Original Article

Circulation of bovine viral diarrhea virus – 1 (BVDV-1) in dairy cattle and buffalo farms in Ismailia Province, Egypt

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Abstract

Introduction: Bovine viral diarrhea (BVD) is one of the most economically significant diseases in the bovine industry causing losses due to diarrhea, reproductive disorders, immunosuppression and mortalities. The aim of our investigation was to detect and subtype BVDV from calves on two dairy cattle and two buffalo farms in Ismailia province, Egypt as an indicator of BVDV infection status in the province.

Methodology: A total of 298 blood samples were collected and tested using an optimized one-step, real-time multiplex Taqman-based RT-PCR. All the positive samples by the multiplex real-time RT-PCR were tested using conventional RT-PCR to amplify multiple areas of the genome for further phylogenetic analysis and subtyping.

Results: Thirty one (10.4%) of the tested samples were positive for BVDV-1. Only three samples, all from a single dairy cattle farm, had enough viral RNA to be amplified by RT-PCR. The PCR products were sequenced and phylogenetic analysis revealed detection of BVDV-1b. The detected strain is closely related to worldwide BVDV-1b strains, making it difficult to trace its origin. Nucleotide and amino acid alignments of the E2 glycoprotein region of the detected strain with other BVDV-1b strains showed high divergence, with identity ranging from 81.3% to 93.6% and 85.3% to 93.6%, respectively.

Conclusion: To our knowledge, this is the first report describing the circulation of BVDV-1b in Egyptian dairy cattle populations.

Key words: Bovine viral diarrhea virus; typing; phylogenetic analysis; BVDV-1b; Egypt.

Introduction

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus,* family *Flaviviradae*. It is spherical in shape, 40-60 nm in diameter, composed of a nucleocapsid with icosahedral symmetry, surrounded by an envelope [1]. The virus genome is single-stranded RNA, approximately 12.5 kb in length, and composed of a single open reading frame flanked by 5´ and 3´ untranslated regions (UTR) [2]. The single open reading frame is translated as a polyprotein. The polyprotein is processed co- and post-translationally by host and viral proteases into structural and non-structural proteins. The structural proteins include: C, E^{rns}, E1 and E2 [3].

BVDV is classified into two main species (BVDV-1 and BVDV-2) based on nucleotide sequence analysis of the 5´ UTR, N-terminal protease region (N^{pro}) and the envelope glycoprotein (E2). Recently, a third BVDV species, called HoBi-like virus or BVDV3, was reported [4]. BVDV-1 is classified by genetic analysis into about 20 subtypes (1a-1t) [5-10] and BVDV-2 into three subtypes (2a-2c) [11-13]. BVDV subtyping is useful in molecular epidemiological studies, vaccine development and tracing the origin of emerging viruses [14].

The E2 envelope glycoprotein plays a vital role in BVDV antigenicity. It has the major antigenic epitopes that trigger the humoral immune response, and neutralizing antibodies targeting the E2 glycoprotein region are responsible for protection against infection [15,16]. However, the E2 glycoprotein is highly diversified among BVDV strains with different

subtypes resulting in antigenic variation and failure to cross protect against infection [17,18]**.**

BVDV is endemic in most countries where cattle are raised [19]. In some countries, it is the most important pathogen threatening the cattle industry. A successful control program for BVDV is based on the identification and elimination of persistently infected animals (PI), mass vaccination, implementation of biosecurity measures and monitoring of BVDV herd status after removal of PI animals [20]. In Egypt**,** the BVDV control program is based on mass vaccination by commercially available inactivated vaccines. There are no surveillance programs or measures for detection and elimination of PI calves from farms.

In Egypt, BVDV was isolated for the first time in 1972 from a calf suffering from severe enteritis [21]. Most of the BVDV reports from Egypt are based mainly on the detection of virus by isolation and/or detection of viral antibodies [22-25]. There are only a few reports that describe subtyping of circulating BVDV in animal populations [26, 27]. Therefore, the aim of our investigation was to detect BVDV in Ismailia province dairy cattle and buffalo farms and perform molecular characterization of the circulating strains.

