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Comparison of hemagglutinating, receptor-destroying, and acetylesterase activities of avirulent and virulent bovine coronavirus strains

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Summary. Hemagglutinating and acetylesterase functions as well as the 124 kDa glycoprotein were present in the highly cell-culture adapted, avirulent bovine coronavirus strain BCV-L9, in the Norden vaccine strain derived from it, and in 5 wild-type, virulent strains that multiplied in HRT-18 cells but were restricted in several types of cultured bovine cells. The BCV-L9 and the wild-type strain BCV-LY-138 agglutinated chicken and mouse erythrocytes. The acetylesterase facilitated break-down of the BCV-erythrocyte complex with chicken but only to a minimal extent with mouse erythrocytes in the receptor-destroying enzyme test. Purified preparations of the vaccine and the wild-type strains agglutinated chicken erythrocytes at low titers and mouse erythrocytes at 128 to 256 times higher titers whereas receptor destroying enzyme activity was detectable only with chicken erythrocytes. When wild-type strains were propagated in HRT cells at low passage levels, they produced 5×10^5 to 4.5×10^6 plaque forming units per 50 µl which agglutinated erythrocytes from mice but not from chickens. Diisopropylfluoro-phosphate moderately increased the hemagglutination titers, but completely inhibited the receptor destroying enzyme of purified virus of all strains. It had virtually no influence on the plaque-forming infectivity of the different BCV strains. The acetylesterase of strain BCV-L9 reacting in the receptor-destroying enzyme test was stable for 3 h at 37 and 42 °C. It was inactivated within 30 min at 56 °C while the hemagglutinin function of this strain was stable for 3 h at 37, 42, and 56 °C, but it was inactivated at 65 °C within 1 h.

Introduction

Bovine coronavirus (BCV) has a hemagglutinin (HA) for some erythrocytes with an approximate molecular mass of 62 kDa and 124 kDa in the reduced

and the nonreduced forms. This structural protein composes the short spikes of the viral envelope [4, 9, 11]. Acetylesterase activity (AE) was found associated with this glycoprotein of a BCV strain isolated from calves in the Netherlands and of the strain BCV-L9 [15, 16, 28]. This glycoprotein is now referred to as the hemagglutinin-esterase (HE) of BCV [2]. The AE inactivates the receptors for BCV of susceptible cells by hydrolyzing an ester bond to liberate acetate from C-9 of sialic acid [15, 16, 29], an enzyme function first detected in the influenza C virus by Herrler and coworkers [5]. The gene of the HE glycoprotein of BCV is located upstream of the S gene and predicts a protein with 424 amino acids [7, 12, 33].

As an enteropathogen BCV causes severe diarrhea in neonatal calves, and it is also considered to be etiologically involved with winter dysentery of adult cattle [4, 11, 14]. BCV represents one of the better characterized coronaviruses with HA properties. Four major structural proteins are associated with infectious BCV. The HE as well as the spike or S glycoprotein (190 kDa) and the integral membrane glycoprotein M (23–26 kDa) are associated with the viral envelope while the phosphorylated N protein (50–54 kDa) functions as a nucleocapsid [3, 8, 20, 23]. Proteolytic cleavage of the S glycoprotein precursor into S 1 and S 2 of 100 and 110 kDa is required for cell fusion activity [20, 22]. The S 1/S 2 glycoproteins facilitate virus attachment to susceptible cells, and also binding to erythrocytes, cell fusion, and induction of neutralizing antibodies [17, 20, 22, 24, 25]. The exact functions of HE and S 1/S 2 and their interplay in the infectious process in vitro and in vivo are not fully defined [17, 24].

The presence and function of the HE was analyzed in two cell-adapted BCV strains [15, 24, 28, 29]. Some of the BCV isolates from winter dysentery agglutinated rodent erythrocytes and others did not [1]. The objective of our investigations was to identify the HE of wild-type BCV strains at low passage levels in HRT cell cultures and to compare it with the HE of prototype BCV-L9 and vaccine strains by relating its functions to the interaction with different erythrocytes, the effects of enzyme inhibitors, and to plaque-forming infectivity.

