

# The sialate-4-O-acetyl esterases of coronaviruses related to mouse hepatitis virus: a proposal to reorganize group 2 *Coronaviridae*

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Group 2 coronaviruses are characterized within the order *Nidovirales* by a unique genome organization. A characteristic feature of group 2 coronaviruses is the presence of a gene encoding the haemagglutinin–esterase (HE) protein, which is absent in coronaviruses of groups 1 and 3. At least three coronavirus strains within group 2 expressed a structural protein with sialate-4-O-acetyl esterase activity, distinguishing them from other members of group 2, which encode an enzyme specific for 5-*N*-acetyl-9-*O*-acetylneuraminic acid. The esterases of mouse hepatitis virus (MHV) strains S and JHM and puffinosis virus (PV) specifically hydrolysed 5-*N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac<sub>2</sub>) as well as the synthetic substrates *p*-nitrophenyl acetate, 4-methylumbelliferyl acetate and fluorescein diacetate. The  $K_m$  values of the MHV-like esterases for the latter substrates were two- to tenfold lower than those of the sialate-9-*O*-acetyl esterases of influenza C viruses. Another unspecific esterase substrate,  $\alpha$ -naphthyl acetate, was used for the *in situ* detection of the dimeric HE proteins in SDS–polyacrylamide gels. MHV-S, MHV-JHM and PV bound to horse serum glycoproteins containing Neu4,5Ac<sub>2</sub>. De-*O*-acetylation of the glycoproteins by alkaline treatment or incubation with the viral esterases resulted in a complete loss of recognition, indicating a specific interaction of MHV-like coronaviruses with Neu4,5Ac<sub>2</sub>. Combined with evidence for distinct phylogenetic lineages of group 2 coronaviruses, subdivision into subgroups 2a (MHV-like viruses) and 2b (bovine coronavirus-like viruses) is suggested.

## Introduction

Viruses belonging to the family *Coronaviridae* are divided into three groups (Enjuanes *et al.*, 2000). A typical representative of group 1 is porcine transmissible gastroenteritis virus. Group 3 consists of avian infectious bronchitis virus and turkey coronavirus. Members of group 2 are human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), porcine haemagglutinating encephalomyelitis virus (HEV), murine hepatitis virus (MHV), rat coronavirus (RtCoV) and

sialodacryoadenitis virus (SDAV). Available data indicate that puffinosis virus (PV) may also belong to group 2 (Nuttall & Harrap, 1982; Klausegger *et al.*, 1999). A major characteristic of group 2 viruses is the presence of a haemagglutinin–esterase (HE) gene, which encodes a surface glycoprotein of approximately 60–65 kDa. The HE gene is located immediately upstream of the spike gene encoding the surface peplomer present in all coronaviruses. It has been proposed that the HE gene is derived from an ancestral gene of unknown origin, which was acquired by coronaviruses during a non-homologous recombination (Luytjes *et al.*, 1988). Other viruses known to possess an HE gene are the influenza C viruses and toroviruses. In influenza C viruses, the HE gene is located on RNA 4 (Nakada *et al.*, 1984; Pfeifer & Compans, 1984). It encodes a protein that becomes post-translationally processed by trypsin-like proteases into an HE-1 and an HE-2 domain (Herrler *et al.*, 1979; Sugawara *et al.*, 1981). In toroviruses, the gene is located between the envelope and the nucleoprotein gene (Cornelissen *et al.*, 1997; Duckmanton *et al.*, 1999). The

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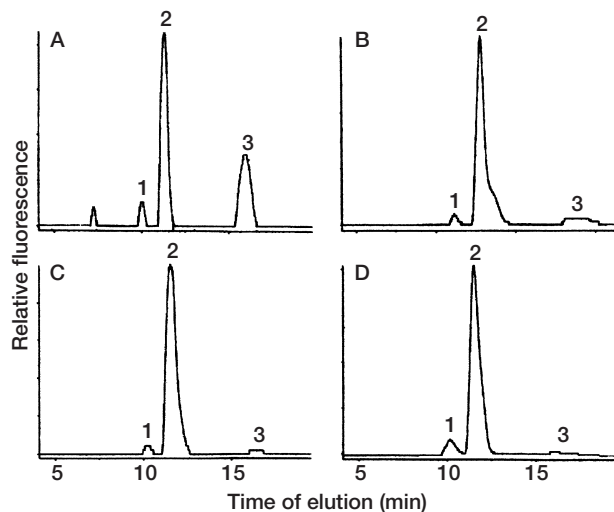


