

Expression and Self-Assembly of Grimsby Virus: Antigenic Distinction from Norwalk and Mexico Viruses

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A cDNA obtained from Grimsby virus (GRV), a Norwalk-like virus, purified from a stool sample of a symptomatic adult associated with a gastroenteritis outbreak in the United Kingdom, was used to obtain the complete nucleotide sequence of the second open reading frame (ORF2). The ORF2 sequence of GRV predicts a capsid of 539 amino acids (aa) which exhibits aa identities of 96% to Lordsdale virus, 67% to Mexico virus (MXV), and 43% to Norwalk virus (NV). The GRV capsid protein was expressed in insect cells by using a recombinant baculovirus, and the resulting virus-like particles (VLPs) possessed a protein with an apparent molecular weight of 58,000. Hyperimmune antisera raised against purified GRV, MXV, and NV VLPs were tested in an indirect enzyme-linked immunosorbent assay (ELISA) against GRV, NV, and MXV VLPs, revealing that GRV is antigenically distinct from both NV and MXV. The antigenic specificity of the GRV-hyperimmune antiserum was confirmed in an antigen capture ELISA using GRV-, NV-, or MXV-containing fecal specimens. The expression of the GRV capsid protein has, for the first time, allowed the antigenic comparison of three distinct recombinant Norwalk-like viruses.

The genetic characterization of Norwalk virus (NV) and other human caliciviruses (HuCVs), previously called small round-structured viruses or classical HuCVs, has revealed a high degree of diversity within the family *Caliciviridae* (1–3, 8, 9, 12, 14, 19, 21–23, 27, 30). Phylogenetic analyses indicate an ancestral division of the family into four genera. Two of these genera, called Norwalk-like viruses and Sapporo-like viruses, currently contain viruses from humans (8). Viruses in the Norwalk-like virus genus were previously divided into two genogroups based on phylogenetic analyses of both structural (capsid region) and nonstructural (polymerase) genes (1–3, 7, 8, 20, 21, 33), although the biological significance of this subgrouping was unknown. For the polymerase region, viruses within a genogroup show >80% amino acid (aa) similarity. For the capsid region, viruses within a genogroup share >64% aa homology. The cutoff value for the two genogroups was arbitrary (2, 3, 12). Overall comparative analyses of most Norwalk-like strains (reviewed in references 2, 3, and 10) have been hindered by the lack of published sequence data and type-specific reagents. Viruses from the Sapporo-like virus genus include viruses with a Star of David or classical HuCV appearance by electron microscopy (EM), as represented by the prototype classical HuCV Sapporo virus (30).

Prior to the production of recombinant HuCV (rHuCV) capsid proteins, the antigenic diversity of these noncultivable agents was investigated by immune EM (IEM) and cross-protection studies in volunteers (18, 35). NV, Hawaii virus (HV), and Snow Mountain agent (SMA) were found to be antigenically distinct in volunteer challenge studies, and these three strains can also be differentiated by solid-phase IEM (SPIEM)

(26, 35). In the United States, there are at least four antigenic groups of Norwalk-like viruses, represented by NV, HV, SMA, and Taunton virus, based on SPIEM (12). The scheme described by Lewis (26) has now been extended to include seven types: UK1 (Taunton virus), UK2, UK3 (HV), UK4 (SMA), NV, Oklahoma virus, and Mexico virus (MXV) (10, 25). Independently, nine antigenic types have been determined by IEM in Japan (31), but the relationship of these antigenic types to Lewis' scheme is not clear because the antigenic characterization was performed with different immunological reagents.

The observation that expression of NV capsid protein in insect cells results in self-assembly into virus-like particles (VLPs) has allowed the production of reagents to further study antigenic relationships among the Norwalk-like viruses and other HuCVs (15). The use of antisera prepared against expressed VLPs has shown that NV is antigenically distinct from MXV (16). However, despite reports of the self-assembly of a number of other HuCVs, few data on the specificity of hyperimmune sera raised against these VLPs have been published (1, 7, 20, 21). Antigen detection enzyme-linked immunosorbent assays (ELISAs) based on the use of hyperimmune antisera to rNV and rMXV are mainly type specific (10, 29). In contrast, antibodies to VLPs in individuals infected with HuCVs appear to be more broadly reactive, especially between strains belonging to the same genogroup (11, 24, 28). We now report the expression of Grimsby virus (GRV), a virus in the same genogroup as MXV, using recombinant baculovirus technology and the production and use of hyperimmune antiserum raised against self-assembled GRV VLPs to characterize the antigenic relatedness of this virus to other prototype HuCVs.

