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Molecular Diversity and Histopathological Findings of Novel Bovine Viral Diarrhea Virus Strains Isolated from Bull Semen

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is one of the most common viral pathogens affecting the cattle industry worldwide. The present study aimed to molecularly characterize BVDV isolates that are currently circulating in breeding bulls farmed with cattle suffering from reproductive disorders, and also to assess the consequences of BVDV infection on bulls' semen quality and conception, and its pathological effects on the structure of testicular tissue and spermatozoa. For this purpose, semen, serum, and testicular samples were collected from four breeding bulls in four private dairy farms in the governorates of Kafr-El Sheik, Beni-Suef, Giza, and Assuit, in Egypt from April 2019 to May 2020. An evaluation of sperm abnormalities was carried out by assessing the integrity of the plasma and acrosomal membranes where severe damage and abnormalities were found. Ultrastructure analysis of the spermatozoa by transmission electron microscopy revealed the presence of a swollen plasma membrane with segmented outer acrosomal membrane of spermatozoa and vacuolar degenerated mitochondria. Histopathological examination of testicular and epididymal tissues indicated moderate to severe degenerative effects of virus infection on seminiferous tubules with hypospermatogenesis. By detection of virus antigen in the serum samples using ELISA, bulls were identified as persistently infected with BVDV. Virus isolation revealed four noncytopathic (NCP-BVDV) strains that were confirmed by fluorescent antibody technique (FAT) and amplification of the 5′ untranslated genomic region (5'UTR) and molecularly typed by amplification of the E^{rns} glycoprotein region. Isolates' Phylogenetic analysis revealed two subgenotypes: BVDV-1b (Genbank accession numbers; LC634512, LC634513, LC634515) and BVDV-1d (LC634516). According to the knowledge of the authors of the present study, the circulation of the BVDV-1d subgenotype is not reported in Egypt. Therefore, it would be of great importance to track circulating strains in specific countries for successful vaccination programs or accurate diagnostic tests, and this necessitates regular updates.

Keywords: BVDV, Isolation, Spermatozoa ultrastructure, Sperm abnormalities, Testicular histopathology

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the most important viruses affecting cattle worldwide, which has been recorded for more than six decades, however, it remains a crucial hazard in the cattle industry (Hou et al., 2019; Al-Kubati et al., 2021).

Bovine viral diarrhea virus is classified as a Pestivirus within the Flaviviridae family (Simmonds et al., 2017). Its genomic RNA is non-polyadenylated and single-stranded (ss RNA) with positive polarity and an average of 12.5 Kb in length. It has a long open reading frame (ORF) flanked at the 5' and 3' termini with a highly conserved untranslated region (UTR) for translation and replication controls, respectively. The ORF encodes polyproteins that are cleaved into four structural (C, E^{rns}, E1, and E2) and eight nonstructural (Npro, p7, NS2-3, NS4A, NS4B, NS5A, and NS5B) proteins by viral and cellular proteases during and after translation (Neill, 2013; Tautz et al., 2015). More than two decades ago, BVDV isolates were classified as BVDV-1 or BVDV-2, based on phylogenetic analysis of incomplete sequences (Ridpath et al., 1994; Simmonds et al., 2017). Later investigations revealed an expanding number of BVDV-1 and

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BVDV-2 subgenotypes, and it is now widely understood that pestiviruses are genetically highly variable, even within single subgenotypes (Yeşilbağ et al., 2017).

Bovine viral diarrhea virus affects the immune, respiratory, reproductive, and enteric systems. Reproductive problems caused by BVDV include early embryonic death, fetal mummification, abortions, and the birth of deformed calves (Stott et al., 2012; Scharnböck et al., 2018). In bulls, BVDV can produce persistent localized testicular infection (Givens et al., 2003; Newcomer et al., 2014) with testicular hypoplasia, degeneration of seminiferous tubules, and specific dissemination of BVDV antigen throughout the male genital tract.

The seroprevalence of infection with BVDV in non-vaccinated cattle herds ranges from 28% to 66% depending on geographical areas, and from 40% to 90% in individual cattle. The persistently infected (PI) cattle percentage is between 0.5% and 2.5% in most countries without any control program being in place against the virus (Velasova et al., 2017; Scharnböck et al., 2018; Oguejiofor et al., 2019). Vaccination can be used to prevent infection but, without the elimination of PI animals, it is impossible to eradicate the virus in a susceptible population. Diagnostics and vaccines that act well against analogous strains may be less effective against genetically different viruses. Therefore, considering the genetic variants described for BVDV-1 and BVDV-2 may play a role in disease prevention (Vilcek et al., 2001; Bauermann et al., 2013).

In Egypt, the prevalence of BVDV infection in cattle is about 40-47.4%, and the percentage of PI animals ranges from 1.6% to 18% based on the following factors; the variance observed in the regions where populations were investigated, the management system in the analyzed herd, and the diagnostic tests used in each study (El-Bagoury et al., 2014; Selim et al., 2018; Atwa et al., 2019). Only a few studies have been conducted on the characterization and sub-genotyping of the circulating virus (El-Kholy et al., 2005; Abdel-Latif et al., 2013; Soltan et al., 2015b). Thus, the primary goals of this study were to molecularly characterize BVDV isolates that are currently circulating in breeding bulls. Moreover, the study sought to assess the effect of BVDV infection on bulls' semen quality and conception as well as its pathological effects on the structure of testicular tissue and spermatozoa.

