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RESEARCH ARTICLE

Molecular Detection and Characterization of Bovine Noroviruses from Cattle in Konya, Turkey

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A B S T R A C T

This study was carried out to investigate the presence of BNoV in cattle and its phylogenetic analysis. The fecal samples from a total of 80 cattle (ages 2 to 5 years old) brought to a slaughterhouse in Konya for slaughter (between 2019-2020) were collected, regardless of clinical symptoms and gender, and examined for the presence of nucleic acid by RT-PCR. Six samples out of the tested fecal samples (7.5%) were found to be positive for the presence of BNoV nucleic acid. DNA sequences were analyzed by comparing the nucleotide gene sequences specific to BNoV isolates with GIII genotype and the RdRp gene region of the virus in different regions of Turkey and the world. As a result of the phylogenetic analysis in line with the data obtained, it was determined that the virus genotype circulating in the sampling region was included in the GIII.2 cluster-2. It can be concluded that the BnoV infection exists in the country, therefore, it can be stated that protection and control measures should be applied within the scope of the struggle.

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INTRODUCTION

Caliciviruses belonging to the *Caliciviridae* family are non-enveloped, single-stranded RNA viruses with a positive-stranded genome of 7.4-8.3kb. Based on genomic organization and genetic analysis, *Caliciviridae* has been divided into at least four genera: These are *Vesivirus, Lagovirus, Norovirus (NoV)* and *Sapovirus* (Lee *et al.*, 2019). The *Norovirus* and *Sapovirus* genera include several viruses that primarily cause enteric diseases in other animals, such as the Murine and Canine noroviruses, as well as the human enteric viruses of the same name (Robilotti *et al.*, 2015).

Noroviruses, the most common etiologic agent of acute gastroenteritis, responsible for approximately 20% of all acute gastroenteritis cases worldwide (Nordgren and Svensson, 2019).

The viral genome encodes two capsid proteins and six nonstructural proteins regulated in the ORF 1-3 framework (Woodward *et al.*, 2017). ORF-1 encodes a large polyprotein that is cleaved into 6 nonstructural proteins (N-terminal-NTPase-3A-like-VPg3C-like polymerase). ORF-2 encodes the main capsid protein, VP1 (Shi *et al.*, 2019), while ORF2 is translated as VP1, a 55-60kDa protein involved in self-assembly and capsid formation, receptor recognition, host specificity, lineage antigenic diversity, and immunogenicity (Di Felice *et al.*, 2016). ORF 3 encodes a small structural protein, VP2 (Shi *et al.*, 2019). There is tremendous variation within and between strains, with six broad genotypes (GI-VI) defined based on the amino acid sequence of the VP1 capsid protein (differing by as much as 38% between GI and GII isolates) (Woodward *et al.*, 2017). VP2, which is not required for virion formation in Noroviruses, is mainly responsible for virion stability and genome encapsulation. Furthermore, VP2 interacts with VP1 and in turn increases the expression of capsid proteins (Mohamed *et al.*, 2018). In the Murine norovirus (MuNoV) genome, ORF4 produces virulence factor 1 (VF1), which regulates the innate immune response (Di Felice *et al.*, 2016). ORF 1 is translated as a large polyprotein of 1740 amino acids (aa), which is cleaved by the viral protease (3CLPro) to encode 6 nonstructural (NS) proteins. N-terminal nonstructural protein NS1-2 (p48), NS3 nucleoside triphosphatase (NTPase)/RNA helicase, NS4 protein (p22), NS5 protein (VPg), NS6 protease (3CLPro) and NS7 RNA-dependent RNA polymerase (RdRp), respectively are copied from the 5' end to the 3'end of the

ORF-1 (Di Felice *et al.*, 2016). Phylogenetic studies based on a partial coding regions for the RNA-dependent RNA polymerase (RdRp) and partial capsid (VP1) (Mohamed *et al.*, 2018).

