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ISOLATION AND IDENTIFICATION OF BOVINE VIRAL DIARRHEA VIRUS IN SEMEN SAMPLES

(With 2 Figures)

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عزل وتصنيف فيروس الاسهال البقرى من عينات السائل المنوى

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تهدف هذة الدراسة الى عزل و تصنيف فيروس الأسها ل البقرى من السائل المنوى لطلا ئق مـــن مـــرا كز تلقيح أصطناعى مختلفة, من عدد ٤٠ عينة سا ئل منوىّ وبا ستخدام الأجسام المضادة متعددة القسيلة ضد فيروس الأسهال البقرى وأجسام مناعية للماشية محملة بالصبغة الفلورسنتية وجد عدد ٢٣(٥٧,٥%) ايجابى حيث توجد الحبيبات الفلورسنتية فى السيتوبلازم حـــول الـــنواة, باســـتخدام البلمرة المتسلسل المتعاكس للكشف عن الحامض النووى وعلى خطوتين منفصلتين وجد عدد٢٩ عينة بنسبة٧٢,٢٥ % ايجابية حيث اعطت خطوط واضحة عـــند p288 . هذة الدراسة هى البداية حيث يحتاج هذا المجال الى اهتمام لتحسين الأنتاجية فى المزارع الحلابة والتسمين.

SUMMARY

The objective of this study is to isolate and identify bovine viral diarrhea virus (BVDV) from semen of persistently infected (PI) bulls collected from different artificial insemination (AI) centers as start point. From a total 40 seminal fluid samples and by using polyclonal anti-BVDV and FITC conjugated anti-bovine IgG, 23 (57.5%) were positive. The pattern of fluorescence granules distribution was perinuclear, surrounding the nucleus and extending to most of the cytoplasm. Reverse transcription-polymerase chain reaction (RT-PCR) was impact on the viral RNA at a high conserved region 5`UTR among known BVDV sequences. On two steps, RT- PCR were carried out and revealed that 29 /40 (72.25%) are highly specific that gave a sharp bands at 288 base pair (bp). This study is pin point the area that requires greatest impact to improve the efficiency of dairy and beef production.

Key words: Bovine viral diarrhea, virology, semen, RT-PCR.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is one of the leading viral pathogens that is an economically significantly among cattle population (Brock *et al.*, 1997). The viral genome is a single stranded R N A of positive polarity with 12.5 kilo base (kb) in length and molecular weight 4.3 X 10^6 (Collett *et al.*, 1988 b and c and Rumenpaf *et al.*, 1993). There is common ancestry of BVDV and both border disease virus (BDV) and classical swine fever virus (CSFV) (Sullivan *et al.*, 1994). The three members form the genus *Pestivirus*, family *Flavivirdae* (Francki *et al.*, 1991). According to cell culture behavior, two forms of the virus exist. Cenotaphic (CP) biotype is associated predominantly with animal that develop mucosal disease (MD) or non cytopathic (NCP) biotype that is widely distributed and account for than 90% of infection so consider the standard BVDV-biotype that responsible mainly for persistent infection (PI) (Dubovi, 1992).

The effect of BVDV infection on bovine fertility and reproductive diseases has greatly evolved in the past decade. Currently, reproductive disease is one of the primary consequences of BVDV infection in both beef and dairy cattle.Nevertheless, once the infection is established in a herd, there is a potential for its amplification through recycle transmission (Kirkland *et al.*, 1997; Stringfellow *et al.*, 1997 and 2000).

Bulls PI with BVDV regularly yielded BVDV-contaminated semen of significantly reduced quality (Kirkland *et al.*, 1991, 1994 and 1997). Although, there have been conflicting finding between some studies, the risk of infection has been reported to be highest from natural service (Grahn *et al.*, 1984; Barlow *et al.*, 1986 and Revell *et al.*, 1988). Knowledge in this area continues to evolve. Some of the lesions are not specific for BVDV, reflecting the importance of laboratory diagnosis. A final diagnosis must rest on isolation of the virus for detection of specific antigen in appropriate samples (Ohmann, 1995).

