Detection of Bovine Viral Diarrhea Virus by Conventional RT-PCR: A comparative Study

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Abstract

Fifty serum and buffy coat samples were collected from bovine from illegal abattoirs around Baghdad. RNA extraction was performed on buffy coat samples. These samples were subjected to conventional RT-PCR for the detection of BVDV. The result showed that the BVDV was detected by RT-PCR in three samples only. A comparison study was also conducted on the sensitivity of RNA extracted and non-extracted buffy coat and serum samples in conventional RT-PCR. The result showed that RNA extraction is the method of choice when conventional RT-PCR was performed on blood samples from BVDV infected animals. In a final conclusion, BVDV was detected by RT-PCR in local bovine. The findings suggested and proposed epidemiological studies on BVDV in Iraq in conjunction with isolation of the virus and identification of its genotype and biotype.

الكشف عن فيروس الاسهال البقري الفيروسي بواسطة (RT-PCR) التقليدي: دراسة مقارنة

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الخلاصة

جمع خمسون نموذجا من مصل وخلايا دم ابقار ذبحت في مجازر غير قانونية في المناطق المحيطة ببغداد. استخلص الحمض النووي الريبي من نماذج خلايا الدم ثم عرضت النماذج المستخلصة إلى اختبار (RT-PCR) التقليدي لغرض الكشف عن فيروس الإسهال البقري فيها. واظهرت النتائج وجود الفيروس في ثلاث نماذج فقط. ولغرض المقارنة في تقنية (PCR) لنماذج استخلص الحمض النووي منها وأخرى لم يستخلص الحمض النووي منها تم اختيار 10 نماذج من خلايا الدم تشتمل على الموجبة لوجود الفيروس و10 نماذج مصل لنفس نماذج خلايا الدم العشرة السابقة. استخدمت النماذج في نفس الاختبار ولكن بدون استخلاص الحمض النووي .ظهرت النتائج اكثر وضوحا في حالة استخلاص الحمض النووي مقارنة بنفس النماذج التي لم يستخلص الحمض النووي منها. يستنتج من هذه الدراسة ان فيروس الإسهال البقري موجود في الأبقار في العراق وان استخلاص الحمض النووي من نماذج المصل وخلايا الدم أفضل من عدم الاستخلاص في حالة استخدام (RT-PCR) التقليدي للكشف عن الفيروس. ان النتائج تظهر وجوب اجراء دراسات وبائية عن مدى انتشار مرض الاسهال الفيروسي في الابقار تقترن بعزل الفيروس ومعرفة نوعه البايولوجي والجيني.

Introduction

Bovine viral diarrhea virus (BVDV) is belonging to the genus *Pestivirus* of the family *Flaviviridae.* It is spherical enveloped virus carrying single stranded positive sense RNA genome (1). There are two distinct species of the virus, BVDV1 and BVDV2; both exist as

one of two biotypes, cytopathic and noncytopathic. Furthermore, the noncytopathic biotype is the predominant biotype in both BVDV species (2). Persistent infection may result from infection with noncytopathic virus during the first 70-125 days of gestation; the cytopathic strain may cause hemorrhagic syndrome with high morbidity and mortality (3). Some other cases may develop mucosal disease that characterized by low morbidity and high mortality (4). The virus can infect not only bovine, as many studies reported the infection in goats (5, 6) chamois (7) white deer (8) sheep (9,10) and wild cervids (11). Pigs also were susceptible naturally and experimentally (12). Many laboratory tests were used for the diagnosis of BVDV like serum neutralization test (13) immunoperoxidase and immunofluorescent tests (14,15,16),ear-notch antigen capture enzyme-linked immunosorbent assay (ACE) (17,18) and RT-PCR (19). PCR techniques were mainly based on the nucleic acid sequence of the virus, so that they may be considered as better method of BVDV detection than other conventional methods. Multiplex PCR was used for typing of BVDV isolates (20). In the same study the researchers used infected culture supernatant and whole blood samples without RNA extraction. In another study and by the use of the same multiplex PCR, a comparison on the PCR sensitivity was performed on RNA extracted and non-extracted samples (21). Multiplex PCR is more sensitive technique than conventional PCR technique (22). In area around Baghdad no data were available on detection of BVDV by the use of RT-PCR. Furthermore, no data were available on comparing of the sensitivity of conventional RT-PCR techniques depending on RNA extracted and non-extracted serum and whole blood samples. To achieve the above-mentioned targets the present study was performed.

