MOLECULAR TYPING OF OVINE PESTIVIRUSES

By

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SUMMARY

The genus pestivirus of the family flaviviridae comprises three viruses, namely bovine viral diarrhea virus (BVDV), border disease virus (BDV) and classical swine virus (CSFV). In this study, we applied RT-PCR for direct detection of pestiviruses RNA in leukocyte samples collected from apparently healthy and clinically diseased sheep and goats. This was followed by molecular typing of pestivirus using specific BVDV and BDV primers.

Our results indicated that 13 out of 75 (17.3 %) examined samples were positive for pestivirus infection. 9 out of 13 (69.2 %) pestivirus positive samples were positive for BDV and the remaining 4 samples (30.8 %) were positive for BVDV when we used specific BDV and BVDV primers, respectively. The specificity and the lack of cross reactivity of BVDV and BDV primers used were also confirmed.

In conclusion, RT-PCR is rapid, accurate and specific assay for direct detection of pestivirus nucleic acid as well as typing of pestiviruses in clinical samples collected from sheep and goats. Moreover, molecular typing of pestiviruses by RT-PCR using specific primers for each virus allows good understanding molecular epidemiology of pestivirus infection in sheep and goats in Egypt.

INTRODUCTION

Pestiviruses are enveloped, single stranded RNA viruses which belong to the Flaviviridae family. The genus Pestivirus includes bovine viral diarrhea virus (BVDV), with two genotypes BVDV1 and BVDV2, Border disease virus (BDV) and classical swine fever virus (CSFV) (Becher et al. 1995 and Murph et al. 1999). All pestiviruses are important veterinary pathogens causing economic losses among cattle, sheep and pigs (Moennig and

Plagemann, 1992). Recently, it has been demonstrated that ovine pestiviruses are divided into BDV, BVDV1 and BVDV2, both at the antigenic and genetic level (Panton et al. 1995, Becher et al. 1997, Van Rijn et al. 1997 and Vilcek et al. 1997).

BDV is a causative agent of border disease of sheep and goats which was first reported in 1959 from the border region of England and Wales (Murph et al. 1999). Border disease is characterized by abortion, stillbirth and birth of live but abnormal lambs characterized by the "hairy shaker" syndrome (Nettleton et al. 1998). Although BDV infects mainly sheep, the virus can also cross the host-species barrier and infects cattle and pigs (Roehe et al. 1992). Distribution of border disease virus is worldwide. The prevalence rates vary from 5 to 50% between countries and from region to region within countries (Nettleton et al. 1998).

Vertical transmission plays an important role in the epidemiology of the disease. Spread of pestivirus from animals to animals with persistent infection being the most potent source of infection (Murph et al, 1999). Control of pestivirus depends on detection and removal of persistently infected animals (Harkness, 1987). Diagnosis of pestivirus depends on isolation of virus in tissue cultures followed by immunoperooxidase or immunofluorescence assay. These techniques are time consuming and can't detect low level of virus. Moreover, isolation of virus is unreliable in animals younger than 2 months old that have received colostral antibody (Nettlenton et al. 1998). Differentiation between pestiviruses has been achieved by a panel of monoclonal antibodies (Wensvoort et al. 1989 and Panton et al. 1994).

The close antigenic relationship between pestiviruses and their ability to cross host-species barriers emphasise the value of laboratory typing of pestivirus strains. Several RT-PCR assays have been developed for detection of all pestiviruses, however, there are few genetic assays for specific detection BDV (Wirz et al. 1993, Vilcek et al.1994, Sullivan and Akkina., 1995 and McGoldrick et al. 1999).The main difficulty in the designing such assays has been the lack of nucleotide sequence data available for BDV, either in literature or in international databases. Therefore, the aim of the present study was to apply RT-PCR for a) direct detection of ovine pestiviruses RNA in leukocytes samples collected from sheep and goats, and b) Molecular typing of ovine pestiviruses using specific BDV and BVDV primers.

