Molecular, Virological and Pathological diagnosis of Bovine Viral Diarrhea (BVD) virus infection in calves

By

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SUMMARY

A dairy cattle farm at Monofia governorate, Delta, Egypt, showed high mortality rate (31.8%) among newly born calves from birth and up to 2 months of age during October and November 2008. Weakness, severe mouth ulceration and diarrhea were seen during clinical examination of ill calves with hemorrhagic enteritis during autopsy. Bovine viral diarrhea (BVD) virus antigen was detected in buffy coats of blood samples of living calves as well as lymph nodes and some internal organs of dead animals using antigen-capture enzyme-linked immunosorbent assay (ELISA) and direct fluorescent antibody technique (DFA). Real-time reverse transcription -PCR technique revealed positive results in four samples out of six tested pooled samples. Severe lymphocytic depletion in spleen and lymph nodes, destruction of intestinal villi and intestinal glands were seen during histopathological examination.

INTRODUCTION

Bovine viral diarrhea (BVD) is one of the most imperative world wide 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 Ridpatb, 2005; Ahmed and Zaher, 2008). Economic losses are directly related to multiple clinical forms of the infection that vary from subtle enteric infection to fatal mucosal disease caused by combination of cytopathic (cp) and noncytopathic (ncp) biotypes of the virus (Aykut et al., 2002). The BVD pathogen has been associated with a variety of syndromes, such as diarrhea, abortion, stillbirth, and other reproductive problems (Houe, 1999). The virus has also been shown to decrease the resistance of
infected cattle to other pathogens (Fray, et al., 2000).

BVD virus is a member of the family Flaviviridae, genus Pestivirus. The positive sense RNA genome is single stranded and ranges in length from 12.2 to 15.5 kilobases (kb). The genome contains one reading frame coding for a poly protein of about 3900 amino acid and is flanked at the 5' and 3' termini by untranslated regions (5' UTR, and 3' UTR). The 5' UTR is highly conserved and is usually used in studying the differences between and within pestivirus species and genotypes, while the 3' UTR is highly variable (Ridpath et al., 1994; Mahony et al., 2005). On the basis of the 5' UTR sequence, BVDV was initially divided into two major genotypes (types 1 and 2), and each genotype may appear as 2 biotypes (cytopathic or noncytopathic). More extensive analysis later divided them into 11 groups (Carrie et al. 2002; Niranjan et al., 2007).

The use of contaminated cells (due to the presence of nonspecific nutrient fetal calf serum contaminated with BVD virus) act as a cause for contaminated vaccines, which may lead to seroconversion or disease in vaccinated animals (Wessman and Levings, 1999). Also RNA viruses, since they exhibit higher sequence variability than DNA viruses and since it is not always possible to identify regions of the genome which are highly conserved (Papin et al., 2004), therefore the aim of our work is directed towards the diagnosis of BVD virus in field clinical samples by molecular, virological and pathological methods and a trial to compare between field strain and the vaccine strain using real-time RT-PCR technique.

**MATERIAL and METHODS**

**Animals:**
A total number of 1360 Friesian cattle (750 dairy cow, 500 growing animal and 110 suckling calves) vaccinated with BVD vaccine in a private farm at Monofia Governorate were investigated during October and November 2008. During this period, the mortalities in newly born calves were very high. The number of deaths was 35 from 110 calves born during the period of the study with a mortality rate of 31.8%.

**Sampling:**
A Total number of 68 random representative samples from both living calves showing clinical signs of illness and two dead calves during necropsy were used for this work. The sample were: 10 Nasal and 10 fecal swab samples, 10 serum samples, 10 buffy coat samples, 28 tissue specimens from different internal organs (lung, heart, liver, spleen, kidney, small intestine and mesenteric lymph
node), either frozen or preserved in 10% buffered formalin. Two blood samples were collected from the jugular vein of each examined animal, a sample was collected on EDTA as anticoagulant to obtain buffy coat samples and the other sample was collected in clean dry centrifuge tubes, left to clot, centrifuged at x1500g for 20 minutes for serum separation which kept at -20°C until analyzed.