MATERIALS AND METHODS

Ethical approval

All procedures for samples collection and animals' manipulation were performed following the protocol approved by the Ethical Research Committee of the Animal Reproduction Research Institute, Agriculture Research Center ARC with Code No.2 11429.

Investigation of the reproductive problem and data collection

A history of poor conception rates in dairy cows in four private farms in the governorates of Kafr-El Sheik, Beni-Suef, Giza, and Assuit, in Egypt, was reported from April 2019 to May 2020. Data records showed 125 cases of abortion in cows between the third and fifth month of gestation, 17 cases of early fetal deaths and reabsorption, and 74 normal parturitions with some calves displaying congenital malformations. These herds had received only the official vaccines (foot-and-mouth disease and Rift Valley fever vaccines). For laboratory investigation conducted in the present study, semen and serum samples from four local hybrid Holstein breeding bulls, with the age range from 4 to 6 years, and body weights range from 600 to 700 Kg (one per farm) were collected for semen evaluation and detection of pathogenic agents. Serum samples were taken again 3 weeks after sampling. In this regard, one bull was slaughtered (Beni-Suef farm), the testes were subjected to gross examination and were then processed for virus isolation and histopathological examination.

Sample collection and preparation

Two ejaculates were obtained in the early morning (average volume 4 to 6 ml) from each bull with a pre-warmed artificial vagina (40-42°C). The ejaculates were tested for sperm abnormalities as soon as they were collected. Then, 1 ml of each semen sample was transferred to the laboratory on ice for virus isolation. From testis, 1 g was homogenized in 5 ml of phosphate-buffered saline (PBS) for virus isolation, and the other part was immersed and fixed in formal saline solution (10% formalin in 0.9% NaCl) in "1:10 proportion" for histopathological examination. The blood samples were taken twice, 14 days apart, from the coccygeal vein of a properly secured bull and centrifuged (Beckman, USA) at 3000 rpm for 15 minutes in clean sterilized tubes. No medication was used before sampling; Serum samples were collected and preserved at -20°C for serological examination.

Sperm abnormalities assessment

Plasma membrane integrity

The plasma membrane integrity of bull spermatozoa was evaluated using the hypo-osmotic swelling test, as described in Revell and Mrode (1994).

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Acrosomal defect

Acrosome integrity was examined using silver nitrate staining based on the procedure described in Chinoy et al. (1992).

Histopathological examination of testicular tissue and ultrastructure analysis of spermatozoa

Formalin-fixed tissue specimens (testicular and epididymal, after gross examination) were processed in an automated tissue processor. The tissue sections were stained with hematoxylin and eosin and examined microscopically (Bancroft and Gamble, 2018). The ultrastructural changes in spermatozoa were examined and evaluated using transmission electron microscopy (TEM, JEOL-EM-100 S at 80 Kv Tokyo, Japan) at the Electron Microscopy Unit, Faculty of Agriculture, Cairo University, Giza, Egypt) and photographed for further analysis as mentioned in Boonkusol et al. (2010).

Detection of the causative agent

After exclusion of bacterial and parasitic infection, viral diagnosis was performed with a focus on BVDV infection.

Detection of bovine viral diarrhea virus antigen by ELISA

Four Serum samples were used for the detection of BVDV infection by anti-p80/p125 monoclonal antibody-coated double sandwich ELISA plate (INGEZIM BVD DAS) kits (Ingensasa, Madrid, SPAIN) following the manufacturer's instructions.

Virus isolation

A reference strain National Animal Diseases Laboratory (NADL) supplied from Ames Iowa, USA was used as a positive control during the isolation and identification of the virus. Semen and testicular samples were processed and diluted as 1.0 ml^{-1} in PBS (with antibiotic/antimycotic at the recommended concentration) 100 IU Penicillin, 100 µg dihydrostreptomycin) as mentioned in OIE (2018). The supernatant of each sample was inoculated three successive passages on mycoplasma and BVDV-free MDBK monolayer cell culture (6 days each) Supplied by the national animal disease center, Ames Iowa, USA. Daily microscopic examinations with an inverted microscope (Zeiss, USA) were performed to monitor the cytopathic effects (CPE).

Virus identification using fluorescent antibody techniques

On coverslips, monolayer MDBK cells were inoculated with the second passage and after 24 hours of the third passage, the inoculated cells were air-dried and fixed with chilled acetone. Viral antigens were captured and detected by direct fluorescence isothiocyanate (FITC) conjugated anti-BVDV as recommended manufacturer dilution (CJ-F-BVD; VMRD, Pullman, WA 99163 USA). The mounted coverslips were examined using an inverted epifluorescence trinuclear Nikon microscope (Nikon ECLIPSE-TS 100, Japan with 10× plan achromatic lens and a digital camera DS-US and it is NIS elements software, Lens 10X).

Viral genome detection

RNA isolation

Total RNA was extracted from the tissue culture suspension of the third passage after three successive freezing and thawing cycles for each sample using QIAzol Lysis Reagent (QIAGEN, Germany) according to the manufacturer's instructions, and it was kept at -80°C. Complementary DNA (cDNA) was synthesized with a RevertAid First Strand cDNA Synthesis kit (Thermoscientific, Germany) following the manufacturer's instructions, and was stored at -20°C. The positive (BVDV-NADL strain inoculated cell culture) and negative (BVDV-free cell culture) controls were included in all the assay steps, from RNA extraction to amplification.

