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Genomic characterization of the unclassified bovine enteric virus Newbury agent-1 (Newbury1) endorses a new genus in the family *Caliciviridae*

S.L. Oliver^{a,1}, E. Asobayire^a, A.M. Dastjerdi^b, J.C. Bridger^{a,*}

^a Department of Pathology and Infectious Diseases, Royal Veterinary College, 4, Royal College Street, Camden, London NW1 0TU, UK

^b Department of Craniofacial Development, King's College London, Guy's Tower, Floor 27, London Bridge, London SE1 9RT, UK

Received 8 December 2005; returned to author for revision 20 December 2005; accepted 16 February 2006

Available online 30 March 2006

Abstract

The pathogenic bovine enteric virus, Newbury agent-1 (Bo//Newbury1/1976/UK), first identified in 1976, was characterized as a possible calicivirus by morphology, buoyant density in CsCl and the presence of a single capsid protein but genomic sequence could not be obtained. In the present study, the complete genome sequence of Newbury1 was determined and classified Newbury1 in a new genus of the *Caliciviridae*. The Newbury1 genome, of 7454 nucleotides, had two predicted open reading frames (ORFs). ORF1 encoded the non-structural and contiguous capsid proteins. ORF2 encoded a basic protein characteristic of the family *Caliciviridae*. Compared to the 4 recognized *Caliciviridae* genera, *Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus*, Newbury1 had less than 39% amino acid (47% nucleotide) identity in the complete 2C-helicase, 3C-protease, 3D-polymerase and capsid regions but had 89% to 98% amino acid (78% to 92% nucleotide) identity to the recently characterized NB virus in these regions. By phylogenetic analyses, Newbury1 and NB viruses formed a distinct clade independent of the 4 recognized genera. However, amino acid identities showed that Newbury1 and the NB virus were distinct polymerase types (90% amino acid identity), but their complete capsid proteins were almost identical (98% amino acid identity). Analyses of contemporary viruses showed that the two polymerase genotypes, Newbury1 and NB, were circulating in UK cattle and antibody to Newbury1-like viruses was common in cattle sera. The present study defined the existence of a new genus in the *Caliciviridae* that we propose be named *Becovirus* or *Nabovirus* to distinguish the new clade from bovine noroviruses.

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Keywords: Calicivirus; Bovine; Diarrhea; Antigenic; Genome organization; Newbury1; Newbury agent; *Becovirus*; *Nabovirus*

Introduction

The study of the viral causes of diarrhea has revealed a number of previously unrecognized viruses including viruses now classified in the two calicivirus genera *Norovirus* and *Sapovirus* (Green et al., 2001). In the UK before 1984, studies into the viral causes of calf diarrhea identified at least two pathogenic calici-like viruses, Newbury agent 1 (Newbury1 virus) and Newbury agent 2 (Newbury2 virus), using electron

microscopy and animal cross-protection experiments (Bridger et al., 1984; Woode and Bridger, 1978). Newbury2 was classified recently by genomic approaches as a member of a third genogroup in the genus *Norovirus* (Dastjerdi et al., 1999; Oliver et al., 2003). Newbury1 remained unclassified despite numerous failed attempts to amplify any region of its genome using calicivirus-specific oligonucleotides (A. M. Dastjerdi and S. Oliver personal observations).

In experimental calves, Newbury1 was more pathogenic than the Newbury2 virus, causing fecal color change and increased fecal output, anorexia and xylose malabsorption (Bridger et al., 1984). The most severe small intestinal lesions caused by Newbury1 infection were in the anterior small intestine where villi were atrophied with complete loss of enterocytes that exposed parts of the lamina propria (Hall et al., 1984). Virus particles were seen by electron microscopy in feces (Woode and

* Corresponding author. Fax: +44 207 468 5306.

E-mail addresses: sloliver@stanford.edu, soliver@rvc.ac.uk (S.L. Oliver), [easobayire@rvc.ac.uk](mailto: easobayire@rvc.ac.uk) (E. Asobayire), alex30stuk@aol.com (A.M. Dastjerdi), jbridger@rvc.ac.uk (J.C. Bridger).

¹ Current address: Department of Pediatrics, Stanford University School of Medicine, Grant Building Room S366, 300 Pasteur Drive, Stanford, CA 94305, USA.

Bridger, 1978). Viral antigen was found in the cytoplasm of enterocytes in the anterior small intestine up to 3 days post-infection (Hall et al., 1984). All of these features were consistent with an enteropathogenic virus. The Newbury1 virion had several properties consistent with the *Caliciviridae*: a typical morphology showing cup-like depressions, a particle diameter of 36.6 nm, a buoyant density of 1.34 g/cm³ in CsCl and a single capsid protein with a molecular mass of 49 kDa (Dastjerdi et al., 2000).

There are currently 4 genera recognized in the family *Caliciviridae* (Green et al., 2000). Each one has been named after the disease symptom (vesicular lesions—*Vesivirus*), hosts infected (lagomorphs—*Lagovirus*) or the geographical location where the type species was first isolated (Sapporo, Japan—*Sapovirus*; Norwalk, USA—*Norovirus*). All of these small, non-enveloped, icosahedral viruses contain positive-sense, single-stranded, polyadenylated RNA genomes that range from 7.3 to 8.5 kbp in length flanked by 5' and 3' untranslated regions (reviewed by Green et al., 2001). The division of the caliciviruses into the 4 distinct genera has been based on differences in their genomic organization and extensive genomic sequence diversity of the polymerase and capsid genes that form 4 clades by phylogenetic analyses (reviewed by Green et al., 2000). The genomes of the lagoviruses and sapoviruses are organized into two open reading frames (ORFs) whereas those of the noroviruses, into which Newbury2 has been classified, and the vesiviruses are organized into three. ORFs 1 and 2 of the noroviruses overlap but those of the vesiviruses are separated by a few nucleotides. In addition, the capsid proteins of the vesiviruses have a leader sequence that is proteolytically cleaved during maturation (Matsuura et al., 2000; Sosnovtsev et al., 1998). In all the genera, the 3' terminal ORF, ORF2 for the sapoviruses and lagoviruses and ORF3 for the noroviruses and vesiviruses, overlap with the capsid gene (reviewed by Green et al., 2001). The proteins encoded by the genome follow the order NH₂-terminal, 2C-helicase, 3A, 3B, 3C-protease, 3D-polymerase, capsid and a basic protein (3' terminal ORF), which distinguished the *Caliciviridae* from other families of positive-sense, single-stranded RNA viruses. The translated proteins of all the genera have at least one conserved amino acid motif for the 2C-helicase (GXXGXGKS/T), 3C-protease (GDCG), 3D-polymerase (GLPSG, YGDD) and capsid (PPG) proteins.

In the present study, the complete genomic sequence and genome organization of the Newbury1 virus were determined and analyzed to characterize Newbury1 and verify its relationship with established members of the 4 *Caliciviridae* genera. Further Newbury1-like viruses were sought in contemporary samples to determine whether a distinct extant calicivirus genus was circulating in UK cattle.