Materials and methods

Bovine coronavirus isolates, their propagation, quantitation and purification

The cell culture-adapted prototype BCV-L9 was originally isolated in bovine fetal kidney (BFK) cells from diarrhea fluid of a calf [11, 18]. Our virus strain had been passaged 42 times in BFK cells, 16 times in bovine fetal brain cells, 15 times in bovine fetal spleen (BFS) cells, and 5 to 10 times in human rectal tumor (HRT-18) cells [20–22]. Five other wild-type BCV isolates, initially maintained by calf inoculation, were adapted to HRT-18 cells from diarrhea fluid or intestinal mucosal scrapings of calves with clinical diarrhea and electron microscopic evidence of coronavirus infection [4, 21]. These strains are: BCV-LY-138, BCV-C-50, BCV-Miller, BCV-Meeker, and BCV-Fisher. The vaccine strain of BCV was cultured in HRT-18 cells from the vaccine of Norden Laboratories, Omaha, Nebraska [11, 18]. Importantly, cultivation of the BCV wild-type strains remained impossible for us until it was demonstrated that the cytopathic expression of BCV-L9 in cultured bovine cells was enhanced by trypsin [22] and that HRT-18 cells were susceptible

to BCV [10]. These wild-type strains were completely restricted in a variety of cultured bovine cells even in the presence of trypsin [21].

The HRT-18 cells were cultured with Dulbecco's modified Earle's medium (DMEM) with 5% bovine fetal calf serum which was omitted from cultures used for virus propagation. A plaque assay in HRT-18 cells overlaid with 3 ml of 1.2% (w/v) agarose in DMEM containing 4 µg/ml final trypsin concentration was used to measure infectivity [22, 27]. Samples of the 7 BCV strains were propagated in HRT cells and purified as described [6, 20, 30].

BCV-L9 infected cell lysate

The BCV-L9-infected cell sediment of the first step in the virus purification was suspended in 7.5 ml phosphate buffered saline (pH of 7.4), treated with sound 3 times for 15 s at a power setting of 4 (Branson Sonifyer), and centrifuged at 3000 × **g** for 30 min. The supernatant fluid represented the BCV-L9-infected cell lysate. Uninfected HRT-18 cells were treated the same way and were used in control tests.

Acetylesterase assay

Acetylesterase activity of purified BCV preparations was determined according to Vlasak et al. [28]. Purified preparations of the different BCV strains were added in 5 µl quantities to 1 ml of phosphate buffered saline containing 1 mM p-nitrophenylacetate (PNPA). The PNPA was initially dissolved in ethyl alcohol. Hydrolysis of the substrate was monitored at 405 nm in a Beckman spectrometer with multiple cuvette sets and a chart recorder. The reaction was measured at 1 min intervals for 5 min. The 5 min value was used for comparisons [15, 16].

Assays for hemagglutination (HA) and receptor-destroying enzyme (RDE)

The HA test was employing 0.5% suspensions of erythrocytes from adult chickens or mice [5]. The tests with 2-fold BCV dilutions in 50 µl quantities were initially incubated at 4 °C for 1 h to assess the HA titers. Thereafter, the microtiter 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 extent of disappearance of the BCV-erythrocyte aggregates was recorded in a final evaluation made following an additional 4 to 6 h at room temperature when unequivocal interpretation of the results was possible [24].