5' untranslated genomic region amplification

Amplification of a 208 bp fragment within the 5' UTR of the viral genome was based on a primer set outlined in Hoffmann et al. (2006) and reported in OIE (2018). The reaction mixture (20 µl) consisted of DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific, Lithuania), 20 pmol of each forward (190-F) and reverse (V326) primers and 4 µl of cDNA. The reaction was performed in a thermocycler (Nexus Gradient Eppendorf, Germany). The primer sequences, thermal conditions, and expected PCR products were outlined in Table 1.

Virus genotyping by nested multiplex RT-PCR within the E^{rns} region

As mentioned by Sullivan and Akkina (1995) and shown in Table 1, the first round of the cDNA amplification was carried out in a single tube reaction using P1 and P2 primers. Then the nested amplification was performed using the type-specific primers (TS1 for BDV, TS2 for BVDV-2, and TS3 for BVDV-1) and P2 on amplified products within the first round. The amplified products were analyzed using 1.5% agarose gel electrophoresis on a UV transilluminator.

Partial sequencing and analysis of the E^{rns} region

The genotype E^{rns}-specific PCR products were subjected to purification by Gene JET Gel Extraction Kit (ThermoScientific K0691, Germany) following the manufacturer's instructions. The purified PCR products were subjected to sequencing in an automated ABI 3730×l DNA sequencer (Applied Biosystems, USA) using forward and

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reverse primers. Sequencing was performed only by combining the traditional Sanger technology with the new 454 technology. The CLUSTAL W was used to align the obtained nucleotide and amino acid sequences with each other and with sample sequences from Genbank. Then, a phylogenetic tree was generated using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993); the evolutionary analyses were carried out in MEGA X (Kumar et al., 2018).

Statistical analysis

The semen evaluation and a number of service/conception values were expressed as means ± standard error for all variants using one-way ANOVA followed by Bonferroni multiple comparison tests to determine the significance of differences, which was set at p value ≤ 0.05

Table 1. The oligonucleotide primers and amplification conditions used in bovine viral diarrhea virus detection and typing

Primer name	Primer sequence	PCR Conditions	Amplicon size (bp)	Reference
190-F V326	5'-GRAGTCGTCARTGGTTCGAC-3' 5'-TCAACTCCATGTGCCATGTAC-3'	95°C for 5 min and 35 cycles of 45 sec at 95°C, 45 sec at 58°C, 45 sec at 72°C and final extension for 10 min at 72°C	208	Hoffmann et al. (2006)
P1-F P2-R	5'-AACAAACATGGTTGGTGCAACTGGT-3' 5'- CTTACACAGACATATTTGCCTAGGTTCCA-3'	(First round) 94°C for 5 min and 30 cycles of 60 sec at 94°C, 60 sec at 55°C, 60 sec at 72°C and final extension for 10 min at 72°C	826	Sullivan and Akkina (1995)
TS1-F TS2-F TS3-F P2-R	5'-TATATTATTTGGAGACAGTGAATGTAGTAGCT-3' 5'- TGGTTAGGGAAGCAATTAGG-3' 5'- GGGGGTCACTTGTCGGAGG-3' 5'- CTTACACAGACATATTTGCCTAGGTTCCA-3'	(Nested amplification) 95°C for 5 min and 35 cycles of 60 sec at 94°C, 45 sec at 55°C, 45 sec at 72°C and final extension for 10 min at 72°C	(TS1-F, P2) 566 (TS2-F, P2) 448 (TS3-F, P2) 223	

RESULTS

Services/conception and conception rates of *bovine viral diarrhea virus* infected farms

Based on the collected data, cows fertilized with semen from BVDV-infected bulls showed a significantly (p ≤ 0.05) lower conception rate with a mean percentage of 24.42% ± 0.98% (53/216) and 6.12 ± 0.53 service per conception, compared to those bred with BVDV-free semen, for which the respective values were 60.74% ± 2.04% (95/158) and 2.67 ± 0.75.

Assessment of sperm abnormalities

Microscopical examination of the stained spermatozoa revealed a highly damaged plasma membrane (53-59.67%) and elevated acrosomal defect (27.66-36%). The mean and standard deviation for acrosomal defect and plasma membrane integrity for each bull were calculated and are shown in Table 2.

Histopathological findings and ultrastructure of spermatozoa

Grossly, the examined testes and epididymis were slightly congested and had a small size (atrophy). The pathological changes observed in the testicular sections were characteristic of chronic orchitis with moderate to severe degeneration, Orchitis represented by moderate infiltration of mononuclear inflammatory cells (mostly lymphocytes and/or plasma cells and few histiocytes) in the interstitial tissues along with oedema and congestion of blood vessels (Figure 1 A). Also, many giant multinucleated cells of eosinophilic cytoplasm were seen in seminiferous tubules (Figure 1 B), while those observed in the epididymal sections were characteristic of chronic epididymitis with mild to moderate degeneration, some ductal tubules showing homogenous eosinophilic masses of lysis epididymal spermatozoa in lumen and absence of spermatozoa in the lumen of other tubules. Moreover, hyperplasia of the lining epithelial cells of the tubules was seen in more than one columnar cell layer as shown in Figure 2. Transmission electron microscopy images of the sperm's sagittal sections indicated the presence of swollen plasma membranes with segmented outer acrosomal membranes. The nucleus content was not homogenous in terms of electron density. Transverse sections of the neck region showed vacuolar degenerated mitochondria with electron-translucent spaces and absence of mitochondrial cristae (Figures 3 A and B).