Fig. 1. Hydrolysis of Neu4,5Ac<sub>2</sub> by MHV-JHM and PV. Free 4-*O*-acetylated sialic acids of guinea pig serum glycoproteins, containing approximately 25% Neu4,5Ac<sub>2</sub> and 75% Neu5Ac, were incubated with 4 mU viral esterases at 37 °C for 2 h. Sialic acids were incubated subsequently with DMB and subjected to reverse-phase HPLC analysis. Fluorescent DMB-labelled sialic acids were detected at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. Incubations of sialic acids were carried out with (A) PBS, (B) MHV-JHM, (C) PV and (D) MHV-S. Peak 1 represents 5-*N*-glycolylneuraminic acid (Neu5Gc), peak 2 represents 5-*N*-acetylneuraminic acid (Neu5Ac) and peak 3 represents 5-*N*-acetyl-4-*O*-acetylneuraminic acid (Neu4,5Ac<sub>2</sub>).

viral HE proteins are evolutionarily equidistant, with approximately 25–30% identity on the amino acid level, while those of MHV and BCoV share approximately 60% identity.

BCoV, HEV and HCoV-OC43 use 9-*O*-acetylated sialic acid (Neu5,9Ac<sub>2</sub>) as their receptor determinant (Schultze *et al.*, 1991a, b; Vlasak *et al.*, 1988a, b). The acetyl esterases of those coronaviruses hydrolyse the 9-*O*-acetyl group of sialic acid and are, therefore, receptor-destroying enzymes (Schultze *et al.*, 1991b; Vlasak *et al.*, 1988a).

Recently, we have shown that the HE protein of PV, which is closely related to MHV-S, exhibits a substrate specificity different from those of influenza C viruses and BCoV (Klauegger *et al.*, 1999). In addition, we have shown that the HE protein of MHV-S specifically hydrolyses 5-*N*-acetyl-4-*O*-acetylneuramic acid (Neu4,5Ac<sub>2</sub>). In contrast, Neu5,9Ac<sub>2</sub> is no substrate for MHV-S (Regl *et al.*, 1999).

These results prompted us to investigate the substrate specificities of other coronaviruses closely related to MHV-S. In this study, we compared substrate specificities of MHV-S, MHV-JHM and PV for natural and synthetic low molecular mass substrates. We now show that Neu4,5Ac<sub>2</sub> is indeed the natural substrate of MHV-JHM and PV.

## Methods

■ **Viruses and cells.** MHV-S was kindly supplied by M. Buchmeier (Scripps Research Institute, La Jolla, CA, USA). MHV-JHM was supplied

by M. C. Lai (Howard Hughes Medical Institute, University of Southern California, CA, USA). PV was supplied by P. A. Nuttall (NERC Institute of Virology and Environmental Microbiology, Oxford, UK). All viruses were tested for the presence of acetyl esterase activity in a plaque assay, as described previously (Klauegger *et al.*, 1999).

■ **Enzyme assays.** The acetyl esterase activity of MHV-S, MHV-JHM and PV was determined with *p*-nitrophenyl acetate (*p*NPA), as described previously (Vlasak *et al.*, 1987). One unit of viral esterase activity was defined as the amount of enzymatic activity resulting in the cleavage of 1 μmol *p*NPA per min.

The relative fluorescence of 4-methylumbelliferone or fluorescein released by the enzyme was measured using a TECAN SPECTRAFluor spectrofluorometer. The substrates were solubilized in acetone [5 mM 4-methylumbelliferyl acetate (4-MUAc) or 1 mM fluorescein diacetate (FDA)]. Hydrolysis of the substrates was monitored continuously during a period of 30 min at 25 °C (excitation wavelengths of 485 and 360 nm; emission wavelengths of 535 and 465 nm for FDA and 4-MUAc, respectively). All assays were performed with blanks of heat-denatured enzyme. Each assay was carried out five times to determine the *K<sub>m</sub>* and *V<sub>max</sub>* values.

For assays involving glycosidically bound sialic acids, virus (4 mU esterase activity) was incubated at 37 °C either with guinea pig or horse serum, both containing approximately 30% Neu4,5Ac<sub>2</sub>. Heat-inactivated virus was used as a control. Reactions were stopped by heating for 10 min at 96 °C.

■ **Fluorimetric high pressure liquid chromatography (HPLC) analysis.** Reverse-phase HPLC analysis of sialic acids was performed essentially as described previously (Regl *et al.*, 1999). Briefly, samples containing glycosidically bound sialic acids were first hydrolysed with 2 M propionic acid for 4 h at 80 °C. The hydrolyzed mixtures were centrifuged for 10 min and the supernatants were lyophilized. Samples were then incubated with 1,2-diamino-4,5-methylene-dioxybenzene (DMB) reagent for 1 h at 56 °C. After centrifugation for 10 min, 100 μl of a 1:50 dilution (in water) of the supernatant was injected onto an RP-18 column and eluted isocratically by water–methanol–acetonitrile (86:7:9 by volume) at a flow-rate of 0.9 ml per min. Fluorimetric detection occurred at an excitation wavelength of 373 nm and an emission wavelength of 448 nm.