Neonatal calf diarrhea is one of the most important issues that the livestock industry should consider and take the necessary precautions worldwide. Although neonatal calf diarrhea affects both feeder cattle and dairy cattle, it is of particular importance in dairy farming as it is the main cause of death in calves before weaning (Castells *et al.*, 2020). Considering the morbidity and mortality records related to calf diarrhea, it is known that it causes significant economic losses in the cattle industry worldwide (Di Felice *et al.*, 2016). Deaths in the first 1 month of age constitute 80-85% of the total, and this occurs mostly in the 3rd week. These neonatal causes of death have been reported to be caused by parasitic and bacterial infections as well as viral agents. *Bovine rotavirus*, Bovine coronavirus, and Bovine viral diarrhea virus have been identified as the main diarrhea-causing viruses. However, there is little interest in *Neboviruses* and *Noroviruses* for calf diarrhea (Guo *et al.*, 2018).

This study was carried out to obtain the first data on BNoV infection in cattle in Konya region, to determine the molecular characterization of the virus circulating in our region by phylogenetic analysis.

MATERIALS AND METHODS

For this study, fecal samples were collected from 80 cattle aged between 2 and 5 years, brought for slaughter in a special meat and meat products integrated facilities in Konya, regardless of clinical signs and gender.

Preparation of fecal samples: Fecal samples were diluted 10% with PBS and centrifuged at 4000rpm for 15 min. The obtained supernatant was filtered through 0.22µm pore diameter cellulose acetate filters and transferred to 1ml polystyrene tubes and used for RNA extraction.

Molecular studies: Conventional Nested reverse transcriptase-polymerase chain reaction (Nested RT-PCR) method was used to determine the presence of BNoV. Commercially available kits were used for PCR analyzes and RNA extraction processes. PCR was performed by sequencing the primer pair selected from the conserved gene region (RdRp) specific for BNoV (Table 1).

RNA extraction: For this purpose, RNA extraction of *Norovirus* was performed as per the manufacturer's instructions (QIAamp Viral RNA Mini Kit, Cat. No: 52906, Germany).

Nested RT-PCR: ABM MegaFI one-step rt-PCR kit was used in the 1st step of the reaction for the detection of BNoV Nested RT-PCR, which is a two-step method. 50µl reaction mixture: 25µl 2X One-Step RT-PCR Buffer, RT-PCR enzyme mix 4µl, 10ppm forward (F) primer and reverse (R) primer 2µl, RNase free water 12µl were prepared, 5µl of RNA was added to this mixture and the total mixture was made up to 50µl. The prepared reaction mixtures were introduced into the 1st PCR cycle as described below.

cDNA synthesis was performed at 60◦C 15 min, initial denaturation 98◦C 30 sec, denaturation 98◦C 10 sec, annealing 52◦C 30 sec extension 72◦C 30 sec was carried out for reaction of 30 cycles, the final elongation was performed at 72 ◦C for 2 min and the 1st step PCR process was terminated.

For the 2nd PCR cycle process, 1st PCR products were used. The Qiagen Taq PCR Master Mix Kit was used for this purpose. The reaction mixtures and thermal cycle were applied following the optimal intervals determined by the manufacturer.

50µl reaction mixture: 25µl 2x Taq PCR Master Mix, 2µl F primer, 2µl R primer, 18µl nuclease-free water and 3µl of 1st step PCR products were added to each of the mixtures. At the end of the mixing, the samples were placed on the thermal cycler and the 2nd reaction of the Nested PCR method was carried out as described below.

Initial denaturation was performed at 94◦C 3 min, denaturation 94◦C 45 sec, annealing 52◦C 50 sec extension 72◦C 45 sec were carried out for reaction of 30 cycles, the final elongation was performed at 72◦C for 2 min and the 2nd step PCR process was terminated.

At the end of the reaction, PCR products were transferred to agarose gel prepared with 2% 1x TBE buffer containing Ethidium Bromide and subjected to electrophoresis under 90volts for 50 min. At the end of the process, the products were examined in a gel imaging device (UVP Inc., Upland CA, USA).

Sequencing operations and phylogenetic analysis: The samples that were considered positive for the presence of BNoV nucleic acid were sequenced by the PCR method. The obtained raw DNA sequences were processed with the Aliview sequencing program. The processed data were analyzed by comparing the nucleotide gene sequences specific to BNoV isolates with GIII genotype in different regions of Turkey and the world, and a 286bp part of the RdRp gene zone of the virus. In line with this objective, the phylogenetic tree was formed using the p-distance nucleotide model making 1000 repetitions of bootstrap in MEGA 7 software.

