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A recent non cytopathogenic bovine viral diarrhea virus detected by immunofluorescence and reverse transcriptase polymerase chain reaction

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ABSTRACT

In the present study, a non-cytopathogenic bovine viral diarrhea virus (BVDV) field strains from bovine semen samples were detected by immunofluorescence (IF) and reverse transcriptase polymerase chain reaction (RT-PCR). Two out of five semen samples tested positive for the presence of BVDV antigen employing antigen capture ELISA kits. The two semen samples were injected in Madin-Darby bovine kidney (MDBK) cells in a trial for BVDV isolation. The chosen two samples were not cytopathic and subjected for further identification and genotyping using one step reverse transcriptase polymerase chain reaction (RT-PCR) assay. The isolated BVDV field strains from semen were found to be BVDV genotype 1.

1. INTRODUCTION

Bovine viral diarrhea virus (BVDV) in cattle are characterized by a variety of symptoms, including unapparent, respiratory, digestive, reproductive, and especially persistently infected (PI) calves as a result of foetal infections (Fulton, 2015). BVDV belongs to *flaviviridae*, genus *pestivirus* which is naked, positive sense single stranded the length of genomic RNA is about 12.3 kb, and it is made up of a single open reading frame (ORF) flanked by untranslated regions at both ends (5 and 3 UTR)(Smith et al., 2017).

Four structural proteins (C, E^{rns}, E1 and E2), as well as seven to eight non-structural proteins (N^{pro}, p7, NS2-3, NS4A, NS4B, NS5A and NS5B), are encoded by the ORF (Ridpath, 2005). BVDV can be divided into two genotypes based on the nucleotide sequence of the 5'untranslated (UTR) region: BVDV-1 and BVDV 2 (Ridpath et al., 1994). BVDV appears in two biotypes form, cytopathic(CP) or non-cytopathic (NCP) according to their effects on tissue cell culture (Ridpath and Flores, 2007). NCP viruses can induce persistent infection in cells and have an intact NS2/3 protein. In CP viruses the NS2/3 protein is either cleaved to NS2 and NS3 or there is a duplication of viral RNA containing an additional NS3 region (Qi et al., 1998).

Persistently infected bulls shed large amounts of virus in their seminal fluid, and the virus may survive both cryopreservation and processing of semen for artificial insemination (McGowan and Kirkland, 1995; Walz et al., 2008). The virus was found in the semen of both acutely and persistently infected bulls (Wrathall et al., 2006). These facts directed the need to develop continuous screening of whole semen samples for the presence of BVDV. BVDV has been diagnosed using enzyme linked immunosorbent assay (ELISA), virus isolation (VI), immunohistochemistry (IHC), serum neutralization (SN), direct and indirect fluorescent assay (Cornish et al., 2005). The target of this study was identification of BVDV biotypes and genotypes from bovine semen using immunofluorescence and reverse transcriptase polymerase chain reaction.

2. MATERIAL AND METHODS

2.1. Specimen collection

Five semen samples were collected from bovine farms of Egyptian provinces during the year 2019. Electro-ejaculation was used to obtain these samples. Using collection funnels, all ejaculates were collected into newly graduated sample tubes. Cross-contamination between animals was avoided by collecting all samples in separate sterile funnels for each animal. Semen samples were transported under ice and stored at -20ºC until used.

2.2. Preparation of suspected samples

The semen samples were subjected to two cycles of freezing and thawing. To equal volume of the suspension phosphate buffered saline containing 0.2% (v/v) of 10 000 IU/ml penicillin, 0.2% (v/v) of 10 mg/ml streptomycin and 0.4% (v/v) of 12.5 mg/ml mycostatin. It was centrifuged at 10.000 rpm for 30min. The supernatant fluid was filtered through a membrane filter with a pore diameter of 0.45 mm and kept at -80ºC until use in antigen capture ELISA.

