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APPLICATION OF NESTED REVERSE TRANSCRIPTION PCR FOR DETECTION OF BOVINE VIRAL DIARRHEA VIRUS IN SEMEN

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SUMMARY

A nested reverse transcription-polymerase chain reaction (RT-PCR) was employed for detection of bovine viral diarrhea virus (BVDV) in 59 semen samples collected from farms of known history of BVDV infection. The results indicated that 6 (10.2 %) and 8 (13.6 %) net and extended semen samples, respectively, were culture-positive and 53 (89.8 %) and 51 (86.4 %) were culture-negative. On the other hand, 10 (16.9 %) and 10 (16.9 %) net and extended semen samples, respectively, were nested RT-PCR-positive and 49 (83.1 %) and 49 (83.1 %) were nested RT-PCR-negative. Ten semen samples were collected from BVDV-free animals to be used as negative control samples and they tested negative by culture, nested RT-PCR and double PCR. The revised sensitivity and specificity of nested RT-PCR in net and extended semen samples were 100.00 %. On the other hand the sensitivity of culture technique in net and extended semen samples were 60.00 % and 80 %, respectively, while the specificity were 100 % in both net and extended semen.

The results indicated that nested RT-PCR is reliable, rapid, highly sensitive, and specific for accurate detection of BVDV in semen samples. According to the best of our knowledge, our work is the first to report a nested RT-PCR assay that has proved to be efficient in detecting the presence of BVDV in semen. Finally we recommend the use of nested RT-PCR assay as a supplemental diagnostic tool for detection and identification of BVDV in semen.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a small enveloped-RNA virus that, together with classical swine-fever virus and border-disease virus, belongs to the genus *Pestvirus* within the family *Flaviviridae* (Tautz et al., 1994 and Wengler et al, 1995). The new pestivirus species was isolated from cattle and sheep (Becher et al., 1995). BVDV are classified into two different genotypes known as type 1 and type 2. Within each genotype there are several different strains of BVDV that can differ significantly in their ability to cause disease (Pellerin et al., 1994). In addition there are two different biotypes of BVDV called noncytopathic and cytopathic based on effect observed in infected monolayers and both of them may be virulent in cattle (Brownlie et al., 1987). The genome of BVDV consists of a single strand positive sense RNA which is about 12.300 nucleotides long (Collett et al., 1988).

BVDV is an economically important pathogen of all ages of cattle. The infection is associated with lesions in the alimentary tract, which occurs in two forms, bovine viral diarrhea and mucosal disease (Barker et al., 1993). Infection of the bovine fetus may result in abortions, stillbirths, teratogenic effects or persistent infection in the neonatal calf (Barker et al., 1993). The persistent infection form, the most important one (Holland et al., 1993), begins with transplacental infection of the fetus during the first 120 days of gestation with noncytopathic BVDV prior to the complete maturation of the fetal immune system (Brownlie et al., 1987). The persistently infected calves remain seronegative to all tests and shed large amounts of virus in most body secretion and excretions over prolonged periods (Afshar and Eaglesome, 1990). High viral titers ($1X10^{4.7-7.6}$ $TCID_{50}/g$) can be isolated from the semen of infected bulls (Barlow et al., 1986). Transmission of BVDV can occur following the use of raw, extended or cryo-preserved semen from the serologically negative infected bulls and they are often associated with poor fertility (Meyling and Jensen, 1988 and Kirkland et al., 1991). Nevertheless, persistently infected bulls can sire clinically normal offspring. These calves that are born persistently infected may arise following recirculation of the virus in susceptible dams or through viral persistence within the urogenital tract (Meyling and Jensen, 1988). To prevent transmission of BVDV by artificial insemination, only BVDV-free semen should be used.

The most common method for laboratory diagnosis of BVDV infection in clinical samples is culture technique. Cell culture is considered the "gold standard" for detection of the virus in semen of infected bulls but it is laborious and gave variable results depending on the presence of sufficient numbers of virus particles in the samples. In addition, it is time consuming since isolation of BVDV from any sample requires more than one passage which takes several days or even weeks

(Afshar and Eaglesome, 1990 and Hains et al., 1992). On the other hand, the sensitivity of this method is low because semen is cytotoxic, and may cause cytotoxicity after inoculation directly onto cell monolayers (Brownlie, 1996). Because of these difficulties, the development of new rapid and sensitive diagnostic tests for detection of BVDV in semen is increasingly drawing interest.

