

Identification of a Bovine Enteric Calicivirus, Kırklareli Virus, Distantly Related to Neboviruses, in Calves with Enteritis in Turkey

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A calicivirus was detected in neonatal calves with enteritis in Kırklareli, Thrace, Turkey. In the full-length genome, Kırklareli virus was related (48% nucleotide identity) to bovine enteric caliciviruses (*Nebovirus* genus). The virus was also detected in a herd in Ankara, Central Anatolia, but not in other Turkish prefectures.

Caliciviruses (family *Caliciviridae*) are important pathogens of humans and animals. Caliciviruses are nonenveloped, small round viruses with a single-strand positive-sense RNA genome of 7 to 8.5 kb in size, polyadenylated at the 3' end (1). The family includes the genera *Vesivirus*, *Norovirus*, *Sapovirus*, *Lagovirus*, and *Nebovirus*. In recent years, novel, yet unclassified caliciviruses have been identified in mammals, birds, and fishes (2).

Caliciviruses of at least three distinct genera (*Nebovirus*, *Norovirus*, and *Vesivirus*) have been detected in cattle. However, on the basis of either experimental infections or observational studies, only neboviruses and noroviruses have been associated with enteric replication and with enteric signs (3, 4).

In early 2012, an outbreak of enteritis occurred in Kırklareli, Thrace, Turkey. The herd consisted of 250 cows and 200 calves, with enteric disease affecting about 60% of the calves, resulting in 30% mortality. A total of 17 fecal samples, collected from the affected calves, were tested and found to be positive for either species A rotavirus or coronavirus. All animals also tested positive for *Cryptosporidium* spp. Interestingly, upon electron microscopy observation, small round viruses (SRVs) could be observed in 3 stool samples. Using reverse transcription (RT)-PCR with consensus primers (5) and the sequence analysis of a short (330-nucleotide [nt]) fragment of the RNA-dependent RNA polymerase (RdRp), the SRVs were characterized as caliciviruses, with limited homology (<65% nt identity), to the prototype strain Nebraska (*Nebovirus* genus) (6). By combining RT-PCRs with a primer walking strategy, 5' and 3' RACE (rapid amplification of cDNA ends) protocols and next-generation sequencing, the complete genome of the calicivirus, named Kırklareli virus, was determined (GenBank accession no. [KT119483](#)). The genome was 7,484 nt long, excluding the poly(A) tail, with a ribonucleoside composition of 20.4% A, 30.9% C, 27.5% G, and 21.2% U. The 5' untranslated region (UTR) was 67 nt long, while the 3' UTR was 80 nt long. Two open reading frames (ORFs), of 6,581 nt (ORF1) and 657 nt (ORF2) in length, were mapped. ORF1 encoded a large polyprotein of 2,226 amino acids (aa) in length, containing the replicative proteins and, at the 3' end, the 1,629 nt-long (542 aa) capsid region, with a potential cleavage site, EGD, between the nonstructural proteins and the capsid protein. ORF2 encoded a 218-aa long protein (Fig. 1). Conserved amino acid motifs characteristic of caliciviruses, including the 2C-like helicase-nucleoside triphosphatase DNA-binding (GXGXGKS/T) and the ATP hydrolysis (KXXFXSXXXXXS/TTN) motifs, the 3D pol

(GLPSG and YGDD) motifs and the VP1 capsid (PPG) motifs, were present in the ORF1 polyprotein. In the 3C-like protease of Kırklareli virus, cytosine (C) replaced aspartic acid (D) in the GDDG motif, which is conserved in caliciviruses. By sequence comparison of the full-length genome, the highest nucleotide identity was to neboviruses (48%), followed by lagoviruses (38%), while the lowest nucleotide identity (25%) was to recoviruses and to Atlantic salmon caliciviruses. In the ORF1, the highest identity (51% nt and 42% aa) was to neboviruses, while identity to other caliciviruses was lower than 39% nt and 23% aa. In the capsid precursor coding region of ORF1, Kırklareli virus displayed 44% nt identity (41% aa) to neboviruses and less than 36% nt (21% aa) to other caliciviruses. In the ORF2, the highest identity (39% nt and 27% aa) was to neboviruses (Table 1). Upon phylogenetic analysis, the virus clustered closest to the *Nebovirus* taxon (Fig. 2).

