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An Agent Forgotten in the Diagnosis of Calf Diarrhea: Bovine Noroviruses and Molecular Characterization

Buzağı İshallerinde Teşhiste Unutulan Bir Etken: Sığırların Norovirusları ve Moleküler Karakterizasyonu

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ABSTRACT

The role of viruses in the diarrhea algorithm is very important. In addition, losses due to diarrhea are quite high for calves, and herd control and protection cannot be performed in cases where a rapid etiological diagnosis cannot be made. Group A rotaviruses and coronaviruses, which are the main viral etiological agents of enteric diseases in calves, are frequently detected in these infections. However, the presence/prevalence of other agents in single or mixed infections is still up-to-date and under investigation. Two genetically distinct bovine enteric caliciviruses are known: genogroup III noroviruses (NoVsGIII), which are genetically related to human noroviruses, and neboviruses, which represent a new genus of caliciviruses. In this study, it was aimed to elucidate the role and genotypes of noroviruses (bovine norovirus) in the diarrheal paradigm in calves. In order to determine the presence of norovirus and genogroups, a total of 92 diarrheal stools from calves were included in the study from the collection material in our laboratory. In the study, reverse transcription nested polymerase chain reaction was performed with specific primer pairs. Polymerase chain reaction (PCR) test results were positive in 3 samples (3/92, 3.2%) and were subjected to sequence analysis. According to the results of bioinformatics analysis, noroviruses are divided into 7 main genogroups in light of recent literature. Among these genogroups, bovine noroviruses were included in genogroup III (GIII). There are also subtypes within GIII. Of the 3 strains identified in our study, 2 were grouped in GIII-2a (TR/ERZ/25 and TR/ELZ/23) and 1 strain was grouped in GIII-2b (TR/ERZ/9). Conducting more comprehensive molecular prevalence studies on noroviruses, focusing on the detected genogroups in vaccine studies to be developed and determining their prevalence, the effects on the pathogenesis and clinical picture should be revealed. Determination of genogroup type profiles in the world and in our country and, if vaccines can be developed, elucidating different genogroup types on protection will be beneficial in diarrhea and norovirus dilemmas.

Keywords: Calf, diarrhea, genogroup, norovirus, Türkiye

ÖZ

İshal algoritması içerisinde virusların rolü oldukça önemlidir. Ayrıca buzağılar için; ishallere bağlı kayıplar oldukça fazla olmakta ve hızlı bir etiyolojik teşhis yapılamadığı durumlarda sürü kontrolü ve korunması gerçekleştirilememektedir. Buzağılarda enterik hastalıkların ana viral etiyolojik ajanlarından olan grup A rotaviruslar ve coronaviruslar bu enfeksiyonlarda sıklıkla tespit edilmektedir. Ancak tekli veya miks enfeksiyonlarda başka etkenlerin varlığı/yaygınlığı da güncelliğini korumakta ve araştırılmaktadır. Genetik olarak farklı iki sığır enterik calicivirusu bilinmektedir: Bunlar genetik olarak insan norovirusları ile ilişkili olan genogrup III norovirusları (NoVsGIII) ve yeni bir calicivirus cinsini temsil eden neboviruslardır. Bu çalışmada buzağılarda ishal paradigması içerinde norovirusların [bovine norovirus (BNoV)] rolü ve genotiplerinin aydınlatılması amaçlanmıştır. Norovirus varlığı ve genogruplarını belirlemek için ishalli buzağılardan toplam 92 adet dışkı örneği çalışmaya dahil edildi. BNoV spesifik nested primerleri kullanılarak RT-nested-PCR gerçekleştirildi. Polymerase chain reaction sonucu 3 örnekte (3/92, %3.2) pozitiflik tespit edildi ve sekans analizine tabi tutuldu. Biyoinformatik analiz sonuçlarına göre son literatürler ışığında norovirüslar 7 ana genogruba ayrılmaktadır. Bu genogruplar içerisinde de sığır norovirusları genogrup III (GIII) içinde yer almıştır. GIII içerisinde ayrıca alt tipler bulunmaktadır. Çalışmamızda tespit edilen 3

suştan 2'si GIII-2a (TR/ERZ/25 ve TR/ELZ/23) ve 1 suş GIII-2b (TR/ERZ/9) içerisinde gruplanmıştır. Noroviruslar üzerine daha kapsamlı moleküler prevalans çalışması yapılarak yaygınlığının ortaya konulması ve mücadele için geliştirilecek aşı çalışmalarında, tespit edilen genogruplar üzerine yoğunlaşılarak; patogenez ve klinik tablo üzerine etkilerinin ortaya konulması gerekmektedir. Dünyada ve ülkemizde genogrup tip profillerinin belirlenmesi ve eğer aşılar geliştirilebilirse koruyuculuk üzerine farklı genogrup çeşitlerinin aydınlatılması, ishal ve norovirus ikileminde fayda sağlayacaktır.

