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DETECTION AND FIRST MOLECULAR CHARACTERISATION OF THREE PICORNAVIRUSES FROM DIARRHOEIC CALVES IN TURKEY

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The involvement of picornaviruses in calf diarrhoea was evaluated by the analysis of 127 faecal samples collected from diarrhoeic calves during 2014–2016. Virus detections were carried out by PCR using generic or specific primer pairs. One-third of the faecal samples (33.86%) were found to be positive for one or more of the studied viruses. Bovine kobuvirus was detected in 22.83%, bovine hungarovirus in 11.02%, while bovine enterovirus 1 in 5.51% of the samples. The sequences of the PCR products indicated the existence of novel variants in all the three virus species. When comparing the partial sequences, the nucleotide sequence identities between our newly detected viruses and those previously deposited to the GenBank ranged between 76 and 99%. Phylogenetic analyses revealed a novel lineage within the species *Hunnivirus A*. Our findings suggest that these viruses should be regarded as possible aetiological agents of calf diarrhoea. Based on the newly determined sequences, we designed and tested a new generic PCR primer set for the more reliable detection of bovine hungaroviruses. This is the first report on the molecular detection of the presence of bovine hungarovirus, bovine kobuvirus and bovine enterovirus 1 in the faecal samples of diarrhoeic calves in Turkey.

Key words: Bovine hungarovirus, bovine kobuvirus, bovine enterovirus 1, diarrhoea, calf, Turkey

Neonatal diarrhoea of calves is one of the most significant health problems in the cattle industry. Numerous aetiological agents including parasites (*Cryptosporidium parvum*, etc.), bacteria (*Salmonella enterica*, *Escherichia coli*, *Clostridium perfringens*, etc.) and viruses [bovine rotavirus (BRV), bovine coronavirus (BCoV), bovine viral diarrhoea virus (BVDV), etc.] have been shown to be responsible for the diarrhoea of young calves (Bartels et al., 2010; Cho and Yoon, 2014). In addition, newly emerging viruses, including bovine torovirus (BToV),

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bovine norovirus and nebovirus, as well as bovine picornaviruses from the species *Enterovirus E*, *Aichivirus B* and *Hunnivirus A* have also been reported as enteric disease agents in calves (Pham et al., 2007; Reuter et al., 2011; Cho and Yoon, 2014).

Together with *Dicistroviridae*, *Iflaviridae*, *Marnaviridae*, *Secoviridae* and some unassigned viruses, the family *Picornaviridae* is a member of the order *Picornavirales* (MacLachlan and Dubovi, 2017). Members of the family *Picornaviridae* share basic characteristics such as the shape and the small size (approximately 30 nm in diameter) of the non-enveloped virion, as well as the single-stranded, positive sense RNA genome of approximately 7.5 to 8.5 kb in size (Zell, 2018). *Picornaviridae* is a large virus family currently containing more than 35 distinct genera according to the International Committee on Taxonomy of Viruses, ICTV (http://www.ictvonline.org). Three classified genera of the family are *Hunnivirus*, *Kobuvirus* and *Enterovirus*, members of which have been reported to cause gastrointestinal infections in cattle (Zell et al., 2017).

The genus *Enterovirus* contains about 15 species (*Enterovirus A* to *L* and *Rhinovirus A to C*) at present (Zell et al., 2017). These occur in humans and different animal species including cattle and swine. The species *Enterovirus E* and *F* which contain bovine enterovirus 1 and 2 (BEV-1 and 2), respectively, are regarded as aetiological agents in bovine gastrointestinal, respiratory and reproductive diseases (Knowles and Mann, 1990; Blas-Machado et al., 2007).

The genus *Kobuvirus*, being also a member of the family *Picornaviridae*, currently includes six species named as *Aichivirus A* to *E,* out of which *Aichivirus B* contains bovine kobuvirus (BKV) (Zell et al., 2017). Kobuviruses/Aichiviruses were first detected in human diarrhoeic stool specimens obtained from an oyster-associated gastroenteritis outbreak in Japan (Yamashita et al., 1991). Since then, kobuviruses have been detected worldwide in humans as well as in many domestic animal species including cattle, sheep, ferret and goat (Yamashita et al., 2003; Di Martino et al., 2012; Smits et al., 2013; Melegari et al., 2016; Otomaru et al., 2016; Pankovics et al., 2016). Since BKV has been detected in connection with calf diarrhoea, it can be considered an emerging bovine virus (Khamrin et al., 2008; Park et al., 2011). More recent studies have revealed that aichiviruses may also occur in different laboratory-bred or free-living rats (*Rattus norvegicus*, *R. tanezumi*, *R. argentiventer* and *Bandicota indica*) in the USA (Firth et al., 2014) as well as in China (Du et al., 2016).