This study was carried out with the approval of the local ethics committee of SÜVETFAK, dated 27.03.2020 and numbered 2020/31.

RESULTS

In PCR analyzes, detection of Nested PCR 1st step 532 bp and Nested PCR 2nd step 326bp DNA segment specific to the gene region encoding RdRp was investigated. With the 2nd PCR reaction, 326 bp gene segments in 6 samples (7.5%) were visualized on the gel imaging device (Fig. 1).

Phylogenetic analysis results: In the created phylogenetic tree, it was determined that 3 study samples marked with black square were included in the BoNV GIII.2 cluster. Besides, it was determined that field isolates, which were found to be closely related to Belgium (EU794907) and Turkey (KF218822) isolates, were placed in a sub-cluster in the phylogenetic tree, including isolates from Italy (KC896784), France (FJ974134), Tunisia (JN418491 and Belgium (EU877974) (Fig. 2).

Table 1: Primer pair sequences used for BNoV.

Primer direction	Sequence (5'-3')	Zone	Product (bp)	Source
Forward	AGTTAYTTTTCCTTYTAYGGBGA	4543-5074	532	Smiley *et al.* 2003
Reverse	AGTGTCTCTGTCAGTCATCTTCAT	4543-5074	532	Smiley *et al.* 2003
nForward	GTCGACGGYCTKGTSTTCCT	4690-5015	326	Park *et al.* 2007
nReverse	CACAGCGACAAATCATGAAA	4690-5015	326	Park *et al.* 2007

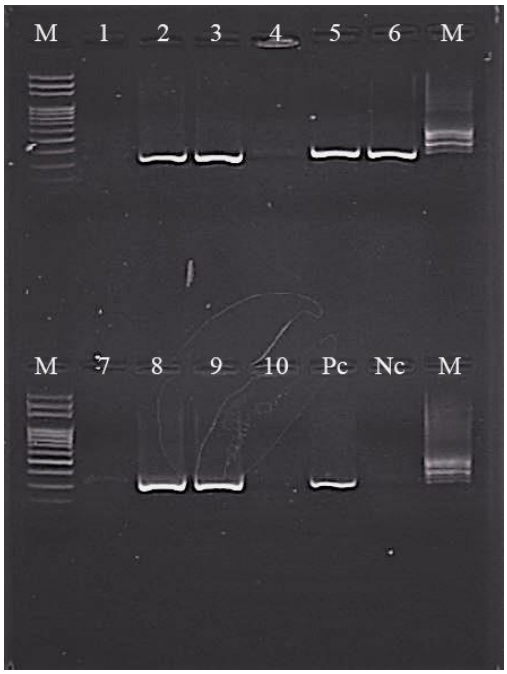

Fig. 1: Nested PCR 2. Step Results Samples that gave bands in 326 bp regions were considered positive. M: Marker 100bp. 1-10 sample sequence. PC: Positive control. NC: Negative control.

DISCUSSION

Based on the genetic divergence in the capsid genes and RNA-dependent RNA Polymerase (RdRp), the NoVs have been phylogenetically, noroviruses (NoVs) are divided into five genogroups (GI–GV) (Park *et al.*, 2007a). While those that infect humans are GI, GII, and GIV NoVs, animals are mostly infected by the genogroups GII (pigs), GIII (ruminants), GIV (lions and dogs), and GV (mice) (Scipioni *et al.*, 2008b).

In this study, fecal samples were taken from 80 animals aged between 2 and 5 years in a slaughterhouse in Konya, regardless of clinical signs and gender, and the presence of BNoV was investigated by the Nested PCR method and sequence analysis of the partial RdRp fragment of samples determined positive for BNoV was compared with previously reported reference strains. In the phylogenetic tree formed from the samples determined positive by Nested PCR (Fig. 1), it was determined that the samples belonging to the current study were included in the BNoV GIII.2 cluster (Fig. 2).

