NOTES

Multiplication of Bovine Parvovirus in Two Cell Strains

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Received for publication 14 November 1975

Bovine parvovirus has been found to infect two cell strains: buffalo lung fibroblasts and embryonic bovine tracheal cells. Infectivity titers were best determined by plaque assays. Immunofluorescence studies revealed intranuclear staining, whereas complement fixation tests confirmed bovine parvovirus antigen production. These cell strains represent economical and convenient cell types which can be used in studies on bovine parvovirus.

Bovine parvovirus (BPV), also known as the bovine hemadsorbing enteric virus (HADEN), was isolated from the gastrointestinal tract of calves by Abinanti and Warfield (1) and has been identified as a parvovirus (8). Cultivation of bovine parvovirus isolates is difficult since host cell range and conditions for optimal viral growth have not yet been well defined. Initial BPV isolates were grown in bovine embryonic kidney (BEK) cells (1). Spahn et al. (7) later tested nine cell culture systems to determine the variety of cell types capable of supporting BPV replication and detected replication in only BEK and bovine lymph node cells. Bates and Storz (2) found BPV to replicate well in bovine fetal lung and spleen cells; however, bovine line cells and line cells of other animal species tested did not support viral growth. In past BPV investigations, primary BEK cell culture has become the preferred cell type for growth and production of this virus. The purpose of this study was to find a sensitive cell strain that would support BPV growth so that quantitative amounts of infectious virus could be economically produced.

The virus used in this study was the original strain of BPV, which was a gift of M. D. Hoggan, who obtained it from F. R. Abinanti.

Two cell strains obtained from the American Type Culture Collection were tested for susceptibility to viral growth. A buffalo lung fibroblast strain, Bu (IMR-31), cultivated in 1964 from a male yearling American buffalo, was used. This near-diploid strain was frozen at passage level nine and is reported by the American Type Culture Collection to have a finite life expectancy of at least 30 passages. An embryonic bovine tracheal cell strain, EBTr (NBL-4), was also tested for susceptibility to virus infection. This cell strain was cultivated in 1964 from the minced whole trachea of a male bovine fetus. It was frozen at the 25th serial passage and supports growth of a number of viruses. Optimal growth is obtained in the passage range 30 through 55. Growth, morphological characteristics, and ability to support viral growth decline beyond the 55th passage. This strain contains a considerable number of hypodiploid cells. Primary cultures of BEK cells were obtained from Flow Laboratories, Inglewood, Calif.

Bu and EBTr cells were grown in autoclavaable minimal essential medium with Earle salts (Flow Laboratories) containing 0.1% sodium bicarbonate, 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 20% fetal calf serum (Grand Island Biological Co., Santa Clara, Calif). BEK cells were grown in a similar medium containing 5% fetal calf serum.

For tissue culture infectivity assays (50% tissue culture infective dose (TCID₅₀)), the Bu and EBTr cells were seeded at 200,000 cells per tube and allowed to grow to 70 to 80% confluency. The cells were then placed in 1.0 ml of serumless medium and infected with 0.1 ml of BPV dilution. Two hours postinfection, 0.5 ml of medium containing 6% fetal calf serum was added. The medium was renewed every 5 days during the course of the infectivity assay with 1.0 ml of minimal essential medium containing 2% fetal calf serum. Viral replication was allowed to proceed to complete cytopathic effects (CPE), whereupon the cultures were stored frozen and later tested for BPV complement fixation (CF) antigen.

The plaque assay technique was also used to determine viral infectivity. Bu and EBTr cells (400,000) were planted in plastic petri dishes (20 cm²). At 60 to 70% confluency the cells were washed with 5 ml of serumless media, and 0.25
ml of serial 10-fold dilutions of virus was absorbed for 1 h at 37 C. After absorption, 6 ml of overlay consisting of 0.75% Noble agar, 20% fetal calf serum, and 80% minimal essential medium was added to each plate. The cultures were incubated for 9 to 11 days at 36 C in a CO2 incubator. The cells were fixed with 10% neutralized formalin and stained with crystal violet. The indirect method of fluorescent antibody staining was used to detect BPV virion antigens. Cover slips with monolayers of BEK, Bu, and EBTr cells infected with BPV were stained and observed as previously described (3).

Virus antigen was assayed by the microtiter CF test using guinea pig antiserum prepared against purified CsCl-banded virus. The procedure outlined by Sever was used (6).

Purified virus was obtained by three-times banding in isopycnic CsCl gradients as previously described (5).

Bu and EBTr cells were both found to support BPV replication. Typical CPE was observed in both Bu and EBTr BPV-infected cells. At 24 to 48 h after inoculation, infected cells became swollen and refractive. As the infection proceeded, the cells became rounded and soon detached from the culture vessel surface. The cytopathic effects proceeded until all cells were involved, usually by 72 to 96 h postinfection. Total CPE developed if cell sheets were infected at 60 to 75% confluency. Cell sheets infected too near confluency frequently had to be passaged once before total cell sheet destruction would occur.