The genetic diversity between Kırklareli virus and other bovine caliciviruses (neboviruses) appeared within the ranges observed among members of the same genus (7, 8); therefore, Kırklareli virus could represent an ancestor of the *Nebovirus* genus. Although neboviruses are relatively highly conserved and segregate into two major clades, Nebraska-like or Newbury-1-like, genetic heterogeneity due to recombination (9) or genetic drift (10) has also been reported. However, it is interesting to highlight that the Kırklareli virus differed in its genome organization from neboviruses. There was a 1-nt overlap between ORF1 and ORF2, while members of the *Nebovirus* genus have a 1-nt interval between the two ORFs. ORF1 was 48 nt (16 aa) longer, while ORF2 was 21 nt (7 aa) shorter. Also, the 5' UTR was shorter (67 versus

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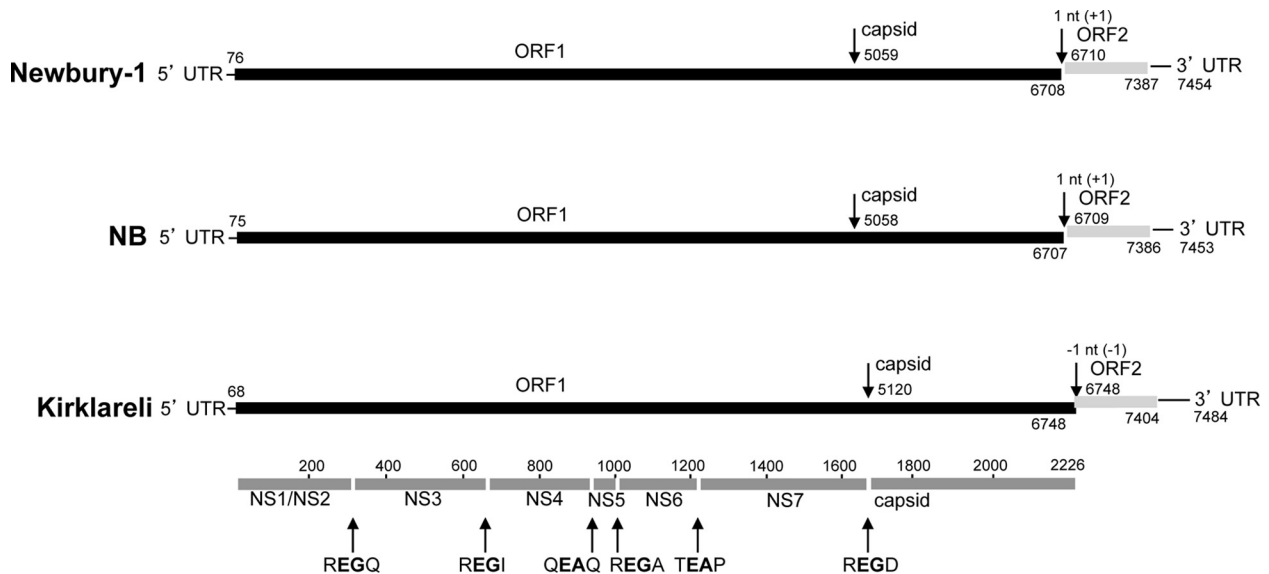


FIG 1 Genome comparison of Kirklareli virus with prototypes of the *Nebovirus* genus, strains Nebraska (NB) (AY082891) and Newbury-1 (DQ013304). Arrows indicate the putative start codon of the capsid region and the position of the ORF1/ORF2 junction. Arrows are also used to indicate the putative cleavage sites on the ORF1-encoded polyprotein.

74/75 nt), and the 3' UTR was longer (80 versus 67 nt) than in neboviruses (Fig. 2).

