Detection of bovine viral diarrhea virus by amplification on polycation-treated cells followed by enzyme immunoassay

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ABSTRACT

A bovine viral diarrhea virus (BVDV) amplification method combined with an enzyme immunoassay was developed to detect BVDV antigens in seropositive cattle. Reconstitution assays conducted by adding decreasing amounts of BVDV (Singer strain) to Madin-Darby bovine kidney (MDBK) cells showed that the sensitivity threshold of the combined assay was $10^{-7}$ TCID$_{50}$. BVDV amplification was carried out in polycation (DEAE-Dextran and polybrene)-treated MDBK cells. Treated cells were able to replicate both ether-treated virus and neutralizing antibody-coated virus. Ammonium chloride decreased virus replication in polycation-treated cells, suggesting viral penetration by endocytosis. BVDV detection was tested in leukocytes from 104 seropositive cattle from 2 unvaccinated commercial closed dairy herds with high seroprevalence. Lysates and co-cultures of peripheral blood leukocytes (PBL) were tested, directly or after up to 6 blind passages in normal or polycation-treated cells. BVDV was detected in 10/104 cattle after only one co-culture of PBL in treated cells. No virus was detected in whole blood or plasma samples. BVDV positive and negative cattle were retested three times, achieving consistent results. The finding of immune carriers supports the possibility that these animals may constitute an epidemiological risk.

Key words: bovine viral diarrhea virus (BVDV), epidemiology, detection, amplification, polycations, cELISA

RESUMEN

Detección del virus de la diarrea viral bovina por amplificación sobre células tratadas con policationes seguido de enzimoinmunoensayo. Se desarrolló un método de detección de antígenos del virus de la diarrea viral bovina (BVDV) combinando amplificación viral con enzimoinmunoensayo. El método combinado presentó una sensibilidad de $10^{-7}$ TCID$_{50}$ en ensayos con diluciones decrecientes de BVDV cepa Singer sobre la línea celular MDBK. La amplificación del título viral se efectuó sobre células MDBK tratadas con policationes Estas células replicaron tanto el BVDV tratado con éter como el unido a anticuerpos. La replicación viral en las células tratadas disminuyó ante la presencia de cloruro de amonio, lo que sugiere la penetración viral por endocitosis. El BVDV se determinó en leucocitos de 104 bovinos seropositivos de dos roedores en producción, cerrados y con alta seroprevalencia. Los leucocitos de sangre periférica (LSP) fueron lisados y analizados directamente o luego de hasta 6 pasajes ciegos sobre células normales o tratadas con policationes. El BVDV se detectó en 10 de los 104 animales después de solamente un cultivo de LSP en células tratadas. No se pudo detectar presencia viral en las muestras de sangre o plasma. Los estudios se repitieron tres veces en animales BVDV positivos y negativos, con resultados consistentes. El hallazgo de bovinos seropositivos portadores del virus indica la posibilidad de que estos animales puedan significar un riesgo epidemiológico.

Palabras clave: virus de la diarrea viral bovina (BVDV), epidemiología, detección, amplificación, policationes, cELISA

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a Pestivirus of the Flaviviridae family, is one of the most insidious cattle pathogens throughout the world. In immunocompetent cattle, BVDV infection induces a mild disease, and a fast immune response against the virus leads to healing and seroconversion in about 2 weeks, and the virus is eliminated. It is considered that healed seropositive cattle do not harbor the virus and are immune to further infection with the same strain of BVDV. Thus, they are not considered an epidemiological risk (15). In contrast, when a fetus is infected in utero between the 30th and 120th day of gestation, immunotolerance is installed. In this case the fetus becomes persistently infected (PI) with BVDV. PI cattle play a key role in the epidemiology of this complex disease because they are an important viral source (16). Therefore, detection of cattle harboring virus to identify viral carriers is an important tool in BVDV control measures (2).

In the Argentine Pampas, infected and healed seropositive animals are extremely common in most herds (17, 18). Even in closed herds, where infection from foreign cattle is practically non-existent, most calves are in-
fected when young, many of them before they are six months old (Gogorza L.M. et al., unpublished results). This indicates an internal source of infection. Using several techniques, free virus has not been detected in plasma of seropositive animals (2), nor has BVDV been detected in their peripheral blood leukocytes (PBL) using polymerase chain reaction (PCR) (3).

