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

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**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.


*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

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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/cm$^3$ 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, icosohedral 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 $NH_2$-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 ($NH_2$-terminal, 2C-helicase, 3A, 3B, 3C-protease and 3D-polymerse) 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 ($^{5059}$ATG$^{5061}$) for the capsid gene that encoded a 58 kDa protein. A second possible initiation codon ($^{5326}$ATG$^{5328}$) 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 $NH_2$-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 (pH11.1). 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).