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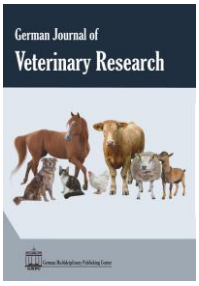
Research article

# *Pestivirus bovis* is implicated in repeated abortions and subfertility of vaccinated cattle in Egypt

**Ahmed Orabi[1*], Hany Abdalla[2], Mrigendra Rajput[3] and Shimaa M.G. Mansour[1*]**

[1] Department of Virology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
[2] Department of Theriogenology, Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt
[3] Department of Biology, University of Dayton, Dayton, Ohio, USA

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***Corresponding author:**
Ahmed Orabi
ah_orabi222002@yahoo.com
Shimaa M.G. Mansour
shimaagalal@gmail.com

## Abstract

The study of *Pestivirus bovis*, formerly known as Bovine Viral Diarrhea Virus (BVDV), is of significant importance due to the economic challenges it poses in cattle herds in several countries. This virus leads to decreased productivity, reproductive failures, and increased susceptibility to secondary infections. The current study aimed to investigate the role of BVDV in abortion and infertility among cattle in Egypt using advanced diagnostic techniques. We employed a one-step multiplex real-time reverse transcription polymerase chain reaction (rRT-PCR) to detect BVDV, and the results showed an overall prevalence of 11.2% among 178 tested samples. Notably, 11.2% tested positive for BVDV-1, with higher detection rates in adult cows (12.1%) compared to calves (9.85%). Importantly, 13.8% of samples from dams with subfertility and repeated abortions were positive for BVDV-1. The results of rRT-PCR guided virus isolation using Madin–Darby bovine kidney (MDBK) cells. Identifying cytopathic effects (CPEs) in 15% of the samples, consistent with cytopathic-BVDV (CP-BVDV). An indirect fluorescent antibody assay (IFA) confirmed the presence of CP-BVDV in these samples. For non-cytopathic (NCP)-strains, the immunoperoxidase (IP) test using bovine turbinate (BT) cells was more effective, detecting NCP-BVDV in samples without CPE in MDBK cells. The presence of mixed infection was indicated by an isolate from a diarrheic calf, showing positive results for both CP-BVDV and NCP-BVDV. Plaque assays further confirmed CP-BVDV and NCP-BVDV isolation from mixed infections, highlighting the selective amplification of both biotypes. These findings underscore the significant role of BVDV-1 in reproductive disorders in cattle and the importance of employing comprehensive diagnostic methods for effective control and management strategies. Our study provides valuable insights that can guide future studies and improve herd health and productivity.

**Keywords:** BVDV, Abortion, Infertility, Cytopathic, Non-cytopathic

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## Introduction

*Pestivirus bovis*, earlier referred to as Bovine Viral Diarrhea Virus (BVDV), a member of the genus *Pestivirus* within the family *Flaviviridae* (ICTV, 2023), constitutes one of the most important pathogens infecting cattle worldwide (Pinior et al., 2017). BVDV is a small, enveloped virus with a positive single-stranded RNA genome of about 12.3 kb. The genome contains a single open reading frame (ORF) that encodes all BVDV structural and non-structural proteins (Liu et al., 2009; Cerutti et al., 2016). This ORF is flanked by two untranslated regions (UTRs) at the 5′ and 3′ ends. The 5′-UTR region is particularly useful for viral differentiation due to its high conservation among members of each *Pestivirus* species (Berry et al., 1992; Hofmann et al., 1994). Analysis of the 5′-UTRs enabled the classification of BVDV into three genotypes: BVDV-1, BVDV-2, and BVDV-3 (van Rijn et al., 1997, Smith et al., 2017, ICTV, 2023)

BVDV genotypes are further divided into two biotypes: cytopathic (CP) and non-cytopathic (NCP). They are distinguished by their effect on cell cultures, with the CP biotype causing visible cytopathic effects and the NCP biotype not

causing such effects (Ridpath et al., 1994; Ammari et al., 2010). The NCP biotype of BVDV is the most common in natural settings and is responsible for the more severe forms of the disease. NCP viruses can cause persistent infections (PI) in unborn calves, particularly between 18 and 125 days of pregnancy (Grooms, 2004; Lanyon et al., 2014). These persistent infections can sometimes change into the CP biotype, resulting in the development of fatal mucosal disease (Baker, 1995; Meyers et al., 1997). PI calves, which are the result of exposure to BVDV before birth, are crucial in spreading the virus within and between herds (Brodersen, 2014).

