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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Detection of bovine viral diarrhea virus in bovine and buffalo milk thorough conventional and real-time reverse transcriptase polymerase chain reaction

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Abstract

Bovine viral diarrhea virus is a *Pestivirus* that causes a range of symptoms in bovine and buffalo, leading to widespread economic losses worldwide. Milk of the infected animals plays an important role in distribution of disease. The conventional PCR and real-time PCR were developed for detection of Bovine Viral Diarrhea Virus in bovine and buffalo milk samples. A total of 4500 (2400 cows and 2100 buffalo) milk samples were tested for conventional and real-time PCR. We found that in cows, that the percentage of positive samples for BVDR was 28.37 and 28.75 on the basis of conventional and real-time PCR respectively. In buffalo milk samples, 18.00 and 18.23% samples were found to be positive for conventional and real-time PCR respectively. We conclude that real-time PCR is better option than conventional PCR for detection of BVDV in milk samples.

Keywords: Bovine viral diarrhea virus; milk samples; conventional PCR; real-time PCR

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Introduction

Bovine viral diarrhea virus (BVDV) is a pestivirus belongs to family Flaviviridae (Heinz et al., 2000) that cause Bovine Viral Diarrhea (BVD) in mammals including Bovidae, Antilocapridae, Cervidae, Camelidae, Suidae, Tragulidae, Giraffidae families and small ruminants (Nettleton, 1990; Loken, 1995; Van Campen et al., 2001; Grondahl et al., 2003). Pestiviruse is a small, enveloped virus that has a single-strand positive sense RNA molecule of approximately 12.5 kb. The genome is transcribed as a single open reading frame, flanked by 5'- and 3'- untranslated regions (UTRs). Distribution of the virus is a world-wide and causes various clinical syndromes in bovine and buffalo including reproduction dysfunctions (abortion, embryonic resorption, fetal teratogenesis, stillbirth and mummification), mucosal disease, diarrhea and hemorrhagic syndrome (Baker, 1995; Passler et al., 2007; Coetzer and Tustin, 2004). Cattle and buffalo of all ages are susceptible to infection of BVDV and in many cases pneumonia, diarrhea and abortions are the main signs of the disease.

Basically the virus spreads through contact between animals but vertical transmission plays an important role in its epidemiology and pathogenesis. In addition, the milk of the infected animals plays an important role in its distribution. On the other hand, the immunotolerant animals can shed the virus via secretion and excretion (milk, feces, vaginal secretions and urine), thus being a major source of BVDV infections in herds.

The traditional diagnosis was based on an isolation of virus in cell cultures but it was difficult, time consuming and lengthy process. The serological methods usually employed for diagnosis of BVDV in clinical specimens but usually cross immunity with other pathogens causes its low sensitivity and specificity. Today, the new diagnostic methods have been used. On the top of all diagnostic methods,

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conventional and real-time PCR have been developed for rapid and sensitive detection of BVDV (Bhudevi and Weinstock, 2001; Letellier and Kerkhofs, 20003).

So the two-fold purpose of the current study was to detect BVDV in cow and buffalo milk through conventional and real time PCR.

Materials and Methods

A total of 2100 buffalo and 2400 cow milk samples were randomly collected from different parts of Iran. Samples were collected from 201 and 217 dairy herds randomly in 2012. The animals from which the milk samples were collected were clinically healthy and the milk samples showed normal physical characteristics. Samples were collected under sterile hygienic conditions and were immediately transported at 4°C to the laboratory in a cooler with ice packs. All milk samples were kept at –20°C until processing.

RNA extraction

RNA purification was performed using the RNX™ Plus Kit (Sinagen, Iran) according to the manufacturer's instructions. Briefly 100-150 µl of viral suspension (abomasal contents and water control) were mixed with 1 ml of RNX and left for at least 5 min at 4ºC. After the addition of 200 µl chloroform and mixing, the liquid was clarified by centrifugation at 12,000 x g for 15 min at 4ºC. The supernatant was transferred to a new tube and mixed with an equal volume of isopropanol followed by centrifugation at 12,000 g for 15 min at 4ºC. The pellet was washed with 1 ml of 70% ethanol. Finally, RNA was eluted in 50 µl of 1 mM RNase free EDTA.

