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ISOLATION, IDENTIFICATION, MOLECULAR CHARACTERIZATION AND TISSUE CULTURE ADAPTATION OF BOVINE VIRAL DIARRHEA (RELATED VIRUS) RECENTLY ISOLATED IN EGYPT

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ABSTRACT

This study reports a wave of abortions, diarrhea and a sharp drop in milk production at a dairy farm. The farm was routinely vaccinated with an imported vaccine against BVDV, containing the typical strains of Pestiviruses (BVDV-1 and BVDV-2). A total of 13 serum samples, 5 vaginal discharge samples, and 5 fecal samples were collected from aborted cows and from cows showing persistent diarrhea. All the samples were screened for potential microbial causes of abortion (viral or bacterial) using PCR. Out of 23 samples tested, only one vaginal discharge sample yielded a positive PCR result at the expected size of 288 bp. The primers used were designed with high sensitivity to 5'-UTR–based-RTPCR assays for the detection of Pestiviruses. The PCR product was sent for sequence analysis, and results were submitted to GenBank Accession Number #OR425033, and designed as GERD/VSVRI/pesti-Giraffe/2022. The virus was then successfully isolated and propagated in MDBK cells through three successive blind passages. A distinct cytopathic effect (CPE) was observed 2–3 days after an incubation period post-virus inoculation, characterized by vacuolation, cell rounding, and cluster formation 72 hours after infection. PCR was carried out on each passage and gave a specific band at the expected size. Further analysis through sequence alignment and phylogenetic analysis revealed that the isolate is closely related to the Pestivirus giraffe, especially Pestivirus PG-2. This marks the first record of detection, isolation, and characterization of this strain in Egypt. Therefore, this epidemic was caused by a newly introduced strain recorded in Egypt. So, the imported vaccines would not provide protection, and the local vaccine need to be updated to include this Pestivirus strains.

Keywords: Pestivirus PG-2, PNS, MDBK, 5`UTR, CPE, Phylogenetic analysis, PCR, BDV, BVDV, Abortion

INTRODUCTION

The Genus Pestivirus, part of the family *Flaviviridae,* affects a wide variety of animal species causing devastating economic losses in livestock. This genus includes border disease virus (BDV), classical swine fever virus (CSFV), bovine viral diarrhea virus-1 (BVDV-1), BVDV-2, and several newly identified Pestivirus species like antelope Pestivirus, atypical porcine Pestivirus (APPV), giraffe Pestivirus (Yuan *et al.,* 2022). Pestivirus includes a wide variety of highly relevant animal pathogens like bovine viral diarrhea virus, which is divided into BVDV-1 and

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BVDV-2 and known as Pestivirus A and Pestivirus B), first described in 2004 (Leveringhaus *et al.,* 2022). Another newly emerging virus is HoBi-like Pestiviruses (HoBi-PeV), classified as a distinct species (Pestivirus H), sharing some similarities with BVDV-1 and BVDV -2.Additionally, two distinct giraffe Pestiviruses (GPeV), originating from Kenya and classified as Pestivirus G, have emerged (Leveringhaus *et al.,* 2022). Taxonomy of the genus Pestivirus has been revised, family *Flaviviridae*, adding seven new species to the original four. The species are now named in a host-independent manner using the Pestivirus X format. While virus isolates retain their original names, species designation have changed with BVDV-1, BVDV-2, CSFV, and BDV now referred to as Pestivirus A (original designation Bovine viral diarrhea virus 1), Pestivirus B (Bovine viral diarrhea virus 2), Pestivirus C (Classical swine fever virus) and Pestivirus D (Border disease virus), respectively. The seven newly recognized Pestivirus species, along with example isolates, include: Pestivirus E (pronghorn Pestivirus), Pestivirus F (Bungowannah virus), Pestivirus G (giraffe Pestivirus), Pestivirus H (HoBi-like Pestivirus), Pestivirus I (Aydin-like Pestivirus), Pestivirus J (rat Pestivirus), and Pestivirus K (atypical porcine Pestivirus). Additionally, a bat-derived virus and Pestiviruses from sheep and goats, such as the Tunisian sheep Pestiviruses, have been identified (Smith *et al.,* 2017).

Another classification of Pestiviruses distinguishes between typical and atypical species. The typical Pestiviruses include four classical species: classical swine fever virus, bovine viral diarrhea virus types 1 and 2, and border disease virus. Atypical Pestiviruses include giraffe virus, pronghorn virus, and HoBi virus, along with several novel identified species, such as Bungowannah virus, bat Pestivirus, Norway rat Pestivirus, atypical porcine Pestivirus, and LINDA virus (Blome *et al.,* 2017). Until the early 1990s, only three species were recognized as classical Pestiviruses: classical swine fever virus (CSFV), bovine viral diarrhea virus (BVDV), and border disease virus (BDV). Bovine viral diarrhea virus was further classified into types 1 and 2. Since then, new putative Pestivirus species have been identified through phylogenetic analysis, with discoveries reported in chronological order. These include HoBi (first isolated from fetal bovine serum originating in Brazil and later from samples in Southeast Asia), giraffe Pestivirus (isolated from a giraffe in Kenya's Nanyuki District suffering from mucosal disease-like symptoms), pronghorn Pestivirus (isolated from a blind pronghorn antelope in the USA), and Bungowannah virus (isolated during a pig outbreak in Australia that caused stillbirths and neonatal deaths) (Ridpath *et al.,* 2015). Both classical and emerging Pestiviruses infect domestic and wildlife hosts, showing changes in virulence, which has prompted the need to update disease control programs for both domestic and wild populations (Ridpath *et al.,* 2015).

A novel method for genotyping Pestivirus RNA is based on bioinformatics modeling of palindromic nucleotide substitutions (PNS) in the 5' untranslated region (UTR). This approach is simple, sensitive, and practical, utilizing software specifically designed for PNS genotyping. The method has been validated through the analysis of over 500 sequences in the 5' UTR of Pestivirus (Giangaspero *et al.,* 2023; Ridpath *et al.,* 2015). The first isolation of a newly emerging HoBi-like Pestivirus (BVD-3) in Egypt was recorded between 2019 and 2020. Sequence analysis and comparison with other sequences submitted to GenBank, along with phylogenetic analysis, indicate the presence of an atypical Pestivirus circulating in the Egyptian field. This strain may serve as a candidate for vaccine updates (Afify *et al.,* 2022).

