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A chimeric bovine enteric calicivirus: evidence for genomic recombination in genogroup III of the *Norovirus* genus of the *Caliciviridae*

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Abstract

The *Norovirus* genus of the *Caliciviridae* encompasses viruses that cause outbreaks of gastroenteritis in human and viruses that have been associated with diarrhea in cattle. The two bovine noroviruses, Bo/Newbury2/76/UK and Bo/Jena/80/DE, represent two distinct genetic clusters in the newly described genogroup III. In the present study, Jena-like polymerase sequences were identified for the first time in the UK, but one of these, Bo/Thirsk10/00/UK, was a chimeric virus. Bo/Thirsk10/00/UK had a Jena-like polymerase gene but Newbury2-like capsid and ORF3 genes by comparison of their genome organization, nucleotide, and amino acid identities and phylogenetic analyses. The present study is one of few studies to clearly demonstrate the existence of chimeric genomes in the *Norovirus* genus and the first, to our knowledge, to identify a chimeric genome in genogroup III. It provides additional support that genomic recombination is part of the natural evolution of noroviruses and is relevant to the diagnosis and immunological control of norovirus diarrhea outbreaks.

Keywords: Bovine; Calicivirus; Capsid; Chimeric; Genomic; Human; *Norovirus*; Polymerase; Recombination; RNA

Introduction

The *Norovirus* genus of the *Caliciviridae* has recently been shown to encompass viruses associated with diarrhea in cattle as well as the most frequently identified pathogens of adult human nonbacterial gastroenteritis (Anon, 2000; Bridger et al., 1984; Dastjerdi et al., 1999; Fankhauser et al., 1998; Liu et al., 1999; Mead et al., 1999; Oliver et al., 2003; Reynolds et al., 1986; Smiley et al., 2003; van der Poel et al., 2000; Woode and Bridger, 1978). The noroviruses have positive-sense, single-stranded RNA genomes that range from 7.2 to 7.7 kb, which are organized into three open reading frames (ORFs) that encode for the nonstructural proteins (ORF1), a capsid protein (ORF2) and the ORF3 protein (Jiang et al., 1993; Lambden et al., 1993). Genomic analyses of the bovine enteric caliciviruses (BoCVs) showed that they formed a third norovirus genogroup distinct from genogroups I and II to which the human noroviruses belong (Ando et al., 2000; Dastjerdi et al., 1999; Liu et al., 1999; Oliver et al., 2003). In contrast to the human genogroup I and II noroviruses (reviewed by Green et al., 2001), the extent of genomic and antigenic diversity among the BoCVs is not well characterized. The two human norovirus genogroups each contain at least seven genetic clusters (Ando et al., 2000; Green et al., 2001). Ando et al. (2000) proposed two genetic clusters of BoCVs based on limited analysis of short regions of the capsid genes from two BoCVs. This suggestion was confirmed by Oliver et al. (2003) by phylogenetic analyses of the RNA-dependent RNA polymerase (RdRp), capsid, and ORF3 genes of three additional viruses from the UK. The two clusters were represented by viruses similar to Bo/Newbury2/76/UK, identified in the UK in 1976, and a single virus from Germany, Bo/Jena/80/DE, identified in 1980. In addition to phylogenetic analyses, Oliver et al. (2003) identified differences between the two genogroup III clusters. The complete capsid (ORF2) proteins of four Newbury2-like viruses were predicted to be 522 amino acids long, 3 amino acids longer than the capsid protein of Bo/Jena/80/DE. The third ORF of the Newbury2-like viruses was predicted to be 282 amino acids long, 59

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amino acids longer than Bo/Jena/80/DE, from an initiation codon 209 nucleotides upstream of the ORF2 termination codon. Other differences, such as insertions and deletions in the capsid and ORF3 genes and differences in the lengths of the 3′ nontranslated regions, were also observed between the two genetic clusters.

Epidemiological studies of human noroviruses throughout the world have predominantly used the RdRp for genetic typing (Foley et al., 2001; Green et al., 1995a, 1995b; Greening et al., 2001; Jiang et al., 1999b; Reuter et al., 2002; Vinje and Koopmans, 1996). A few studies have used both the RdRp and the capsid genes, and on occasion, human noroviruses belonged to a different genetic cluster when the two regions were analyzed (Hardy et al., 1997; Jiang et al., 1999a; Katayama et al., 2002; Vinje et al., 2000). This suggested the existence of chimeric genomes, but in two of these studies, the RdRp, capsid, and ORF3 sequences were not derived from a contiguous cDNA spanning the RdRp and capsid genes (Hardy et al., 1997; Vinje et al., 2000). This is important to demonstrate chimeric genomes as mixed virus infections can occur (Gray et al., 1997).

In the present study, the genomic diversity of BoCVs circulating in the UK was investigated. To date, Jena-like viruses have not been identified in the UK and have been identified rarely elsewhere (Smiley et al., 2003). Short amplicons were generated, using the reverse transcription-polymerase chain reaction (RT-PCR), from the RdRp of viruses in bovine diarrheic fecal samples. These were prioritized for sequencing by identification of heterogeneity to Bo/Newbury2/76/UK using a heteroduplex mobility assay (HMA). Such an approach has been used by the Health Protection Agency (formerly the Public Health Laboratory Service) in the UK to type human noroviruses (Mattick et al., 2000). Continuous cDNAs spanning the three ORFs, from the poly-A tail to the RdRp gene in ORF1, were generated and sequenced. We describe for the first time a chimeric genome, Bo/Thirsk10/00/UK, from a genogroup III BoCV.