The current techniques for BVDV detection are viral isolation, antigen capture ELISA (cELISA), immunofluorescence (IFI) or immunoenzymatic staining tests (IPA), and RT-PCR (7, 11). Although cELISA per se has lower sensitivity than RT-PCR, it has several advantages. It is more suitable for large scale testing, automatization eliminates the confusion between weakly positive and negative tests in IFI or IPA, and the use of monoclonal antibodies (MAbs) gives it a great specificity, which virtually eliminates background reactions. Moreover, two limiting factors rule the successful performance of RT-PCR: the reliability of nucleic acid purification methods and the oligonucleotide-specific priming of the reverse transcriptase reaction. As in RNA hybridization, RT-PCR performance can be affected by BVDV genome variability (13).

The interference of neutralizing Abs and the barrier of cell receptor/viral anti-receptor specificity can be a main problem for wild type BVDV detection and isolation. Ferrer J.F. & Diglio CA (1976) showed a great increase in susceptibility of target cells to bovine leukemia virus after treating cells with polycations, as diethylaminoethyl (DEAE) dextran and polybrene (Hexadimethrine bromide). Polycations are often used in transfection methods and to enhance DNA and plasmid entry into cells (1,12,14). In contrast, some weak basic compounds, such as ammonium chloride, can inhibit the in vitro viral infection by decreasing the lysosomal pH with negative influence on the viral replication (9).

In order to study the possibility that seropositive cattle may somehow be harboring the virus and so be the source of infection in closed herds, a very sensitive method for the detection of BVDV was developed. The method combines BVDV amplification by co-cultures of PBL in polycation-treated cells, with an antigen capture enzyme immunonassay cELISA-FCV (Facultad de Ciencias Veterinarias).

MATERIALS AND METHODS

Animals and clinical samples
A total of 104 seropositive animals were selected from two unvaccinated commercial dairy herds with high seroprevalence against BVDV. Blood samples were collected by venipuncture from the jugular vein, and the sera were kept at –20 °C until use. Serum BVDV Abs were measured by the virus neutralization (VN) test (4) using Singer strain BVDV. Only healthy seropositive cattle were included in this study.

In order to collect PBL blood was collected at the same time on 10% sodium citrate and centrifuged at 800 x g at 18 °C for 15 min. Theuffy coat was collected, diluted with Ca ++ /Mg ++ free phosphate buffered saline (PBS) pH 7.4, treated with ammonium chloride for haemolysis, resuspended in PBS, and the resulting PBL were collected by centrifugation at 800 x g at 20 °C for 20 min. Whole blood and plasma were also used as clinical samples.

Viruses and cell cultures
The Madin Darby bovine kidney (MDBK) cell line (Virology Lab, INTA, Balcarce, Argentina) was used (Carbrey E.A., 1971). Cell monolayers were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS). FCS, medium and cells were tested to be BVDV-free at first using a commercial capture ELISA assay (Merieux Labs, France) and then, on each new batch of FCS and periodically in all media, by the cELISA-FCV assay, that proved to be more sensitive than RT-PCR (see Results). BVDV Singer strain (INTA Castelar, Argentina) was cloned three times by limiting dilution and a virus stock was prepared on MDBK cells. The 50% tissue culture infectious dose (TCID50) was assessed by the Reed & Muench test (19): a BVDV suspension was seeded on MDBK cells in 48-well plates (COSTAR, Cambridge Ma, Cat # 3548) in serial dilutions of log10 to 10. The cell monolayers were incubated for 48 h at 37 °C, and checked for the appearance of cytopathic effect. This short 48 h incubation time was chosen for all titrations to avoid loss of viral particles after its first round of in vitro replication (Gogorza L. et al, unpublished results). A stock solution of BVDV was titrated, diluted to 10 TCID50 x, fractionated and kept at –196 °C until use. Working dilutions of this 10 TCID50 were freshly prepared as needed.