Full genome sequence analysis carried out in Egypt in 2014 and characterized a strain of bovine viral diarrhea virus-1b (BVDV-1b), designated as strain Egy/Ismailia/2014. This analysis contributes to understanding the epidemiological situation of Pestiviruses circulating in Egypt (Soltan *et al.,* 2015).

Pestiviruses can be transmitted through small ruminants and may play a significant role in the transmission of viruses among cattle and buffaloes. Sequence analysis of the 5'-UTR region from samples isolated from two goat kids identified BVDV-1b, marking the first isolation in Egypt and the second report worldwide (Abdel-Latif *et al.,* 2013). Pestiviruses exhibit diverse mechanisms of cell entry, which have been investigated through the role of bovine complement regulatory protein 46 (CD46). Research using bovine CD46-knockout and CD46-rescue cell lines showed that CD46 is the major entry factor for HoBi-like Pestiviruses, but not for giraffe Pestivirus. This mechanism was observed in BVDV-1 and BVDV-2 as well (Leveringhaus *et al.,* 2022).

Dromedary camels, goats, and sheep have been identified as potential risk factors for the transmission of BVDV and Pestiviruses in general, causing varying symptoms across different populations and serving as sources of infection for cattle (Kandeel *et al.,* 2022). Additionally, Pestiviruses and Rift Valley fever virus are considered transboundary diseases. One significant risk factor for their transmission is the unofficial smuggling of camels from Sudan to Egypt. To prevent the spread of these diseases, which could lead to catastrophic economic losses in Egypt, strict quarantine measures must be enforced (El Bahgy *et al.,* 2018).

It has also been reported that bovine viral diarrhea virus (BVDV) can be found in the semen of infertile camels, potentially contributing to camel infertility (Al-Busadah *et al.,* 2017). In China, HoBi-like Pestiviruses in sheep and goats may pose a risk for transmission to cattle populations, as these small ruminants show evidence of natural infections with HoBi-like Pestiviruses. This was supported by sequence analysis of partial 5'-UTR nucleotides of Pestivirus (Shi *et al.,* 2016).

Bovine viral diarrhea virus (BVDV) was identified in dromedary she-camels experiencing repeated conception failures or abortions in Al Ahsa Province, Kingdom of Saudi Arabia (KSA), between 2013 and 2015. Uterine swabs were tested by PCR for various potential pathogens, revealing that BVDV is a significant contributor to reproductive disorders, particularly uterine infections and abortions, commonly observed in dromedary camels in KSA (Khalafalla *et al.,* 2017).

Bovine viral diarrhea virus (BVDV) is always associated with secondary bacterial infection resulting from immune dysfunction, as it suppresses neutrophil activity through down-regulation of neutrophil L-selectin and CD18 (Abdelsalam *et al.,* 2023). BVDV may also lead to early embryonic deaths, stillbirth, and the birth of persistently infected offspring (Garoussi *et al.,* 2019).

Immune evasion is a critical strategy employed by BVDV to hijack the host immune system. BVDV-E2 is particularly significant in this process, playing a crucial role in pathogenesis and viral infection, (Al-Kubati *et al.,* 2021).

A HoBi-like Pestivirus was first detected in Germany in 2004 in fetal bovine serum exported from Brazil. This virus had previously been identified in 1990 in buffaloes of Brazilian origin. The clinical manifestations of the disease included reproductive and respiratory symptoms, followed by mucosal disease symptoms (Cruz *et al.,* 2018).

Recently, new candidate Pestiviruses have been identified to expand the growing tree of Pestiviruses, particularly those related to

atypical Pestiviruses such as giraffe virus, pronghorn virus, and HoBi virus. Additionally, a series of novel Pestiviruses characterized through metagenomics and next-generation sequencing include Bungowannah virus, bat Pestivirus, Norway rat Pestivirus, atypical porcine Pestivirus, and LINDA virus (Blome *et al.*, 2017).

Aim of work:
Trials for isolation, identification and molecular characterization of microbial agent causing either abortion or diarrhea in dairy farm in Egypt.

MATERIALS AND METHODS

* **Samples** were collected from 13 affected cows exhibiting the enzootic form of abortion, characterized by continuous excessive mucous discharge. Blood samples were drawn from all 13 cows to separate the serum; the blood samples were placed in clean, dry centrifuge tubes, allowed to clot, and then centrifuged at 1500 × g for 20 minutes. The serum was collected and stored at −20 °C until further testing (Weinstock *et al.*, 2001). Additionally, five vaginal discharge samples were collected from animals with severe excessive discharge, and another five fecal samples were taken from cows showing persistent diarrhea. All samples were stored at −80 °C. The samples collected from the aborted cows were screened for potential microbial causes of abortion, including viral and bacterial pathogens such as Rift Valley fever virus and possible Brucella species (including B. abortus strain 19 and RB 51), as well as other causes of persistent diarrhea, such as Bovine Rota Virus and Bovine Corona Virus. The selected primers, target sequences, and cycling conditions are detailed in Table 1.

* **Fecal samples** were collected and stored at -80 °C until needed. Each sample was mixed thoroughly with 3 mL of feces and 7 mL of sterile PBS. The mixture was then clarified by centrifugation at 2500 rpm for 30 minutes. The clarified supernatant was filtered through a 0.45 µm membrane (Sigma) and stored at −80 °C until further use.

* **DNA extraction** was performed using the Gene JET Viral DNA/RNA Purification Kit (Catalog number: K0821) following the manufacturer's instructions. This process aimed to detect viral RNA and bacterial DNA, particularly from Brucella species, which are known to cause epidemic forms of abortion in cattle.