Results

Identification of genomically diverse BoCVs in the UK

HMA and sequence analysis of 15 NI/E3 amplicons showed that three BoCVs (Bo/Aberystwyth58/00/UK, Bo/Carmarthen10/99/UK and Bo/Thirsk10/00/UK) were genomically diverse to the UK reference virus Bo/Newbury2/76/UK and a recently identified Newbury2-like virus, Bo/Aberystwyth65/00/UK (Oliver et al., 2003). The clear separation of the heteroduplexes (Fig. 1, lanes D–F, solid box) from the homoduplexes (Fig. 1, lanes A–F, dotted box) showed that Bo/Thirsk10/00/UK was genomically diverse to the UK reference virus Bo/Newbury2/76/UK and the Bo/Aberystwyth65/00/UK virus in the RdRp (lanes E and F). Sequencing of the short sequence from the NI/E3

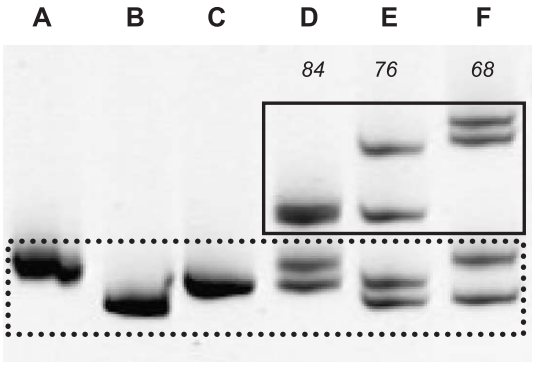

Fig. 1. HMA with the 316 bp M13-forward/M13-reverse amplicons from plasmids containing the 114 bp NI/E3 amplicons from the RdRp of BoCVs Bo/Newbury2/76/UK, Bo/Aberystwyth65/00/UK, and Bo/Thirsk10/00/UK. Lane A—Bo/Newbury2/76/UK, B—Bo/Thirsk10/00/UK, C—Bo/Aberystwyth65/00/UK, D—Bo/Newbury2/76/UK with Bo/Aberystwyth65/00/UK, E—Bo/Aberystwyth65/00/UK with Bo/Thirsk10/00/UK, F—Bo/Newbury2/76/UK with Bo/Thirsk10/00/UK. The dotted box outlines homoduplexes and the solid box outlines heteroduplexes. The figures in italics show nucleotide identities determined by sequence analysis between the 76-nucleotide fragments of the RdRp of the BoCVs in the relevant lane.

amplicon (76 nucleotides excluding the primer annealing regions) showed that Bo/Thirsk10/00/UK was most closely related in its RdRp to Bo/Jena/80/DE (84% nucleotide and 100% amino acid identity) than to Bo/Newbury2/76/UK (68% nucleotide and 96% amino acid identity). Of the 15 samples tested by HMA, the two BoCVs, Bo/Aberystwyth58/00/UK and Bo/Carmarthen10/99/UK, were also identified as diverse from Bo/Newbury2/76/UK. This was confirmed by sequence analysis as they were more closely related to Bo/Jena/80/DE (81% and 82% nucleotide, and, 100% amino acid identity) than to Bo/Newbury2/76/UK (75% and 76% nucleotide, and 96% amino acid identity). The three BoCVs (Bo/Aberystwyth58/00/UK, Bo/Carmarthen10/99/UK and Bo/Thirsk10/00/UK) had the same substitution in the 25 translated amino acids from an aspartic acid in the Bo/Newbury2/76/UK sequence (amino acid 87 in the partial ORF1) to an alanine as seen for the Bo/Jena/80/DE sequence (amino acid 1500 in the complete ORF1). Thus, Bo/Aberystwyth58/00/UK, Bo/Carmarthen10/99/UK, and Bo/Thirsk10/00/UK had a Jena-like RdRp showing that by polymerase type, both genetic clusters of genogroup III noroviruses were present in UK cattle.

Bo/Thirsk10/00/UK has a chimeric genome

Comparison of the partial RdRp, capsid, and ORF3 genes showed that Bo/Thirsk10/00/UK had a bipartite relationship with Bo/Newbury2/76/UK and Bo/Jena/80/DE when five independent clones of the 2.8 kbp amplicons spanning the partial RdRp, complete capsid, and ORF3 genes were analyzed. In contrast to the similarities of the Bo/Thirsk10/00/UK RdRp gene to Bo/Jena/80/DE, the complete capsid gene coded for a protein that was three

Table 1
Genome organization of Bo/Thirsk10/00/UK compared with the representatives of the two genetic clusters of genogroup III

	ORF1-2 overlap[a]	Capsid[b]	ORF2-3 overlap[a]	ORF3[b]	3′ NTR[a]
Bo/Newbury2/76/UK[c]	14	522	209 [11[d]]	282 [216[d]]	43
Bo/Jena/80/DE	14	519	11	223	70
Bo/Thirsk10/00/UK	14	522	11	216	43

[a] Length in nucleotides.
[b] Length in amino acids.
[c] Representative of the UK BoCVs identified by Oliver et al. (2003).
[d] Figures in the square brackets are the number of residues when the Jena-like ORF3 initiation codon is used.

amino acids longer than Bo/Jena/80/DE (Table 1), but the same length as Newbury2-like viruses identified in the UK, the Netherlands, and the USA (Dastjerdi et al., 1999; Oliver et al., 2003; Smiley et al., 2003; van der Poel et al., 2000). The Bo/Thirsk10/00/UK capsid gene had the same two insertions of three and six nucleotides present in the four UK Newbury2-like viruses when compared to Bo/Jena/80/DE, resulting in the same single and double amino acid insertion in the capsid protein at positions 291 and 424–425 (data not shown). The 3′ nontranslated region was also the same length as for the Newbury2-like viruses (43 nucleotides long) and was shorter than Bo/Jena/80/DE.

