins appeared highly conserved among BCoV strains associated to various clinical symptoms.

Although differences were found by sequencing analysis and patterns could be correlated somewhat with pathogenic properties of the BCoV strains characterized, it remains that the region of the genome studied represents only 25% of the entire BCoV genome. Therefore, data obtained do not eliminate the fact that differences among strains that might give rise to differences in pathogenic properties could lie anywhere within the genome, including non-coding regions. For example, previous investigators have demonstrated the existence of nt variations in the genomic region located in the N-terminus of the ORF2 encoding a 32 kDa non-structural protein, highly expressed in BCoV-infected cells, or in cells infected by other members of the hemagglutinating coronaviruses (Cox et al., 1989, 1991; Labonté et al., 1995). In comparison to the EBCoV attenuated BCoV.Que strain, two virulent RBCoV strains isolated in Louisiana, as well as the virulent LY-138 EBCoV strain, have been found to contain two frame-shift mutations which resuted in a nine aa segment near the carboxy-terminus which was different from the corresponding aa sequence specified by the avirulent BCoV.Que strain (Chouljenko et al., 1998). Sequencing of the 32 kDa non-structural protein of the attenuated and virulent Mebus stains, as well as the same region of other virulent strains should substantiate these differences between virulent and avirulent strains.

Other regions may also be involved, namely the large ORF1 region encoding the RNA-polymerase, replicase, helicase and other proteases involved in virus replication and maturation (Spaan et al., 1988). Such findings have been recently demonstrated in the case of the porcine reproductive and respiratory syndrome virus, a member of the *Arteriviridae* family recently grouped in the order *Nidovirales*, together with members of the *Coronaviridae* family (De Vries et al., 1997). The genome length of Arterivirus is 15 kb rather than 28 kb, which makes genomic comparison studies more easier than in case of coronaviruses, particularly for analysis of the ORF1 gene representing near 66% of the entire gemone for both types of viruses. The ORF1 is separated into ORF1a and ORF1b regions by a ribosomal frameshift; six putative Nsps (non-structural proteins) are predicted to result from ORF1a polyprotein processing, whereas ORF1b is cleaved by ORF1a proteases into four proteins: RdPp (RNA dependent RNA polymerase) and putative cleavage products CP2, CP3 and CP4 (De Vries et al., 1997; Allende et al., 1999a). The Nsp2 protein has been shown highly variable among arteriviruses, e.g. PRRSV (740 and 861 residues long for European and North American strains, respectively), LDV (733 residues long) and EAV (401 residues long), with similarity only in the amino- and carboxy-terminal domains, and is thought to be involved in species-specific functions. The low degree of identity and the size differences between Nsp2 of European and North American isolates of PRRSV suggests that variability within this protein may have other than species-specific functions. Whether this involves biological differences observed between virus isolates, such as the degree of pathogenicity in the swine host, remains to be determined. Furthermore, aa changes distributed in the different regions of the ORF1 gene have been identified which may provide the molecular bases for loss of virulence of the live-attenuated PRRSV vaccines (Allende et al., 1999b). To our knowledge, such variability in the various regions of the ORF1 of BCoVs associated with different clinical symptoms has not been yet demonstrated, but could give rise to differences in pathogenic properties, tropism and antigenic variabilities of field isolates.

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