Materials and Methods

- **Collection of samples**
- **Blood samples:** 50 blood samples were collected from calves, adult cows or bulls slaughtered in private illegal abattoirs around Baghdad from the period of December 2008 to June 2009. Ten milliliters of blood were collected from each animal, 5 ml with EDTA anticoagulant and 5 ml without anticoagulant. Serum samples were obtained from coagulated blood samples and kept at -20 °C until use, while buffy coat cells were collected from blood samples with EDTA anticoagulant after centrifugation at 1500 rpm/minutes for 10 minutes.
- **Processing of samples**

A. Samples with RNA extraction: RNA from each buffy coat cells samples were extracted by the use of SV Total RNA Isolation System Kit (Promega, Madison, USA) and according to the manufacturer instructions as shown in Fig.1. The extracted RNA was then subjected to RT-PCR as described later. Furthermore, ten serum samples including those of buffy coat BVDV RT-PCR positive same sample were used with RNA extraction for RT-PCR.

B. Samples without RNA extraction: According to Deregt, et al., (21), ten of buffy coat samples including the BVDV RT-PCR positive samples were diluted into 1:10 in 1M Tris HCl (pH 7.4). Frozen at -20 °C and thawed for several times before taking off 2µl to be used in RT-PCR reaction sets as described later. Furthermore, ten serum samples including those of buffy coat BVDV RT-PCR positive same sample were used without RNA extraction for RT-PCR.

- **Primers and RT-PCR test:** Conversion of RNA to cDNA followed by amplification of the DNA was carried out with PROMEGA® RT-PCR system. The cDNA was

amplified at region that is specific to BVDV using primers that are selected from the sequence of BVSV nucleic acid. The primers sequences used in this study were:

Upstream 5' TAGCCATGCCCTTAGTAGGAC 3'

Downstream 5' ACTCCATGTGCCATGTACAGC 3'

- **Protocol For RT-PCR:** A 10 -11 µl of RT master mix per reaction tube was prepared. The mixture was mixed carefully and allowed to stand at room temperature for 10 minutes. This was followed by reverse transcription cycle of 42 °C for 1 hour, 99 °C for 5 minutes to inactivate the reverse transcriptase enzyme, and 5 °C for 5 minutes.

For each RT master mix a PCR master mix of 40 -41 µl was prepared and subjected to PCR cycles as follows: 95 C for 3 minutes for 1 cycle, and 40 cycles of 94 °C for 1 minute, 53 °C for 1 minute, 72°C for 2 minutes. The cycle 40 was passed into a cycle of 72 °C for 5 minutes. The PCR cycle was done in Eppendorf-Thermocycler.

- **Detection of Amplified PCR products:** Following amplification, the PCR products were loaded into wells of agarose and electrophorised. For this purpose, approximately 25 µl of PCR product was mixed with 1/10 volume of loading buffer (0.25%bromophenol blue, 0.25% Xylene cyanol FF and 30% glycerol) (23). The mixture was electrophorised in 2% agarose prepared in electrophoresis buffer (40 mM Tris-acetate, pH 7.8, 5 mM sodium acetate, 1 mM EDTA and 0.5 µg/ml ethidium bromide). The processed samples were flanked by 3 µl of BVDV positive control fragment of 300 base pair (bp). The gel was run 30-50 volts for an indicated period of time. The gel was then visualized on ultraviolet light and photographed. The band of 300 bp was regarded positive for BVDV.

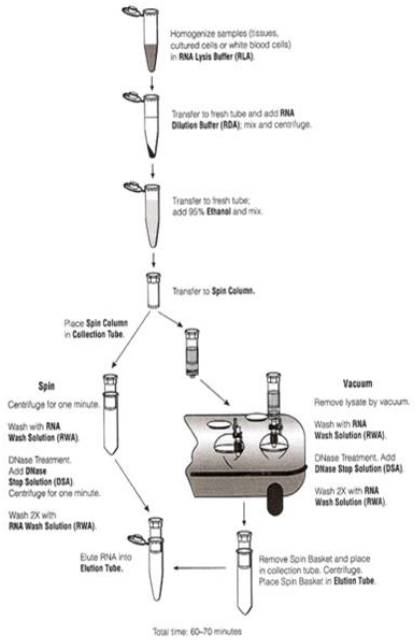

Figure 1. Schematic representation of the SV Total RNA Isolation System.

Results

The RT-PCR of the present study showed that only three samples out of 50 samples were positive when tested by the BVD primers of RT-PCR on buffy coat samples. The product was appeared as of 300 bp as compared to the positive control of BVD PCR

product designed by the manufacturer. BVDV-RNA extracted from buffy coat or serum of the same samples gave very clear PCR product as shown in Fig. (2 and 3). Buffy coat same blood samples without RNA extraction, tested by the BVD RT-PCR showed equivocal results, but in more complicated manner, when many extra bands of different bp size were appeared (Fig. 4). Some of these bands appeared in a size similar to that of positive control, while some others were not. Serum samples that used for RT-PCR but without RNA extraction also gave equivocal results. The product appeared wide and diffused, and some of them appeared with negative result when compared to positive BVD-RNA extracted of the same samples (Fig. 4).