Inhibitors of HA and RDE

Diisopropylfluorophosphate (DFP) was used as an inhibitor of serine esterase activity to assess the effect of AE from different BCV strains on the HA and RDE functions as well as on BCV infectivity. A sample of 50 µl of each purified BCV strain was treated with 1 mM of DFP in 10 µl for 10 min at room temperature. The virus samples were then diluted in 2 fold steps and tested for HA and RDE activities. Untreated samples were tested in parallel. Similarly, 10 µl aliquots of the purified BCV strains were treated with 1 mM of DFP in 10 µl for 10 min at room temperature. Samples were then mixed with 980 µl of PBS, and further decimal dilutions were made. The infectivity of DFP-treated and untreated BCV preparations was assayed in the plaque test [22]. Bovine submaxillary mucin (BSM) was tested with 2-fold dilutions of the purified virus preparations by adding 50 µl of the 1 mg/ml BSM. Alternatively, the indicated inhibitor concentrations was diluted 2-fold and 8 HA units of the strains BCV-L9 or BCV-LY-138 were added. Chicken erythrocytes as well as mouse erythrocytes were employed in these tests.

Thermal inactivation of HA and RDE

Aliquots of the BCV-L9-infected cell lysate having HA and RDE titers of 128 with chicken erythrocytes were incubated at 37 °C, 42 °C, 56 °C and 65 °C. Samples were withdrawn at intervals of 10 min for 1 h and every 30 min for the next 2 h to be tested for HA and RDE activities.

Detection of the 124 kDa glycoprotein

Sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% slab gels under denaturing and under mildly denaturing conditions. Detailed procedures for the analysis of BCV proteins by SDS-PAGE were described [6, 20].

Results

Infectivity and detection of AE, HA, and RDE activities of purified virus preparations from different BCV strains

The different viral functions were assessed on purified BCV samples containing infectivities of 2.5×10^6 to 1.2×10^8 PFUs per 50 µl (Table 1). AE activity was detected in the PNPA test and the gp 124 HE structural protein was seen in PAGE of all BCV strains tested. The highest AE activity was detected in the BCV-LY-138 strain and this strain had also the highest infectivity even though it was only in the 5th passage in HRT-18 cells. Purified virus preparations of the strains BCV-L9 and BCV-LY-138 agglutinated chicken erythrocytes at titers of 1024 and 4096, respectively, but the HA titers were 8 and 4 fold higher with mouse erythrocytes. In contrast, the vaccine and three wild-type BCV strains had HA titers of 32 to 128 with chicken erythrocytes, and 128 to 256 fold higher titers were recorded with mouse erythrocytes. RDE activity was detected at titers of 16 to 64 with chicken erythrocytes whereby HA : RDE ratios of 1 : 1 were evident for the strains BCV-Meeker, BCV-Fisher and BCV-Calf-50. The RDE activity was minimal or not detectable in tests involving mouse erythrocytes. The PFUs per HA unit ranged from 2.6×10^4 to 1.5×10^6 for chicken erythrocytes and 3×10^2 to 7.3×10^3 for mouse erythrocytes (Table 1).

Infectivity and detection of HA and RDE functions released from HRT cell-cultures infected with different BCV strains at high and low passage levels

The HA pattern of the highly cell-culture adapted and the wild-type BCV strains differed. The BCV-L9 strain was in the 78th cell culture passage while the wild-type strains had been passed only 3 to 7 times in HRT-18 cells. Strains BCV-L9 and BCV-LY-138 agglutinated chicken erythrocytes while the other BCV strains did not (Table 2). All strains agglutinated mouse erythrocytes. The infectivity of the preparations ranged from 5×10^5 to 4.5×10^6 PFUs. RDE activity for receptors on chicken erythrocytes was detected in samples from BCV-L9 and BCV-LY-138.