In Egypt, several studies have been carried out on isolation, antigenic and genomic characterization of BVDV (Hafez, 1975; Baz, 1982; Baz *et al.*, 1990; Abd El-Hafeiz, 1997; Allam, 2000; Hussein, 2001; Abd El- Hafeiz, 2002; Abd El-Hafeiz *et al.*, 2003; Amin, *et at.*, 2003 and Hassanein *et al.*, 2003). However, this study aimed to isolate and identify BVDV isolates from semen of PI bulls.

MATERIALS and METHODS

Semen samples:

A total of 40 fresh semen samples were collected from different artificial insemination (AI) centers, submitted directly to our laboratory and processed by standard method as described by Kirkland *et al*, 1991. In 1.8 ml cell culture growth media, 200 µl of each row semen sample was diluted. At 800 *xg*, 4 °C, the sample was centrifuged for 10 minutes. The seminal fluid was then removed and frozen at-80 °C. The cells from the row semen were washed twice in phosphate-buffered saline (PBS) pH 7.4 before freezing in growth media at the original volume.

Viruses and cell culture:

Mycoplasma and BVDV- free Madin – Darby bovine kidney (MDBK) cells were supplied from (National Animal Diseases Center, Ames, Iowa, USA). Eagle's minimum essential medium (MEM, Gibco, Life Technologies, Scotland, UK) supplemented with fetal bovine serum (FBS, Biochrom) and antibiotic /antimycotic (Sigma, Ames, Iowa, USA) were used. A newly isolated, Behera-CP/58, and Behera- NCP/53 as genotype 2 strains, from purified milk somatic cells (PMSC) by Abd El-Hafeiz, 2002, an international NADL strain and Egyptian strains, Iman and Kenna, were used as positive control.

Virus isolation:

Virus isolation from the total 40 seminal fluid samples was done in 24 tissue culture plate as described by Schweizer and Peterhans, 1999.

Immune fluorescence assay (IFA):

Antigen detection was performed as in Dubovi, 1990. After 48 hours of the 3 rd passage, the supernatant was discarded and the cover slips washed twice with PBS. The inoculated cells were fixed immediately in 50 v/v acetone /methyl alcohol at room temperature for 30 minutes then air dried. On cell side cover slips, two drops of polyclonal anti-BVDV serum (against reference NADL strain) were added and incubated at humidified atmosphere for 60 minutes. Then, thoroughly washing for 3 times with PBS before adding two drops of anti-bovine conjugated with fluorescence isothiocyanate (FITC). The cover slips were re-incubated for 60 minutes under the same above condition. After thoroughly washing and drying, pre-mounted cover slips with pre-warmed mounting fluid (1 part glycerol + 9 part PBS) were examined under a fluorescence microscope.

Extraction of RNA:

Total RNA was extracted from inoculated samples and mock infected cells, as negative control, using guanidin thiocyanate as described by Chomczynski and Sacchi, (1987) and Sambroock *et al.* (1989). Briefly, to 100 µl of sample in 1.5ml DNase / RNase free eppendorf tube, 400 µl of denature solution (360 µl β-mercaptoethanol+50 ml of guanidin solution {50 gm guanidin thiocyanate crystals +3.52 ml of 0.75M sod. citrate pH 7.0 + 5.28 ml of 10% sarkosyl + 58.6 ml of ultra pure water}) was added and mixed. To the mixture, 40, 400 and 80 µl of sod. acetate pH 4.0, water saturated phenol pH 4.3 and chloroform / isoamyl alcohol (24/1) were added respectively and mix after each addition. The mixture was cooled on ice for 15 minutes before centrifugation at 10000 *xg* for 20 minutes. Transfer the upper aqueous phase to a fresh eppendorf tube and add 500 µl cooled isopropanol and storied at –20 °C over night. The sample was centrifuged at 10000 *xg* / 20 minutes to precipitate the RNA. For more purification, 300 µl of denature solution, 30 µl sod. acetate pH 5.4 and 600 µl 95% cooled ethanol were added and freezing at –70 °C over night. Next, the sample was centrifuged at full speed for 20 minutes and washing by 500 µl 75% ethanol and 250 µl 95% ethanol for 10 and 5 minutes at 10000 *xg* respectively. The RNA was dried for up to 1 hour before dissolving in 20 µl diethyl pyrocarbonate (DEPC) treated-water.