Anahtar Kelimeler: Buzağı, ishal, genogrup, norovirus, Türkiye

INTRODUCTION

Calf diarrhea causes serious economic losses worldwide.[1] This situation creates a significant economic problem for cattle farms. The primary source of profitability on a cattle farm is not acquiring and selling milk but acquiring and selling calves.[2,3] There are many reports about calf diarrhea in our country. Different viral agents, including rotavirus[4-6] and coronavirus[7], have been detected in diarrhea cases rather than factors forgotten in the diagnosis, such as noroviruses. Diarrhea caused by bovine norovirus (BNoV) is seen in cattle alone or in combination with other viral enteropathogens.[8] There are 11 genera in the *Caliciviridae* family. These genera are *Norovirus, Nebovirus, Sapovirus, Lagovirus, Vesivirus, Bavovirus, Minovirus, Nacovirus, Recovirus, Salovirus*, and *Valovirus*. Bovine norovirus belongs to the Norovirus genus (NoV) and is a nonenveloped, positive single-stranded ribonucleic acid (RNA) virus. The viral genome is 7.3 to 7.5 kb in size and consists of 3 open reading frames (ORF1, ORF2, and ORF3). Open reading frames 1 encodes a large polyprotein that is posttranslationally and cotranslationally cleaved by a virus-encoded protease (NS6), producing 6 nonstructural proteins: NS1.2, NS3 (NTPase), NS4, NS5 (VPg), NS6 (Protease), and NS7 (RNA-dependent RNA polymerase, RdRp). Open reading frames 2 encodes the major capsid protein (VP1), and ORF3 encodes the minor capsid protein (VP2). Noroviruses are classified into 10 genogroups (GI-GX) based on the phylogenetic analysis of amino acid sequences encoded by the VP1 gene. Of these, GI, GII, and GIV infect humans, while GIII infects cattle and sheep. BNoVs are classified in genogroup III (GIII), and this genogroup is divided into 4 different genotypes (GIII.1, GIII.2, GIII.3, and GIII.4).[9-11] Although GIII.2 strains are the most common variant worldwide, GIII.1 strains are reported to be more virulent than GIII.2 strains. In 2009, a new NoV strain was detected in sheep feces, and this strain was named GIII.3.[12,13] Recombination occurs frequently in caliciviruses within or between genogroups, and this is considered an important mechanism in the continuous emergence of new variants.[9,10,14] Today, polymerase chain reaction (PCR), genomic sequencing, and bioinformatics tools are frequently used to understand the phylogenetic relationships between viruses and to characterize viruses.

Analysis of partial capsid (VP1) or RNA-dependent RNA polymerase (RdRp) gene sequences is routinely used in NoV genotyping, and genotyping is based on data from these regions.[15] However, although the recombination event in NoVs can also occur in different gene regions, errors may occur in epidemiological data due to the fact that it commonly occurs at the ORF1–ORF2 junction. In other words, the genotype may be similar to a different norovirus strain in the analysis for ORF1, and it may be similar to a different genotype in the phylogenetic analysis for ORF2.[16-17] It has been reported that an epidemiological data and naming system that includes both polymerase and capsid regions would be more appropriate to eliminate inconsistencies in NoV genotyping.[18] The inability to adapt NoVs to cell culture systems prevents the pathogenesis of the virus from being fully understood. Rotavirus (BRoV) and coronavirus (BCoV) are frequently investigated as viral etiological agents in calf diarrhea, and other infectious agents are ignored as coinfections.[19,20] The fact that NoV is not yet included in routine diagnostic activities used in calf diarrhea results in the inability to fully elucidate the role of BNoV in cattle farms.[10,15] Although BNoV is seen worldwide, it is an overlooked agent in routine diagnostic activities, and there are limited studies on BNoV in Türkiye.[12,21-24] In this study, it was aimed to examine the presence of BNoV in calves with diarrhea and make molecular characterizations of these viruses.