Table 1. Infectivity, acetylesterase, hemagglutinating, and receptor-destroying enzyme activities of purified preparations of avirulent and virulent bovine coronavirus strains

BCV strain	PFU per 50 µl	Acetyl-esterase per 5 µl[a]	HA titer[b]		RDE titer[c]		PFU per unit of	
			chicken	mouse	chicken	mouse	HA chicken	HA mouse
L9-78	2.7×10^7	0.2250	1,024	8,192	32	8	2.6×10^4	3.2×10^3
Vaccine-4	5×10^6	0.2590	128	16,384	16	< 2	3.9×10^4	3×10^2
LY-138-5	1.2×10^8	2.3320	4,096	16,384	32	< 2	2.9×10^4	7.3×10^3
Meeker-6	5×10^7	2.1560	32	8,192	32	2	1.5×10^6	6.1×10^3
Fisher-8	2.5×10^6	1.2550	64	8,192	64	2	3.9×10^4	3×10^2
Calf 50-3	3×10^7	nt	32	8,192	32	2	9.3×10^5	3.6×10^3

PFU Plaque forming units
nt Not tested
[a] Optical density at 405 nm after 5 min of reaction with 1 mM p-nitrophenyl acetate
[b] Reciprocal value of highest dilution with complete hemagglutination (HA) by 50 µl of virus preparation
[c] Reciprocal of highest dilution with complete disaggregation of BCV-erythrocyte complexes after 1 h at 37 °C

Table 2. Correlation between infectivity, hemagglutination and receptor-destroying enzyme activities of different BCV strains at various passage levels in HRT cells

BCV strain and HRT-passage level	PFU/50 µl	Chicken RBC		Mouse RBC	
		HA[b]	RDE[c]	HA	RDE
L9-79[a]	4.5×10^6	256	8	512	< 2
LY-138-5	1.5×10^6	128	8	32	< 2
Vaccine-3	5×10^5	< 2	< 2	32	< 2
Meeker-7	7×10^6	< 2	< 2	64	< 2
Fisher-6	3.5×10^6	< 2	< 2	64	< 2
Calf 50-3	5×10^5	< 2	< 2	64	< 2
Miller-6	1.5×10^6	< 2	< 2	64	< 2

[a] 5 passages in HRT and numerous passages in cultured bovine cells
[b] Reciprocal value of highest dilution with complete hemagglutination by 50 µl of virus preparation
[c] Reciprocal of highest dilution with complete disaggregation of BCV-erythrocyte complexes after 1 h at 37 °C

Effect of inhibitors on infectivity, HA and RDE activities of purified virus preparations of different BCV strains

The BCV infectivity was virtually unaffected by DFP (Table 3). In contrast, the RDE activity for chicken erythrocytes was completely inhibited. The HA titers for chicken erythrocytes were 2 to 64 times higher after DFP treatment. The RDE activity for mouse erythrocytes was eliminated by DFP and an enhancing effect on HA was not noticed. BSM inhibited HA by BCV-L9 and the vaccine strain, while it reduced the HA titers of the other strains for chicken erythrocytes (Table 4). Mouse erythrocytes became aggregated in the control tests by BSM at the concentration used.

Thermal inactivation of HA and RDE activities of BCV-strain BCV-L9

The BCV-L9-infected cell lysate was used to assess thermal inactivation of the HA and RDE functions for chicken erythrocytes. As documented in Figs. 1 and 2 these activities remained unaffected at temperatures of 37 °C and 42 °C for 3 h. The RDE titer was reduced from 256 to < 2 within 30 min at 56 °C while HA remained constant. This activity was lost within 60 min at 65 °C.

Discussion

All BCV strains tested had AE activities when purified preparations were assayed in the PNPA test [28]. This enzyme was inhibited by the serine esterase inhibitor DFP when assayed in the AE-mediated breakdown of BCV-chicken erythrocyte complexes yet the HA titers of purified preparations of wild-type BCV strains were increased 2 to 64 fold at 4 °C. The infectivity of identically DFP-treated

Table 3. Effect of diisopropylfluorophosphate (DFP) on infectivity, hemagglutination, and receptor-destroying enzyme activities of purified virus preparations of different BCV strains at high and low passages in HRT-18 cells

