Comparison of Flow Cytometry and Virus Isolation in Cell Culture for Identification of Cattle Persistently Infected with Bovine Viral Diarrhea Virus

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Detection of bovine viral diarrhea virus in 143 blood samples by virus isolation in cell culture and flow cytometry was performed. The material included 37 samples later shown to originate from persistently infected cattle. Thirty-three samples were positive by virus isolation, and all 37 samples were positive by the flow cytometric assay.

Bovine viral diarrhea virus (BVDV) induces a wide variety of fetal and postnatal disorders in cattle. Prenatal infections can lead to the birth of persistently infected (PI) calves, which are important transmitters of viruses to other susceptible cattle (1). Demonstration of BVDV in blood samples is usually done by isolation of the virus in cell culture and subsequent identification by immunostaining (4). This technique is time-consuming, as the virus has to be multiplied in living cells. Therefore, we investigated whether flow cytometry could be used for the detection of BVDV directly in blood cells of PI cattle. Recently, a study indicated this possibility (5).

Porcine antiserum to BVDV was a gift from L. Rønsholt (State Veterinary Institute for Virus Research, Lindholm, Denmark). The antiserum was prepared against the Danish field strain UG 59 and the Allfort strain of hog cholera virus essentially as described by others (3). Immunoglobulins were purified and biotinylated as described earlier (5). One milliliter of heparinized blood was added to 9 ml of lysis buffer containing 155 mM ammonium chloride, 130 mM potassium chloride, and 0.1 mM EDTA, pH 7.3. This mixture was incubated for 3 min and then centrifuged at 400 × g for 5 min. The cell pellet was transferred to duplicate wells in a microtiter plate (96-well microwell plate; Nunc, Roskilde, Denmark). The leukocytes were fixed in solution, the membranes were permeabilized, and the BVDV antigens were detected as described before (5). Briefly, the cells were washed in phosphate-buffered saline containing 0.5% normal rabbit serum (PBS-0.5 NRS), fixed for 5 min in 1% paraformaldehyde, and then preincubated with PBS-10.0 NRS containing 1% saponin (Sigma) for 30 min. Biotinylated immunoglobulins prepared from porcine antiserum to BVDV or normal serum were added, and after incubation for 45 min bound primary antibody was demonstrated by incubation for 15 min with fluorescein isothiocyanate conjugated to streptavidin (Dakopatts, Glostrup, Denmark). The fixed and stained cells were washed three times, resuspended in a small volume of diluid (J. T. Baker, Deventer, The Netherlands), and analyzed by flow cytometry. Samples were analyzed by using a fluorescence-activated cell analyzer (FACScan; Becton Dickinson, Mountain View, Calif.). Data were collected and processed as described elsewhere (5). Gates were set to exclude cells binding normal porcine immunoglobulins. Data for 10,000 cells passing through this gate were collected and visualized in a histogram displaying fluorescence intensity versus number of cells. A window located proximate to the peak of nonfluorescent cells was defined. The percentages of cells within this window, i.e., the fluorescent cells obtained with immunoglobulins from BVDV antiserum (F1) and normal serum (F0), were calculated. The test result of the specimen was calculated as the difference between the two percentages. Serum was analyzed for BVDV by virus isolation in cell culture (4) and for antibodies by neutralizing peroxidase-linked antibody assay (2). Heparinized as well as unstabilized blood samples were obtained from 143 cattle originating from seven herds. All herds had cattle with clinical symptoms suspected to arise from infection with BVDV. If BVDV was demonstrated by either virus isolation or flow cytometry, unstabilized blood samples were re-collected 3 weeks later for isolation of BVDV. The persistent infection was diagnosed after two successive isolations of virus.

