Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

The Veterinary Journal 206 (2015) 317–321

Contents lists available at ScienceDirect

The Veterinary Journal

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

Isolation and molecular characterization of bovine enteroviruses in Egypt

N.M. Sobhy [a], S.K. Mor [b], M.E.M. Mohammed [a], I.M. Bastawecy [c], H.M. Fakhry [d], C.R.B. Youssef [e], N.Z. Abouzeid [a], S.M. Goyal [b,*]

[a] Department of Animal Medicine, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Sharkia 44511, Egypt
[b] Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN 55108, USA
[c] Department of Virology, Animal Health Research Institute, Dokki, Giza 12618, Egypt
[d] Veterinary Serum and Vaccine Research Institute, East of Cairo 131, Egypt
[e] Department of Microbiology, Faculty of Pharmacy, Zagazig University, Zagazig, Sharkia 44519, Egypt

ARTICLE INFO

Article history:
Accepted 4 October 2015

Keywords:
Bovine enterovirus
Cattle
Complete sequence
Egypt
Phylogenetic analysis

ABSTRACT

Enteroviruses belong to the *Picornaviridae* family and infect a wide range of mammals including cattle. Bovine enterovirus (BEV) has recently been reclassified into E and F serotypes. BEV was first isolated in Egypt in 1966 although it has been known in other countries since the 1950s. In this study, BEV-F2 was isolated from calves with severe diarrhea and the isolated viruses were subjected to molecular characterization. Illumina sequencing of one of the isolates revealed the presence of a complete BEV-F genome sequence. The phylogenetic analysis revealed nucleotide substitutions along the genome in comparison with other known strains of BEV-F (HQ663846, AY508697 and DQ092795). Two primer sets were designed from the 3D and 5′NTR regions and used for the examination of the remaining isolates, which were confirmed to be of the BEV-F2 serotype. The availability of the complete genome sequence of this virus adds to the sequence database of the members of *Picornaviridae* and should be useful in future molecular studies of BEV.

© 2015 Elsevier Ltd. All rights reserved.

Introduction

Bovine enterovirus (BEV) belongs to the genus *Enterovirus* in the family *Picornaviridae*, which currently consists of 46 species grouped into 26 genera. The *Enterovirus* genus consists of 12 species; nine enteroviruses (A, B, C, D, E, F, G, H, and J) and three rhinoviruses (A–C) (Knowles et al., 2012). *The* enteroviruses are small (~25–30 nm in diameter), non-enveloped, icosahedral viruses consisting of a single stranded positive sense RNA genome surrounded by capsid proteins. The genome has approximately 7500 nucleotides (nt). The 5′ non-translated region (NTR) contains an internal ribosome entry site, which is the initiation site for translation. The genome contains a single open reading frame (ORF) that encodes a polyprotein (P), which is further processed to yield structural capsid proteins (VP1-VP4) and non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D) (Jiang et al., 2011).

As more information on nt sequences has become available, the classification of picornaviruses has become more confusing and is continually changing (Kaku et al., 2001). For example, the BEVs are now classified into two subgroups E (1–4) and F (1–6) while they were previously named as BEV-A (1–4) and BEV-B (1–6) (Shaukat et al., 2012; Adams et al., 2013). The BEVs were first isolated in the late 1950s (McFerran, 1962). From then on, the virus has been found to exist widely in cattle herds worldwide without causing a specific disease (Gur et al., 2008). However, BEVs have also been isolated from cattle with a wide range of clinical signs including respiratory disease (pyrexia, cough, and dyspnea), enteric infections (diarrhea, high morbidity and moderate mortality), reproductive disorders, and infertility (Ze-Li et al., 2014; Zhang et al., 2014). Zhang et al. (2014) reported a novel BEV-E2 from faecal samples of cattle with severe diarrhea and haemorrhagic intestinal mucosa. Calves experimentally infected with BEV-1 manifested symptoms varying from respiratory illness and moderate diarrhea to subclinical infection with the virus being localized mainly in the digestive tract (Blas-Machado et al., 2011). In mice, the virus was able to infect intestine, liver and lung (Zhang et al., 2014).

Large scale recombination has been shown to occur in enteroviruses (Simmonds and Welch, 2006). The genetic changes tend to accumulate over time and can occur between viruses of different species. For example, ovine enterovirus type 1 (OEV-1) is a natural interspecies recombinant between bovine and porcine enteroviruses (Boros et al., 2012). This type of recombination occurs on multiple occasions resulting in a complex set of sequences in the nonstructural region of the viral genome (Oberste et al., 2004).