Methodology

Farm historical data, clinical examination and samples

A total of two dairy cattle farms and two dairy buffalo farms were investigated in Ismailia province, Egypt. The investigated farms have previous histories of mortalities in newborn calves. Historical data was collected from farm records including the BVDV vaccination program, mortality rate in newborn calves and history of calf respiratory tract infection and/ or calf scours. A total of 298 newborn calves less than two months of age from the four dairy farms were clinically examined and whole blood samples were collected in EDTA tubes and tested for BVDV.

Detection of BVDV by probe based multiplex real-time RT-PCR

A multiplex real-time RT-PCR assay, targeting the 5′ UTR, was used for detection of BVDV [28]. The assay sensitivity was optimized before testing the clinical samples using RNA standards produced by cloning PCR products from BVDV-1 (strain NADL) and BVDV-2 (strain 125) with the TA Cloning Kit with pCR2.1 Vector and One Shot INVαF' Chemically Competent E. coli (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The purified plasmids were used as a template for synthetizing of in vitro transcribed RNA by MEGAscript T7 (Ambion, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions. RNA copy numbers were calculated and standard curves were generated from Ct values produced by ten-fold serial dilution of the RNA standard. Total RNA was extracted from 300 µl of buffy coat samples, using Ribozol RNA extraction reagent (Amersco, Solon, OH, USA) according to the manufacturer's instructions. Each RNA pellet was eluted in 50 µl nuclease free water. Five microliters of extracted RNA was used as a template for multiplex real time RT-PCR using Superscript III Platinum Taq One Step qRT-PCR Mix (Invitrogen, Carlsbad, CA, USA) in Step One Real-Time PCR System (Life Technologies). Strict laboratory procedures were performed to avoid any cross contamination.

Table 1. Primer pairs and probes used in this study

Primers and probe	Target Gene	Sequence	Amplicon size	References
Pesti-F	5′ UTR	5′-CTAGCCATGCCCTTAGTAG-3′	104 bp	[28]
Pesti-R		5′-CGTCGAACCAGTGACGACT-3′		
BVDV1- probe		5′-FAM-TAGCAACAGTGGTGAGTTCGTTGGATGGCT-BHQ-3′		
BVDV2- probe		5′-VIC-TAGCGGTAGCAGTGAGTTCGTTGGATGGCC-BHQ-3′		
324	5′ UTR	5′-ATG CCC WTA GTA GGA CTA GCA-3′	288 bp	[29]
326		5′-TCA ACT CCA TGT GCC ATG TAC-3′		
BD1	N^{pro}	5′-TCT CTG CTG TAC ATGGCA CAT G-3′	428 bp	[30]
BD3		5′-CAT CCA TCT ATR CAY AYA TAA ATR TGG TAC-3′		
E2-1 F	E2	5′-GAAGAGGTGGGTCAGGTAA-3′	873 bp	This study
E2-1 R		5′-GTCTATAGCCACTCTCATTCTTC-3′		
E2-2 F	E2	5′-CCCAATHGGCAAATGCAG-3′	500 bp	This study
E2-2 R		5′-AGTTGCCCATCATCACTATTT-3′		

RT-PCR and sequencing

All the positive samples by multiplex real-time RT-PCR were tested by conventional RT-PCR for amplification of a 288 bp fragment of the 5′ UTR and a 428 bp fragment of the N^{pro} region using Superscript III Platinum Taq One Step RT-PCR Mix for further sequencing and subtyping [29, 30]. Primers used in this study are shown in Table 1. The PCR products were purified using ExoSAP-IT (USB Corporation, Cleveland, OH, USA), according to the manufacturer's instructions, and then sequenced (University of Tennessee Molecular Biology Resource Facility, Knoxville, TN, USA). The resulting nucleotide sequences of the 5′ UTR and N^{pro} regions were aligned with previously published BVDV-1b genomes and two overlapping primer sets were designed to amplify the E2 glycoprotein region using a two-step RT-PCR assay, primers sequences are shown in Table 1. Briefly, five microliters of the extracted RNA were reverse transcribed using MMLV reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The PCR reaction was composed of 20 μM of each primer, 10 μl of 5X reaction buffer, 200 μm dNTPs mixture, one unit of Promega Taq polymerase and 5 μl cDNA in a final volume of 50 μl. The cycling conditions were 94°C for 2 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at 50°C and 1minute at 72°C. The amplified E2 PCR products were also sequenced as previously described. The resulting nucleotide and amino acid sequences were assembled using Geneious Software (http://www.geneious.com) and aligned with representative sequences from GenBank using MAFFT and MUSCLE software, respectively [31,32].