In recent years, the importance of BNoV as an etiological agent of diarrhea in calves has increased. Therefore, many researchers have focused on clinical results of infection and phylogenetic analysis of BNoVs (Deng *et al.*, 2003; Milnes *et al.*, 2007). The presence, incidence, and molecular characterization of BNoV have been demonstrated by various studies conducted all over the world (Deng *et al.*, 2003; Park *et al.*, 2008; Mauroy *et al.*, 2009; Di Bartolo *et al.*, 2011; Gomez and Weese, 2017; Mohamed *et al.*, 2018; Wang *et al.*, 2019). On the other hand, it is thought that the data on BNoV will increase over time in Turkey (Yilmaz *et al.*, 2011; Gülaçti *et al.*, 2016; Turan *et al.*, 2018; Karayel-Hacioglu and Alkan, 2019).

The prevalence of *norovirus* in cattle varies according to the countries and times of the study. In the study conducted by Smiley *et al.* (2003) in the US, they determined 47 (18%) as positive by RT-PCR from the fecal samples collected from 260 calves aged 0-6 weeks, while Mauroy *et al.* (2009) examined a total of 133 samples, including 74 fecal samples from animals with diarrhea symptoms and 59 necropsy material from animals with gastroenteritis lesion with necropsy, aged between 1 week and 6 months in Belgium, with one-step RT-PCR, they found a positive rate of 7.5%. In a study Deng *et al.* (2003) determined 34 (8.9%) of 381 animals with diarrhea symptoms, aged 1-4 weeks, to be positive when they were examined by ELISA, while Machnowska *et al.* (2014) collected 120 fecal samples from feeder pigs (6 to 9 months old) and determined positivity in 17 (14.2%) samples by real-time RT-PCR. When Park *et al.* (2008) examined 629 Korean domestic cattle fecal, aged between 3 and 70 days, by RT-PCR and nested PCR, they found that 9 were positive at stages 1 and 59 (9.3%) at stage 2. Yilmaz *et al.* (2011) examined the fecal of 70 diarrheal animals aged 1 to 60 days in the Marmara region by real-time RT-PCR and determined 6 (8.5%) as positive. In the study by Turan *et al.* (2018), fecal samples were collected from 127 diarrheal cattle aged between 1-30 days from three cities in the central region of Turkey. They found BNoV positive in five samples (3.93%) by RT-PCR. In the phylogenetic analysis, they reported that this new *norovirus* Turkish strain belongs to genotype III.2. Karayel-Hacioglu and Alkan (2019) collected fecal samples from 167 diarrheal animals aged 1 day to 7 months and reported that 56 (33.5%) were positive for BNoV. Also, by sequence analysis of the RdRp and capsid gene of BNoVs, they stated that the GIII.1 and GIII.2 genotypes were circulating in Turkey. They added that both of the BNoV strains are recombinant strains (GIII.1/GIII.2). In this study, the BNoV positivity rate was determined as 7.5%. When the determined positivity rate was compared with other study data, it was determined that the positivity rates obtained varied according to the age, clinical signs, and diagnostic method of the animals sampled.

Studies have shown that calves can shed virus between 1 and 4 weeks, but this shedding is not strongly associated with diarrhea symptoms (Otto *et al.*, 2011). Although the animals in this study were sampled regardless of age, all animals were over 2 years old and had no diarrhea symptoms. In line with the results obtained, 7.5% positivity supports that diarrhea symptom is not obligatory in BNoV shedding.

Due to the specificity, sensitivity, speed, and low risk of cross-contamination of the RT-PCR method, it is recommended to be used for the determination of noroviruses in the fecal, especially during periods when virus shedding is high (Mauroy *et al.*, 2009). When Mauroy