The genome organization had similarities to both Bo/Newbury2/76/UK and Bo/Jena/80/DE. The 3′ end of the Bo/Thirsk10/00/UK ORF1 gene overlapped with the capsid gene (ORF2) by 14 nucleotides, identical to both Bo/Newbury2/76/UK and Bo/Jena/80/DE, but the ORF2-3 junction (nucleotides 6600–6610 of Bo/Jena/80/DE) had an 11-nucleotide overlap, identical to Bo/Jena/80/DE (Table 1). The ORF3 initiation codon, which was found 209 nucleotides upstream of the ORF2 termination codon for the Newbury2-like viruses in two studies (Oliver et al., 2003; Smiley et al., 2003), was present in Bo/Thirsk10/00/UK, but termination codons at nucleotide positions 67–69 and 196–198 of the ORF3 gene, in the region between the Newbury2- and Jena-like initiation codons, suggested premature termination of the extended ORF3 protein for Bo/Thirsk10/00/UK, as seen with Bo/Jena/80/DE (Fig. 2). The conservation of the initiation codon 11 nucleotides upstream of the ORF2 termination codon implies that this is the likely initiation site for the ORF3 protein of the Newbury2-like viruses.

The Bo/Thirsk10/00/UK ORF3 gene was the same length as the four UK Newbury2-like viruses, 648 nucleotides (216 amino acids), when the size of the Newbury2-like ORF3 was calculated from the Bo/Jena/80/DE initiation codon, but 21 nucleotides (7 amino acids) shorter than Bo/Jena/80/DE (Table 1). In addition, the Bo/Thirsk10/00/UK ORF3 gene had the same two deletions as the Newbury2-like viruses of 18 and 3 nucleotides (at amino acid positions 203–208 and 245 of the Bo/Jena/80/DE ORF3 protein) when compared with Bo/Jena/80/DE (Fig. 2). Thus, the genome organization for the Bo/Thirsk10/00/UK capsid and ORF3 genes was consistent with the two known types of BoCVs.

Comparisons of nucleotide and amino acid identities from the partial RdRp (583 nucleotides and 193 translated amino acids), complete capsid, and complete ORF3 genes confirmed that Bo/Thirsk10/00/UK had a chimeric genome. The relationship of the Bo/Thirsk10/00/UK RdRp identified using HMA and the 76-nucleotide sequence was confirmed with the 583-RdRp nucleotide sequence, which had 98% amino acid identity to Bo/Jena/80/DE (Table 2). In addition, 18 of the 20 amino acid substitutions found in the Bo/Thirsk10/00/UK RdRp were identical in position and residue with those of Bo/Jena/80/DE, not Bo/Newbury2/76/UK (data not shown). In contrast to the RdRp, the Bo/Thirsk10/00/UK capsid and ORF3 genes were more closely related to Bo/Newbury2/76/UK with the capsid gene having 94% amino acid identity to Bo/Newbury2/76/UK and only 68% to Bo/Jena/80/DE.

Phylogenetic analyses also confirmed the relationship of Bo/Thirsk10/00/UK with Bo/Newbury2/UK and Bo/Jena/

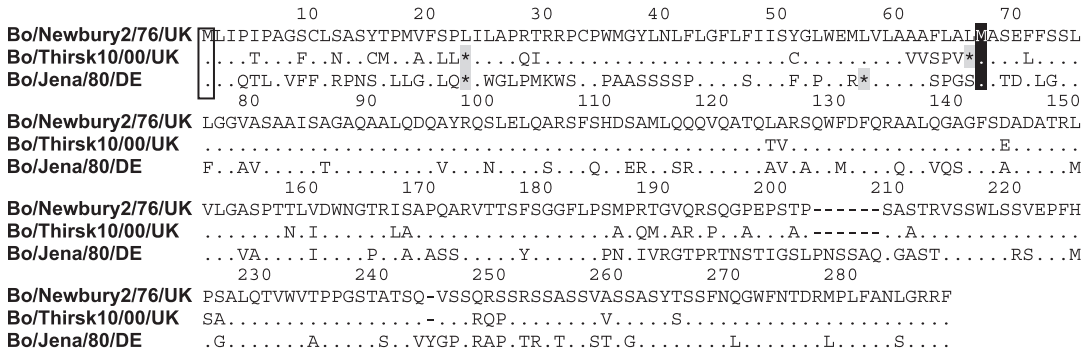

Fig. 2. Multipie alignment of the BoCV ORF3 proteins starting at the methionine of the Bo/Newbury2/76/UK initiation codon (boxed). The methionine for the initiation codons of Bo/Thirk10/00/UK and Bo/Jena/80/DE is highlighted with a black background. Asterisks (highlighted with a gray background) represent noncoding residues caused by premature termination codons in the Bo/Thirsk10/00/UK and Bo/Jena/80/DE ORF3 genes. Dots represent identical amino acids to Bo/Newbury2/76/UK. Dashes represent gaps in the aligned ORF3 proteins. Numbers represent amino acid position in the Bo/Jena/80/DE ORF3 protein.