Using specific primers designed for the capsid-coding region (primer CapFor, CCACCATTATCACCAAATTGC, and primer CapRev, CATAATCAGAATAGAAGGCGC), fecal samples obtained from calves of the Kirklareli enteritis outbreak were re-screened, identifying Kirklareli virus RNA in 5 of 17 (29.4%) calves. In addition, an archival collection of samples available in our laboratory was screened. This collection included an additional 33 calves with enteritis from 28 herds located in 3 Turkish prefectures. Kirklareli virus RNA was only detected in 1 additional herd in the Ankara region (Table 2). If this can be accounted for by nucleotide polymorphisms in the primer binding sites or if it reflects a limited/diverse geographical distribution remains to be assessed. It is important from this perspective to underline that, although Kirklareli virus is genetically related to neboviruses, oligonucleotides specific for neboviruses available in the literature

(11, 12) failed to recognize Kirklareli virus RNA in RT-PCR. Upon alignment with the Kirklareli virus genome, we observed several nucleotide mismatches in the primer binding regions that likely prevented correct annealing.

Attempts were made to cultivate the virus in bovine cells lines, e.g., MDBK and PEB, but the virus failed to replicate *in vitro*, as observed by monitoring the onset of cytopathic effect in serial passages and viral replication by RT-PCR. Failure to propagate the virus *in vitro* was not unexpected, as neboviruses and other enteric caliciviruses are not cultivatable, with the exceptions of the porcine sapovirus strain Cowden (13) and of murine noroviruses (14).

Livestock constitutes a large part of agricultural production in Turkey and contributes to the economic development of rural households, with several farmers relying on livestock for their incomes. In this study, we identified a bovine calicivirus, distantly related to other bovine enteric caliciviruses, e.g., neboviruses, with

TABLE 1 Sequence identities between Kirklareli virus and other caliciviruses in the genome and in the RdRp and capsid region^a

Species (GenBank accession no.)	Genus	Sequence identity (%) for:				
		Genome		RdRp		Capsid
		nt	nt	aa	nt	aa
Bo/NB (AY082891)	<i>Nebovirus</i>	48	59	59	44	41
Le/EBHSV (NC002615)	<i>Lagovirus</i>	38	46	38	36	21
Go/strain N (KJ473715)	<i>Nacovirus</i> ^b	32	43	36	33	20
Ch/V0021/Bayern/2004 (HQ010042)		30	42	36	32	21
Hu/Manchester (X86560)	<i>Sapovirus</i>	32	44	39	36	21
Fe/FCV/CFI/68 (U13992)	<i>Vesivirus</i>	32	44	37	34	21
Hu/Norwalk (NC001959)	<i>Norovirus</i>	28	39	34	29	15
Si/Tulane (EU391643)	<i>Recovirus</i> ^b	25	38	28	28	13
Po/St-Valerien (AB863586)	<i>Valovirus</i> ^b	27	37	28	31	15
ASC Nordland/2011 (KJ577139)	<i>Salmonid CV</i> ^b	25	36	25	30	15

^a Distance values were calculated after alignment with ClustalW, without distance correction.

^b Candidate genera.

