International Journal of Veterinary Science and Medicine 6 (2018) 48–52

HOSTED BY

Contents lists available at ScienceDirect

International Journal of Veterinary Science and Medicine

journal homepage: www.elsevier.com/locate/ijvsm

Full Length Article

Phylogeny of bovine norovirus in Egypt based on VP2 gene

Fakry F. Mohamed[a,b,*,1], Gamelat K.F. Ktob[a], Mohamed E.A. Ismaeil[a], Ahmed A.H. Ali[a], Sagar M. Goyal[b]

[a] Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia 44511, Egypt
[b] Department of Veterinary Population Medicine and Veterinary Diagnostic Laboratory, University of Minnesota, St. Paul, Minnesota 55108, USA

ARTICLE INFO

Keywords:
Caliciviruses
Diarrhea
Gastroenteritis
Genogroups
ORF3
Phylogeny

ABSTRACT

Bovine norovirus (BNoV) has emerged as a viral pathogen that causes a gastrointestinal illness and diarrhea in cattle. Despite its worldwide distribution, very little information is known about BNoV in Africa. In this study, BNoV was detected in 27.6% (8/29) of tested fecal materials, collected from sporadic cases of diarrheic calves, using the reverse transcription-polymerase chain reaction (RT-PCR) and primers that target RNA dependent RNA polymerase gene. Additionally, one primer pair was designed to flank the BNoV-VP2 (small capsid protein) gene for molecular analysis. Study VP2 sequences were phylogenetically-related to BNoV-GIII.2 (Newbury2-like) genotype, which is highly prevalent all over the world. However, they were separated within the cluster and one strain (41FR) grouped with recombinant GIII.P1/GIII.2 strains. Compared to reference VP2 sequences, 14 amino acid substitution mutations were found to be unique to our strains. The study confirms that BNoV is currently circulating among diarrheic calves of Egypt and also characterizes its ORF3 (VP2) genetically. The status of BNoV should be continuously evaluated in Egypt for effective prevention and control.

1. Introduction

Calf diarrhea is considered a significant contributor to economic losses worldwide. Bovine norovirus (BNoV) is an important cause of diarrhea in cattle [1] either alone or with other viral entero-pathogens. BNoV was found in the feces of both diarrheic and healthy animals [2–4]. On experimental infection, gnotobiotic calves infected with BNoV showed various degrees of diarrhea and anorexia as a result of gastroenteritis [5,6].

The BNoV is a small, non-enveloped, and positive sense single-stranded RNA virus, which belongs to the family *Caliciviridae*. The viral genome is 7.3 to 7.5 kb in size and consists of three open reading frames (ORF1, ORF2, and ORF3), which encode for large polyprotein, major capsid protein (VP1) and small basic protein (VP2), respectively [7]. Based on the phylogeny of deduced amino acids (aa) of VP1 protein, noroviruses are classified into seven genogroups (GI–GVII); GI, GII, and GIV infect humans while GIII infects bovine and sheep [8]. Each genogroup is further divided into several genotypes. For example, GIII is divided into GIII.1 (Bo/Jena/1980/DE) and GIII.2 (Bo/Newbury-2/1976/UK) originally discovered in Germany [9] and England [10], respectively. Ovine norovirus and recombinant BNoV strains represent newer genotypes in GIII [11,12].

The reverse transcription-polymerase chain reaction (RT-PCR) is commonly used for norovirus detection. The RT-PCR usually targets the RNA dependent RNA polymerase (BNoV-RdRp) gene because it contains highly conserved regions. For phylogenetic classification, RT-PCR targets the BNoV-VP1 gene. Recently, several recombination events have been detected at the ORF1/ORF2 junction, thus imposing a new genotyping strategy within the *Norovirus* genus. It was reported that GIII.P1/GIII.2 had a GIII.1-related RdRp and a GIII.2-related VP1 while GIII.P2/GIII.1 had a GIII.2-related RdRp and a GIII.1-related VP1. This indicates that both BNoV-RdRp and BNoV-VP1 should be determined for phylogenetic purposes [13].

In noroviruses, VP2 is not essential for virion production but it is mainly responsible for virion stability and genome encapsidation. Besides, VP2 interacts with VP1 and, in turn, enhances the expression of capsid proteins [14]. At the molecular level, VP2 is highly variable and can express different types of mutations [15,16]. Little is known about BNoV-VP2 due to the limited number of available sequences.