**Direct fluorescent antibody (DFA):**

Twenty four samples (smears of buffy coats, lymph nodes and frozen tissues samples) were used in this test according to (Bezek, et al. 1988). The samples were fixed in cold acetone and stained with fluorescein-conjugated anti BVD antibody and then examined under fluorescent microscope.

**Enzyme-Linked Immunosorbent assay:**

Fifty four samples were investigated using antigen-capture ELISA according to Bottcher, et al. (1993). The BVD viral antigen was detected in samples by using anti BVD monoclonal antibody followed by detection of antigen-antibody complex with enzyme conjugated antibody.

**Real Time-Reverse Transcription-PCR (real time-RT-PCR) technique:**

The samples were pooled and classified into six samples (each one represented: pooled fecal swabs, pooled nasal swabs, pooled buffy coat samples, pooled serum samples, pooled lymph node samples and pooled samples from different internal organs). These six samples were run with one BVD vaccine sample that contain inactivated virus for the detection of viral RNA genome by real-time RT-PCR technique.

**Extraction and purification of viral nucleic acid:**

Viral RNA from prepared samples (200ul each) was automatically extracted and purified on the Magna pure LC instrument (soft ware version 2.1) with the Magna pure LC total nucleic acid isolation Kit (Roche Diagnostics Gmbtt) according to the manufacture's instruction. The purification runs were performed with the Magna pure LC protocol of total nucleic acid.

**Real-time RT-PCR assay using SYBR Green 1:**

One step RT-PCR on the light Cycler instrument was performed with LC RNA amplification Kit SYBR Green 1 according to the manufacture's instruction. Real-time PCR for RNA templates (1ul) was performed in 19ul master mix in a total volume of 20 μl in LC capillaries. The oligonucleotide primers sequences were: UTR-DL1F (5’- GCC ATG CCC TTA GTA GGA CTA GC-3’)
UTR-DL4R (5’- CAA CTC CAT GTG TGT ACA GC-3’)

The primers were designed from 5UTR BVDV genome which is identical for both genotype I and II strains (Kim and Duboni, 2003). The primers were supplied by TIB MOL BTOL, Berlin, Germany. The cycling protocol was initiated with the template RNA at 42°C for 30 minutes, then denaturation at 95°C for 2 minutes followed by 45 cycles of denaturation at 94°C for 5 seconds, annealing at 57°C for 10 seconds, and extension at 72°C for 10 seconds. After the final cycle, analysis of melting temperature (TM) was carried out in all the amplified samples including the controls by continuous recording of fluorescence at gradual increase of a temperature (0.1°C/second) over the range 55-95°C. The reaction were carried out on a capillary system of light Cycler (Roche Diagnostics) and data analyzed using the light Cycler software version 3.5.

Pathological examination:

Post-mortem examination of dead calves was done and the observed gross findings were recorded. Tissue specimens from lung, heart, liver, spleen, kidney, small intestine and mesenteric lymph node from two post-mortem examined animals were fixed in 10% buffered formalin solution, processed for paraffin sections and stained with haematoxylin & eosin for histopathological examination (Bancroft et al., 1996).

RESULTS

Four buffy coat samples, one lymph node and two samples of the internal organs showed obvious positive intracytoplasmic apple green fluorescence by direct FA technique for the detection of BVD virus antigen (figure 1 and table 1).

Also antigen-capture ELISA were positive for BVD virus antigen in the ten examined buffy coat samples, one lymph node sample and five samples from internal organs. BVD viral antigen could not detected in any of the nasal swabs, fecal swabs and serum samples (table 1).