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Table 2. Evaluation of sperm abnormalities in bulls infected with Bovine viral diarrhea virus

Bull number (Governorate and Year)	Acrosomal defect (%)	Plasma membrane integrity (%)
1 (Kafr-El Sheik, 2019)	36.00 ± 3.61	54.00 ± 4.36
2 (Beni-Suef, 2019)	31.67 ± 3.53	59.67 ± 5.61
3 (Giza, 2019)	27.66 ± 4.41	53.00 ± 6.09
4 (Assuit, 2020)	32.00 ± 1.73	54.67 ± 5.51

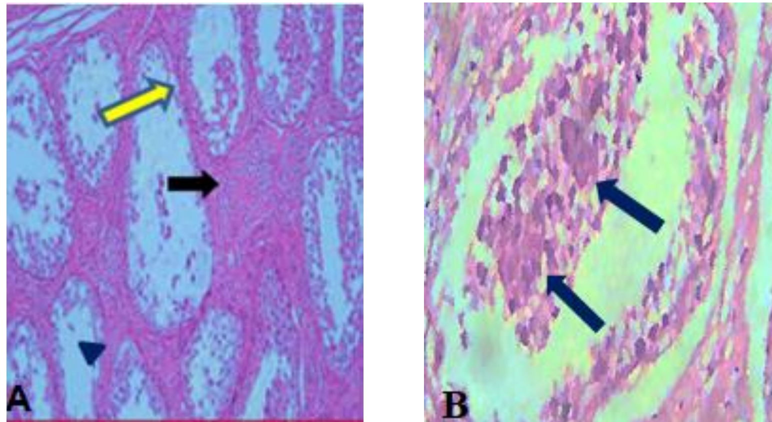

Figure 1. Histopathological alterations associated with Bovine viral diarrhea virus infection in bull testis. **A:** Severe degeneration with thickened basement membranes of seminiferous tubules (yellow arrow) and hypospermatogenesis (blue triangle). Also, mononuclear inflammatory cells infiltration (mainly lymphocyte and plasma cells) with congested blood vessels and oedema (black arrow) in interstitial tissues were seen. (H&E, X100). **B:** Giant cells of multinucleated eosinophilic cytoplasm in seminiferous tubules, indicated by the arrows (H&E, X400).

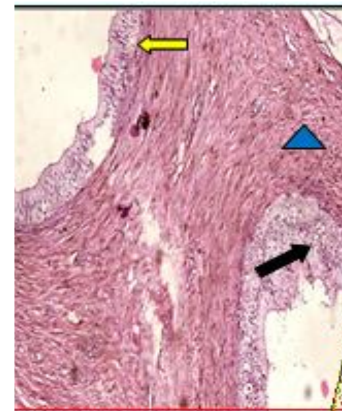

Figure 2. Histopathological alterations associated with Bovine viral diarrhea virus infection in bull epididymis. The epididymis affected by Bovine viral diarrhea virus infection showing moderate vacuolar degenerated tubules (yellow arrow) with cellular debris and reduced sperm in lumen, and epithelial cell hyperplasia in many tubules (black arrow). Lymphocytic inflammatory infiltration (blue triangle) in interstitial tissues is also visible (H&E, X100).

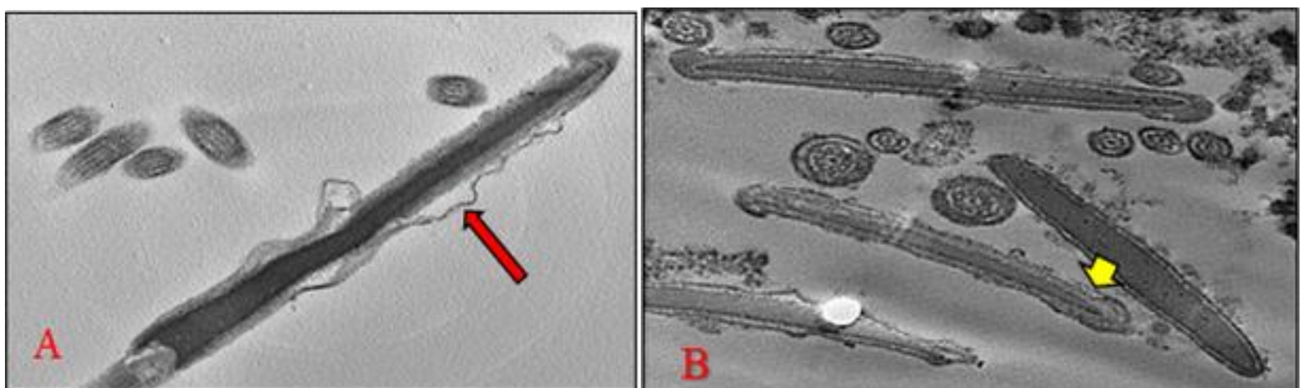

Figure 3. The sagittal section of the sperm head from bull semen under transmission electron microscopy **A:** sperm head sagittal section shows swollen plasma membrane (red arrow). **B:** The segmented outer acrosomal membrane (yellow arrowhead); the nucleus content is not homogenous in terms of electron density. The transverse sections of the neck region show vacuolar degenerated mitochondria with electron-translucent spaces and the absence of mitochondrial cristae (X15000 and X12000).

