Molecular diagnosis and genotyping of Bovine viral diarrhea virus

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Abstract:

In the present study, isolation, biotyping, antigenic and genomic characterization of BVDV isolates was carried out on buffy coat samples obtained from apparently healthy cattle. The samples were inoculated in MDBK cells for three successive passages and the CPE was recorded indicating that 48%, 62% and 52% were positive CPE in the 1st, 2nd and 3rd passages respectively. The virus was additionally detected in cell culture after propagation by direct FAT and revealed that (62%) showed positive result. The mean virus titer was recorded after each passage showing values of \(<10^2,10^2\) and \(10^4\) TCID_{50}/ml by 1st, 2nd and 3rd passages respectively. The virus was identified by VNT using the reference anti-BVDV-1 serum on cell cultures infected fluids at the third passage. It was found that all samples showed a characteristic CPE of BVDV were neutralized by the used antiserum confirming that they are BVDV-1. Nine buffy coat samples were selected to carry out the nested RT-PCR for detection and genotyping of suspected BVDV. The results indicated the presence of BVDV genotype-I. The positive samples for FAT, VNT and RT-PCR in infected cell culture were stained by H&E for biotyping indicating the presence of the two biotypes (CP& NCP) in the samples. The SNT was carried out on serum samples from the same animals indicating the prevalence of antibodies in 62% by mean titer ranged from \(<\frac{1}{2}\) to 1/8. In conclusion, the present study reports the identification of BVDV genotype – I in apparently healthy cattle and presence of P.I. animals.

Keywords: Bovine viral diarrhea virus, biotyping, reverse transcriptase polymerase chain reaction (RT-PCR).
Introduction:

Bovine viral diarrhea (BVD) is one of the most imperative worldwide diseases in domestic and wild ruminants, leading to substantial damage in infected herds as well as extensive economic losses for the cattle industry (Goyal and Ridpath, 2005; Ahmed and Zaher, 2008). Causing multiple disease and clinical syndromes including embryonic mortalities, abortion, fetal mummification, stillbirths, congenital deformities, respiratory disease (Flores et al., 2002) and hemorrhagic syndrome (Walz et al., 1999). An important condition for maintenance of BVDV in bovine populations is the immunotolerant and persistent infection (PI) that result from transplacental infection of the fetus before onset of immunological maturity. Animals persistently infected with BVDV not only transmit the virus effectively to susceptible in-contact animals, but they harbour clones of BVDV which through serial transplacental passages may be able to replicate for years without immunological selective pressure (Toplak et al., 2004). It is well established that persistently vireamic animals may later succumb to fatal mucosal disease (Brownlie, 1985).

BVDV is a small enveloped RNA virus which together with classical Swine fever virus (CSFV) and Border disease virus (BDV) form the genus pestivirus in the family Flaviviridae (Pringle, 1999). BVDV consists of a single stranded positive-sense RNA genome, of approximately 12.5Kb long (Collett, 1992).

BVDV strains have two biotypes, cytopathic (cp) and non-cytopathic (ncp) which can be distinguished on the basis of their effect on cultured bovine cells (Gillespie et al., 1960). The two biotypes are important for the occurrence of MD. The MD is either induced by super infection of persistently infected animals with an antigenically closely related cp BVDV or by generation of a cp mutant from the persisting ncp virus (McClurkin et al., 1985).

According to the analysis of the basis of the 5' UTR, BVDV was segregated into two genotypes, BVDV-1 and BVDV-2. Sequence homology within each genotype was over 93%, while between genotype 1 and 2 it dropped to 74 % (Ridpath et al., 1994).

According to the phylogenetic tree constructed from 420 nt of the E2 glycoprotein gene fragment; the BVDV-1 can be subdivided into BVDV-1 a, c, d, e, f, g and 1 b which is divided into 1b1 and 1b2 and the BVDV-2 is subdivided into BVDV-2a, 2b and 2c. (Motoshi et al., 2001)

Due to the complex pathogenesis of BVDV infections and presence of many genovares, laboratory diagnosis of BVDV becomes important in the strategy of control and prevention BVDV infections. Moreover, the knowledge of the type of strains occurring in the field can help to establish effective vaccine and effective control (Kabango, 2005).