Phylogenetic analysis

Phylogenetic trees were constructed using the nucleotide sequences of the 5′UTR, N^{pro} and E2 glycoprotein region using the UPGMA method [33] employing the Jukes-Cantor model [34]. The tree topology was evaluated with 1000 bootstrap replicates.

Results

Farm historical data and clinical examination

The histories from farm 1 and farm 3 revealed severe respiratory tract infections in newborn calves three weeks prior to our sample collection, with mortality rate approaching 20%. Furthermore, historical data from the other two farms revealed newborn calf mortality, economic losses from underweight calves, calf pneumonia and calf diarrhea.

Figure 1. Clinical manifestation of BVDV infection in farm one. Ulceration in upper gum, hard palate and lower gum (black arrows).
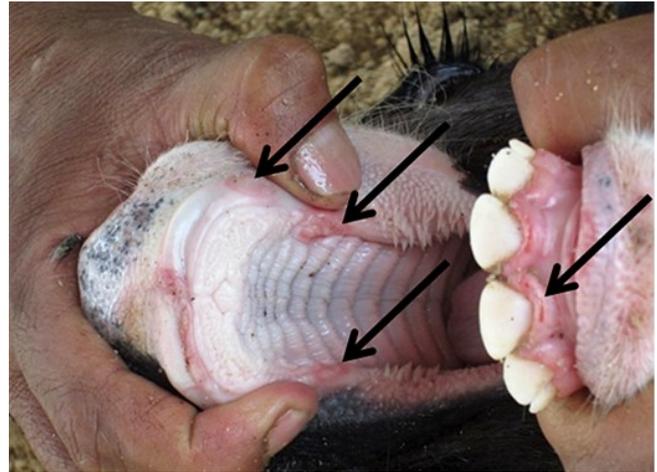

Figure 2. Clinical manifestation of BVDV infection in farm one. Necrosis in lower tongue. (black arrow)

The BVDV vaccine used by all the investigated farms was the Cattle Master 4 vaccine (Pfizer, inc., New York, NY), which contains inactivated BVDV-1a strain. Vaccine was administered to pregnant dams only in the late stage of pregnancy in two doses, according to the manufacturer's instructions. Clinical examination of 87 newborn calves on farm 1 showed normal body temperature in all calves, oral lesions in 16 (18.3%) calves, including erosions on mucosa, necrotic foci in the tongue and hyperemia of cheek papillae (Figure 1 and 2). About 10 (11.5%) of the calves were underweight and 8 (9%) had diarrhea. Calves on the other 3 farms were clinically normal.