Hunnivirus A containing bovine hungarovirus (BHuV) is the only species in the genus *Hunnivirus*. It was first detected from sheep and cattle in Hungary in 2008 and 2009 and had been named initially as bovine and ovine hungarovirus 1, respectively (Reuter et al., 2012).

Evidence for the presence of BEV-1 in Turkey has already been reported based on serological surveys without direct virus detection (Yilmaz et al., 2011). Our aim was to screen faecal samples of diarrhoeic calves for selected picorna-

viruses. Partial sequence characterisation of any newly detected viruses was also planned.

Materials and methods

Samples and RNA isolation

A total of 127 faecal samples (rectal swabs) from diarrhoeic calves, up to one month old, on 76 small farms (with less than 20 animals) were collected during 2014–2016 from three provinces (Sivas, Malatya and Elazığ) located in the central Turkish inland. The collected faecal samples were transported to the laboratory as soon as possible, then stored in an ultra-deep freezer at –80 °C until the RNA isolation step. The samples had been tested for the presence of common viral enteric pathogens (BRV, BCoV, BVDV and BToV) previously.

To remove large cellular debris, faecal samples were diluted in 1 M phosphate buffered saline solution (1/10) and centrifuged at 5000 rpm for 5 min. The supernatants were submitted to the nucleic acid extraction procedure by using the Vivantis GF-1 Viral Nucleic Acid Extraction Kit according to the manufacturer's instructions (Vivantis Technologies, Malaysia). Eluted nucleic acids were stored in –80 °C until use.

Genomic data analysis and primer design

Because of the genetic diversity of picornaviruses, we designed new PCR primers in order to broaden the detection limits for BEVs, BKV and BHuV. Nucleotide sequences of these viral agents (94 for BEV-1 and 2 entries, 20 BHuV entries and 158 individual BKV sequences) were downloaded from the GenBank, and multiple alignments were prepared by using Cluster W plugin of Unipro UGENE software version 1.21. (Okonechnikov et al., 2012). A generic detection primer pair which covers all the sequences deposited in GenBank, for BEV-1 5' UTR region was designed using primer3 plugin of BENCHLING online molecular analysis software (http://www.benchling.com). A new detection primer pair amplifying the highly conserved 5' UTR region of the BHuV genome was also designed using the same software. For the detection of BKV, we used the primer pair 'UNIV-kobu-F/R' designed and published previously (Reuter and Egyed, 2009). All primers used in this study are listed in Table 1.

Reverse transcription polymerase chain reaction (RT-PCR)

The cDNA synthesis was carried out in a 25 µl final volume containing 4 µl of RNA extract, 10 mM deoxy-nucleoside triphosphate (dNTP), 2.5 µl 10 × RT buffer (50 mM Tris-HCl, pH 8.3 at 25 °C, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT), 50 ng of random hexamer, 40 U RNasin, 200 U M-MuLV Reverse-Transcriptase (Vivantis, Germany). The reverse transcription was per-

formed at 37 °C for 1 h. The PCR was conducted in a 50 µl final volume using 5 µl of the RT reaction mixture as template, along with 5 µl 10 × PCR buffer, 10 mM dNTP, 10 pmol/µl of each sense/antisense primer, and 5 U of Taq DNA Polymerase (Vivantis, Germany). The following PCR program was used: an initial step at 95 °C for 2 min followed by 40 cycles of denaturation at 94 °C for 40 s, primer annealing (49 °C for BKV, 57 °C for BHuV and 59 °C for BEV) for 30 s, and elongation at 72 °C for 1 min. The final elongation step was at 72 °C for 10 min.

Table 1

PCR primers used in this study

Primer name	Target	Sequences (5'-3')	Amplicon length	References
Entero1-F Entero1-R	5' UTR region of bovine enterovirus	GTACCTTTGTACGCCTGTT AGGATTAGCCGCATTCA	488 bp	Knowles and Mann, 1990
Entero274-F Entero564-R	5' UTR region of bovine enterovirus	TCAAGCACT**Y**CTGT**Y**TCCCCGG CTCGGAGGTT**R**GGATTAGCAGC	291 bp	This study
UNIV-kobu-F UNIV-kobu-R	3D Region of bovine kobuvirus	TGGA**Y**TACAAGTGTTTTGATGC ATGTTGTT**R**ATGATGGTGTTGA	216 bp	Reuter and Egyed, 2009
Hungaro-3D-F Hungaro-3D-R	3D Region of bovine hungarovirus	GA**Y**TATTC**K**GGATTTGATGC CAT**Y**AC**Y**GGGCGAACAAG	465 bp	Reuter et al., 2012
Hunni166-F Hunni477-R	5' UTR region of bovine hungarovirus	TCAGTCGAAGCCGCTTGGAATA GTGCTGT**W**AACACCGTGGCTTT	312 bp	This study

Sequencing and phylogenetic analysis

The PCR amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). All of the sequence outputs were aligned with use of MUSCLE algorithm.