MATERIALS AND METHODS

1. Blood buffy coat samples:

A total of 75 blood samples were collected from sheep (50 samples) and goats (25 samples) at Matrouh and EL-Behera Governorates. The samples were collected from apparently healthy and clinically diseased animals (Table, 1). 10 ml of EDTA-blood sample was centrifuged at 1500 rpm for 15 – 20 minutes (Abd El-Rahim et al.1997). The buffy coat was collected and transferred into eppendorff tubes and kept at -70 C until be used.

2. Reverse transcription-polymerase chain reaction (RT –PCR) (Vilcek and Panton, 2000):

a. RNA extraction:

Total RNA was extracted from buffy coat samples using Quickprep RNA extraction kit (Amersham Bioscince Co.).

b. Primer design:

The sequences of all the following primers were described according to Vilcek and Panton (1999). The Primers were synthesized by DNA synthesizer, (Amershame Bioscience Co.)

1. General pestivirus primers:

They used for detection of any pestivirus nucleic acid. The sequence of primer pair is a) upstream primer: 5'-GGGCATGCCCTCGTCCAC-3' and b) downstream primer: 5'-CATGCCCGTAGGACTAGC-3'. These primers amplify a 288 bp DNA fragment.

2. Border disease virus (BDV) primers:

Pair of specific primers targeting the 5'noncoding region (5'NCR) of BDV was used. The sequence of the primer is a) upstream primer (PBD1): 5"-TCGTGGTGAGAT CCTGAG-3') and down stream primer (PBD2)(5'-GCAGAGATTTTTTATACTAGCC TT-3'). These primers specifically amplify a 225 bp DNA fragment of BDV nucleic acid.

3. Bovine viral diarrhea virus (BVDV) primers:

The sequence of upstream primer is 5'-CGACACTCCATTAGTTGAGG-3' and the downstream primer is 5'-GTCCATAACGCCACGAATAG-3'. These primers specifically amplify a 200 bp DNA fragment of BVDV nucleic acid.

3. Reverse transcription (RT) (synthesis of complementary DNA): 2-ul volume of total RNA was used in RT/PCR using ready to go RT/PCR beads (Amershame Bioscience). The RNA was

denatured at 95 C for 5 minutes and then used in RT reaction. The reaction mixture was incubated at 42 C for 1 hour and then at 95 C for 5 minutes for inactivating reverse transcriptase.

4. Polymerase chain reaction (PCR):

PCR was conducted using 2.5 units Taq DNA polymerase in a reaction buffer containing 50 mM Kcl, 10 mm tris-Hcl (pH, 9.0) and 200 uM each of dATP, dCTP, dGTP and dTTPP. The PCR cycle was denaturation at 94 C for 1 min., annealing at 56C for 1 min. and extension at 72 C for 1 min. The cDNA was amplified for 35 cycles. Following the last cycle, a final extension for 7 at 72 C was done.

5. Gel electrophoresis:

Following amplification, the PCR products were run on 2% agarose gel in Tris-acetate EDTA (TAE) buffer at 100 voltages for one hour. The gel was stained with ethidium bromide and then visualized by UV-illuminator.

RESULTS AND DISCUSSION

Many RT-PCR assays have been developed for detection of pestivirus nucleic acid, however, there are only a few of them specifically detected BDV (Sullivan et al. 1995 and McGoldrick et al. 1999).The main difficulty in the designing such assays has been the lack of nucleotide sequence data available for BDV, either in literature or in international databases. The BDV-specific primers used in this work originate from the evolutionarily conserved 5"NCR of the pestivirus genome (Vilcek and Panton ,1999).

In this study, a complementary DNA (cDNA) of RNA extracted from buffy coat samples collected from sheep and goats, was synthesized by reverse transcription (RT) using random oligonucleotides primer. The cDNA was amplified by PCR using pair of general pestivirus primers. This was followed by specific amplification using BDV and BVDV primers.

The specificity and the lack of cross reactivity of primers used were confirmed by testing BDV positive samples using specific BVDV primers and BVDV positive samples using specific BDV primers. Our results indicated that BDV and BVDV primers were specifically amplified BDV and BVDV RNA, respectively and there was no cross reactivity between them. Vilcek and Panton

(2000) applied RT-PCR for rapid typing of laboratory pestiviruses strains using specific BDV and BVDV primers..