* Corresponding author. Tel.: +1 612 625 2714.
E-mail address: goyal001@umn.edu (S.M. Goyal).

http://dx.doi.org/10.1016/j.tvjl.2015.10.011
1090-0233/© 2015 Elsevier Ltd. All rights reserved.

Recombination may lead to the emergence of virulent enteroviruses which may cause severe disease as was the case with human enterovirus D68 outbreaks in the USA (Midgley et al., 2014; Pastula et al., 2014). In Egypt, BEV was first isolated from calves in1966 (Singh et al., 1966) but there have been no further reports of this virus in the Egyptian cattle population. The present study reports the isolation and molecular characterization of BEV from diarrheic calves in Egypt.

Materials and methods

Samples and virus isolation

Bovine calves from a private farm of 60 cattle in the El-Fayoum governorate showed signs of severe diarrhea; infection with bovine coronavirus (BCV), bovine rotavirus (BRV) or bovine viral diarrhea virus (BVDV) was also suspected. Faecal samples were collected from 28 calves and diluted 1:5 in phosphate buffer saline (PBS, pH 7.4) followed by centrifugation at 2500 *g* for 10 min. The supernatants were filtrated through 0.22 μm filters and the filtrates were inoculated in three different cell lines for the isolation of BCV, BRV, and BVDV. For BCV isolation, the filtrates were inoculated in human rectal adenocarcinoma (HRT-18) cells maintained in MEM (Fisher) supplemented with 10% donor horse serum (Fisher), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma), and 2.5 μg/mL fungizone (Fisher). For isolation of BRV, the samples were inoculated in African Green monkey kidney (MA104) cells maintained in modified Eagle's medium (MEM) (Fisher) supplemented with 1.5 mg/mL trypsin (Fisher), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma) and 2.5 μg/mL fungizone (Fisher). For BVDV, Madin-Darby bovine kidney (MDBK) cells were used.

Inoculated cells were observed for the appearance of cytopathic effects (CPE) daily under a light microscope. The infected cells were harvested when the CPE involved about 80% of the monolayer. The cell cultures were subjected to three freeze-thaw cycles followed by centrifugation at 1200 *g* for 10 min to remove cellular debris. The supernatants were loaded on fast technology analysis (FTA) cards (Liang et al., 2014) and sent to the University of Minnesota, Veterinary Diagnostic Laboratory (UMVDL), St. Paul, MN, USA, for further processing.

Next generation sequencing (Illumina sequencing)

At the UMVDL, viral RNA was extracted from loaded FTA cards using a QIAamp Viral RNA mini kit (Qiagen) followed by testing for BCV, BRV, and BVDV by real time reverse transcriptase (RT)-PCR. RNA from one of the isolates was submitted to the University of Minnesota Genomic Center (UMGC) for Illumina HiSeq. The RNA passed sequencing quality assessment in which total RNA was quantified using a fluorimetric RiboGreen assay. The library was created using Illumina's Truseq RNA sample preparation kit (RS-122-2001). Once clustering was complete, the flow cell was loaded on the HiSeq 2000 and sequenced using Illumina's sequencing by synthesis (SBS) chemistry. Finally, the library fragments were re-synthesized in the reverse direction and sequenced from the opposite end of the read 1 fragment thus producing the template for paired end read 2.

The sequence reads were analyzed by CLC Genomic Workbench 6.0.[1] Trimming and sequence quality were tested followed by preparation of contigs by de novo assembly. Extracted contigs were analyzed by BLAST (tBlastx) analysis on NCBI.[2] The ORF finder tool[3] was used to find ORFs in the obtained sequences. Prediction of structural and non-structural proteins was done based on amino acid (aa) alignments with reference picornavirus sequences from GenBank. In addition, the presence of cleavage sites was determined by the NetPicoRNA program,[4] which distinguishes known cleavage sites from non-cleavage sites (Blom et al., 1996).

Phylogenetic analysis and genome characterization

Sequences obtained in this study were aligned with published sequences of different members of *Picornaviridae* family. All sequences were aligned using Clustal W method in MEGA 6.0 software (Tamura et al., 2013). The best substitution model of the maximum-likelihood method for analysis of DNA and protein sequences was selected on the basis of the lowest BIC score (Bayesian Information Criterion) in MEGA 6.0. The complete ORF sequence of BEV/Egypt/2014/KM667941 isolate was aligned with members of different genera of *Picornaviridae*. This was followed by alignment with sequences of different virus species of genus *enterovirus* by using LG+G+F and LG+G+I+F models, respectively. The VP1, VP2 and VP3 genes of the study sequence were also compared with two groups of bovine enteroviruses; groups BEV-E and BEV-F included only common subgroups (1–3) by using the GTR (General Time Reversible) +G (gamma distribution with five rate categories) model for VP1 and the GTR+G+I model for VP2 and VP3 genes in MEGA 6.0 software (Tamura et al., 2013). The maximum likelihood phylogenetic trees were statistically validated by using 1000 bootstrap replicates.