2.3. Reference BVDV strain and antiserum

The cytopathic BVDV, NADL strain was propagated in MDBK cell line with a titer $10^{6.5}$ $TCID_{50}$/ml and used as

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positive control in RT-PCR. BVDV polyclonal antiserum and Rabbit Anti-bovine IgG conjugated with fluorescence isothiocyanate were used in viral detection by isolation-immunofluorescent test (IFX). The BVDV reference strain and antiserum was supplied by the Animal Health Research Institute's (AHRI), Department of Virology, Dokki, Giza, and the conjugate was supplied by Sigma.

2.5. Antigens capture ELISA kit

Five semen samples were investigated using antigen capture ELISA of commercial ELISA BVD/MD antigen mix screening kit (INSTITUTE POURQUIER, France). Monoclonal antibodies (MAbs) against the nonstructural BVD virus protein p125/p80 were used in antigen-detecting ELISA according to manufacturer instructions.

2.6. Isolation of BVDV (Isolation —IFX)

The semen suspension was inoculated into culture tubes after they had been seeded with MDBK cell culture. To achieve efficient viral development, the culture medium was replaced 24 hours after inoculation and the cells were kept for another 4 days. The culture drums were frozen and thawed twice at the end of the period, and the culture fluids were then injected onto MDBK cells cultured in a 24-well plate. Immunofluorescence (IFX) staining was used to confirm the isolations (Roberts et al., 1991)

2.7. Extraction of BVDV RNA

BVDV RNA was extracted using the QIAamp® Viral RNA mini Kit (Qiagen, United States), according to the manufacturer's instructions

2.8. Detection of BVDV RNA using one step RT-PCR

The One-Step RT-PCR Master Mix, Qiagen Operon Technologies, Alameda, CA, was used to perform one-step RT-PCR using commercial reagents according to manufacturer's instructions. The primer sequences were based on the sequence of BVDV 5'un- translated region (5'-UTR) gene (Vilcek et al., 1994). The specific primers were manufactured by Metabion international, Germany. The sequences of oligonucleotides are: F 5'–ATGCCCWTAGTAGGACTAGCA- 3' (forward primer) R 5'- TCAACTCCATGT GCCATGTAC - 3' (reverse primer) targeting 288bp sequence. The reactions were carried out in the following order on a programmable thermocycler: 94°C for 7 min; 35 cycles of 94°C for 10 sec, 53°C for 30 sec, 68°C for 30 sec; and final elongation at 68°C for 7 min. the target amplicon were analyzed on agarose gel 1% stained with ethidium bromide.

3. RESULTS

From five prepared semen samples, two samples were positive by antigen capture ELISA. By isolation and biotyping, the two non-cytopathic samples were positive for IFX (Fig1 and Fig 2). The One step RT-PCR produce target amplicon 288bp characteristic for BVDV genotype 1 was similar to reference NADL strain (Fig 3).

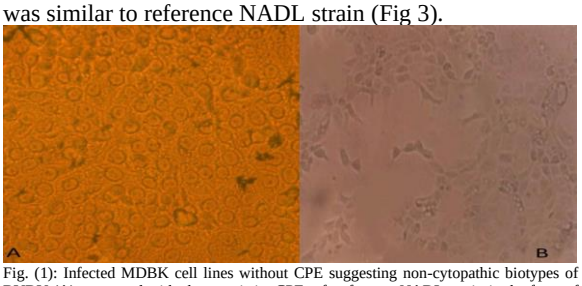

Fig. (1): Infected MDBK cell lines without CPE suggesting non-cytopathic biotypes of BVDV (A) compared with characteristic CPE of reference NADL strain in the form of cell rounding, aggregation, vacuolation followed by cellular darkness and cluster formation and cell detachment (B)

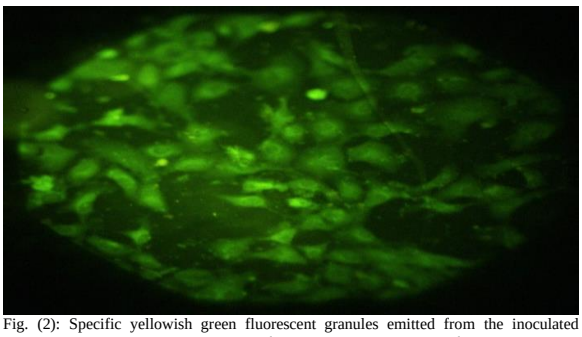

Fig. (2): Specific yellowish green fluorescent granules emitted from the inoculated MDBK cell culture indicating presence of non-cytopathogenic strain of BVDV