There are several methods used for diagnosis of BVDV infection which include virus isolation (Dubovi, 1990), virus neutralization (Brock 1995), immunoperoxidase (Castro et al., 1997), ELISA

Chu et al., 1985), FAT (Dubovi, 1990) and AGPT (Gutekunst and Malmquist, 1963) but all these tests suffer several disadvantages where virus isolation may requires as long as three weeks especially when more than one passage in cell culture is required to recover the virus. It’s expensive and unsuccessful when antibodies are present in the examined samples. Moreover, cell cultures in many laboratories are contaminated with low levels of BVDV which interferes with the recovery of field strains of the virus (Bolin et al., 1985).

FAT and IP detection are rapid but their specificity and sensitivity are dependent on the quality of the reagents used. Although reagents used in the immunoassays apparently react with group specific antigens, some strains may be missed (Bolin et al., 1985).

The results of virus neutralization test may vary widely depending on the strains of the virus used (Hassan and Scott, 1986).

The use of molecular techniques has been increased because of the existing rapidity and accuracy. The nucleic acid based techniques are useful tools for detecting and simultaneously genotyping of BVDV without isolation and propagation in cell cultures. Several researchers reported the use of reverse transcriptase polymerase chain reaction (RT-PCR) (Hooft et al., 1992), multiplex RT-PCR (Gilbert et al., 1999) and nested RT-PCR (Sullivan and Akkina, 1995) for detection and typing of BVDV.

Materials and methods:

Animals: -

A total of 750 apparently healthy Friesian cattle housed under bad conditions in two farms present in Behera Governorate were investigated.

Samples: -

Two sets of blood samples were collected from 50 randomly selected cattle by jugular vein puncture under sterile conditions. One set with EDTA and another set without EDTA for separation of the buffy coat according to Rossmanith et al. (2001) and sera according to Lannette (1964), respectively.

BVD virus isolation:-

The obtained buffy coat samples were inoculated on BVDV free cell line of Madine Darby Bovine Kidney (MDBK) supplied by Veterinary Serum and Vaccine Research Institute, Abassia, Cairo, Egypt according to Marcus and Moll (1968). These cells were used in virus isolation, virus titration, SNT, and FAT.

Direct fluorescent antibody technique (FAT): -

Fluorescent antibody technique was applied on the buffy coat and on cell culture infected with the 3rd viral passage according to Fernelius and Ritche (1964).

Detection of viral RNA and genotyping of BVDV by nested RT-PCR.

This technique was applied on 9 buffy coat samples from the 50 collected samples. Total RNA was extracted from both reference strain (Iman Strain) and field samples (Buffy coat) using commercial total RNA extraction kit according to the instruction of the manufacturer. Reverse transcription and nested polymerase chain reaction
was carried out according to Sullivan and Akkina (1995).

The nested-PCR oligopimer sequences are presented in Table 1.

The RT/PCR was performed with ready to Go RT/PCR beads (Amersham Pharmacia Biotech) according to the manufactures instruction.

The first round of PCR was performed in a 50 µl reaction mix containing 5 µl of P1 primer, 5 µl of P2 primer, 5 µl of ready to go RT-PCR beads and 35 µl RNAse free water. Reactions were also performed in Biometra personal cycler.

Conditions for thermal cycler were as follows: one cycle at 94°C for 1 min, thirty cycles in 3 continous phases which included: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, a final extention at 72°C for 10 min. The PCR products were then stored at 4°C

The second round of PCR was performed also in a 50 µl reaction mix containing 5 µl of product of the first round (diluted 1 in 100 µl distilled water), 5 µl of P2 primer, 5 µl of TS1 primer, 5 µl of TS2 primer, 5 µl of TS3 primer, and 22 µl RNAse free water. Reactions were also performed in the same automated thermal cycler.

Conditions for thermal cycler were as follows: one cycle at 94°C for 30 sec, twenty five cycles in 3 continous phases which included: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, a final extention at 72°C for 10 min. The PCR products were then stored at 4°C until gel electrophoresis were performed. The nested PCR products were separated on 3% agarose gel. The agarose gels contained 10 µg/ml of ethidum bromide to allow visualization of the products under an ultraviolet transiluminator.