Results

Characterization of the Bo/Newbury1/1976/UK virus

The Newbury1 genome had a G + C content of 56.2% and was 7454 nucleotides in length. It had a 5' untranslated region

of 75 nucleotides and a 3' untranslated region of 67 nucleotides flanking 2 ORFs (Table 1). ORF1 was 6633 nucleotides (2210 amino acids) long, started from nucleotide 76 and was terminated by the ochre stop codon UAA. The short 3' terminal ORF (ORF2) was 678 nucleotides (225 amino acids) long. It did not overlap with ORF1 but was separated by a single nucleotide so that the gene was in a +2 frame-shift. The non-structural proteins (NH₂-terminal, 2C-helicase, 3A, 3B, 3C-protease and 3D-polymerase) and the capsid protein were predicted to be encoded by ORF1 with a combined molecular mass predicted to be 239 kDa. The organization of the ORF1 polyprotein was determined by the presence of amino acid motifs (conserved amino acids underlined) for the 2C-helicase (GPPGHGKS), 3C-protease (GYCG), 3D-polymerase (GLPSG and YGDD) and capsid (PPG) proteins. The observation that the 5' end of the Newbury1 genome had 12 nucleotides conserved within a 22-nucleotide region of ORF1 (nucleotides 5036 to 5047) predicted a possible internal initiation codon (⁵⁰⁵⁹ATG⁵⁰⁶¹) for the capsid gene that encoded a 58 kDa protein. A second possible initiation codon (⁵³²⁶ATG⁵³²⁸) was predicted to produce a 49 kDa capsid protein similar to that detected previously for Newbury1 by Western blot (Dastjerdi et al., 2000). However, it is unknown, at present, if translation occurs from a subgenomic RNA.

The polyprotein translated from ORF1 had 6 protease cleavage sites as predicted from those identified experimentally for the 3 genera *Lagovirus*, *Norovirus* and *Vesivirus* (Fig. 1). The dipeptides at the predicted cleavage sites consisted of E/QG/A. Increased hydrophilicity of the polyprotein, as determined by the method of Kyte–Doolittle, corresponded with the position of the cleavage dipeptides. The predicted NH₂-terminal, 2C-helicase and 3A cleavage products for the Newbury1 polyprotein contained both a hydrophobic and a hydrophilic region. The predicted 3B cleavage product was exclusively hydrophilic with the 3C-protease predominantly neutral. Whether alternative cleavage sites exist is unknown at present. The capsid protein could be divided into 2 hydrophobic and a hydrophilic region that coincided with the S, P1 and P2 domains based on the crystal structure determined for the capsid protein of the Norwalk virus (Prasad et al., 1999). In contrast to viruses in the 4 recognized *Caliciviridae* genera, cysteine, important for disulfide bridge formation, was not present in the capsid protein. Consistent with a calicivirus, the translated protein from the 3' terminal ORF, ORF2, had a predicted basic isoelectric point (pI11.1). Many of the above findings were consistent with viruses classified in the family *Caliciviridae*.

Newbury1 amplicons from the polymerase gene were detected by RT-PCR in the feces of 2 clinically affected, experimental calves using the primers NBU(F)/NA1ORF1_07 (data not shown). In the one calf (1424) for which daily fecal samples were available, an amplicon was not produced on the day of inoculation, a weak amplicon was produced the day after inoculation, before clinical signs commenced, and strong amplicons were produced from day 2 to at least day 4 post-inoculation when diarrhea, increased temperature and diminished appetite were present. The morphology of Newbury1 virions resembled classical calicivirus morphology in contrast to the genogroup III bovine norovirus Newbury2 (Fig. 2).

Table 1
Bo/Newbury1/1976/UK compared to representative viruses for the 4 genera in the family *Caliciviridae*

Genus	Virus	Genome length (nt)	Length of the 5' UTR (nt)	Length of the 3' UTR (nt)	Number of ORFs	ORF overlap (nt)		Length of ORF (nt ^a [translated aa])		
						1–2	2–3	ORF1	ORF2	ORF3
New	Newbury1	7454	75	67	2	1 ^b	NA	6633 [2210] ^c	678 [225] ^d	NA
	NB	7453	74	67	2	1 ^b	NA	6633 [2210] ^c	678 [225] ^d	NA
Lagovirus	RHDV-FRG	7437	9	59	2	8	NA	7035 [2344] ^c	354 [117] ^d	NA
	EBHSV-GD	7442	8	92	2	20	NA	7005 [2334] ^c	345 [114] ^d	NA
Sapovirus	Manchester	7431	12	82	2 (3) ^e	4	NA	6843 [2280] ^c	498 [165] ^d	NA
	Dresden	7429	12	80	2 (3) ^e	4	NA	6843 [2280] ^c	498 [165] ^d	NA
	Cowden	7320	9	55	2	4	NA	6765 [2254] ^c	495 [164] ^d	NA
Vesivirus	VESV-48	8284	19	179	3	5 ^b	4	5646 [1881] ^f	2106 [701] ^g	333 [110] ^d
	SMSV-1	8284	19	182	3	5 ^b	4	5640 [1879] ^f	2109 [702] ^g	333 [110] ^d
	FCV-CFI/68	7677	19	43	3	2 ^b	4	5289 [1762] ^f	2007 [668] ^g	330 [109] ^d
	FCV-Urbana	7683	19	46	3	2 ^b	4	5292 [1763] ^f	2007 [668] ^g	321 [106] ^d
	CCV	8513	11	235	3	2 ^b	4	5790 [1929] ^f	2076 [691] ^g	402 [133] ^d
Norovirus	Norwalk	7654	4	66	3	17	1	5370 [1789] ^f	1593 [530] ^h	639 [212] ^d
	Southampton	7708	4	78	3	17	1	5367 [1788] ^f	1641 [546] ^h	636 [211] ^d
	SMV	7537	4	45	3	20	1	5100 [1699] ^f	1629 [542] ^h	780 [259] ^d
	Lordsdale	7555	4	45	3	20	1	5100 [1699] ^f	1620 [539] ^h	807 [268] ^d
	Newbury2	7311	21	43	3	14	209 (11) ⁱ	5055 [1684] ^f	1569 [522] ^h	849 [282] ^d
	Jena	7338	21	67	3	14	11	5043 [1680] ^f	1560 [519] ^h	672 [223] ^d

See Materials and methods for references and accession numbers. RHDV: Rabbit Hemorrhagic Disease Virus; EBHSV: European Brown Hare Syndrome Virus; VESV: Vesicular Exanthema of Swine Virus; SMSV: San Miguel Sea Lion Virus; FCV: Feline Calicivirus; CCV: Canine Calicivirus; SMV: Snow Mountain Virus. nt—nucleotides; UTR—Untranslated Region; ORF—Open Reading Frame; aa—amino acids.

^a Including the termination codon.

^b ORF1 and 2 do not overlap but are separated by the number of nucleotides shown.

^c ORF1 encodes the non-structural and major capsid protein.

^d 3' terminal ORF proposed to be a minor structural protein.

^e Sapoviruses have been reported to have a 3rd ORF that is encoded within ORF1 but is not the canonical ORF3 typical of the genera *Vesivirus* and *Norovirus*.

^f ORF1 encodes only for the non-structural proteins.

^g ORF2 encodes for a precursor for the capsid protein.

^h ORF2 encodes for the major capsid protein.

ⁱ The initiation codon is unknown for the bovine norovirus Newbury2 and could be either 209 or 11 nucleotides upstream of the ORF2 termination codon.

Surface depressions were visible on many Newbury1 virions. Antigenically, Newbury1 was distinct from Newbury2 using an ELISA specific for Newbury2 antibody. Sera from 3 experimental calves inoculated orally with Newbury1 (Bridger et al., 1984), and shown to have Newbury1 antibodies by SPIEM (Dastjerdi et al., 2000), failed to react in the Newbury2 ELISA which detected homologous IgG titers of log₁₀ 3.0 to 4.0 in Newbury2 convalescent calf sera.