In a field study conducted by Roeder et al. (1986), it was found that introducing BVDV into a susceptible herd as a single source resulted in a 21% abortion rate over six months. Additionally, in herds with endemic BVDV infections and lacking control measures such as vaccination, biosecurity protocols, or eradication policies, BVDV is estimated to account for 7% of fetal deaths (Rufenacht et al., 2001). In addition, congenital BVDV infection, which occurs between 100 and 150 days of gestation, is often associated with various congenital defects (Grooms, 2004). Also, acute BVDV infection can affect ovarian hormone secretion, which has been proposed as a potential mechanism for BVDV-induced infertility (Fray et al., 2002).

In Egypt, both genotypes of BVDV have been identified. The NCP biotype predominates and is frequently associated with severe disease manifestations in natural infections (Ammari et al., 2010). Challenges persist in controlling BVDV spread among vaccinated cattle in Egypt despite efforts to vaccinate against the virus. Studies conducted across various Egyptian regions have detected BVDV in cattle herds, indicating widespread exposure to the virus (Soltan et al., 2015; Lotfy et al., 2020; Afify et al., 2022). Due to the scarcity of data on BVDV's effects on abortion and infertility in Egypt, this study examined its impact on vaccinated cattle farms experiencing these reproductive issues.

## Materials and methods

### Cells and viruses

Madin–Darby bovine kidney (MDBK) and bovine turbinate (BT) cells were sourced from VACSERA (Cairo, Egypt) and cultured in Eagle's Minimal Essential Medium (EMEM) with 10% fetal bovine serum (FBS) (Gibco, Grand Island. NY, USA). These cells were verified to be free from *Mycoplasma* contamination using the LookOut™ *Mycoplasma* PCR Kit (Sigma, Rockville, MD, USA) and also free from the NCP strain of BVDV using reverse transcription polymerase chain reaction (RT-PCR). The FBS tested negative for both BVDV and BVDV -antibodies and was heat-inactivated at 56°C for 90 min as an extra precaution against potential low-level BVD virus contamination (Gomez-Romero et al., 2021). BVDVs (Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, USA), including NADL (CP type 1), A125 (CP type 2), and 890 (NCP type 2), were propagated in MDBK cells and harvested after two freeze-thaw cycles. Virus titers were determined by Tissue Culture Infective Dose 50 ($TCID_{50}$ ) (Reed and Muench, 1938).

### Samples

A total of 178 samples were collected, consisting of 142 buffy coat samples (100 from dams and 42 from calves) and 36 fecal samples (5 from dams and 31 from calves). The samples were obtained from apparently healthy animals (n= 81; 70 buffy coat and 11 fecal samples) in contact with diseased ones, as well as from diseased animals experiencing repeated abortion and/or subfertility issues (n=72) and diarrhea (n=25) within four cattle farms in Sharkia Province, Egypt. The cattle on these farms were vaccinated against BVDV using an inactivated BVDV vaccine at 6 months of age and received annual booster shots.

### rRT-PCR

Total RNA was extracted from buffy coat and fecal samples and also from reference BVDVs (Type 1 and 2) using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Differential detection of BVDV-1, BVDV-2, and BVDV-3 in the 5′ untranslated region (UTR) was conducted using one-step multiplex rRT-PCR with specific primers and probes (Mari et al., 2016). The thermal profile involved an initial activation of *Taq* DNA polymerase at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute (Mari et al., 2016). Strict laboratory procedures were followed to prevent cross-contamination.

### Virus isolation on MDBK cells

MDBK cells, at a concentration of $2\times10^6$

cells/mL, were distributed into 24-well cell culture plates with 750 µL per well and incubated at 37°C until 80% confluency was achieved. The growth medium was decanted, and the cells were inoculated with positive buffy coat samples and supernatants prepared from fecal samples, using 200 µL/well in triplicates for each sample. Cell and virus controls were included. The plates were incubated at 37°C for one hour in 5% $CO_2$, with rotation every 15 min. The excess inoculum was then removed and replaced with maintenance media containing 2% horse serum, followed by incubation for 5-7 days. The plates were examined daily under the inverted microscope. After 3× blind passages (if no CPE was detected in subsequent passages), the inoculated cells were examined for BVDV using an indirect immunoperoxidase assay. Samples showing CPE consistent with CP-BVDV were further examined using an indirect fluorescent antibody (IFA) assay. The biotype was determined based on the visual cytopathic effects (CPEs) observed in cell culture.