In Egypt, few data are available on the newly emerging enteric viruses of cattle, including BNoV. Only one study described BNoV infection in two cattle farms in Egypt [17]. The present study was conducted to (i) report the existence of BNoV among diarrheic calves in Sharkia province of Egypt and (ii) to study the phylogenesis of these

Peer review under responsibility of Faculty of Veterinary Medicine, Cairo University.
* Corresponding author at: Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia 44511, Egypt.
E-mail address: ramad021@umn.edu (F.F. Mohamed).
[1] Current position: Teaching-assistant (T/A) of Virology, Faculty of Veterinary Medicine, Zagazig University (MVSc, Virology).

https://doi.org/10.1016/j.ijvsm.2018.04.005
Received 23 February 2018; Received in revised form 6 April 2018; Accepted 6 April 2018
Available online 13 April 2018
2314-4599/ © 2018 Faculty of Veterinary Medicine, Cairo University. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

BNoV strains on the basis of VP2 gene.

2. Materials and methods

2.1. Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

2.2. Samples and RNA extraction

Twenty-nine fecal specimens were collected aseptically from sporadic cases of diarrheic calves in Sharkia province of Egypt between late 2016 and early 2017. Calves had diarrhea, dehydration, and weakness with different degrees. The age of calves ranged from 3 weeks to 3 months. No deaths were observed at the time of sample collection. According to the case history, animals were not vaccinated against locally-identified enteric viruses, including bovine rotavirus (BRV) and bovine viral diarrhea virus (BVDV). All samples were diluted in phosphate buffer saline (10×) and centrifuged at 2500×*g* for 10 min. The viral RNA was extracted from the supernatant fluid of each sample (140 μL) by following the manufacturer's instructions of the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA).

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Initially, the one-step RT-PCR (QIAGEN, Valencia, CA, USA) was carried out to detect the BNoV RNA in tested fecal samples by targeting a conserved area in BNoV-RdRp gene (4543–5074) using the primer pair CBECu-F/R [18]. The PCR cyclic conditions were 50 °C/30 min for reverse transcription, 95 °C/15 min for PCR activation, and 35 cycles of 94 °C/1 min for denaturation, 51 °C/45 s for annealing, and 72 °C/1 min for elongation followed by a final cycle of extension at 72 °C/10 min. Then, another primer pair (BNoV-VP2-F/R) was designed to flank the BNoV-VP2 gene from base 6432 to base 7257 (826 bp) in the positive samples. The design of these primers was made based on the VP2 region of the GenBank reference sequence (Newbury2/UK/AF097917). The previous PCR cycle was used except for annealing that was changed to 55 °C for 1 min. The PCR reactions were 25 μL in volume (1 μL enzyme mix, 12.5 μL buffer, 1 μL forward primer, 1 μL reverse primer, 2.5 μL of extracted RNA, and 7 μL RNase-free water). In negative controls, a 9.5 μL of RNase-free water was added with no RNA. The PCR amplicons were allowed to run through agarose gel by electrophoresis and then were visualized on UV transilluminator to visualize the band size. The oligonucleotide primers used in this study are listed in Table 1.

2.4. Sanger sequencing

The PCR products of BNoV-VP2 were purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) and sequenced, directly in both directions using the same primers previously used in RT-PCR, at the University of Minnesota Genomics Center (UMGC). Using Sequencher 5.1 software (http://genecodes.com/), contigs were generated using forward and reverse sequences. The obtained sequences were further identified by online nucleotide BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Sequence and phylogenetic analysis

The study sequences had the accession numbers of MF784575 and MF784576 in GenBank. Using the MEGA 6 software [19], nucleotide (nt) sequences of BNoV-VP2 were comparatively aligned to reference strains using the Clustal W option. The phylogenetic tree was constructed using the neighbor-joining statistical method (1.000 bootstrap replicates). The identities of nt and aa were calculated using the p-distance method. The aa were deduced from BNoV-VP2 nt sequences and analyzed using the DNASTAR Lasergene 7.2 software [20].