Phylogenetic trees were reconstructed in Unipro UGENE package version 1.21. (Okonechnikov et al., 2012) using neighbour-joining tree building method and Tamura-Nei's genetic distance model with 1,000 bootstrap replicates. The sequences used in the phylogenetic analyses are listed in Tables 2, 3 and 4.

Table 2

Description of bovine kobuviruses used in the phylogenetic analysis (Fig. 2)

No.	Designation	Country	Year	Host	Accession number
1	D40	China	2010	Bovine	KF728677.1
2	GN1155	China	2010	Bovine	KF728693.1
3	B1213	China	2007	Bovine	KF728676.1
4	SZ7	China	2008	Bovine	KF728698.1
5	T0158	China	2010	Bovine	KF728700.1
6	X1-07078	China	2007	Bovine	KF728703.1
7	X3-23	China	2007	Bovine	KF728708.1
8	H18	China	2010	Bovine	KF728689.1
9	CMB02	Thailand	–	–	EF659450.1
10	U-1	Japan	–	–	NC_004421.1
11	N-2	Japan	–	Bovine	AB097157.1
12	K-38	Japan	–	Bovine	AB097157.1
13	K-49	Japan	–	Bovine	AB097159.1
14	K-3	Japan	–	Bovine	AB097152.1
15	K-44	Japan	–	Bovine	AB097158.1
16	K-60	Japan	–	–	AB097161.1
17	K-55	Japan	–	Bovine	AB097160.1
18	08KB680	South Korea	2008	Bovine	JQ026109.1
19	bovine/CPF3419/2008/Korea	South Korea	2008	Bovine	HQ234905.1
20	bovine/CPF3423/2008/Korea	South Korea	2008	Bovine	HQ234906.1
21	bovine/CPF7030/2009/Korea	South Korea	2009	Bovine	HQ234910.1
22	KB40	South Korea	2010	Bovine	HQ650176.1
23	KB8	South Korea	2008	Bovine	HQ650166.1
24	KB96	South Korea	2010	Bovine	HQ650196.1
25	BoKoV-164-BRA-BU	Brazil	2012	Bovine	KP164581.1
26	1830	Northern Ireland	2011	Bovine	KC928134.1
27	17497	Northern Ireland	2009	Bovine	KC928132.1
28	17511	Northern Ireland	2009	Bovine	KC928130.1
29	17518	Northern Ireland	2009	Bovine	KC928133.1
30	20021	Northern Ireland	2009	Bovine	KC928131.1
31	104TE-11 ITA	Italy	2011	Bovine	JX070084.1
32	106R/IT	Italy	2014	Roe deer	KP864078.1
33	109TE-11-ITA	Italy	2011	Bovine	JQ900632.1
34	118TE-11-ITA	Italy	2011	Bovine	JQ900633.1
35	21TE-11-ITA	Italy	2011	Bovine	JQ900630.1
36	81TE-11-ITA	Italy	2011	Bovine	JQ900631.1
37	8TE-11-ITA	Italy	2011	Bovine	JQ900629.1
38	95R/IT	Italy	2014	Roe deer	KP864077.1
39	HC818	UK	2013	Bovine	KR911601.1
40	HC832	UK	2013	Bovine	KR911608.1
41	SC1	UK	2013	Bovine	KT003671.1
42	SC840	UK	2013	Bovine	KR911609.1
43	SC848	UK	2013	Bovine	KR911611.1
44	SC863	UK	2013	Bovine	KR911617.1
45	AIV-BOLAT2-16-TUR	Turkey	2016	Bovine	KY695136.1
46	AIV-BOLAT8-16-TUR	Turkey	2016	Bovine	KY695137.1
47	AIV-BOLAT13-16-TUR	Turkey	2016	Bovine	KY695138.1
48	AIV-BOLAT14-16-TUR	Turkey	2016	Bovine	KY695139.1
49	AIV-BOLAT16-16-TUR	Turkey	2016	Bovine	KY695140.1
50	AIV-BOLAT33-16-TUR	Turkey	2016	Bovine	KY695141.1
51	AIV-BOLAT55-16-TUR	Turkey	2016	Bovine	KY695142.1
52	AIV-BOLAT69-16-TUR	Turkey	2016	Bovine	KY695143.1
53	AIV-BOLAT110-16-TUR	Turkey	2016	Bovine	KY695144.1
54	AIV-BOLAT112-16-TUR	Turkey	2016	Bovine	KY695145.1
55	AIV-BOLAT113-16-TUR	Turkey	2016	Bovine	KY695146.1
56	AIV-BOLAT114-16-TUR	Turkey	2016	Bovine	KY695147.1
57	AIV-BOLAT117-16-TUR	Turkey	2016	Bovine	KY695148.1
58	AIV-BOLAT125-16-TUR	Turkey	2016	Bovine	KY695149.1