In some publications in Egypt, polymerase chain reaction assay is the most promising development in detection of some pathogens in semen, which has been proved to have high degree of sensitivity and specificity (Amin and Ibrahim, 1998, Amin et al., 2001 and Amin, 2003). Although, there are several studies on detection of BVDV-RNA from different samples (tissue suspensions, blood leucocytes, milk, semen and serum) by RT-PCR (Brock, 1991, Hertig et al., 1991, Lopez et al., 1991, Gruber et al., 1993, Ridpath et al., 1993, Gruber et al., 1994, Schmitt et al., 1994, Da Silva et al., 1995, Laamanen et al., 1997, Sandvik et al., 1997, Weinstock et al., 2001 and Abd-El-Hafeiz, 2002), not enough data are available to assess the performance of the nested RT-PCR assay on semen samples.

The purpose of this study was to apply nested RT-PCR assay for detection of BVDV in semen samples and to determine its sensitivity and specificity in comparison with culture.

MATERIALS AND METHODS

Semen samples:

Fifty-nine bulls' semen samples were collected from farms of known history of BVDV infection. Another semen sample from each bull was diluted in a Tris-buffered-fructose-glycerol-yolk extender to 30-80 million spermatozoa per ml, and the mixture was divided into 200 µl aliquots (Foote, 1970) and stored at -70° C until used. At the same time, 10 semen samples were collected from BVDV-free herds to be used as negative control. Cultural and nested RT-PCR amplification assays were applied for detection of BVDV in net and extended semen samples (Gruber et al., 1993 and Brownlie, 1996) .

Preparation of semen samples:

Different seminal fractions were separated from each semen sample as described by Von Berold-ingen et al. (1990). Briefly, two volumes of lysis buffer 1 (0.15 M NaCl, 0.75% sodium-N-lauroylsarcosine, and 1.5 mg of proteinase K [Boehringer Mannheim, Germany] per ml) were added to each semen sample and the mixture was incubated at 60°C for 1 h. The pooled fraction of nonsperm cells and seminal fluid was obtained from the supernatant. A pooled fraction of sperm heads and non-sperm cells was isolated by centrifugation of semen, resuspending the cell pellet in 100 µl of PBS, and lysis in buffer 2 (buffer 1 in

addition to 40 mM dithiothreitol [DDT; Sigma Chemical Co., USA]). To obtain BVDV from whole semen, each semen sample was directly lysed in buffer 2. All prepared samples were stored in small aliquots at -70°C until used.

Virus template control:

BVDV strain NADL was kindly supplied by Prof. Dr. Saber, M.S., Department of Virology, Faculty of Veterinary Medicine, Cairo University. It was grown in BVDV-free MDBK cells in modified Eagle's minimum essential medium (MEM)(GIBCO BRL, USA) supplemented with 0.5 % inactivated BVDV-free fetal calf serum (FCS) (GIBCO BRL, USA) and 50 mg/l gentamycin for seven days. The virus was stored at -70°C until used.

Virus isolation:

The procedure described by Brownlie (1996) was followed for virus isolation by culture technique. Briefly, shell vials were seeded with 1 ml of BVDV-free MDBK cells in modified MEM (GIBCO BRL, USA) supplemented with 5 % inactivated BVDV-free FCS (GIBCO BRL, USA) and 50 mg/l gentamycin and incubated at 37°C. The tissue culture medium was aspirated from each shell vial; 0.1 ml of each diluted semen sample (1/10 in culture medium) was inoculated directly to each of three shell vial monolayers and incubated for 1 h at 37°C. After the absorption period, the shell vial monolayers were washed with tissue culture medium and 1 ml of MEM supplemented with 2 % inactivated BVDV-free FCS and 50 mg/l of gentamycin was added to each vial. None inoculated shell vials were used as controls. The shell vials were incubated at 37°C for 5 days and were examined daily for evidence of signs of cytotoxicity before being stained. After incubation, shell vial monolayers were washed twice with PBS, fixed in cold acetone for 10 min, stained with fluorescein-conjugated bovine antiserum to BVDV (National Animal Disease Center, Ames, Iowa, USA) and examined under a fluorescent microscope. Passages were considered negative if a lack of staining by DFA was observed. Each sample was passaged three times before being considered negative.