**Indirect fluorescent antibody (IFA) assay for the detection of CP-BVDV**

An 80% confluent sheet of MDBK cells was inoculated with a positive buffy coat and fecal samples in triplicates for each sample. Cell and virus controls were included. The plates were incubated at 37°C for 36 hours, after which the cells were fixed in 80% cold acetone for 15 min. The fixed cells were incubated with anti-BVDV monoclonal antibody (mAb) KD-80 antiserum (Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, USA) for 1 hour at 37°C in a humid chamber and then washed with PBS. Subsequently, the cells were stained with goat anti-mouse FITC conjugate (Sigma, Rockville, MD, USA). After 1 hour of incubation at 37°C in a humid chamber, the cells were washed three times with PBS and examined for specific fluorescence using a fluorescent microscope (Leica DM ILM with MPS30 Camera System, Wetzlar, Germany).

**Immunoperoxidase (IP) assay for the detection of NCP-BVDV**

An 80% confluent sheet of BT cells was inoculated with a positive buffy coat and fecal samples, with triplicates for each sample, along with cell and virus controls. The plates were then incubated at 37°C for 4 days. Following incubation, the cells were fixed in 80% cold acetone for 15 min. The fixed cells were then stained with anti-BVDV mAb 20.10.6 anti-p80/p125 (Animal Disease Research and Diagnostic Laboratory, South Dakota State University, Brookings, USA) for 45 min at 37°C in a humid chamber, followed by washing with PBS. A biotinylated rabbit anti-mouse IgG was added, followed by streptavidin-horseradish peroxidase (Gibco, Grand Island. NY, USA), and then washed with PBS. Finally, a 3-amino-9-ethyl carbazole (Sigma, Rockville, MD, USA) substrate with $H_2O_2$ was applied following the manufacturer's instructions to visualize the staining.

**Plaque assay to distinguish CP- and NCP-BVDV in mixed infections**

To test for the presence of mixed CP and NCP-BVDV infections, the three IFA-positive samples were subjected to successive passages using the plaque assay. Monolayers of MDBK cells, grown to confluency, were used for the assay. After washing the cultures twice with PBS, 1 mL of the inoculum was added. After a two-hour adsorption period, the inoculum was removed, and the cell monolayers were covered with a 2× media overlay containing 0.8% SeaPlaque agarose. The cultures were then placed in an incubator at 37°C with 5% $CO_2$ for three days. After the three-day incubation, the cells were covered with agarose containing 0.01% neutral red and further incubated for an additional two days. Subsequently, the plates were inspected for plaques, and all identified plaques were picked and suspended in MEM for the next passage. To detect the NCP virus, the plaques were picked from the first passage. The agarose was removed, and the underlying cells (without CPEs) underwent two successive freeze-thaw cycles. Then, the mixture was centrifuged to collect the supernatant. This supernatant was inoculated into MDBK cells and inspected for CPEs. This process of inoculation and harvesting was repeated until CPEs completely disappeared. Once CPEs were definitely absent, the cells were subjected to the IP assay to detect the NCP virus.

**Statistical analysis**

The detection rate of BVDV types and biotypes was calculated as the proportion of positive samples in which infection was detected by rRT-PCR and cell culture, respectively. Both the detection rate and the 95% confidence interval

(CI) were computed using WinPepi software, Version 11.65 (Abramson, 2011). Statistical analysis was carried out using SPSS version 11 software (IBM). Each variable was tested in triplicate per experiment, with all experiments conducted at least twice. A one-way analysis of variance was used to assess significance, which is indicated as *p<0.05 and **p<0.01.

## Results

### Detection and typing of BVDV using one-step multiplex rRT-PCR

The rRT-PCR results revealed that 11.2% of the tested RNA samples were positive for BVDV-1. Specifically, the BVDV-1 genome was detected in 13 samples from adult cows and 7 samples from calves. BVDV-1 was found in 10 samples from dams exhibiting subfertility and repeated abortion and in 9 samples from apparently healthy animals. Only one sample from a diarrheic calf tested positive for BVDV-1. Regarding the type of sample, 18 buffy coat samples tested positive for BVDV-1, while only 2 positive fecal samples were from apparently healthy calves (Table 1).

