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GENOTYPING THE EGYPTIAN ISOLATES OF BOVINE VIRAL DIARRHEA VIRUS FROM MILK USING RESTRICTION ENDONUCLEASE ENZYME-PST1

(With One Figure)

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التوصيف الجينى للمعزولات المصرية لفيروس الأسهال البقرى من الألبان باستخدام أنزيم القطع- Pst1

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تهدف هذة الدراسة الى أستخدام الطرق الجزيئية الحيوية للتوصيف الجينى لفيروس الأسهال البقرى من الخلايا الجسيمية للألبان باستخدام انزيم القطع Pst1 بعد أكثار الحامض النووى فى المنطقة 5`UTR بواسطة أختبار البلمرة المتسلسل المتعاكس. تم أستخلاص الحامض النووى RNA من عدد ٢٩ معزولة محقونة على الخلايا النسيجية MDBK (مختبرة ضد الأصابة بفيروس الأسهال البقرى). وبقياس الكثافة الضوئية للحامض النووى RNA باستخدام الأشعة الفوق بنفسيجية بجهازالسبكتروفوتوميتر عند التردد الموجى ٢٦٠نانوميتر، وجد أنها عند المتوسط ٠,٨- ٠,٩. باستخدام بادئ التفاعل (فى المنطقة 5` UTR والمحصورة بين القواعد النيكلوتيديه ١٠٨- ١٢٨ ، ٣٩٥- ٣٧٥) وانزيم البوليميريز، تم اكثار المنطقة وأعطت نتيجة ايجابية عند الوزن الجزيئى ٢٨٨ زوج قاعدة نيكلوتيدية لكل المعزولات. تم تعريض ناتج أختبار البلمره المتسلسل المتعاكس لعدد ٢٩ معزولة للتقطيع بواسطة أنزيم القطع Pst1 وأعطت نتيجه أيجابيه عند الوزن الجزيئى ٢٣٠ ، ٥٨ زوج قاعدة نيكلوتيدية. تم استخدام العترة العالمية NADL ممثلا للسلالة ١ لفيروس الأسهال البقرى وتم تقطيعها بنفس الأنزيم كعينة ايجابية ضابطة. ومن هذة الدراسة نستخلص أنه يمكن باستخدام انزيم واحد لقطع الحامض النووى الذى تم اكثاره فى المنطقة 5`UTR بسرعة وسهولة التعرف على السلالة الخاصه بالفيروس. ويعتبر ذلك هاما فى أجراء الدراسات الوبائية والجزيئية كمعلم هام لفيروس الأسهال البقرى. كما أن التحسين فى طرق التشخيص والخطط الأستراتيجيه للتحكم فى المرض هامه جدا للاقلال من الخسائر الناتجه عن الأصابه بفيروس الأسهال البقرى.

SUMMARY

The objective of this study was to use the molecular methods for genotyping the Egyptian isolates of bovine viral diarrhea virus (BVDV) from purified milk somatic cells (PMSC) samples using restriction enzyme (RE)-Pst1 after amplification the viral RNAs at 5`untranslated region (5`UTR) by reverse transcription-polymerase chain reaction (RT-PCR). A purified and biological active RNAs were extracted from 29 inoculated isolates on Madin-Daby bovine kidney (MDBK) cells tested against latent infection with BVDV. By using UV spectrophotometer at wavelength 260 nm, the optical density (OD) of the extracted RNAs was an average 0.8-0.9. A primer sequence within 5`UTR flanked the region (108-128th nucleotide, nt, as upstream and 395-375th nt as downstream) and Taq DNA polymerase enzyme were used to amplify and gave highly specific bands at 288 bp. The 29 isolates were clearly identified by ethidium bromide staining of the amplified DNA specific bands at (288 bp) in the gel. The RT-PCR products of the isolates in this study were digested with RE-Pst1 and gave 2 sharp bands at molecular weight 230 and 58 bp. A positive control, NADL strain, represented genotype I was included allover this study. In conclusion, the single RE digestion of RT-PCR-amplified 5`UTR products may be a quick and easy method for identification of BVDV genotype. Understanding the molecular epidemiology and molecular biology of BVDV is an important milestone. Improved diagnostic and control strategies are essential to reduce losses inflected by BVDVs.