Bovine viral diarrhea virus detection

ELISA

Using a double-sandwich ELISA, all serum samples (at the time of collection and three weeks later) tested positive for the presence of BVDV antigen. The optical density obtained was 15% greater than the cut-off value considered positive according to the kits used.

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Virus isolation

After three successive serial passages, no specific CPE was detected for BVDV on MDBK cells inoculated with previously prepared semen samples and testicular tissues.

Fluorescent antibody techniques

Perinuclear and intracytoplasmic fluorescence granules were detected in the MDBK cells inoculated with the four semen samples and testicular one after the third passage (Figure 4).

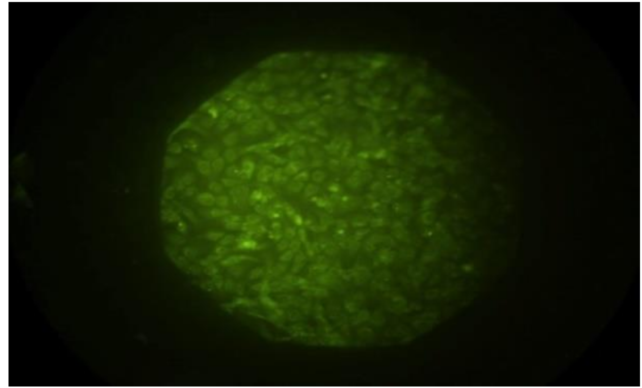

Figure 4. Identification of Bovine viral diarrhea virus isolated from bull semen using FAT. Bovine viral diarrhea virus-free MDBK cells inoculated with the third cell culture passage of the samples, Perinuclear intracytoplasmic fluorescence granules were observed (X100).

Detection of bovine viral diarrhea virus genome

5' untranslated genomic region amplification

As an additional confirmation of the isolated virus, amplicons with the size of 208 bp were clearly detected as a result of the 5'UTR amplification of the four isolates (Figure 5).

Nested multiplex Reverse Transcription -PCR within the E^{rns} glycoprotein region

The nested PCR products showed specific size for BVDV-1 at 223 bp. Specific fragments for genotype BVDV-2 and BDV were not detected with a predicted size of 448 and 566 bp, respectively (Figure 6).

Partial E^{rns} glycoprotein region sequencing and phylogenetic analysis for virus subgenotyping

The sequences of the E^{rns}-amplified fragments of the four isolates (B2, C2, F2, and G2), as well as the reference one (E2), were submitted to Genbank with the following accession numbers: LC634512 (strain ID Sakha-ARRI-eg2019), LC634513 (strain ID Suef-ARRI-eg2019), LC634515 (strain ID Giza-ARRI-eg2019, LC634516 (strain ID Assuit-ARRI-eg2020), and LC634514 (NADL reference strain). Nucleotide sequences were aligned with each other to determine sequence identity among the isolated strains (figures 7 and 8), and with the corresponding sequences of BVDV-1 strains in Genbank. The identity percentage among the Sakha-ARRI-eg2019, Suef-ARRI-eg2019, and Giza-ARRI-eg2019 strains was 100% in the E^{rns} glycoprotein region, and the phylogenetic relationship revealed clustering of these strains with BVDV-1b (showing the highest sequence identity of 98%) and noncytopathic BVDV-1 strains originally isolated from alpaca, in the USA in 2015 (accession nos. JX297515.1, JX297512.1, JX297520.1, JX306012.1, JX297521.1, and JX297519.1). The Assuit-ARRI-eg2020 strain was found to be different from the other isolates with an identity percentage of 90% and phylogenetic analysis confirmed the clustering of this strain with the BVDV-1d subgenotype. It shared the highest homology of 98% with the BVDV-1d LV01-2018 strain (MG923683.1) originally isolated from cattle in Brazil, and with three other BVDV-1d strains (MF166858.1, KT951841.1, and KT943518.1) isolated from yak and cow in China (Figure 9).

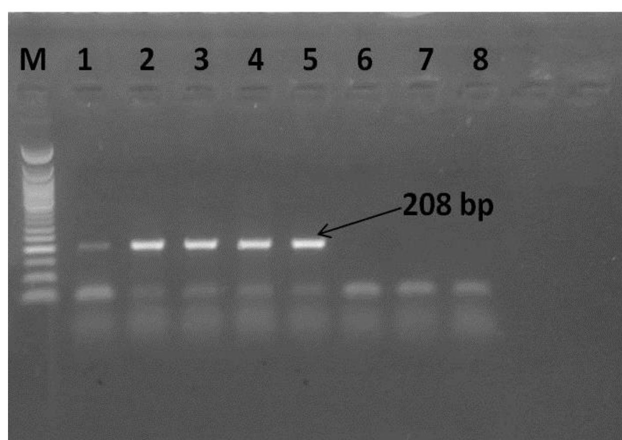

Figure 5. Reverse transcription-PCR within the 5' UTR for detection of Bovine viral diarrhea virus in bull semen. Agarose gel (1.5%) analysis of RT-PCR products for genomic detection within 5' UTR. Lane M represents the 50 bp DNA ladder, lane 1 is a positive control (NADL strain), lanes 2–5 show the amplified products of the novel isolates (208 bp), lanes 6 and 7 are negative controls, and lane 8 is a non-target control.