The relationship of Bo/Newbury1/1976/UK with members of the *Caliciviridae*

Although many features of the Newbury1 genome organization were consistent with the *Caliciviridae*, the Newbury1 genome had low nucleotide ($\leq 47\%$) and amino acid ($\leq 39\%$) identities to all representative viruses of the 4 *Caliciviridae* genera by comparison of complete non-structural genes (2C-helicase, 3C-protease, 3D-polymerase), complete capsids, capsid subdomains and the 3' terminal ORF genes (Table 2). The highest amino acid identity (39%) was seen with the *Lagovirus* polymerase. The lowest amino acid identity ($\leq 9\%$) was seen with the 3' terminal ORF for the sapoviruses (ORF2) and vesiviruses (ORF3). In contrast, the entire Newbury1 ORF1 polyprotein had $\geq 89\%$ amino acid ($\geq 78\%$ nucleotide) identity

compared to the previously characterized NB virus (Smiley et al., 2002). Surprisingly, the least variation was seen between the P-domains of the capsid protein of the 2 viruses.

Newbury1 failed to group phylogenetically with the 4 recognized *Caliciviridae* genera but formed a separate clade with NB virus by UPGMA, Fitch–Margoliash, parsimony and maximum likelihood analyses of the nucleotide and translated amino acids of ORF1 (excluding the capsid gene), the individual regions that encoded the 2C-helicase, 3C-protease, 3D-polymerase and complete capsid genes (not shown). The NH₂-terminal, 3A and 3B regions were not used for phylogenetic analyses because of the poor nucleotide and amino acid alignments caused by the extensive diversity in these sequences between the 4 calicivirus genera. Both Newbury1 and NB viruses consistently grouped more closely with the lagoviruses but the branch lengths were very long, indicative of evolutionary diverse viruses.

The distant relationship of Newbury1 to the 4 recognized *Caliciviridae* genera was confirmed using additional phylogenetic analyses based on amino acid alignments generated from homology models of structurally conserved regions of the partial polymerase and the capsid S-domain (Fig. 3). Homology models are useful to compare sequences with low levels of identity, as observed in the present study. Newbury1 and NB

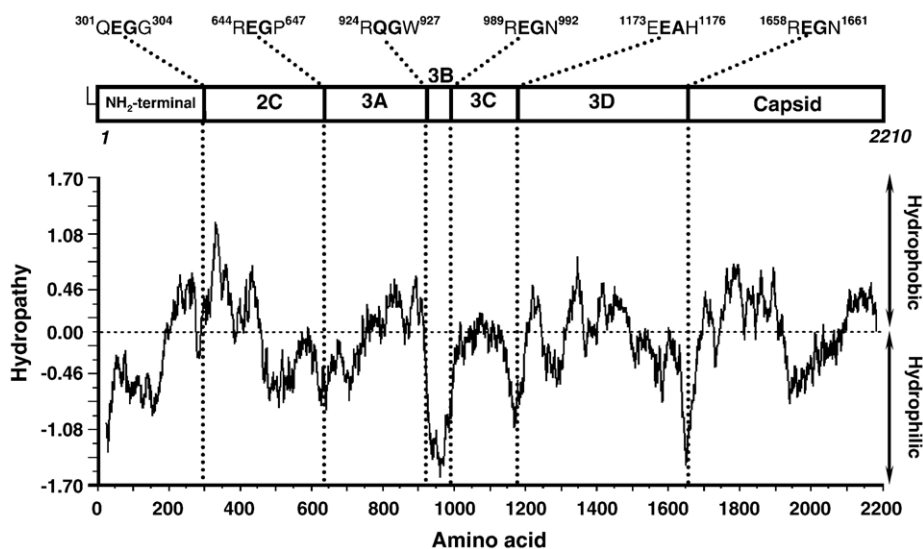


Fig. 1. Protease cleavage map and hydropathy plot (Kyte–Doolittle) of the bovine enteric calicivirus Newbury1. The protease cleavage sites and cleavage products, NH₂-terminal, 2C (helicase), 3A, 3B (VPg), 3C (protease), 3D (polymerase) and capsid proteins, were predicted from those determined experimentally for the genera *Lagovirus*, *Norovirus* and *Vesivirus*, and conserved in the Newbury1 ORF1 polyprotein. The horizontal dotted line shows the neutral point and the vertical dotted lines show the locations of the predicted cleavage sites on the hydropathy plot.

were again phylogenetically closer to the lagoviruses in their polymerase sequences (Fig. 3A) but were more closely related to the noroviruses in their conserved capsid S-domain sequences (Fig. 3B). However, the statistical support at the node between the Newbury1 and NB viruses and the noroviruses was weak (72%), indicating that this relationship was uncertain.

The divergence of the Newbury1 and NB genomes from the other calicivirus genera was confirmed by higher mean values for maximum likelihood distances, a measure of evolutionary divergence for nucleotide or amino acid sequences. Newbury1 and NB had twice the nucleotide maximum-likelihood distances when compared to the 4 recognized calicivirus genera for the non-structural genes ($\bar{x} = 1.419$; $\sigma = 0.158$) and the complete capsid genes ($\bar{x} = 1.377$; $\sigma = 0.214$) than the means within each of the 4 genera (non-structural genes: *Lagovirus*—0.357; *Norovirus*— $\bar{x} = 0.551$; $\sigma = 0.158$; *Sapovirus*— $\bar{x} = 0.615$; $\sigma = 0.445$; *Vesivirus*— $\bar{x} = 0.536$; $\sigma = 0.185$. Capsid genes: *Lagovirus*—0.345; *Norovirus*— $\bar{x} = 0.660$; $\sigma = 0.137$; *Sapovirus*— $\bar{x} = 0.542$; $\sigma = 0.411$; *Vesivirus*— $\bar{x} = 0.645$; $\sigma = 0.189$).

Similar differences were obtained for the Newbury1 and NB amino acid sequences compared to the 4 genera for the non-structural proteins ($\bar{x} = 2.499$; $\sigma = 0.485$) and for the complete capsid proteins ($\bar{x} = 2.283$; $\sigma = 0.418$). These were >3 times the mean values within each of the 4 genera (non-structural proteins: *Lagovirus*—0.238; *Norovirus*— $\bar{x} = 0.544$; $\sigma = 0.246$; *Sapovirus*— $\bar{x} = 0.735$; $\sigma = 0.624$; *Vesivirus*— $\bar{x} = 0.538$; $\sigma = 0.257$. Capsid proteins: *Lagovirus*—0.219; *Norovirus*— $\bar{x} = 0.747$; $\sigma = 0.215$; *Sapovirus*— $\bar{x} = 0.642$; $\sigma = 0.548$; *Vesivirus*— $\bar{x} = 0.726$; $\sigma = 0.323$). The inability to classify Newbury1 and NB genomically into any of the 4 recognized genera using all of the above approaches provided strong support for a new genus within the family *Caliciviridae*.