* The PureLink™ Viral RNA/DNA Mini Kit (Thermo Fisher, USA) was utilized to extract viral RNA from field samples, including serum, vaginal discharge, and fecal matter. Fecal samples are particularly challenging for nucleic acid extraction and amplification due to the presence of PCR inhibitors that can lead to false-negative results. Following the manufacturer's instructions, 200 µL of clarified supernatant from virus-infected tissue culture cells was added to 25 µL of proteinase K in a sterile microcentrifuge tube. Then, 200 µL of lysis buffer containing 5.6 µg of carrier RNA was added, and the mixture was incubated at 56 °C for 15 minutes. To precipitate the nucleic acid, 250 µL of absolute molecular biology grade ethanol was added to the tube and mixed by vortexing for 10 seconds, followed by incubation at room temperature for 5-10 minutes. The lysate was then transferred to a viral spin column and centrifuged for 1 minute at 6800 x g. Afterward, the spin column was washed twice with washing buffer (WII) before the captured nucleic acid was eluted using 50 µL of RNase-free water. The eluted nucleic acid was stored at -80 °C.

* The primers used in this study were designed and selected by the staff members of the Genetic Engineering Research Department at the Veterinary Serum and Vaccine Research Institute for the routine diagnosis of these organisms. These primers were synthesized at a scale of 0.5

nm by Thermo Fisher, as listed in Table 1. The cycling conditions are also provided, with 0.5 µg of DNA or RNA used for the initial detection of potential bacterial or viral causative agents in the samples.

***PCR**

RT-PCR was conducted following RNA extraction, during which complementary DNA (cDNA) was synthesized using Superscript III reverse transcriptase (Thermo Fisher Scientific, Cat Number #K1621) according to the manufacturer's instructions. Primers 324-326, positioned at 108–395, were utilized, targeting a size of 288 bp. The forward primer sequence is ATGCCCW (A, T) TAGTAGGACTAGCA, while the reverse primer is TCAACTCCATGTGCCATGTAC (Baxi *et al.*, 2006).

In brief, approximately 450 ng of total RNA and about 50 ng of primers were mixed with the annealing buffer and incubated at 65°C for 5 minutes before being placed on ice for 1 minute. Next, 10 µL of 2× First-strand reaction mix and 2 µL of Superscript III were added, followed by incubation at 25°C for 5 minutes, then at 50°C for 50 minutes, and finally at 85°C for 5 minutes. The PCR conditions were as follows: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C, annealing at 50°C, and extension at 72°C, each for 30 seconds, with a final extension at 72°C for 10 minutes. **b-** PCR was performed using 5 ng of the extracted DNA to implement the Bruce ladder technique with multiplex primers, following the cycling conditions outlined in Table 1. This method was utilized to detect potential causes of abortion associated with Brucella species (López-Goñi *et al.*, 2008).

- **Median Derby Bovine Kidney (MDBK)** cell cultures were utilized for the primary isolation and adaptation of the virus obtained from a positive PCR sample. The isolation and propagation of the BVD isolate on the MDBK cell line were conducted according to the method described by Darweesh *et al.* (2018). The virus isolates were propagated through three blind serial passages on MDBK cell cultures. Distinct cytopathic effects (CPE) were observed in cell monolayers infected with BVD following an incubation period of 2–3 days post-inoculation. These effects included vacuolation, cell rounding, and the formation of cell clusters by 72 hours post-infection. By the fifth day post-inoculation, complete detachment of the cell sheet was noted (Fig. 1).

***Gel purification**

The PCR product was then loaded onto a 1% low melting agarose, and the correct amplicon size was sliced and purified using Quiaquick Gel Extraction Kit (cat No #.28704) according to the manufacturer instructions.

***Sequencing**

After amplifying the target fragment, the entire volume of the amplified PCR product was subjected to electrophoresis on a 1% agarose gel. The DNA concentration was measured using a Qubit 4 fluorometer along with a DNA High Sensitivity (HS) Quantification Kit (Thermo Fisher Scientific, USA). For sequencing, the purified sample was sent to GATC Biotech AG (Germany). Nucleotide sequences were retrieved from the NCBI database and aligned with the 5' UTR region segment sequences from GenBank and other Pestivirus families using the nucleotide Basic Local Alignment Search Tool (BLASTn). Sequence homology, identity percentage, and phylogenetic analysis were conducted using the MEGA-X software package.

Suspected disease	primer name	primer sequence	product length	Cycling conditions	Reference	Target
Brucellosis	BMEI0998f	ATC CTA TTG CCC CGA TAA GG		Denaturing at 95 °C temperature for 4 min, then 35 cycles at 94 °C for 45 s, 45 s at 60 °C, 60 s at 72 °C then 72 °C for 7 min for final extension.		Glycosyltransferase, gene *wboA*
B. abortus strain 19 and RB 51	BMEI0997r	GCT TCG CAT TTT CAC TGT AGC	1682 bp			
	BMEII0843f	TTT ACA CAG GCA ATC CAG CA			(López-Goñi et al., 2008)	Outer membrane protein, gene *omp31*
B. abortus	BMEII0844r	GCG TCC AGT TGT TGT TGA TG	1071 bp			
	BMEII0428f	GCC GCT ATT ATG TGG ACT GG				Erythritol catabolism, gene *eryC*
B. abortus S19	BMEII0428r	AAT GAC TTC ACG GTC GTT CG	587 bp			
	Primer name 324-326	ATGCCCW(A,T)TAGTAGGACTA GCA		cDNA synthesis carried out at 50°C for 50 min and 85°C for 5 min then followed by PCR condition as follows 94°C for 5 min, 35 cycles of 94°C, 50°C), and 72°C, 30 s each, with a final extension at 72°C for 10 min	(Monteiro et al., 2019)	5' (UTR) of Pestivirus
BVDV		TCAACTCCATGTGCCATGTAC	288 bp			
	RVF-ForI (597-617)	GTCTTGCTTGAAAAGGGAAAA	745 bp	cDNA synthesis carried out at 50°C for 50 min and 85°C for 5 min then followed by PCR condition as follows 40 cycles of 94°C for 15 seconds, annealing at55°C for 30 seconds, and extension of amplified fragment at 68°C for 30 seconds; one cycle for final extension at 68°C for 5 minutes		M segment
Rift Valley Fever Virus	RVF-REVE (1326-1342)	CCTGACCCATTAGCATG			(Shoemaker et al., 2002)	
	F	TGGATCAAGATTAGAGTTGGC	236 bp	cDNA synthesis carried out at 50°C for 50 min and 85°C for 5 min then followed by PCR condition as follow 1 cycle at 95 °C for 5 minutes; followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and then a final cycle at 72 °C for 7 min	(Takiuchi, Stipp, Alfieri, & Alfieri, 2006)	N gene
Bovine corona virus	R	CCTTGTCCATTCTTCTGACC				
	F	ATGTATGGTATTGAATATACCAC	884 bp	PCR condition as follow 1 cycle at 95 °C for 5 minutes; followed by 35 cycles at 94 °C for 30 s, 52 °C for 60 s, and 72 °C for 30 s; and then a final cycle at 72 °C for 7 min	(Simmonds et al., 2008)	VP7 gene
Bovine Rota Virus	R	AACTTGCCACCATTTTTTCC				