Anti BVDV antisera
An ovine anti-BVDV serum (Ov-073) was obtained in an ove by 3 intraperitoneal doses each of 6 ml of BVDV stock, every 15 days, followed by a booster inoculation 30 days later. A rabbit anti-BVDV serum (CON-4 serum) was obtained in rabbits by 6 intradermical doses of 0.5 ml each of ether-treated BVDV in incomplete Freund adjuvant, every 7 days. An anti-BVDV MAb, MAB # 20.10.6 anti-p80 (5), was kindly supplied by E. Dubovi, New York State College of Veterinary Medicine, Cornell University, Cornell, NY.

For some experiments, stock BVDV virus was treated with sulfuric ether, 10 min at room temperature. Naked virus was centrifuged at 20000 x g, fractionated and vacuum dried, and resuspended in MEM as needed.

For other experiments, stock virus was incubated with high titer seroneutralizing bovine sera, 15 min at 37 °C, before seeding virus and serum on MDBK cells.

Target cell lysis
Hypotonic buffer (0.02 M Tris - 0.02 M NaCl - 0.001 M EDTA) and lytic buffer (0.02 M NaTris - 0.001 M EDTA - Np40 1%), 100 µl each, were added to infected MDBK cells or PBL, incubated for 10 min at room temperature, vortexed for 1 min (15000 x g) and frozen and thawed twice. Cells were centrifuged and the supernatant was stored at -20 °C until use. A similar lystate of non-infected MDBK cells was used as negative control.

BVDV antigen capture ELISA (cELISA)
A sensitive cELISA for the detection of BVDV antigen was developed and standardized: 96 well plates (COSTAR ELISA Cambridge Ma, Cat # 3590) were coated with different dilutions (1:500 to 1:15000) of Ov-073 anti BVDV serum (CON-4 serum) was obtained in rabbits by 6 days, followed by a booster inoculation 30 days later. A rabbit anti-BVDV serum (CON-4 serum) was obtained in rabbits by 6 intradermical doses of 0.5 ml each of ether-treated BVDV in incomplete Freund adjuvant, every 7 days. An anti-BVDV MAb, MAB # 20.10.6 anti-p80 (5), was kindly supplied by E. Dubovi, New York State College of Veterinary Medicine, Cornell University, Cornell, NY.

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BVDV detection by amplification and cELISA

second Ab, and the plates were incubated at 37 °C for 30 min. The plates were washed, and 100 µl of substrate solution (3.3’5’-tetra-methyl-benzidine (TMB) (Moss Labs. Inc) were added. Color development was stopped after 30 min by adding 30 µl of stop solution (4 M sulfuric acid) and the absorbance was measured on an ELISA reader at 450 nm. All tests were done in triplicate wells.

Cut-off value was defined by the range difference between optical records of positive and negative samples. No difference (> one dilution) in sensitivity was found between MAb # 20.10.6 and rabbit CON-4 anti-BVDV serum.

Virus amplification

In order to increase the cELISA sensitivity, very high BVDV dilutions were preamplified on cell cultures. Stock virus 10^7 TCID₅₀ suspensions were diluted from 10⁻² to 10⁻30 and inoculated into MDBK cells in 48-well plates. After incubation, medium was removed, cells were lysed as indicated above, and the lysates were analyzed by cELISA.

The serial dilutions were screened first directly by cELISA and then, after amplification, by culturing once in MBDK cells, in 48-well plates (Table 1).

<table>
<thead>
<tr>
<th>Stock Virus dilution (TCID 50)</th>
<th>cELISA (1)</th>
<th>cELISA-FCV (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-BVDV (serum CON-4)</td>
<td>Monoclonal Ab anti-p80 (MAb 20.10.6)</td>
<td></td>
</tr>
<tr>
<td>10⁻³</td>
<td>1.280 ± 0.04</td>
<td>1.350 ± 0.04</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>1.210 ± 0.04</td>
<td>1.320 ± 0.04</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>1.190 ± 0.04</td>
<td>0.820 ± 0.06</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>0.650± 0.02</td>
<td>0.614 ± 0.04</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>0.350 ± 0.02</td>
<td>0.300 ± 0.08</td>
</tr>
<tr>
<td>10⁻⁸</td>
<td>0.110 ± 0.02</td>
<td>0.095 ± 0.02</td>
</tr>
<tr>
<td>10⁻⁹</td>
<td>0.110 ± 0.03</td>
<td>0.070 ± 0.02</td>
</tr>
</tbody>
</table>