Simplot analysis of the complete Newbury1 genome confirmed a close genomic relationship with the NB virus. The region of ORF1 that encoded for the non-structural proteins of the 2 viruses had a mean nucleotide identity of 81%. This increased to 92% for the capsid and ORF2 proteins (Fig. 4). The sharp increase in nucleotide identity, from 72% in the polymerase to 97% in the NH₂-terminal region of the capsid

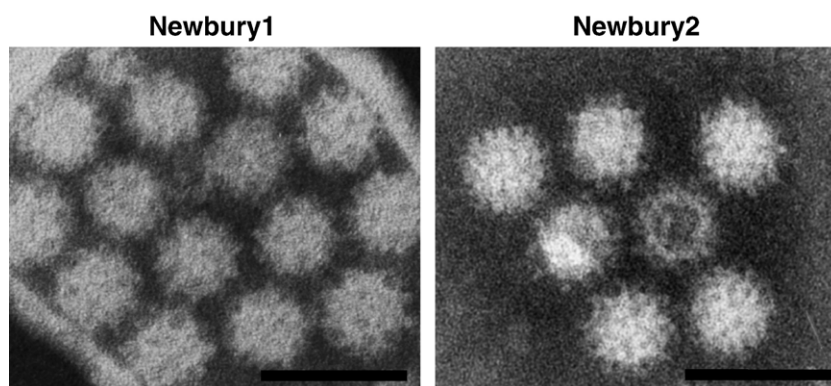


Fig. 2. Electron microscopy of Newbury1 and Newbury2 virions negatively stained with 2% potassium phosphotungstate (pH 6.0). Scale bar, 50 nm.

Table 2
Amino acid and nucleotide identities of the bovine enteric calicivirus Bo/Newbury1/1976/UK compared to Bo/NB/1980/US and representative viruses from the 4 recognized *Caliciviridae* genera

Genus	Virus	Amino acid [nucleotide] identities								
		ORF1 NS	2C-hel	3C-pro	3D-pol	Capsid			3' term ORF	
					Complete	S-domain ^a	P-domain			
<i>New</i>	NB	91 [80]	93 [81]	89 [80]	90 [78]	98 [92]	98 [93]	99 [92]	96 [91]	
<i>Lagovirus</i>	RHDV	27 [40]	29 [43]	27 [41]	39 [46]	27 [39]	36 [45]	20 [34]	22 [40]	
	EBHSV	28 [40]	30 [43]	28 [42]	39 [46]	27 [38]	36 [45]	19 [33]	22 [38]	
	<i>Norovirus</i>	Norwalk	18 [33]	23 [38]	23 [38]	28 [39]	20 [34]	30 [42]	11 [28]	10 [32]
<i>Norovirus</i>	Southampton	18 [34]	22 [37]	21 [37]	27 [39]	19 [33]	30 [40]	10 [28]	9 [31]	
	Snow Mountain	16 [33]	20 [35]	20 [35]	27 [39]	18 [32]	28 [41]	10 [26]	13 [26]	
	Lordsdale	17 [32]	19 [32]	19 [36]	27 [38]	18 [33]	26 [40]	12 [27]	11 [27]	
	Jena	18 [34]	23 [39]	21 [38]	30 [40]	19 [35]	30 [42]	13 [29]	13 [26]	
	Newbury2	18 [34]	23 [38]	21 [35]	29 [41]	21 [35]	32 [43]	11 [28]	11 [28]	
	<i>Sapovirus</i>	Manchester	23 [36]	26 [40]	33 [41]	34 [43]	25 [39]	33 [46]	19 [33]	4 [24]
	Dresden	23 [37]	26 [39]	33 [41]	34 [45]	25 [39]	33 [45]	19 [34]	4 [24]	
<i>Vesivirus</i>	PEC	23 [37]	27 [40]	26 [39]	35 [43]	25 [40]	36 [46]	16 [35]	7 [26]	
<i>Vesivirus</i>	FCV CFI68	25 [37]	29 [41]	26 [36]	34 [44]	26 [40]	35 [45]	19 [36]	9 [25]	
	FCV Urbana	25 [37]	28 [39]	27 [36]	34 [44]	26 [41]	36 [46]	19 [37]	9 [23]	
	CCV	25 [38]	28 [40]	28 [37]	32 [43]	27 [41]	38 [46]	21 [36]	9 [23]	
	SMSV-1	25 [38]	30 [41]	31 [39]	33 [41]	27 [39]	37 [46]	18 [34]	4 [30]	
	VESV-A48	25 [37]	30 [41]	31 [39]	32 [41]	27 [39]	36 [47]	20 [33]	4 [26]	

See Materials and methods for references and accession numbers. RHDV: rabbit hemorrhagic disease virus; EBHSV: European Brown Hare Syndrome Virus; PEC: Porcine Enteric Calicivirus; FCV: Feline Calicivirus; CCV: Canine Calicivirus; SMSV: San Miguel Sea Lion Virus; VESV: Vesicular Exanthema of Swine Virus; nt—nucleotides; UTR—untranslated region; ORF—open reading frame; aa—amino acids; ORF1 NS—the complete ORF1 gene excluding the capsid gene for genera *Lagovirus* and *Sapovirus*; 2C-hel—helicase gene, 3C-pro—protease gene, 3D-pol—polymerase gene; 3' term ORF—the 3' terminal ORF of the genome; ORF2 for the bovine enteric caliciviruses, lagoviruses and sapoviruses and ORF3 for the noroviruses and vesiviruses.

^a The S-domain was based on the 1 to 225 amino acids for the Norwalk virus capsid protein as determined by X-ray crystallography (Prasad et al., 1999).

was a feature that has been seen for caliciviruses in the genera *Norovirus* and *Sapovirus*.

The levels of identities seen in the 2C-helicase (93% amino acid; 81% nucleotide), 3C-protease (89% amino acid; 80% nucleotide) and 3D-polymerase (90% amino acid; 78% nucleotide) suggested that Newbury1 and NB were different genotypes in 3 of their non-structural proteins but not in their capsid proteins, which had 98% amino acid (92% nucleotide) between the two viruses (Table 2). The lack of capsid genotypes for the Newbury1 and NB viruses was evident in their capsid P-domains that were almost identical (99% amino acid; 92% nucleotide). The 3' terminal ORF (ORF2), which usually shows high levels of variability between genotypes within the same genus, had a remarkably high amino acid identity of 96% (91% nucleotide).

Characterization of contemporary Newbury1-like viruses from UK cattle

Newbury1 was identified in a fecal sample taken in 1976 but serological evidence showed that Newbury1 virus or Newbury1-like viruses were circulating in UK cattle in 1990 and 1993. Fourteen of 18 sera (78%) were positive for Newbury1 antibody at 1:10 dilution by SPIEM, trapping up to 45 particles per field. Subsequently, Newbury1-like viruses were identified in feces of diarrheic farm calves in 2000 from 2 geographically distant locations in the UK, Penrith and Starcross. Out of 107 samples examined, 7 samples from Penrith and 2 from Starcross (a mean prevalence of 8.4%)

produced amplicons of the expected size for the polymerase (509 bp for primer pair NBU(F)/NA1ORF1_07) and polymerase-capsid (522 bp for primer pair NA1ORF1_12/NA1ORF1_29) regions of the ORF1 gene.