■ **Solid-phase binding assay.** Virus binding assays were performed on coated 96-well microtitre plates as described previously (Klauegger *et al.*, 1999). Horse serum was diluted in PBS and allowed to bind at 4 °C overnight (100 μl per well). For assays involving de-*O*-acetylation, serum glycoproteins were incubated with virus (4 mU esterase activity) or 0.2 M NaOH. The viral esterase was heat-inactivated prior to the coating of the microtitre wells and NaOH was neutralized by the addition of HCl. Wells were then washed with PBS and the remaining binding sites were blocked with 2% BSA in PBS for 2 h at room temperature. Pre-incubated and control wells were incubated with virus (1–2 mU esterase activity) at 4 °C overnight. For inhibition assays, virus preparations were pre-incubated with antiserum K134, which is specific for MHV-A59 (Bos *et al.*, 1996). This antiserum was kindly provided by W. Spaan (Leiden University, Leiden, The Netherlands). Unbound virus was removed by washing with ice-cold PBS. Bound virus was detected by incubation with 100 μM 4-MUAc. Hydrolysis of substrate was monitored at an excitation wavelength of 365 nm.

■ **Analysis of phylogenetic relationships.** Amino acid sequences of coronavirus proteins were aligned using the CLUSTAL method (Higgins & Sharp, 1989) and phylogenetic trees were constructed using

the MEGALIGN program (DNASTAR) by the neighbour-joining method (Saitou & Nei, 1987). The following protein sequences (with accession numbers) were used for the alignments: HE proteins, BCoV-F15 (P33468), BCoV-Mebus (P15776), HCoV-OC43 (P30215), MHV-DVIM (AAC63044), MHV-JHM (AAA46442), MHV-S (AAA46460), PV (CAA06776) and SDAV (AAF97737); spike proteins, BCoV-F15 (P25190), BCoV-Mebus (P15777), HCoV-OC43 (P36334), MHV-A59 (P11224), MHV-DVIM (BAA23719), MHV-JHM (P11225) and SDAV (AAF97738); E proteins, BCoV-F15 (P15775), BCoV-Mebus (P15779), HCoV-OC43 (Q04854), MHV-DVIM (AAC36597), MHV-JHM (P06591), MHV-S (P29076) and SDAV (AAF97741); M proteins, BCoV-F15 (P10526), BCoV-Mebus (AAA66396), HCoV-OC43 (Q01455), MHV-A59 (P03415), MHV-JHM (P08549) and SDAV (AAF97742); and N proteins, BCoV-F15 (VHIHNI), BCoV-Mebus (VHIHBC), Equine coronavirus (EqCoV) (AAG39339), HCoV-OC43 (P33469), MHV-A59 (NP\_045302), MHV-DVIM (AAA474734), MHV-JHM (P03417), MHV-S (AAA46468) and SDAV (BAA01591).

## Results

### The HE proteins of MHV-JHM and PV specifically hydrolyse 4-O-acetylated sialic acids

Recently, we have shown that the HE protein of MHV-S exhibits a novel substrate specificity towards Neu4,5Ac<sub>2</sub> (Regl *et al.*, 1999). In contrast, other viral HE proteins, including those of influenza C viruses, BCoV and HEV, have been shown to specifically hydrolyse Neu5,9Ac<sub>2</sub>. For other murine coronaviruses and related strains, the precise substrate specificities of their esterases have not been determined yet. For PV, we have shown that the esterase is capable of hydrolysing low molecular mass substrates, but not 9-O-acetylated sialic acids. Therefore, we tested the enzymatic activities of MHV-JHM and PV for their specificities towards different O-acetylated substrates. Since the HE of MHV-S exhibits a specificity for Neu4,5Ac<sub>2</sub>, we first incubated 4-O-acetylated sialic acids with PV or MHV-JHM. The free sialic acids were derived from guinea pig serum glycoproteins by acidic hydrolysis. Reverse-phase HPLC analysis of the DMB-derivatized sialic acids revealed a complete conversion of Neu4,5Ac<sub>2</sub> into Neu5Ac (Fig. 1).