B. After 48 h amplification on MBDK cells

<table>
<thead>
<tr>
<th>Stock virus dilution (TCID 50)</th>
<th>cELISA-FCV (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-BVDV (serum CON-4)</td>
<td>Monoclonal Ab anti-p80 (MAb 20.10.6)</td>
</tr>
<tr>
<td>10⁻³</td>
<td>1.284 ± 0.27</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>1.220 ± 0.22</td>
</tr>
<tr>
<td>10⁻⁵</td>
<td>1.080 ± 0.20</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>1.076 ± 0.20</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>0.447 ± 0.30</td>
</tr>
<tr>
<td>10⁻⁻²</td>
<td>0.430 ± 0.25</td>
</tr>
<tr>
<td>10⁻⁻³</td>
<td>0.110 ± 0.02</td>
</tr>
</tbody>
</table>

The cloned stock BVDV Singer strain virus (see M&M) was log 10 serially diluted from 10⁻² to 10⁻30. A: stock virus dilutions were directly analyzed by cELISA. B: dilutions were inoculated in MBDK cells and then analyzed by cELISA.

(1) Optical Density (OD), mean value and standard deviation (SD) of triplicate results.

Polycation treatment of MBDK cells

In order to sensitize and adapt the amplification method to neutralizing Ab containing samples from seropositive animals, a modified Ferrer & Diglio (1976) technique was used. MBDK cells were seeded on 24 (COSTAR, Cambridge Ma., Cat # 3424) or 48 well plates. After 80% confluence, cells were incubated with 200 µl of DEAE-dextran chloride form (SIGMA cat. D-9885 Lot 27H0936) at different concentrations (50, 75 and 100 µg/ml) in MEM, for 30 min at 37 °C. The medium was removed and cells were washed with 0.15 M NaCl. BVDV dilutions in MEM with 12 µg/ml of Polybrene (SIGMA H-9268, lot 106H3691) were added and incubated for 48 h at 37 °C. Cell treatment with DEAE-dextran or polybrene alone was also evaluated.

Ammonium chloride effect on BVDV infection

DEAE-Dextran treated and untreated MBDK cells were incubated with 5 mM ammonium chloride in MEM, for 15 min at 37 °C. The supernatant was discarded and the monolayers were inoculated with whole BVDV, ether-treated BVDV or Ab coated-BVDV, in 5 mM ammonium chloride and polybrene in MEM. After 48 h incubation, the supernatant was discarded and the cells were lysed and analyzed by cELISA.

Virus amplification combined with cELISA (cELISA-FCV)

PBL from seropositive animals were aliquoted. One aliquot was lysed and tested with cELISA, the others were co-cultivated with normal or DEAE-dextrane / polybrene-treated MBDK cells on 24 well microplates, and cells were lysed and tested by the cELISA technique. All supernatants were blind passed 6 times on normal or polycation-treated MBDK cells and all cells were lysed and tested by cELISA. Animals giving a positive detection of BVDV with this technique are now referred to as virus positive animals; animals giving negative detection are referred to as virus negative animals. In the following experiments PBL were co-cultured only once with polycation- treated cells.

Test controls - cELISA FCV reproducibility control and cut-off value

In order to test the cELISA-FCV reproducibility, and to reject the possibility that positive results could have been due to contamination, all virus positive cattle were retested 3 times each (a total of 29 samples) together with 55 samples from virus negative cattle. Results were evaluated by the “kappa index” statistical test, analyzing record concordance.

Cut-off value was established using 88 samples from the same virus positive cattle and 80 samples from virus negative samples in 62 cELISA-FCV tests. Samples with 4 standard deviation (SD) values above the top negative values were considered positive.