Table 1
Predicted polyproteins in Egyptian BEV-F genotype.

Gene	Nucleotide sequence			Amino acid sequence			Predicted cleavage site
	Start	End	Size	Start	End	Size	
5′ NTR	1	822	822				
VP4	823	1029	207	1	69	69	
VP2	1030	1767	738	70	315	246	K/S
VP3	1768	2496	729	316	558	243	Q/G
VP1	2497	3318	822	559	832	274	Q/G
2A	3319	3768	450	833	982	150	T/G
2B	3769	4065	297	983	1081	99	Q/G
2C	4066	5054	287	1082	1410	329	Q/A
3A	5053	5319	267	1411	1499	89	Q/G
3B	5320	5388	69	1500	1522	23	Q/G
3C	5389	5937	549	1523	1705	183	Q/G
3D	5938	7320	1383	1706	2167	461	Q/G
′3 NTR	7321	7417	97				

NTR, non-translated region; K/S, lysine/serine; Q/G, glutamine/glycine; T/G, threonine/glycine; Q/A glutamine/alanine.

Primer design and RNA testing by reverse transcription PCR

From BEV/Egypt/2014/KM667941 sequence obtained by Illumina, we designed primers from the 3D gene and the 5′NTR region. The 3D forward primer was 5′-ATGGACAAGAGGTAYGTCGTCGT-3′ while the reverse primer was 5′-GGGCACACTCCGGATTTTCTCC-3′ yielding an amplicon of 450 base pairs (bp). The 5′NTR forward primer was 5′-TTTAAAACAGCCTGGGGGTTGT-3′ and reverse primer was 5′-GGTTTGTCAATGGCTGTGGCAT-3′; the amplicon size was 1180 bp. Extracted RNA was subjected to RT-PCR using one step RT-PCR kit (Qiagen). The amplification protocol used 25 μL reaction mixture incubated in a thermocycler at 50 °C for 30 min for the RT step and then at 95 °C for 15 min for *Taq* activation. This was followed by 35 cycles consisting of 94 °C for 1 min for denaturation, annealing for 1 min at 55 °C, and then elongation at 72 °C for 1 min followed by final extension cycle at 72 °C for 10 min. The RT-PCR products were analyzed in ethidium bromide stained 1.2% agarose gel. A single band of expected product size confirmed the presence of target BEV.

Sequencing of amplified RT-PCR products

The RT-PCR products were purified using QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. The purified products were sequenced using the same forward and reverse primers as used in RT-PCR. The obtained sequences were curated and aligned using 'Sequencher 5.1' software[5] followed by BLAST analysis in GenBank data base for comparing with other BEV sequences.

Results

The CPE in various cells (MA104, HRT-18 and MDBK cells) appeared on the third day of incubation and consisted of rounding and detachment of cells. Real time RT-PCR of infected cell culture fluids indicated the absence of BCV, BRV, and BVDV. However, BLAST analysis of Illumina sequence contigs gave match with BEV and the complete nt sequence of BEV/Egypt/2014/KM667941 was 7417 nt. The organization of nt was typical of the BEV genome including 822 nt in 5′NTR, 6498 nt for ORF, and 97 nt in 3′NTR. The detailed nt and aa content of each gene with their predicted cleavage sites were estimated (Table 1). The single ORF encoded a polypeptide of 2166 aa beginning with methionine codon that initiates translation dividing ORF into P1, P2 and P3 subgenomic regions. The complete genome sequence of BEV/Egypt/2014 isolate was submitted in GenBank with accession number KM667941.

[1] See: http://www.clcbio.com/products/clc-genomics-workbench/ (accessed 22 September 2015).
[2] See: http://www.ncbi.nlm.nih.gov/ (accessed 22 September 2015).
[3] See: http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi (accessed 22 September 2015).
[4] See: http://www.cbs.dtu.dk/services/NetPicoRNA/ (accessed 22 September 2015).
[5] See: http://genecodes.com/download/external-tools-download (accessed 22 September 2015).

