

Short Communication

Genotyping of bovine viral diarrhea virus using multiplex PCR, with and without RNA extraction

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Bovine viral diarrhea virus (BVDV) is a very important viral disease, which constitutes a major problem worldwide due to the carrier state and due to its misdiagnosis with other viruses. The goal of the current study was to detect and serotype BVDV through multiplex PCR with and without RNA extraction, due to the fact that extraction of RNA may be laborious. Blood samples were taken from 100 randomly-selected animals, containing diseased animals suffering from diarrhea and respiratory manifestation. One part of the sample was subjected to RNA extraction and the other part was not, before performing reverse transcriptase (RT)-PCR. The assay succeeded to type BVDV with and without extraction RNA, as well as in detecting carrier animals. BVDV-2 was detected in a slightly higher number of animals than BVDV-1 (27 and 25, respectively), while 47 buffalo were identified as carrier animals.

Key word: Buffaloes, BVD, Multiplex PCR, Serotyping, RNA.

INTRODUCTION

Bovine viral diarrhea virus (BVDV), an economically important viral pathogen of cattle, belongs to the genus *Pestivirus* of the family *Flaviviridae* (van Regenmortel et al., 2000). It also affects buffalo, causing severe economic losses (Ghazy et al., 2007; Ghazi et al., 2008). The genome of BVDV is a single-stranded (+) RNA of about 12.5 kb in length, without a poly(A) tail (Collett et al., 1988). BVDV isolates consist of two different genotypes, that is, BVDV-1 and BVDV-2.

BVDV is associated with several clinical symptoms, including mild diarrhea, respiratory disease, congenital malformations, reproductive disorders and mucosal disease (Baker, 1987). Infection of bovine fetuses with BVDV-2 during the first 120 days of pregnancy can result in the birth of persistently-infected offspring that are immuno-tolerant to the virus (Charleston et al., 2001; Givens et al., 2003). Persistently-infected animals are difficult to identify, because of their normal appearance. Serum samples from these animals fail to react in most conventional serological tests and these animals shed large amounts of virus in all body secretions and excretions over prolonged periods (Barlow et al., 1986). The prevalence of BVD has been mainly reported on the basis of the detection of antibody against BVDV (Daliri et al., 2007). Investigations reported a range of 20 - 90% of BVD incidence (Mirshamsy et al., 1970).

Reverse transcriptase (RT)-PCR has become an increasingly popular procedure for detecting BVDV and other pestiviruses (Andre et al., 1995). Two limiting factors that control the successful performance of RT-PCR are dependent on nucleic acid purification methods and the oligonucleotide-specific priming of the RT reaction (Erlach et al., 1988). Other important considerations include the thermocycling parameters and the reagents that are to be used in the PCR (Sambrook et al., 1989). Similar to RNA hybridization, RT-PCR performance can be affected by BVDV genome variability (Alansari et al., 1993). All existing BVDV RT-PCR protocols require separate RT reactions and PCRs, which may increase the potential for contamination. The methods use total RNA extracted from cell-cultured specimens, and only a few use RNA extracted directly from the original specimens (Schmitt et al., 1994). None of these methods use total RNA obtained directly from whole blood specimens. Instead, total RNA obtained from the purified lymphocytes is used, because the available RNA extraction methods fail to consistently eliminate PCR-inhibiting compounds present in blood (Sambrook et al., 1989).

Recent refinements in PCR and nucleic acid purification techniques have provided opportunities for developing more rapid, sensitive and specific BVDV detection assays. By using the cationic surfactant tetradecyltrimethyl-

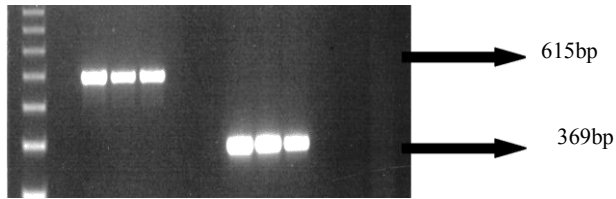


Figure 1. PCR of extracted RNA reveals bands at 615 and 369 bp. Lane 1 contains 123 bp ladder.

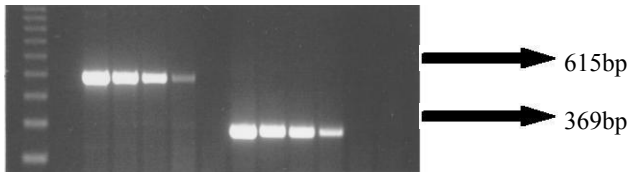


Figure 2. PCR from samples of which RNA was not extracted reveals bands at 615 and 369 bp. Lane 1 contains 123 bp ladder.

thylammonium oxalate (Catrimox-14 [Cat-14]), RT-PCR quality RNA can be extracted directly from whole blood in 1 h (Andre et al., 1995). In addition, recently published protocols combine the reagents for both RT and PCR in one tube and RT-PCR can thus be performed by a single, uninterrupted thermal cycling program (Andre et al., 1995). The current study aimed to demonstrate the serotype of BVD, using multiplex RT-PCR with and without extracting viral RNAs from lymphocytes of blood samples from infected and apparently healthy buffaloes.

MATERIALS AND METHODS

Samples

The present investigation was carried out at the National Research Centre, during January - June 2007. Blood samples were collected through a jugular vein puncture from 100 randomly-selected buffaloes in the Sharkia governorate. The buffaloes suffered from diarrhea and respiratory symptoms. Samples were collected with EDTA as anticoagulant. A similar volume of Ficol (Sigma) was added to the whole blood sample, centrifuged at 1 500 rpm for 15 min and the buffy coat was separated (Quinn et al., 1994).

