CONTAMINATION OF FOETAL BOVINE SERUM WITH BOVINE VIRAL DIARRHOEA VIRUS (BVDV)

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Abstract

Twelve batches of commercially available foetal bovine sera were tested for the presence of bovine viral diarrhoea virus (BVDV) and BVDV antibodies. The antibodies were found in three batches coming from two suppliers. Fragments of BVDV genome were present in five of these batches when tested by RT-PCR. All of them were type 1 BVDV according to direct sequencing of PCR products. However, the further analysis of the samples by virus isolation showed that all RT-PCR positive batches contained non-infectious BVDV. Hence, inactivation methods used during the production process proved to be efficient making the tested sera safe for the use in cell culture and for vaccine production. Results of RT-PCR detection of viral genome must be treated with caution since not in every case positive results indicate that the virus is live and infectious. Genetic typing based on sequence analysis of PCR products confirmed wide geographic spread of donor cattle.

Key words: foetal calf serum, bovine viral diarrhoea virus, contamination, 5'UTR typing.

Foetal bovine serum (FBS) is widely used in diagnostic laboratories in cell culture and in vaccine production as cell growth promoting factor. Its quality is crucial for proper diagnosis and the safety of biologicals used in control programmes (1, 4, 5, 9). Therefore, any viral contaminants or antibodies present in FBS may hamper the proper diagnosis and the efficient use of vaccines. Vaccine contamination may not only influence the results of vaccination but may also lead to new infections, causing serious economic problems in a herd. Such a detrimental effect after the use of bovine viral diarrhoea virus (BVDV)-contaminated live vaccine was observed in 1999 first in the Netherlands and then in Italy (4). About 500 animals either died or have been slaughtered on several Dutch farms and 20 animals went through the acute infection in one Italian herd where only 10 animals were vaccinated. In diagnostic laboratories testing for BVDV, the presence of neutralising antibodies in FBS used for cell culture might influence the virus isolation test. Such antibodies might neutralise the virus leading to a false negative result. Therefore, it was suggested that RT-PCR should be a test of choice when the presence of antibodies is suspected (e.g. in colostrum fed calves) to avoid such confusing results. However, not all RT-PCR positive results mean that the infectious virus was found since that method detects only small parts of viral genome, depending on primer set used in the reaction. In such circumstances, virus isolation remains the test of choice, since it allows the detection of infectious virus multiplying in susceptible cell culture.

The aim of the study was to evaluate foetal bovine serum from nine suppliers for the presence of BVDV and BVDV antibodies. Additionally, all RT-PCR positive batches were sequenced to genotype the viruses found.

Material and Methods

Tested batches of sera. Twelve batches of commercially available foetal bovine serum were used in the study. Among them, two batches of the same sera from three suppliers were tested. Altogether, the sera from nine suppliers were analysed.

Detection of antibodies. Serological testing was done with three commercial ELISA kits detecting antibodies against E2 glycoprotein of BVDV. Antigen detection, genome analysis, virological testing. Antigen detection was done with a commercial ELISA kit according to manufacturer’s instructions. All sera samples were additionally tested with RT-PCR using RNA extracted by standard phenol/chloroform extraction method. Primers encompassed the conserved 5' untranslated region (5'UTR) of viral genome and enabled the amplification of all pestiviruses (288 base pair amplicon was expected) (12). RT-PCR was performed in one-tube with a
commercial kit (Roche) according to manufacturer’s instructions. Briefly, reverse transcription was done at 50°C for 30 min followed by 30 cycles of PCR with the following profile: 94°C for 15 s, 54°C for 30 s, and 68°C for 30 s, terminated with the final elongation at 68°C for 10 min. Type 2 positive control was CS8644 strain of BVDV (kind gift of Dr Horst Schirrmieier from the Friedrich-Loeffler-Institut, Germany). Another method employed to distinguish two types of BVDV was RFLP with PstI enzyme (7). BVDV type 1 can be characterised by the presence of the restriction site for PstI, while type 2 BVDV is lacking this restriction site. The 5’UTR amplicons were sequenced with the same set of primers used for RT-PCR (12) in both directions using Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3730XL Genetic Analyzer (Applied Biosystems). The sequences were analysed and consensus sequences were produced after sequence comparison using the ClustalW software. Phylogenetic analysis was performed using the MEGA software based on the Neighbor-joining method. Bootstrap analysis was carried on 1,000 replicates and phylogenetic trees were drawn in MEGA software. Reference sequences for the known type 1 and type 2 viruses were taken from the GeneBank. While all these methods encompassed 5’UTR fragment of viral genome, additional RT-PCR followed by nested PCR within the coding sequence of non-structural protein NS5B was used to distinguish the both types (6). The types 1 and 2 could be distinguished in this method by different amplicon sizes of 369 base bp and 615 bp, respectively.