3. Results

3.1. Molecular detection of BNoV in diarrheic fecal samples

Using the RT-PCR with BNoV-RdRp specific primers, 8 of 29 fecal samples (27.6%) were confirmed positive for BNoV-RNA. Positive samples showed specific bands of 532 bp on 1.5% agarose gel under UV rays (Fig. 1). The previous step aimed to rule out the negative samples for BNoV as the RdRp is highly conserved and commonly used for BNoV detection. Furthermore, positive RNA samples were retested by RT-PCR but with BNoV-VP2 targeting primers in order to characterize the VP2 gene molecularly. The designed primers annealed successively to their sites; nt 6432–6449 on the plus strand for the forward primer and nt 7257–7237 on the minus strand for the reverse primer and the expected bands (826 bp) were demonstrated on the gel. Samples tested negative for BRV, BVDV, and bovine coronavirus (data not shown).

3.2. Sequence and phylogenetic analysis of study BNoV-VP2 strains

The molecular characters of BNoV-VP2 strains were investigated by sequencing an 826 bp region. Homogenous hits were obtained from GenBank upon blasting and used in the phylogenetic tree, which revealed that study strains (41FR and 42FR) clustered within BNoV-GIII.2 genotype (Newbury2-like). However, they were separated from each other within the same major cluster. The 41FR strain grouped with BV416/Belgium and Thirsk10/UK (recombinant GIII.P1/GIII.2 strains) but the 42FR strain grouped with CV186-OH/USA and Penrith55/UK (Fig. 2).

At nt and aa levels, 41FR and 42FR had identities of 81.8% and 88.5%, respectively. The 41FR was closely related to BV416/Belgium (84.3% nt identity and 89.7% aa identity) while 42FR was more related to CV186-OH/USA (identities of 89.5% and 89.9% at nt and aa levels, respectively). In relation to Newbury2/UK/AF097917, 41FR and 42FR shared nt identities of 79.1–86.5% and aa identities of 86.7–88.9% (Table 2).

The VP2 regions from BNoV-GIII.2 reference strains (n = 13) were obtained from blast hits. Their deduced aa sequences were aligned with those of 41FR and 42FR to detect substitution mutations in our strains.

Table 1
The oligonucleotide primers used in this study.

Primer name	Primer sequence (5′-3′)[3]	Amplicon size (bp)	Target gene/s	Reference
CBECu-F[1]	AGTTAYTTTTCCTTYTAYGGBGA	532	RdRp	[18]
CBECu-R	AGTGTCTCTGTCAGTCATCTTCAT			
BNoV-VP2-F[2]	CCAATACCGGCCGGGTCC	826	VP2	This study[4]
BNoV-VP2-R	CAAGGTTTGCGAACAATGGCA			

[1] The BNoV-RdRp targeting primers were used in one step RT-PCR for the purpose of BNoV detection.
[2] The BNoV-VP2 targeting primers were used in one step RT-PCR for the purpose of sequencing and molecular analysis.
[3] F; forward (sense) primer, R; reverse (antisense) primer.
[4] The VP2 specific primers were designed based on the GenBank reference sequence (Bo/Newbury2/1976/UK/ AF097917).

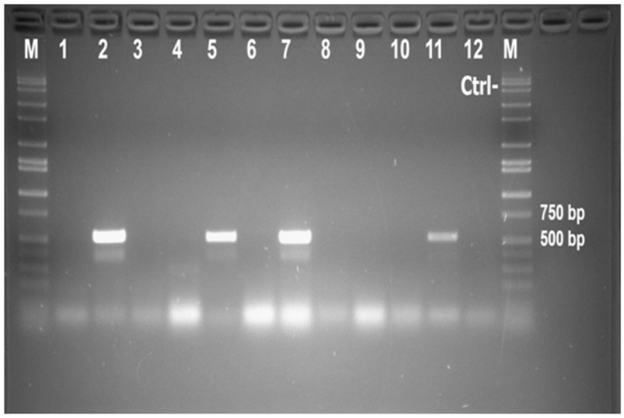

Fig. 1. The RT-PCR amplification of BNoV-RdRp genes from study fecal RNA samples. Lane M; DNA marker (50–10.000 bp). Lane 12 (Ctrl-); negative control. Lanes 2, 5, 7, and 11; BNoV positive samples (532 bp).

Table 2
The nucleotide (nt) and amino acid (aa) identities between study strains (41FR and 42FR) and reference BNoV-GIII.2 strains.