Virus isolation

BVDV wild type isolations from the positive cELISA-FCV animals were obtained by co-culture of their PBL on polycation-treated MBDK cells. After one blind passage on treated cells, the supernatant was stored at −196 °C, and cells were lysed and kept at −196 °C.

RT-PCR and c-ELISA FCV specificity control

RNA was extracted from 100 µl of peripheral blood cells or MBDK lysates with 900 µl of a specific commercial reagent (Trizol, Life Technologies Inc, Grand Island NY14072, USA) following the suppliers protocol. RNA pellets were resuspended in 10 µl of H₂O and immediately used for reverse transcription. Reverse transcription was carried out with 1 µl of Moloney murine leukemia virus reverse transcriptase and random hexanucleotide primers with RNase inhibitors (Promega Corp, WI 53711-5399, USA) at 37 °C for 1 hour and at 94 °C for 10 min.

PCR of the 5'-UTR was carried out using the primer set 324 and 326 (20) and Pfu DNA polymerase (BioRad Labs, Richmond CA 94804) to ensure maximum fidelity of nucleotide incorporation. The reaction mix was subjected to 35 cycles of 94 °C 1 min, 55
C 1 min and 72 °C 1 min. Reverse transcription and PCR cycling conditions were as described in Jones et al. (2001).

PBL from the same virus positive and virus negative cattle used in this study were directly tested with this RT-PCR. Also, in order to test the specificity of the cELISA-FCV, BVDV wild type isolations were analyzed by RT-PCR.

RESULTS

Standardization of the combined amplification/cELISA test (cELISA-FCV)

Optimization of the BVDV antigen capture cELISA was carried out as described in M & M. Briefly, the optimal cELISA protocol, used in all further experiments, was as follows: plates were coated with 100 µl / well of anti-BVDV ovine # 073 serum 1:10000 in carbonate buffer (pH 9.0), and incubated overnight at 4 °C. Several tests showed that dilutions 1:2 for virus samples or cell lysates, 1:800 for the MAb, 1:2000 for anti-BVDV Con-4 and 1:10000 for the conjugate were optimal. Optimal time for substrate development was 30 min. Optimal concentration of polycations was 75 µl /ml of DEAE-Dextran and 12 µl /ml of Polybrene.

cELISA-FCV sensitivity

This standardized c-ELISA FCV was able to detect BVDV carrying samples: cell lysates of BVDV infected MDBK cells gave positive results, while normal cell lysates were negative. A standard sensitivity curve of c-ELISA FCV was assessed by infecting MDBK cells with serial dilutions of the 10−7 TCID 50 BVDV Singer strain and testing lysates after 48 hours of incubation. Table 1 shows that cELISA could detect a 10−7 dilution of the stock virus while the combined cELISA-FCV assay was able to detect a 10−11 dilution of the stock virus.

Table 2 shows that DEAE and polybrene treated MDBK cells were able to replicate ether- treated BVDV. But neither of these polycations per se were able to allow naked virus replication.

Table 2 also shows that treated MDBK cells were able to replicate BVDV even when it was coated with specific bovine Abs. BVDV replication in treated or untreated cells was inhibited by the lysosomal agent ammonium chloride (Table 3).

Use of cELISA-FCV for screening clinical samples

Whole blood, plasma and PBL from 104 healthy seropositive cattle were aliquoted and evaluated using cELISA and cELISA-FCV and 10/104 gave positive results. Table 4 shows that by direct testing of PBL lysates, BVDV antigen was detected in two animals. Two samples were positive after one co-culture on normal MDBK cells, and another sample turned positive after two blind passages on normal cells. Another 4 samples were positive after the 3rd blind passage on normal cells. No further positive animals were detected after the 3rd passage. When co-cultures and blind passages were carried out on polycation-treated cells, these 9 animals gave positive results after the first co-culture. Another animal that gave negative results after the 2nd blind passage on treated MDBK cells, showed positive results after one more passage. On later experiments this animal gave positive results after only one co-culture on polycation-treated cells. All other 94 seropositive animals remained negative even after the 6th blind passage. Neither whole blood nor plasma ever gave positive results.