Table 2
Percentage identity of the Bo/Thirsk10/00/UK partial RdRp, complete capsid, and complete ORF3 genes to the UK and German reference BoCVs Bo/Newbury2/76/UK and Bo/Jena/80/DE

	RdRp[a]		Capsid		ORF3	
	Nucleotide	Amino acid	Nucleotide	Amino acid	Nucleotide	Amino acid
Bo/Newbury2/76/UK	76	89	**85**	**94**	**84**[b]	**88**[b]
Bo/Jena/80/DE	**88**	**98**	67	68	60	61

Identities closest to either Bo/Newbury2/76/UK or Bo/Jena/80/DE are in bold text.
[a] The partial RdRp sequence was 583 nucleotides and 193 translated amino acids.
[b] The Bo/Jena/80/DE and Bo/Thirsk10/00/UK ORF3 initiation codon was used.

80/DE in the RdRp, capsid, and ORF3 genes. Previously published nucleotide and amino acid maximum likelihood phylograms showed that BoCVs formed two distinct genetic clusters in all three ORFs, one represented by the UK reference virus Bo/Newbury2/UK and the other by Bo/Jena/80/DE (Oliver et al., 2003). Analysis of the Bo/Thirsk/10/00/UK partial RdRp (193 amino acids) grouped Bo/Thirsk/10/00/UK with Bo/Jena/80/DE, with strong statistical support (quartet puzzling value of 95) at the internal branch node between the Bo/Newbury2/UK and Bo/Jena/80/DE clusters (Fig. 3A). In contrast, the complete capsid and partial ORF3 phylograms grouped Bo/Thirsk10/00/UK with the Newbury2-like viruses, not with Bo/Jena/80/DE, and was strongly supported by the quartet puzzling statistics of 100 and 99 at the internal branch nodes (Figs. 3B and C). Additional phylogenetic analyses of the three ORFs using UPGMA, Fitch–Margoliash, or parsimony methods showed the same relationships between Bo/Thirsk10/00/UK, Bo/Newbury2/76/UK, and Bo/Jena/80/DE as described above and were supported by strong bootstrap values of 86–100 (not shown).

The above data suggested that a recombination event had occurred at the overlap between the Bo/Thirsk10/00/UK ORF1 (RdRp) and ORF2 (capsid) genes to produce a chimeric virus. This was confirmed using SimPlot and LARD analyses. The nucleotide identity plot for 400 nucleotides towards the 5′ end of the partial RdRp showed an average identity of approximately 70% between Bo/Thirsk10/00/UK and Bo/Newbury2/76/UK and approximately 90% between Bo/Thirsk10/00/UK and Bo/Jena/80/DE (Fig. 4). Around the region where ORF1 and 2 overlap, between nucleotide positions 400 and 600, the identity of Bo/Thirsk10/00/UK and Bo/Newbury2/76/UK rose sharply to a peak of 90%, whereas nucleotide identity with Bo/Jena/80/DE dropped to 75%. This showed that the region between 400 and 600 nucleotides was where the putative recombination event had occurred in the Bo/Thirsk/10/UK genome. The point of recombination, predicted using LARD, occurred upstream of nucleotide 586 of the Bo/Thirsk10/00/UK 2818-nucleotide sequence. This was the first nucleotide substitution for a region of 29 completely conserved nucleotides (positions 557–585) among Bo/Thirsk10/00/UK, Bo/Newbury2/76/UK, and Bo/Jena/80/DE that spanned the ORF1-2 overlap (Fig. 5). The nucleotide identity of Bo/Thirsk10/00/UK remained higher to Bo/Newbury2/76/UK (80–87%) for the S, P1, and P2 domains of the capsid gene (ORF2) (Fig. 4). Between Bo/Thirsk10/00/UK and Bo/Jena/80/DE, nucleotide identity dropped to 70% in the S domain and fell sharply in the P1 domain at nucleotide positions