The Newbury1 and NB polymerase genotypes circulating in UK cattle were determined by amino acid and nucleotide sequence identities of 7 partial polymerase sequences (amino acids 1490 to 1662 [nucleotides 4541 to 5058]) from the Penrith and Starcross isolates of 2000 (Table 3). The 5 Newbury1-like viruses (Penrith142, Penrith143, Penrith150, Penrith151 and Starcross117) had 95% amino acid (88 to 89% nucleotide) identity to Newbury1 and 88% amino acid (76 to 77% nucleotide) identity to the NB virus. The 2 NB-like viruses (PenrithC39 and Starcross93) had 88% amino acid (78% nucleotide) identity to Newbury1 and 96 to 97% amino acid (89 to 90% nucleotide) identity to NB. In contrast, evidence was not obtained for more than one capsid type. Sequence data generated for the NH₂-terminal of the capsid protein (amino acids 1663 to 1794 [nucleotides 5059 to 5454]) from 8 of the UK isolates showed a close relationship with both Newbury1 and NB, having 96 to 99% amino acid (92 to 94% nucleotide) identity. The partial polymerase sequence of the NB-like virus, Starcross93, had 19 (11.1%) amino acid substitutions compared with Newbury1, of which 15 were identical in position and composition to those of the NB virus (Fig. 5). In contrast to Starcross93, the Newbury1-like virus Penrith150 had just 8 (4.7%) amino acid substitutions in the polymerase when compared with Newbury1. The remaining Newbury1-like viruses (Penrith142, Penrith143, Penrith151 and Starcross117)

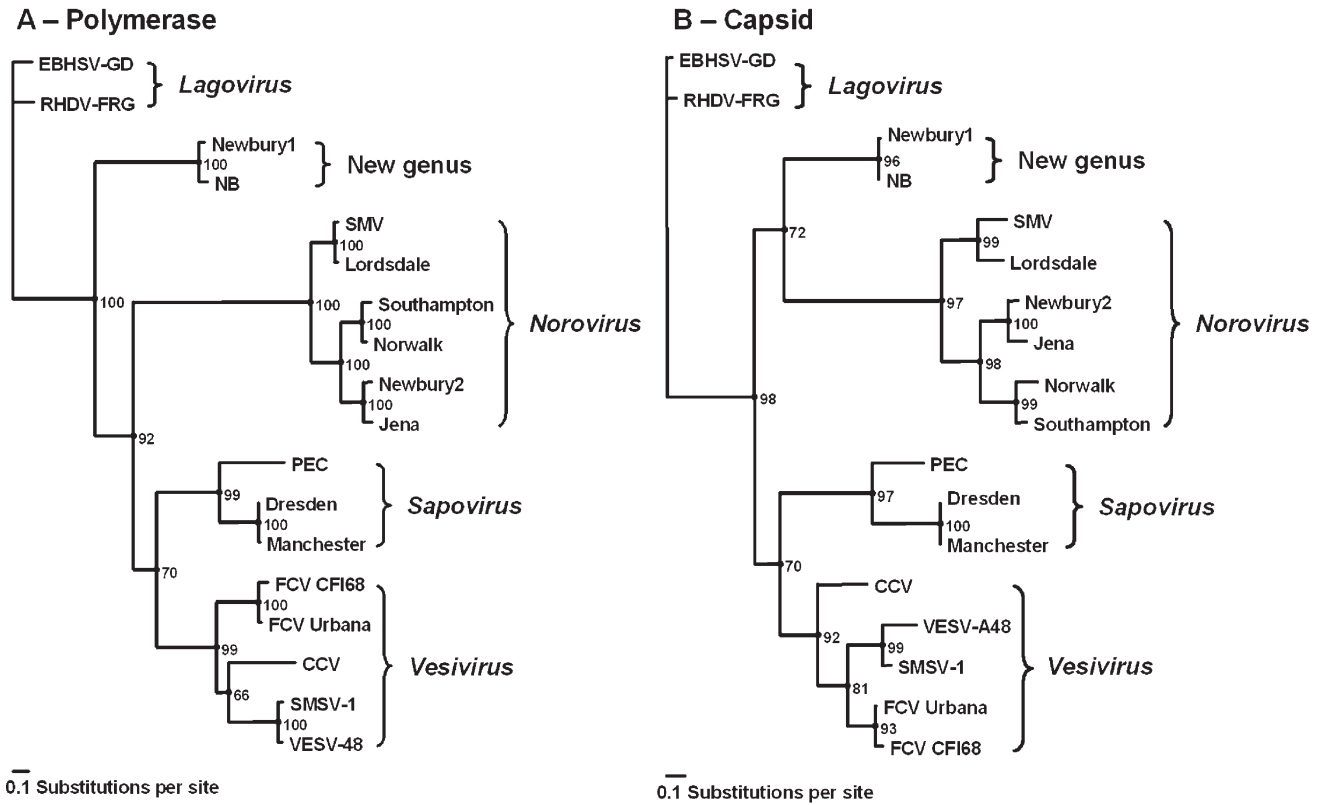


Fig. 3. Phylogenetic relationships of the *Caliciviridae* by maximum likelihood analyses of structure-based alignments that were generated based on homology models (Swiss-model) of the complete polymerase (A—464 amino acids) and partial capsid (B—S-domain; 141 amino acids) proteins. Quartet puzzling statistics are given at the internal branch nodes for 10,000 replicate trees. The scale indicates substitutions per site. EBHSV: European brown hare syndrome virus; RHDV: rabbit hemorrhagic disease virus; SMV: snow Mountain virus; PEC: porcine enteric calicivirus; FCV: feline calicivirus; CCV: canine calicivirus; SMSV: San Miguel sea lion virus; VESV: vesicular exanthema of swine virus. See Materials and methods for references and accession numbers.

had similar substitutions in frequency (7 to 8; 4.1 to 4.7%), location and composition to Penrith150 when they were compared to Newbury1, whereas the NB-like virus PenrithC39 had 20 (11.6%) substitutions almost identical in location and

composition to Starcross93 (not shown). Few amino acid substitutions (1 to 4; 0.8 to 3.0%) were seen in the genomic sequence generated for the NH₂-terminal of the capsid protein. The relationship of UK isolates from Penrith and Starcross with

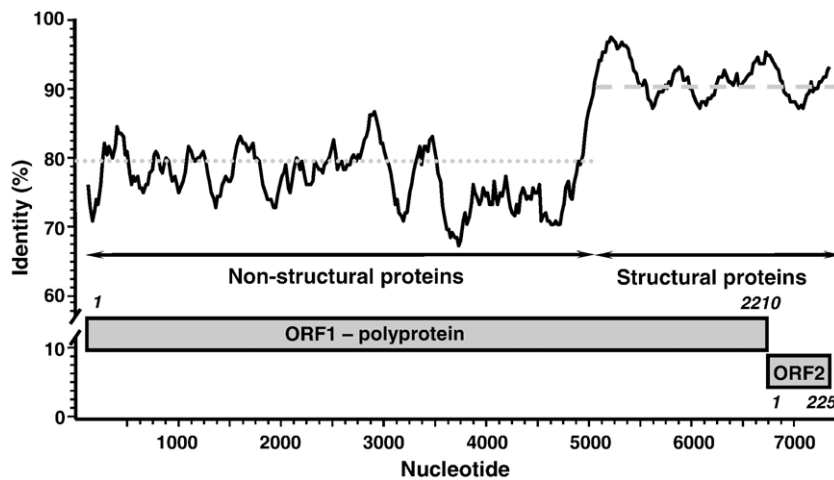


Fig. 4. Nucleotide identity plot between the two bovine enteric caliciviruses Newbury1 and NB generated using Simplot with a 250-nucleotide window moved along in 20 nucleotide steps. The two horizontal grey lines show the mean nucleotide identity for the non-structural (dotted—81%) and structural (dashed—92%) proteins encoded by ORF1 and ORF2. ORFs 1 and 2 encoded by the Newbury1 genome are shown by the two shaded boxes (grey). The numbers in italics show the predicted length of the ORF1 and ORF2 proteins.