Positive amplification by RT-PCR was observed in each pooled sample of buffy coat, serum, lymph node and internal organs. The result of the four positive samples was a specific PCR product and a fluorescent signal while negative fluorescent signals were present qualitatively in both rectal and nasal swab samples, negative control of amplification, as well as BVD virus vaccine (figure 2). After the amplification, all the samples were subjected to melting temperature analysis. Melting temperatures of amplicons ™ were presented by plotting the values of
the negative derivation of the fluo-
rescence signal against tempera-
tures. The analysis of melting
curves of the amplified positive
samples yielded one expected dis-
sociation peak of Tm 88.34°C, in-
dicating specific amplification Fig-
ure (3).

Post-mortem examination of
dead calves revealed emaciated
carcasses with obvious signs of re-
cent diarrhea. Examinations of
buccal cavity showed severe ul-
ceration on the upper and lower
surface of the tongue, gum and
hard palate. Severe congestion in
the intestine and associated mesen-
teric lymph nodes, bloody fluid in-
filtration in the peritoneum and
distended gall bladder were ob-
served during the examination of
the abdominal cavity.

Histopathological examina-
tion revealed that lymph nodes,
spleen, jejunum, ileum and lung
were the most affected tissues. The
lymph nodes and Spleen revealed
marked lymphocytic depletion
within the lymphoid follicles
(figure 4) with large number of
haemosederin loaded macro-
phages. Mesenteric lymph nodes
showed congestion and replace-
ment of lymphocytes with macro-
phages that appeared mostly ne-
crosed and occupied the medullary
sinuses (figure 5). Small intestine
(jejunum and ileum) showed en-
teritis, marked necrosis of the in-
testinal villi and intestinal glands
(figure 6). Lungs revealed suppu-
rate bronchopneumonia with pul-
onary alveoli filled with red
blood cells and haemosederin
loaded macrophages (figure 7).
Liver showed mild fibrosis around
central vein which contained
haemolysed red blood cells. Con-
gestion in the interstitial blood ves-
sels in both cortex and medulla
was seen in the kidneys. No obvi-
ous histopathological lesions were
observed in the heart.
Figure (1): Showing positive intracytoplasmic apple green fluorescence by direct FA technique for the detection of BVD virus antigen.

Table (1): Results of DFA assay and ELISA technique.

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>Number</th>
<th>Results of ELISA</th>
<th>Results of DFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swab</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Fecal swab</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Buffy coat</td>
<td>10</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Serum</td>
<td>10</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Lymph node</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Internal organs</td>
<td>12</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Total NO.</td>
<td>54</td>
<td>16</td>
<td>38</td>
</tr>
</tbody>
</table>
Figure (2): Amplification curve showing positive amplification of four of the tested samples and positive control.

Figure (3): Melting curve analysis of amplified samples and controls. The melting points of the amplified samples and controls were Tm= 88.34°C.
Figure (4): Spleen showing marked lymphocytic depletion (H&E, A: X 100, B: X 200).
Figure (5): Mesenteric lymph node showing congestion with macrophages filling most of the medullary sinuses, some of the macrophages are necrosed (H&E X400).
Figure (6): Jejunum (A) showing marked necrosis of intestinal villi and ilium (B) showing variable degrees of necrosis of intestinal glands (H&E X 200).
Figure (7): Lung showing bronchopneumonia with most of the alveoli, bronchi and bronchiols are filled with polymorph inflammatory cells (H&E, A: X100, B: X200).
I
nfection with noncytopathic BVDV in uterine life may result in a lifelong persistent infection in the fetus (PI) (Baker, 1987; De2
regt and Loewen, 1995). PI animals may be born as weak, un-thrifty calves or small and are known as “poor doers (Deregt
and Loewen, 1995), or may appear as normal healthy calves and be unrecognized clinically. Some of these animals may later develop mucosal disease with anorexia, gastrointestinal erosions and profuse diarrhea leading invariably to death. Mucosal disease can arise only in persistently infected animals (OIE Terrestrial manual, 2008). PI animals also lack neutralizing and non-neutralizing antibodies to BVDV or have low levels of these antibodies (Werdiv et
al., 1989).