	BNoV-VP2 strains	41FR		42FR	
		nt%	aa%	nt%	aa%
1	41FR/Egypt/MF784575	.....	.....	81.8	88.5
2	42FR/Egypt/MF784576	81.8	88.5	.....	.....
3	Thirsk10/UK/AY126468	83.1	87.8	82.8	85.4
4	CV186-OH/USA/AF542084	82.6	87.6	89.5	89.9
5	CV95-OH/USA/AF542083	81.2	89.4	87.9	88.5
6	Aberystwyth24/UK/AY126475	81.7	88.5	86.9	87.5
7	Dumfries/UK/AY126474	81.7	88.5	86.6	87.5
8	Penrith55/UK/AY126476	81.4	87.6	89.3	89.7
9	11MSU-MW/USA/AY274820	80.5	88.5	87.9	88.9
10	BV15/Belgium/EU794905	79.8	87.2	86.1	87.1
11	BV416/Belgium/FJ946859	84.3	89.7	82.1	86.8
12	B309/Belgium/EU794907	80	88.5	87.1	89.3
13	BV24/Belgium/EU794906	79.4	88.5	85.7	87.4
14	Adam/Norway/JX145650	79.8	86.2	86.8	89.2
15	Newbury2/UK/AF097917	79.1	86.7	86.5	88.9

Newbury2/UK/AF097917 was selected as a reference base for building the alignment (282 aa in length). The consensus sequence for VP2 proteins was determined based on the major aa present at each position in the 15 (13 reference and 2 study) strains. Overall, study strains (41FR and 42FR) had 41 substitutions compared to the consensus sequence. Among them, 14 substitutions were found to be unique to our strains. Substitutions at positions 171 (Q to R), 173 (R to Q), 209 (S to T), 255 (S to T) and 277 (N to K) were found only in 41FR. Meanwhile, mutations at positions 29 (T to N), 100 (S to N), 139 (G to E), 144 (D to G), 197 (P to L), 214 (S to T) and 269 (T to V) were found only in 42FR. Both 41FR and 42FR shared two unique substitutions at positions 275 (F to C) and 276 (A to Q) as shown in Fig. 3.

4. Discussion

Noroviruses are one of the leading causes of human and animal gastroenteritis. The circulation of BNoV has been reported in Europe, America, Asia, and Africa [21]. However, BNoV is usually not included in diagnostic algorithms of calf diarrhea. Accordingly, its impact on livestock industries is unknown. In Africa, the reports of BNoV-infections came only from Egypt and Tunisia [17,22].

The results of this study confirm those of Mohamed et al. [17], who found the detection rates of BNoV at 24% in Egyptian cattle as compared to 27.6% in this study. The results of both studies collectively indicate the endemic nature of BNoV infection in Egyptian cattle. The detected BNoV strains were genetically-related to BNoV-GIII.2