GRV was associated with a hospital outbreak of gastroenteritis which occurred in February 1995 at Grimsby District General Hospital, Grimsby, United Kingdom. Prospective molecular typing of outbreak strains indicated that this was the predominant strain circulating in the United Kingdom during

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GRV	MKMASNDANP	SDGSTAN...	LVPEVN	NEV	MALEPVVGAA	IAAPVAGQON	46
LRV	-----	-----A-----	-----	-----	-----	-----	46
MXV	-----R-A-	NDGA-C	-----I-	-----A-	-----	-----LT	46
NV	-M---K--TS	-VDGASGAGQ	-----ASDP	L-MD--A-SS	T-VAT--V-	-----	50
GRV	VIDPWIRNPF	VQAPGGGEFTV	SPRNAPGEIL	WSAPLGPDLN	PYLSHLARMY	-----	96
LRV	-----	-----	-----	-----	-----S---	-----	96
MXV	I-----M---	-----	-----S--V-	LNLE--EI-	-----A---	-----	96
NV	P-----I---	-----Q---I	-N-T--DV-	FDLS---H--	-F-L--SQ--	-----	100
GRV	NGYAGGFEVQ	VILAGNAFTA	KKIIFAAVFP	NFPAEGLSFS	QVTMPFHIV	-----	146
LRV	-----	-----	-----A-----	-----	-----	-----	146
MXV	-----	-V-----	-----I--	---IDN--AA	-I--C--V--	-----	146
NV	--WV-NMR-R	IM-----	-----VSCI--	G-GSHN-TIA	-A-L---V-A	-----	150
GRV	DVRQLEPVLII	FLPDVRRNFY	HYNQSDSTI	KLIAMLYTFL	RANNAGDDVF	-----	196
LRV	-----	-----	-----A-----	-----	-----	-----	196
MXV	-----	-INL-M-----	-----GS--RL	R-----	-----S---	-----	196
NV	---T-D-IEV	--E---VLF	-N-DR-QQ-M	R-VC-----	-TGGTG-S-	-----	200
GRV	TVSCRVLTRP	SPDFDFIFLV	PPTVESRTKP	FTVPILTVEE	MSNSRFPIPL	-----	246
LRV	-----	-----	-----	-----V-----	-----	-----	246
MXV	-----	-----L-N---	-----K---	-L--IS-	-----V-I	-----	246
NV	V-AG--M-C-	-----N-L---	-----QK-R-	--L-N-PLSS	L---A-L-I	-----	250
GRV	EKLYTGPSA	FVVQPQNGRC	TTDGVLLGT	QLSAVNICTF	RGDVTHIAGS	-----	296
LRV	-----	-----	-----	-----N-----	-----	-----	296
MXV	DS-H-S-TEN	I---C---V	-L-E-M---	---PSQ--A-	-TL-GSTSR	-----	296
NV	SSIGIS-DNV	QS--F-----	-L-R-V---	FV-LSHVAKI	--TSNGTV..	-----	298
GRV	HDYTMN	LASQNNVVD	PTEETIAPLG	TPDEFVKIQG	-----	332
LRV	-----	-----S-----	-----	-----	-----	332
MXV	ASDQADTFPT	RLFN-HWHIQ	-DNL-GTP--	-A-D-----	-----R--VF-	-----	346
NVIN	-TELDGTFFH	-F-G---T-	F--LG-CDWH	-----	329
GRV	MLTQTTREDG	STRAHKATVS	TGSVHFIPKL	GSVQYTTDTN	NDFQTCQNTK	-----	382
LRV	L-----A-	-----	-----T-----	-----F-----	-----A---	-----	382
MXV	VAG-RNP--S	T---E-K-D	-T-GR-T---	--LET--ESD	--LDLS-P--	-----	394
NV	I	NMTQF-	HSSQTQYD-D	-TPDT-V-H-	--I-ANGIGS	.GNYV-VLSW	375
GRV	FTFVGVIQDG	NNHQNEPQQW	VLPNYSGRTG	HNVHLAPAV	APTFFPGEQLL	-----	431
LRV	-----	DH-----	S-----	-----	-----	-----	431
MXV	-----	I...V	VDNRA-F---	S--D---QFT	--MN-----	--N-----	440
NV	IS-P.....S	HPFSQVLDL-	KI--GSSIT	EAT---S-Y	P-G-...V-V	-----	419
GRV	FFRSTMPGCS	GYPNMLDCL	LQEQWVQHEF	QEAAPAQSDV	ALLRFVNPDT	-----	481
LRV	-----	-----	-----L-----	-----	-----	-----	481
MXV	-----	-QL-SSG	-RS-GV---	V-----	-----S--TQ-	-V-----	490
NV	--M-K---PG	A---P---	-----YIS-LA	S-Q--TVGEA	-----HY-D---	-----	466
GRV	GRVLFECKLY	KSGVVHVAHT	GPH..DLVIP	PNGYFRFDSW	VNQFYTLAPM	-----	529
LRV	-----	-H-----	-----I-----	-----Y..-L-	-----	-----	529
MXV	-----	-A--H	-L-FM--KN	-DS	PITV-----	-E--P-----	538
NV	--N-L-F-A-	PD-FL-CVFN	-ASSGPQQL-	I--V-V-V--	--SR--Q-K-V	-----	516
GRV	GNGAGRRRAL	-----	-----	-----	-----	539
LRV	-T-----	-----	-----	-----	-----	-----	539
MXV	-T-N-----	IQ....	-----	-----	-----	-----	548
NV	-TASSA-GR-GLRR	-----	-----	-----	-----	530

