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Border Disease Virus Infection as Recurrent Field Problems among Sheep and Goats

[1]Hala A. Salem, Jehan [2]A. Gafer and [3]Nahla Sh. Kotb

[1]Department of Virology, Animal Health Research Institute, Dokki, Giza, Egypt
[2]Department of Biotechnology, Animal Health Research Institute, Dokki, Giza, Egypt
[3]National Organization for Drug Control and Research (NODCAR), Egypt

Abstract: During three successive months in 2009 a flock of sheep and goats at El Fayoum governorate, Egypt showed 60% abortion cases and 10% birth of small week lambs died just after delivery in addition to diarrhea, hairy fleece and nasal discharges in the other affected flock mates. Theses findings implicated BDV as the primary cause. BVDV antigen was detected in 58.3% of samples collected from the affected animals using ELISA and FAT. While antiviral antibodies were detected in 78.9 and73.6% of serum samples collected from contact apparently healthy animals by using ELISA and SNT, respectively. Nested RT-PCR assay were used for confirmation, typing and differentiation of the BVDV by using type specific primers which yielded DNA products of unique size characteristic for each ovine pestivirus genotype. Result revealed positive amplification of 223bp specific for BVDV type 1 while negative results with other two primers specific for BVDV type 2 and border disease virus were evident.

Key words: BDV · ElISA · FAT · SNT · Nested RT-PCR

INTRODUCTION

Border disease virus (BDV) is an important sheep pathogen causing significant losses in sheep farming worldwide [1]. The economic costs of endemic infection have been estimated as a loss of ~20% of expected income [2].

First report of border disease in sheep was in 1959 from border region of England and Wales, and since that time it was recorded on world wide scale [3]. It is a viral disease together with Bovine viral diarrhoea virus (BVDV) and Classical swine fever virus (CSFV). It belongs to the genus Pestivirus within the family Flaviviridae. The term BDV is ambiguous, because it has also been used to refer to any ovine pestivirus which may include viruses of the BVDV genotypes [4]. BDV-3 was also detected in a cow in Austria; in that case sheep were suspected as the source for BDV infection [5].

The causative virus has positive stranded RNA genomes of an average length of 12.3 kb. The genome contains a large open reading frame flanked by 5' and 3' untranslated regions (UTR). The viral polypeptides and the genes encoding them are (from the N to C terminus): Npro, C, Erns, E1, E2, p7, NS2-3 (or NS2, NS3), NS4A, NS4B, NS5A and NS5B [6].

The disease is characterized by barren ewes, abortion, stillbirth and the birth of small, weak lambs showing tremor, abnormal body conformation and hairy fleeces and in lambs persistently infected (PI) with Border Disease Virus (BDV) there is an enteric disease characterized by diarrhoea and ill thrift [1, 7].

Control of infection is depends upon accurate diagnosis of PI animals; so direction towards conventional diagnosis relies upon virus isolation and also on ELISA, FAT and SNT testing to demonstrate viral antigen in samples of clinically ill animals and detecting viral antibodies in samples of apparently healthy sheep and goat, beside the application of nested RT-PCR using type specific primer to confirm the presence of the virus as well as typing and differentiation of this causative agent.

The current work aimed to detect BDV in field clinical samples and distinguished it from the other ruminant pestiviruses, using nested RT-PCR assay.

MATERIALS AND METHODS

Samples: Sixty different types of samples (from clinically diseased animals) were examined for viral antigen detection and RT-PCR assay to confirm the presence of

Corresponding Author: Jehan Gafer, Department of Biotechnology, Animal Health Research Institute, Dokki, Giza, Egypt, E-mail: jehan.gafer@gmail.com.

Table 1: Samples collected from both clinically diseased & contact apparently healthy animals

	Clinically diseased animals					
Samples Animals	N.S	B.C	V.S	F.S	Total No.	Apparently healthy Serum samples
Sheep	7	10	14	11	42	13
Gaots	2	5	6	5	18	9
Cattle	-	-	-	-	-	16
Total No.	9	15	20	16	60	38

N.S: Nasal swab samples B.C: Buffy coat samples
F.S: fecal swab samples V.S: Vaginal swab samples

virus. Beside 38 sera sample (from the apparently healthy sheep and goats as well as contact cattle) were examined for viral antibody detection. The samples were collected from a flock found at El-Fayoum governorate, Egypt with a total number of 112 sheep and goats. The details of collected samples are shown in table 1.

Trial for Virus Isolation: On trail for virus isolation, two blind passage were made on MDBK cells with modified Eagle's minimum essential medium (MEM) (GIBCO BRL, USA).