Key words: *Virology, bovine viral diarrhea, milk, endonuclease enzyme-Pst1*

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is an enveloped, widely distributed, single stranded RNA, of positive polarity with 12.5 kilo base (kb) in length (Renard *et al.*, 1985; Collett *et al.*, 1988 a and b). Currently, the genus *Pestivirus* comprises the four approved species BVDV-1, BVDV-2, classical swine fever virus (CSFV) and border disease virus (BDV), in the family *Flaviviridae* (Francki *et al.*, 1991 and Pellerin *et al.*, 1994). Up date, BVDVs are classified on the basis of nucleotides sequence analysis into genotype I which further subdivided into 11 subgroups and genotype II which also contains 2 subgroups (Becher *et al.*, 1999 and Vilcek *et al.*, 2001). The potential of BVDV genetic and antigenic diversity are far ranging (Bolin and Grooms, 2004).

The 5`untranslated region (5`UTR) is one of the highest conserved regions among BVDV strains. In this region, the homology percentage is high (86-93%) in different strains of genotype I either cytopathic or noncytopathic (Deng and Brock, 1992). On the other hand, the homology percent in 5`UTR between genotype I and II is about 95% (Ridpath and Bolin, 1995).

The virus has been known to infect the mammary glands which considered as an important source in virus excretion in somatic cells (Shin and Acland, 2001). Therefore, milk samples would be good criteria for BVDV isolation and diagnosis (Drew *et al.*, 1999; Abd El-Hafeiz *et al.*, 2003; Heath *et al.*, 2003; Kim and Dubovi, 2003 and Robert *et al.*, 2004).

Reverse transcription-polymerase chain reaction (RT-PCR) is an in vitro technique that is increasingly being used for diagnosis of viral animal pathogens. Due to its high sensitivity, the RT-PCR within 5`UTR will provide a more accurate picture of bovine infection by BVDVs (Hyndman *et al.*, 1998).

The recognition sequence of restriction enzyme (RE)-PstI CTGCAG is located in between 329-348th nt within 5`UTR of BVDV-genotype I while not present in genotype II. So, a positive RE-PstI digestion indicates that the isolate is a BVDV genotype 1, whereas a negative one means that it is a BVDV genotype II (Harpin *et al.*, 1995).

In Egypt, several studies have been conducted to isolate and characterize both genotypes and biotypes from different clinical samples (Abd El-Hafeiz *et al.*, 2003; Hussein *et al.*, 2003; Abd El-Hafeiz, 2004 and Abd El-Hafeiz *et al.*, 2005).

The objective of this study was to use the molecular methods for genotyping the Egyptian isolates of BVDV from purified milk somatic cells (PMSC) samples using RE-PstI after amplification the viral RNAs at 5`UTR by RT-PCR.

MATERIALS and METHODS

Viruses and cell culture:

A total of 29 BVDV isolates from individual milk samples (Abd El-Hafeiz *et al.*, 2003) were used in this study. These milk samples were collected from 3.5-7 years old multiparous dairy cattle from different localities in Lower Egypt. Milk somatic cells were prepared and purified as described by Radwan *et al.* (1995). The PMSC samples were inoculated on Madin-Darby bovine kidney (MDBK) cells tested against

latent infection with BVDV in 24 wells tissue culture plates as standard method (Schweizer and Peterhans, 1999). A reference international NADL strain (genotype I) was used as positive control.

Extraction of RNA:

Acid guanidin-phenol-chloroform (AGPC) method developed by Chomczynski and Sacchi (1987) was used to extract intact RNA from each inoculated isolate, NADL strain, and mock infected cells as negative control.

Quantitation of RNA product:

The quantity of extracted RNAs was estimated using UV spectrophotometer at wavelength 260 nm and at dilution factor 10^2 in diethyl pyrocarbonate (DEPC) -treated water. The optical density (OD) of the extracted RNAs was read and the concentration was calculated according to the formula: RNA conc. (µg/µl) = [OD_{260} X 100 (dilution factor) X 40 µg/ml]/1000 (Weigand *et al.*, 1993).