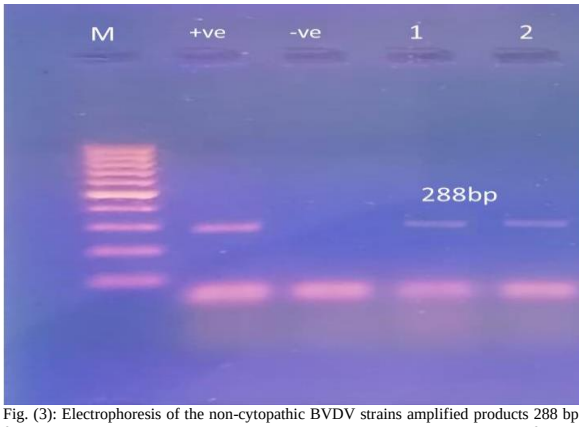

Fig. (3): Electrophoresis of the non-cytopathic BVDV strains amplified products 288 bp from bovine semen. Lane M: base pair marker (100 bp), Lane +ve: RT-PCR reference NADL strain, Lane -ve: negative control, lane 1&2: Positive 288bp product of non-cytopathic BVDV strains

These problems were attributed to high antigenic diversity of BVDV (Fulton et al., 2002). According to reports, bulls affected with BVDV, either acutely or persistently, can shed the virus in their semen or transfer it to cows during insemination (Givens et al., 2006; Gard et al., 2007; Walz et al., 2008). So, in this study a trial for identification of BVDV biotypes and genotypes from bovine semen were adopted.

Screening of BVDV antigen in semen by antigen capture ELISA emphasis presence of BVDV antigen in two samples indicating BVDV infection (Wrathall et al., 2006) as ELISA is considered one of the laboratory methods has been used for rapid detection of BVDV antigen and antibodies (Fulton et al., 2006). There are 2 biotypes of BVDV, non-cytopathogenic (NCP) and cytopathogenic (CP) based on its growth behavior in cell culture, but not related to virus virulence. The NCP biotype has no effect on cultivated bovine cells. CP biotype, on the other hand, causes substantial cell damage and apoptosis in cell cultures (Ridpath and Flores, 2007). Although acute BVDV infections are the most common, when a fetus is exposed to a non-cytopathic biotype before gaining immune competence, persistent infections can occur (Ridpath et al., 2006, Gard et al., 2009).

The result of IFAT was agreed with Goyal and Ridpath (2005) who reported that isolation of BVDV in cell cultures followed by identification by IF or RT-PCR is the most reliable method for detecting BVDV infection in young calves and Fulton et al., (2006) who mentioned that MDBK cell line is considered one of the most suitable cells for isolation of BVDV. Direct fluorescent antibody assay, or RT-PCR is used to confirm the isolated virus.

The detection of a non-cytopathogenic biotype of BVDV supports the development of persistent infection of BVDV among the cattle population (Ridpath et al., 2006; Gard et al., 2009).

Previously, several PCR-based assays for typing BVDV have been reported (Ridpath et al., 1994). In the current study rapid detection and typing of BVDV field isolates, RNA extracted from cytopathic and non-cytopathic biotypes were amplified by one step RT-PCR using primers complementary to the sequences in untranslated region of the 5´ end of the BVDV RNA genome (Vilcek et al., 1994, Brian, 2007). PCR product was identical to the reference NADL strain and was characteristic of BVDV type 1 indicated distinct PCR product at the (288 bp). The obtained results come in agreement with (Ridpath 2003) who reported that genotype 1 may exist as cytopathic or non-cytopathic.

5. CONCLUSION

Recent non-cytopathogenic BVDV genotype 1 was detected by IF and RT-PCR in bovine semen will be enhancing the persistence infection among cattle population and this required continuous screening of semen before natural and artificial insemination.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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