Virus was initially isolated from 34 samples originating from six herds (Table 1). The remaining samples, except three, were antibody positive (data not shown). Re-collection of blood from these 34 cattle demonstrated that 33 were persistently infected. One animal was found to be virus negative and antibody positive. Investigating heparinized blood samples from the 143 cattle by flow cytometry demonstrated that PI cattle had F1 > 1% (Fig. 1). Therefore, the cutoff in the flow cytometric assay was determined as 1%. Flow cytometric detection of BVDV in the 143 samples yielded 41 positive test results (Table 1). These samples had F0 values in the range 3.1 to 28.9% (geometric mean ± standard deviation, 17.2% ± 6.7%) and F1 values in the range 0.7 to 12.3% (mean, 4.3% ± 2.5%) (data not shown). Samples with negative test results by flow cytometry had F0 values in the range 0.3 to 10.0% (mean, 2.8% ± 1.9%) and F1 values in the range 0.4 to 12.3% (mean, 4.2% ± 3.0%). Initially, eight blood samples (1 to 8) were negative by virus isolation but positive by flow cytometry (Table 1). Blood samples (1 to 4) from four of these eight animals were virus negative by the virus isolation method and antibody positive in repeated tests after 3 weeks. Two (1 and 2) were retested by flow cytometry and found to be negative (data not

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shown). The remaining four samples (5 to 8) had $F$ values of 1.9, 6.2, 17.9, and 18.5%. Two samples (5 and 6) were antibody positive and originated from cattle younger than 1 week. All four samples (5 to 8) were found to originate from PI cattle, as virus was isolated in blood samples obtained 3 and 6 weeks later. These data are summarized in Fig. 1.

Initially, blood samples (5 to 8) from four PI cattle were negative by virus isolation and positive by flow cytometry. Antibodies to BVDV were demonstrated in two samples (5 and 6), suggesting that neutralization of virus was responsible for the negative findings. One additional sample (no. 7) originated from a 4-day-old calf also likely to have colostral antibodies in the blood. This, however, could not be demonstrated by the neutralizing peroxidase-linked antibody assay. The reason for the remaining blood sample (no. 8) to be negative in cell culture was unknown. Four other samples (1 to 4) were negative by virus isolation but positive by flow cytometry. The presence of serum antibodies to BVDV in these four animals could indicate previous acute infection. However, the time of infection was unknown. It is therefore possible that virus was present in the blood samples but remained undetected by virus isolation because of BVDV antibodies. One of the animals had a rather high percentage of fluorescent cells (11.5%), an indication of recent infection, while the others were only weakly positive. The preparation of the blood cells for the flow cytometric assay described here is a modification of a procedure described earlier (5). The major differences are (i) omission of Ficoll-Paque gradient centrifugation for purifying and concentrating the leukocytes and (ii) introduction of the microtiter plate as an incubation chamber during fixation and immunostaining. Consequently, the assay is now much quicker and a large number of samples can be handled simultaneously. It was, however, noted that the percentage of fluorescent cells obtained by incubation with normal porcine immunoglobulins varied more than was previously observed (5). This could be due to inability to wash out all of the unbound antibody from the cells or contamination with Fc receptor bearing monocytes in some preparations. Apparently, this problem does not interfere with the performance of the assay, which depends on the difference between $F_1$ and $F_0$.

The detection limit is assumed to be lower for virus isolation in cell culture than for flow cytometry. However, the study described here demonstrates that flow cytometry is a more sensitive technique for detection of BVDV in blood cells of PI cattle.

**TABLE 1. Detection of BVDV in 143 blood samples by virus isolation in cell culture and flow cytometry**

<table>
<thead>
<tr>
<th>Flow cytometry resulta</th>
<th>No. of virus isolation resultsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>33 (33)</td>
</tr>
<tr>
<td>Negative</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (4)</td>
</tr>
<tr>
<td>Negative</td>
<td>101 (101)</td>
</tr>
</tbody>
</table>

a Flow cytometric detection of BVDV in peripheral blood leukocytes. A sample was considered positive by flow cytometry if $(F_1 - F_0) \approx 1.0%$.  
b Virus in serum was demonstrated by isolation on monolayers of MDBG cells and subsequent detection by an immunoperoxidase technique. The number of blood samples originating from cattle later shown to be persistently infected with BVDV is given in parentheses.  
c Blood samples from these animals were not re-collected and analyzed.

REFERENCES