Table 3

Description of hunniviruses used in the phylogenetic analysis (Fig. 3)

No.	Designation	Country	Year	Host	Accession number
1	83GR-70-RAT106	Vietnam	2012	*Rattus argentiventer*	KT944212.1
2	83GR-70-RAT130	Vietnam	2012	*Rattus argentiventer*	KT944213.1
3	05VZ-75-RAT099	Vietnam	2013	*Rattus tanezumi*	KT944214.1
4	NrHuV/NYC-E21	USA	2012	*Rattus norvegicus*	NC_025675.1
5	BHUV1/2008/HUN	Hungary	2008	Bovine	NC_018668.1
6	OHUV1/2009/HUN	Hungary	2009	Ovine	HM153767.3
7	HUV-BOLAT37-16-TUR	Turkey	2016	Bovine	KY974326.1
8	HUV-BOLAT55-16-TUR	Turkey	2016	Bovine	KY974327.1
9	HUV-BOLAT95-16-TUR	Turkey	2016	Bovine	KY974328.1
10	HUV-BOLAT89-16-TUR	Turkey	2016	Bovine	KY974329.1
11	HUV-BOLAT83-16-TUR	Turkey	2016	Bovine	KY974330.1
12	HUV-BOLAT75-16-TUR	Turkey	2016	Bovine	KY974331.1
13	HUV-BOLAT58-16-TUR	Turkey	2016	Bovine	KY974332.1
14	HUV-BOLAT1-16-TUR	Turkey	2016	Bovine	KY974333.1

Results and Discussion

The PCR screening allowed the detection of some representatives of all the three picornavirus species in the faecal samples of diarrhoeic calves tested in this study. The most prevalent was BKV, present in 29 samples (22.83%). We found seven samples (5.51%) positive for the RNA of BEV-1 by both primer sets. Interestingly, for BHuV, 6 (4.72%) and 14 samples (11.02%) were found positive by the use of the Hungaro-3D-F/R or the Hunni166-F/477-R primer sets, respectively. The overall detection results are summarised in Fig. 1.

The aim of this study was to clarify the potential role of picornaviruses (namely members of the species *Enterovirus E*, *Hunnivirus A*, and *Aichivirus B*) in clinical disease. More than one-third (33.86%) of the 127 samples collected from calves with diarrhoea was found to contain one or more of the three agents.

We designed two generic primer sets for the detection of BEVs and BHuV, based on alignments of the large sequence data available in the GenBank at present. For the detection of BEV-1, both primer pairs gave identical results in our study. However, for the detection of BHuV, the newly designed primers (Hunni166-F/477-R) that target the 5' UTR region, gave more positive results than the Hungaro-3D-F/R primer set (Reuter and Egyed, 2009). Nonetheless, for phylogenetic studies, this primer set that targets the 3D region of the virus is perhaps a better choice than the novel primer set. For screening faecal samples for the presence of BHuV, the use of our more robust primer set can be recommended.

Table 4

Description of bovine enteroviruses used in the phylogenetic analysis (Fig. 4)