### Virus isolation and IFA assay using MDBK cells

The rRT-PCR-positive samples (n=20) were inoculated into MDBK cells. CPEs, including cell lysis, aggregation, and rounding (Figure 1), were observed in both the CP-BVDV reference viruses and 3 of the tested samples (15%; one from a dam and two from calves). No CPE was noted in the negative control and the remaining 17 tested samples (85%) (Table 2). The presence of CP-BVDV was further confirmed using IFA staining. The positive samples (n=3) displayed bright greenish-yellow fluorescence, whereas the absence of this fluorescence indicated a negative result (Figure 1). Among dams, this fluorescence was detected in one buffy coat sample but not in fecal samples. In calves, the fluorescence was observed in two fecal samples only.

### Detection of NCP - BVDV by IP test using BT cells

All BVDV-positive samples that exhibited no CPE in MDBK cells (n=17) displayed strong intracytoplasmic reddish-brown signals (Figure 2). Furthermore, one isolate from a diarrheic calf that tested positive for CP-BVDV in IFA also showed a distinct positive result in the IP assay in cells without CPE, and after collecting all CP-BVDV plaques over four successive passages until no further plaques appeared, indicating the presence of a mixed NCP-BVDV infection (Table 2).

### Isolation of CP- and NCP-BVDVs from mixed infections using plaque assay in MDBK cells

During the four successive passages of plaques from the plaque assay for the three IFA-positive samples, the plaques became increasingly prominent and more numerous. By the fourth passage, the plaques had become massive, covering almost the entire cell sheet (Figures 3 A and C). Additionally, plaques appeared more quickly in the third and fourth passages compared to the first and second. Out of the three samples, only one, from a diarrheic calf, showed a distinct positive result in the IP assay (Figure 2), indicating a mixed NCP-BVDV infection (Table 2). In contrast to the four successive passages of CP-BVDV plaques, the harvest of underlying cells showed a decrease in the intensity and percent of CPE in MDBK cells from the first to the third passage, eventually disappearing completely by the fourth passage (Figures 3 B and D).

**Table 1:** Molecular detection of BVDV-1 using one-step multiplex rRT-PCR.

| Criteria | Number of samples examined | Number of positive samples | % of positive samples | 95% CI confidence interval |
|---|---|---|---|---|
| **Age** | | | | |
| Dams | 107 | 13 | 12.1 | 6.6%-19.8 |
| Calves | 71 | 7 | 9.9 | 4.0%-19.3 |
| **Sample type** | | | | |
| Buffy coat | 142 | 18 | 12.7 | 7.7%-19.3 |
| Feces | 36 | 2 | 5.6 | 0.68%-18.7 |
| **Disease condition** | | | | |
| Abortion/infertility | 72 | 10 | 13.9 | 6.9%-24.1 |
| Diarrhea | 25 | 1 | 4 | 0.1%-20.4 |
| Apparently healthy | 81 | 9 | 11.1 | 5.2%-20.1 |
| **Total** | **178** | **20** | **11.2** | **7.0%-16.8** |

**Table 2:** Determination of BVDV biotypes in BVDV-positive samples using virus isolation and IFA and IP assays.

| Criteria | No. of isolates | CP | NCP |
|---|---|---|---|
| **Age** | | | |
| Dams | 13 | 1 | 12 |
| Calves | 7 | 2 | 5 |
| **Sample type** | | | |
| Buffy coat | 18 | 3 | 15 |
| Feces | 2 | 0 | 2 |
| **Disease condition** | | | |
| Abortion/infertility | 10 | 1 | 9 |
| Diarrhea | 1 | 1 | 1* |
| Apparently healthy | 9 | 1 | 8 |
| **Total** | **20** | **3** | **17** |

* A sample from a diarrheic calf showed mixed infection of both BVDV biotypes (CP and NCP)