In the present investigation, the number of deaths among newly born calves was 35 calves out of 110 calves born (from one day to 2 month of age) during the period of the study with a mortality rate of 31.8%. Post-mortem examination of dead calves revealed emaciated carcasses with obvious signs of recent diarrhea associated with severe ulceration on the upper and lower surface of the tongue, gum and hard palate. The clinical signs and the high mortality rate are similar to the mucosal form of BVDV infection in persistently infected animals (OIE Terrestrial manual, 2008).

The present work for BVD virus detection was directed towards the methods of direct antigen detection (DFA&ELISA) in clinical samples without using cell culture system because the standard BVDV detection protocols, which use the combination of cell culture and fluorescein-labelled immunoassay, are hampered by the problem of false-positive results, as BVDV is a widespread contaminant of fetal calf serum (FCS) and cell culture (Wessam and Levelings, 1999). In addition, cell culture assays are labor-intensive and lengthy, usually requiring several days for completion. These problems prevent many laboratories from simultaneously processing large numbers of samples (Andre, et al. 1995). Our results were positive in 7 samples out of 24 samples tested by direct FA technique and 16 samples out of 54 samples tested by ELISA. These two assays are often as sensitive as some of the other methods. Although, the presence of viral antigen in tissues is often not associated with lesions, particularly in subclinical and persistent infection, but the pathological findings beside the clinical signs observed indicate a true infection.
Due to the high specificity of the PCR assays, PCR techniques are considered as an alternative to current standard methods for detecting BVDV especially in pooled samples (Goyal and Ridpath, 2005). Previous work has shown that reverse transcription polymerase chain reaction (RT-PCR) amplification of the 5'-untranslated region of BVDV is a reliable alternative to other methods of virus detection (Carrie et al., 2002). The present work developed an alternative real-time RT-QPCR assay with the intercalating dye SYBR green. This assay is less expensive than TaqMan or beacon-based real-time quantitative PCR but is still faster than gel-based, single, or nested RT-PCR and should be economical for large-scale routine testing of clinical samples and blood products. Furthermore, through dissociation curve analysis, the SYBR green-based RT-QPCR assay allows for the identification of novel strains (Papin et al., 2004). The results of real-time RT-PCR revealed four positive samples out of six pooled samples (buffy coat, serum, lymph node and internal organs). Serum samples showed positive result to the presence of BVDV with RT-PCR; however the same samples were negative with ELISA which may be due to the presence of neutralizing antibodies in the serum samples. This result is in agreement with Mackay (2004) who reported that the advantages of RT-PCR method are that the presence of antibodies in serum samples does not affect the outcome of the test. In addition to be rapid and sensitive, RT-PCR test can detect all BVDV strains and has some advantages compared with the conventional PCR, it is an important diagnostic tool yielding reliable and reproducible results and does not require post PCR analysis (Gel electrophoresis or hybridization) as gel-based RT-PCR testing is labor intensive. It is also frequently compromised by the RT-PCR products from the opened tubes of previous amplifications, this PCR product can subsequently become the erroneous template for future RT-PCR reactions and create false-positive results. Also, the advantages of real-time PCR assay based on SYBR Green include simple detection of false or non-specific amplification and its ability to detect non-described variants (Papin et al., 2004; Richards et al., 2004 and Young et al., 2006). However, instead of using a specific fluorescence-labeled probe (TaqMan) for the detection of amplicons, we use SYBR green dye, Since SYBR green binds only to double-stranded and not to single stranded DNA molecules. PCR product concentrations can be recorded at each cycle, yielding a real-time amplification curve suit-
able for automated threshold analysis and quantification (Morrison et al., 1998).

The result of BVD vaccine sample was unexpected; it was negative for the presence of BVDV genome using Real-time Rt-PCR. This result was really confusing because it should contain BVDV genome even if it is inactivated or dead vaccine.