Conventional RT-PCR assay

The forward primer sequence is 5'- CAT- GCC-CCT-AGT-AGG-ACT-AGC-3', and the reverse primer sequence is 5'- TCA-ACT-CCA-TGT-GCC-ATG-TAC-3', which is used for BVDV screen in milk samples. All oligonucleotide primers were obtained from a commercial source (Cinna Gen, Iran). Total RNA (3 µl) was mixed with 1.5 µl of reverse primer (10 µM / µl) and incubated at 70ºC for 5 min followed by chilling on ice. The rest of the reaction mixture contained 4 µl of 5X first strand buffer, 2 µl of dNTPs (10 mM), 20 U of RNasin (20 U/ µl), 200U (200 U/ µl) of Moloney Murine Leukemia Virus (M-MuLV) (Fermentas) and 7.5 µl d-H20 was added, followed by an incubation at 42°C for 60 min cDNA synthesis was terminated by incubation at 70ºC for 10 min. PCR was performed in a 25 µl reaction mix. The final concentration of the reagent was as follows: PCR buffer (1×time) (Cinagen, Iran), dNTP 0.2 mM), MgCl2 (1.5 mM), each primer (0.5 µM), Taq DNA polymerase (0.625). Reactions were performed in an automated thermal cycler (Bio-Rad gradient Thermal Cycle). Cycle parameters for PCR were as follows: one cycle at 95ºC for 5 min followed by thirthy five cycles in 3 continous phases including 94ºC for 30 sec, 55ºC for 100 sec, and 72°C for 2 min, and finally terminated by a single cycle of a final extention at 72ºC for 10 min. The RT-PCR-amplified products were examined by electrophoresis in a 2% agarose gel, stained with a 1% solution of ethidium bromide, and examined under UV illumination. In this study, A negative control (sterile water), and a positive control RNA from BVDV (Cinna Gen, Iran), were included in each amplification run.

Real-Time PCR

The method used by Young et al. (2006) was employed to run real-time RT-PCR. SYBR® Green I dye was used in this real-time RT-PCR method, which binds to any double stranded DNA produced in the reaction. Each reaction contained 10 µl SYBR® Green JumpStart *Taq* ReadyMix for QPCR (Sigma), 2.5 µl forward primer (10 pmol/ µl), 2.5 µl reverse primer (10 pmol/µl) and 5 µl template cDNA. Samples were analyzed in triplicate. The PCR conditions were 94ºC for 10 min followed by 35 cycles of 94 ◦C for 10 s; 53ºC for 20 s; 72ºC for 20 s; 80ºC for 10 s after which a plate read was taken. The reactions were carried out on a DNA Engine Opticon 2 (MJ Research) and data analysed using the Opticon Monitor Analysis Software version 2.02. The primers were designed to amplify a 156 bp region of BVDV 5'- UTR (sense 5'-TAGTCGTCAGTGGTTCACGCC; antisense 5'-CCTCTGCAGCACCCTATCAG), or a 280 bp region of CECov nucleocapsid gene (sense 5'-CTCGTGGYCGGAAGAGTAAT; antisense 5'-GCAACCCAGAMRACTCCATC) and were tested to confirm sensitivity and efficiency over a range of DNA concentrations.

Sensitivity of conventional and real-time PCR assays

Solutions of purified BVDV were prepared ranging from 1x106 to 1 microorganisms/100 µl. DNA was extracted by a genomic DNA purification kit (Invitrogen, Paisley, U.K.), according to the instruction manual. 200 µl of the solution at microorganism concentration were used, and the extracted DNA was dissolved in 100 µl of distilled water. 300 µl of the DNA solution was used in the conventional and real-time PCR assays. Results are shown as number of microorganisms in one PCR-tube (microorganisms/ PCR-tube) and in 200 µl of sample (microorganisms/ sample).

Results

Results indicated that out of total milk samples, 1059 (23.53%) and 1073 (23.84%) were positive for

presence of BVDV by conventional and real-time PCR respectively (Table 1).

Table 1: Frequency of Bovine viral diarrhea virus in cow and buffalo milk samples by conventional and real-time PCR methods

Species	No of milk samples	Conventional PCR (%)	Real-Time PCR (%)
Cows	2400	681 (28.37)	690 (28.75)
Buffalo	2100	378 (18.00)	383 (18.23)
Total	4500	1059 (23.53)	1073 (23.84)

Sensitivity was compared between the conventional and real-time PCR assays in the detection of BVDV DNA. The conventional PCR assay with 5'-CAT- GCC-CCT-AGT-AGG-ACT-AGC-3', and 5'-TCA-ACT-CCA-TGT-GCC-ATG-TAC-3' pair primers, detected 63 microorganisms/PCR-tube, equivalent to 2x103 microorganisms/sample, whereas the SYBR® Green I assay with the specific primers to BVDV DNA, detected 36 microorganisms/PCR-tube equivalent to 2x102 microorganisms/sample (data not shown), indicating that the real-time PCR assay was 1.75 times more sensitive than the conventional PCR assay for detection of BVDV in bovine and buffalo milk samples.

The present study showed that the real-time RT-PCR assay was more sensitive and accurate than conventional RT-PCR method for detection of BVDV in bovine and buffalo milk samples. Therefore, the incidence of BVDV in bovine and buffalo milk samples of Iran were estimated 28.75 and 18.23% by application of real-time RT-PCR, respectively.