Oligonucleotide specific primers:

The primers (Gruber et al., 1993) were chosen from the sequence of the gene coding for the non-structural protein p125 (Collett et al., 1988), synthesized using a DNA synthesizer 392 (Applied Biosystems) and purified chromatographically (NAP-10 Sephadex column, Pharmacia). The sequences of oligonucleotide primers were as follows: Outer sense primer GAA, AGC, AAA, GGC, TTA, AAG, AAG, TT

Outer antisense primer TGA, CGC, CAT, ACT, CTG, TCT, CAT

Inner sense primer GCA, GAT, TTT, GAA, GAA, AGA, CAC, TA

Inner antisense primer TTG, GTG, TGT, GTA, AGC, CCA

RNA extraction:

Extraction of RNA from each prepared semen sample was carried out as previously described (Chomczynski and Sacchi, 1987). Briefly, a 100 µl-aliquot of each sample was mixed with two volumes of extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, 30 µl of 2 M sodium acetate [pH 4.0], 300 µl of phenol saturated with diethyl pyrocarbonate [DEPC]-treated water). The mixture was then vortexed and left at room temperature for 15 min. A total of 60 µl of a chilled chloroform-isoamyl alcohol mixture (49:1) was added and the mixture was vortexed and placed on ice. After 15 min, the mixture was centrifuged at 15,000 rpm for 5 min at 4°C and the aqueous phase was transferred to a tube with 600 µl of chilled isopropanol and was then left at -20°C overnight. On the following day, the sample was centrifuged at 15,000 rpm for 30 min at 4°C and the final pellet was washed once with 70 % ethanol. The RNA pellet was briefly dried and was resuspended in 10 µl of DEPC-treated water. The nucleic acid from negative control semen samples collected from BVDV-free bulls (previously tested) and positive samples (NADL strain) were extracted at the same time as the test samples and were used in 1- to 5-µl volumes in the amplification protocol.

Nested RT-PCR:

The synthesis of cDNA and the nested PCR assay conditions were performed as previously described by Gruber et al. (1993). Briefly, 5 µl of extracted RNA was added to15 µl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 500 µM (each) deoxynucleotide triphosphates (Boehringer Mannheim, Mannheim, Germany), 0.5 µg of random hexamers (Promega, USA), and 200 U Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega, USA). The mixture was incubated at 37°C for 60 min and was then heated for 2 min at 94°C for inactivation of the RT enzyme and cooled on ice for 5 min.

The first round of PCR amplification was carried out in a 100-µl reaction volume containing 5 µl of the cDNA product and 95 µl of the PCR mixture [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.001% (wt/vol) gelatin, 0.1% Triton X-100, 0.25 mM (each) deoxynucleotide triphosphates, 90 ng (each) of outer primers, and 1.5 U of Taq polymerase (Perkin-Elmer Cetus, Langen, Germany)]. The reaction mixture was amplified for 25 cycles in a programmable thermal cycler (model 2400; Parkin-Elmer Cetus) by using the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, plus a final extension step at 72°C for 5 min.

For second round, 2 µl from the first PCR product was further amplified with inner pairs in a 100-µl reaction-mixture containing the same PCR mixture, but with inner primers instead of outer primers. These mixtures were amplified for 30

cycles of 94°C for 1 min, 55°C for 45 s, and 72°C for 30s, followed by a final extension step at 72°C for 10 min. To monitor the PCR, the negative and positive control samples were included in each run. The 402-bp PCR products were analyzed by electrophoresis on a 2 % agarose gel (Tris-borate-EDTA buffer) and were visualized by staining the gel in an ethidium bromide (0.5 µg/ml) solution. To avoid false-positive PCR results, the precautions for PCR described by Kwok and Higuch (1989) were strictly followed.