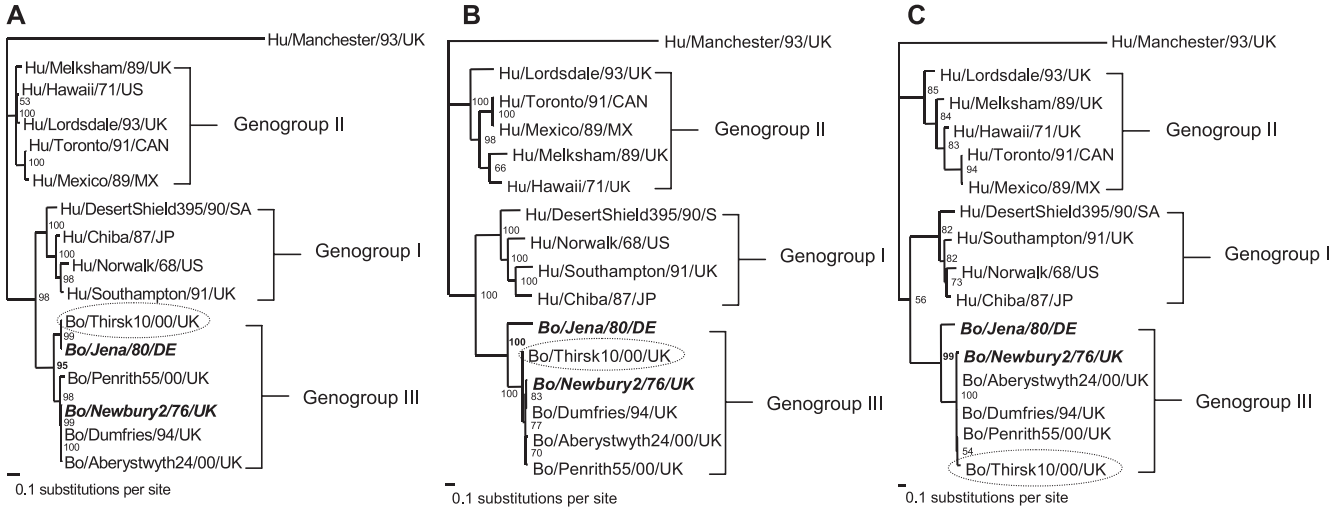

Fig. 3. Phylogenetic analyses generated using the maximum likelihood method of TreePuzzle for the translated amino acid sequences of (A) the partial RdRp sequence (193 amino acids), (B) the complete capsid, and (C) 113 amino acids from the 5′ end of the ORF3 gene (starting from the Bo/Jena/80/DE ORF3 initiation codon) of the UK BoCVs, the German Bo/Jena/80/DE, and representatives of the human genogroups I and II noroviruses. The Sapovirus Hu/Manchester/93/UK was used as the out-group. The UK and German reference viruses that represent the two genetic clusters of genogroup III are highlighted in bold italicized text. The numbers at the nodes (quartet puzzling values) indicate the frequencies of occurrence for 1000 replicate trees.

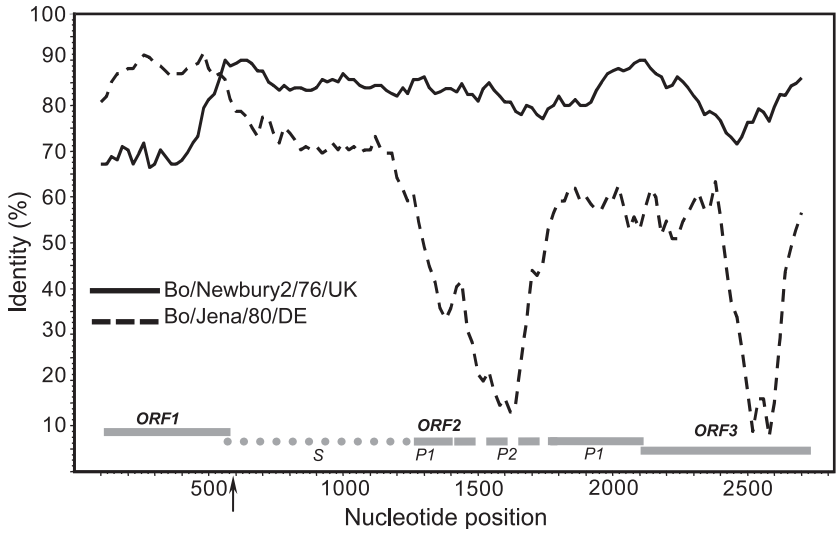

Fig. 4. Nucleotide identity plot of the 3′ end (2818 nucleotides) of the Bo/Thirsk10/00/UK genome compared with the UK (Bo/Newbury2/76/UK) and German (Bo/Jena/80/DE) reference BoCVs. The bars below the plot represent the three ORFs. The capsid gene (ORF2) is divided into the S (dotted line), P1 (solid line), and P2 (dashed line) domains of the Hu/Norwalk/68/US as determined by X-ray crystallography (Prasad et al., 1999). The arrow indicates the point after the recombination site (nucleotide 586) identified by LARD analysis. The Bo/Jena/80/DE and Bo/Thirsk10/00/UK ORF3 initiation codon was used.

1225–1375. Nucleotide identity of Bo/Thirsk10/00/UK and Bo/Jena/80/DE reached a low of 15% at nucleotide position 1600 in the P2 domain but rose to an average of 55% in the P1 domain between nucleotide positions 1875 and 2000. The 3′ terminal nucleotides of the ORF3 gene (2100 and 2800) showed a small drop and rise in nucleotide identity to Bo/Newbury2/76/UK (from 80% to 73% then 73% to 85%), whereas nucleotide identity fell sharply then rose sharply (from 60% to 10% then 10% to 53%) compared with Bo/Jena/80/DE.