Table 3

Amino acid and nucleotide identities of the partial polymerase and the NH₂-terminal region of the partial capsid genes from bovine enteric calicivirus isolates from the UK and NB-like viruses from the USA compared to Bo/Newbury1/1976/UK and Bo/NB/1980/US

Isolate	Accession number	Amino acid (nucleotide) identities to			
		Newbury1		NB	
		Polymerase ^a	Capsid ^b	Polymerase ^a	Capsid ^b
Bo/Newbury1/76/UK	DQ013304			89 (79)	98 (96)
Bo/Penrith140/00/UK	DQ228159	NA	96 (93)	NA	97 (93)
Bo/Penrith142/00/UK	DQ228160	95 (89)	99 (94)	88 (77)	98 (93)
Bo/Penrith143/00/UK	DQ228161	95 (89)	97 (93)	88 (76)	96 (92)
Bo/Penrith150/00/UK	DQ228157	95 (88)	99 (93)	88 (76)	98 (93)
Bo/Penrith151/00/UK	DQ228158	95 (89)	NA	88 (76)	NA
Bo/PenrithC39/00/UK	DQ228162	88 (78)	97 (94)	97 (90)	99 (94)
Bo/PenrithC70/00/UK	DQ228163	NA	97 (94)	NA	99 (94)
Bo/Starcross93/00/UK	DQ228165	88 (78)	96 (94)	96 (89)	98 (94)
Bo/Starcross 117/00/UK	DQ228164	95 (89)	98 (94)	88 (76)	97 (93)
Bo/NB/80/US ^c	AY082891	89 (79)	98 (96)		
Bo/CV23-OH/00/US ^c	AY082890	88 (78)	97 (93)	95 (86)	98 (95)
Bo/CV504-OH/02/US ^d	AY549168	NA	98 (94)	NA	99 (95)
Bo/CV519-OH/02/US ^d	AY549169	NA	96 (93)	NA	97 (94)
Bo/CV526-OH/02/US ^d	AY549170	NA	98 (93)	NA	99 (95)
BoCV531-OH/02/US ^d	AY549171	NA	98 (93)	NA	99 (95)
Bo/CV548-OH/02/US ^d	AY549172	NA	98 (93)	NA	99 (96)
Bo/CV562-OH/02/US ^d	AY549173	NA	98 (94)	NA	99 (95)

The bovine enteric calicivirus isolates in bold had a Newbury1-like polymerase. NA—genomic sequence for region not available.

^a The sequence analyzed was 489 nucleotides (162 translated amino acids) at the 3' end of the polymerase gene.

^b The sequence analyzed was 396 nucleotides (132 translated amino acids) at the 5' end of the capsid (NH₂-terminal region) gene.

^c Smiley et al. (2002).

^d Han et al. (2004).

the Newbury1 and NB viruses was supported by phylogenetic analyses of the partial polymerase and NH₂-terminal region of the capsid genes and endorsed the existence of 2 polymerase but not capsid genotypes (data not shown).

Discussion

The genomic analyses reported in the present study, plus previously published physicochemical studies, showed that Newbury1 virus was a calicivirus in its morphology, virion size, buoyant density, possession of a single major capsid protein,

genome organization and the presence of conserved amino acid motifs (Bridger et al., 1984; Dastjerdi et al., 2000; Hall et al., 1984; Woode and Bridger, 1978). The finding that Newbury1 and the Newbury1-like viruses failed to group phylogenetically with any of the known viruses of the *Caliciviridae*, apart from the recently described bovine enteric calicivirus NB and NB-like viruses from the US (Han et al., 2004; Smiley et al., 2002), endorsed the existence of a new genus within the *Caliciviridae* as proposed by Smiley et al. (2002). This was in agreement with the calicivirus study group's definition of a new calicivirus genus: "A genus in the *Caliciviridae* will be defined as a genetically distinct clade of viruses" (Green et al., 2000). We propose that the new genus be named either *Becovirus* or *Nabovirus* to distinguish them from the bovine noroviruses that are also associated with bovine enteric disease. *Becovirus* is based on sigla for 'bovine enteric calicivirus' and provides information on the species infected, the disease caused and follows precedents used for the calicivirus genera *Lagovirus* and *Vesivirus*. *Nabovirus* is based on Newbury1 and NB, the first two viruses to be identified in the new genus.

Newbury1 was evidently not a member of the families *Picornaviridae*, *Astroviridae* or *Hepeviridae* (Matsui and Greenberg, 2001; Purcell and Emerson, 2001; Racaniello, 2001) as there were major differences in the number of ORFs and gene order that were consistent with those of the family *Caliciviridae* (Green et al., 2001). The genomic organization was typical of the calicivirus genera *Lagovirus* and *Sapovirus*, not the genera *Norovirus* or *Vesivirus*, as a single ORF encoded the non-structural (NH₂-terminal, 2C-helicase, 3A, 3B, 3C-protease, 3D-polymerase) and single major capsid protein (Green et al., 2001). However, three significant differences in the Newbury1 genome organization distinguished it from the 4 recognized calicivirus genera. Firstly, the 5' UTR was at least 3 times longer than any other calicivirus. Secondly, ORF1 and ORF2 did not overlap but were separated by a single nucleotide. A similar separation between ORFs has been seen for the vesiviruses but between the non-structural and major capsid proteins (Glenn et al., 1999; Matsuura et al., 2002). Thirdly, the 3C-protease had a GYCG motif that was identical to some members of the *Picornaviridae* (Carrillo et al., 2005; Doherty et al., 1999; Skern et al., 1985; Wutz et al., 1996), but not the typical calicivirus GDCG motif. The Newbury1 ORF2 protein was at least 60 residues longer than those for the lagoviruses, sapoviruses and vesiviruses but was of a similar size to the analogous 3' terminal norovirus proteins (ORF3). All of these genomic features and the low levels of sequence identities, combined with phylogenetic analyses and the maximum likelihood distances, proved that Newbury1 was a calicivirus distinct from those of the 4 recognized genera of the *Caliciviridae*.

The evidence for classification of the Newbury1 and NB viruses in the same genus was their identical genome organization, identical composition and location of predicted ORF1 protease cleavage sites and almost identical capsid proteins. The latter was surprising as the two viruses were isolated from diarrheic calves on different continents and 4 years apart (Smiley et al., 2002; Woode and Bridger, 1978). The

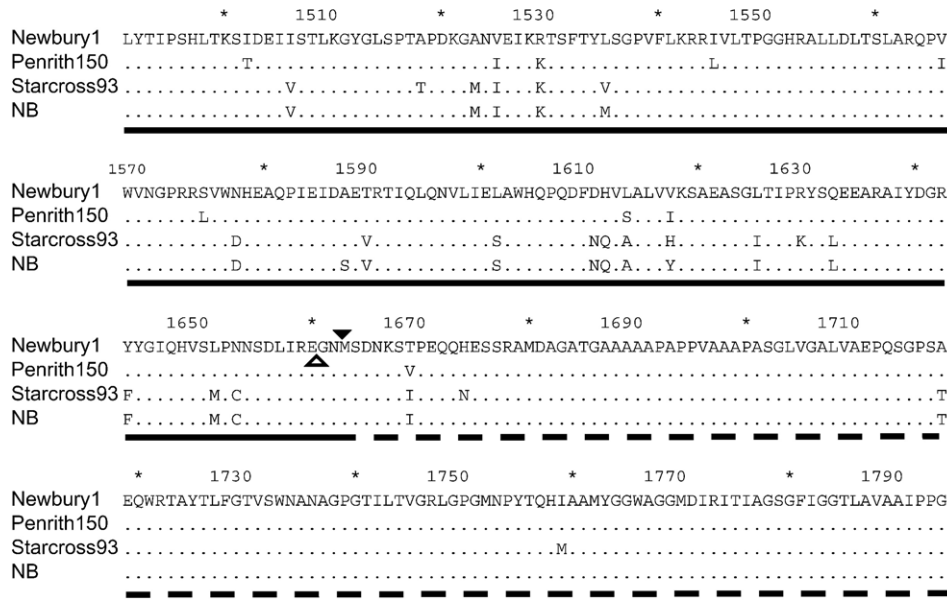


Fig. 5. Amino acid alignment of bovine enteric caliciviruses Newbury1 and NB with contemporary UK viruses Penrith150 (Newbury1-like) and Starcross93 (NB-like). The numbers above the alignment show the amino acid location in the Newbury1 ORF1 and the dots represent identical amino acids. The lines beneath the alignment show the partial polymerase (solid line) and partial capsid (dashed line) regions from where the sequences were generated. The open triangle shows the predicted cleavage site between the end of the polymerase and the beginning of the capsid protein. The solid triangle shows the methionine of the proposed initiation codon for the capsid protein.