Table (1) showing the Oligonucleotides used in the PCR assays for detection of both possible viral and bacterial causes of abortion in cattle used in this study.

RESULTS

All samples utilized in this study, including serum, fecal, and vaginal discharge samples, underwent a panel of screening primers to detect potential microbial causes of the enzootic abortion or diarrhea observed on the farm. Initially, the Bruce ladder multiplexing system was employed to identify possible Brucella species associated with abortion in cattle; however, all samples tested negative, indicating that Brucellosis was not responsible for the abortions. Following this, viral causes were investigated, including the Rift Valley Fever Virus (RVFV), using specific conserved primers aimed at detecting various strains of RVFV. Unfortunately, these tests also yielded negative results. Next, primers specific for Bovine Rota Virus (BRV) and Bovine Corona Virus (BCV) were used to identify potential causes of persistent diarrhea in both young calves and adult cattle, but again, the results were negative. Finally, attention shifted to Bovine Viral Diarrhea Virus (BVDV), known to cause both abortion and diarrhea. Primers flanking the 5' UTR were utilized for the detection of a broad range of Pestivirus species, based on the identification of palindromic nucleotide substitutions in the 5' untranslated region (PNS).

Out of the 23 tested samples, only one sample from the vaginal discharge returned a positive result in the RT-PCR, matching the expected size of 288 bp, as illustrated in Figure 2. This positive PCR product was subsequently gel-purified and submitted for sequence analysis. The sequenced product was then deposited in GenBank, assigned the accession number GERD/VSVRI/pesti-Giraffe/2022

(#OR425033). A BLAST analysis was performed in GenBank to evaluate the differences and similarities with other Pestiviruses, particularly those isolated in Egypt. Multiple sequence alignment, presented in Figures 3, 4, and 6, revealed that the local isolate is closely related to Pestivirus Giraffe, specifically Pestivirus Giraffe-2 (PG2). This represents the first record of this group of atypical Pestiviruses detected in Egypt, as it has not been previously recorded in the region.

Phylogenetic analysis of the 5' UTR was conducted using the maximum likelihood method with bootstrap probabilities from 1,000 replicate trials, rooted with sequences from different genotypes (see Figure 5). The analysis showed that the isolate GERD/VSVRI/pesti-Giraffe/2022

(#OR425033) clusters in a distinct group that includes all Pestivirus Giraffe variants, as illustrated in Figure 7. Furthermore, it exhibited the highest identity percentages of 85.185% and 84.848% with JQ994202 and KJ660072, respectively, as detailed in the identity matrix in Table 2.

Virus isolation and propagation were conducted using MDBK cell cultures. The positive sample was utilized for virus isolation on the MDBK cell line, as depicted in Figure 1. The virus isolates underwent three blind serial passages on MDBK cell cultures. Distinct cytopathic effects (CPE) in the cell monolayers infected by BVD were observed after an incubation period of 2–3 days post-inoculation, characterized by vacuolation, rounding of cells, and the formation of cell clusters 72 hours post-infection. Complete detachment of the cell sheet was noted by the fifth day post-inoculation (Fig. 1).

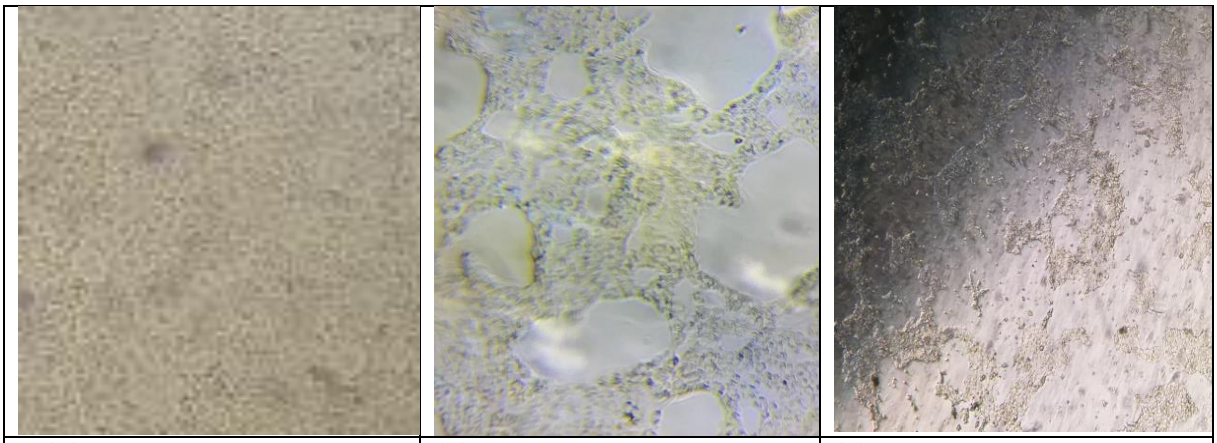

Figure. (1): Showing normal MDBK cells (A), Infected MDBK cells 72hr post inoculation showing vacculation and cluster formation cells (B) and Infected MDBK cells 5th day post inoculation showing cells rounding and sheet detached (C)

RT-PCR

Figure (2): PCR showing PCR amplification of the isolated virus strains showing clear band at 289 bp where M (100 bp DNA ladder) lane one isolated virus strain GERD/VSVRI/pesti-Giraffe/2022 lane two the tissue culture adapted isolate after three blind serial passages on MDBK cell cultures.