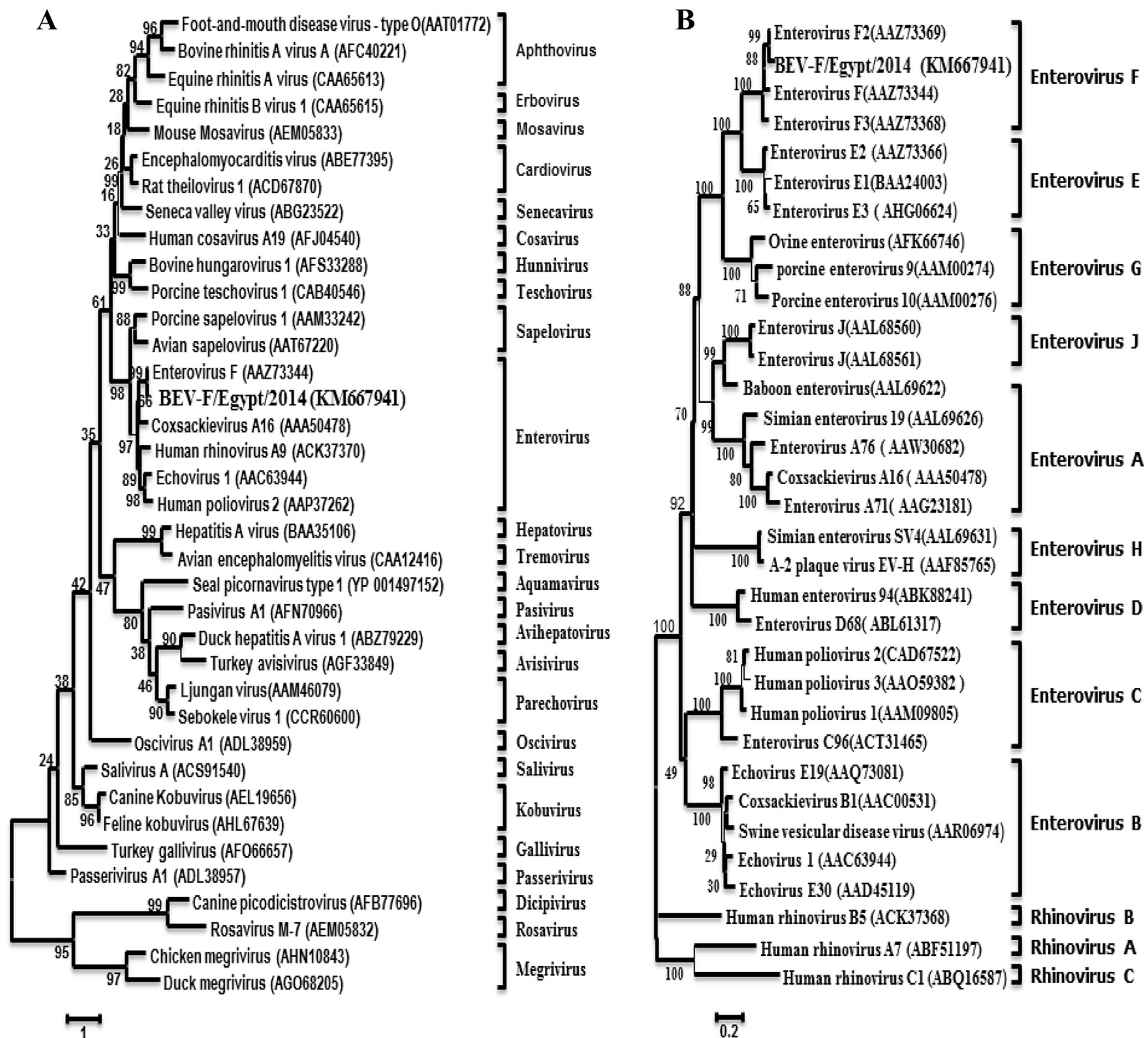

Fig. 1. Phylogenetic relationships of BEV/Egypt/2014/KM667941 amino acid to: (A) the other members of *Picornaviridae* and (B) the other Enteroviruses. Each identified genus/species is represented by at least one virus. Amino Acid sequences were aligned with the Clustal W program. Branch lengths are proportional to the genetic divergence. The evolutionary history was inferred using maximum Likelihood method in MEGA 6.0.