predicted molecular mass of 58 kDa for the Newbury1 and NB capsid proteins contrasted with the 49 kDa previously determined for Newbury1 by Western blot analysis (Dastjerdi et al., 2000). The smaller mass might be due to cleavage of a precursor capsid protein, as described for the genus *Vesivirus* (Matsuura et al., 2000; Sosnovtsev et al., 1998), or translation of a subgenomic RNA of the Newbury1 capsid gene at a predicted initiation codon located at nucleotide 5326 in the Newbury1 genome (5327 for the NB genome). The molecular mass of the NB capsid protein has not been reported. As the viruses have not been propagated in cell culture, in vitro molecular studies are required to determine the biologically active ORF1 polypeptide protease cleavage sites and translation of the capsid genes for this new calicivirus genus.

The present study and one previous study demonstrated that Newbury1-like viruses are currently circulating in cattle on two continents (Smiley et al., 2003). For the first time, the present study indicated that two polymerase, but not capsid, genotypes exist. It was unlikely that analysis of the NH₂-terminal regions of the Newbury-like and NB-like viruses failed to recognize different capsid types as capsid genotypes within the genera *Norovirus* and *Sapovirus* can be distinguished by analysis of the conserved NH₂-terminal region of the capsid protein (Kageyama et al., 2004; Okada et al., 2002). It is uncertain whether both polymerase types exist in the USA as, to date, polymerase sequence data are available only for a single contemporary NB-like virus, Bo/CV23-OH/00/US. There are precedents within the genera *Norovirus* and *Sapovirus* for different polymerase genes being associated with similar capsid genes (Bull et al., 2005; Han et al., 2004; Jiang et al., 1999a; Katayama et al., 2004; Katayama et al.,

2002; Lochridge and Hardy, 2003; Oliver et al., 2004). This is thought to be due to genomic recombination. Further studies are required to examine the diversity of the caliciviruses within the new genus.

Our data has substantially advanced the knowledge about caliciviruses that cause diarrhea in cattle. Outbreaks of calf diarrhea cause significant economic losses in the UK (Stott and Gunn, 1995). Enteropathogens are not identified for approximately 30% of calf diarrhea cases (Andrews, 2000; Reynolds et al., 1986; Snodgrass et al., 1986), even though the presence of rotaviruses, coronaviruses, *Cryptosporidia*, *Coccidia*, *Salmonella* spp. and *Escherichia coli* are sought. However, caliciviruses are not routinely tested for. In the present study, Newbury1- and NB-like viruses were found with similar frequency (8.4%) to the bovine noroviruses (Oliver et al., 2003; Smiley et al., 2003; van der Poel et al., 2003; Wise et al., 2004) although this was substantially lower than the frequency of 28% for NB-like viruses found in the USA (Smiley et al., 2003). Hence, in the UK, our data indicate that caliciviruses (noroviruses and the caliciviruses in the new genus) are associated with about 17% of calf diarrhea cases, leaving about 13% of cases still undiagnosed. It seems likely that Newbury1- and NB-like viruses are responsible for a proportion of diarrhea in farm calves as Newbury1 and NB caused enteric disease after oral inoculation of experimental calves and produced small intestinal lesions typical of enteropathogenic viruses (Bridger et al., 1984; Hall et al., 1984; Smiley et al., 2002). Further studies are required to assess the antigenic diversity of caliciviruses within the proposed new genus, their incidence and prevalence in calf diarrhea outbreaks, their species specificity and whether similar viruses exist in man.

Materials and methods

Fecal samples, viruses and sera

Fecal samples from experimentally inoculated calves contained either the Newbury1 virus (formerly named SRV-1 and identified in 1976 in diarrheic feces from a farm calf, [Woode and Bridger, 1978](#)), the genogroup III bovine norovirus Bo/NV/Newbury2/1976/UK or the genogroup III bovine norovirus Bo/NV/Dumfries/1994/UK ([Oliver et al., 2003](#)). For Newbury1, feces were available from two experimentally infected calves (1424 and 2010) ([Dastjerdi et al., 2000](#)). Fecal samples from 107 diarrheic farm calves collected in 2000 from two geographically distant locations in the UK, Penrith ($n = 57$) and Starcross ($n = 50$), were kindly provided by the Veterinary Laboratories Agency. Sera from 18 cattle were collected from two herds; 8 were from mixed sexes of 6-month-old cattle at the Institute for Animal Health, Compton, Berkshire, UK in 1990 and 10 were from yearling female cattle collected in 1993 from Wiltshire, UK, kindly provided by Dr. Ian Thompson, Royal Veterinary College, UK.

Amplification and sequence analysis of the Bo/Newbury1/1976/UK genome

RNA extraction and reverse transcription were performed as described previously ([Oliver et al., 2003](#)). Reverse transcription was performed at 42 °C using 200 IU of Moloney murine leukemia virus (H-) reverse transcriptase (Promega). The PCR primers used to initially amplify Newbury1 were those designed to anneal with viruses in the family *Caliciviridae* BoCVpol001 (5'-ATCAAGGAGCACG-TGGTGGAG-3')/YGDD ([Green et al., 1995](#)), 290/289 ([Jiang et al., 1999b](#)), NORO(F)/NORO(R) ([Smiley et al., 2003](#)) or the NB-like viruses NBU(F)/NB(R) ([Smiley et al., 2003](#)). To generate amplicons that spanned the entire Newbury1 genome, PCR was performed with HotStar Taq (Qiagen) using primers partly based on Newbury1 sequence and the NB virus ([Smiley et al., 2002](#)) that overlapped the entire length of the Newbury1 genome. An initial denaturation at 95 °C for 15 min, then, dependant on the primer pairs, 40 cycles of 95 °C for 30 s, 50 °C to 55 °C for 30 to 60 s and 72 °C for 1 to 2 min were performed, with final extension at 72 °C for 10 min. Amplicons from 0.2 to 1.7-kbp in length were generated and gel purified using a QIAquick gel extraction kit (Qiagen). Purified amplicons were cloned using a TOPO-TA pCR2.1 cloning kit (Invitrogen Ltd.). Nucleotide sequencing of 3 to 5 clones for each PCR amplicon from Newbury1 was performed by MRCgeneservice, Hinxton, UK. The consensus nucleotide sequence of Newbury1 was prepared using the STADEN sequencing package ([Staden et al., 2000](#)). ORFs were predicted using the NCBI ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The GC content of the Newbury1 genome was calculated, the molecular masses and isoelectric points of proteins were predicted and hydropathy plots were generated using the bioinformatics software Jemboss ([Carver and Bleasby, 2003](#)).