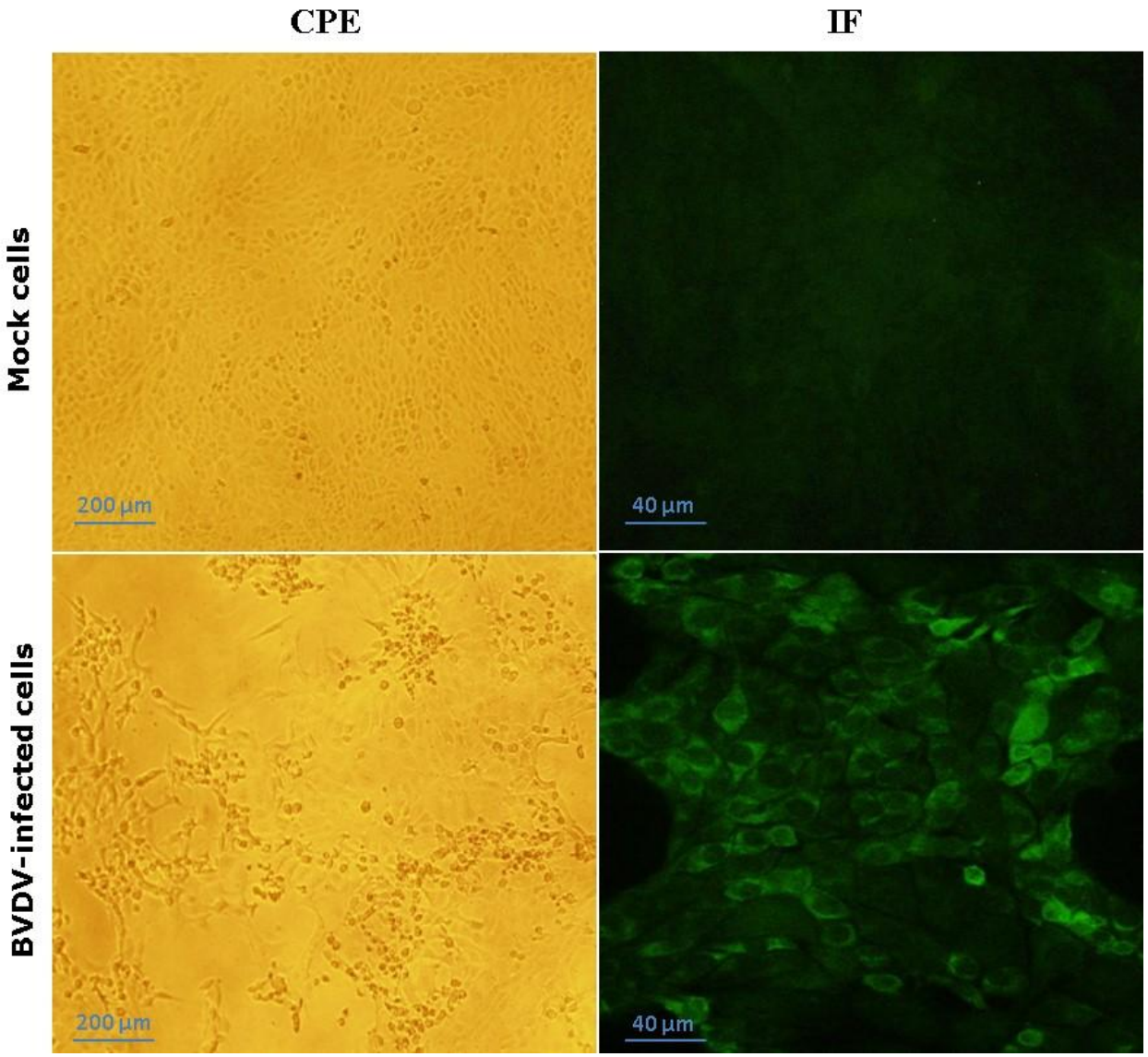


**Figure 1**: Isolation and immunofluorescence detection of cytopathic Bovine Viral Diarrhea Virus (CP-BVDV)-positive samples in MDBK cells. The left panel displays normal mock cells (top) and CP-BVDV-infected cells exhibiting prominent cytopathic effects such as cell lysis, aggregation, and rounding (bottom). The right panel shows the negative control of non-infected cells without fluorescent signals (top) and CP-BVDV-infected cells with strong immunofluorescent signals (bottom).