No.	Designation	Country	Year	Host	Accession number
1	BEV/Thailand/E5	Thailand	2013	Bovine	KT992109.1
2	BEV/Thailand/D5	Thailand	2013	Bovine	KT992103.1
3	BEV/Thailand/E18	Thailand	2013	Bovine	KT992111.1
4	BEV/Thailand/F11	Thailand	2013	Bovine	KT992114.1
5	BEV/Thailand/F17	Thailand	2013	Bovine	KT992116.1
6	BEV/Thailand/F22	Thailand	2013	Bovine	KT992117.1
7	HY12	China	2012	Bovine	KF748290.1
8	BEV IS1/Bos taurus/JPN/1990	Japan	1990	Bovine	LC150009.1
9	BEV IS2/Bos taurus/JPN/1991	Japan	1991	Bovine	LC150010.1
10	BEV Ho12/Bos taurus/JPN/2014	Japan	2014	Bovine	LC150008.1
11	VG-5-27	–	–	–	NC_001859.1
12	Vir 404/03	–	–	–	DQ092771.1
13	D 8/01	Germany	–	–	DQ092782.1
14	E 6-82	Germany	–	–	DQ092776.1
15	SD 1182 II	Germany	–	–	DQ092784.1
16	D 14/3/96	Germany	–	–	DQ092786.1
17	D 58/96-V2130	Germany	–	–	DQ092772.1
18	VD 2860/1-99	Germany	–	–	DQ092774.1
19	56/59/1	Germany	–	–	DQ092778.1
20	Jena 38/02	Germany	–	–	DQ092788.1
21	D 3/98	Germany	–	–	DQ092790.1
22	D 14/1/96	Germany	–	–	DQ092780.1
23	RM-2	–	–	–	X79369.2
24	PA12-24791	USA	2012	Bovine	KC667561.1
25	LC-R4	USA	–	Bovine	DQ092769.1
26	PS 42	USA	–	Bovine	DQ092792.1
27	PS 83	USA	–	Bovine	DQ092793.1
28	IL/alpaca	USA	2007	Alpaca	KC748420.1
29	MexKSU/5	Mexico	2015	Bovine	KU172420.1
30	BEV/Egypt/1	Egypt	2014	Bovine	KM887136.1
31	BEV/Egypt/2	Egypt	2014	Bovine	KM887137.1
32	BEV/Egypt/3	Egypt	2014	Bovine	KM887138.1
33	BEV/Egypt/4	Egypt	2014	Bovine	KM887139.1
34	BEV/Egypt/5	Egypt	2014	Bovine	KM887140.1
35	BEV/Egypt/6	Egypt	2014	Bovine	KM887141.1
36	BEV-Bolat12-16-TUR	Turkey	2016	Bovine	MF667937.1
37	BEV-Bolat20-16-TUR	Turkey	2016	Bovine	MF667938.1
38	BEV-Bolat42-16-TUR	Turkey	2016	Bovine	MF667939.1
39	BEV-Bolat63-16-TUR	Turkey	2016	Bovine	MF667940.1
40	BEV-Bolat64-16-TUR	Turkey	2016	Bovine	MF667941.1
41	BEV-Bolat86-16-TUR	Turkey	2016	Bovine	MF667942.1

In several previous reports, it has been demonstrated that BKV can be detected from clinically healthy animals as frequently as from diarrhoeic ones (Reuter and Egyed, 2009; Jeoung et al., 2011). Some other studies have reported a higher rate of positivity in diarrhoeic calves (Barry et al., 2011; Jeoung et al., 2011; Park et al., 2011; Candido et al., 2017). BKV has been detected in 5% (2/40) of adult cattle, and in 20.9% (38/182) of diarrhoeic calf samples in Brazil

(Blas-Machado et al., 2011; Ribeiro et al., 2014). It has also been detected in 16.7% (12/72) and 6.2% of healthy cattle in Japan and in Hungary, respectively (Khamrin et al., 2008; Reuter and Egyed, 2009), as well as in 25.8% (16/62) of diarrhoeic cattle in South Korea (Park et al., 2011). On the other hand, the highest proportion of samples from diarrhoeic cattle has been found to be positive for BKV, namely 34.6% (37/107) and 77.8% (7/9) in South Korea (Park et al., 2011) and in the Netherlands (Barry et al., 2011), respectively. In our study also, BKV was detected in the highest proportion of faecal samples collected from young diarrhoeic calves up to 1 month of age.

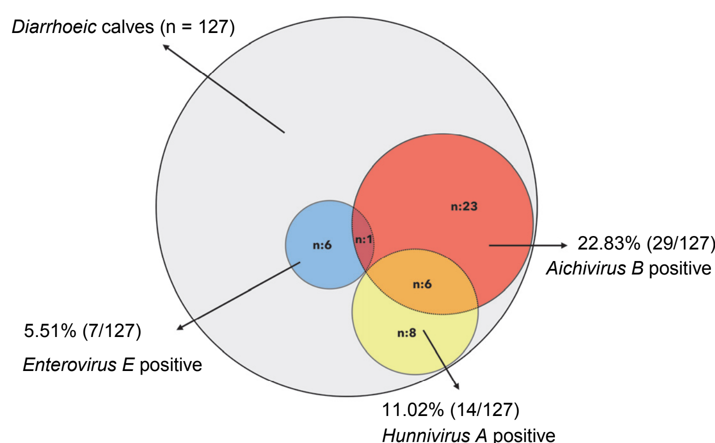

Fig. 1. Overall detection results of three picornaviral agents (*Bovine enterovirus E*, *Aichivirus B* and *Hunnivirus A*) by RT-PCR analysis