Discussion

The present study is, to our knowledge, the first to identify a chimeric genome in the bovine genogroup III of the noroviruses and one of the few studies to identify chimeras in the *Norovirus* genus of the *Caliciviridae*. It validates the study by Hardy et al. (1997), which first suggested that chimeric noroviruses exist. Substitutions and deletions in the RdRp, capsid and ORF3 genes, amino acid and nucleotide identities, phylogenetic analyses, and

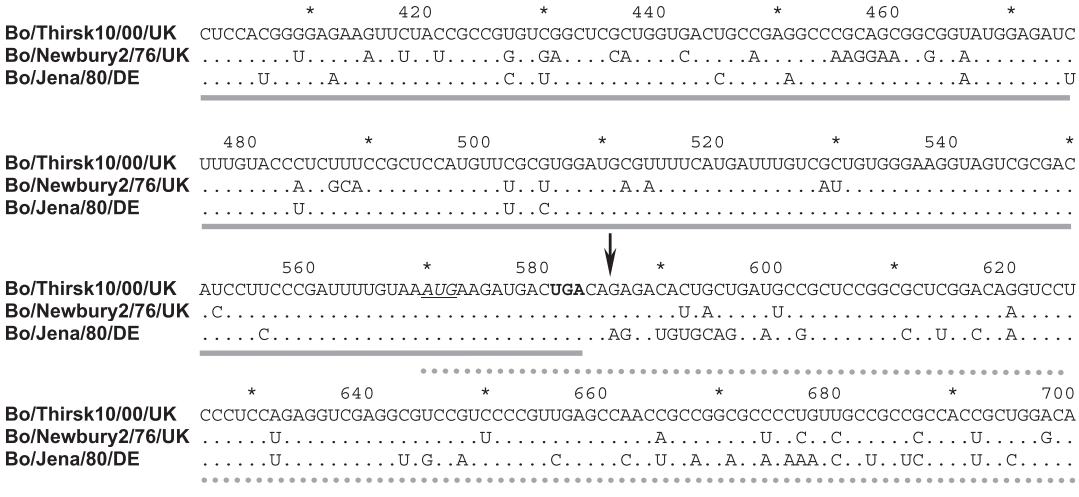

Fig. 5. The genome recombination site of Bo/Thirsk10/00/UK identified at the ORF1 (solid line)–ORF2 (dotted line) overlap. The scale indicates the nucleotide position in the 2818 nucleotides from the 3′ end of the Bo/Thirsk10/00/UK genome. The point of recombination as determined by LARD analysis is indicated by the arrow. The ORF1 termination codon is in bold text and the ORF2 initiation codon is shown in italic underlined text. The arrow indicates the point after the recombination site (nucleotide 586) identified by LARD analysis.

the supporting data from SimPlot and LARD analyses showed that Bo/Thirsk10/00/UK had a bipartite relationship with Bo/Newbury2/76/UK and Bo/Jena/80/DE, representatives of the two genetic clusters established in genogroup III to date (Dastjerdi et al., 1999; Liu et al., 1999; Oliver et al., 2003). The Bo/Thirsk10/00/UK genome grouped with the Bo/Jena/80/DE cluster in its RdRp gene, but after the ORF1-2 overlap, the Bo/Thirsk10/00/UK genome grouped with the Bo/Newbury2/76/UK cluster. The bipartite relationship is unlikely to be explained by a series of point mutations but by the process of genome recombination.

The chimeric genome of Bo/Thirsk10/00/UK had similarities to three chimeric genomes, Hu/Mendoza320/95/ARG (Arg 320), Hu/SaitamaU1/97/JP (Saitama U1), and Hu/WakayamaWUG1/00/JP (WUG1), identified previously in genogroup I and II human noroviruses (Jiang et al., 1999a; Katayama et al., 2002). In common with the present study, both these studies described sequences generated from single amplicons spanning the three ORFs. In the study with human genogroup II noroviruses (Jiang et al., 1999a), the RdRp gene of Hu/Mendoza320/95/ARG virus had 95% amino acid identity to the Hu/Lordsdale/93/UK virus (genetic cluster 4) but 87% identity to the Hu/Mexico/89/MX virus (genetic cluster 3). However, the amino acid identity of the Hu/Mendoza320/95/ARG capsid gene was more closely related to the Hu/Mexico/89/MX virus than to Hu/Lordsdale/93/UK (95% vs. 68% amino acid identity), indicating that recombination had occurred between the two genetic clusters. Katayama et al. (2002) confirmed genomic recombination between genogroup II noroviruses and also provided the first evidence for chimeric genomes in norovirus genogroup I. In both these studies with the three human noroviruses, the point of recombination was at the ORF1-2 overlap, the same region identified for the bovine genogroup III Bo/Thirsk10/00/UK virus. Additional studies support the existence of human noroviruses with chimeric genomes (Hardy et al., 1997; Lochridge and Hardy, 2003; Vinje et al., 2000). However, in contrast to Jiang et al. (1999a), Katayama et al. (2002), and the present study, a contiguous cDNA of the polymerase and complete capsid genes was not used, but regions from the RdRp, capsid, and ORF3 genes were amplified independently. These could be generated from the genes of different viruses, rather than from a single virus, if a mixed population of viruses was present in the original fecal sample. Mixed populations have been described with human diarrhea samples (Gray et al., 1997). This led Vinje et al. (2000) to conclude that their data did not strictly show that noroviruses with chimeric genomes were identified. Hardy et al. (1997) predicted that further chimeric viruses would be identified and this has proved to be true.

Genome recombination has been reported for viruses from several other positive-sense, single-stranded RNA viruses and include the enterically transmitted astroviruses, coronaviruses, enteroviruses, and polioviruses (Belliot et al., 1997; Kew et al., 2002; Oprisan et al., 2002; Walter et al., 2001). Of the three classes of RNA recombination proposed by Nagy and Simon (1997), the limited evidence so far (Jiang et al., 1999a; Katayama et al., 2002), including the present study, suggests that similarity-essential recombination (Class 1) is likely for the noroviruses. This requires a region of identity to allow for base pairing between the nascent-strand and the acceptor RNA within a complementary region such as the ORF1-2 overlap, the site of recombination for the chimeric noroviruses.