All RT-PCR positive samples were subjected to virus isolation in MDBK cell line. Incubations were done at 37°C/5% CO₂. Two subpassages for 5 d each were done in 24-well plates followed by the third subpassage in 96-well plate for 2 d with subsequent cell fixation and virus detection using immunoperoxidase test with a commercial monoclonal antibody pool WB103/105 at 1:100 dilution (VLA Weybridge, UK).

**Results**

The results of all tests carried out on FBS samples are shown in Table 1. BVDV antigen was found in three samples from two suppliers using antigen ELISA while in RT-PCR, BVDV genome fragment from 5’UTR region was found not only in the same samples as in antigen ELISA but additionally also in two other samples (Fig. 1).

<table>
<thead>
<tr>
<th>Serum I.D.</th>
<th>Supplier/ batch</th>
<th>ELISA Ag</th>
<th>RT-PCR 5’UTR</th>
<th>RFLP 5’UTR type 2</th>
<th>Nested PCR NS5B type 1/2</th>
<th>Virus isolation</th>
<th>ELISA Ab</th>
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<tbody>
<tr>
<td>FBS 1</td>
<td>A</td>
<td>-</td>
<td>-</td>
<td>n.t.</td>
<td>n.t.</td>
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<tr>
<td>FBS 2</td>
<td>B/1</td>
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<td>+</td>
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<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FBS 3</td>
<td>B/2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
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<td>-</td>
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<tr>
<td>FBS 4</td>
<td>C</td>
<td>-</td>
<td>+</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>FBS 5</td>
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<td>n.t.</td>
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<td>-</td>
</tr>
<tr>
<td>FBS 6</td>
<td>E</td>
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</tr>
<tr>
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<td>n.t.</td>
<td>n.t.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FBS 8</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>doubtful</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>n.t.</td>
<td>n.t.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FBS 10</td>
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<td>-</td>
<td>-</td>
<td>n.t.</td>
<td>n.t.</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FBS 11</td>
<td>H</td>
<td>-</td>
<td>-</td>
<td>n.t.</td>
<td>n.t.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FBS 12</td>
<td>I</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

n.t. – not tested

**Fig. 1.** Results of RT-PCR on 12 serum samples with 5’UTR primers.

C+ - positive control; MW - molecular weight marker; 1-12 - samples of foetal calf serum; NY-1 - New-York 1 - reference strain of BVDV; C- - negative control

### Table 1

Cumulative results of testing twelve bovine serum batch samples for BVDV and BVDV antibodies
Fig. 2a. Results of RFLP analysis with Pst I enzyme of five serum samples positive in RT-PCR.

MW - molecular weight marker;
2-12 - serum samples positive in RT-PCR;
C+1 - positive control, Singer strain (type 1);
C+2 - positive control, CS8644 strain (type 2).

Fig. 2b. Results of nested PCR with type 1 and type 2 specific primers for NS5B region of five serum samples positive in RT-PCR.

MW - molecular weight marker;
2-12 - serum samples positive in RT-PCR;
C- - negative control;
C+1 - positive control, NY-1 strain (type 1);
C+2 - positive control, CS8644 strain (type 2).

Since RT-PCR, RFLP, and sequencing enable BVDV typing, all the methods were applied to distinguish BVDV type 1 and type 2 among RT-PCR positive serum samples. Using the same amplicons from the 5’UTR fragment of viral genome as those after RT-PCR, RFLP with PstI confirmed type 2 BVDV only in one sample, while additional amplifications within NS5B coding sequence identified four positive samples (sample No. 3 was weak positive) and all of them of type 1 (figs. 2a and 2b). Direct sequencing of both strands of 5’UTR amplicons using the same set of primers as the ones used for the RT-PCR confirmed that all of those positive samples were contaminated with type 1 BVDV, but three subtypes could be identified (fig. 3). One FBS isolate was of type 1a, another one was of type 1c while remaining three isolates were grouped as type 1b BVDV.

Virus isolation test was negative for all samples tested. Antibodies against BVDV were found in three samples (two of them comprised two batches from the same supplier). One sample gave doubtful result for antibodies against BVDV. All RT-PCR positive samples were antibody negative.

Discussion

Ubiquitous presence of BVDV in cattle may have a detrimental effect on diagnostic work and the vaccine production due to the contamination of foetal bovine serum not only with the virus but also with neutralising antibodies. Live virus contamination of foetal bovine serum may have adverse effects of utmost importance in vaccines produced, since animals may not only develop no immunity expected but moreover they may succumb to unexpected infections due to immunosuppressive effect of BVDV. For diagnostic laboratories, viral contamination and the presence of virus neutralising antibodies may affect proper diagnosis.
Fig. 3. Phylogenetic tree of five positive serum samples within 5’UTR region and comparison with other isolates available in GeneBank. Labels include accession numbers and country of origin. Other names refer to FBS serum samples and reference strains (NADL, Singer, Oregon, Osloss and NY-1 for type 1 and 890 with CS8644 for type 2).