FIG. 1. Amino acid sequence alignments of the GRV, LRV, MXV, and NV putative capsid proteins. Alignments were generated by the Clustal method. The hypervariable region of the GRV sequence is underlined, and residues matching GRV exactly are indicated by dashes. GenBank accession numbers for the sequences used in the alignments are as follows: LRV, X86557; MXV, U22498; and NV, M87661.

the HuCV season of 1995–1996, and similar strains had also been seen in The Netherlands at the same time (32). Viral nucleic acid was extracted and purified from a fecal specimen of a symptomatic adult by the guanidinium thiocyanate-silica method. A single-stranded cDNA was produced by using SuperScript II reverse transcriptase (Life Technologies, Paisley, United Kingdom) after priming of the polyadenylated positive-strand RNA genome with the primer Linker-T20VN (27). The entire second open reading frame 2 (ORF2) of GRV, flanked by small regions of ORF1 and ORF3, was amplified by a series of seminested PCRs, using the Expand Long Template PCR System (Boehringer Mannheim UK Limited, Lewes, East Sussex, United Kingdom) with forward primers GII and GV4 and reverse primers Linker and GV7 (4, 5). The resulting amplicon of approximately 1.6 kb was cloned into the pTag vector (R&D Systems, Abingdon, United Kingdom). The ORF2 sequence of GRV predicted a capsid of 539 aa which exhibited 96% aa identity to Lordsdale virus (LRV) within the capsid region. In contrast, GRV exhibited only 67 and 43% aa identity to MXV and NV, respectively, over this same region. An alignment of

the capsid regions of GRV, LRV, MXV, and NV is shown in Fig. 1.

The GRV capsid cDNA was recloned into pFastbac1 (Bac-to-Bac; Life Technologies, Gaithersburg, Md.), and a recombinant “bacmid” vector containing ORF2 of GRV downstream from the polyhedron promoter was produced by site-specific transposition between this pFastbac1 recombinant and a baculovirus shuttle vector in the presence of a helper plasmid in *Escherichia coli*. The resulting composite bacmid was transfected into *Spodoptera frugiperda* 9 (Sf9) cells, and recombinant baculovirus was isolated by plaque purification. VLPs were obtained by infecting Sf9 cells with recombinant baculovirus at a multiplicity of infection of between 5 and 10, with harvesting of cell cultures at 7 days postinfection. VLPs were concentrated by ultracentrifugation (20,000 × g) of culture supernatants followed by purification in 20 to 60% sucrose gradients in 10 mM Tris-HCl (pH 6.0). VLPs were finally pelleted by ultracentrifugation (120,000 × g) and the protein content was estimated by using a bicinchoninic acid kit (Bio-Rad, Hercules, Calif.) and bovine serum albumin as a standard. The purity and capsid integrity of rGRV preparations were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining, endotoxin quantification (Associates of Cape Cod Inc., Woods Hole, Mass.), and EM.