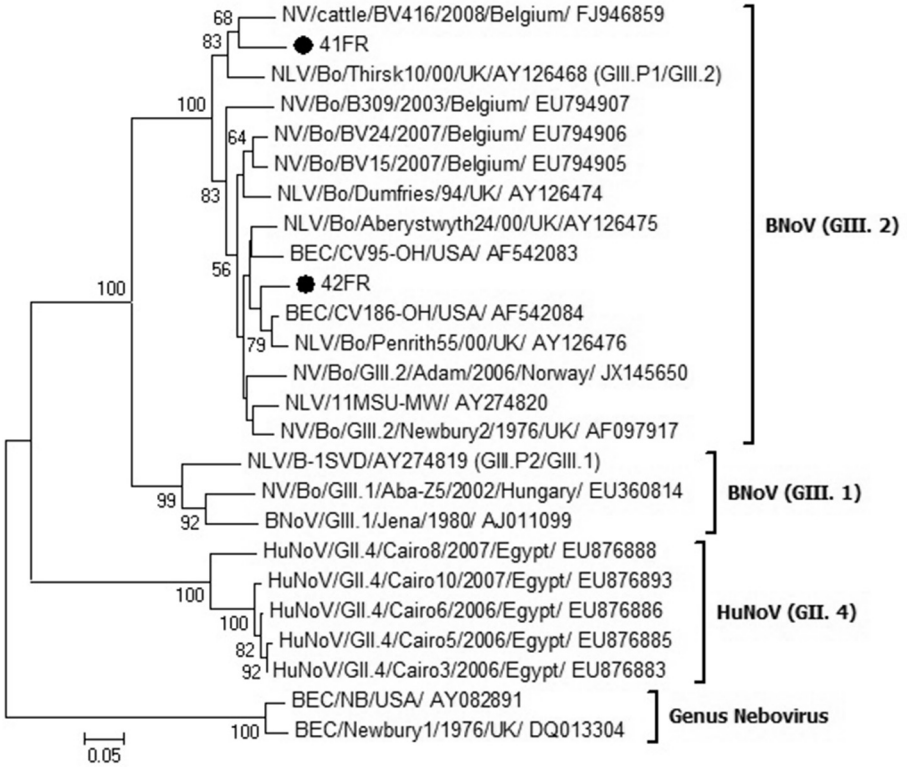

Fig. 2. The neighbor-joining phylogenetic tree of BNoV-VP2 nt sequences. Bootstrap values of > 50% are shown above the branches. The study strains (41FR and 42FR) are indicated with black circles. Strains of *Nebovirus* genus were chosen as an outgroup.