Nested RT-PCR

The external primers for primary PCR, 5'-AAGATCCACCCCTTATGAGC-3' and 5'-AAGAAGCCATCATCACCCACA-3', were derived from nucleotides 10,385 to 10,404 and 11,528 to 11,547, respectively (Collett et al., 1988). The multiplex PCR primers for secondary PCR, 5'-TGGAGATCTTTCACACAATAGC-3' (BVDV-1 specific), 5'-GGGAACCTAAGAACTAAATC-3' (BVDV-2 specific), and 5'-GCTGTTTCACCCAGTTAGTACAT-3', were derived from nucleotides 10,758 to 10,779, 10,514 to 10,533, and 11,096 to 11,117, respectively (Gilbert et al., 1997).

BVD virus RNAs were obtained either by extraction from the isolated buffy coats with a commercial RNA extraction kit (Promega

BioSciences Co.) and RT-PCR was performed, or RT-PCR was performed directly on the isolated buffy coat according to Hamel et al. (1995). Reverse transcription was carried out at 37°C for 30 min, followed by denaturation at 94°C for 3 min. The primary PCR reactions were cycled 25 times at 94°C for 20 s, 50°C for 30 s and 72°C for 30 s, with a final extension step of 72°C for 15 min. The products were then used as template in a secondary PCR for 40 cycles. This was performed in the same manner as the primary PCR, but with multiplex primers and without reverse transcriptase, RNase inhibitor and external primers. The amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

RESULTS

Using the primers described in this study, amplification products of 604 and 360 bp were predicted for BVDV-2 and BVDV-1, respectively. By using RNA extracted from the blood samples for RT-PCR, products were obtained of which the sizes are consistent with the predicted sizes (Figure 1). The same sized products were also obtained for the samples from which the RNA was not extracted before use in the RT-PCR (Figure 2).

A total of 100 BVDV isolates were typed by PCR and tested against multiplex PCR. BVDV-2 was detected in a slightly higher number of animals than BVDV-1 (27 and 25, respectively), while 47 buffaloes were identified as carrier animals. Also, the multiplex RT-PCR of non-extracted RNA was sensitive and detected the virus in all the infected animals, as evidenced by extracting the virus.

DISCUSSION

Infections with bovine viral diarrhea virus (BVDV) occur globally and are the cause of substantial economic and genetic losses to the beef and dairy industries. Two distinct BVDV genotypes exist, that is, BVDV-1 and BVDV-2. There are different symptoms of the disease, which may lead to its misdiagnosis (Charleston et al., 2001; Givens et al., 2003). Unfortunately, conventional serological tests fail to accurately diagnose and serotype BVDV (Daliri et al., 2007).

In this study, a PCR that can be used to type BVDV from infected blood was described. This PCR could be used on samples from which RNA was extracted or not before use. This is an important advance, since RNA extraction procedures can be very laborious, expensive and time-consuming.

The PCR results obtained without prior RNA extraction suggest that a fraction of BVDV RNA in the blood of infected buffaloes may be unpacked (naked) and resistant to degradation, and were thus readily available for RT. Alternatively, viral RNA may have been released from virus and/or cells during sample handling or during RT-PCR. Definitive experiments are planned to distinguish between these possibilities, but initial experiments showed that incubation in the RT-PCR mixture had a dra-

matic negative effect on the titer of infective virus which lends some support to the second hypothesis. Infected blood that was known to be either citrated or heparinized should yield products by PCR. However, citrate and EDTA are considered more suitable as anticoagulants than heparin, since the latter has been shown to inhibit PCR (Holodniy et al., 1991). Previously, several PCR-based assays for typing BVDV have been reported (Harpin et al., 1991; Ridpath et al., 1994; Sullivan and Akkina, 1995). Typing of BVDV with the assay of Harpin et al. (1991), however, involves restriction endonuclease digestion of the PCR products for typing. The assay of Ridpath et al. (1994) relies on the use of specific amplification of BVDV-2 for typing, with a negative result indicating BVDV-1. The advantage of the multiplex PCR is that a specific product is produced for both genotypes. The multiplex PCR of Sullivan and Akkina (1995) could type border disease virus (BDV), another pestivirus, in addition to BVDV-1 and BVDV-2. This is an advantage for typing pestiviruses from sheep, which can be infected with all three viruses. However, BDV does not appear to readily infectious in cattle BDV and has not been isolated from North American or European cattle (Ridpath et al., 1994; Sullivan and Akkina 1995; Paton et al., 1996). Only one bovine BDV isolate has ever been reported; its original isolation is thought to have been made in the 1960s (Becher et al., 1997).

Because colostral antibodies may interfere with BVDV detection by virus isolation and antigen capture enzyme-linked immunosorbent assay (Shannon et al., 1992; Brinkhof et al., 1996; Saliki et al., 1997), additional studies to evaluate the PCR for screening the carrier status of young calves in herds are planned. PCR may be especially useful in screening beef herds, since early and accurate identification of carriers avoids excessive rounding up of cattle from pasture for testing. The results obtained in this study indicated that the PCR may have a role in screening herds for carriers and that it is a valuable tool for typing BVDV directly from blood. Also, it proves that PCR may be used without RNA extraction, which may reduce both time and expenses, and may be useful as an initial rapid screening method for detection of BVDV.

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