

Genotyping of Different *Pestivirus* Isolates by RT-PCR and RFLP techniques

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Summary

1320 blood samples were collected from herds showing clinical signs of pestiviral infections. 39 samples were positive in Ag-ELISA assay. All these 39 samples in addition to 5 cytopathic strains were cultured in MD-BK cell line and presence of pestiviral antigens was confirmed by direct-immunofluorescent test. 27 out of 44 BVDV suspected isolates were detected by RT-PCR using a primer set. Differentiation among the viruses was achieved by cutting the PCR products with restriction endonucleases enzymes *Ava*1, *Bgl*1 and *Alu*1. Using this procedure it was possible to distinguish at least two genogroups, 1 and 2 BDV containing 14 and 3 isolates, respectively.

Key words: Bovine viral diarrhea, RT-PCR, RFLP, genotyping

Introduction

Bovine viral diarrhea (BVD) is an important widespread disease of cattle with a diverse spectrum of clinical signs including reproductive losses, diarrhea and usual fatal mucosal disease (Paton 1995). The causative agent, BVDV, is a member of the genus *Pestivirus* in the family Flaviviridae. The presence of variability among BVDV isolates has been shown, however, the extent of its genetic variability in

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most countries remains unknown (Becher *et al* 1999, Hamers *et al* 2001, Vilcek *et al* 1999). Two genetically distinct forms of BVDV type I and II have been identified. Based on genetic comparisons, BVDV type I has been subdivided into BVDV type Ia represented by reference strain NADL and BVDV type Ib represented by reference strain Osloss (Becher *et al* 2003, Ridpath 2003). BVDV type II appears to be a newly emerging variant that has become increasingly common in North America since the 1980s (Ridpath 2003, Ridpath & Bolin 1998), which has rarely been described in Europe (Wolfmeyer *et al* 1997, Baule *et al* 1997, Ridpath & Bolin 1998). Two further categories, BVDV, Ic and BVDV Id, have recently been identified (Baule *et al* 1997).

Several reverse transcription-polymerase chain reaction (RT-PCR) methods for detection of BVDV at the genetic level have been applied (Pfeffer *et al* 2000, Brock *et al* 1992, Letellier *et al* 1999, Vilcek *et al* 1994, el-Kholy *et al* 1998). Variety sets of primers have been used for genotyping of BVDV mainly due to high mutation levels in some parts of its genome (Donis *et al* 1995, Saliki & Dubovi 2004). The designed primers should be able to detect conserved regions of BVDV genome (el-Kholy *et al* 1998, Brock *et al* 1992, Vilcek *et al* 1994). In this study, we used RT-PCR and restriction fragment length polymorphism (RFLP) methods for genotyping of different pestiviruses isolated from variety regions of Iran.

Materials and Methods

Sample. 1320 blood samples of cattle showing clinical signs of pestiviral infection including congenital defects, enteric and/or respiratory symptoms as well as mucosal disease were collected from different parts of Iran. Buffy coats were separated and run for Ag-ELISA using a commercial kit (Moredun Diagnostics, Midlothian, Scotland) according to the manufacturer's instruction. The positive samples were cultured in MD-BK cell line using RPMI 1640 Medium (Gibco BRL) with 2% fetal calf serum (Gibco BRL). In addition four cytopathic (CP) pestiviruses

isolated in diagnostic virology laboratory of Razi Institute and a reference strain NADL (Waybridge, UK) were cultured under the same condition. In order to detect noncytopathic (NCP) strains in cell cultures direct-immunofluorescence (FA) test was performed using conjugated antibody (bio-X GmbH, Ebersberg, Germany).

RNA extraction. Two techniques were employed for extraction of total RNA from the infected cell cultures: phenol/guanidine thiocyanate method TriPure isolation reagent (Roche diagnostic GmbH, Mannheim, Germany) and Silica filter based method using HighPure Viral RNA kit (Roche diagnostic GmbH, Mannheim, Germany). The isolated RNA of either method was then utilized for cDNA synthesis.

DNA synthesis. Each cDNA reaction consisted of 5 μ l of extracted total RNA, 2 μ l random hexamer (Fermentas, Germany)(1 μ g/1 μ l) and 4 μ l DDW. Mixture denatured at 70°C for 5min and chilled on ice. Then 4 μ l reaction buffer 5X, 2 μ l dNTP mix (1mM), 1 μ l ribonuclease inhibitor (Fermentas, Germany) and sterile deionized water were added to give a final volume of 19 μ l. The reaction was incubated at 25°C for 5min and then 200U of M-MuLV reverse transcriptase (Fermentas, Germany) was added. The reaction mixture was incubated at 25°C for 10min and then at 42°C for 60min. The reaction was stopped by heating at 70°C for 10min and chilled on ice.