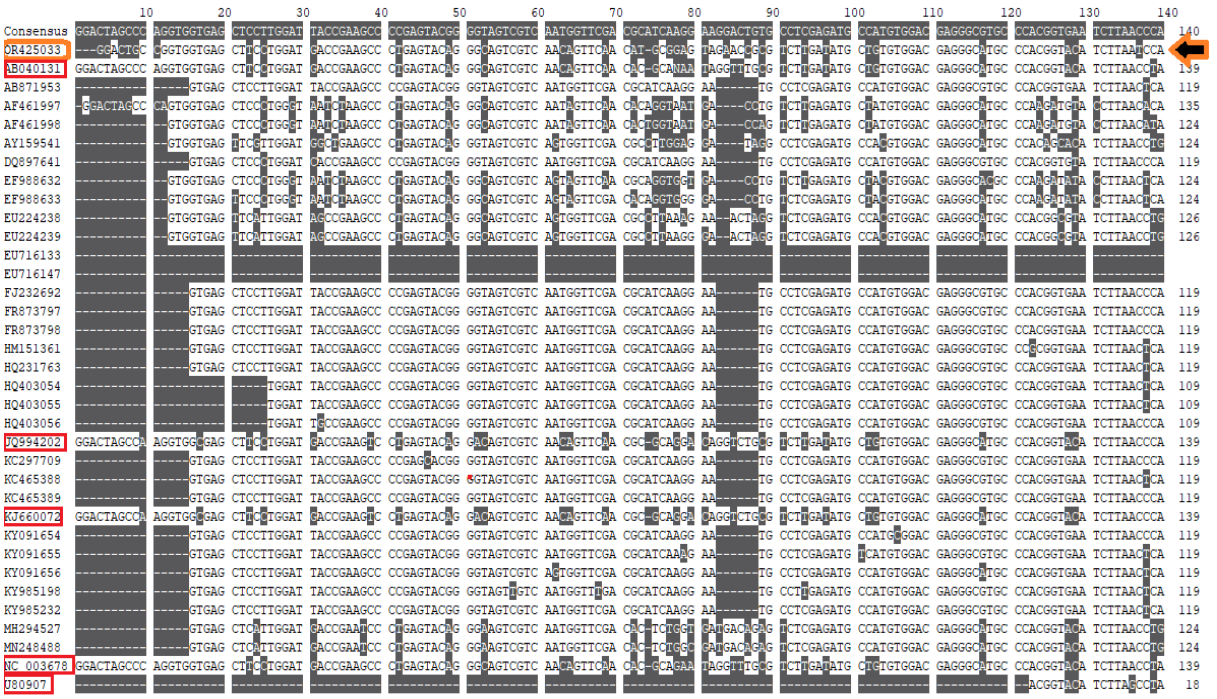

Figure (3): Schematic representation of the nucleotide sequence alignment of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 which is marked with orange arrows and rectangles and other PestiGiraffe strains marked by red rectangles and other Pestiviruses from base 1 to base 140 of the sequenced part of the 5`UTR.

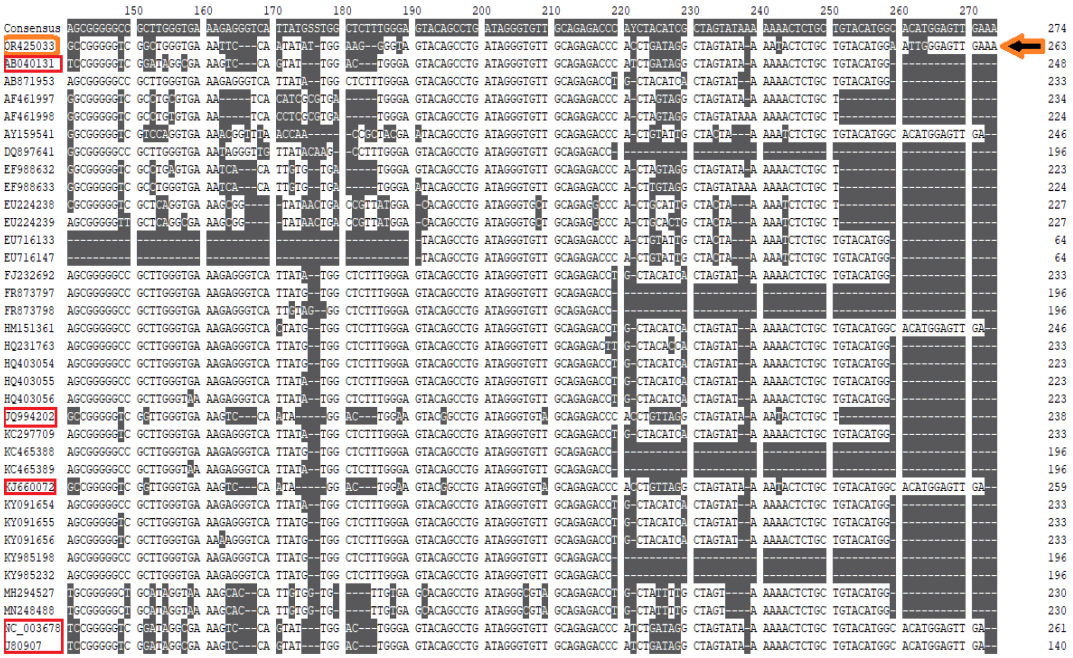

Figure (4): Schematic representation of the nucleotide sequence alignment of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 which is marked with orange arrows and rectangles and other PestiGiraffe strains marked by red rectangles and other Pestiviruses from base 141 to base 274 of the sequenced part of the 5`UTR