### Hydrolysis of synthetic substrates

Since viral esterases commonly hydrolyse synthetic substrates (Garcia-Sastre *et al.*, 1991; Schauer *et al.*, 1988; Vlasak *et al.*, 1987), we determined the  $K_m$  and  $V_{max}$  values of the three different HE proteins with FDA, 4-MUAc and *p*NPA. The  $K_m$  values of the coronavirus esterases were two- to tenfold lower than that for the influenza C virus HE protein. The lowest  $K_m$  values were observed with FDA, while *p*NPA was hydrolysed with the highest velocity (Table 1). We also developed an assay for direct *in situ* detection of the HE proteins in SDS-polyacrylamide gels. In a non-reducing gel, the enzymatic activity of the coronavirus esterases could be restored after the removal of SDS by a 1 h incubation in Triton X-100. Esterase activity was detected by incubation with a commercial  $\alpha$ -naphthyl acetate kit. Staining was observed at a position slightly below the 130 kDa molecular mass marker, indicating that the HE proteins of MHV-S and MHV-JHM are present as homodimers (Fig. 2A). In order to demonstrate that the viral hydrolysis of  $\alpha$ -naphthyl acetate is caused by the viral HE proteins, a Western Blot was performed in parallel. HE-specific monoclonal antibodies (originally provided by H. Wege, University of Würzburg, Würzburg, Germany) reacted specifically with the dimeric HE proteins, which migrated at the same position as the proteins that hydrolysed  $\alpha$ -naphthyl acetate. It should be mentioned that the esterase activities could not be restored when the viral proteins were subjected to electrophoresis under reducing conditions (data not shown). Presumably, incubation with  $\beta$ -mercaptoethanol resulted in an irreversible loss of enzymatic activity of the HE proteins.

### Hydrolysis of 4-O-acetylestere by MHV-S, MHV-JHM and PV results in loss of virus binding to glycoproteins

MHV-S specifically binds to glycoproteins containing Neu4,5Ac<sub>2</sub> (Regl *et al.*, 1999). To determine whether the O-acetylestereases of MHV-JHM and PV are able to destroy binding domains for MHV-S, we incubated 4-O-acetylated

**Table 1.** Comparison of the kinetic characteristics of coronavirus sialate-4-O-acetylestereases and the sialate-9-O-acetylesterease of influenza C virus

Substrate	MHV-S		MHV-JHM		PV		Influenza C/JHG/66 virus	
	$K_m$ (mM)	$V_{max}$ (pmol/ml/min)	$K_m$ (mM)	$V_{max}$ (pmol/ml/min)	$K_m$ (mM)	$V_{max}$ (pmol/ml/min)	$K_m$ (mM)	$V_{max}$ (pmol/ml/min)
FDA	0.0013 ± 0.0003	739 ± 71	0.0058 ± 0.0007	470 ± 23	0.0019 ± 0.0002	1090 ± 37	0.011 ± 0.002	354 ± 27
4-MUAc	0.0205 ± 0.0011	380 ± 26	0.053 ± 0.008	200 ± 12	0.094 ± 0.005	1147 ± 52	0.21 ± 0.003	1290 ± 84
<i>p</i> NPA	0.103 ± 0.014	4390 ± 314	0.33 ± 0.017	6150 ± 530	0.176 ± 0.009	3620 ± 207	0.98 ± 0.045	9120 ± 850

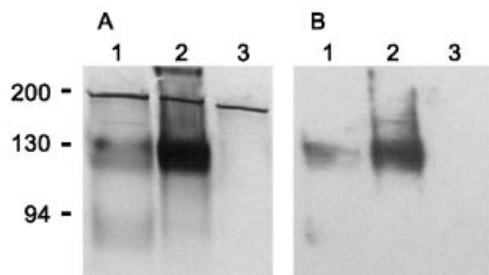


Fig. 2. Detection of viral acetyltransferase activity in SDS–polyacrylamide gels. Tissue culture supernatants of infected cells (5  $\mu$ l MHV-S and 20  $\mu$ l MHV-JHM) were mixed with sample buffer, incubated for 10 min at room temperature and resolved by 8% SDS–PAGE on duplicate gels run in parallel. One gel was washed three times for 20 min in PBS and 1% Triton X-100, followed by an additional wash with ddH<sub>2</sub>O. Then the gel was incubated for 1 h with  $\alpha$ -naphthyl acetate/Fast Blue BB solution (Sigma). Reactions were stopped by rinsing the gel in ddH<sub>2</sub>O. The second gel was blotted onto nitrocellulose and incubated with monoclonal antibodies specific for the HE protein of MHV-JHM. After the removal of unbound antibodies, the blot was incubated with goat anti-mouse serum coupled to horseradish peroxidase. Then the blot was washed and developed with ECL substrate (Pierce). Positions of molecular mass markers (kDa) are indicated on the left. Lane 1, MHV-S; lane 2, MHV-JHM; lane 3, control.