Results:

**BVDV isolation:-**

The buffy coat samples were inoculated into MDBK cell culture. Three successive passages were done in the same cells. It was noticed that 24 samples induced CPE by the first passage, 31 samples induced CPE by the second passage, and 26 samples induced CPE by the third passage. As shown in table (2).

The observed CPE was characterized by onset on second day post infection. The signs of CPE were early rounding and granulation of the infected cells in scattered areas of monolayer then vaculation and foamy appearance were observed in 90% of the cells followed by complete detachment of the monolayer.

**Detection of BVDV in infected cell using the direct FAT:-**

Direct FAT was applied on 50 infected cell cultures with the 3rd viral passage using anti-BVDV antibodies conjugated with FITC. The results of this technique revealed that 31 samples showed specific fluorescent green reaction

<table>
<thead>
<tr>
<th>Primer (A)</th>
<th>Sequences</th>
<th>Genome position (b)</th>
<th>Size of amplified DNA product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>5'-AAC AAA CAT GGT TGG TGC AAC TGG T-3'</td>
<td>1424-1449</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>5'-CTT ACA CAG ACA TAT TTG CCT AGG TTC CA -3'</td>
<td>2221-2250</td>
<td></td>
</tr>
<tr>
<td>TS1</td>
<td>5'-TAT ATT ATT TGG AGA CAG TGA ATG TAG TAG CT-3'</td>
<td>1648-1716</td>
<td>566 (TS1 &amp; P2) BDV</td>
</tr>
<tr>
<td>TS2</td>
<td>5'-TGG TTA GGG AAG CAA TTA GG-3'</td>
<td>1802-1821</td>
<td>448 (TS2 &amp; P2) BVDV-II</td>
</tr>
<tr>
<td>TS3</td>
<td>5'-GGG GGT CAC TTG TCG GAG G-3'</td>
<td>2027-2045</td>
<td>223 (TS3 &amp; P2) BVDV-I</td>
</tr>
</tbody>
</table>

Indicating positive results with a total percentage of (62%). These results are tabulated in table (2) as shown in photo (1).

Detection and genotyping of suspected BVDV by nested RT-PCR.

Nine buffy coat samples were randomly selected to carry out the nested RT-PCR for the detection and genotyping of suspected BVDV. In addition, the technique was including a positive control sample (IMAN strain). This technique indicated the presence of BVDV-RNA type-1 in the examined samples as shown in photo (2&3).

Photo (1): Direct immunofluorescence. The photo show specific fluorescent green reaction indicating positive results.

Photo (2): Polyacrylamid gel electrophoresis stained with ethidium bromide. Showing the specific amplification products with specific size (826 bp) of the first round of the nested RT-PCR with primers (P1and P2) indicating that all examined samples are pestiviruses. Lane (1): 100bp DNA ladder, lane (2) positive control (IMAN strain), lanes 3 till 11 are examined buffy coat samples.
In the present study the direct FAT was carried out on 50 buffy coat samples. 26 samples out of them (52%) were found to be positive to BVDV. The results of FAT were obtained within 3 hours. FAT was also carried out on all samples propagated in cell culture where 31 samples (62%) showed positive reaction (apple green fluorescent color). Trials of virus isolation through the infection of MDBK cell culture with the same buffy coat samples revealed that 26 samples (52%) showed CPE for 3 successive passages. The difference between the FAT results and detected CPE in cell cultures could be attributed to the presence of 5 samples which did not show CPE. These results agree with those obtained by (Gerda et al., 1970) who concluded that the FAT is efficient test in the diagnosis of non-cytopathic BVDV. Moreover, Ruckerbauer et al. (1971) concluded that the FAT was of a value to detect both cytopathic and non-cytopathic BVDV antigen in primary fetal kidney tissue cultures inoculated with field specimens. They proved that FAT was faster than virus neutralization test. In addition, similar results were obtained by (Snyder et al., 1979) who stated that BVDV could be detected 3 days post inoculation into tissue cultures in case of virus isolation, while the virus could be detected within 24 hours post infection in using FAT. They added that FAT sensitivity reached 80% agreement with virus.

Serum neutralization test (SNT):

Both screening and quantitative SNT were carried out on 50 serum samples obtained from the same animals from which the buffy coats were obtained using the microtiter technique. Screening SNT showed that 31 samples were found to be positive for BVDV antibodies. From these samples the quantitative SNT showed that 12 samples had a titer of <2; 7 samples had a titer 2; 6 samples had a titer 4 and 6 samples had a titer 8. These results are tabulated in table (3).