Table (1) The PCR cycle of BVDV of the RT cycle

Step	Temperature	Time	No.of cycles
Denaturation	95 °C	3 minutes	1
Denaturation	94 °C	1 minute	40
Annealing	53 °C	1 minute	
Extension	72 °C	2 minutes	
Extension	72 °C	5 minutes	1
Hold	4 °C		

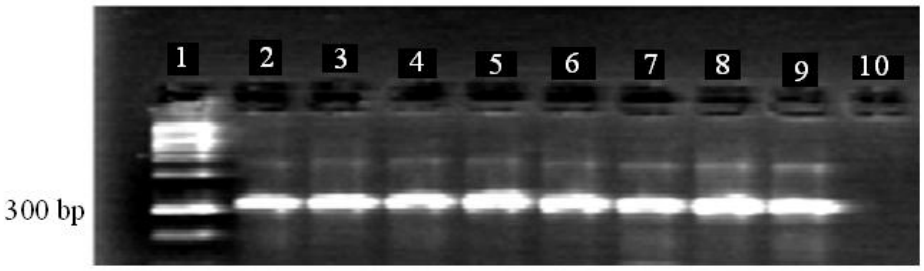

Fig. (2) The PCR products of BVDV from serum and buffy coat samples after RNA extraction electrophorized in 2% agarose at 4v/cm for 2 hourse. Lane 1 molecular weight marker. Lane 2 and 9 are the BVDV positve control. Lane 10 is the negative control. Lanes 3, 4 and 5 are the postive BVDV serum samples. Lanes 6, 7 and 8 are the buffy coat BVDV postive same samples.

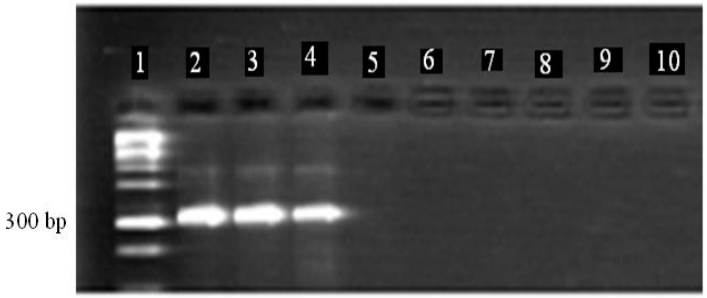

Fig. (3) The PCR products of BVDV from serum samples after RNA extraction electrophorized in 2% agarose at 4v/cm for 2 hourse. Lane 1 is the DNA molecular weight marker, Lanes, 2,3 and 4 are the PCR-BVDV positive serum samples.

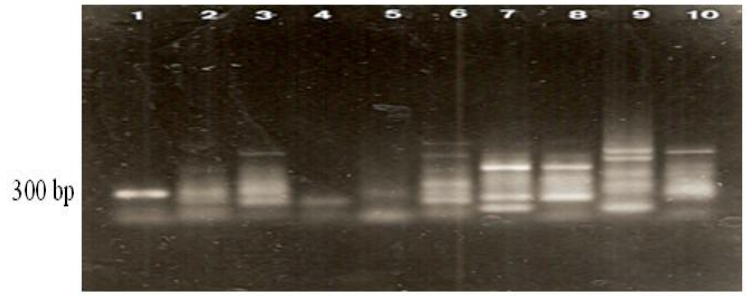

Fig. (4) The PCR products of BVDV from buffy coat samples without RNA extraction electrophorized in 2% agarose at 4v/cm for 4 hourse. Lane 1 is the complementary DNA BVDV positive sample, Lanes, 2to 10 are the PCR-BVDV buffy coat samples. Lanes 7, 8 and 9 are of the same PCR postive buffy coat RNA extracted samples.

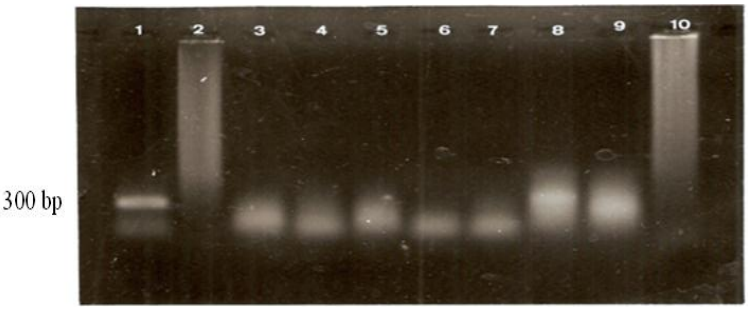

Fig. (5) The PCR products of BVDV from serum samples before RNA extraction electrophorized in 2% agarose at 4v/cm for 2 hourse. Lane 1 is the complementary DNA BVDVpositive control, Lanes 2 to 10 are the PCR-BVDV serum samples. Lanes 7, 8 and 9 are same postive of RNA extracted samples.