MATERIALS AND METHODS

Samples and Viral RNA Isolation

Ethical committee approval was received from the Unit Ethics Committee of Atatürk University (Date: 26.01.2023, Number: 2023/02). A total of 92 stool samples from calves with diarrhea between 0 and 1 year of age were included in the study. Stool samples were transported to the laboratory immediately after sampling and kept at −80°C until RNA isolation. Stool samples were diluted 1 : 10 with 1 M phosphate buffered saline and centrifuged at 5000 rpm for 5 minutes. After centrifugation, 300 µL of supernatant was isolated using the GF-1 Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia), and the resulting suspension was kept at −80°C until use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used as prescribed by the company for the synthesis of complementary DNA (cDNA) from the obtained RNAs. For PCRs with BNoV RdRp gene-specific primer pairs, first round forward (5′-AGTTAYTTTTCCTTYTAYGGBGA-3′), Reverse (5′-AGTGTCTCTGTCAGTCATCTTCAT-3′) primer and second round nestedF (5′-GTCGACGGYCTKGTSTTCCT-3′) and nestedR (5′-CACAGCGACAAATCATGAAA-3′) were used[25]. As a result of PCR, amplicons with product sizes of 532 bp and 326 bp were evaluated as BNoV-positive.

Sequence and Phylogenetic Analyses

Bidirectional sequence analyses of BNoV amplicons were performed by sanger sequencing. Bioinformatics analyses of the obtained raw data were performed. Partial sequences of reference RdRp genes were compared with other norovirus sequences provided by the National Center for Biotechnology Information (NCBI: https://www.ncbi.nlm.nih.gov/). Phylogenetic trees were created using BioEdit version 7.2.5[26] and MEGA version 11[27] bioinformatics programs. The phylogenetic tree was constructed using 1000 bootstrap datasets (replication value, repeat value) based on the maximum likelihood method and the clustalW algorithm. Phylogenetic distance values were calculated using the Kimura 2 parameter model.

RESULTS

Open reading frame 1 encodes viral nonstructural proteins, including RNA-dependent RNA polymerase (RdRp). RdRp is a key enzyme responsible for transcription, replication of the viral genome, and the correct initiation of RNA synthesis, which is necessary to prevent the loss of viral genetic information.[15] As a result of PCR analysis performed by targeting the BNoV partial RdRp gene region, positivity was detected in 3 (3/92, 3.2%) of 92 stool samples, and the minimum infection rate (MIR) was detected at 32,60. The nucleotide sequences of the BNoV positive amplicons are entered into the NCBI GenBank system, and accession numbers are received as OP615128 (NorV-GIII-Erz25), OP615129 (NorV-GIII-Elz23), and OP615130 (NorV-GIII-Erz9). According to the bioinformatic analysis results of our positive BNoV sequences and GenBank reference strains, noroviruses were collected in 10 genogroups (GI-GX). Among these genogroups, bovine noroviruses were included in genogroup III. Although Cho[28] categorizes genogroup III as GIII-1, GIII-2a, and GIII-2b at the capsid gene level in his study, he separated GIII-1 as GIII-1a and GIII-1b in order to better understand the genotypic structure of the virus at the level of the RdRp gene. In addition, Cho[28] divided phylogenetic branches into GIII-2a and GIII-2b, and reported GIII-2b in smaller groups as GIII-2b$_1$ and GIII-2b$_2$ in MEGA 4 software-based phylogenetic analysis using the neighbor joining method. In our study, the GIII genogroup was grouped in 3 different phylogenetic branches: GIII-1, GIII-2a/b, and GIII-3.[28] Of the 3 strains identified in our study, 2 were grouped in GIII-2a (TR/ERZ/25 and TR/ERZ/23) and 1 strain was grouped in GIII-2b (TR/ERZ/9) (Figure 1).

The similarity rates of the strains identified in our study with the strains obtained from other studies conducted in Türkiye (Table 1) were found to be between 77.4 and 95.5%. The nucleotide similarity rates of the strains detected in our study were determined as 85.6%-100%. Figure 2 illustrates the amino acid similarities between the strains obtained in the study and the reference strains included in the phylogenetic analysis.