Phylogenetic analysis based on complete ORF of BEV/Egypt/2014/KM667941with other genera of *Picornaviridae* revealed that the virus belonged to the enterovirus genus (Fig. 1A). Within enteroviruses, the study sequence clustered with BEV-F with 95% aa identity with BEV-F2 (AAZ73369). The isolate grouped differently from BEV-E strains with maximum aa identity of 75% (Fig. 1B).

Sequence analysis of P1 region, which encodes four genes (VP4, VP2,VP3 and VP1), revealed that the BEV-VP1 gene is the most variable among the four structural genes and shared only 75–76% nt identity (92–94% aa identity) with BEV-F2 strains (HQ663846, AY508697, and AY508696). The VP4 gene had the least variation as the nt identity was 80–84% (93–97% aa identity) with other strains of BEV-F2 (see Appendix: Supplementary Table S1). The P2 region that encodes three genes (2A, 2B and 2C) showed high identity with HQ663846 (BJ001/China) sharing 82% nt and 98% aa identity. The P2 region (genes 2A and 2B) had high variation among BEV-E and BEV-F strains as compared the 2C gene. The identity was 66–69% nt (75–76% aa), 65–67% nt (70–71% aa) and 71% nt (79% aa) in 2A, 2B and 2C genes, respectively (see Appendix: Supplementary Table S2).

The P3 region consists of four genes (3A, 3B, 3C and 3D). The variation in P3 region was the least among the three polypeptides and the 3D gene was highly conserved. Alignment of the conserved gene (3D) with other BEV-E and BEV-F viruses in GenBank revealed that the BEV/Egypt/2014/KM667941closely matched with HQ663846 (BJ001/China) sequence with nt identity of 85% (98% aa) (see Appendix: Supplementary Table S3). In relation to the known BEV-E and BEV-F strains, there were several variable regions in the capsid protein especially in the VP1 and VP2 genes of BEV/Egypt/2014/KM667941. The aa substitutions in the genome of the study strain are shown in Table 2.

All remaining viral isolates were positive by RT-PCR yielding the expected amplicon size of 450 bp for 3D gene and 1180 bp for 5′NTR. The 3D and 5′NTR sequences of six representative isolates were 100% identical with each other and all clustered with BEV/Egypt/2014/KM667941. The GenBank accession numbers for 3D sequences are: KM887136, KM887137, KM887138, KM887139, KM887140 and KM887141. For 5′NTR the numbers are KM887130, KM887131, KM887132, KM887133, KM887134 and KM887135.

Table 2
Nucleotide and amino acid substitutions in BEV/Egypt/2014/KM667941 genome in comparison with the closest strains (HQ663846, AY508697 and DQ092795).

Genes	Region	Nucleotide substitution	Amino acid substitution	Amino acids site in translated ORF
5 NTR		21		
VP4	P1	11	2	S (24), A (66)
VP2		26	6	K (147), A (205), S (208), D (220), S (222), T (278)
VP3		22	2	R (356), L (428)
VP1		42	6	S (599), I (614), N (681), N (818), A (819), N (820)
2A	P2	9	1	R (898)
2B		12	1	N (990)
2C		32	1	D (1334)
3A	P3	10	1	A (1426)
3B		–	–	–
3C		16	2	V (1575), A (1576)
3D		12	2	S (2066), T (2100)

NTR, non-translated region; P, polyprotein; S, serine; A, alanine; K, lysine; D, aspartic acid; T, threonine; R, arginine; L, leucine; I, isoleucine; N, asparagine; V, valine.

Discussion

Cattle infected with BEV show varying degrees of diarrhea, mild to severe respiratory signs, and sometimes abortion of pregnant heifers (Zhang et al., 2014). Animals infected with BEV shed large amounts of virus in their faeces leading to environmental contamination (Jimenez-Clavero et al., 2005). After its first report from water buffaloes in Egypt in 1966 (Singh et al., 1966), the virus has not been reported from Egypt. The isolates of BEV in this study were from calves with severe diarrhea. On the basis of clinical signs, it was suspected that BCV, BRV, or BVDV were involved, but only BEV was isolated from these cases indicating the role and importance of laboratory diagnosis in confirming the aetiology. Although Zhang et al. (2014) and Zhu et al. (2014) have reported on the isolation of BEV from cases that were also positive for other viruses, we did not isolate any other virus in this study. Relatively few reports are available on the pathogenesis and molecular biology of BEV (Goens et al., 2004) and our study appears to be the first on molecular characterization of BEV in Egypt.