Figure 3. Phylogenetic analysis of a 245 bp of the 5´UTR region using UPGMA method and Jukes-Cantor model in Geneious Software (http:// www.geneious.com).The tree topology was evaluated by 1000 bootstrap replicates. The Ismailia strain (red dot) groups with BVDV-1b (Highlighted in red).
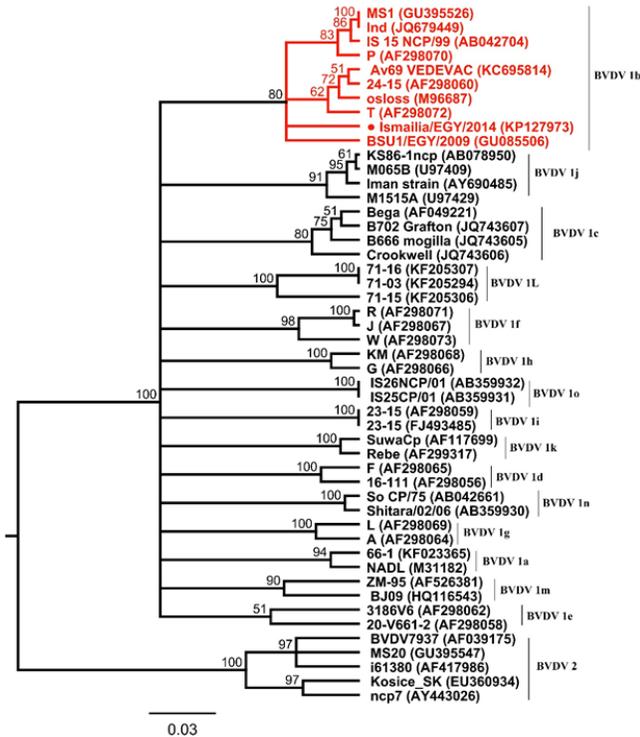
Figure 4. Phylogenetic analysis of a 380 bp of the N^{pro} region using UPGMA method and Jukes-Cantor model in Geneious Software (http:// www.geneious.com).The tree topology was evaluated by 1000 bootstrap replicates. The Ismailia strain (red dot) groups with BVDV-1b (Highlighted in red).
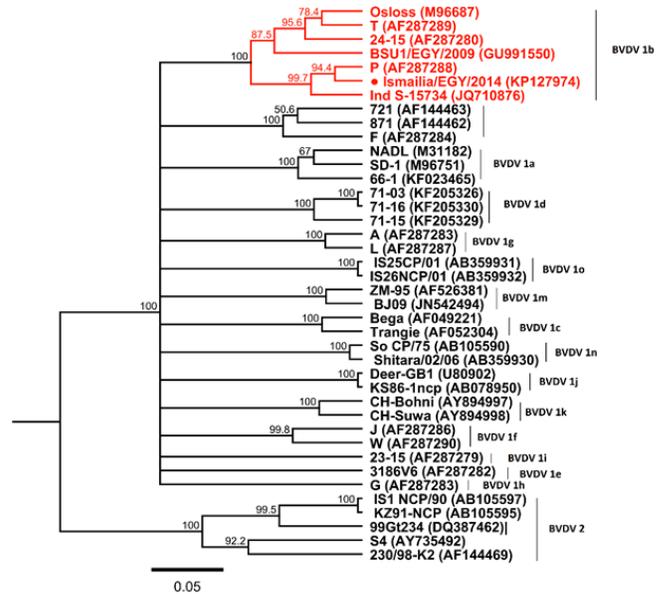

Multiplex real-time RT-PCR and RT-PCR

Following optimization, the sensitivity of the multiplex real time RT-PCR for detection of BVDV-1 and BVDV-2 was 55 and 20 genome equivalents per reaction, respectively. Of the 298 tested samples, 31 (10.4%) were positive, and all were BVDV-1. Only three samples, all from farm 1, had enough viral RNA that enable amlificaton of the 5´UTR,N^{pro} and the E2 glycoprotein regions by conventional RT-PCR (Table 2).

Percent of identity and phylogenetic analysis

The percent of identity of the characterized BVDV from farm 1 was 100% in the 5´UTR, N^{pro} and E2 glycoprotein regions indicating circulation of a single strain on this farm. The nucleotide sequences were submitted to GenBank (accession numbers: KP127973, KP127974 and KR014249 for 5´ UTR, N^{pro} and E2 glycoprotein regions, respectively). Phylogenetic analysis revealed clustering of this strain with BVDV-1b (Figures 3, 4 and 5).