Since the vaccine sample was one only we couldn’t interpretate its result, but some explanation might be worth to be mention. Such negative RT-PCR may be due to the fact of the possibility of false negative results due to the mutation of the genomic RNA virus (Papin, et al. 2004) or perhaps due to improper storage and transport which also change the genomic structure. Further investigation should be done in the near future for immunological evaluation of this vaccine, its efficacy as well as the RT-PCR for the genome detection of the virus.

Gross and histopathological findings observed in our study were previously detected by many authors; however, some other lesions could not be detected. Our results were in agreement with Jones et al. (1997) who mentioned that except for general dehydration and emaciation of the carcass, the principle gross lesions are found in the gastrointestinal tract with ulcers and erosions in the mucosa of dental pad, palates, tongue, inside cheeks, muzzle and external nares. They mentioned that the mucosa of small intestine is reddened and may contain small hemorrhages and ulcers particularly over peyer's patches with necrosis of intestinal glands and lymphoid tissue. Also, Stoffregen et al. (2000) mentioned that the most severe lesions observed in the digestive tract were in the peyer's patches and were characterized by depletion of lymphocytes and proliferation of crypt cells. These observations resemble our findings of white bulb depletion of spleen and mesenteric lymph nodes. Similarly, Pratelli et al. (1999) recorded slight depletion of thymic medullary lymphocytes associated with an increase in reticular cells in small ruminants with pestivirus infection. On the other hand, Stoffregen et al. (2000) mentioned that there were no erosions or ulcerations in the upper digestive tract of BVD virus infected cattle. The necrosis observed in the lymph nodes and spleen may be contributed to immunosuppressive role of the virus (Kapil et al., 2005). Field outbreaks of BVD virus infection in cattle herds in three Egyption provinces (El-Monofya, El-Fayoom and El- Behira) showed bronchopneumonia as well as enteritis, the study reported that the
immunosuppression effect of BVD virus had predisposed the animals to secondary infection with bovine herpes virus and parainfluenza-3 virus (Aly et al., 2003).

CONCLUSION:
Nowadays, there is much interest in the control strategies for BVDV infection of cattle. Rapid and reliable diagnosis of both persistently and acutely infected cattle is imperative. Molecular diagnostic methods are being increasingly utilized as tools for the detection of numerous viral pathogens. The use of Real-Time RT-PCR methods to establish the presence or absence of BVD viral RNA in cattle specially the PI animals may have important implications for future diagnostic screening and control strategies.

REFERENCE


التشخيص الجزيئي والفيروجي والباثولوجي لعدوى مرض الإسهال الفيروسي البقرى في العجل

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معهد بحوث الصحة الحيوانية - الدقي

الملخص العربي

أظهرت إحدى مزارع الأبقار الحلاب في محافظة المنوفية نسبة عالية من النفوق (31.8%) بين العجل حديثة الولادة في الفترة خلال شهري أكتوبر ونوفمبر 2008 وقد أظهر الفحص الإكلينيكي وجود ضعف عام وفرق بالفم وإسهال مع وجود التهابات معوية دموية أثناء إجراء الصفة التشريحية. تم اكتشاف أنثيئين فيروس الإسهال الفيروسي البقرى في طبقة الخلايا البيضاء المنضدة كم الدم للعجلة الحية ومن الغدد الليمفاوية والأعضاء الداخلي للحيوانات النافقة باستخدام اختبار الإلزارد الاختبار الفيروسي المستمر المباشر كما أظهر اختبار إنزيم البلمرة المتسلسل ذو الوقت الحقيقي وجود الفيروس في عدد 4 عينات من إجمالي 6 عينات مجمعة وقد شهد نقص شديد في الخلايا الليمفاوية بحيوصلات الطحال و الغدد الليمفاوية وتركز في خ말ات وغدد الأمعاء أثناء الفحص.

المحكمون:

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