Antigen Detection

Fluorescent Antibody Technique: The test was performed according to Bezek *et al.* [8]. Briefly, the inoculated MDBK cell cultures were fixed with cold acetone and overlaid with anti BVDV serum conjugated with FITC incubation for 1 hr and washing by PBS then examined under fluorescent microscope.

Double Sandwich ELISA: ELISA test was adopted by using commercial kit, is based on double antibody sandwich enzymatic immunoassay (DAS or capture ELISA). The plate is coated with monocolonal antibody to the non-structural protein p80/p125 of BVDV.

Detection of BVDV antibodies

Blocking ELISA Kit: This commercial Kit is based on competitive enzymatic immunoassay. The antigen is fixed in polystrene plate after incubation with serum samples a specific monoclonal antibody (peroxidase conjugated) against this virus was added. If the serum sample contains antibodies against the virus, it will not permit the binding of the labeled Mab to the antigen, but if it doesn't contain specific antibodies the Mab will bind to the antigen on the plate.

Serum Neutralization Test: It was carried out according to Murakami *et al.* [9] using BVDV (NADL strain) was kindly supplied from Department of Virology, Faculty of Veterinary Medicine,Cairo University. The virus was stored at -70°C untill used.

RT-PCR: Positive BVDV antigen samples of sheep were pooled in one sample also the positive samples of goats were pooled in another sample. The two samples were subjected to testing by RT-PCR assay.

RNA Extraction and PCR of the Pestivirus Genome: Total RNA was extracted from the two samples as well as control using the TRIzol reagent (Gibco, USA), according to the manufacturer's instructions. RNA samples were reverse transcribed and the P1 (5` AAC AAA CAT GGT TGG TGC AAC TGG T 3`) / P2 (5` CTT ACA CAG ACA TAT TTG CCT AGG TTC CA-3`) 826-bp region of the Erns was amplified according to the method described by Sullivan and Akkina [10]. Briefly, primers and viral RNA were incubated at 70°C for 10 minutes and quickly cooled on ice for 10 min. The RT-PCR reaction contained 1 X PCR buffer (10 X buffer contains 500 mM KCl; 100 mM Tris-Cl, pH 8.3), 15 mM MgCl2, 1mM of each deoxynucleotide triphosphate (dNTP), 20 U of RNase Inhibitor, 2.5 U of Taq polymerase, and 100 pmole from each of the P1 and P2 primers in a reaction volume of 100 il. PCR tubes were initially incubated for 35 min. at 42°C to allow cDNA synthesis. This was followed by a five-minute hold at 95°C to inactivate the reverse transcriptase, and then cooling at 5°C for 5 min. The PCR reaction was carried out in 35 cycles using these parameters: template denaturation, 94°C for 1 min; primer annealing, 55°C for 1 min; and extension, 72 °C for 1 min. A final extension was allowed at 72°C for 10-min. PCR products were separated by electrophoresis on 1.5 % agarose gels containing ethidium bromide stain, illuminated, and visualized using a transilluminator.

Nested PCR with Type-specific Primers: A nested PCR reaction was performed using the type-specific primers, TS1 (5'-TAT ATT ATT TGG AGA CAG TGA ATG TAG TAG CT-3'), TS2 (5` TGG TTA GGG AAG CAA TTA GG

3`) and TS3 (5` GGG GGT CAC TTG TCG GAG G 3`) which were designed to amplify BDV, BVDV type II and BVDV type I, respectively. The bp count of the amplified DNA for the respective T primers was TS1, 566 bp; TS2, 488 bp and TS3, 223 bp. Two µl of 1:100 dilution of the first PCR product in nuclease-free distilled water were added to 98 µl of PCR master mix II composed of the same reaction components as before without RNase or reverse transcriptase and with the substitution of P1 with 100 pmole of each of primers TS1, TS2 and TS3. Twenty-five cycles of amplification were done using the following reaction conditions: template denaturation at 94°C for 1 min., primer annealing at 50°C for 45 sec., and extension at 72°C for 1 min. The final extension was at 72°C for 10 min. Fifteen µl of the PCR product were electrophoresed in 2% agarose gel containing 0.5 µg/ml ethidium bromide, and visualized as before.

RESULTS

Case History and Clinical Observations: There were 60% abortion cases and 10% birth of small week lamb died just after delivery during three successive months during 2009. Other clinical symptoms were diarrhea, hairy fleece and nasal discharges.

Result of FAT was shown in fig 1 whereas obvious green intracytoplasmic fluorescence were detected in the positive samples.

Results of detection of BVDV antigen are presented in table 2. 35 out of 60 (58.3%) samples collected from sheep and goats showing sign of illness by using both tests ELISA and FAT were positive.