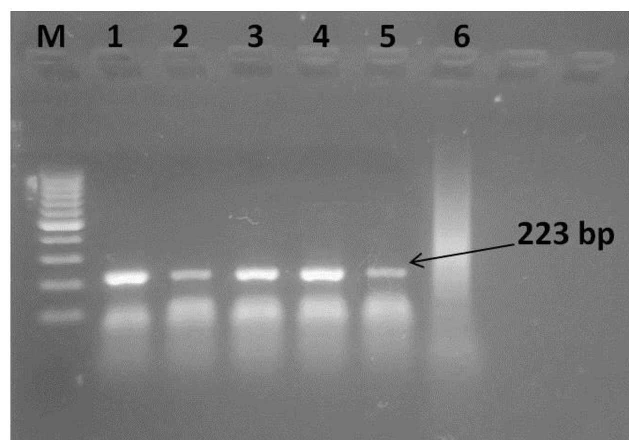

Figure 6. Nested multiplex RT-PCR within the E^{rns} region for typing of BVDV isolated from bull semen. Agarose gel (1.5%) analysis of nRT-PCR products with type-specific primers. Lane M represents the 100 bp DNA ladder, lane 1 is a positive control (NADL strain as genotype 1), lanes 2–5 are the amplified products of the novel isolates (223 bp; genotype 1), and lane 6 is a negative control.

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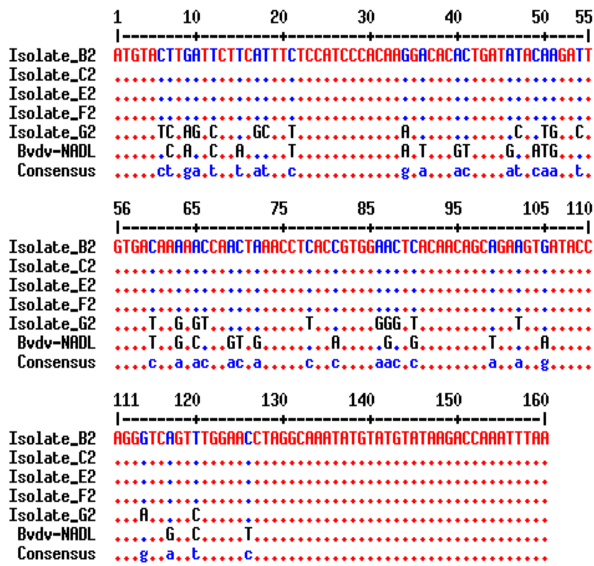

Figure 7. Multiple sequence alignment of nucleotide sequences of polyproteins from Bovine viral diarrhea virus isolates and NADL strains.

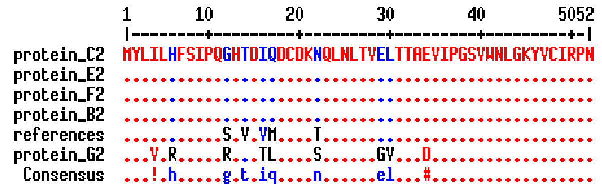

Figure 8. Multiple sequence alignment of predicted amino acid sequences of polyproteins from Bovine viral diarrhea virus isolates and NADL strains.