<table>
<thead>
<tr>
<th>MDBK cells</th>
<th>BVDV</th>
<th>cELISA FCV(1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>Untreated</td>
<td>1.284 ± 0.27</td>
</tr>
<tr>
<td>Untreated</td>
<td>None</td>
<td>0.170 ± 0.09</td>
</tr>
<tr>
<td>Treated w/DEAE and polybrene</td>
<td>Untreated</td>
<td>1.620 ± 0.42</td>
</tr>
<tr>
<td>Treated w/DEAE and polybrene</td>
<td>None</td>
<td>0.062 ± 0.02</td>
</tr>
<tr>
<td>Untreated</td>
<td>Ether treated</td>
<td>0.018 ± 0.03</td>
</tr>
<tr>
<td>Treated w/only DEAE</td>
<td>Ether treated</td>
<td>0.347 ± 0.30</td>
</tr>
<tr>
<td>Treated w/only polybrene</td>
<td>Ether treated</td>
<td>0.030 ± 0.02</td>
</tr>
<tr>
<td>Treated w/DEAE and polybrene</td>
<td>Ether treated</td>
<td>1.297 ± 0.25</td>
</tr>
<tr>
<td>Untreated</td>
<td>Ab coated</td>
<td>0.060 ± 0.02</td>
</tr>
<tr>
<td>Treated w/DEAE and polybrene</td>
<td>Ab coated</td>
<td>1.095 ± 0.19</td>
</tr>
</tbody>
</table>

MDBK cell monolayers were treated or not with DEAE-dextran and/or polybrene, and inoculated with ether denuded or Ab-coated BVDV. As positive and negative controls, whole BVDV was inoculated or not on treated and untreated cells. Cells were lysed and analyzed by cELISA (see M & M). The experiments were repeated twice giving similar results.

(1) Optical Density (OD), mean value and standard deviation (SD) of triplicate results.
Reproducibility was assessed by repeatedly testing the virus negative animals together with the 10 virus positive animals: all positive cattle repeatedly gave positive results after one co-culture while negative cattle consistently gave negative results (Figure 1). The Kappa index was 0.675. Cut off value was established at 4 SD above the highest negative record.

In order to compare RT-PCR and cELISA-FCV sensitivity, PBL samples from these virus positive and virus negative cattle were simultaneously tested on RT-PCR and cELISA-FCV. All samples were negative in the former test while the above 10 virus positive cattle were positive in the latter, thus showing that cELISA-FCV has higher sensitivity than RT-PCR.

Wild type virus isolation and RT-PCR

As stated above, using RT-PCR directly on PBL, BVDV could not be detected in either positive or negative cattle. Using a previous co-culture of PBL on polycation-treated cells, BVDV was not detected in the 94 virus negative animals but was repeatedly isolated from the above 10 virus positive animals. On these isolations, a RT-PCR which detects viral sequences from 5'UTR of the BVDV genome was applied to identify the genome. All these wild BVDV isolates were positive to the test (Figure 2). These viral isolates were also positive to cELISA using MAb # 20.10.6 anti-p80 as first Ab (not shown), corroborating the specificity of the isolated virus.

DISCUSSION

A very sensitive method, able to detect BVDV in PBL from infected and cured allegedly non virus carrying seropositive cattle is presented. The method is based on the amplification of the virus by co-culture of bovine PBL samples with polycation-treated MBDK cells. All supernatants were blind passed up to 6 times on normal or polycation-treated MBDK cells. The blind-passaged cells were tested again by cELISA-FCV.

Table 3. Effect of 5 mM ammonium chloride on BVDV penetration to target cell.

<table>
<thead>
<tr>
<th>BVDV virus</th>
<th>cELISA-FCV test (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated MDBK cells</td>
</tr>
<tr>
<td></td>
<td>Without NH₄Cl</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.596 ± 0.22</td>
</tr>
<tr>
<td>Ether-treated</td>
<td>0.0240 ± 0.01</td>
</tr>
<tr>
<td>Ab-coated</td>
<td>0.660 ± 0.03</td>
</tr>
<tr>
<td>None</td>
<td>0.0830 ± 0.04</td>
</tr>
</tbody>
</table>

Polycation treated and untreated MDBK cells (see M&M) were incubated with 5 mM ammonium chloride in MEM, 15 min at 37 °C. The supernatant was discarded and the monolayers were inoculated with whole BVDV, ether-treated BVDV or Ab coated-BVDV, in 5mM ammonium chloride and polybrene in MEM. After 48 h incubation, the cells were lysed and analyzed by cELISA-FCV. The experiment was repeated twice giving similar results.