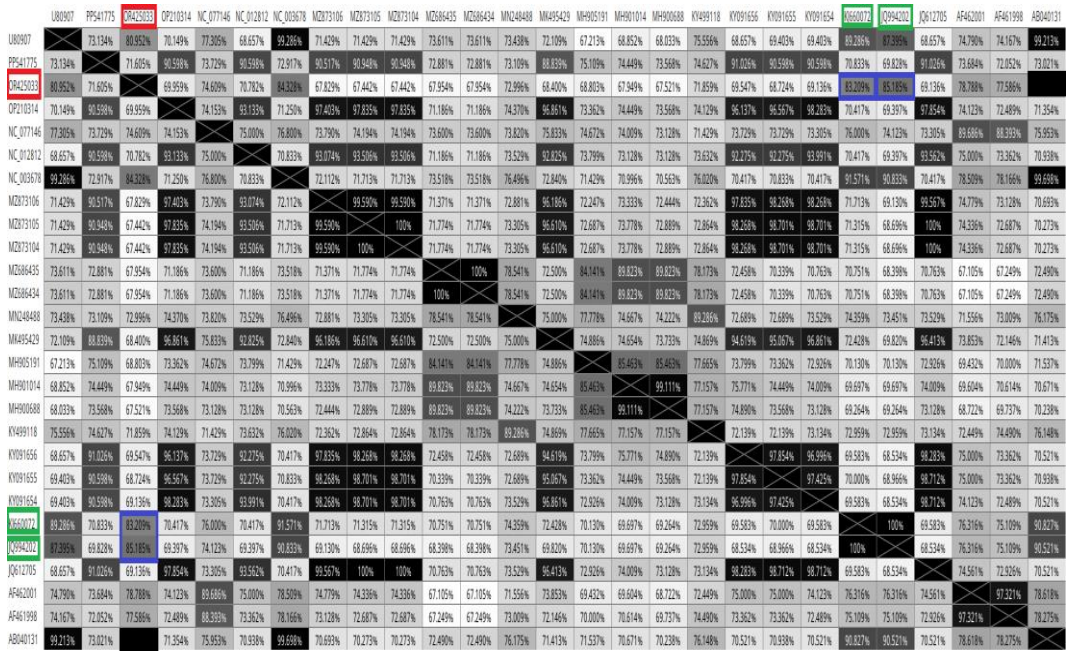

Table (2): Showing the sequence identity percent based on the maximum like hood method of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 and the rest Pestiviruses deposited on genbank, where the Accession Number where it is marked by Red rectangles and that one marked by Green rectangle is that one Related to pesti-Giraffe 2 (PG2) KJ660072 and JQ99202 that shown the highest Identity percent between 83.209 % and 85.185 % respectively.

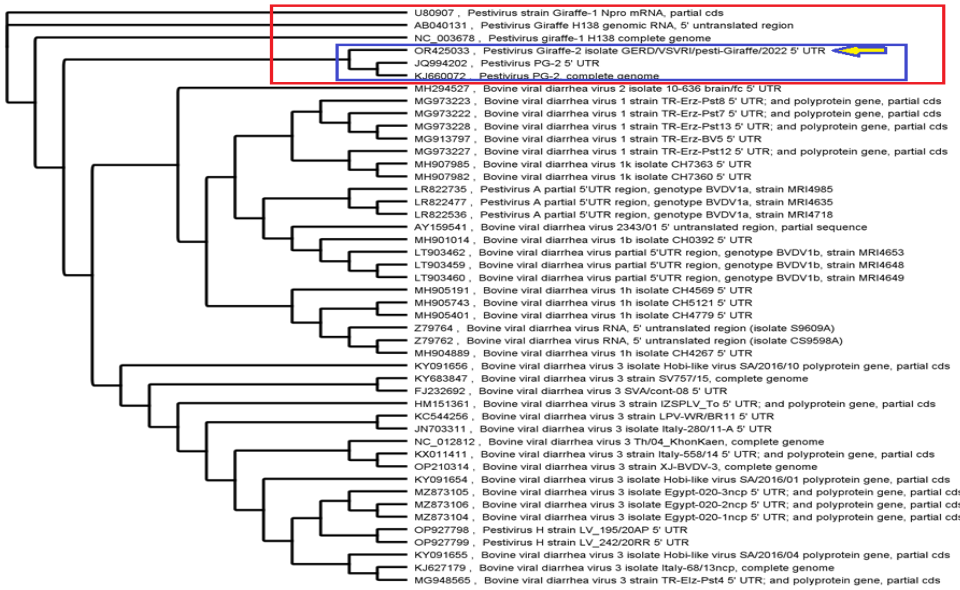

Figure (5): Showing Phylogenetic analysis by maximum like hood method of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 using bootstrap probabilities after 1,000 replicate trials and rooted with sequences of different genotype and other isolates and strains around the world and In Egypt as well