Reverse transcription-polymerase chain reaction (RT-PCR):

The RT-PCR reaction was carried out in one tube with a single reaction buffer as mentioned by the manufacturer (Aßgene, UK) using a primer sequence set as Vilcek *et al.* (1994). All steps were set up on ice. Each reaction mixture (50 µl) contained the following: 5 µl RNA template, 5 µl of 10X one-step buffer contains 15 mM $MgCl_2$ and RNase inhibitor (Aßgene, UK), 1 µl dNTPs (10 mM/µl, Amresco, OH, USA), 1 µl of each sense primer (UTR1) 5`---ATG CCC WTA GTA GGA CTA GCA---3`(108-128th nt) where W = A or T and anti-sense primer (UTR2) 5`---TCA ACT CCA TGT GCC ATG TAC---3`(395-375th nt), 1 µl RT enzyme (100 U/µl, Aßgene, UK), 0.25 µl Taq DNA polymerase (5 U/µl, Aßgene, UK). RT-PCR was performed on a programmable thermocycler as follows: 47 °C/30 minutes as one cycle for the first strand synthesis, 94 °C /2 minutes as one cycle for RT enzyme inactivation and initial denaturation, and then 35 cycles of denaturation at 94 °C /1 minute, primer annealing at 56 °C /1 minute and extension at 72 °C /1 minute. A final extension as one cycle at 72 °C /7 minutes was done.

Concentration of 5` UTR amplified products:

Concentration of RT-PCR products was performed according to standard procedure (Sambrook and Russell, 2001). To 50 µl of 5`UTR amplicons, 5 µl of 3M sod. acetate pH 5.2 and 125 µl of 95% chilled ethanol were added and stored at -20 °C overnight. In the next day, the mixture was centrifuged at 13 000 *Xg*, 4 °C for 15 minutes to precipitate the amplified DNA products. The precipitate then washed with 70% ethanol, dried and redissolved in 20 µl DEPC-water.

Restriction digestion of concentrated amplified products:

On ice and in a total volume 20 µl, the digestion of concentrated amplicons with RE-Pst1 was carried out as described by manufacturer (Promega, USA) and Harpin *et al.* (1995). To 4.5 µl of concentrated RT-PCR products, a mixture of 2 µl 10X RE buffer (900 mM Tris-HCl pH 7.5, 500 mM NaCl, 100 mM $MgCl_2$ and 10 mM DTT at 37 °C, Promega, USA),0.2 µl acetylated bovine serum albumin (BSA) and 12 µl ultra pure water was added, mixed and spun to be collected at the bottom of microfuge tube. Finally, 1.3 µl (10 U/µl, Promega, USA) of RE-Pst1 was added and mixed gently. In 37 °C water bath, the mixture was incubated for 3 hours. The reaction was stopped by removal the tube from the water bath. Ten µl of the digested products were mixed with 2 µl of 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 was run through 2.5% agarose gel stained with 0.5 µg/ml ethidium bromide for 3 hours at 70 volts. The results were visualized by UV transilluminator and photographed by a Polaroid camera.

RESULTS

Bovine viral diarrhea-viral RNAs were extracted from the 29 isolates and NADL strain as well as RNA of mock infected cells. By measuring the OD of extracted RNAs at UV wavelength 260 nm, the OD average was 0.8-0.9. The 29 isolates and the NADL strain were clearly identified by ethidium bromide staining of the amplified DNA specific bands of 5`UTR (288 bp) in the gel as illustrated in the figure while not in negative control. The RT-PCR products of the isolates in this study were digested with RE-Pst1 and gave 2 sharp bands at molecular weight 230 and 58 bp. The NADL strain represented genotype I was digested with the same enzyme and gave 2 sharp bands at the same expected size (230 and 58 bp) as shown in the figure.

DISCUSSION

The past 20 years have witnessed a dramatic improvement in laboratory methods for diagnosis BVDV infections (Saliki and Dubovi, 2004). Several key factors influence the success of BVDV diagnosis. Current knowledge and an understanding the problem and the impact of BVDV associated diseases will facilitate the organization and tremendous effort that required to control BVDV infection (Brock, 2004).

Bovine viral diarrhea virus infects an array of different cell types including milk somatic cells which are mixture of secretary epithelial and leucocytic cells where replication of the virus takes place (Shin and Acland, 2001). Recent diagnosis have centered on milk samples as a material for virus diagnosis (Drew *et al.*, 1999; Abd El-Hafeiz *et al.*, 2003; Heath *et al.*, 2003; Kim and Dubovi, 2003 and Robert *et al.*, 2004).