The results presented in tables (3&4) showed the relationship between the presence of BVDV in the samples and BVDV antibodies in the same animals sera. The results revealed that 20 samples were positive for both BVDV and BVDV antibodies, 11 samples were positive for BVDV and negative BVDV antibodies, 12 samples were negative for BVDV and positive for BVDV antibodies and 8 samples were negative for both BVDV and BVDV antibodies.

Discussion and conclusions
Table (1): BVDV isolation on MDBK cell cultures.

<table>
<thead>
<tr>
<th>No. of examined samples</th>
<th>Passage number</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>First passage</td>
<td>Second passage</td>
<td>Third passage</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No. of positive samples</td>
<td>Percent of positive samples</td>
<td>No. of positive samples</td>
<td>Percent of positive samples</td>
</tr>
<tr>
<td>50</td>
<td>24*</td>
<td>48%</td>
<td>31*</td>
<td>62%</td>
</tr>
</tbody>
</table>

* Number of samples which gave CPE.

Table (2): Detection of BVDV antigen in the infected cell using direct FAT.

<table>
<thead>
<tr>
<th>No. of tested samples</th>
<th>No. of positive samples</th>
<th>% of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>31</td>
<td>62%</td>
</tr>
</tbody>
</table>

Table (3): Detection and titration of BVDV antibodies in bovine serum samples:

<table>
<thead>
<tr>
<th>No. of examined samples</th>
<th>Mean of BVDV antibody titer* for positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples had titer &lt;2</td>
</tr>
<tr>
<td></td>
<td>No.</td>
</tr>
<tr>
<td>50</td>
<td>31</td>
</tr>
</tbody>
</table>

* Antibody titer = the reciprocal of the final serum dilution which neutralized 100 TCID₅₀ of the BVDV.
isolation. In the present work the FAT sensitivity reached 100% and such difference in the 2 results could be attributed to the number of the examined samples from which these samples were obtained. In addition to the inferior health conditions of the animals due to bad conditions of housing, and the method of sample preparation. Furthermore (Tarable et al., 1980) recommended the use of FAT for detection and diagnosis of BVDV in field tissues specimens collected during the epidemics in Argentina. Among trials of virus isolation from the collected samples in cell culture it was found that the MDBK cell line was considered as one of the most suitable cells for propagation of BVDV (Marcus and Moll, 1968; Philip, 1973 and Allam, 2000).

In the present study, the buffy coat samples were inoculated on BVDV free MDBK cells and BVDV was detected by CPE and direct immunofluorescence. The recorded CPE was characterized by onset after 48 hours post infection, scattered early cell rounding and cytoplasmic granulation followed by lysis and cell detachment as shown in Photo (3&4). These finding agree with the results obtained by (Marcus and Moll, 1968) who tried to adapt NADL- BVDV cytopathic strain to MDBK and recorded the CPE. The first observed CPE was recorded by the third day post infection while the severe CPE occurred by the 4th or 5th day post-infection. We reordered the same CPE and accordingly MDBK cell culture was proved for BVDV detection by isolation and serodiagnosis using serum neutralization test. In addition, Brock (1995) reported that the cytopathic strains of BVDV cause characteristic changes in vitro cell changes such as cytopathic vaculations that are evident in inoculated cell cultures within 24-48 hours.

The results of BVDV isolate propagation in the present study revealed that 24 samples (48%), 31 samples (62%), and 26 samples (52%) showed CPE after the first, second and third successive cell passages, respectively.

Table (4): - Results of detection of BVDV and/or BVDV antibodies in examined animals:

<table>
<thead>
<tr>
<th>No. of examined samples</th>
<th>Group A</th>
<th></th>
<th>Group B</th>
<th></th>
<th>Group C</th>
<th></th>
<th>Group D</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
<td>Percent</td>
<td>Number</td>
<td>Percent</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>40%</td>
<td>11</td>
<td>22%</td>
<td>12</td>
<td>24%</td>
<td>8</td>
<td>16%</td>
</tr>
</tbody>
</table>

*Group (A)*: Samples show positive virus and positive antibody.