Enteric, respiratory and reproductive diseases have been described in association with BEV infections in cattle (Jiménez-Clavero et al., 2005). As the main route of transmission is the faecal-oral route, BEV has been proposed as an indicator of faecal pollution originating from animals (Ley et al., 2002; Li et al., 2012). According to a study conducted in Spain in 2004, 78.0% (78 of 100) of the tested faecal samples were BEV positive (Ley et al., 2002). In China, 24.6% of faecal samples (17/69) from diarrhoeic and healthy cattle have been found positive for the presence of BEV RNA (Li et al., 2012). Although there is no report on direct virus detection, different epizootiological studies have indicated the presence of BEV-specific antibodies in 41.8–67.7% of goats, in 3.9% of water buffalos and 64.8% of cattle, detected by virus neutralisation assay in Turkey (Acar and Gur, 2009; Gür et al., 2006; Gür et al., 2008). In this study, we detected the presence of BEV-1 RNA for the first time in Turkey.

We submitted 14 novel partial BKV sequences to the GenBank (accession numbers: KY695136–KY695149). According to the results of blast, our newly acquired sequences of the 217-nt-long fragment shared 89.4–99.0% identity

among each other, and 74.8–97.1% nucleotide identity with those previously deposited in the GenBank. Phylogenetic analysis was applied to the data and these clustered on distinct branches among the 182 other sequences retrieved from the GenBank. We furthermore limited the phylogenetic tree to 58 strains to simplify (Fig. 2), which revealed that some of them appeared close to the Brazilian sequences, while others were closer to those found in Japan or South Korea. This result indicates that a moderate level of genetic diversity exists among Turkish strains.