The putative capsid protein of GRV had a predicted molecular weight of 59,000, and Coomassie blue staining of 10% polyacrylamide gels following electrophoresis of purified rGRV revealed migration of the capsid protein to a position slightly above that of the 58,000-molecular-weight capsid protein of rNV (Fig. 2). In addition, similar to rNV, high-molecular-weight forms of rGRV capsid protein were observed when purified VLPs were not boiled prior to electrophoresis (data not shown). These more-slowly migrating bands are thought to represent oligomeric forms (13). EM of purified rGRV particles revealed characteristic VLPs of 38 nm in diameter (Fig. 3). Some preparations of rGRV particles revealed both 38- and 23-nm VLPs by EM, similar to those observed in rNV preparations (data not shown) (34).

Two New Zealand White rabbits and four CD-1 mice were immunized with purified rGRV (four doses of 50 µg/dose for rabbits and 20 µg/dose for mice), using Freund’s complete adjuvant for the first dose and Freund’s incomplete adjuvant for the subsequent doses. Indirect ELISAs were performed as described previously for rNV except that VLPs were used at a concentration of 1 µg/ml to coat ELISA plates (6). Rabbit (n = 2) and mouse (n = 4) hyperimmune sera raised against purified rGRV VLPs and rabbit hyperimmune serum raised against purified rNV or rMXV (10, 15, 17) VLPs were tested

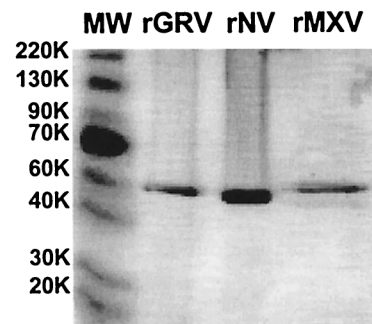


FIG. 2. Coomassie blue-stained 10% polyacrylamide gel following sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified rGRV, rNV, and rMXV capsid proteins. Preparations were boiled prior to electrophoresis.

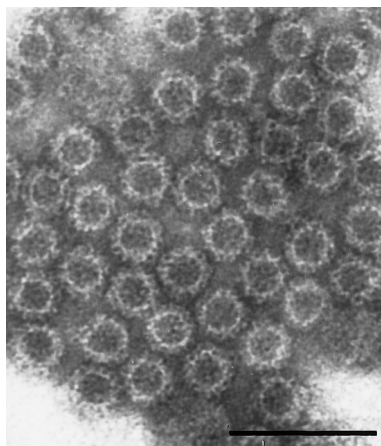


FIG. 3. Electron micrograph of rGRV VLPs showing 38-nm-diameter VLPs. Bar, 100 nm.

by indirect ELISAs against rGRV, rNV, and rMXV VLPs (Table 1 and data not shown). Titers of the hyperimmune sera to control preparations from insect cells infected with a wild-type baculovirus were ≤ 100 (data not shown). Titers of the anti-rGRV rabbit and mouse hyperimmune sera against individual VLPs differed by up to 16-fold between animals, but in all cases, titers to rGRV were at least ≥ 256 -fold higher than those to heterologous rNV or rMXV VLPs (Table 1 and data not shown). The titers of the anti-rMXV hyperimmune serum when tested against rGRV and rNV were < 8 - and < 128 -fold lower, respectively, than that obtained against the homologous rMXV (Table 1). The titers of the anti-rNV hyperimmune serum when tested against rMXV and rGRV were > 512 -fold lower than that against rNV (Table 1). The low cross-reactivity of the anti-rGRV hyperimmune sera with rMXV was rather unexpected because GRV and MXV exhibited 68% aa identity over the capsid region, although the level of aa identity was 47% within the hypervariable region. The titers of anti-rGRV hyperimmune sera obtained when tested against rNV and rMXV were similar, although NV exhibited only 43 and 20% aa identities to rGRV over the complete and hypervariable capsid regions, respectively.