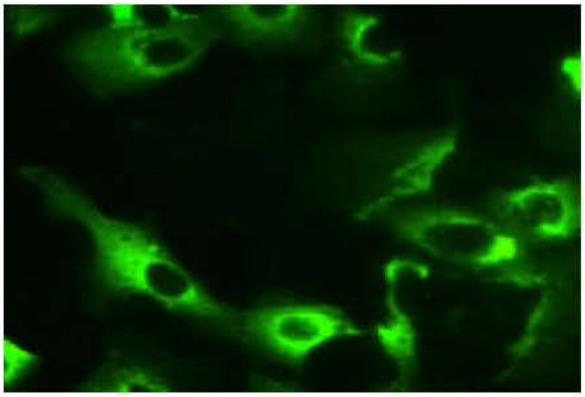

Fig. 1: Inoculated MDBK cell show observed intracytoplasmic fluorescence

Results for detection of antibodies in sera sample of the contact cattle and apparently healthy sheep and goats were recorded in table 3. Positive results in 30 out of 38 sample collected with a percentage of 78.9% by using ELISA test were evident. While by using SNT the results were positive for 28 out of 38 (73.6%) samples collected. Tables 2 and 3 indicated a lower percentage of infection in goats.

Results of RT-PCR: DNA products of the expected size (826 bp) were obtained for the two pooled samples of sheep and goats after amplification with the consensus primers P1 and P2 (Fig.2). The DNA products from the RT-PCR were then subjected to a second round of amplification with the nested, type-specific primers. The only 223bp sized product specific for BVDV type 1 was obtained (Fig.3). While neither 556bp nor 448bp sized products specific for (BVDV type 2 respectively) were amplified.

Table 2: Results of detection of viral antigen using ELISA & FAT

	ELISA			FAT		
Animals	+Ve	-Ve	+Ve %	+Ve	-Ve	+Ve %
Sheep	29	13	69%	30	12	71%
Goats	6	12	33.3%	5	13	27.7%
Total	35	25	58.3%	35	25	58.3%

Table 3: Results of detection of BVDV antibody in apparently healthy and contact animals using SNT and ELISA

	ELISA				SNT		
Animals	Total No.	+Ve	-Ve	+Ve %	+Ve	-Ve	+Ve %
Cattle	16	15	1	93.7%	13	3	81%
Sheep	13	10	3	76.9%	10	3	76.9%
Goats	9	5	4	55.5%	5	4	55.5%
Total	38	30	8	78.9%	28	10	73.6%

N.B Titre of SNT > 1/16 consider +ve results

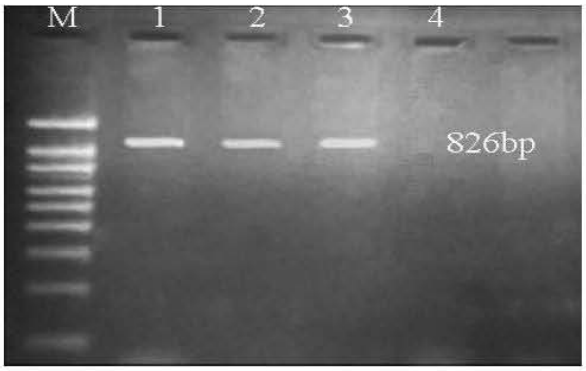

Fig. 2: Gel electrophoresis show amplification products with primers P1 and P2. M, 100 bp marker. Lane 1, positive control (NADL strain). Lane 2 & 3, amplification from pooled samples of sheep and goats, respectively (826 bp). Lane 4, negative control

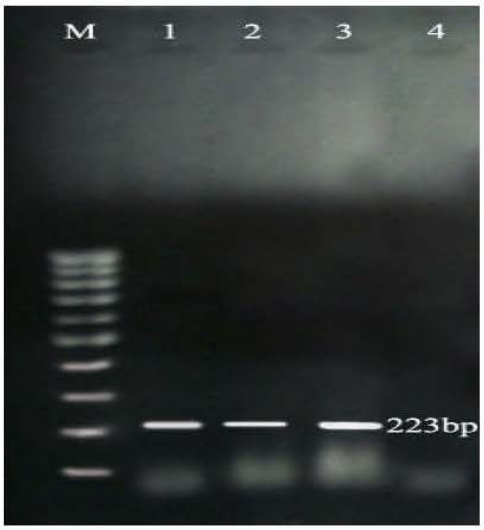

Fig. 3: Gel electrophoresis analysis of the DNA products with type specific primer TS3 of DNA product from primers P1 and P2. M, 100 bp marker. Lane 1, positive control (BVDV-1). Lane 2 & 3, amplification from sheep and goat samples, respectively. Lane 4, negative control