BCV strain	Infectivity[a]		Chicken erythrocytes				Mouse erythrocytes			
	− DFP	+ DFP	HA[b]		RDE[c]		HA		RDE	
			− DFP	+ DFP	− DFP	+ DFP	− DFP	+ DFP	− DFP	+ DFP
L9-78	7×10^7	2.5×10^7	1,024	4,096	32	< 2	8,192	4,096	< 2	< 2
Vaccine-4	5×10^6	5×10^6	64	2,048	64	< 2	2,048	1,024	8	< 8
LY-138-5	1.2×10^8	nt	2,048	4,096	32	< 2	16,384	8,192	16	< 16
Meeker-6	5×10^7	2.5×10^7	64	1,024	32	< 2	8,192	4,096	64	< 16
Fisher-6	1×10^8	2.5×10^8	16	1,024	16	< 2	nt	nt	nt	nt
Calf 50-3	3×10^7	2×10^7	128	2,048	128	< 2	8,192	8,192	32	< 16

[a] Per volume of 50 μl which was employed in all tests
[b] Reciprocal value of highest dilution with complete agglutination by 50 μl of virus preparation
[c] Reciprocal of highest dilution with complete disaggregation of BCV-erythrocyte complexes after 1 h at 37 °C
nt Not tested

Table 4. Inhibition of hemagglutination and receptor-destroying enzyme functions of purified virus from different BCV strains by bovine submaxillary mucine (BSM)

BCV Strain	Hemagglutination[a]		Receptor-destroying enzyme[a]	
	control	+ BSM[b]	control	+ BSM
L9-78	256	< 2	8	< 2
Vaccine-4	16	< 2	16	< 2
LY-138-5	256	8	8	2
Meeker-6	32	16	32	16
Fisher-8	64	8	32	8
Calf 50-5	32	32	32	32

[a] With chicken erythrocytes; mouse erythrocytes were agglutinated by BSM; the volume was 50 µl for all components in these tests

[b] 1 mg/ml

BCV samples was not significantly reduced when compared with the untreated samples in the plaque test employing HRT-18 cells (Table 1). These results indicate that the AE of the BCV is probably not directly involved in viral uptake during the infectious process. Receptor binding and viral attachment to susceptible cells in infections of non-HA and HA coronaviruses are mediated by the S glycoprotein [19]. The HE protein is not expressed in some murine coronavirus strains [31]. Our finding contrasts a previous report of a 100-fold reduction of infectivity by DFP of another BCV strain plaque-assayed in Madin-Darby bovine kidney cells [28]. Infectivity assayed by the plaque test does not address the potential significance of AE in viral spread within an infected cell culture or animal. The AE activity of the HE protein may play a role in facilitating virus release from infected cells and viral spread. BCV particles were seen adsorbed abundantly to the plasmalemma or microvilli. Adsorbed BCV covered the entire exposed surfaces of enterocytes in infections of calves [4] and HRT cells [13]. Further insight into the role of HE will be gained through investigations of the interactions of HE with cellular receptors in vitro as well as with the neuraminidate-containing glycokalyx protecting mucous membranes of the respiratory and intestinal tracts of hosts. The strongly inhibitory function of BSM on HA points in this direction (Table 4).

One difference between the BCV-L9 and BCV-LY-138 strains and the vaccine, Meeker, Fisher and Calf-50 strains involves their interaction with different erythrocytes. Chicken erythrocytes were agglutinated by BCV-L9 and BCV-LY-138 and the RDE functioned. These strains also agglutinated mouse erythrocytes, but elution through RDE activity was minimal. The approximately 100-fold concentrated purified preparations of the other BCV strains agglutinated chicken erythrocytes at low titers which were increased by DFP. The HA titers with mouse erythrocytes were 128 to 256 fold higher, but virtually no RDE function was detectable (Table 1). This difference was further substantiated