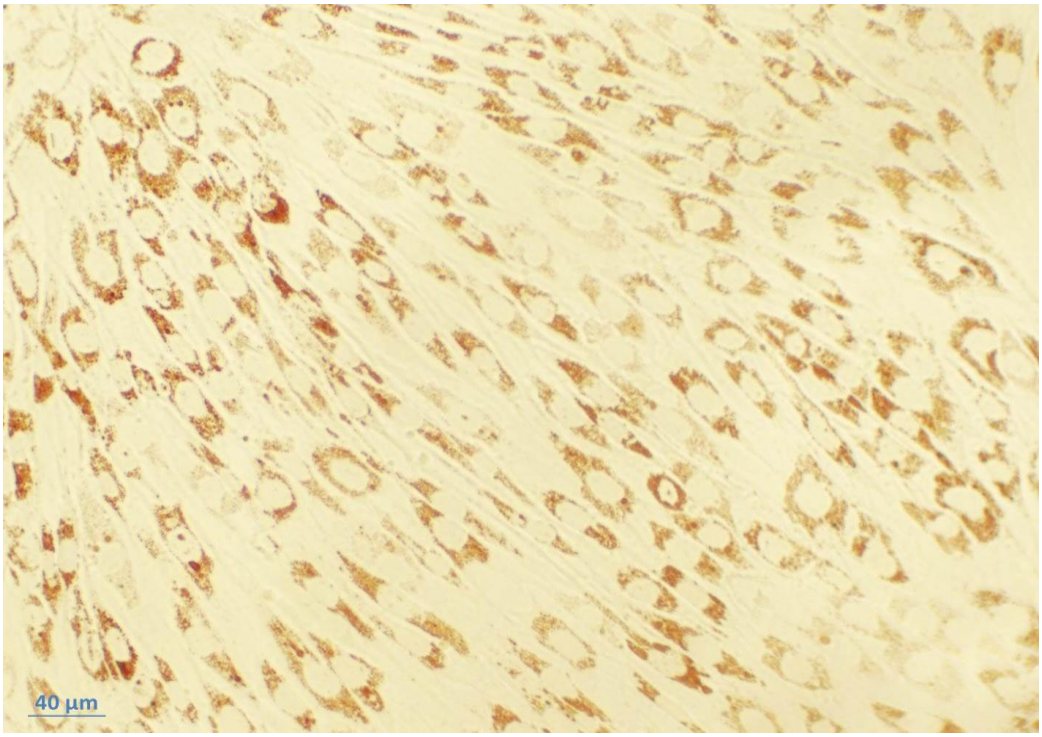


**Figure 2**: IP detection of non-cytopathic Bovine Viral Diarrhea Virus (NCP-BVDV)-positive samples in BT cells. NCP-BVDV-infected cells displayed distinct intracytoplasmic reddish-brown staining.