Next generation sequencing is one of the most advanced techniques for the detection and complete genome amplification of all known and unknown pathogens present in a sample. In this study Illumina sequencing was used for the identification of viruses isolated in MDBK cells. For accurate classification of the isolated virus, phylogenetic analysis was done on three logical levels (family, genus, and species). The study strain (BEV/Egypt/2014/KM667941) was classified into *Picornaviridae* under genus *enterovirus* and BEV-F2 species.

Studies on sequencing and phylogenetic analysis of BEVs have revealed the presence of two clusters, namely BEV-E and BEV-F (Shaukat et al., 2012; Zhang et al., 2014; Zhu et al., 2014). The most prominent difference between BEV-F and BEV-E sequences relates to the presence of cleavage sites at VP3/VP1 and 2B/2C (Goens et al., 2004). BEV-F has glutamine-glycine at the proposed cleavage site for VP3/VP1, whereas BEV-E has glutamine-asparagine. At the 2B/2C cleavage site, BEV-F has glutamine-alanine while BEV-E has glutamine-serine. All other predicted polyprotein cleavage sites are glutamine–glycine type, which are commonly found in most picornaviruses (Earle et al., 1988).

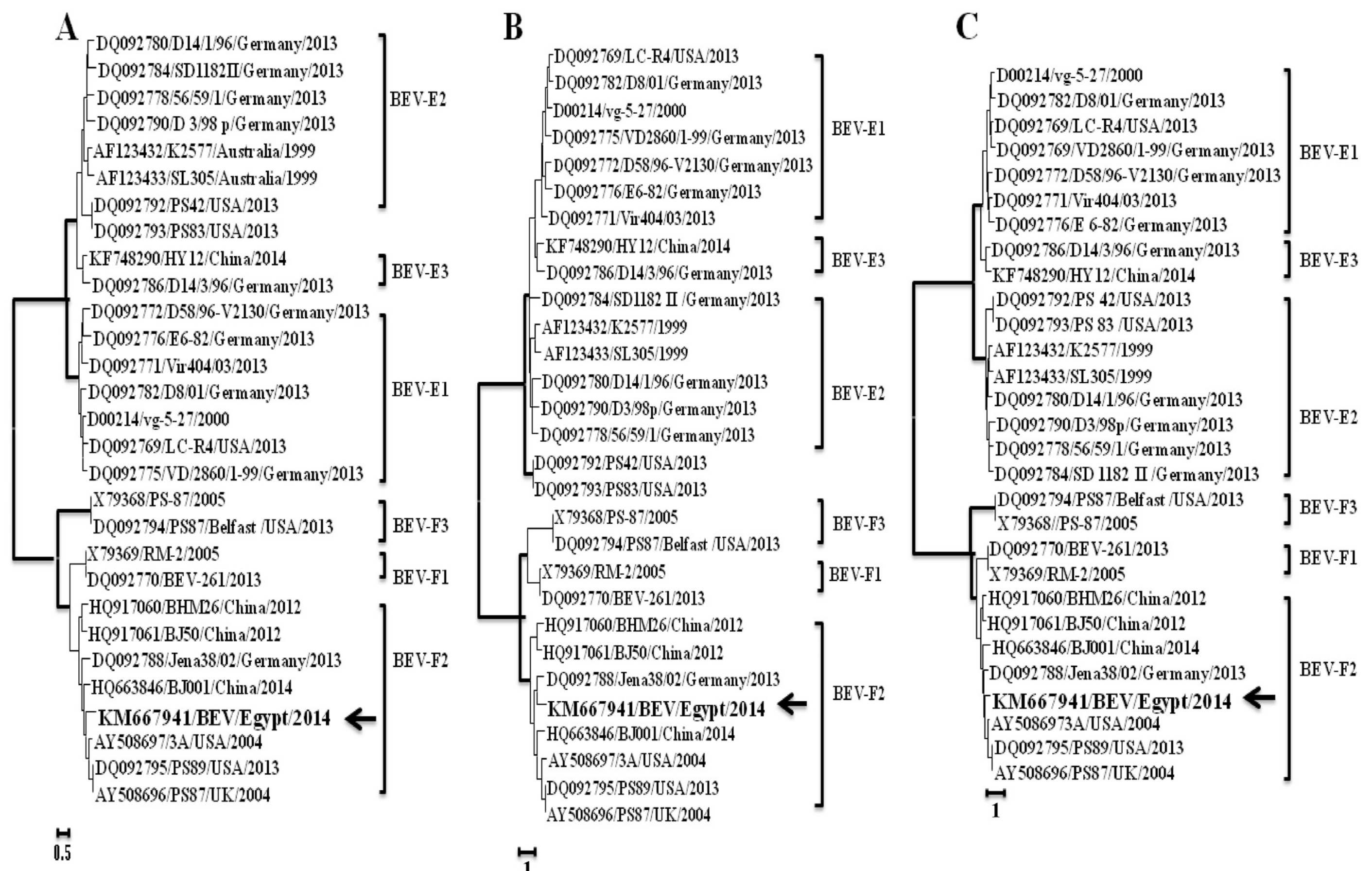

Fig. 2. (A, B, and C) Phylogenetic analysis of BEV/Egypt/2014/KM667941 with other related strains comparing the nucleotide sequence of VP1, VP2, VP3 regions.

The analysis of deduced aa sequences of the study strain revealed a few highly conserved regions including VP4 in the structural protein and 3B and 3D in non-structural proteins as compared to other BEV-E and BEV-F groups. In BEV-F viruses, the highly conserved part is in the P3 gene. The phylogenetic analysis of VP1, VP2, and VP3 revealed a close relationship between the study strain and a German strain (Jena 38/02) (GenBank accession number DQ092788) (Figs. 2A–C). In GenBank, the German strain (Jena 38/02) is reported to be BEV-E, which appears to be a mistake and should be corrected.

There was a larger number of nt substitutions than aa substitutions in the study strain indicating that synonymous substitutions are higher than non-synonymous ones (Table 2). A maximum of 16 non-synonymous substitutions were observed in the P1 region followed by five and three in P3 and P2, respectively. The significance of these substitutions is not known but previous studies have reported a high rate of recombination in enteroviruses that can be interserotypic and/or intraserotypic thereby leading to the generation of new strains capable of causing severe disease outbreaks (Yozwiak et al., 2010; Boros et al., 2012; Kim et al., 2013).