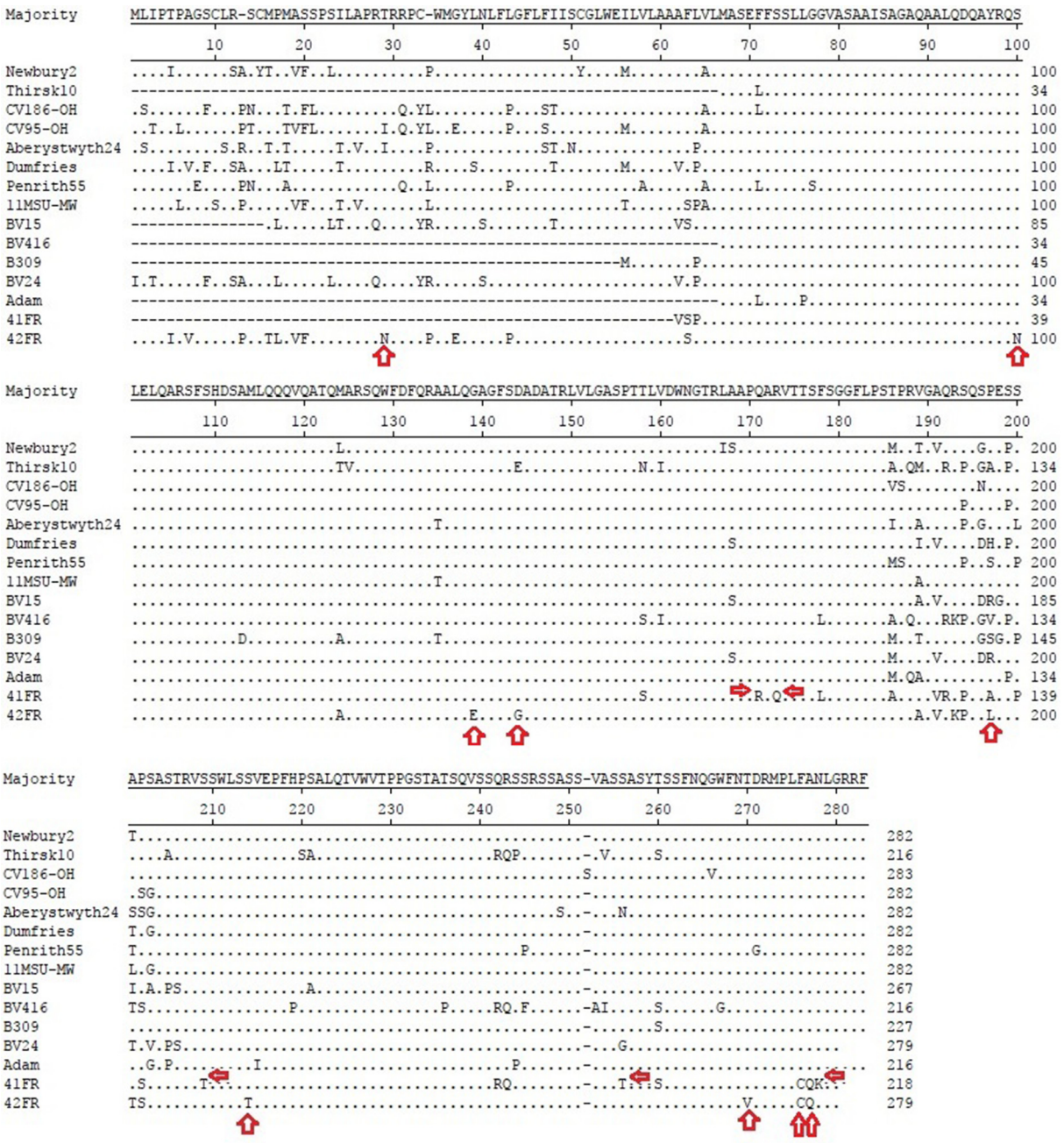

Fig. 3. Residue substitutions in deduced amino acids (aa) of BNoV-GIII.2 strains compared to the consensus sequence. Majority is the consensus represented on the ruler. Points mean identical aa while dashes mean unresolved aa. Unique aa substitutions in the study strains are marked with red arrows.

genotype (Newbury2-like), either based on BNoV-RdRp [17] or based on BNoV-VP2 (this study). BNoV-GIII.2 has a worldwide distribution, unlike BNoV-GIII.1 [23]. Calves under the age of one month were the most affected with BNoV (data not shown).

To address BNoV-VP2 molecularly, representative strains from GenBank (n = 23) were used to construct a neighbor-joining phylogenetic tree (Fig. 2). BNoV strains were phylogenetically-distinct from human norovirus (HuNoV) strains, excluding the possibility of interspecies transmission. Although being classified as BNoV-GIII.2, study strains (41FR and 42FR) were separated from each other. Studies on HuNoVs in Egypt suggested that ORF3 (VP2) followed ORF2 (VP1) in its genetic and phylogenetic characters. However, due to the slightly different topologies between ORF2 and ORF3, BNoV-VP2 may have distinct evolutionary strategies [24]. Interestingly, the 41FR was related to recombinant GIII.P1/GIII.2 strains (BV416/Belgium and Thirsk10/UK), raising questions about the presence of intra-genotypic recombinations. We are planning for complete genome sequencing of 41FR strain to detect possible recombination breakpoints.

Fourteen unique aa substitution mutations were detected in both strains (Fig. 3). Neither insertions nor deletions were observed. Such variations may play a role in classifying closely-related BNoV strains. Study strains shared identities of 81.8% and 88.5% at nt and aa, respectively. The nt and aa identities (Table 2) further confirm the clustering made by the phylogenetic tree. It is expected for norovirus capsid proteins, particularly the VP2, to exhibit broad sequence variability [15]. The hypervariability of VP2 sequences was assumed to be functionally-related to those occurring in the P2 domain of VP1 protein [25] and this may affect the viral pathogenesis.

5. Conclusions

The study reported the presence of BNoV-GIII.2 for the second time in Egypt as well as the phylogenetic properties of BNoV-VP2. It is concluded that BNoV is circulating among diarrheic cattle of Egypt. The genetic diversity of BNoV strains should be monitored to prevent future economic losses related to the virus.

Competing interests

The content of this paper is not related personally or financially to other people or organizations.

Author contributions

FM provided the study specimens (sample source) and initiated their preparation. SG supervised the project. FM, SG conceived and planned the experiments, contributed to reagents/materials/analysis tools. FM, GK, SG contributed to the interpretation of the results. FM carried out the experiments and took the lead in writing the manuscript in consultation with others.

Acknowledgments

The authors thank the Ministry of Higher Education and Scientific Research (MHESR) in Egypt for providing a visiting grant to FFM.