The percent identity in the 5´ UTR (95.5% to 97.8%) revealed that this strain is related to other widely circulating BVDV-1b strains. Furthermore, the percent of identity was 89.8% and 90.5% with the BVDV-1b strain previously characterized from Egyptian goats [27] and the local Egyptian vaccinal

Table 2. Detection of BVDV by multiplex Real-time RT-PCR and RT-PCR

Herds	Animal species	Number of tested animals	No. of positive samples by multiplex real time RT-PCR	Ct value		No. of positive samples by RT-PCR for 5´UTR , N^{pro} and E2 glycoprotein regions
				< 30	>30	
Farm 1	Cattle	87	15 (17.2%) BVDV1	3/15	12/15	3/15
Farm 2	Cattle	103	6 (5.8%) BVDV1	0/6	6/6	0/6
Farm 3	Buffalo	46	4 (8.7%) BVDV1	0/4	4/4	0/4
Farm 4	Buffalo	62	6 (9.7%) BVDV1	0/6	6/6	0/6
Total		**298**	**31 (10.4 %)**	**3/31 (9.7%)**	**28/31 (90.3%)**	**3/31 (9.7%)**

strain (Iman strain, BVDV-1j), respectively. The nucleotide and amino acid sequence alignment of the E2 glycoprotein from the characterized Egyptian strain and other BVDV-1b strains showed high diversity, with identity ranging from 81.3% to 93.6% and 85.3% to 93.6%, respectively. In addition, the nucleotide and amino acid identity with BVDV-1a, strains used mainly in commercial vaccines, ranged from 73.1% to 76.6% and 76.7% to 79.7%, respectively.

Discussion

In this study, BVDV-1 was detected in blood samples from newborn calves less than two months of age on two dairy cattle and two buffalo farms in Ismailia province, Egypt. It has been previously reported that BVDV-1 is distributed worldwide in comparison to BVDV-2, which is reported mainly in the USA and Canada [35], Japan [36], South America [37] and in some European countries such as Austria [38]. The detected BVDV strain was further subtyped as BVDV-1b from one of the investigated dairy cattle farms. To our knowledge, this is the first study to confirm circulation of BVDV-1b in an Egyptian cattle population.

A multiplex real-time RT-PCR was optimized for detection of BVDV from clinical samples. Optimization of the real time RT-PCR protocol is a crucial step in ensuring high assay sensitivity and consistent results. The advantage of using real-time RT-PCR for BVDV screening is relatively high sensitivity in comparison to other diagnostic assays that enable detection of transiently infected animals where the viral load is relatively low [28, 39]. The real-time RT-PCR Ct values were >30 in 28/31 (90.3%) of positive samples indicating a low viral load which is consistent with BVDV transient infection or convalescent stage of infection. None of those positive samples had enough viral RNA to be amplified by the conventional RT-PCR assays used in this study. The inconsistent results between real-time RT-PCR and the RT-PCR assays are likely attributed to the very low viral load in most of the positive samples that could only be detected by the highly sensitive real-time RT-PCR assay.

The overall percentage of positive samples by real-time RT-PCR in our investigation was (10.4%). A high percentage of positive samples (17.2%) was detected in farm 1, which may be attributed to the presence of the active infection (clinical signs were consistent with BVDV) during the sample collection period. Three calves from this farm had Ct values ranging from (13-17.5) which indicate high viral loads

Figure 5. Phylogenetic analysis of a 1122bp region of the E2 glycoprotein region using UPGMA method and Jukes- Cantor model in Geneious Software (http:// www.geneious.com).The tree topology was evaluated by 1000 bootstrap replicates. The Ismailia strain (red dot) groups with BVDV1b (Highlighted in red).
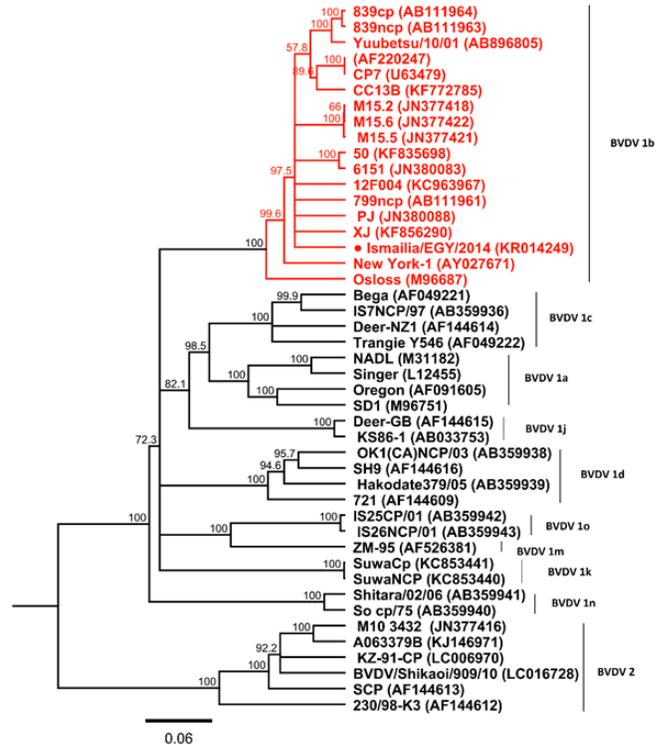
that may be associated with BVDV persistent infection.