PCR. One primer set, forward (5'-ATGCCCTTAGTAGGACTAGCA-3') and reverse (5'-TCAACTCCATGTGCCATGTAC-3') specific for all of pestiviruses was used for RT-PCR according to Vilcek *et al* (1994). The amplification of cDNA by PCR was carried out in a total volume of 25 μ l containing 2.5 μ l 10X reaction buffer (final concentration of 10mM Tris-HCl, 1.5mM MgCl₂, 50mM KCl, 0.1mg/ml gelatin; pH8.3), 2 μ l of each primer (final concentration of 1 μ l), 2 μ l of each 2.5mM dNTP (Fermentas, Germany), 2 μ l cDNA and 2 μ l (1U) *Taq* DNA polymerase (Fermentas, Germany). The reaction was overlaid with 30 μ l of mineral oil (Fermentas, Germany) and heated in thermocycler (Mastercycler, eppendorf, Germany) for 35 cycles of 95°C (1min), 56°C (1min), and 72°C (1min). In each

PCR process one positive cDNA derived from NADL strain and one negative cDNA obtained from non-infected cell culture was used as positive and negative controls, respectively. The electrophoresis analysis of the PCR products was run in 2% agarose gel using TAE buffer at 100V for 45min. Visualization of ethidium bromide stained DNA bands was performed by a UV transilluminator and gel images were printed using video camera and thermal printer.

RFLP. The amplified products were digested by *AvaI*, *AluI* and *BglII* (Roche diagnostic GmbH, Mannheim, Germany) according to manufacturer's instruction and were analyzed on 10% PAGE gel run in electrophoresis buffer at 15V/cm of gel for 1h. DNA bands were visualized by silver staining method.

Results

Out of 1320 buffey coats tested by Ag-ELISA 39 samples were positive. All of these positive samples were also positive by FA test. Five CP viruses (according to observation of CPE after 96h in cell culture) were used for RNA extraction. Tripure and Highpure (Silica based) methods varied in RNA yield. Silica based method yielded more quantities of RNA. Amplification of 27 out of 44 positive samples resulted in a 288bp band common (Figure 1). 4 out of the 27 samples belonged to the CP strains (3 Razi strains and NADL strain) and the rest belonged to the NCP field isolates.

BglII, *AvaI* and *AluI* restriction endonucleases enzymes digested amplified DNA from all of the 27 positive samples. PCR products of four CP strains digested by *AvaI* resulted in two 112bp and 167bp bands in silver stained PAGE. 18 out of 23 NCP isolates were digested by *AvaI* resulted in two 112bp and 167bp bands. This enzyme did not digest 5 isolates. *BglII* and *AluI* enzymes did not digest any of 27 isolates.

M 1 2 3 4 5

Figure 1. Gel electrophoretic analysis of PCR products using a specific primer for pestiviruses. Lanes 1-5: positive samples with 288bp band, lane 6: negative control and M:100bp marker

Discussion

From 44 positive results in Ag-ELISA and FA, only 27 were positive by RT-PCR. Although based on cell culture experiments, the 27 positive samples belong to two biotypes of pestiviruses, CP (n=4) and NCP (n=23) all of them were shown the same results in the RT-PCR. Presumably, different factors can affect virus viability and RNA integrity and also due to high mutation frequencies among pestivirus isolates it is difficult to predict primers that will amplify all virus strains. RT-PCR technique is not appropriate for routine diagnosis of pestiviruses and PCR is mostly used for genotyping of the virus following primary diagnosis. So, it prefers to diagnose the virus by using one of the routine laboratory methods such as Ag-ELISA or FA test (Vilcek *et al* 1994, Beer *et al* 2002, Ridpath 2003, Sullivan & Akkina 1995). The common laboratory method of antigen capture ELISA has restricted value for diagnosis of persistently infected animals. It has been reported, following virus clearance procedures in some herds, cases of seroconversion were detected indicating possible false negative Ag-ELISA results (Saliki & Dubovi 2004). The problem of insufficiently sensitive commercial Ag-ELISA systems has been noted (Shannon *et al* 1993), especially in cases where maternal antibodies from young calves interfere with the assay (Evermann & Ridpath 2002).

Eradication programs against fatal mucosal form of BVD are based on the strict control of cattle movements and on the elimination of persistently infected animals from herds. The experience with eradication program against the disease in a number of countries has shown that the RT-PCR method is a useful tool to reveal persistently infected animals in cattle herds (Hurtado *et al* 2003, Falcone *et al* 2003). In this study, using the specific primer pair a 228bp fragment of pestivirus DNA is amplified. Our results showed that 22 out of 27 PCR products digested by *Ava*1, which were belong to NADL or Osloss strains. As none of the PCR products were digested by *Bgl*1 enzyme, it might be suggested that none of our isolates were of porcine origin. The remaining 4 PCR products, which were not digested by any enzymes, might belong to ovine group (Becher *et al* 1999, Vilcek *et al* 1997).

In our case, only one primer was used for amplification of pestivirus DNA from serum samples, thus other primers and biological fluids such as semen, FCS or pestivirus vaccines would probably be used in future studies to increase the sensitivity for detecting of the virus strains involved in BVD.

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