A high quality RNA is critical for the success of RT-PCR analysis. In this study, a purified and biologically active RNAs were extracted from the total 29 isolates as well as the NADL strain. By measuring the OD of extracted RNAs at UV wavelength 260 nm, the OD average was 0.8-0.9. It is constant that OD 1 = 40 µg/ml (Weigand *et al.*, 1993).

It is difficult to ignore the potential detection of BVDV by RT-PCR assays (Brock and Potgieter, 1990). For amplification and genotyping the BVD-viral RNA, the choice of primers is critical and based on conserved motifs within the genome. Also, the primers should span a region with an appropriate level of variability according to the aim of study. The 5`UTR in the viral genome is one of the highest conserved regions among different strains of the pestiviruses (Deng and Brock, 1992 and Ridpath and Bolin, 1995). According to NADL strain sequence (Collett *et al.*, 1988 c) and as in Vilcek *et al.* (1994), a primer sequence within 5`UTR flanked the region (108-128th nt as upstream and 395-375th nt as downstream) was used. The 29 isolates were clearly identified by ethidium bromide staining of the amplified DNA specific bands (288 bp) in the gel. The NADL strains gave the same sharp bands at the same size (288 bp).

Several studies have been focused and used the REs digestion of the amplified 5`UTR products to distinguish between different species of genus *Pestivirus*. The RT-PCR-based assays in this region capable of detecting and differentiate abroad range of pestiviruses (Vilcek *et al.*, 1994; Harpin *et al.*, 1995 and Paton *et al.*, 1995).

The RT-PCR products of the isolates in this study were digested with RE-Pst1 and gave 2 sharp bands at molecular weight 230 and 58 bp. These results were agreement with results recorded by Harpin *et al.* (1995) who found, positive RE-Pst1 digestion in all tested BVDV genotype I while not true in BVDV genotype II were occur. A positive control, NADL strain represented genotype I was digested with the same enzyme and gave 2 sharp bands at the same expected size (230 and 58 bp).

In conclusion, the single RE digestion of RT-PCR-amplified 5`UTR products may be a quick and easy method for identification of BVDV genotype. Understanding the molecular epidemiology and molecular biology of BVDV is an important milestone. Improved diagnostic and control strategies are essential to reduce losses inflected by BVDVs.

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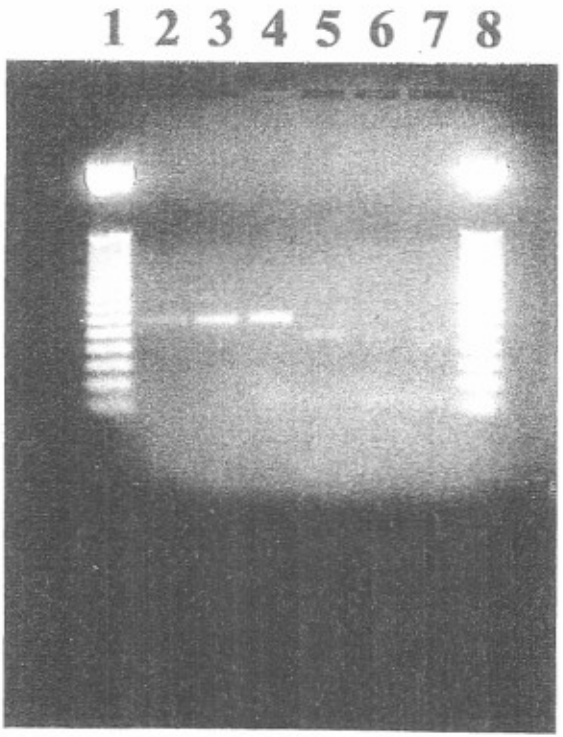

Fig.1: Agarose gel (2.5%) analysis of BVD-viral RNA amplicons of 5` UTR and their digestion by RE-Pst1. Lanes 1 and 8 are DNA ladder (Promega, Cat. No G 4521), lane 2 a positive control (NADL strain) by RT-PCR amplification, lanes 3 and 4 are represented tested isolates, lanes 5 and 6 are represented RT-PCR amplicons digested with RE-Pst1 and lane 7 is a positive control (NADL strain) digested with RE-Pst1.