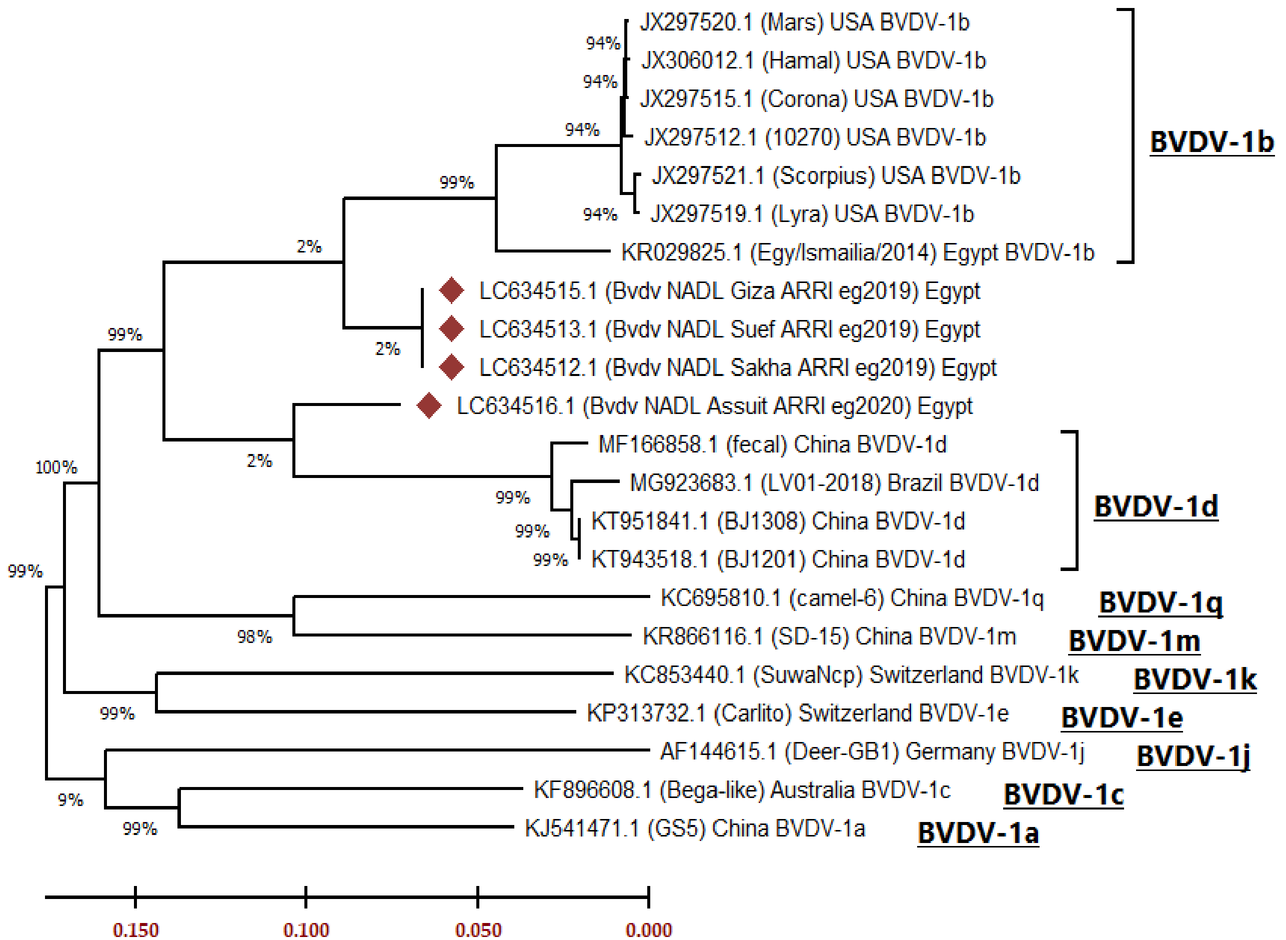

Figure 9. Phylogenetic analysis of Bovine viral diarrhea virus strains isolated from bull semen Phylogenetic tree was constructed using the Maximum Likelihood method and Tamura-Nei model with 1000 bootstrap replicates in MEGA X. Sequencing was conducted on the second step nested multiplex PCR positive samples to confirm the Bovine viral diarrhea virus genotype based on the partial E^{rns} gene. The accession number, isolate name, and country are listed at the beginning of each isolate. The horizontal bar indicates the substitutions per site, and the vertical bars and letters indicate different BVDV-1 subgenotypes. Isolates identified in this study are marked by the red diamond symbol.

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DISCUSSION

It is widely known that maintaining animal health is crucial for improving livestock performance. Among a variety of diseases, BVDV is a difficult challenge for any cattle producer. Understanding the pathogenesis of this virus represents a powerful weapon against the threat of infection.

Persistently infected animals are usually virus carriers for the rest of their lives, shedding substantial amounts of the virus through most of their body excretions and secretions (Alenius et al., 1997; Garoussi et al., 2019). In particular, PI bulls were shown to regularly shed BVDV-contaminated semen of significantly reduced quality (El-Mohamady et al., 2020). In the present study, the assessment of semen collected from breeding bulls, either by direct microscopic examination or TEM, revealed damage of the sperm plasma membrane with high acrosomal defect (Table 2), which was consequently reflected in low conception rates. As a result, BVDV infection has the potential to affect testicular function as well as producing sperm abnormalities. This could have an impact on cows' conception rates and fertility after natural breeding or artificial insemination. Moreover, the semen of these infected bulls could be a source of infection for receptive cows (Oguejiofor et al., 2019; Montoya-Monsalve et al., 2021).

A consistent finding in PI bulls is the very high titer of the virus in the semen, usually at least 100 times higher than in the blood, which may lead to replication of the virus within the male genital tract, for example, in the prostatic and vesicular glands, and epididymides (Selim et al., 2018). In previous studies, the BVDV antigen was detected in the media of arterial walls in the male genital organs. The BVDV antigen was also identified in the testicular Sertoli cells and to a limited extent, in the intact spermatogonia, but not in Leydig cells (Barlow et al., 1986; Brodersen, 2004; Borel et al., 2007). Moreover, Givens et al. (2003) reported that BVDV antigen was found within the seminiferous tubules near the basement membrane. The above-mentioned studies reported reproductive tract lesions, such as testicular hypoplasia and (to a greater or lesser extent) degeneration of seminiferous tubules. This came in accordance with findings of the current study in the testicular and epididymal tissues which presented different degrees of degenerative changes.

Novel pestivirus isolation and characterization will have far-reaching implications for epidemiology and vaccine development research. Since various genotypes of pestiviruses can be identified in different hosts, diagnostic tests that can discriminate between known pestivirus genotypes are required (Booth et al., 2013). Differences among BVDV strains can be assessed using a variety of approaches, including monoclonal antibody reactions, cross-neutralization assays, and nucleotide sequence comparisons (Yeşilbağ et al., 2017). In the present study, the bulls were identified as being infected with BVDV by the anti-p80/p125 monoclonal antibody-coated double sandwich (DAS) ELISA in serum samples. The BVDV p80 antibody is a nonstructural protein (NS3) that can be identified 2 to 3 weeks after natural infection (Sayers et al., 2015). Antibodies against the NS2-3 BVDV were detected in dairy cattle that had a history of reproductive problems and did not receive the BVDV vaccine (Curti and Jaeger, 2013). This indicated that dairy cattle were exposed to the circulating BVDV because they had not been vaccinated (Wuryastuti et al., 2016).