RT-PCR assay:

Reverse transcription of viral RNA was carried out as mentioned by the manufacture (Aßgene, UK). In a final volume 20 µl, RT of RNA was done. To 10 µl of RNA, a mixture of 4 µl of 5X RT-buffer (250 mM Tris-HCl pH 8.3; 375 mM KCl; 15 mM $MgCl_2$ and 50 mM DTT; Aßgene, UK), 1 µl hexameric primer (0.5 µg /µl, Promega, USA), 0.5 µl RNase inhibitor (40 U /µl, Amresco, OH, USA), 0.4 µl dNTPs (10 mM /µl, Amresco, OH, USA), and 0.5 µl Molony Murine Leukemia Virus-Reverse Transcriptase (MMLV-RT) enzyme (250 U /µl, Aßgene, UK) was added. The mixture was incubated at 37 °C for 1 hour and stop the reaction by heating the mixture at 65 °C for 5 minutes.

Amplification of RT products was done in a total volume 50 µl as optimized previously by Abd El-Hafeiz (2002) using a primer sequence within 5`UTR as in Vilcek, *et al.* (1994). To 5 µl of RT product, a mixture of 5 µl 10X Tag buffer (750 mM Tris-HCl pH 8.8; 200 mM $(NH_4)_2 SO_4$ and 0.1% v/v Tween 20; Aßgene, UK), 1 µl of each sense primer (UTR 1) 5`--ATG CCC WTA GTA GGA CTA GCA--3` (108~128) and antisense primer (UTR 2) 5`-TCA ACT CCA TGT

GCC ATG TAC-3`(395~375), 1 µl dNTPs (10 mM /µl, Amresco, OH, USA), 3 µl $MgCl_2$ (25mM) and 0.25 µl Tag polymerase (5 U /µl, Aßgene, UK) was added. The reaction was initially denature at 94 °C for 1 minute followed by 35 cycles of denature at 94 °C /1minute, primer annealing at 56 °C /1minute and extension at 72 °C /1 minute. A final extension as one cycle at 72 °C / 7minutes was done.

Analysis of RT-PCR products:

Analysis of RT-PCR products was formed as in Weigand, *et al.* (1993). The amplified products were mixed with 6X gel loading buffer (50% sucrose, 2 mM EDTA pH 8.0, 0.1% bromo phenol blue, 0.1% xylene cyanole) 1: 5 v/v and electrophoresed through 1% agarose gel in Tris-acetate-EDTA (TAE) containing 0.5 µg /ml ethidium bromide at 70 volts for 2 hours. After that, the amplified bands were visualized by UV transilluminator and photographed by a Polaroid camera.

RESULTS

From all 40 seminal fluid samples inoculated on MDBK cells, a fluorescence granules distributed perinuclure, surrounding the nucleus and extending to most of the cytoplasm were observed in 23 samples (57.5%) (Fig.1). In a previous experiments, we tested the PCR conditions and seemed to work well on BVD viral-RNA extracted from different clinical samples. We decided to analyze seminal fluids against viral RNA. Forty inoculated seminal fluid samples on MDBK cells for one time were analyzed for the presence of infectious viral RNA. Only 29 (72.5%) samples were clearly identified by ethidium bromide staining of the amplified DNA bands (288bp) in the gel (Fig.2). Also, PCR can detect 6 samples more than IFA. However, PCR analysis confirmed the presence of BVD viral-RNA in seminal fluids of PI bulls.

DISCUSSION

Bovine viral diarrhea virus (BVDV) is an important infectious disease agent of cattle that can potentially have a negative effect on all phases of reproduction. The effect of BVDV infection on bovine fertility and reproductive diseases has greatly evolved in past decade (Kirkland *et al.*, 1997; and Grooms, 2004). There have been conflicting findings between some studies on the effect of BVDV infections on reproduction performance. Knowledge in this area continues to evolve (Grahn *et al.*, 1984 and Sheldon and Dobson, 2003).