References

[1] Scipioni A, Mauroy A, Vinje J, Thiry E. Animal noroviruses. Vet J 2008;178:32–45.
[2] Van der Poel WH, van der Heide R, Verschoor F, Gelderblom H, Vinjé J, Koopmans MP. Epidemiology of Norwalk-like virus infections in cattle in The Netherlands. Vet Microbiol 2003;92:297–309.
[3] Jor E, Myrmel M, Jonassen CM. SYBR Green based real-time RT-PCR assay for detection and genotype prediction of bovine noroviruses and assessment of clinical significance in Norway. J Virol Methods 2010;169:1–7.
[4] Di Martino B, Di Profio F, Di Felice E, Melegari I, Ceci C, Mauroy A, et al. Genetic heterogeneity of bovine noroviruses in Italy. Arch Virol 2014;159:2717–22.
[5] Otto PH, Clarke IN, Lambden PR, Salim O, Reetz J, Liebler-Tenorio EM. Infection of calves with bovine norovirus GIII. 1 strain Jena virus: an experimental model to study the pathogenesis of norovirus infection. J Virol 2011;85:12013–21.
[6] Jung K, Scheuer KA, Zhang Z, Wang Q, Saif LJ. Pathogenesis of GIII. 2 bovine norovirus, CV186-OH/00/US strain in gnotobiotic calves. Vet Microbiol 2014;168:202–7.
[7] Oliver SL, Asobayire E, Charpilienne A, Cohen J, Bridger JC. Complete genomic characterization and antigenic relatedness of genogroup III, genotype 2 bovine noroviruses. Arch Virol 2007;152:257–72.
[8] Green KY. Caliciviridae: the noroviruses. In: Knipe DM, Howley PM, editors. Fields virology. Sixth Edvol. 2. Philadelphia, USA: Lippincott Williams & Wilkins; 2013. p. 582–608.
[9] Günther H, Otto P. Diarrhea in young calves. 7.“ Zackenvirus” (Jena agent 117/80)–a new diarrhea pathogen in calves. Arch Exp Veterinarmed 1987;41:934–8.
[10] Woode GN, Bridger JC. Isolation of small viruses resembling astroviruses and caliciviruses from acute enteritis of calves. J Med Microbiol 1978;11:441–52.
[11] Wolf S, Williamson W, Hewitt J, Lin S, Rivera-Aban M, Ball A, et al. Molecular detection of norovirus in sheep and pigs in New Zealand farms. Vet Microbiol 2009;133:184–9.
[12] Bull RA, Tanaka MM, White PA. Norovirus recombination. J Gen Virol 2007;88:3347–59.
[13] Ferragut F, Vega CG, Mauroy A, Conceição-Neto N, Zeller M, Heylen E, et al. Molecular detection of bovine Noroviruses in Argentinean dairy calves: Circulation of a tentative new genotype. Infect Genet Evol 2016;40:144–50.
[14] Vongpunsawad S, Prasad BV, Estes MK. Norwalk virus minor capsid protein VP2 associates within the VP1 shell domain. J Virol 2013;87:4818–25.
[15] Seah EL, Gunesekere IC, Marshall JA, Wright PJ. Variation in ORF3 of genogroup 2 Norwalk-like viruses. Arch Virol 1999;144:1007–14.
[16] Bok K, Abente EJ, Realpe-Quintero M, Mitra T, Sosnovtsev SV, Kapikian AZ, et al. Evolutionary dynamics of GII. 4 noroviruses over a 34-year period. J Virol 2009;83:11890–901.
[17] Mohamed FF, Mansour SM, El-Araby IE, Mor SK, Goyal SM. Molecular detection of enteric viruses from diarrheic calves in Egypt. Arch Virol 2017;162:129–37.
[18] Smiley JR, Hoet AE, Tråvén M, Tsunemitsu H, Saif LJ. Reverse transcription-PCR assays for detection of bovine enteric caliciviruses (BEC) and analysis of the genetic relationships among BEC and human caliciviruses. J Clin Microbiol 2003;41:3089–99.
[19] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013;30:2725–9.
[20] Burland TG. DNASTAR’s Lasergene sequence analysis software. Methods Mol Biol 2000;132:71–91.
[21] Di Felice E, Mauroy A, Dal Pozzo F, Thiry D, Ceci C, Di Martino B, et al. Bovine noroviruses: a missing component of calf diarrhoea diagnosis. Vet J 2016;207:53–62.
[22] Hassine-Zaafrane M, Kaplon J, Sdiri-Loulizi K, Aouni Z, Pothier P, Aouni M, et al. Molecular prevalence of bovine noroviruses and neboviruses detected in central-eastern Tunisia. Arch Virol 2012;157:1599–604.
[23] Mauroy A, Scipioni A, Mathijs E, Ziant D, Daube G, Thiry E. Genetic and evolutionary perspectives on genogroup III, genotype 2 bovine noroviruses. Arch Virol 2014;159:39–49.
[24] Kamel AH, Ali MA, El-Nady HG, De Rougemont A, Pothier P, Belliot G. Predominance and circulation of enteric viruses in the region of Greater Cairo, Egypt. J Clin Microbiol 2009;47:1037–45.
[25] Chan MCW, Lee N, Ho WS, Law COK, Lau TCK, Tsui SKW, et al. Covariation of major and minor viral capsid proteins in norovirus genogroup II genotype 4 strains. J Virol 2012;86:1227–32.