(1) Optical Density (OD), mean value and standard deviation (SD) of triplicate results.

Table 4. BVDV detection in peripheral blood leukocytes from seropositive cattle.

| PBL lysates co-culture 1st passage 2nd passage 3rd passage 4th to 6th passage |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| Untreated cells           | + / total                | + / total                | + / total                | + / total                |
| Treated cells             | 2 / 104                  | 4 / 104                  | 5 / 104                  | 9 / 104                  |
|                          | 2 / 104                  | 9 / 104                  | 9 / 104                  | 10 / 104                 |
|                          | 2 / 104                  | 9 / 104                  | 9 / 104                  | 10 / 104                 |

Peripheral blood leukocytes (PBL) from 104 seropositive cattle were aliquoted and either lysed and tested by cELISA, or co-cultured with normal or polycation-treated MBDK cells. All samples were negative in the former test while the above 10 virus positive cattle were positive in the latter, thus showing that cELISA-FCV has higher sensitivity than RT-PCR.
Cut-off value of cELISA-FCV. Eighty-eight samples from the 10 virus positive animals and 80 samples from virus negative cattle were analyzed in 62 assays by c-ELISA-FCV, and the highest and lowest values of optical density of triplicate records are shown. Positive and negative controls were infected and non-infected MDBK cells. Cut-off value (250) was arbitrarily chosen as the 4 SD above the highest negative records.

RT-PCR amplification of the 5'UTR end of wild BVDV isolates. Wild type BVDV was isolated from virus positive animals after only one co-culture on polycation-treated cells and amplified on MDBK cells. RNA was extracted and submitted to RT-PCR (see M & M). All isolates were positive for the typical 288 bp band of the Singer strain BVDV.

Virus attachment and penetration into polycation-treated cells may be using alternative ways. Ammonium chloride, an antagonist to endocytic pH levels, decreased up to 6 times viral penetration into treated host cells. This may suggest that viral penetration into polycation-treated cells is by endocytosis.

No virus could be detected by RT-PCR in PBL lysates from seropositive cattle, but when these same lysates were tested by cELISA, 2 animals were positive. When PBL were co-cultured with MDBK followed by blind passages, a total of 9 cattle gave positive results. When co-culture of PBL was carried out on polycation treated cells, 10 animals gave positive results, without the need of blind passages. It should be noted that when these 10 virus positive animals were repeatedly retested, they consistently gave positive results while virus negative cattle consistently gave negative results even after repeated blind passages. This would confirm that BVDV was actually present in the PBL of virus positive animals and that results were due neither to contamination nor to a technical error. What is more, cELISA-FCV had higher sensitivity when compared with RT-PCR: PBL samples that
were concurrently tested gave negative results in RT-PCR and positive results with cELISA-FCV. BVDV could not be detected in either whole blood or plasma, suggesting that the virus is “hidden” in leukocytes of seropositive animals.

BVDV was then isolated from these virus positive animals by co-culture of their PBL on treated cells and a later passage on them, and its specificity was confirmed by its simultaneous recognition with a reference MAb and by RT-PCR.

All this points to a very high sensitivity of the cELISA-FCV combined method, even in the presence of neutralizing Abs in the PBL donors. The presence of virus in the PBL of seropositive animals could be due to an acute re-infection with a mutant strain, but the latter should also disappear with healing. Thus, the data here presented points to the actual existence of hidden virus in healthy cattle, even in the presence of specific Abs. This virus would only be detectable in PBL by this very sensitive test.

The finding of BVDV in about 10% of healthy, seropositive cattle raises the possibility of their role of being the source of infection in closed herds and so having an important role in the epidemiology of BVDV infection.

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