Reproductive efficiency is imperative for maintenance of profitability in both dairy and cow-calf enterprises. There have been reports of the excretion of BVDV in semen of bulls which had experienced an acute transient and persistent infection (Paton *et al.*, 1989; Kirkland *et* al., 1991 and Voges *et al.*, 1998). A consistent finding with PI bulls is the very high titer of the virus in the semen, usually at least 100 times higher than the titer in the blood and this may encounter to replication of the virus in prostatic and vesicular glands (Kirkland *et al.*, 1991 and Kirkland *et al.*, 1994). The virus continue excreted in semen of acutely infected bulls for several days after the last day of viremia and ceased with production of serum neutralizing antibodies while in PI bulls, shedding of the virus continue up to 11 months post pubertal. The effect of the virus on semen quality is of questionable, but generally affects on conception rates, seroconvertion of dams and subsequently effects on fetus and reproductive tracts (Kirkland *et al.*, 1991).

This study aimed to isolate and identify BVDV from semen of PI bulls collected from different AI centers to investigate the incidence of virus spread as start point. By using polyclonal anti-BVDV and anti-bovine IgG-conjugated with FITC from a total 40 samples, 23 (57.5%) were positive. The pattern of fluorescence granules distribution in BVDV-infected MDBK cells were perinuclear, surrounding the nucleus and extending to most of the cytoplasm. This distribution of viral antigens is most consistent with its localization in the rough endoplasmic reticulum (Ng *et al.*, 1983). As mentioned previously, this technique has been responsible for many incorrect diagnosis of BVDV, mainly because of the poor quality of the antisera and high antigenic variability among viral strains. To overcome the disadvantages of IFA technique that less vulnerable in diagnosis, a more sensitive and specific assay, RT-PCR, was impact on the viral RNA at a high conserved region 5'UTR among known BVDV genomic sequences (Collett *et al.*, 1988a; Deng and Brock, 1992 and Dubovi, 1992) to amplify a target region 288 bp as recommended in Vilcek *et al.* (1994). The RNA was extracted from the 40 samples using guanidin thiocyanate as potent denature solution (Chomczynski and Sacchi, 1987). Reverse transcription and amplification were carried on two steps and revealed that PCR are highly specific that gave sharp bands at 288 bp for 29/40 (72.25 %). Positive and negative controls were included all over the study. This study is pin point the area that requires greatest impact to improve the efficiency of dairy and beef production.

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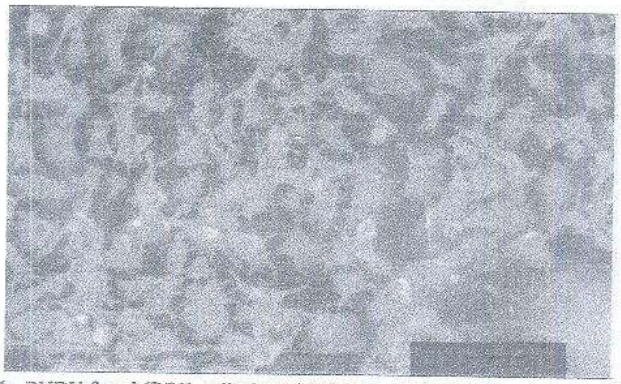

Fig.1: BVDV-free MDBK cells inoculated with seminal fluids stained with anti-BVDV hyperimmune serum followed by FITC conjugated anti-bovine IgG. Perinuclear intracytoplasmic fluorescence granules (X 200).

M 1 2 3 4 5 6 7

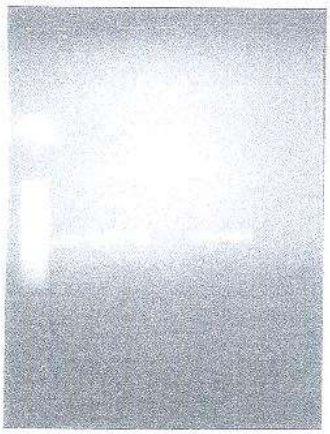

Fig. 2: Agarose gel (1%) analysis of RT-PCR amplified products of 5 UTR viral RNA: M is 50 bp DNA ladder (Promega, Cat. No. G 4521). Lane 1 is a positive control, lanes 2, 3, 5 and 6 are some represented positive samples, lane 4is represented a negative sample and lane 7 is a negative control.