The clinical examination of those three calves revealed stunted growth and curly hair coat as expected in PI calves. Due to feasibility reasons, we could not collect additional samples from those animals three weeks later to confirm their infection status. A commercially available real-time RT-PCR assay has been previuosly used to differentiate between BVDV transient infection and persistent infection [39]. Based on the study, a Ct cut-off value of 24.79 was suggested for the differentiation between the transiently infected and PI animals. While a specific value might have to be established for each laboratory and protocol used, the disparity between the Ct values obtained from PI suspect calves compared to other animals in this study suggests that Ct values may have value for this application.

In this study, the BVDV subtyping was performed through phylogenetic analysis of the 5′ UTR, N^{pro} and E2 glycoprotein regions. The detected BVDV strain was BVDV-1b. It has been previously reported that BVDV subtyping should be performed using at least

two areas of the genome and the results should be in agreement to confirm the subtype [40].

In Egypt, few articles discussing BVDV subtyping have been published. About nine strains of BVDV-1a have been previously detected in El-sharquia province, Egypt [26]. Unfortunately, the nucleotide sequences of those strains were not published in GenBank to include in our phylogenetic analysis. The BVDV-1b was detected before in two Egyptian goat kids [27]. The nucleotide alignment of the 5´ UTR of the BVDV-1b strain characterized in this study with the BVDV-1b strain detected in those goat kids showed a greater difference than the comparison with other worldwide bovine BVDV-1b strains. The close genetic relationship among worldwide BVDV-1b strains in the 5´ UTR in comparison to the strain characterized in this study make it difficult to trace the origin of the detected strain**.** It has been previously speculated that the origin of the BVDV-1a strains circulating in Egypt may be from the importation of foreign cattle breeds or using contaminated imported biological products, such as commercial vaccines [26].

The vaccination program implemented to control BVDV on all four dairy farms was the Cattle Master 4 vaccine (Pfizer, inc., New York, NY). The most common BVDV subtype used in BVDV vaccines, including the cattle master 4 vaccine, is BVDV-1a [41]. The nucleotide and amino acid sequences of the E2 glycoprotein region of the characterized strain in this study showed significant divergence in comparison to BVDV-1a strains. It has been previously reported that BVDV-1b could infect calves that had been vaccinated with a BVDV-1a strain, which raises many questions about the efficacy of BVDV-1a vaccines against BVDV-1b infection [41]. Ideally, each country should use vaccines containing strains similar to circulating field strains. The local Egyptian BVDV vaccine contains an inactivated BVDV-1j strain (Iman strain). Unfortunately, the nucleotide sequence of the E2 glycoprotein of the Iman strain was not published in GenBank to include in our phylogenetic analysis, but considering other BVDV-1j strains, the Iman strain is expected to have a significant variation in comparison to the BVDV-1b strain detected in this study.

In conclusion, we confirmed circulation of BVDV-1 in dairy cattle and buffalo farms in Ismailia province, Egypt. Continuous monitoring and updating the vaccine with currently circulating strains is crucial for control of BVDV in Egypt.

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