Studies on experimental reproduction of disease and surveillance of apparently healthy and enteritis-affected cattle should be conducted in Egypt to understand the exact status and importance of this virus in the cattle population. The absence of BCV, BRV, and BVDV in this study is in agreement with the findings of Zhang et al. (2014) who were also not able to detect these enteric viruses even when using highly sensitive and specific RT-PCR methods. Our study has shown the association of BEV with calf diarrhea in Egypt. The RT-PCR designed in this study should be useful in the early detection of BEV and in conducting surveillance studies on the prevalence of BEV in the Egyptian cattle population.

Conclusions

This study reports on the isolation of BEV from calves suffering from severe diarrhea in Egypt. Illumina sequencing of the isolate revealed the complete BEV genome identifying the isolated virus as BEV-F2. The newly designed RT-PCR assays from 3D and 5′NTR regions should be useful in future BEV surveillance studies.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Acknowledgements

NMS's Fellowship was provided by the Egyptian Ministry of Higher Education and is gratefully acknowledged.

Appendix: Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tvjl.2015.10.011.

References

Adams, M.J., King, A.M.Q., Carstens, E.B., 2013. Ratification vote on taxonomic proposals to the international committee on taxonomy of viruses. Archives of Virology 158, 2023–2030.

Blas-Machado, U., Saliki, J.T., Sánchez, S., Brown, C.C., Zhang, J., Keys, D., Woolums, A., Harvey, S.B., 2011. Pathogenesis of a bovine enterovirus-1 isolate in experimentally infected calves. Veterinary Pathology 48, 1075–1084.

Blom, N., Hansen, J., Blaas, D., Brunak, S., 1996. Cleavage site analysis in picorna viral polyproteins: Discovering cellular targets by neural networks. Protein Science 5, 2203–2216.

Boros, A., Pankovics, P., Knowles, N.J., Reuter, G., 2012. Natural interspecies recombinant bovine/porcine enterovirus in sheep. Journal of General Virology 93, 1941–1951.

Earle, J.A., Skuce, R.A., Fleming, C.S., Hoey, E.M., Martin, S.J., 1988. The complete nucleotide sequence of a bovine enterovirus. Journal of General Virology 69, 253–263.

Goens, S.D., Botero, S., Zemla, A., Zhou, C.E., Perdue, M.L., 2004. Bovine enterovirus 2: Complete genomic sequence and molecular modelling of a reference strain and a wild-type isolate from endemically infected US cattle. Journal of General Virology 85, 3195–3203.

Gur, S., Yapkic, O., Yilmaz, A., 2008. Serological survey of bovine enterovirus type 1 in different mammalian species in turkey. Zoonoses and Public Health 55, 106–111.