Chimeric genomes between the genetic clusters within the norovirus genogroups might be more frequent than currently perceived. The nucleotide sequence for five BoCV genomes spanning the RdRp, capsid, and ORF3 genes have been reported so far (Liu et al., 1999; Oliver et al., 2003), and of these, 1, Bo/Thirsk10/00/UK, was a recombinant. Two additional amplicons, Bo/Aberystwyth58/00/UK and Bo/Carmarthen10/99/UK, were identified as Jena-like in the present study. However, it was not possible to establish whether these viruses had chimeric genomes similar to Bo/Thirsk10/00/UK, as the generation of amplicons for the capsid gene and four attempts to generate cDNA that spanned the three ORFs for each virus was unsuccessful. With human noroviruses, two chimeric genomes were found in Japan out of 10 new genomes sequenced (Katayama et al., 2002). In Europe, two potentially chimeric genomes were found from 31 viruses (Vinje et al., 2000). Although the frequency of recombination in the *Norovirus* genus cannot be established from the limited number of studies so far, available data do suggest that the frequency of recombination might be relatively high and should be investigated further.

With human noroviruses, of the two chimeric (Hu/Mendoza320/95/ARG and Hu/SaitamaU1/97/JP) and three putative chimeric (Hu/SnowMountain/76/US, HU/NLV/Seacroft/90/UK, and HU/NLV/Wortley/90/UK) genomes identified for genogroup II noroviruses to date, four (Hu/SnowMountain/76/US, HU/NLV/Wortley/90/UK, Hu/Mendoza320/95/ARG, and Hu/SaitamaU1/97/JP) (Hardy et al., 1997; Jiang et al., 1999a; Katayama et al., 2002; Vinje et al., 2000), have RdRp genes that belong to genetic cluster 4 (Lordsdale-like) but capsid genes that belong to genetic clusters 1, 2, or 3 (Hawaii-like, Melksham-like, or Mexico-like). The genogroup II noroviruses from genetic cluster 4 are the most predominant global strain of noroviruses, especially those that cause outbreaks in hospitals and residential care homes (Fankhauser et al., 1998; Green et al., 2002; Maguire et al., 1999; Noel et al., 1999; Wright et al., 1998). RT-PCR using the RdRp has been very successful for the rapid detection of norovirus outbreaks, but is unlikely to provide a complete understanding of the capsid types, in the population, if recombinant noroviruses prove to be common.

The results from the present study have implications for the diagnosis and control of BoCVs. BoCVs have been reported in the UK, Germany, The Netherlands, and the USA (Liu et al., 1999; Oliver et al., 2003; Smiley et al., 2003; van der Poel et al., 2000), but their diversity, prevalence, and association with natural disease needs

further investigation. The majority of epidemiological studies of human noroviruses has been performed using RT-PCR with primers targeted at the RdRp (Foley et al., 2001; Green et al., 1995a, 1995b; Greening et al., 2001; Jiang et al., 1999b; Reuter et al., 2002; Vinje and Koopmans, 1996), but the existence of chimeric genomes shows that genetic clusters based on the RdRp gene do not necessarily correlate with the capsid gene. This observation was endorsed by recent surveillance data from the CDC, which showed that only 41% of human norovirus outbreaks typed using the capsid gene were associated with the same genetic cluster when the RdRp gene was used for typing (Anon, 2003). If chimeric BoCVs are not rare, diagnostic methods need to be used that take this into account. Vaccination is used to control calf diarrhea caused by bovine rotaviruses and coronaviruses (Crouch et al., 2001). It is possible that a calicivirus component might be incorporated into these vaccines. Thus, knowledge on the prevalence of the two capsid types in cattle populations is required so that vaccines with the appropriate strains can be produced. The results from the present study suggest it would be unwise to use a diagnostic test based solely on the BoCV RdRp gene.

Materials and methods

Of 38 amplicons of the expected size generated from 476 fecal samples (Oliver et al., 2003), 15 NI/E3 amplicons (114 bp) were selected from different geographical locations and cloned into the pCR2.1-TOPO® vector as previously described (Oliver et al., 2003). Amplicons of 316 bp were generated from 1:100 dilutions of the pCR2.1-TOPO purified plasmids using the High Fidelity PCR system (ROCHE) and the vector specific primers M13-forward and M13-reverse with an initial denaturation at 95 °C for 3 min, 25 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min and a final extension of 72 °C for 7 min. The primer pair M13-forward/M13-reverse amplified a 316 bp region of the cloning vector that included the 114 bp insert of the NI/E3 amplicon.