Analysis of discrepant results:

Semen samples with discrepant results between the nested RT-PCR and culture techniques for the detection of BVDV were resolved by the additional double polymerase chain reaction (Alansari et al., 1993). Briefly, 5 µl of cDNA was placed in a tube contained a final concentration of 200 µM of each dNTP and 0.5 µM of outside primer set (5` TGC, CTC, AAA, TCC, TAT, AGG, CAA, CT 3` and 5` CTA, TCA, GAG, AAG, GAG, TGG, CAC, AA 3`) in PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.0), 0.01% (wt/vol) gelatin and 2.5 mM $MgCl_2$). Two and half units of Taq DNA polymerase (Perkin-Elmer Cetus, Langen, Germany)] was added and the reaction volume was made up to 100 µl with water. The reaction mixture was cycled in a programmable thermal cycler (model 2400; Parkin-Elmer Cetus) using the following temperature profile: 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. This temperature profile was repeated 25 times, ending with a final annealing and extension period of 2 min and 10 min, respectively. Amplified product produced by the outside primer set (5 µl) was used as a target for a second PCR with the inside primer set (5` AAG, CCG, ATC, ATC, CCC, ACA, GAC, GA 3` and 5` AAC, AGC, AGA, TGG, TGA, AGT, ATA 3`. To monitor the PCR, the negative and positive control samples were included in each run. The presence of PCR products (183 bp) was detected by gel electrophoresis in 2% agarose and was visualized by staining the gel in an ethidium bromide (0.5 µg/ml) solution.

Statistical analysis:

The sensitivity and specificity of the nested RT-PCR and culture for detection of BVDV in semen samples were calculated by using, as a reference gold standard, samples that were detected as positive by at least two methods: nested RT-PCR, culture, or double-PCR (Fliess, 1981).

RESULTS AND DISCUSSION

In this study, semen samples were collected from 59 bulls from farms of known history of BVDV infection. Since semen contains some inhibitory components (Von Beroldingen et al., 1990), a protocol was used to extract the RNA from the semen samples (Von Beroldingen et al., 1990). Previous reports suggested that most BVDV are present in non-sperm cell fraction and might be in the seminal fluids (Radostits and Littlejohns, 1988 and Afshar and Eaglesome, 1990). The pre-

pared samples were examined by culture technique for isolation of the virus (Brownlie, 1996) and nested RT-PCR assay (Gruber et al., 1993) for detection of BVDV in the seminal fluid and nonsperm cell fraction from each semen sample. The results indicated that 6 (10.2 %) and 8 (13.6 %) net and extended semen samples, respectively, were culture-positive and 53 (89.8 %) and 51 (86.4 %) were culture-negative (Table 1). On the other hand, 10 (16.9 %) and 10 (16.9 %) net and extended semen samples, respectively, were nested RT-PCR-positive and 49 (83.1 %) and 49 (83.1 %) were nested RT-PCR-negative (Table 1 and Fig 1). The results of the additional RT-PCR assay indicated that 10 (16.9 %) and 10 (16.9 %) net and extended semen samples, respectively, were positive and 49 (83.1 %) and 49 (83.1 %) were negative (Table 1 and Fig. 2). Ten semen samples were collected from BVDV-free animals to be used as negative control samples and they tested negative by culture, nested RT-PCR and the additional RT-PCR techniques. The results indicated that, BVDV was detected in extended semen samples more than in net semen samples. This finding is in agreement with those presented by Revell et al. (1988) and Paton et al. (1989), who reported that the transient seminal shedding of BVDV in infected bulls could be detected more successfully by testing the extended rather than net semen.