DISCUSSION

Border disease is the causative agent of an important congenital disease of sheep and goats and is distributed world wide. Border disease viruses (BDV) together with bovine viral diarrhoea virus (BVDV) types 1 and 2 were originally classified dependant upon the species from which they were isolated. Earlier studies demonstrated that the pestiviruses from sheep can be antigenically subdivided into two groups which include BDV-like and BVDV-like viruses [10, 11-17] Recently genetic typing of several pestivirus isolates of sheep and goats originating from different countries allocated them to at least three different genotypes [18-21]. Sequence and phylogenetic analyses further demonstrated that interspecies transmission of closely related ruminant pestiviruses is possible and occurs in the field. [22-24]. Also, the increasing use of molecular typing techniques has demonstrated that both BVDV and BDV can infect sheep [2].

Regarding the two genotypes; BVDV-1 and BVDV-2, BVDV-1 has a world-wide distribution, while BVDV-2 appears to be relatively rare in other continents [25]. The present clinical observation and the case history of the flock agree with those reported by Nettleton *et al*. [26].

For definitive diagnosis of BDV, isolation of the virus is required. Although highly specific, isolation is time consuming, expensive. Immunohistochemistry (IHC) has been successfully applied to bovine and ovine fetal tissues [27]. However, viral isolation and IHC require expertise and special laboratorial resources.

Antigen and antibody ELISAs are readily available and easy to use, and viral antigen can be detected in serum as well as whole blood and even skin biopsies. Our results using ELISAs and FAT denoted that the lower percentage of BD infection found in goats as previously recorded [3, 24].

In the present study it was clear that BVDV antibody is circulating among contact cattle and goats in examined governorates, so it was concluded that this virus is widely spread due to active infection rather than vaccination, as there is no vaccination program for cattle, sheep and goats in the studied flocks. Also the breeding of cattle, sheep and goats together under Egyptian field conditions were considered as the main factors that helps the transmission of the disease infection from cattle to goats and sheep and vise versa.

In cell culture, ruminant pestiviruses appear as two biotypes, cytopathogenic or non cytopathogenic. Both biotypes have been isolated in bovines, while ovine strains appear almost exclusively to be non-cytopathogenic [3, 28] so we depend mainly on RT-PCR in detecting and typing of the ovine pestivirus.

Reverse-transcription PCR (RT-PCR) has become a routine diagnostic method and is highly sensitive as well as specific, and has the advantage that it allows pooling of samples, and viral RNA can be detected independently of specific antibodies [29].

Regions of the pestivirus genome used for genotyping have included the structural genes (C, Erns and E2) as well as the nonstructural genes or regions (5'UTR, Npro and NS5B) [6, 30-34].

Although many RT-PCR assays have been developed for the detection of pestiviruses, only a few of them specifically detected BDV. The main difficulty in designing such assays has been the lack of nucleotide sequence data available for BDV, either in the literature or in international databases [4].

The primers used in this work (P1 and P2) (826-bp region) were selected from Erns region that share maximum homology with all 3 pestiviruses (BVDV types 1 and 2 and BDV) and have no homology to other regions of the pestivirus genomes. These primers (P1 and P2) were chosen homlogous to the conserved region of pestivirus genomic sequences to ensure that all strain of ovine pestivirus will be amplified in the first-round amplification. In the second reaction, nested PCR 3 type specific primer, TS1, TS2 and TS3, were used for differentiation of the three types of ruminant BVD viruses (BDV, BVDV type 2 and BVDV type 1). The bp count of the amplified DNA for the respective T primers was: TS1, 566; TS2, 488 and TS3, 223. The TS1/P2 was specific for border disease virus; TS2/P2 was specific for BVDV type 2; and TS3/P2 was specific for BVDV type 1 the same primers were previously used [10, 35]. This nested PCR method for typing pestivirus is faster and simpler than other methods for typing since correct typing require only electophoresis of the amplified product on agarose gel In contrast to other may require labeling, purification and standardization of probes which are time consuming and laborious. Our results revealed positive amplification of 223bp specific for BVDV type 1 for both pooled samples of sheep and goats (Photo 2). While did not yield visualized products when the other two primers of (TS1 and TS2 specific for both border disease and BVDV type 2, respectively) were used. This finding that the border disease in sheep and goats can be caused by BVDV type 1 agree with OIE [3] and Evermann and Ridpath[25] who reported that sheep and goats can become infected with BVDV from cattle and in some countries, BVDV can be a more common cause of BD than BDV.

We conclude that nested RT-PCR was suitable for detection of PI sheep and goats and for rapid and easy genotyping of pestiviruses present.

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