Discussion

In the present study, the virus was detected in appearantly healthy animals that brought to illegal private abattoirs for human consumption. This may give an indication that the detected virus was of noncytopathic BVDV type that caused persistent infection in tested animals. Although the number of postive samples for BVDV were small, but this did not exclude the posibility of the spread of the virus a mong bovine or other suceptible animals in Iraq. No data were available to point out the spread of the virus among susceptible animals in Iraq.It is well documented that BVDV fall in two genotypes BVDV1 and BVDV2 which were well known as of two biotypes non-cytopathic and cytopathic (1). The non-cytopathic strains of both BVDV1 and BVDV2 can cross the placenta and establish persistent infections (2). Animals with persistent infection can carry and shed the virus without clinical signs leading to the spread of the virus (1,24,25). The present study cannot point out that the infected animals were from arround Baghdad, as in private illegal abattoirs, they brought such animals from different provinces (South, North, West or East). These illegal abattoirs may play an important role in spreading of BVDV as not all the brought animals were slaughtered; some of them were sold for farmers. Because animals with persistent infection shed large amount of virus through their entire lives, they are considered the major source of BVDV transmission both within and between herds (26). The most efficient mode of transmission is direct contact with body fluids from persistently

infected cattle. The virus has been isolated from nasal swabs, aerosols, saliva, urine, feces, semen and utrine fluids from persistently infected cattle (25). Transmission by contaminated clothing, boots, and equipment including needles and nose tongs. Cattle also can transmit the virus to other animal species such as goats (6,27). We do not know the origin of BVDV virus in Bovine in Iraq. The virus might be brought by some above-mentioned infected animal species or through contaminated vaccines that commercially prepared in cell cultures. Non-cytopathic strain of the virus was reported by many invesigators to cause contamination of vaccines (28,29) and cell cultures (30). BVDV2 was reported to cause acute infections associted with poor conception rates, early embryonic deaths, abortion, various congenital defects (23,31). In the present study no animals with above-mentioned clinical signs were observed as all of them were appearantly healthy. Furthermore, the detected viruses by RT-PCR cannot be mentioned as BVDV1 or BVDV2, as both of them can cause pesrsistent infection (2,32, 33). According to available data the present study might be the first that recorded the detection of BVDV in bovine in Iraq by the use of RT-PCR. Further studies must be performed to point out the spread of BVDV in bovine or other animals species. Moreover, studies that concerning with isolation and identification of the genotype of the isolated viruses seem to be important to point out the circulating strains among susceptible animal species in Iraq. To achief such targets, monoclonal antibodies, hybridization probes and specific primers for both genotypes are availabe and used by many investigators (1,17,19). In comparison of detection of BVDV RNA genome with or without RNA extraction in conventional RT-PCR, the study showed that the samples without RNA extraction did not give clear cut of results as the generated bands appeared equivocal single or multiple specially with those from buffy coat samples. The same result of equivocal single and diffused bands were appeared with PCR product of serum samples subjected to RT-PCR without RNA extraction. This findings may be attributed to the presence of PCR inhibitors in blood samples and to the sensitivity of the sample as RNA extracted samples were 4 folds more sensitive than the non-extracted samples (20,21). The comparison results of the present study came in contrast of Deregt et al (21) findings when they reported that RNA extracted and non-extracted samples were of the same sensitivity in multiplex RT-PCR. In the present study the conventional RT-PCR was followed. It is a conventional method that depends mainly on two primers for the genome under study. The above-mentioned study of Deregt et al had used a nested multiplex RT-PCR that designed to use more than two primers. The nested multiplex RT-PCR technique was more sensitive than the conventiona RT-PCR (22). Accordingly, application of RNA extraction on samples subjeted to conventional RT-PCR seems to be a method of choice. In final conclusion, this study showed the presence of persistent infection with BVDV among local bovine by the use of conventional RT-PCR technique. This lead to a suggestion that epidemiological studies on the prevelance and spread of BVDV in local susceptible animals must be conducted as soon as possible. Further studies on isolation and identification of the genotypes and biotypes of isolated viruses must be achieved. Although the conventional RT-PCR technique was time consuming specially when it was associated with RNA extraction from collected blood samples, but can clearly detect the BVDV in such samples. Furthermore, RNA extraction from suspected BVDV blood sample is essential for conventional RT-PCR.

Acknowledgements: The author would like to express the thanks to Dr. Iman Shaker Mahmood of Medical Teaching Laboratories /Medical City/ Ministry of Health /Baghdad, for her nice cooperation.

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