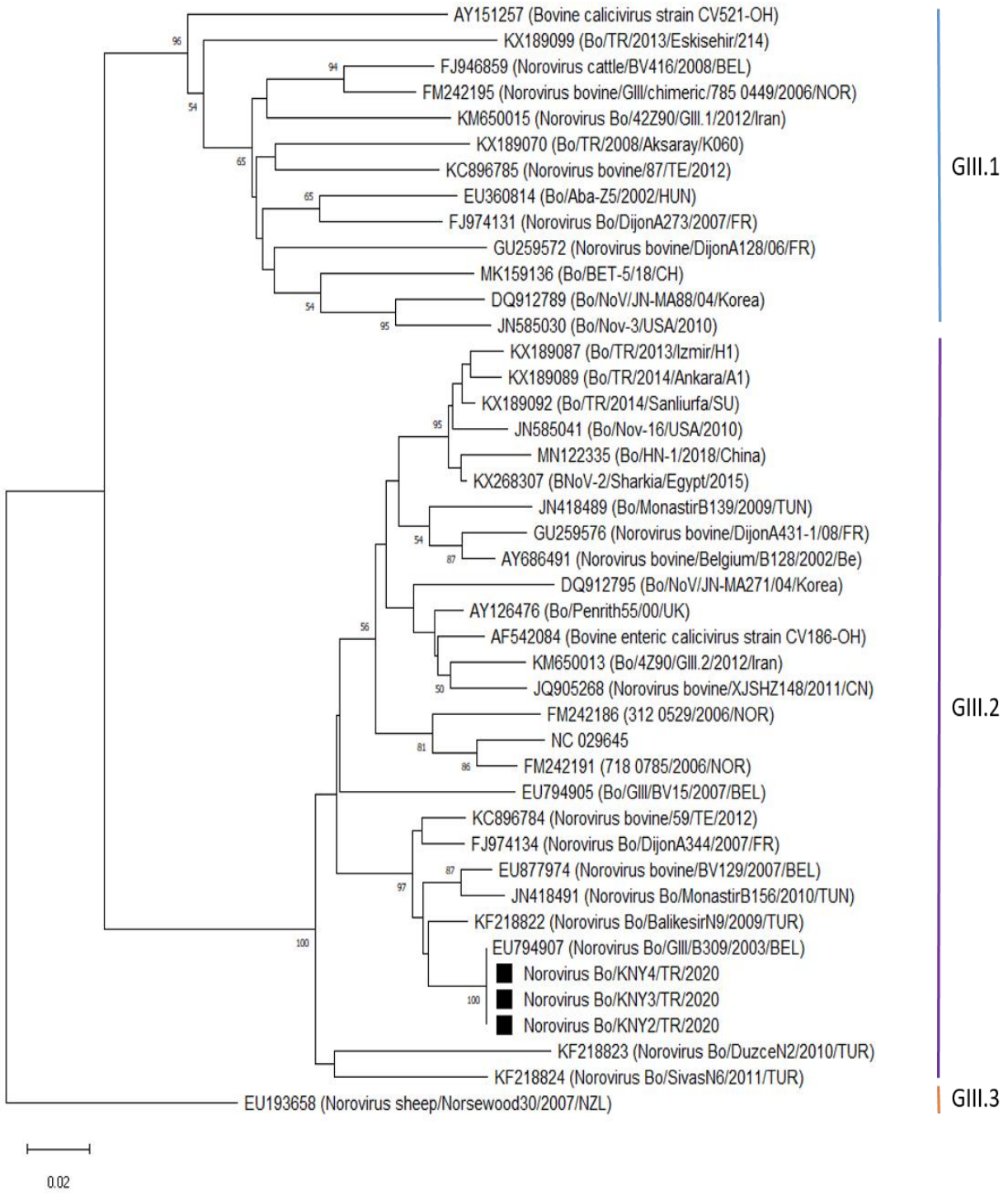

Fig. 2: Phylogenetic analysis of BoNV GIII. nucleotide sequences obtained from the Konya region. Phylogenetic tree including Turkish isolates and nucleotide sequences selected from different countries. Those marked with * are the GIII.2 sequences obtained in this study.

et al. (2009) examined the fecal samples collected from 133 calves with diarrhea by RT-PCR for norovirus, they determined that 10 (13.4%) of them were positive. In their study, Milnes *et al.* (2007) determined that 44 (11.1%) of 398 bovine fecal samples from the Belgian diagnostic laboratory were positive. Since BNoV Genotypes 1 and 2 are antigenically different from each other, (Oliver *et al.*, 2006a), the actual prevalence of BNoV may be higher or a preferential geographic distribution may exist. Conversely, while Mattison *et al.* (2007) found only 1.6% positive in fecal samples from a study in Canadian cattle, Smiley *et al.* (2003) found that up to 72% of samples on two farms in the USA were positive. In a study conducted in the Netherlands, prevalence's of 31.6% and 4.2% were determined in fecal samples from farm animals and individual samples of dairy cattle, respectively. In this study, animals between the ages of 2-5 years in Konya were sampled regardless of clinical signs, and 7.5% positivity was found. Therefore, prevalence results with RT-PCR have been reported to vary depending on the continent, country, sampling strategy, age, clinical symptoms, and especially test conditions (Scipioni *et al.*, 2008a).