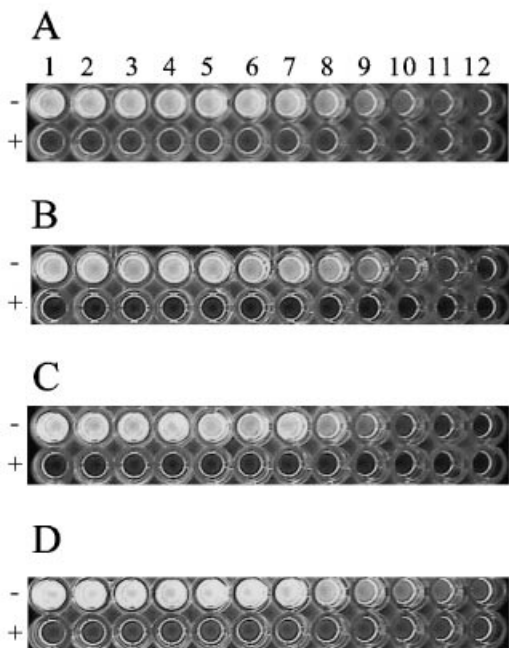


Fig. 3. Hydrolysis of MHV-S receptors by the acetyltransferases of MHV-JHM and PV. 4-*O*-acetylated glycoproteins were incubated with 4 mU viral esterases (+) or mock-treated (–). Then the glycoproteins were coated onto microtitre wells in serial twofold dilutions (32  $\mu$ g in well number 1) and tested for the presence of receptors for MHV-S. Glycoproteins were pre-incubated with (A) MHV-S, (B) PV, (C) MHV-JHM and (D) 0.2 M NaOH.

serum glycoproteins with virus preparations containing 4 mU esterase activity. The viruses were then heat-inactivated and the glycoproteins were coated onto microtitre wells in serial

twofold dilutions. For a control, alkali- or mock-treated glycoproteins were coated. Then, MHV-S was added to the plates and allowed to bind. After the removal of unbound virus, the plates were incubated with 4-MUAc to detect bound MHV-S. As shown in Fig. 3, specific binding was observed in wells containing mock-treated glycoproteins, while pre-incubation with MHV-S, MHV-JHM and PV resulted in a complete loss of binding (Fig. 3, A–C). Pre-incubation with 0.2 M NaOH, which causes complete de-*O*-acetylation, also resulted in a lack of binding sites (Fig. 3 D). In order to verify that glycoproteins that had been saponified with NaOH were still able to bind to solid supports, we stained the sialic acids of the bound glycoproteins with the periodic acid–Schiff technique (Volz *et al.*, 1987). We observed comparable staining of the untreated and NaOH-treated proteins (data not shown).

We also tested whether MHV-JHM and PV exhibit a binding activity to 4-*O*-acetylated glycoproteins. As shown in Fig. 4, both viruses bound to immobilized horse serum glycoproteins to a similar extent as MHV-S. In all cases, approximately 25–50 ng per well of serum glycoproteins was sufficient for virus binding. Again, pre-incubation with the different viral esterases or with NaOH resulted in a complete loss of binding sites.