The 316 bp amplicons were compared to 316 bp amplicons from the UK reference virus Bo/Newbury2/76/UK and the Newbury2-like Bo/Aberystwyth65/00/UK using an HMA adapted from Zou (1997). To tubes containing sample buffer (4-μl nuclease-free H_2O, 2-μl 6× loading buffer, Promega, and 1-μl formamide, Promega), 5 μl of amplicon from Bo/Thirsk10/00/UK was mixed with the Bo/Aberystwyth65/00/UK or Bo/Newbury2/76/UK amplicon to generate heteroduplexes. Samples were denatured for 3 min at 98 °C using a DNA thermal cycler (Perkin–Elmer), placed immediately on ice for 10 min, then loaded on to 8% polyacrylamide gels containing 0.5% polyethylene glycol 6000 (BDH), with 2.5 M urea (Sigma), prepared in a BioRad Protean II apparatus. Gels were run at 300 V until the xylene cyanol band was 1 cm from the bottom of the gel then stained with GelStar stain (BioWhittaker Molecular Applications). Heteroduplexes were identified by their slower migration in the gel compared to the homoduplexes generated by running the Bo/Thirsk10/00/UK, Bo/Aberystwyth65/00/UK, or Bo/Newbury2/76/UK amplicons in separate lanes after denaturation. Sequencing of the 316 bp amplicons showed that the location of the 5′ end of the E3 primer was 98 nucleotides from the M13-forward sequence and that heteroduplexes were not a consequence of the orientation of NI/E3 amplicons in the pCR2.1-TOPO vector.

Long-RT-PCR (Oliver et al., 2003) was used to generate a 2818-nucleotide consensus sequence of Bo/Thirsk10/00/UK. Reverse transcription was performed on separate occasions at 42 °C with either primer NA-2poly-T *Eco*RI (5′-GCGCTTAAGT$_{(15)}$NMCAATGC-3′) or RT-$_{ANCHOR}$ (5′-GACCACGCGTATCGATGTCGACT$_{(16)}$V-3′) (Life-Technologies Ltd.) using 200 IU of Moloney murine leukemia virus (H−) reverse transcriptase (Promega). PCR was performed by using the high-fidelity *Z-Taq* polymerase (BioWhittaker) and the Bo/Thirsk10/00/UK-specific sense primer VIT010 (5′-TTTGTGTGCCCT-TTCTGAAGTCACC-3′) with either NA-2poly-T *Eco*RI or PCR-$_{ANCHOR}$ (5′-GACCACGCGTATCGATGTCGAC-3′). Thirty-five cycles of 98 °C for 5 s, 50 °C for 10 s, and 72 °C for 1 min were performed, with final extension at 72 °C for 5 min. Amplicons, approximately 2.8-kbp long generated using either NA-2poly-T *Eco*RI or RT-$_{ANCHOR}$, were gel purified and cloned using a TOPO-TA XL-PCR cloning kit (Invitrogen Ltd.). Cycle sequencing was performed using a dye terminator cycle sequencing kit and a CEQ 2000 XL DNA analysis system (Beckman Coulter). The Bo/Thirsk10/00/UK consensus nucleotide sequence was prepared using the STADEN sequencing package (Staden et al., 2000). ORFs were predicted using the NCBI ORF finder. Multiple alignments of nucleotide and translated amino acid sequences from the RdRp, capsid, and ORF3 genes were prepared using Clustal X version 1.8 (Thompson et al., 1997), and identities were calculated using GeneDoc (www.psc.edu/biomed/genedoc).

To identify the Bo/Thirsk10/00/UK site of genome recombination, the 2.8 kb nucleotide sequences spanning the RdRp, complete capsid, and ORF3 genes of Bo/Thirsk10/00/UK, the UK reference BoCV Bo/Newbury2/76/UK, and the German BoCV Bo/Jena/80/DE were aligned manually using the RdRp, capsid, and ORF3 amino acid sequence alignments for guidance. The SimPlot computer program (Lole et al., 1999) was used to analyze the alignment of the three BoCVs using a window size of 200 nucleotides that was moved along in 20-nucleotide steps. The percentage identity was calculated for each window and plotted on a line chart. The LARD (likelihood analysis of recombination in DNA) program (Holmes et al., 1999), identified the point of recombination between the BoCV genome sequences.

Phylogenetic analyses were performed with nucleotide and amino acid sequence alignments for the partial RdRp,

complete capsid and partial ORF3 genes from the BoCVs, and representatives of the human genogroup I and II noroviruses. PHYLIP (J. Felsenstein, Department of Genetics, University of Washington, Seattle; Phylogeny Inference Package, version 3.5 c) was used for parsimony (DNApars and Protpars), Fitch–Margoliash and bootstrap analyses, and TreePuzzle 5.0 (Schmidt et al., 2002) for maximum likelihood with quartet puzzling analyses. Phylogenetic trees were prepared by using TreeView (Page, 1996).

The nucleotide sequence accession numbers for sequences referred to in the text for the BoCVs are Bo/Newbury2/76/UK, AF097917; Bo/Jena/80/DE, AJ011099; Bo/Aberystwyth24/00/UK, AY126475; Bo/Aberystwyth58/00/UK, AY126462; Bo/Carmarthen10/99/UK, AY126460; Bo/Dumfries/94/UK, AY126474; Bo/Penrith55/00/UK, AY126476; and Bo/Thirsk10/00/UK, AY126468. The nucleotide sequence accession numbers for human genogroup I noroviruses are Hu/Norwalk/68/US, M87661; Hu/Chiba/87/JP, AB042808; Hu/DesertShield395/90/SA, U04469; Hu/Southampton/91/UK, L07418. The nucleotide sequence accession numbers for human genogroup II noroviruses are Hu/Hawaii/71/US, U07611; Hu/Melksham/89/UK, X81879; Hu/Mexico/89/MX, U22498; Hu/Toronto24/91/CAN, U02030; Hu/Lordsdale/93/UK, X86557. The accession number for the Sapovirus Hu/Manchester/93/UK is X86560.

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