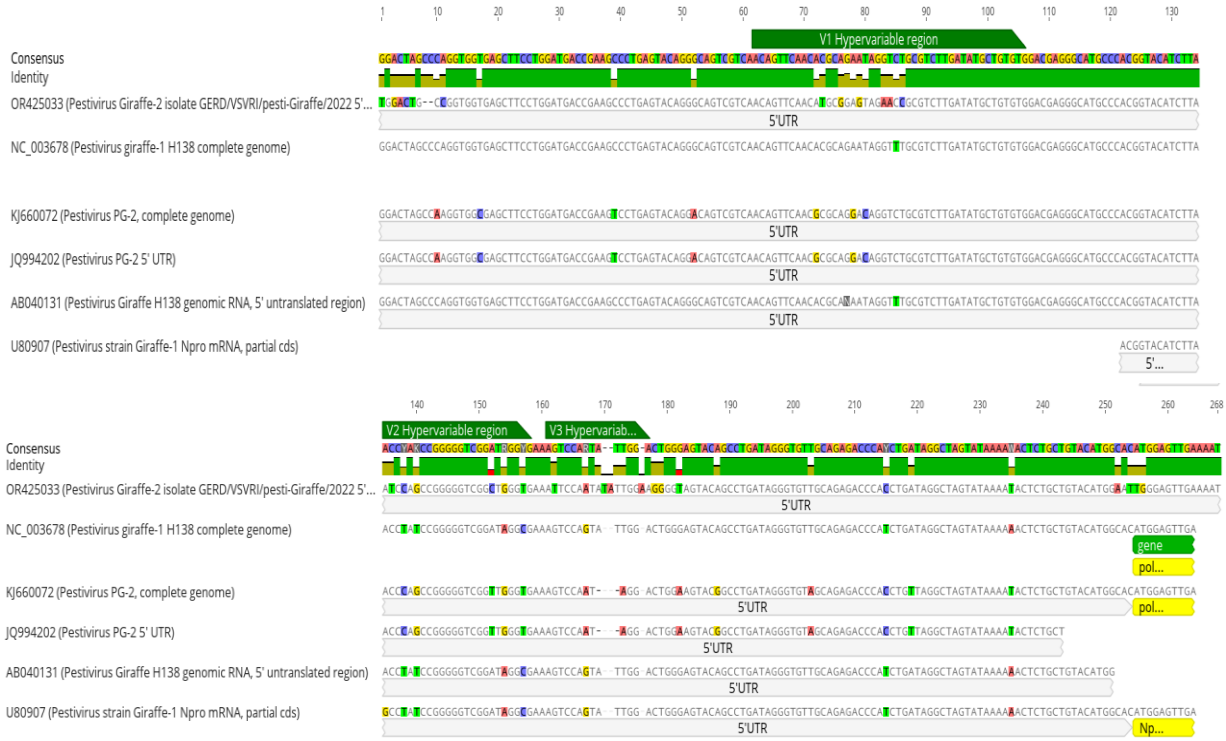

Figure (6): Schematic representation of the nucleotide sequence alignment of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 and other Pestiviruses Giraffe strains isolates showing the main three hyper variable regions (V1,V2 and V3) that used for Pestiviruses typing.

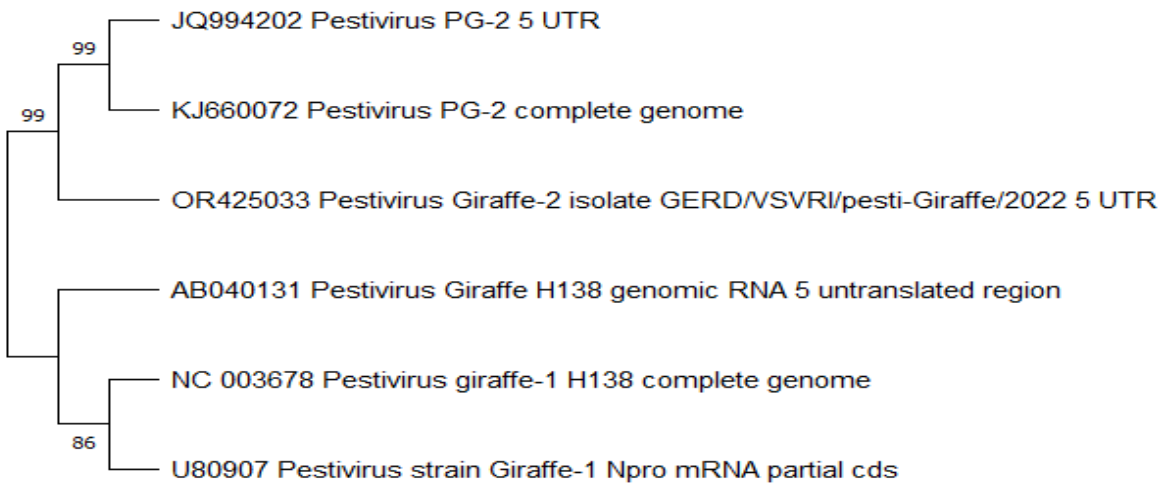

Figure (7): Phylogenetic analysis by maximum like hood method of 5` UTR of the local isolate GERD/VSVRI/pesti-Giraffe/2022 Accession Number #OR425033 and other Pestiviruses Giraffe strains isolates.

DISCUSSION

The enzootic outbreak recorded on a dairy farm in the Wadi El Natron District caused significant economic losses, primarily due to a wave of abortions in pregnant heifers and persistent diarrhea. These losses were exacerbated by the dynamic variations resulting from the genetic diversity of the virus. The best method for classifying and differentiating between different Pestivirus groups is based on palindromic nucleotide substitutions in the 5'-untranslated region (UTR), which occurs in three variable regions within the 5' UTR: V1, V2, and V3

(Giangaspero *et al.,* 2011), as shown in Figure 6. In Egypt, the most common Pestivirus species are BVDV-1 and BVDV-2, with BVDV 1a being the most common subtype. Recently, however, BVDV 1b has also been detected, having been isolated from aborted goat fetuses, indicating that small ruminants play a significant role in transmitting Pestiviruses to cattle and buffalo (Abdel-Latif *et al.,* 2013). In 2022, the first detection of HoBi-like Pestivirus (BVD-3) was recorded, based on sequence analysis of the 5' UTR (Afify *et al.,* 2022), confirming the presence of diverse types and subtypes of Pestiviruses in Egypt. The current study marks the first detection of the pesti-Giraffe strain in Egypt, which caused a severe enzootic outbreak on the affected farm. Despite the farm animals being vaccinated with imported vaccines containing only the classical Pestivirus strains BVDV-1 and BVDV-2, there was no cross-protection or cross-neutralization against the newly isolated strain GERD/VSVRI/pesti-Giraffe/2022 (Accession Number #OR425033). This strain is genetically and antigenically distinct, segregating into a completely different group from the classical Pestivirus strains for which the animals were vaccinated. The severity of the enzootic form recorded in this farm underscores the virulence of this new isolate compared to both the classical forms and the recently isolated HoBi-like Pestivirus (BVD-3) in Egypt.

Cross-species infection has been documented in Pestiviruses, with viruses previously isolated from wild animals such as deer and giraffes shown to infect cattle, sheep, and even swine, based on phylogenetic analysis and palindromic nucleotide substitutions (PNS) (Harasawa *et al.,* 2000). It has been demonstrated that the pesti-Giraffe virus is not cross-neutralized, or only poorly neutralized, by heterologous Pestivirus antisera. Cross-neutralization studies using homologous and heterologous antiserum to assess the antigenic relatedness between Pestivirus species confirm the genetic and antigenic segregation of Pestivirus members (Avalos-Ramirez *et al.,* 2001; Dekker *et al.,* 1995; Paton *et al.,* 1995; Pellerin *et al.,* 1994). Moreover, the entry mechanism of Pestivirus G into bovine cells differs significantly from other Pestiviruses, as demonstrated by studies using CD46-knockout and CD46-rescue cell lines (Leveringhaus *et al.,* 2022).