The results show that, all culture positive samples were nested RT-PCR positive from net and extended semen (Table 1). On the other hand, there were 4 and 2 PCR-positive and culture-negative samples of net and extended semen samples, respectively. All these samples were confirmed as true positives with the additional RT-PCR (Table 1). Presuming that these samples are true positives and culture failures, the revised sensitivity and specificity of nested RT-PCR in net and extended semen samples were 100.00 % (Table 1). On the other hand the sensitivity of culture technique in net and extended semen samples were 60.00 % and 80 %, respectively, while the specificity were 100 % and 100 %, respectively (Table 1). These results indicated that, the PCR was more sensitive than the culture technique compared to the total resolved samples. These findings are in agreement with those presented in published report comparing PCR with culture method for detecting of BVDV in tissue homogenates (Gruber et al., 1994, Laamanen et al., 1997).

In summary, the present research has compared the culture technique with nested RT-PCR and demonstrated the superiority of the latter technique for detecting BVDV in bulls' semen. Here we have demonstrated that nested RT-PCR is reliable, rapid, highly sensitive, and specific for use in the accurate detection of BVDV in semen. According to the best of our knowledge, this work is the first to report a nested RT-PCR that has proved to be efficient in detecting the presence of BVDV in bulls" semen. The results

provide a strong basis for the tentative early diagnosis of BVDV in semen. Finally we recommend the use of nested RT-PCR assay as a supplemental diagnostic tool for detection and identification of BVDV in semen.

Table 1: Comparison between culture and nested RT-PCR assays for detection of BVDV in semen samples.

Samples	No. of samples	Results obtained by:			Sensitivity %[a]		Specificity %[a]	
		nested - RT-PCR	Culture	Double PCR	nested - RT-PCR	Culture	nested - RT-PCR	Culture
Net semen	6	+	+	+	100.00	60.00	100.00	100.00
	4	+	-	+				
	49	-	-	-				
Extended semen	8	+	+	+	100.00	80.00	100.00	100.00
	2	+	-	+				
	49	-	-	-				

+, Positive; -, negative

a The sensitivity and specificity were calculated by using as a reference gold standard samples that were detected as positive by at least two methods: nested RT-PCR, Culture or RT-PCR

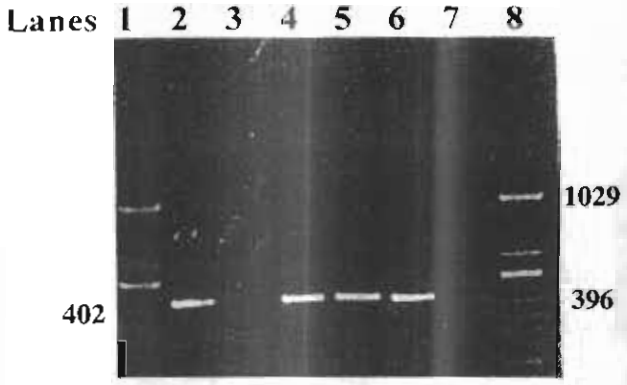

Fig. (1): Nested PCR amplification of viral sequences from BVDV in bulls' semen samples: Lanes 2 and 4, extended semen samples; lanes 3 and 5, net semen samples; lanes 1 and 8, molecular weight marker; lane 6, positive control and lane 7, negative control.

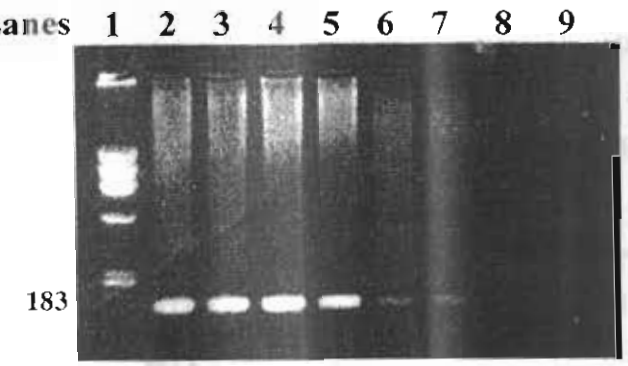

Fig. (2): Double PCR amplification of viral sequences from BVDV in bulls' semen samples: Lanes 3, 4, and 5 extended semen samples; lanes 6, 7, and 8, net semen samples; lane 1, molecular weight marker; lane 2, positive control and lane 9, negative control.

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