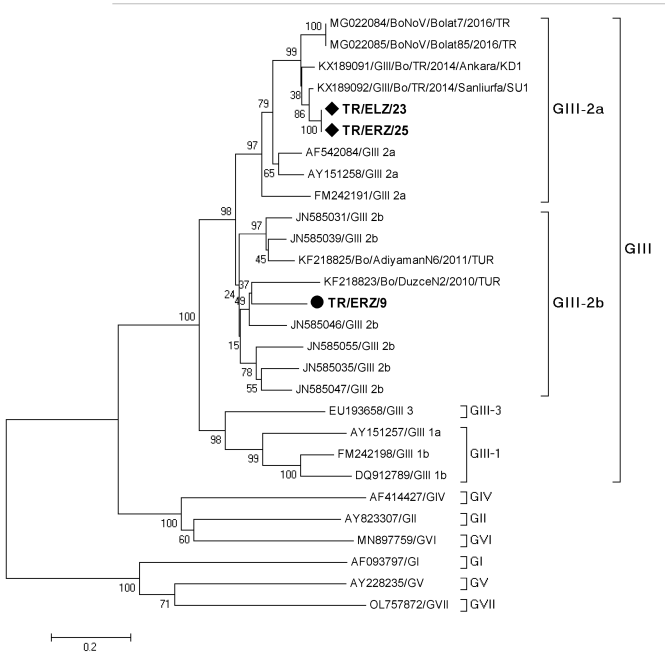

Figure 1. Phylogenetic tree of the RdRp gene sequences of bovine norovirus strains. Among our study strains, the phylogenetic location of genogroup III-2b noroviruses is shown with a diamond shape (◆) and the phylogenetic location of genogroup III-2a noroviruses is shown with a round shape (●).

The 2 strains (TR/ERZ/25 and TR/ELZ/23) obtained in the study were included in the GIII-2a genotype. One of our strains (TR/ERZ/9) was included in GIII-2b. Between GIII-1 and GIII-2 at the relevant gene level (with amino acid), S→P at amino acid level 18th amino acid, S→A at 45th amino acid, M→L at 57th amino acid, and K→R at 64th amino acid and also It can be highlighted by the change of E→S at 65th amino acid and H→F at 75th amino acid. A change of E→S at 26th amino acid and S→A at 57th amino acid was determined between GIII-2 and GIII-3. The C→S change at

Table 1. Bovine Norovirus Studies in Türkiye

Study Year	Regions of Türkiye (Province)	Case Number (Positive/Total, %)	GenBank Accesion Number	References
2011	Marmara	6/70 (%8.5)	–	23
2016	Türkiye	4/235 (%1.7)	..KF218825..	24
2018	East Anatolia	5/127 (%3.93)	..MG022085..	22
2019	Türkiye	56/167 (%33.5)	..KX189100..	12
2019	Central Anatolia	6/80 (%7.5)	–	21
2022	East Anatolia	3/92 (%3.2)	..OP615130..	This study

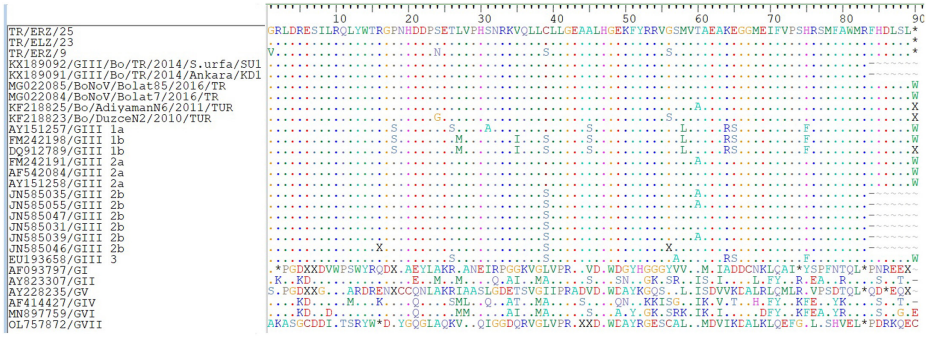

Figure 2. Amino acid presentation of strains included in the study.

39th amino acid between GIII-2a and GIII-2b was determined to be significant (Figure 2).