The virus isolated from semen samples was identified by FAT (Figure 4) and RT-PCR amplification within the 5' UTR (Figure 5). Furthermore, using primers that are homologous to the conserved E^{rns} part of the pestivirus genomic sequences ensured that all strains were amplified (Sullivan and Akinna, 1995). The E^{rns} genomic region nested multiplex PCR is able to differentiate the BVDV-1, BVDV-2, and BDV genotypes during the initial screening of the BVDV-infected samples.

The specificity of this assay is given by the capability of the type-specific primers (TS1, TS2, and TS3) to identify genomic sequences specific to each corresponding pestivirus genotype within the first PCR product amplified by the P1 and P2 primers (Sullivan and Akkina, 1995) In the present study, no cross-reactivity between type-specific primers and heterogeneous pestivirus genotypes was found, only one amplified product was produced. Here, BVDV-1 was the detected genotype in the tested field samples (Figure 7). Based on this result and previous research findings, it is concluded that the worldwide spread of BVDV-1 isolates (88.2%) is substantially wider than the distribution of BVDV-2 isolates (11.8%, Soltan et al., 2015a; Yeşilbağ et al., 2017).

Phylogenetic analysis of partial and whole genomic sequences provides more precise information than antibody-based investigations and allows the quick detection and differentiation of BVDV-1 and BVDV-2 subgenotypes, in addition to the recognition of new subgenotypes. Different genomic areas have been used to genotype and classify BVDV and other pestiviruses. The 5'UTR is a highly conserved region of the viral genome, equipped with an internal ribosome entry site that is primarily involved in the translation of viral polyproteins. Partial 5'UTR sequences have been commonly employed for phylogenetic studies and genotyping of BVDV isolates. However, their limited sequence length and lack of diversity are regarded as the primary drawbacks of their use for phylogenetic analysis (Yeşilbağ et al., 2017). The E^{rns} polyprotein region presents intracellular ribonuclease activity that prevents the production of type I interferons and facilitates the establishment of persistent infection (Shirato et al., 2004; Li et al., 2008). The phylogenetic analysis conducted in the present study, which was based on the E^{rns} glycoprotein region, segregated the isolated BVDV strains into two subgenotypes. Three isolates (Sakha-ARRI-eg2019, Suef-ARRI-eg2019, and Giza-ARRI-eg 2019), with 100% identity, were identified as belonging to the BVDV-1b subgenotype, suggesting the spread of an identical strain in the

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corresponding governorates (Kafr el Sheikh, Beni-Suef, and Giza). The other isolate (Assuit-ARRI-eg2020) was identified as BVDV-1d subgenotype, and this was the first study to report the existence of this subgenotype in Egypt. Previously, the BVDV-1a strain had been detected in Egyptian cattle (El-Kholy et al., 2005) and BVDV-1b in Egyptian goats (Abdel-Latif et al., 2013), dairy cows, and buffalo farms, and these showed 89.04% homology with the BVDV-1b isolate identified in this study (Soltan et al., 2015a).

According to previously published data, BVDV-1b is the most common subgenotype in the world, followed by BVDV-1a and -1c. At the individual continent level, BVDV-1b is the most common subgenotype in the Americas, Asia, and Europe (Yeşilbağ et al., 2017). In comparison, almost all the Australian field isolates have been identified as BVDV-1c. In Africa, the overall number of the studied viral isolates is comparatively low and does not represent the entire continent, however, BVDV-1a is considered the most frequently detected subgenotype in South Africa, and BVDV-1b is the predominant subgenotype circulating in Egypt (Abdel-Latif et al., 2013; Soltan et al., 2015b; Yeşilbağ et al., 2017).

Currently, at least 21 BVDV-1 subgenotypes are either widely accepted or have recently been proposed. It is increasingly probable that further subgenotypes will be reported due to the very diverse structure of pestivirus genomes (Yeşilbağ et al., 2017). Therefore, the tracking of strains circulating in a particular country is a useful indicator for the planning of successful vaccination programs or accurate diagnostic tests, and it necessitates regular updates.

CONCLUSION

In conclusion, the molecular investigation verified the circulation of BVDV-1b, and BVDV-1d in breeding bulls in Egypt for the first time. The isolated BVDV strains had negative effects on semen quality and conception rate and produced pathological changes in the bulls' reproductive organs. Thus, regular screening and vaccine matching with the currently prevalent BVDV strains are necessary to ensure accurate management programs.

DECLARATIONS

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Authors' contribution

Yasser Gamil Mahmoud Abd El-Hafeiz designed the plan of the study and participated in the manuscript writing. Rania Salah El-Mohamady performed virus isolation and identification, and participated in data analysis and manuscript writing. Tahani Salama Behour performed the molecular work and participated in data analysis and manuscript writing. Ahmed Mohamed Saber Mahmoud Azab performed the histopathological examination. Mohamed Ahmed Assi performed the ultrastructure finding of the sperm. Magdy Ramadan Badr evaluated semen abnormalities. Ragab Abdel Monem Dohreig carried out samples and data collection. Inas Mohamed Gamal and Hany Mohamed Hassan performed the ELISA work. All authors have read and approved the data and final draft of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

Consent to publish

The authors agreed to publish the article.

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