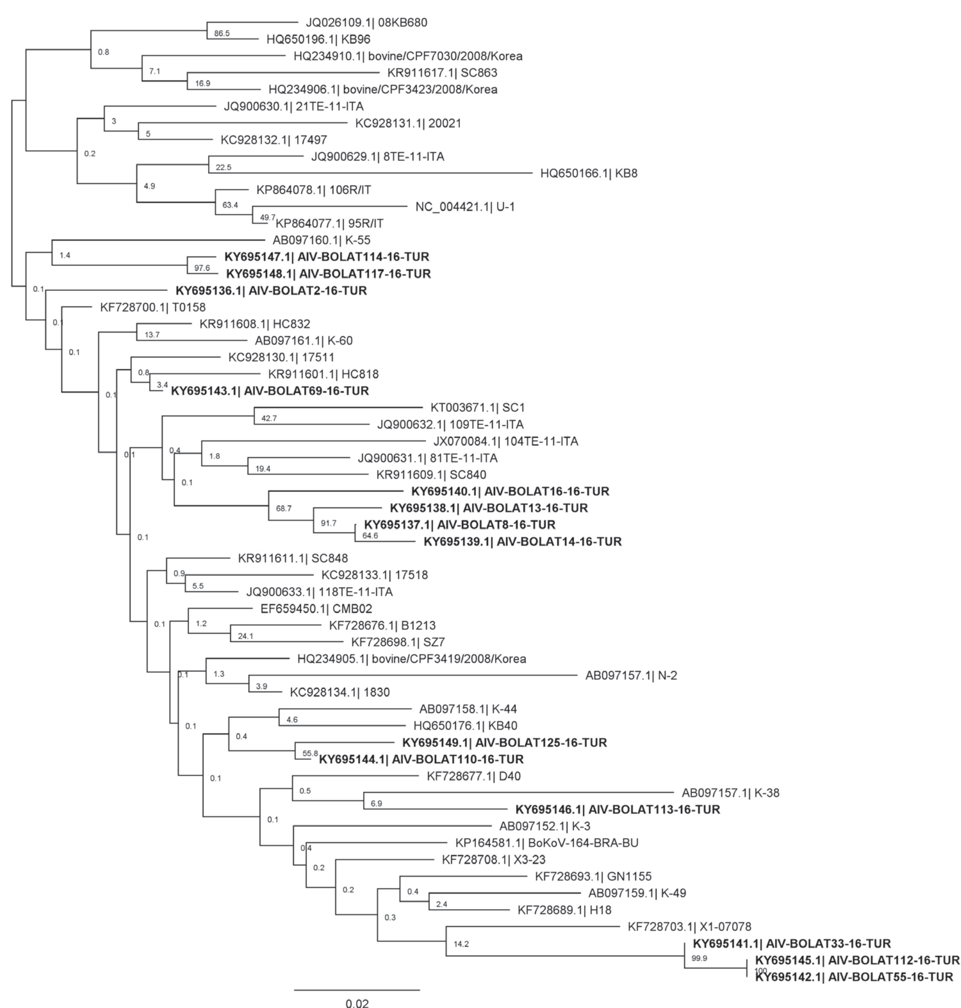

Fig. 2. Phylogenetic analysis based on the 217-bp partial nucleotide sequence of the 3D region of bovine kobuviruses (NJ tree, Tamura-Nei genetic distance model). The bootstrap values were generated with 1,000 pseudoreplicates. The name and description of viruses are listed in Table 2. Novel virus sequences, acquired in the present study, are highlighted in bold

Bovine hungarovirus (species *Hunnivirus A*) is a recently discovered virus (Reuter et al., 2012). According to the phylogenetic analysis, the eight Turkish BHuV sequences (GenBank accession numbers: KY974326–KY974333) clustered with the Hungarian bovine hungaroviruses and some them formed a separate, novel lineage (Fig. 3). Considering the 301-bp PCR fragment, the nucleotide identity among the Turkish hunnivirus sequences ranged between 76.02 and 99.02%. The identity ranged between 75.4 and 91.8% when comparing them to the Hungarian bovine hunnivirus sequence. The nucleotide identity was 75.1–82.3% between the Turkish BHuV and the Hungarian ovine sequence. The partial rat hunnivirus sequences shared 66.4 to 77.9% identity with the Turkish BHuV sequences.

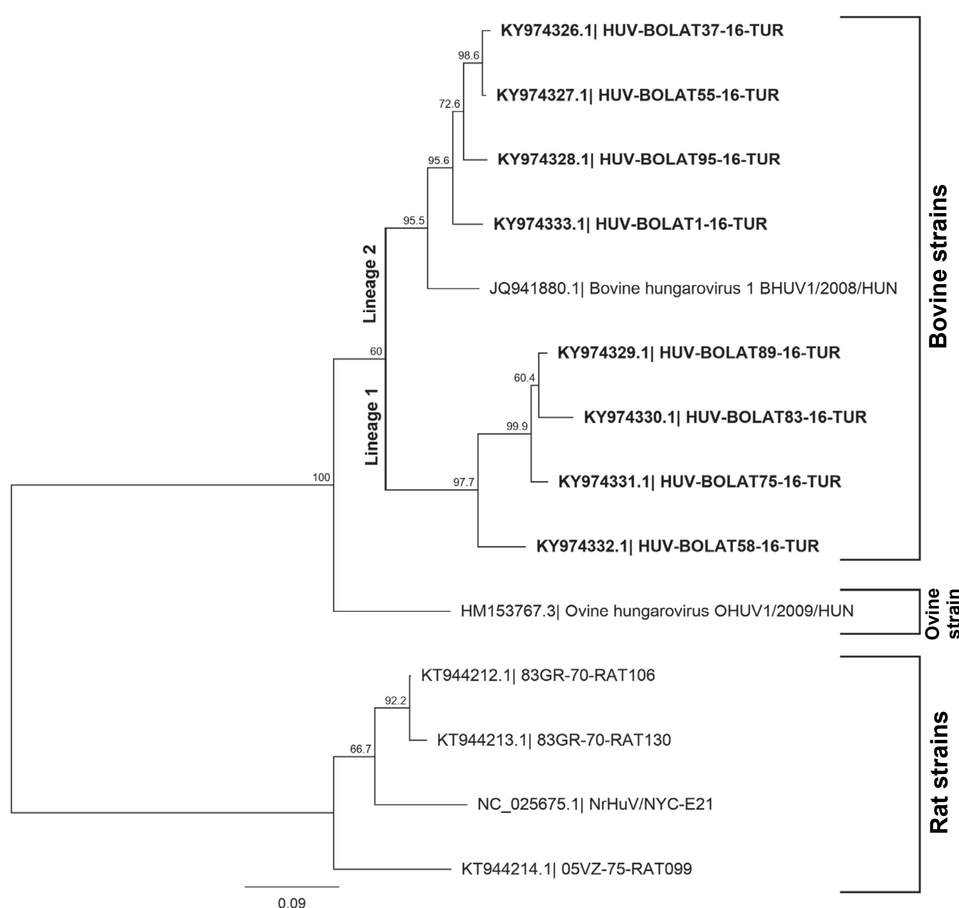

Fig. 3. Phylogenetic analysis based on the 306-bp partial nucleotide sequence of the 5' UTR region of hunniviruses (NJ tree, Tamura-Nei genetic distance model). The bootstrap values were generated with 1,000 pseudoreplicates. The name and description of viruses are listed in Table 3. Novel virus sequences, acquired in the present study, are highlighted in bold

On the other hand, the six novel BEV-1 sequences (GenBank accession numbers: MF667937–MF667942) occupied the same branch of the phylogenetic tree (Fig. 4). The nucleotide identity of the 293-bp partial sequence of the 5' UTR region was found to be 90.14–92.81% among the novel Turkish sequences, and ranged between 72.28 to 92.18% between the Turkish and other, previously sequenced fragments.