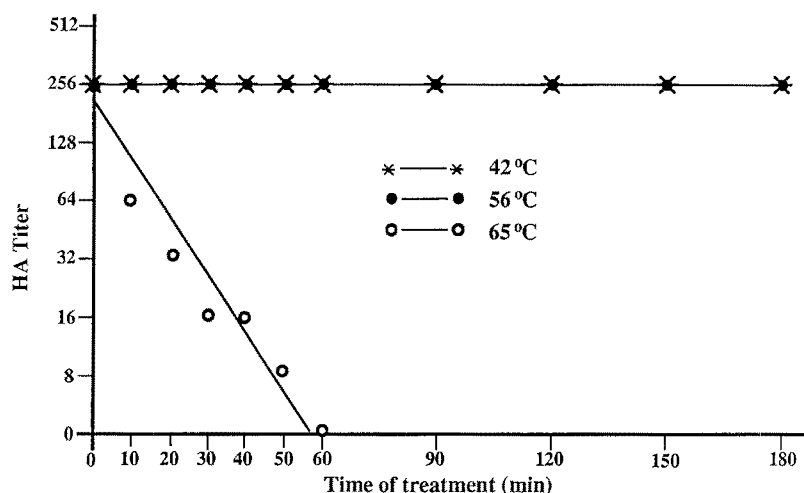

Fig. 1. Thermal inactivation of the hemagglutinating activity of BCV-L9

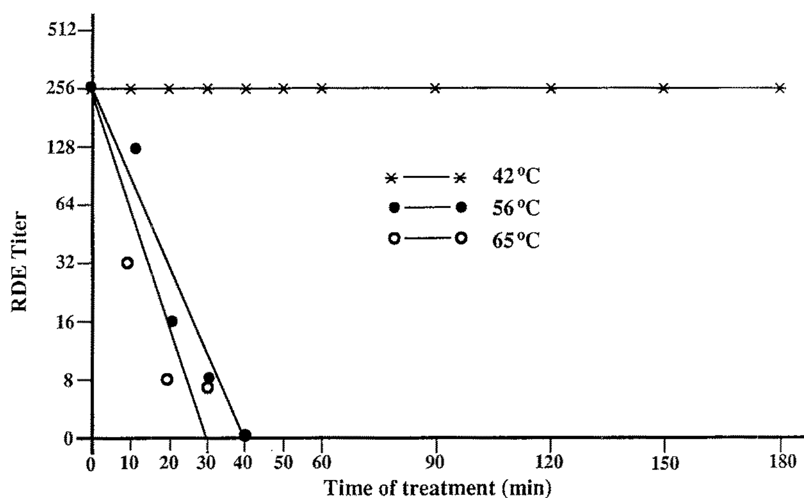

Fig. 2. Thermal inactivation of the receptor destroying activity mediated by the acetylesterase of BCV-L9

in HA tests with fluids from HRT-18 cell cultures infected with the different BCV strains at low passages. The vaccine, Meeker, Fisher, Calf 50 and Miller BCV strains agglutinated mouse but not chicken erythrocytes at infectivity yields of 5×10^5 to 4.5×10^6 PFU s/50 μl (Table 2). The difference in the HA pattern of the avirulent and virulent strains may be determined by strain-dependent receptor binding properties, or possible differences between Neu 5,9 Ac_2-containing receptors on chicken and mouse erythrocytes, or the greater abundance of receptors on mouse erythrocytes [17]. Further differences between the avirulent strain BCV L9 and the virulent strains are host cell restriction [21, 22], epitopes for S-specific monoclonal antibodies [6], and amino acid substitutions among the S and HE proteins [32, 33]. Adaptation

of wild-type BCV strains to cell cultures and the associated viral selection clearly involve significant genetic and functional changes.

The difference in thermal sensitivity separated AE or RDE activity from the HA function. These studies do not define the structural proteins of BCV involved in HA. Monoclonal antibodies against HE as well as against S inhibited HA by BCV-L9. The activity of anti HE monoclonal antibodies was predominately against RDE [24]. Purified HE of BCV-L9 had AE activity but agglutinated only mouse erythrocytes while purified S was a more powerful HA for chicken and mouse erythrocytes [17]. The functions of S and HE, and their interplay in receptor binding, HA, and the infectious process of BCV need to be defined.

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