The negative and positive control in all RT-PCR testing that was performed in this study had a sufficient accuracy and practical diagnostic value. Our results showed that in Iran, buffaloes were more sensitive than bovine to BVDV. On the other hand, the incidence of BVDV in buffalo was more than bovine milk samples. Therefore our results showed that BVDV infection present widely in bovine and buffalo milk samples in the herds of Iran. Figure 1 shows a typical example of RT-PCR assay for detection of BVDV in bovine and buffalo milk samples.

Discussion

BVD is a world-wide disease and has been reported from many parts of the world. The prevalence of BVDV in cows in Iran (28.75%) is higher than Argentina (1.69%) (Camperoet al., 2003), Greek (14%) (Billinis et al., 2005), India (17.31%) (Sudharshana et al., 1999) and Turkey (23.07%) (Seyyal et al., 2002) but is lower than Northern Portugal (35%) (Niza-Ribeiro et al., 2005).

To the author's knowledge, BVDV in addition to induce disorders of reproduction (Abortion and Stillbirth), respiration (Pneumonia) and alimentary disease (Diarrhea in neonatal animals), causes immuno-suppression in cattle and buffaloes which lead to secondary bacterial or fungal (Aspergillosis) diseases. Therefore BVD plays an important role in epidemiological aspects of other bacterial and fungal diseases. One of the most important aspects of BVD is that it causes infertility and abortions. Studies showed that changes in placenta caused by BVDV could allow the other pathogens crossing fetal placenta barrier (Murray, 1991).

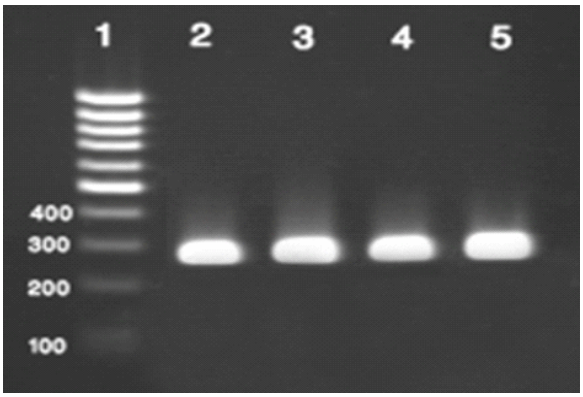

Fig. 1: Electrophoresis of PCR products of BVDV genome obtained by RT-PCR on 2% agarose gel electrophoresis. 1 is 100 bp ladders, 2-4 is positive sample and 5 is positive control. In this study the length of RT-PCR product was 290bp.

To our knowledge, BVD causes significant economic losses in livestock in Iran. In Iran, detection of antibodies against BVDV is used for the prevalence of this virus. For the first time, it was shown that the prevalence of BVD was in the range of 20-90% in Iran (Mirshamsy et al., 1970). Study on slaughtered cattle in Tehran province of Iran showed that 58.51% of animals were seropositive for BVD (Kargar et al., 1995). Study on cattle in Iran showed that 39.6% of young animals were seropositive for BVD (Sedighi-nejad et al., 1996). The reduction of the prevalence of BVD in this study may be due to the control program of this disease in Iran.

In many cases determination of the BVDV in clinical samples is difficult but our present study introduced real-time PCR as a sensitive, safe and accurate diagnostic method for fast determination and identifications of disease.

The real-time PCR assay used in the present study was more accurate, sensitive and faster than conventional PCR for the detection of BVDV. It is an important diagnostic tool yielding reliable and reproducible results, does not require post-PCR analysis (gel electrophoresis, hybridization), and the risk of cross contamination may be more limited than

conventional PCR. This study showed that the conventional PCR method for detecting BVDV in milk samples was more technically time-consuming and labour-intensive than real-time PCR assay. The real-time PCR assay used in this study could simplify the procedure by testing presumptive BVDV genome taken directly from cow and buffalo milk samples. In addition, compared with virus isolation, culture and serological methods, the real-time PCR has the primary advantages of being more sensitive, more rapid and there is no cross-reaction or sub sensitive reaction in samples. Previous studies have indicated that the real time PCR can be adapted to detect BVD viral RNA for diagnostic purposes (Letellier and Kerkhofs, 20003).

Since, real-time PCR assay has been developed for the detection of BVDV in a wide variety of clinical samples such as formalin fixed paraffin embedded tissue sections (Bhudevi and Weinstock, 2003), bulk milk (Kim and Dubovi, 2003), follicular fluid (Kim and Dubovi, 2003), blood (Kosinova et al., 2007) and skin (Passler et al., 2010) and in all of them real time PCR has been introduced as a sensitive, rapid and accurate method for detection of BVDV.

Our study recommended the use of real-time PCR instead of conventional method for rapid, sensitive and safe diagnosis of BVDV in milk samples.

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