Studies on FBS contamination with BVDV were undertaken several times. Bolin et al. (2, 3) evaluated the prevalence of BVDV and antibodies against it in FBS on two occasions in 1991 and in 1998. More than one thousand batches were tested in each study with very similar results. The virus was isolated from more than 20% of all batches while virus neutralising antibodies were found in 13% of all lots of raw foetal calf serum tested. Such a high rate of serum contamination confirmed difficulties when applying control measures for BVDV infection on the herd level and the high rate of foetal infections.

In our study, almost 42% of tested sera were contaminated with the virus while 25% of 12 serum batches tested had antibodies against BVDV. Surprisingly, 38% of all viral isolates in Bolin’s study were identified as type 2 BVDV while in our study all isolates were of type 1. We found BVDV antibodies in three batches from two suppliers. Unexpectedly, the same three batches were contaminated with antibodies against bovine herpesvirus 1 (BHV1) and bovine respiratory syncytial virus (BRSV) (data not shown). This restricted presence of antibodies to three common bovine viruses in sera from two suppliers may indicate that donors were vaccinated with polyvalent vaccine against these three respiratory viruses. The results of antigen and genome detection using ELISA and RT-PCR techniques confirmed superior sensitivity of the latter. While antigen ELISA identified three positive samples, RT-PCR gave positive results in two more batches of sera. Despite positive RT-PCR results in five samples, virus isolation was negative for all these samples, proving that the virus present in sera was inactivated and non-infectious. Similar studies on FBS contamination with BVDV were undertaken by Makoschey et al. (9) on seven batches of serum. However, in that study the
samples were not subjected to an inactivation process. Infectious BVDV was detected in four out of seven batches of FBS. The authors detected also the presence of non-infectious virus of relatively high titre. Safety of such a virus was evaluated by producing an experimental vaccine and injecting it into BVDV seronegative cattle. Four weeks after the second vaccination, no seroconversion was detected. Therefore, FBS containing non-infectious BVDV did not induce any BVDV specific antibodies, which could hamper proper diagnosis under field conditions when eradication steps are undertaken. Additionally, the authors indicated how crucial it is to distinguish between infectious and non-infectious virus present in serum when RT-PCR results are not confirmed by virus isolation in cell culture.

In the present study, only two regions were targeted, namely 5’UTR and NS5B, giving positive results in RT-PCR for five and four samples, respectively. These results also confirmed that partial viral genome was present in serum at least from sample No. 4 (Fig. 2b) probably due to inactivation process used during serum preparation. The negative impact of the inactivation on genome integrity probably influenced RFLP result for FBS12. The sequence of this sample showed the lack of restriction site for PstI, feature typical for type 2 BVDV. However, the following analysis based on direct sequencing of PCR product, although confirmed the absence of this restriction sequence, clearly identified this isolate as type 1 BVDV. Therefore, it can be concluded that direct sequence analysis, although more laborious than RFLP, is more reliable when type of BVDV is determined.

Genetic typing of positive samples based on sequencing of 5’UTR region of viral genome confirmed high genetic diversity of BVDV. Although all positive samples belonged to type 1 BVDV, three subtypes 1a, 1b, and 1c could be distinguished. Type 1b was the most dominant one with three isolates allocated to this group. However, while BVDV isolates from FBS3 and FBS8 were close together in one cluster of 1b isolates (NY-1 strain group), FBS12 isolate was located on the other end of phylogenetic tree of type 1b BVDV (Osloss group). In 2001, Vilcek et al. (12) allocated type 1 BVDV isolates of European origin into at least eleven genetic groups based on sequence analysis of 5’UTR and Npro (autoprotease) regions of viral genome. In our study three genetic subtypes were identified but only 12 serum samples were included in the analysis, while in Vilcek’s study, 78 isolates from seven countries were used.

Geographic clustering of serum samples showed that type 1a BVDV from FBS2 displayed the highest homology with Japanese isolate, while FBS4 isolate, allocated to type 1c subgroup, exhibited the highest sequence identity with Australian isolate (13). Type 1b BVDV from FBS3 and FBS8 showed the highest homology with Spanish and Japanese isolates, while another type 1b isolate from FBS12 had the highest sequence identity with UK and Argentine isolates (8, 10, 12, 13). This serum came from UK, which may justify this type of homology. However, since the collection of foetal bovine serum for the production of cell culture supplement is based on pooling of sera of different origins, one can expect the situation similar to BVDV isolated from FBS3, which showed the highest homology not only with Spanish but also with Japanese isolate.

While the presence of BVDV antibodies may influence the results of virus isolation in the laboratories, the lack of infectious virus in all five batches of sera tested proved that inactivation methods used by all suppliers were sufficient to inactivate the virus, making them safe for vaccine production.

References