*Group (B)*: Samples show positive virus and negative antibody.

*Group (C)*: Samples show negative virus and positive antibody.

*Group (D)*: Samples show negative virus and negative antibody.
The delay of CPE appearance in the third passage of 26 samples less than in the second passage (31) samples may be due to some factors as suggested by (Fernelius et al., 1969) who stated that such delaying may be due to an abortive infection or incomplete multiplication of the isolates on MDBK cells or incomplete adaptation of the isolates to MDBK cells, Johnson et al. (1990) stated that such delaying may be due to an absence of the intracellular secretion which is responsible of CPE evolution. Moreover, Farmer and Frazier (1986) stated that such delaying may be due to sudden reduction in virus titer and/or replication schedule and subsequently BVDV quantity. Petkova et al.(1982) stated that such delaying may be due to repeated freezing and thawing of the viral harvest could lead to denaturation of the viral proteins and reducing the viral titers and viral RNA yields, while Kweon et al. (1997) stated that such delaying may be due to long preservation time of harvest at -70 °C between successive passages could lower the infectivity titer and ice crystals formed could have damage effect on the cell membrane and harmfully affect viral RNA copies, and finally Xue and Minocha (1994) and Xue et al.(1997) stated that such delaying may be due to inhibition of the viral attachment to the MDBK cell membrane due to the presence of receptor blocking substrates.

For detection of BVDV antibodies, the serum neutralization test was carried out on a total 50 serum samples from the same animals (apparently healthy). The results revealed that 31 samples (62%) were positive. These results corresponded to those reported by Baule and Banze (1994) who reported BVDV antibodies prevalence from 7-92% and those reported by David et al. (1994) where they reported that the highest BVDV morbidity was 40% and those reported by Radostits and Littlejohnes (1988) where they reported that the prevalence of BVD antibodies might be as high as 80%-90%.

Regarding the serum neutralization test applied on the collected serum samples, it was found that 12 samples (24%) have a mean titer of <2, 7 samples (14%) have a mean titer 2, 6 samples (12%) have a mean titer 4 and 6 samples (12%) have a mean titer 8. So, it seems that the recorded mean titer of BVDV antibodies was ranged from (<2 to 8) and this result is agree with that of Hopkinson et al. (1979) who detected BVDV neutralizing antibodies in the bovine sera by serum neutralization test showing a titer of 1:4. Also similar results were obtained by McClurkin et al. (1979) who applied serum neutralization test to estimate the seroconversion of cows after mating with persistent infected bulls. The neutralizing antibodies recorded titer was 1:128.

The presence BVDV antibodies in relation to the virus isolation are illustrated in table (10). The animals were classified into four groups. The first group (A): includes animals that were positive to BVD virus isolation and positive antibody. In this case the presence of virus and antibodies is an indication of acute pestivirus infection, as suggested by Brownlie, (1991).

The second group (B): includes animals that were positive to BVD virus and negative antibodies. It
is the group of persistently infected animals, which in most conditions are immune, tolerant and generally lack antiviral antibodies as described by Wolf and Buttner (1994).

The third group (C) includes animals that were negative to BVD virus and positive to its antibodies. These animals were defined as the immune competent ones, which have cleared the virus after infection. The presence of BVD antibodies without virus could be attributed to previous infection or vaccination. Assessment of rise and persistence of neutralizing antibodies to bovine diarrhea virus (BVDV) and border disease virus (BDV) after a two step vaccination using inactivated BVDV/BDV(Mucobovin) and a modified live BVDV vaccine (Vacoviron) was recorded by Oguzoglu, et al. (2001).

The fourth group (D): includes animals that were negative to BVD virus and negative to its antibodies. It is clear that these animals has not exposed to neither pestivirus infection nor vaccination. This group can be considered as basis for a clean herd free from pestivirus infection, which is the basis for control of pestivirus infection Radostitis et al. (2000).

The present study can confirm the presence of the persistent BVDV infection in the tested animals. The persistently infected animals act as reservoir of virus and shed the virus for life exposing other animals in the herd to infection. The same finding was reported by Sozan (2002) who confirmed the presence of persistent infected animals in Egypt. Moreover, RT-PCR provides a new method for rapid detection and genotyping BVDV.

References


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