These findings support the results of this study, where a farm previously vaccinated with an imported vaccine against classical Pestivirus strains, namely BVDV-1 and BVDV-2, experienced a severe enzootic outbreak caused by the newly identified isolate GERD/VSVRI/pesti-Giraffe/2022. The failure of the vaccine to protect against this outbreak can be attributed to the absence of cross-neutralizing antibodies between the classical vaccine strains and the Pesti-Giraffe virus. As Becher *et al.* (2003) noted, the genetic and antigenic differences between the classical strains and the pesti-Giraffe isolate highlight the need for locally produced vaccines incorporating the new strain to ensure adequate protection.

Given that this is the first recorded case of this Pestivirus type in Egypt, it is highly recommended to include the new isolate in locally prepared vaccines to protect against the currently circulating strains. A periodic field survey should also be conducted to monitor the prevalence of different Pestivirus types and ensure that vaccines remain up to date. There is little to no cross-protection between classical and newly emerging Pestivirus strains, such as Pesti-Giraffe, which cluster differently in the Pestivirus family tree and are genetically and antigenically distinct. Careful inspection of imported animals is essential to prevent the introduction of new viruses with potentially catastrophic economic consequences, as these Pestiviruses can be transmitted both vertically and horizontally between generations.

CONCLUSION

It is strongly recommended to conduct periodic field surveys to detect the most prevalent Pestivirus types. Establishing a

national program for the detection and protection against Pestiviruses is crucial due to their significant economic impact. This will help ensure that local vaccines are updated to include newly isolated Pestivirus strains, as there is little to no cross-protection between classical and emerging Pestivirus types, particularly the PestiGiraffe strains. These strains cluster differently in the Pestivirus family tree, exhibiting distinct genetic and antigenic characteristics. Rigorous inspection of imported animals is essential to prevent the introduction of newly emerging viruses, which could have devastating economic effects on local livestock, as Pestiviruses are transmitted both vertically and horizontally across generations.

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عزل وتصنيف وتوصيف جزيئي والتمرير على الخلايا لأحد المعزولات المحلية لأحد الفيروسات شديدة الضراوة المسببة لمرض فيروس الإسهال في للماشية المعزولة حديثا من القطر المصري

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يقوم البحث على توثيق حالة من الإجهاضات بصورة وبائية غير مسبوقة فى إحدى مزارع البقر الحلاب الموجودة على امتداد طريق مصر إسكندرية الصحراوى فى ربيع عام ٢٠٢٠ والتى نتج عنها خسائر إقتصادية فادحة نتيجة الانخفاض الحاد فى انتاج اللبن بالرغم من تلقى الأبقار التحصين المستورد الخاص لمرض إسهال الماشية الفيروسى (BVDV) الذى يعطى مناعة ضد الانواع التقليدية للمرض خاصة النوع الأول والثانى. تمت إصابة المزرعة بالمرض على الرغم من التحصين مما يدل أنه لا يوجد مناعة مشتركة سواءا كانت كلية أو جزئية بين الفيروس المسبب للمرض والتحصين الذى تلقته الأبقار والذى يسبب نفس المرض. جميع العجلات العشر بالمزرعة أجهضت بالكامل واستمرت فى إفراز سوائل مهبلية بنية اللون حتى بعد الأجهاض. تم تجميع ١٣ عينة سيروم من الأبقار المجهضة ومن نفس هذه الأبقار تم تجميع ٥ عينات من الإفرازات المهبلية و٥ عينات أخرى من البراز الخاص بهذه العجلات وتم إجراء تفاعل البلمرة المتسلسل على جميع هذه العينات وقد أعطت عينة واحدة فقط من العينات المجمعة من المخاط البنى المفرز من هذه الأبقارنتيجة إيجابية متطابقة مع حجم المنتج المفروض ظهوره مع إيجابية المرض عند إستخدام بوادىء متخصصة له ليعطى منتج طوله ٢٨٨ قاعدة وهو مطابق تماما للنتيجة الإيجابية عند إستخدام هذه البوادىء المتخصصة للمنطقة الغير مترجمة الموجودة من بداية الجينوم الخاص بالفيروس. ثم تم عمل تحليل التتابع النيوكليوتيدى الخاص بالجزء المراد الكشف عنه ثم بعد ذلك تم رفع التتابع النيوكليوتيدى للعترة المحلية GERD/VSVRI/pesti-Giraffe/2022 برقم تعريفى على بنك الجينات #OR425033 وتم حقن وعزل الفيروس على خلايا الكلى الخاصة بالأبقار وتمريره ٣ تمريرات متتالية وتم عمل تحليل تفاعل البلمرة المتسلسل للتأكد من وجود الفيروس وتم عمل تحليل التتابع النيوكليوتيدى للمعزولة المحلية ومقارنتها بالمعزولات الموجودة ببنك الجينات وتم أيضا عمل شجرة التنسيب الخاصة بالمعزولة المحلية بناءا على هذا التتابع النيوكليوتيدى وقد وجد أن المعزولة المحلية تنتسب اللى مجموعة Pestivirus PG2 والتى تم عزلها من أحد الزرافات أول مرة في دولة كينيا ولذلك يعتبر هذا البحث هو أول توثيق لهذه المعزولة فى مصر. ولذلك تعتبر هذه العترة عترة جديدة فى القطر المصرى والتى لم يسبق تسجيلها فى مصر من قبل. ويثبت ذلك أن اللقاح المستورد المصنع من العترات الكلاسيكية غير مجدى تماما لحماية القطيع من هذه العترة الغير كلاسيكية المستقدمة إلى القطر المصرى ولذلك وجب إضافة العترة الجديدة إلى اللقاح المحلى لحماية الثروة الحيوانية من هذا المرض الذى يسبب خسائر إقتصادية فادحة.