13 0ut of 75 (17.3%) examined samples gave a 288 bp DNA fragment indicating a positive pestivirus infection (Fig. 1 and Table, 2). Moreover, cDNA from the samples that gave positive results for pestiviruses was amplified using specific primers for BDV and BVDV. 9 out of 13 (69.2%) pestivirus positive samples gave 225 bp PCR product when we used specific BDV primers (Fig. 2 and Table, 2). The remaining 4 (30.8 %) pestivirus positive samples gave 200 bp fragment using specific BVDV primers (Fig. 3 and Table, 2).

Hooft Van Iddekinge et al.(1992) suggested that the PCR assay is more sensitive method for the detection of pestivirus nucleic acid than virus isolation. Moreover, Brock (1990) reported that the direct detection of viral nucleic acid has advantages over virus isolation because of the lack of potential interference with neutralizing antibodies and sensitivity and specificity. PCR can detect viral nucleic acid of infectious and defective viruses as well as inactivated virus particles. Abd El-Rahim et al. (1997) used RT_PCCR for detection of BVDV RNA in leukocyte fractions obtained from mucosal disease suspected cattle. Their result revealed that 28 (77.8 %) and 29 (80 %) of 36 buffy coat samples were positive by cell culture method and RT-PCR, respectively.

Azzam (2002) compared PCR, immunofluorscence (IF) and immunoperoxidase (IP) for detection of BVDV and BDV in leukocyte samples from cattle, buffalo and sheep. He reported that PCR was more sensitive than IF and IP. He also mentioned that PCR could detect 100 % of cattle, buffalo and sheep samples that were positive by virus isolation.

In conclusion, RT-PCR is a rapid, sensitive and specific method for direct detection and typing of ovine pestiviruses (BDV or BVDV) in clinical samples collected from sheep and goats. The molecular typing of pestiviruses using specific primers for each virus type allow good understanding molecular epidemiology of pestivirus infection in sheep and in Egypt.

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Table (1): Number of examined buffy coat samples from sheep and goats for pestiviruses by RT-PCR.

Healthy status	Sheep	Goats
Apparently healthy	20	10
Clinically diseased	30	15

Table (2): Results of direct detection of pestiviruses nucleic acid in buffy coat samples collected from sheep and goats.

Virus types	Sheep				Goats			
	Apparently healthy 20 samples		Clinically diseased 30 samples		Apparently healthy 10 samples		Clinically diseased 15 samples	
	No.	%	No.	%	No.	%	No.	%
Pestivirus	2	10	8	26.7	1	10	2	13.4
BDV	1	5	5	16.7	1	10	1	6.7
BVDV	1	5	3	10.0	0	0.0	1	6.7

The percentage was calculated according to the number of samples of each species.

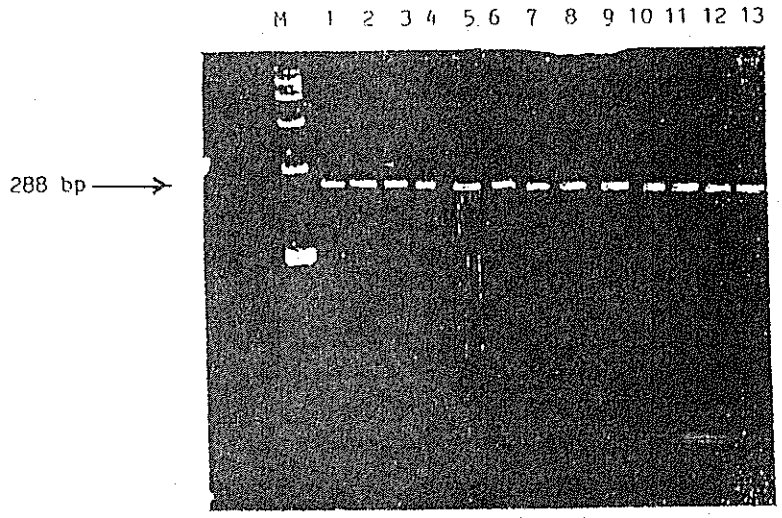

Fig. 1: Agarose gel electrophoresis of PCR amplification products using general pestivirus primers. Lane M: 100 bp ladder as a molecular DNA marker. Lane 1to 13 were positive samples for pestivirus. An arrow indicates the 288 bp amplified products.