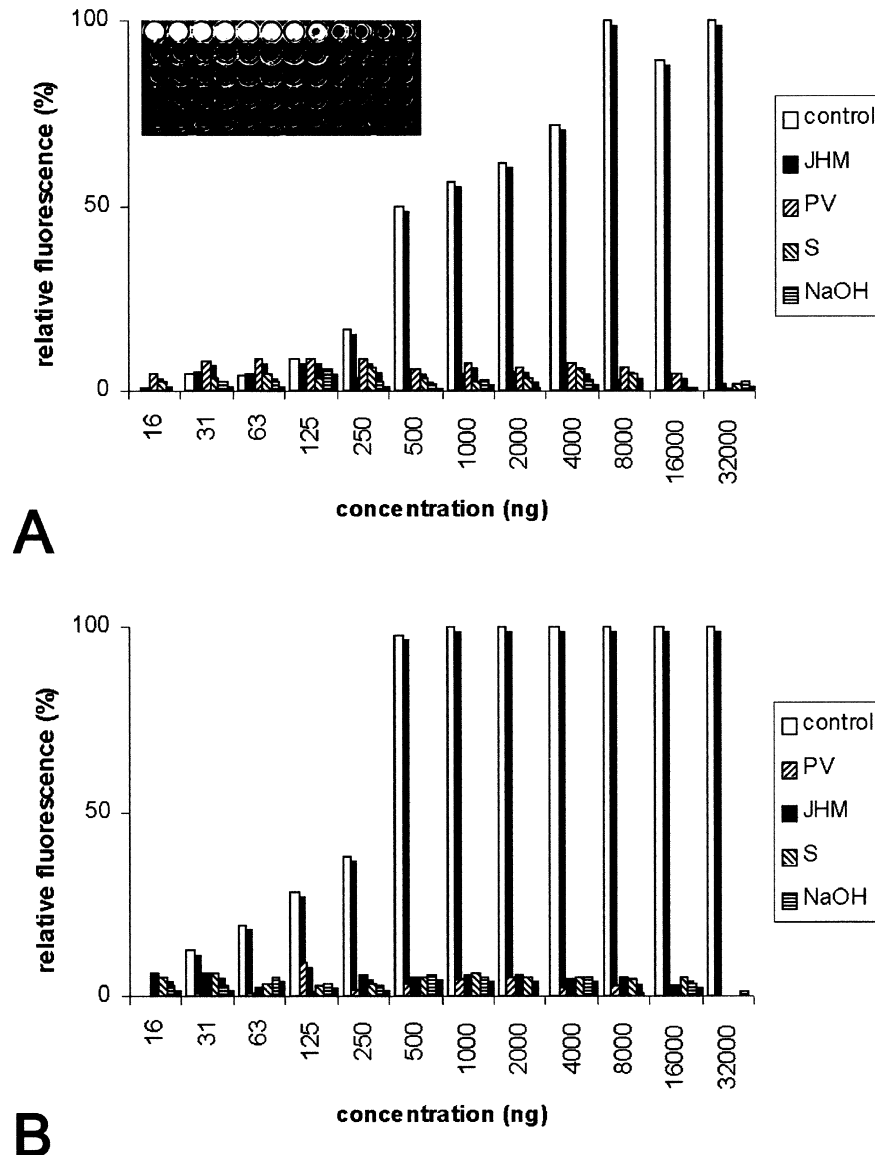
## Discussion

Members of several virus families have in the past been shown to possess receptor-destroying enzymes with sialate-*O*-acetyltransferase activity. Among members of the *Coronaviridae*, viruses of antigenic cluster 2 express this enzyme at their surface. The first coronavirus esterase was identified in BCoV (Vlasak *et al.*, 1988b). Similar to the HE protein of influenza C viruses, the BCoV esterase specifically de-*O*-acetylates Neu5,9Ac<sub>2</sub> (Schultze *et al.*, 1991b; Vlasak *et al.*, 1988a). Recently, we have shown that PV exhibits a substrate specificity different from those of influenza C virus and BCoV (Klauegger *et al.*, 1999), comparable to results obtained for MHV-S which was shown to specifically hydrolyse Neu4,5Ac<sub>2</sub> (Regl *et al.*, 1999).

We now provide evidence that PV and MHV-JHM also exhibit a selective activity towards Neu4,5Ac<sub>2</sub>. Both viruses are capable of de-*O*-acetylating Neu4,5Ac<sub>2</sub>. Subtle differences in substrate turnover by the esterases of the viruses tested were observed with glycosidically bound Neu4,5Ac<sub>2</sub> (data not shown).

In addition to the 'natural' substrate, Neu4,5Ac<sub>2</sub>, synthetic substrates like *p*NPA, FDA and 4-MUAc are hydrolysed by the viral esterases. Compared to the published data for the 9-*O*-acetyltransferase of influenza C viruses, the *K<sub>m</sub>* value of the coronavirus esterases was approximately two- to tenfold lower. Thus, the sialate-4-*O*-acetyltransferases are capable of hydrolysing these synthetic substrates more efficiently.

We present data showing that all coronaviruses tested are also able to bind specifically to horse serum glycoconjugates.



**Fig. 4.** Binding of PV and MHV-JHM to 4-*O*-acetylated sialic acids. Horse serum was incubated with 4 mU esterase of MHV-JHM, (JHM), PV, MHV-S (S) or 0.2 M NaOH and coated onto microtitre wells in serial twofold dilutions. All wells were blocked with 2% BSA. Binding of (A) MHV-JHM or (B) PV was monitored by the detection of 4-methylumbelliferone released from 4-MUAc by viral esterase activity. Data are presented as column charts; the inset in (A) shows the original microtitre plate. Concentrations of glycoproteins bound to the microtitre wells are indicated.

Due to the fact that saponification of the acetyl group abolished binding of MHV-S, MHV-JHM and PV, an involvement of Neu4,5Ac<sub>2</sub> as a determinant for specific binding is clearly suggested. This is corroborated by the fact that pre-incubation of horse serum glycoproteins with the sialate-4-*O*-acylesterases of MHV strains and PV resulted in a complete loss of virus binding. Besides the MHV receptor, also referred to as the biliary glycoprotein, the carcinoembryonic antigen or the carcinoembryonic cell adhesion molecule (Krueger *et al.*, 2001; Robitaille *et al.*, 1999; Wessner *et al.*, 1998), this sialic acid derivative may act as an as yet unexplored and additional receptor determinant for MHV-like coronaviruses. Since,

however, this sialic acid derivative has not been identified in mice, the significance of Neu4,5Ac<sub>2</sub> still awaits further investigation. It should also be mentioned that the viral macromolecule with binding affinity to the sialic acid has not been identified yet. Although the term 'haemagglutinin-esterase' may suggest a binding activity of the HE protein, neither a haemagglutinin activity nor the binding affinity towards Neu4,5Ac<sub>2</sub> can be attributed unambiguously to this viral glycoprotein, at least not in the virus strains tested in this report. In order to shed additional light on the question about the protein involved in binding to Neu4,5Ac<sub>2</sub>, we pre-incubated MHV-S with the polyclonal antiserum K134 (Bos *et*