Phylogenetic analyses and maximum likelihood distances of Bo/Newbury1/1976/UK and viruses of the Caliciviridae

To reduce computation time, 17 viruses from the family *Caliciviridae* (abbreviation, if used; accession number for the complete genome; reference, if available) were used: *Lagovirus*, European brown hare syndrome virus strain GD (EBHSV-GD; NC_002615; [Le Gall et al., 1996](#)) and Rabbit hemorrhagic disease virus strain FRG (RHDV-FRG; M67473; [Meyers et al., 1991](#)); *Vesivirus*, Feline calicivirus strain Urbana (FCV-Urbana; NC_001481; [Sosnovtsev and Green, 1995](#)), Feline calicivirus strain CFI/68 (FCV-CFI/68; U13992), Vesicular exanthema of swine virus serotype A48 (VESV-A48; U76874; [Neill et al., 1998](#)), San Miguel sea lion virus serotype 1 (SMSV; U15301; [Neill and Meyer, 1995](#)), canine calicivirus (CCV; NC_004542); *Sapovirus*, Manchester (X86560; [Dingle et al., 1995](#)), Dresden (NC_006269) and porcine enteric calicivirus (PEC; NC_000940; [Guo et al., 1999](#)); *Norovirus*, Norwalk (NC_001959; [Jiang et al., 1993](#)), Southampton (L07418; [Lambden et al., 1993](#)), Snow mountain virus (SMV; AY134748; [Lochridge and Hardy, 2003](#)), Lordsdale (X86557; [Liu et al., 1995](#)), Newbury2 (AF097917; [Oliver et al., 2003](#)) and Jena (AJ011099; [Liu et al., 1999](#)); and the unclassified viruses, Nebraska virus (NB; NC_004064; [Smiley et al., 2002](#)) and Newbury1 (DQ013304). Multiple alignments of translated amino acid sequences were prepared for the complete ORF1 (excluding the capsid gene), 2C-helicase, 3C-protease, 3D-polymerase and capsid proteins using Clustal X version 1.8 ([Thompson et al., 1997](#)). Multiple alignments of the nucleotide sequences for these proteins, which were generated using tranalign of the bioinformatics software Jemboss, were based on the multiple amino acid alignments. In order to retain the maximum number of conserved evolutionary sites, all columns that contained gaps were removed, then identities were calculated using GeneDoc (www.psc.edu/biomed/genedoc).

Phylogenetic analyses were performed with multiple nucleotide and amino acid alignments generated for the 2C-helicase, 3C-protease, 3D-polymerase and the complete capsid genes. The NH₂-terminal, 3A-B and ORF3 genes were not used due to their extensive diversity between the caliciviruses. PHYLIP (J. Felsenstein, Department of Genetics, University of Washington, Seattle; Phylogeny Inference Package, version 3.5c) was used for parsimony (DNApars and Protpars), UPGMA, Fitch–Margoliash, maximum likelihood and bootstrap analyses. TreePuzzle 5.2 was used for additional maximum likelihood with quartet puzzling analyses ([Schmidt et al., 2002](#)). In addition, phylogenetic analyses were performed using amino acid alignments generated from homology models of the polymerase and the S-domain of the capsid protein. This was done to compensate for the high diversity between Newbury1 and the 4 *Caliciviridae* genera; amino acid alignments were generated based on the 3D-structure of the polymerase and the S-domain of the capsid. Homology models of the polymerase and S-domain of the capsid were generated using the Swiss-model server (<http://swissmodel.expasy.org/SWISS-MODEL.html>). Amino acids

sequences for the 17 caliciviruses plus Newbury1 were aligned by eye using the homology model alignments for the conserved regions of the polymerase and the S-domain of the capsid protein. Phylogenetic trees were prepared by using TreeView (Page, 1996) and edited for clarity with Microsoft Word. To determine the evolutionary divergence of the calicivirus genome sequences, maximum likelihood distances were calculated using TreePuzzle 5.2.

Negative stain electron microscopy

Newbury1 and Newbury2 virus particles were partially purified from feces by differential centrifugation and examined by electron microscopy after staining with 2% potassium phosphotungstate pH 6.0 as described previously (Bridger et al., 1984).

Bo/NV/Newbury2/1976/UK antibody ELISA

The antigenic relationship between Newbury1 and Newbury2 was determined by ELISA as described previously (S.L. Oliver, E. Asobayire, A. Charpilliene, J. Cohen and J.C. Bridger, submitted for publication) using sera from Bridger et al. (1984).

Protein-G solid-phase immuno-electron microscopy (SPIEM)

SPIEM was used to detect Newbury1 antibody as described previously with some modifications (Dastjerdi et al., 2000). All incubations were performed in humidified chambers for 1 h at 37 °C. Formvar, carbon-coated electron microscope grids were incubated with 10 µg/ml protein-G (Sigma, UK), washed 6 times with ultra pure H₂O, then incubated with 1:10 dilution of test serum. Grids were washed again, incubated with 10 µl volume of fecal suspensions containing Newbury1 then negatively stained with 2% potassium phosphotungstate pH 6.0 (PTA; Agar Scientific, UK). Grids were examined with a Jeol 1200EX electron microscope at a magnification of 50,000. Serum taken 39 days post-inoculation with Newbury1 (Bridger et al., 1984) was used as a positive control and trapped an average of 225.6 ± 23.6 Newbury1 particles per field. The Newbury1 serum was free from antibodies to bovine astrovirus, the UK strain of bovine rotavirus, bovine enteric coronavirus, bovine viral diarrhoea virus, feline calicivirus and the Haden strain of bovine parvovirus (J.C. Bridger personal observation). A hyperimmune serum to rotavirus UK was used as a negative control and trapped 0.48 ± 0.85 particles per field. Test sera that trapped more than 2.2 particles per field (the mean number of Newbury1 particles per field trapped by the negative control serum +2 standard deviations) were considered positive for Newbury1 antibody.

Detection by RT-PCR of Newbury1-like viruses in the feces of UK diarrheic calves

RNA extraction and reverse transcription were performed as described previously (Oliver et al., 2003). PCR was

performed with HotStar Taq (Qiagen) using primers NBU(F)/NA1ORF1_07 (5'-TGG CAG ACT CAC ATG TTG GAT GC-3'), which amplified a 509 bp region (nucleotides 4519 to 5026 of the Newbury1 ORF1 gene) of the polymerase gene. A fecal sample from calf 1424 2 days post-inoculation was used as a positive control and H₂O was used as a negative control for RNA extraction and reverse transcription. cDNA generated from fecal samples that produced an amplicon of the expected size for the primer pair NBU(F)/NA1ORF1_07, was used to generate amplicons with primer pair NA1ORF1_12 (5'-CCAGATATTCTCTAGAAGAAGC-3')/NA1ORF1_29 (5'-TTGACACTCTCCGGGTCCACG-3'). NA1ORF1_12/NA1ORF1_29 amplified a 522 bp region that spanned the 3' end of the polymerase and the 5' end of the capsid genes (nucleotides 4954 to 5474 of the Newbury1 ORF1 gene). Thermocycling conditions were an initial denaturation at 95 °C for 15 min followed by 40 cycles of 94 °C for 45 s (NBU(F)/NA1ORF1_07) or 1 min (NA1ORF1_12/NA1ORF1_29), 54 °C for 45 s and 72 °C for 1 min, with a final extension at 72 °C for 10 min. cDNA generated from Newbury1 was used as a positive control and H₂O as a negative control for PCR. Amplicons were cloned, sequenced and compared to Newbury1 as described previously.

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