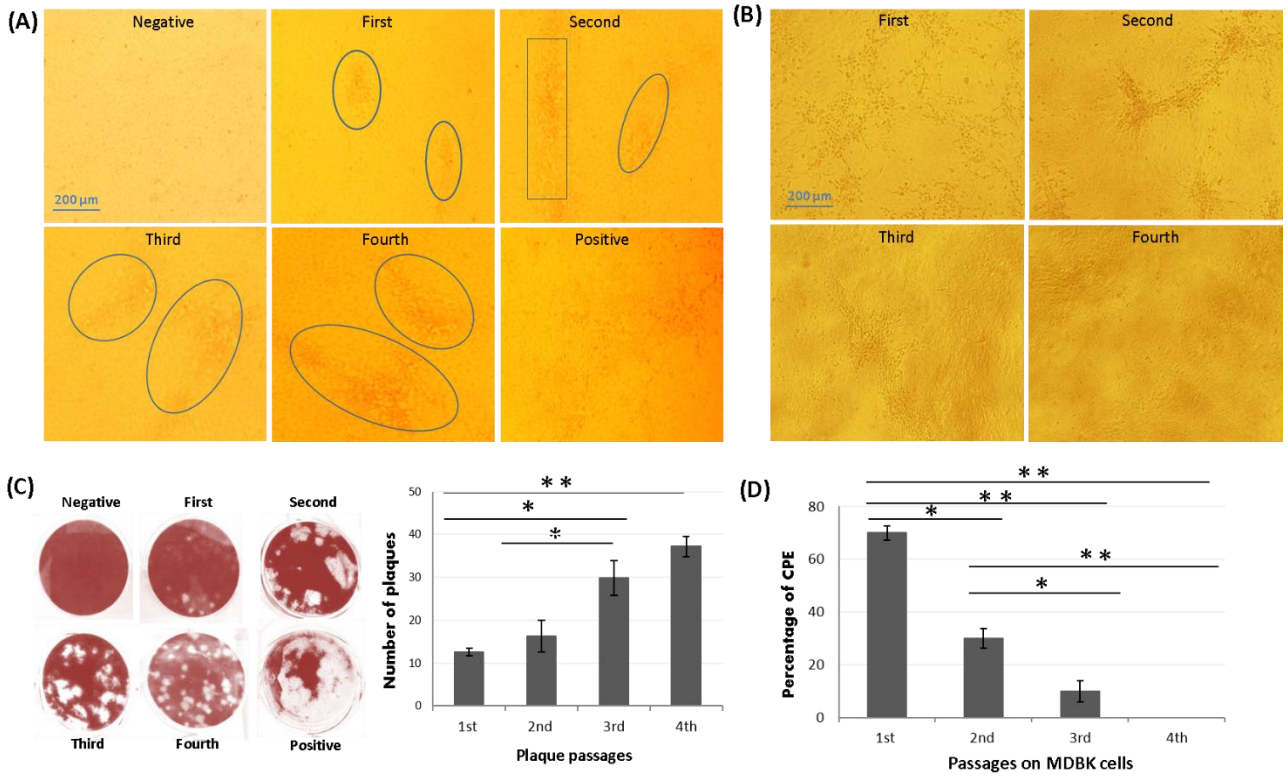


**Figure 3**: Separation of CP- and NCP-BVDV from mixed infections using plaque assay in MDBK cells. The plaques of CP-BVDV increased in intensity and number from the first to the third passage, and by the fourth passage, they covered almost the entire cell sheet (A and C). In contrast, the harvest of underlying cells that showed no CPEs from the first plaque assay passage revealed a decrease in the intensity and percentage of CPEs from the first to the third passage, with CPEs completely disappearing by the fourth passage (B and D). (C) represents the average number of plaques ± standard deviation. Error bars show standard deviation. The asterisk indicates the significant difference (*p<0.05, **p<0.01).

## Discussion

BVDV infections in Egyptian cattle herds have been identified as significant causes of economic losses. These losses result from decreased productivity, reproductive failure, and increased susceptibility to secondary infections (Oguejiofor et al., 2019). Due to limited data on the specific impact of BVDV on abortion and infertility in Egypt, current research aims to investigate its correlation with repeated abortions and subfertility in cattle farms. In order to effectively address these challenges, it is crucial to utilize advanced diagnostic techniques (Werid et al., 2023). One such technique is rRT-PCR, which is essential for screening BVDV due to its high sensitivity, specificity, and rapid results. This molecular technique accurately quantifies viral RNA, allowing for the early detection of BVDV infections, even at low levels. Early detection is crucial for effective control measures to prevent BVDV spread.

Additionally, rRT-PCR can differentiate between BVDV-1 and BVDV-2, aiding in specific vaccination and management strategies. Moreover, rRT-PCR improves the identification of persistently infected (PI) animals and key virus reservoirs (Mari et al., 2016). Therefore, incorporating rRT-PCR into routine screening is vital for comprehensive BVDV control and eradication.

The screening for BVDV in four vaccinated cattle farms that experienced recurrent abortions and infertility revealed that there was a notable presence of BVDV-1, with an overall prevalence of 11.2%. This suggests that the vaccine was ineffective in preventing BVDV infection in the tested cattle farms. Possible reasons for this include the high mutation rate of BVDV and the presence of persistently infected animals (Fulton et al., 2000; Ridpath et al., 2015). Specifically, 20 out of 178 samples were positive; all were identified as BVDV-1. These findings are consistent with studies indicating that all samples from Egyptian cattle infected with BVDV were classified as BVDV-1 (Soltan et al., 2015). Nevertheless, BVDV-3 has also been identified in cattle herds on certain Egyptian farms (Afify et al., 2022). When broken down by age, adult cows had a higher detection rate of BVDV-1 (12.1%) compared to calves (9.9%) (Table 1). These findings suggest that adult cows might be more susceptible or have a higher viral load than calves (Werid et al., 2023).

It is important to note that BVDV-1 was found in 13.8% of dams that experienced subfertility and repeated abortions. This suggests a potential link between BVDV-1 and reproductive issues. For example, a study found a similar prevalence of BVDV-1 in adult cattle, highlighting the virus's impact on reproductive failures, particularly repeated abortions and subfertility (Lanyon et al., 2014). Conversely, the virus was detected in only one diarrheic calf, suggesting that BVDV-1 may not be a primary cause of diarrhea in the studied population. The presence of BVDV-1 in only one diarrheic animal contradicts some studies that have found associations between BVDV infections and gastrointestinal symptoms, indicating that the clinical presentation of BVDV can vary widely and might depend on other co-factors or regional differences (Brock, 2003; Grooms, 2004). A fecal sample from a seemingly healthy calf tested positive for BVDV-1, indicating that the calf had a persistent infection. Among sample types, buffy coat samples had a higher positivity rate (12.67%). This higher detection rate in buffy coat samples is consistent with the findings of Fulton et al. (2000), who emphasized the effectiveness of buffy coat samples in detecting BVDV-1. Fecal samples showed a lower BVDV detection rate (5.6%). This could be due to the smaller sample size (n=36), as the fecal samples were collected from the only farm that exhibited diarrhea along with the main concern (reproductive issues) under investigation. However, fecal samples are effective in detecting BVDV infections and can indicate active viral circulation (Park, 2004).

