Title: A novel bovine enteric calicivirus, Kırklareli virus, in calves with enteritis in Turkey

Running title: Identification of a novel bovine enteric calicivirus

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Abstract (49)

A calicivirus was detected in neonatal calves with enteritis in Kırklareli, Thrace, Turkey. In the full-length genome, Kırklareli virus was more 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.

Keywords: calicivirus; bovine; enteritis; Turkey

Text (1080)

Caliciviruses (Family *Caliciviridae*) are important pathogens of humans and animals. Caliciviruses are non-enveloped small round viruses, with a single strand positive sense RNA genome of 7-8.5 kilobase (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 mammalians, 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 faecal 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 RT-PCR with consensus primers (*5*) and sequence analysis of a short (330 nt) fragment of the RNA-dependent RNA-polymerase, the SRVs were characterized as caliciviruses, with limited homology (<65% nucleotide [nt] identity), to the prototype strain Nebraska (*Nebovirus* genus) (*6*). By combining RT-PCRs with a primer walking strategy, 5' and 3' RACE protocols and next generation sequencing,,the complete genome of the calicivirus, named Kırklareli virus, was determined (GenBank accession no. KT119483). The genome was 7484 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, whilst the 3' UTR was 80 nt long. Two ORFs, of 6581 nt (ORF1) and 657 nt (ORF2) in length, were mapped. ORF1 encoded a large polyprotein of 2226 amino acids (aa) in length, containing the replicative proteins and, at the 3' end, the 1629 nt-long (542 aa) capsid region, with a potential cleavage site,

EGD, between the non-structural proteins and the capsid protein. ORF2 encoded a 218 aa long protein (**Figure 1**). Conserved amino acid motifs, characteristic of caliciviruses, including the 2C-like helicase-nucleoside triphosphatase DNA binding (GXXGXGKS/T), the ATP hydrolysis (KXXXFXSXXXXXS/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 Kirklareli 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 nt identity was to neboviruses (48%), followed by lagoviruses (38%), whilst the lowest nt identity (25%) was to recoviruses and to atlantic salmon caliciviruses. In the ORF1, the highest identity was (51% nt and 42% aa) 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 (**Figure 2**).

The genetic diversity between Kırklareli virus and other bovine caliciviruses (neboviruses) appeared within the ranges observed amongst members of the same genus (*7, 8*) and therefore, Kırklareli virus could represent an ancestor of the *Nebovirus* genus. Although neboviruses are relatively highly conserved and segregate into two major clades, Nebraska- 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, whilst members of the *Nebovirus* genus have a 1-nt interval between the two ORFs. ORF1 was 48 nt (16 aa) longer, whilst ORF2 was 21 nt (7 aa) shorter. Also, the 5' UTR was shorter (67 vs 74/75 nt) and the 3' UTR was longer (80 vs 67 nt) than in neboviruses (**Figure 2**).

Using specific primers designed for the capsid-coding region (primer CapFor CCACCATTATCACCAAATTGC and primer CapRev, CATAATCAGAATAGAAGGCGC),

faecal samples obtained from calves of the Kırklareli enteritis outbreak were re-screened, identifying Kırklareli virus RNA in 5/17 (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. Kırklareli 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 it reflects a limited/diverse geographical distribution remains to be assessed. It is important, from this perspective, to underline that, although being genetically related to neboviruses, oligonucleotides specific for neboviruses available in the literature (*11, 12*) failed to recognize Kırklareli virus RNA in RT-PCR. Upon alignment with Kırklareli 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 cytophatic effect in serial passages and virus 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 exception 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 income. In this study, we identified a bovine calicivirus, distantly related to other bovine enteric caliciviruses, e.g. neboviruses, and with a distinctive genome organization. Experimental infection with the nebovirus prototypes, strain 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 Kırklareli virus also retains these pathogenic properties should be demonstrated by experimental infections. In addition, information on the epidemiological features of Kırklareli-like caliciviruses

should be gathered in larger, structured epidemiological studies in order to assess the relevance of this enteric virus in bovines.

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Table 1: Sequence identities (% values) between Kirklareli virus and other caliciviruses in the genome and in the RdRp and capsid region. Asterisks indicate candidate genera. Distance values were calculated after alignment with ClustalW, without distance correction.

Species	Genus	Genome	RdRp		Capsid	
		nt	nt	aa	nt	aa
Bo/NB AY082891	Nebovirus	48	59	59	44	41
Le/EBHSV NC002615	Lagovirus	38	46	38	36	21
Go/strain N KJ473715	Nacovirus*	32	43	36	33	20
Ch/V0021/Bayern/2004 HQ010042	-	30	42	36	32	21
Hu/Manchester X86560	Sapovirus	32	44	39	36	21
Fe/FCV/CFI/68 U13992	Vesivirus	32	44	37	34	21
Hu/Norwalk NC001959	Norovirus	28	39	34	29	15
Si/Tulane EU391643	Recovirus*	25	38	28	28	13
Po/St-Valerien AB863586	Valovirus*	27	37	28	31	15
ASC Nordland/2011 KJ577139	Salmonid CV*	25	36	25	30	15

Table 2: screening for Kırklareli calicivirus in diarrheal calves in Turkey prefectures.

Prefecture	City	Positive herds	Positive samples
Thrace	Kırklareli	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%)

Figure legends

Figure 1: genome comparison of Kırklareli virus with prototypes of the *Nebovirus* genus, strain 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.

Figure 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 1000 replicates. Abbreviations: Fe, feline; Ca, canine; Po, porcine; 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.