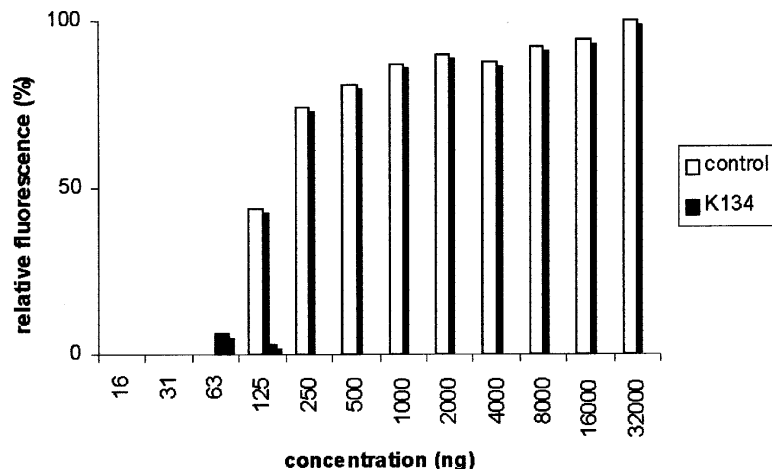


Fig. 5. Inhibition by MHV-A59 antiserum of virus binding to glycoproteins. Horse serum glycoproteins were coated in serial twofold dilutions onto microtitre wells. Then MHV-S (4 mU esterase activity) was added in the presence or absence of 0.5  $\mu$ l per well antiserum K134 and allowed to bind at 4 °C overnight. After removal of unbound virus, binding of MHV-S was determined with 4-MUAC.

*al.*, 1996), which is specific for MHV-A59. Since this strain is devoid of an HE protein, the antiserum does not bind to the HE protein of MHV-S. When we determined enzymatic activity, no inhibition by the antiserum was detected (data not shown). In the presence of K134, we observed, in a solid-phase assay, a complete inhibition of binding of MHV-S to horse serum glycoproteins, indicating that this antiserum interferes specifically with binding to Neu4,5Ac<sub>2</sub> (Fig. 5). Binding of MHV-S to the *O*-acetylated sialic acids apparently involves the viral spike protein and not the HE protein. Thus, a similar situation as that for BCoV may exist. For the latter virus, it was shown that the spike protein is the major receptor-binding protein, whereas the HE protein plays a minor role in binding to sialic acid (Schultze *et al.*, 1991a). A similar situation may exist for MHV. On the other hand, the interference of the MHV-A59-specific antiserum may also be caused by steric hindrance. To finally identify the protein involved in binding to Neu4,5Ac<sub>2</sub>, additional experiments with monoclonal antibodies specific for either the spike or the HE protein are needed. The precise mapping of the binding site for Neu4,5Ac<sub>2</sub> will be a challenging task for the future.

Coronaviruses are currently divided into three groups. Group 2 coronaviruses comprise viruses with an extra surface glycoprotein, termed HE. This HE protein, as well as the corresponding gene, is absent in viruses of antigenic clusters 1 and 3. In addition to the difference in the number of surface glycoproteins, the genome organization and nucleotide sequences of the viral genomes indicate at least three different coronavirus lineages. Although some strains of MHV-like viruses do not express the HE protein, all viruses of antigenic cluster 2 possess the corresponding gene. Due to point mutations, frameshift mutations or deletions, variant strains that do not express the HE protein arise during passage in cell culture and in mouse brain (Luytjes *et al.*, 1988; Yokomori *et al.*, 1991, 1993). It has been proposed that the HE gene was acquired by group 2 coronaviruses via a recombination event during a mixed infection (Luytjes *et al.*, 1988). Since the HE genes of influenza C viruses, toroviruses and coronaviruses are

evolutionarily equidistant, it appears likely that an ancestral gene of unknown origin has been integrated into the genomes during independent recombination events (Cornelissen *et al.*, 1997). The HE proteins of BCoV-like viruses and those of MHV-like viruses are clearly more related to each other than to the corresponding proteins of influenza C viruses and toroviruses. Based on the similarity of the genomes and serological cross-reactivity, the placement of both BCoV- and MHV-like coronaviruses into group 2 appears to be justified. On the other hand, nucleotide sequence data indicate the presence of two distinct lineages of virus evolution within cluster 2. A comparison of representative amino acid sequences of the structural proteins indicates two lineages in all instances (Fig. 6). This alone may justify the proposal for a reorganization of group 2 coronaviruses. In addition, the major difference in the enzymatic properties of the HE proteins further suggests a division of group 2 coronaviruses into subgroups 2a and 2b. It should be stated that the precise substrate specificities of the HE proteins of other MHV-like strains (e.g. MHV-DVIM or SDAV) remain to be determined in the future. However, based on the available nucleotide and protein sequences, we may speculate that they also interact specifically with 4-*O*-acetylated sialic acids. This assumption is based on published biochemical evidence regarding the lack of substantial hydrolytic activity of MHV-DVIM with bovine mucin (Sugiyama *et al.*, 1998), which mainly contains 9-*O*-acetylated sialic acid derivatives. In addition, the HE protein of the rat coronavirus strain SDAV is more closely related to MHV than the esterase of PV, which we have now identified as being sialate-4-*O*-acetyl esterase. Thus, we propose to allocate MHV-like strains, including the rat coronaviruses RtCoV and SDAV, into group 2a. BCoV-like strains, including HCoV-OC43 and HEV, will then belong to group 2b (Table 2).