BVDVs were isolated successfully from all rRT-PCR-positive samples (n=20) using MDBK cells. CPEs, such as cell lysis, aggregation, and rounding, were observed in 15% of the samples identified as CP-BVDV based on their ability to induce these effects (Figure 1). This is consistent with the literature, which indicates that CP-BVDV strains typically exhibit CPE in susceptible cell lines like MDBK due to their high pathogenicity (Fulton and Confer, 2012; Ridpath et al., 2015). The IFA results corroborated the CPE findings. The positive samples displayed bright greenish-yellow fluorescence, confirming the presence of CP-BVDV. This method is effective for identifying CP-BVDV as it relies on specific antibody-antigen interactions that highlight the viral presence within cells (Bezek et al., 1988). Interestingly, the fluorescence was

observed in one buffy coat sample from a dam but not in fecal samples, while in calves, it was detected only in fecal samples. This differential detection might reflect variations in viral shedding or tissue tropism between the two sample types (Brock et al., 1991).

The MDBK cells are useful for detecting some aspects of BVDV. However, they are less sensitive to non-cytopathic strains and may not display the specific immunofluorescence or peroxidase staining needed to confirm the presence of BVDV (Ridpath et al., 1994). This limitation is particularly relevant when dealing with mixed infections or low viral loads. In these cases, the absence of cytopathic effects in MDBK cells can lead to false-negative results (Ridpath et al., 2015). BT cells are often preferred over MDBK cells for IP testing due to their higher sensitivity in detecting BVDV, particularly non-cytopathic BVDV strains. BT cells are derived from the turbinate tissue of cattle, which is a natural site for BVDV infection and replication (McClurkin et al., 1974). This tissue-specific adaptation allows BT cells to support the growth of BVDV strains that might not exhibit cytopathic effects in MDBK cells, making BT cells more reliable for detecting non-cytopathic strains through IP assays (Fulton and Confer, 2012). Therefore, and in contrast to CP-BVDV detection, NCP-BVDV was detected using the IP assay in BT cells, which revealed strong reddish-brown signals in all samples (n=17) that showed no CPE in MDBK cells. This result aligns with previous studies that suggest NCP-BVDV strains often do not produce visible CPE in cultured cells but can be detected by specific assays such as IP (Zhu et al., 2019).

The discovery of a sample from a diarrheic calf that tested positive for CP-BVDV in IFA and also exhibited a clear positive result in the IP assay indicates a mixed infection. This finding is in line with other research that has shown that mixed infections with both CP and NCP strains are not uncommon and can make diagnosis and control efforts more complicated (Brownlie, 1990). The successful isolation of CP-BVDV from a mixed NCP-BVDV infection through plaque assays highlights the usefulness of this method in purifying viral strains. The gradual improvement in visibility and ease of counting of the plaques from the first to the fourth passage indicates that CP-BVDV, when isolated from a mixed infection, becomes increasingly dominant over successive passages (Figure 3). This phenomenon has been observed in other studies where the passage of mixed viral populations often leads to the selective amplification of one strain (Meyers et al., 1997).

## Conclusions

This study emphasizes the significant role of BVDV in causing reproductive problems among Egyptian cattle herds, with BVDV-1 being a notable factor associated with abortion and infertility. The use of advanced diagnostic methods such as rRT-PCR, IFA, IP, and plaque assays is crucial for accurate diagnosis and effective control measures. Additionally, the plaque assay can be an effective method for separating both CP- and NCP-BVDV biotypes in mixed infections. These findings highlight the need for comprehensive BVDV management strategies to address the economic and reproductive impacts of the virus on cattle farms in Egypt. Future analyses should further explore how mixed BVDV infections impact disease outcomes and control measures to enhance herd health and productivity.

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Author contributions. A.O, S.M.G.M: conceptualization and study design. A.O, S.M.G.M, H.A: methodology and data analysis. A.O, S.M.G.M: writing—original draft. A.O, S.M.G.M, M.R: reviewing and editing. All authors contributed to the article and approved the submitted version.

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