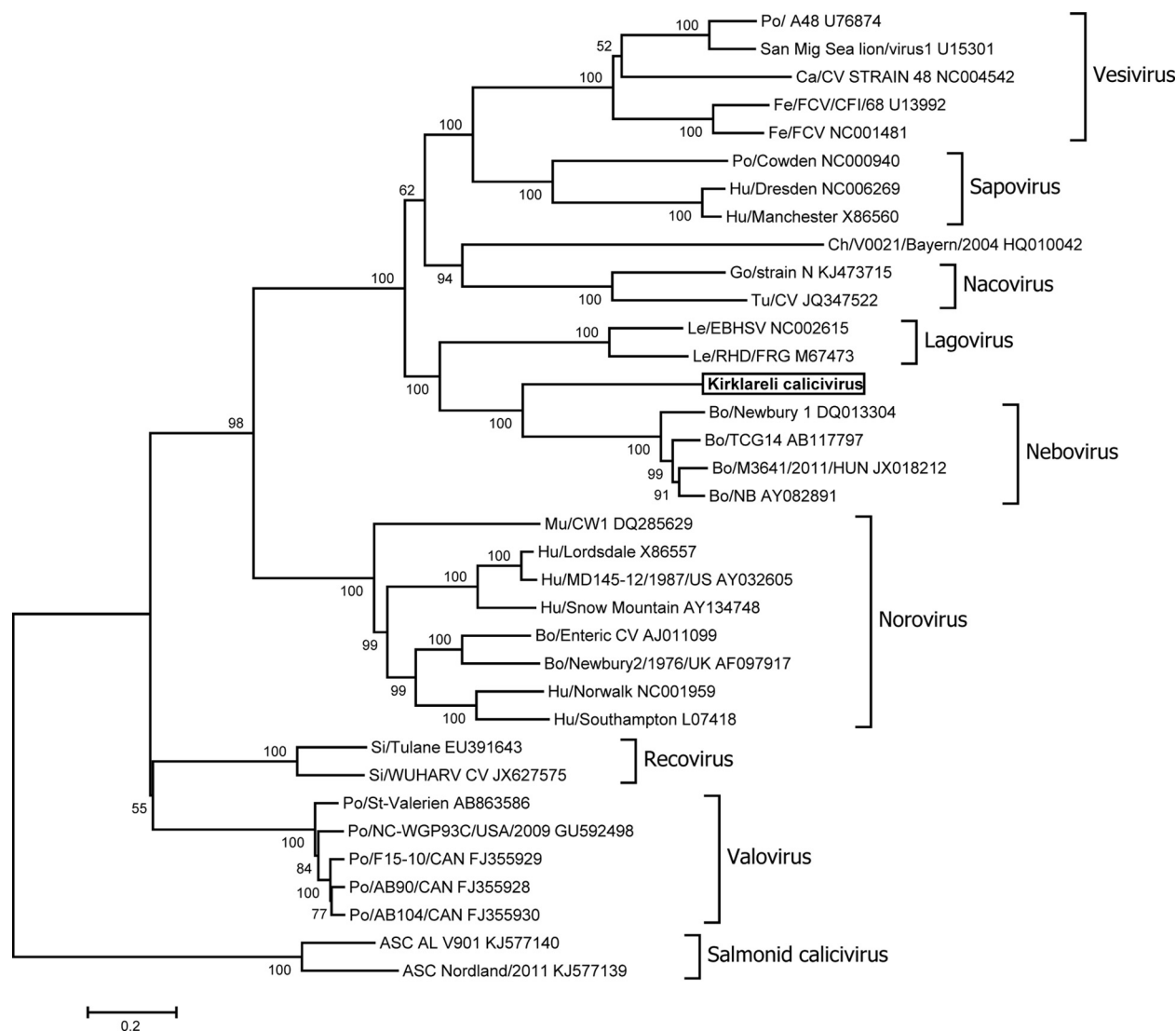


FIG 2 Phylogenetic tree based on the whole genome of representatives of the various established and proposed calicivirus genera. The tree was generated using the neighbor joining method, with the Jukes Cantor algorithm of distance correction, with bootstrapping over 1,000 replicates. Fe, feline; Ca, canine; Po, porcine; Bo, bovine; Le, lapine; Hu, human; Ch, chicken; Go, goose; Tu, turkey; Si, simian; Mu, murine; ASC, Atlantic salmon calicivirus. The scale bar indicates the number of substitution per site.

a distinctive genome organization. Experimental infection with the nebovirus prototypes, strains Newbury-1 and Nebraska, causes anorexia, diarrhea, and xylose malabsorption in gnotobiotic calves, with damage restricted to the anterior half of the small intestine (4, 6, 15), suggesting that neboviruses are able to cause

enteric disease in calves. If Kirkclareli virus also retains these pathogenic properties should be demonstrated by experimental infections. In addition, information on the epidemiological features of Kirkclareli-like caliciviruses should be gathered in larger, structured epidemiological studies in order to assess the relevance of this enteric virus in bovines.

Nucleotide sequence accession number. The new sequence for the Kirkclareli virus genome has been deposited in GenBank under accession no. [KT119483](#).

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TABLE 2 Screening for Kirkclareli calicivirus in diarrheal calves in Turkey prefectures			
Prefecture	City	No. of positive herds/ total no. (% positive)	No. of positive samples/ total no. (% positive)
Thrace	Kirkclareli	1/1 (100)	5/17 (29.4)
Anatolia	Ankara	1/5 (20.0)	1/6 (16.6)
Marmara	Bursa	0/17	0/21
Aegen	İzmir	0/6	0/6
Total		2/29 (6.8)	6/50 (12.0)

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