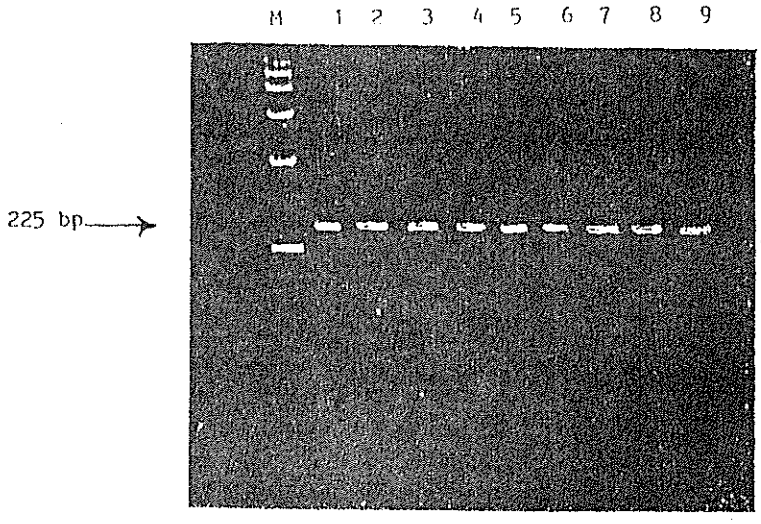

Fig. 2: Agarose gel electrophoresis of PCR amplification products using specific BDV primers. Lane M: 100 bp ladder as a molecular DNA marker. Lane 1 to 9 were positive samples for BDV. An arrow indicates the 225 bp amplified products.

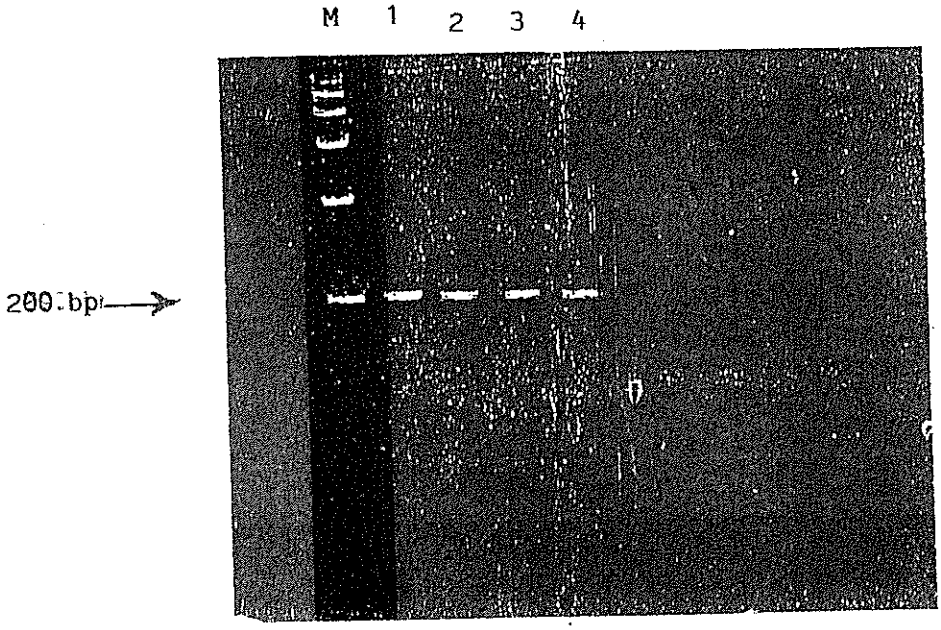

Fig. 3: Agarose gel electrophoresis of PCR amplification products using specific BVDV primers. Lane M: 100 bp ladder as a molecular DNA marker. Lane 1 to 4 were positive samples for BVDV. An arrow indicates the 200 bp amplified products.