Bovine Norovirus GIII.2 genogroup is known as the most common genogroup worldwide. In a study by

molecular analysis of partial RdRp gene sequences, Karayel-Hacioglu and Alkan (2019) indicated that out of 34 BNoV strains, 9 clustered in GIII.1, while the remaining 25 BNoV strains clustered in GIII.2. They reported that the samples for which sequence analysis was performed showed nucleotide (nt) similarity with each other at a rate of 69.5-100%. While they reported that 9 BNoV strains clustered in GIII.1 showed 81.4-90.2% nt sequence identity with Bo/Jena/80/DE, the remaining 25 BNoV clustered in GIII.2 showed 84.2-91.9% nt sequence identity of Bo/Newbury2/76/UK. In the study conducted by Turan *et al.* (2018), two norovirus strains Bo/NoV/Bolat7/2016/TR and Bo/NoV/Bolat85/2016/TR were matched with 96.94% similarity with Bo/November-6/USA/2010 and Bo/ Nov-45/USA/2010 strains previously reported in the USA. The other Bo/November-1, -2, -10, -13, and -16 intersect at the same root. These new strains were reported to belong to the GIII.2 genogroup when compared with reference strains. It was reported that the new strain showed the highest similarity with 90.80% with Bo/Balıkesir/N9/2009/TUR strain and 88.65% with Bo/SivasN6/2011/TUR, 87.42% with Bo/DuzceN2/2010/TUR and 86.81% with Bo/AdıyamanN6/2011/TUR strain. In the study conducted by Gülaçti *et al.* (2016), it was stated that there was 86.3% to 90.1% nt and 96.8% to 98.9% amino acid similarity among Turkish BNoVs. They found Turkish Bo/BalikesirN9/2009/TUR strain similar with 96.1-97.5% nt and 100% aa to Italian and Tunisian strains. At the end of this study, it was determined that the samples were included in the BoNV GIII.2 cluster. Besides, it was determined that field isolates, which were found to be closely related to Belgium and Turkey isolates, were placed in a sub-cluster in the phylogenetic tree, including isolates from Italy, France, Tunisia, and Belgium (Fig. 2).

Due to the limited duration of acquired immunity and cross-protection between different strains, more than one norovirus infection may occur during a lifetime (Parra, 2019). Sherwood *et al.* (2020) demonstrated cross-protection of the vaccine against heterotypic strains in the Takeda bivalent GI.1/GII.4 VLP vaccine trials, it has not been demonstrated that vaccine candidates provide cross-protection against different variants and genotypes (Oliver *et al.* 2006b; Cates *et al.*, 2020) revealed that Jena and Newbury2 share at least one cross-reactive epitope (CM39), although they correspond to two different antigenic types or serotypes. However, they stated that this does not provide a significant degree of cross-protection. Hence, there is a need for 2-way cross-protection studies among these sub-genogroups.

Conclusions: The presence of 7.5% BNoV in cattle in Konya was determined, while the virus circulating in this region was determined to belong to the BNoV (GIII-2) genotype. The 1st year of life is critical for BNoV infection. In this period, the immunological status of cattle against BNoV changes according to both colostrum intake and natural infection. The fact that BNoVs were detected in this study both in animals aged 2 years and older and in animals without clinical signs of diarrhea shows how important the infection is, although this has been overlooked. Therefore, BNoV infections should also be considered in the development of control strategies for calf losses. With this study, it was revealed once again that clinical signs are not necessary for virus shedding. However, sampling to be carried out on animals of different age groups in a wider area is needed for detailed results. More comprehensive and advanced studies are needed to fully determine the epidemiology, clinical findings, and molecular characterization of norovirus infections in calves and cattle in Turkey. It is thought that the data obtained as a result of this study will contribute to the protection and control strategies against this infection, as well as shedding light on the studies planned to be done in the future.

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Authors contribution: Motivation/Concept: ID, OB, OA; Design: ID, OA, OB, MH; Control/Supervision: MH, OB, ZB; Data Collection and / or Processing: ID, HSP, HPA; Analysis and / or Interpretation: ID, HSP, HPA; Writing the Article: ID, OA, OB and Critical Review: ID, OB, OA.

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