DISCUSSION

Noroviruses have an important place in the etiology of diarrhea. There are many factors in the diarrhea algorithm, especially in terms of the host animal type, calves.[1,29] Therefore, it is important to determine the etiological agent of diarrhea in an early diagnosis. However, the role of norovirus as the primary pathogen and the determination of the responsible genotype are important.[30,31] Noroviruses are divided into 10 genogroups. There are subgenotypes within these genogroups. So far, 50 genotypes have been reported in noroviruses. These are 9 GI genotypes, 27 GII genotypes, 4 GIII genotypes, 2 GIV genotypes, 2 GV genotypes, 2 GVI genotypes, and 1 GVII genotype, 1 GVIII genotype, 1 GIX genotype, and 1 GX genotype.[15] GIII genogroup is mostly detected in cattle and sheep.[32-33] Therefore, only the detection of NoV in the diarrhea algorithm is not sufficient; typing at the genotype level is important for good control and protection.[34]

The NoV genome has 3 open reading frames (ORF1, ORF2, and ORF3). Recombination that may occur from time to time with the breaking of ORF1 and 2, which are open reading regions, and the binding of gene regions of different noroviruses have been reported.[16] Therefore, in the genotyping studies mentioned in the introduction, there are studies on working not only the VP1 but also the RdRp gene regions together.[15,20] Although the RdRp gene region is used for diagnosis and typing, it is known that typing with VP1 is a more accurate application in terms of mutation. In a study where this recombination was reported, it was shown that the strains obtained from 2 different animals in the same herd changed between ORF1 and ORF2.[12] Since the number of positivities obtained in our study was limited, no recombination mutation was found. This situation was examined in our study with basic nucleotide and amino acid change graphs and phylogenetic analyses. Therefore, as a result of these analyses, 3 positive samples were detected in our study, so recombination and genetic changes could not be adequately examined. In this study, the RdRp gene region of 3 positive samples was examined with basic nucleotide and amino acid change graphs and phylogenetic analyses. Therefore, as a result of these analyses, recombination and genetic changes could not be adequately examined.

Bovine norovirus studies in our country are quite limited. Yılmaz et al,[23] the first of the studies on this subject, found 6 positivities (8.5%) out of 70 diarrhea cases in the Marmara region and determined that the study strains were GIII-2 type. Gülaçtı et al[24] collected diarrhea samples (n=235) throughout Türkiye. Four positivities (1.7%) were detected in the collected samples, and the importance of including BNoVs in the diagnosis of diarrhea was emphasized. In a study[22] conducted for the Eastern Anatolia region (Elazığ, Malatya, and Sivas), which includes our province, 127 diarrhea samples were collected and 5 positive samples (3.93%) were detected among them. They reported that neboviruses were detected more intensely than noroviruses in this study, in which bovine neboviruses were also investigated together with norovirus, one of the diarrhea agents of the Caliciviridae family. In another study, in which the highest positivity of BNoVs was detected in our country so far, the researchers determined not only the presence of norovirus and nebovirus but also the recombinant character of a strain they detected. In the study of Karayel-Hacioglu et al,[12] 167 stool samples were collected from all over Türkiye, and 56 (33.5%) NoV positivities were detected. At the same time, the researchers emphasized that they detected the GIII-1 type and a recombinant NoV except for the GIII-2 type detected so far in Türkiye (Table 1). In a recent study covering the Central Anatolian region in our country, researchers found 6 (7.5%) positive samples among 80 diarrhea samples. The researchers emphasized that the detected viruses are of the GIII-2 cluster 2 and that the norovirus agent is circulating in our country, and this agent should not be forgotten in prevention and control measures.[21] In our study, which was carried out 11 years after the first study in Türkiye, 3 positivities were detected in 92 diarrhea samples in the Eastern Anatolia region. It is noteworthy that NoV continues to circulate in the calf diarrhea algorithm in Türkiye. It is emphasized that the GIII-2 type is the dominant type both in studies conducted throughout Türkiye and in the Eastern Anatolian region, and that it is the type that should be included in vaccines for Türkiye in future vaccine studies. As far as is known, BNoV isolation in cell culture has not been performed yet. Therefore, we believe that it would be appropriate to conduct vaccine development studies by choosing recombinant vaccine strategies with advancing technology rather than original virus (complete virion)-based vaccines.

It has been reported that NoVs circulate from time to time between animals and humans and have been detected in different hosts at the level of species.[8,9,35] Mutual detection of human and animal NoV strains has also been reported in these studies.[35] However, the exact zoonotic status has not been determined so far. The genotypes detected in our study were the GIII genotype that was originally detected in cattle and sheep. Therefore, the transition status between species has not been determined. However, in terms of subtypes, a very limited genotypic subtype determination was determined as GIII-2a and GIII-2b.

In conclusion, our study will pave the way for both regional and country-based studies to be carried out in the future. As we mentioned before, it is necessary to develop recombinant vaccines for prevention and control of calf diarrhea and to continue carrying out molecular epidemiological studies as regards bovine noroviruses.

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