Jiang, H., Weng, L., Zhang, N., Arita, M., Li, R., Chen, L., Toyoda, T., 2011. Biochemical characterization of enterovirus 71 3D RNA polymerase. Biochimica et Biophysica Acta (BBA) – Gene Regulatory Mechanisms 1809, 211–219.

Jimenez-Clavero, M.A., Escribano-Romero, E., Mansilla, C., Gómez, N., Córdoba, L., Roblas, N., Ponz, F., Ley, V., Sáiz, J.-C., 2005. Survey of bovine enterovirus in biological and environmental samples by a highly sensitive real-time reverse transcription-PCR. Applied and Environmental Microbiology 71, 3536–3543.

Kaku, Y., Sarai, A., Murakami, Y., 2001. Genetic reclassification of porcine enteroviruses. Journal of General Virology 82, 417–424.

Kim, H., Kim, K., Kim, D.W., Jung, H.D., Min Cheong, H., 2013. Identification of recombinant human rhinovirus A and C in circulating strains from upper and lower respiratory infections. PLoS ONE 8, e68081.

Knowles, N.J., Hovi, T., Hyypiä, T., King, A.M.Q., Lindberg, A.M., Pallansch, M.A., Palmenberg, A.C., Simmonds, P., Skern, T., Stanway, G., et al., 2012. Picornaviridae. In: Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report. Elsevier, San Diego, pp. 855–880.

Liang, X., Chigerwe, M., Hietala, S.K., Crossley, B.M., 2014. Evaluation of fast technology analysis (FTA) cards as an improved method for specimen collection and shipment targeting viruses associated with bovine respiratory disease complex. Journal of Virological Methods 202, 69–72.

McFerran, J.B., 1962. Bovine enteroviruses. Annals of the New York Academy of Sciences 101, 436–443.

Midgley, C.M., Jackson, M.A., Selvarangan, R., Turabelidze, G., Obringer, E., Johnson, D., Giles, L., Patel, A., Echols, F., Oberste, M.S., et al., 2014. Severe respiratory illness associated with enterovirus D68 – Missouri and Illinois, 2014. Morbidity and Mortality Weekly Report 63, 798–799.

Oberste, M.S., Maher, K., Pallansch, M.A., 2004. Evidence for frequent recombination within species human enterovirus B based on complete genomic sequences of all thirty-seven serotypes. Journal of Virology 78, 855–867.

Pastula, D.M., Aliabadi, N., Haynes, A.K., Messacar, K., Schreiner, T., Maloney, J., Dominguez, S.R., Davizon, E.S., Leshem, E., Fischer, M., et al., 2014. Acute neurologic illness of unknown etiology in children – Colorado, 2014. Morbidity and Mortality Weekly Report 63, 901–902.

Shaukat, S., Angez, M., Alam, M.M., Sharif, S., Khurshid, A., Malik, F., Rana, M.S., Mahmood, T., Zaidi, S.S.Z., 2012. Molecular identification and characterization of a new type of bovine enterovirus. Applied and Environmental Microbiology 78, 4497–4500.

Simmonds, P., Welch, J., 2006. Frequency and dynamics of recombination within different species of human enteroviruses. Journal of Virology 80, 483–493.

Singh, K.V., Baz, T.I., Osman, O.A., 1966. Isolation of enterovirus from water buffaloes in Egypt. Acta Virologica 10, 372.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA 6.0, Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution 30, 2725–2729.

Yozwiak, N.L., Skewes-Cox, P., Gordon, A., Saborio, S., Kuan, G., 2010. Human enterovirus 109: A novel interspecies recombinant enterovirus isolated from a case of acute pediatric respiratory illness in Nicaragua. Journal of Virology 84, 9047–9058.

Ze-Li, X., Li-Sai, Z., Xin-Ping, W., Su-Jing, L., Chang-Ming, G., Li, Y., 2014. Identification of an enterovirus E isolate from a cattle with respiratory diseases. Chinese Journal of Veterinary Science 34, 390–394.

Zhang, H., Liu, H., Bao, J., Guo, Y., Peng, T., Zhou, P., Zhang, W., Ma, B., Wang, J., Gao, M., 2014. Characterization of an enterovirus species E isolated from naturally infected bovine in China. Virus Research 19, 101–107.

Zhu, L., Xing, Z., Gai, X., Li, S., San, Z., 2014. Identification of a novel enterovirus E isolates HY12 from cattle with severe respiratory and enteric diseases. PLoS ONE 9, e97730.