To confirm the type specificity of rGRV antisera, an antigen capture ELISA similar to those described for NV was developed (13). Preimmune and postimmune rabbit antisera at a dilution of 1:5,000 were used to coat the ELISA plates, and a 1:2,000 dilution of pooled mouse hyperimmune antisera was used as the detection antibody. One percent normal rabbit serum was added to the goat anti-mouse conjugate to reduce background reactivity. NV and MXV antigen capture ELISAs were used as previously described except that normal rabbit or

TABLE 2. Reactivity of fecal specimens containing GRV, MXV, or NV in antigen detection ELISAs using hyperimmune antisera to rVLPs

Specimen	Reactivity in ELISA for ^a :		
	rGRV	rMXV	rNV
85ELH/96/GRV	4.41	0.01	0.00
60RHCH/96/GRV	4.18	0.01	0.00
64RHCH/96/GRV	4.59	0.01	0.00
69Bai/96/GRV	3.77	0.01	0.00
52909/NV/8FIIa	0.01	NT ^b	2.90
54009/NV/8FIIa	0.00	NT	2.45
54504/NV/8FIIa	0.00	NT	3.76
55503/NV/8FIIa	0.00	NT	2.83
55005/NV/8FIIa	0.00	NT	3.64
47RBH/93/MXV	0.08	1.86	NT
67RBH/93/MXV	0.11	3.19	NT
65Car/94/MXV	0.09	2.82	NT
54AGH/94/MXV	0.08	3.45	NT

^a ODs at 450 nm versus those of hyperimmune antisera. Positive specimens are indicated in boldface ($P > 0.1$, $P/N > 2$).

^b NT, not tested because a lack of cross-reactivity between NV and MXV had been demonstrated in previous studies (14, 15).

guinea pig serum was added to the conjugate as deemed appropriate (13, 15). Specimens were considered positive if $P > 0.1$ and $P/N > 2$ (where P is the optical density [OD] with hyperimmune antiserum and N is the OD with preimmune serum). The sensitivity of the assay was approximately 2 ng/ml of purified rGRV. Nevertheless, the assay was unable to detect either rNV or rMXV at concentrations up to 2 μ g/ml.

Five fecal specimens containing NV, obtained from volunteers challenged with NV, were tested in parallel in the GRV and NV antigen ELISAs. Four fecal specimens from symptomatic patients involved in three outbreaks of gastroenteritis associated with MXV-like viruses were tested in both GRV and MXV assays, and four fecal specimens containing viruses closely related to GRV, as determined by sequence analysis (data not shown), were tested in all three antigen ELISAs (Table 2). These ELISAs recognized only homotypic strains. One MXV specimen, 67RBH/93/MXV, was positive in the GRV assay. However, this sample was also highly positive in the MXV assay. Further studies are needed to determine if this specimen contains two viruses. Finally, it will be of interest to test recombinant LRV particles in the GRV ELISA, since we anticipate that LRV particles will react given the high level of predicted aa identity between LRV and GRV.

The demonstration of the type specificity of antigen ELISAs using polyclonal antisera raised against VLPs confirms earlier observations based on NV and MXV assays. However, the expression of GRV has, for the first time, allowed comparison of three antigenically distinct rHuCV ELISAs, two of which are based on viruses within the same genogroup. The high level of sequence similarity in the capsid region of GRV to the corresponding regions of Bristol virus (BV) and LRV suggests that these three viruses are antigenically related. However, neither BV nor LRV has been typed under Lewis' scheme (26); therefore, the antigenic relationship of GRV to LRV and BV awaits confirmation. Antigenic typing of HuCVs has been hampered by the lack of an in vitro culture system. Although SPIEM and IEM studies using human convalescent-phase sera have provided valuable information on antigenic variation within HuCVs, such reagents are poorly defined and cross-reactivity with more than one strain has been observed (7).

TABLE 1. ELISA cross-reactivity of hyperimmune sera raised against rHuCVs NV, MXV, and GRV

rHuCV	Titer with antiserum to rHuCV ^a :			
	NV (rabbit)	MXV (rabbit)	GRV (rabbit)	GRV (mouse)
NV	6,553,600	3,200	1,600	400
MXV	1,600	409,600	1,600	800
GRV	6,400	51,200	1,638,400	6,553,600

^a Values in the table represent the reciprocals of ELISA titers of sera tested with the indicated rHuCVs. Homologous titers are given in boldface type.

ELISAs using hyperimmune antisera raised against VLPs offer an alternative approach for antigenic typing of HuCVs and allow the rapid testing of large numbers of strains with standardized reagents. It is clear, however, that we are still at an early stage in this process. The expression and production of monotypic antisera to VLPs based on genetically distinct HuCVs are essential prerequisites to further our understanding of the antigenic heterogeneity of this group of viruses.

Nucleotide sequence accession number. The EMBL accession number for ORF2 of GRV is AJ004864.

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