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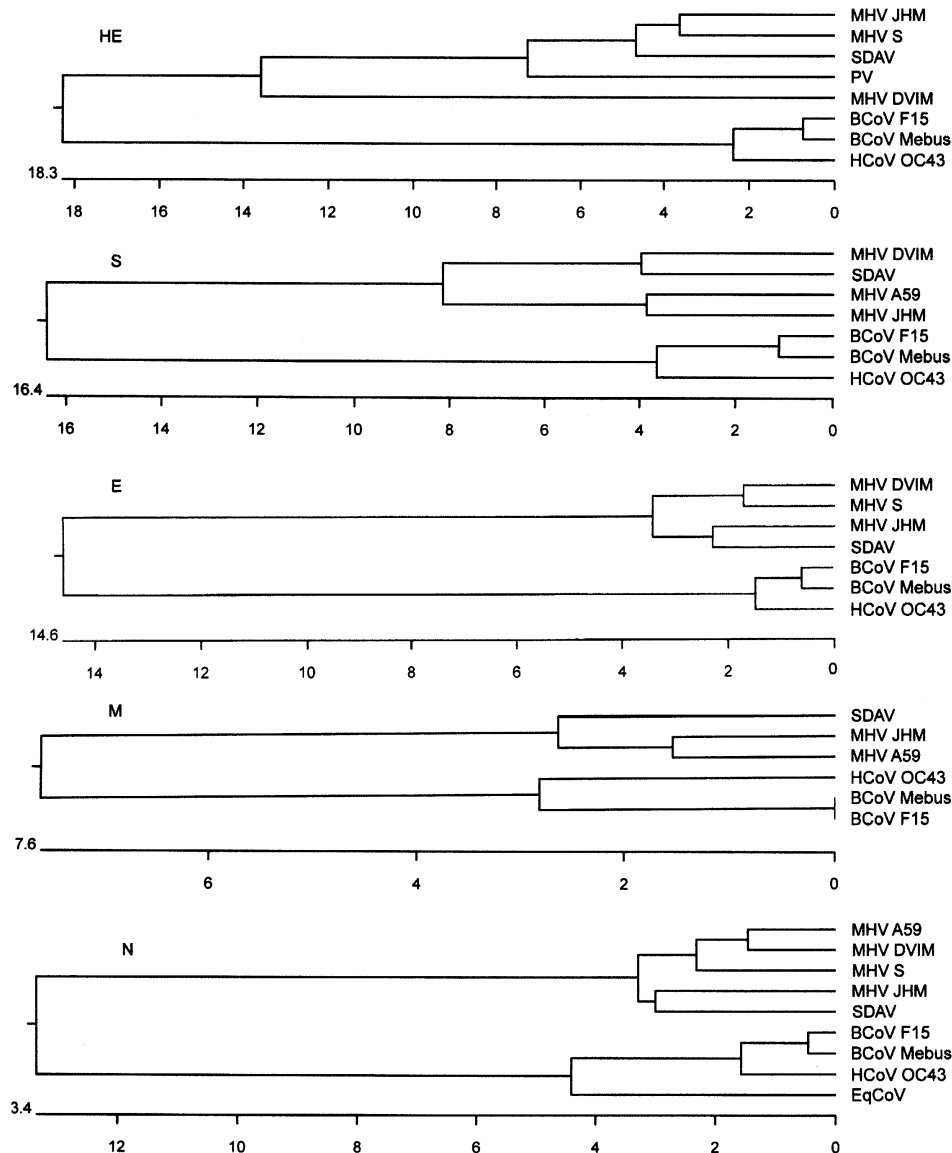


Fig. 6. Phylogenetic relationships of the structural proteins of group 2 coronaviruses. Amino acid sequences of the HE, spike (S), envelope (E), matrix (M) and nucleocapsid (N) proteins of representative coronavirus strains were aligned using the CLUSTAL algorithm. Phylogenetic trees were constructed using the neighbour-joining method.

**Table 2. Proposed reorganization of group 2 coronaviruses**

Group	Virus	Esterase specificity
2a	Mouse hepatitis virus	Neu4,5Ac <sub>2</sub>
	Rat coronavirus	
	Sialodacryoadenitis virus	
2b	Puffinosis virus	Neu5,9Ac <sub>2</sub>
	Bovine coronavirus	
	Human coronavirus OC43	
	Haemagglutinating encephalomyelitis virus	

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