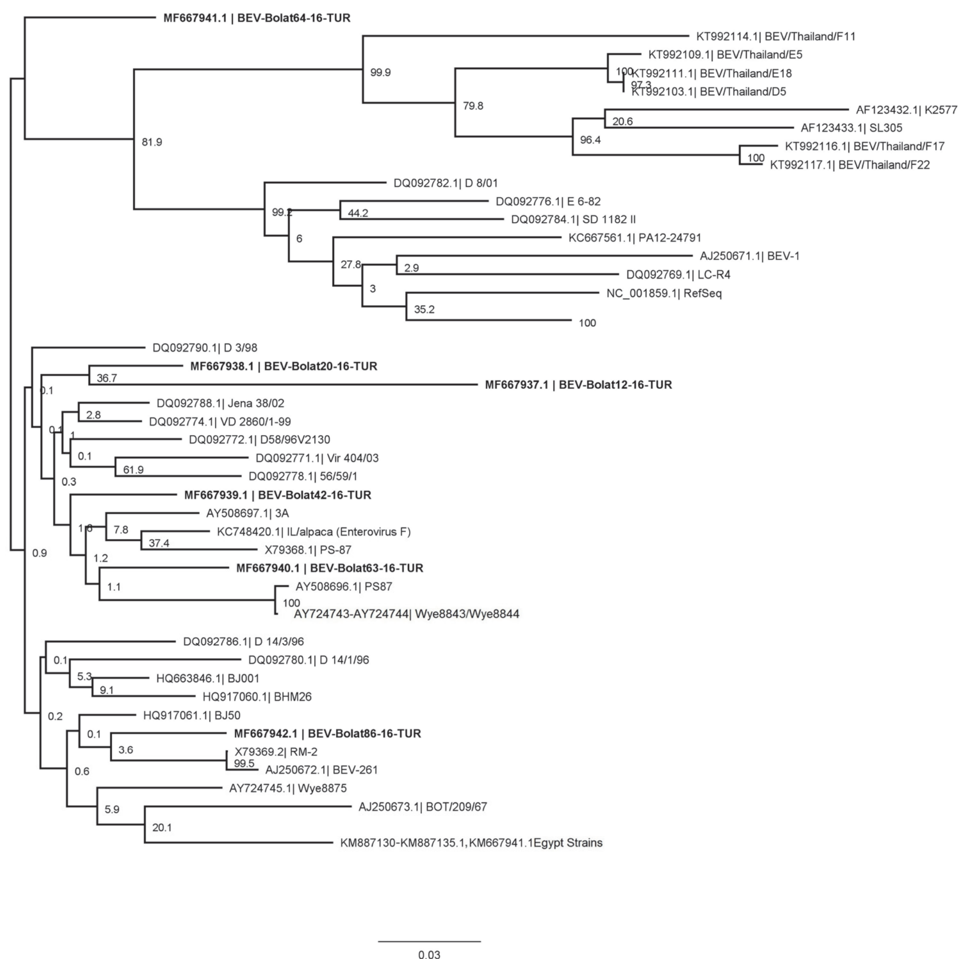

Fig. 4. Phylogenetic analysis based on the 293-bp partial nucleotide sequence of the 5' UTR region of bovine enteroviruses (NJ tree, Tamura-Nei genetic distance model). The bootstrap values were generated with 1,000 pseudoreplicates. The name and description of viruses are listed in Table 4. Novel virus sequences, acquired in the present study, are highlighted in bold

In conclusion, this study indicates that these viral agents can be detected in diarrhoeic calves in Turkey. Based on the PCR results we suggest that the Hun-

ni166-F/477-R primer set should be used instead of Hungaro3D-F/R for the detection of BHuV. The impact of BEV and BHuV in the problem of calf diarrhoea remains questionable unless experimental studies can confirm it. Nonetheless, our results provide a strong evidence for the role of BKV as an enteric pathogen that can cause diarrhoea in calves. We report here a more reliable detection tool for BHuV and also a novel lineage of BHuV. Furthermore, this is the first report on the molecular detection of these three picornaviruses in Turkey.

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