The ability of these cell strains to register infectious virus was tested by both tube dilution assays and plaque assays. TCID50 titers were calculated by the method of Reed and Muench (Table 1). A BPV stock, which titered 10^6.0 TCID50/0.1 ml of virus in BEK cells in two separate trials, titered 10^6.0 TCID50/0.1 ml in both early- and late-passage Bu cells. The stock virus titered 10^5.3 TCID50/0.1 ml in early-passage EBTr cells and 10^5.5 TCID50/0.1 ml in late-passage EBTr. The late-passage EBTr cells were observed to have declined in both morphological characteristics and growth rate, doubling in cell number once every 2 weeks. The stock virus titerved by the plaque assay technique in both early-passage Bu and EBTr cells gave viral titers of 8.0 \times 10^6 plaque-forming units/0.1 ml. The plaque assay method thus appears to be a more sensitive assay technique in titering BPV with these cell strains. The lack of sensitivity of the TCID50 assay is related to the predilection of BPV for actively metabolizing and dividing cells. In the TCID50 assay, cell sheets infected at low multiplicities of infection (MOI) reached confluency before development of extensive CPE; that is, infectious units were incapable of destroying cell sheets that reached confluency soon after infection. Infection of the two cell strains at a relatively high MOI (MOI = 0.1 to 1.0) resulted in the production of trace amounts of CF antigen, whereas infection at a low MOI (10^-4 to 10^-6) resulted in the production of higher quantities of CF antigen (Table 2). Virus produced in Bu and EBTr cells from the tubes containing high and low amounts of CF antigen was assayed for infectious virus by TCID50 and plaque assay techniques.

Plaque assay titers of BPV were higher and more consistent than the titers determined by the TCID50 method. Low-passage Bu cells appeared to produce higher and more consistent plaque titers than the late-passage Bu cells. The plaque assay was used to determine infectious virus production in Bu and EBTr cells.

**Table 1. Infectivity assay of BPV in Bu, EBTr, and BEK cells**

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Passage level</th>
<th>TCID50/0.1 ml</th>
<th>PFU/0.1 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEK</td>
<td>1^a</td>
<td>10^6.0</td>
<td></td>
</tr>
<tr>
<td>Bu</td>
<td>16</td>
<td>10^6.0</td>
<td>8 \times 10^6</td>
</tr>
<tr>
<td>Bu</td>
<td>39</td>
<td>10^6.0</td>
<td></td>
</tr>
<tr>
<td>EBTr</td>
<td>32</td>
<td>10^5.5</td>
<td>8 \times 10^6</td>
</tr>
<tr>
<td>EBTr</td>
<td>48</td>
<td>10^5.5</td>
<td></td>
</tr>
</tbody>
</table>

* PFU, Plaque-forming units.
* Considered early-passage levels.
* Considered late-passage levels.

**Table 2. Infectivity assay of BPV produced in Bu and EBTr cell strains**

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Passage level</th>
<th>MOI</th>
<th>BPV CF antigen produced (units)</th>
<th>Infectious BPV produced (TCID50/0.1 ml)</th>
<th>Infectious BPV produced (PFU/0.1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bu</td>
<td>16</td>
<td>0.1–1.0</td>
<td>TR^a</td>
<td>10^4.5</td>
<td>7.0 \times 10^4</td>
</tr>
<tr>
<td>Bu</td>
<td>16</td>
<td>10^-4–10^-5</td>
<td>4</td>
<td>10^5.5</td>
<td>7.0 \times 10^4</td>
</tr>
<tr>
<td>Bu</td>
<td>35</td>
<td>0.1–1.0</td>
<td>TR^a</td>
<td>10^5.0</td>
<td>5.0 \times 10^4</td>
</tr>
<tr>
<td>EBTr</td>
<td>33</td>
<td>0.1–1.0</td>
<td>0</td>
<td>10^5.5</td>
<td>1.5 \times 10^4</td>
</tr>
<tr>
<td>EBTr</td>
<td>33</td>
<td>10^-5–10^-6</td>
<td>4</td>
<td>10^5.5</td>
<td>3.3 \times 10^4</td>
</tr>
<tr>
<td>EBTr</td>
<td>48</td>
<td>0.1–1.0</td>
<td>0</td>
<td>10^5.5</td>
<td>4.0 \times 10^4</td>
</tr>
</tbody>
</table>

* TCID50 was determined in early passage Bu.
* PFU, Plaque-forming units; plaque assays were determined in early-passage Bu.
* TR, Trace reaction occurred at 1:2.
pear to produce slightly more virus than low-passage EBTr cells. High-passage Bu cells produced relatively high titers of BPV, whereas high-passage EBTr cells produced much less infectious virus. Again, this was attributed to the metabolic decline of the late-passage EBTr cells. The amount of CF antigen produced does not correlate directly with the infectious titer and, therefore, may represent excess unassembled viral components or noninfectious virus.

We have used Bu cells for production of quantitative amounts of BPV which have been purified on isopycnic CsCl gradients and used for studies of virus structural proteins (unpublished data). The cell monolayers were infected as previously described for BEK cells (5) and maintained or passaged one time until complete CPE developed. The cultures were freeze-thawed, and the virus was pelleted and banded on CsCl gradients as previously described (5). Purified virus bands were thus obtained which contained quantities of virus comparable to those isolated from BEK cultures.

Production of virus antigen was determined further by immunofluorescence. BPV-infected BEK cells which are stained with antivirion serum show positive fluorescence in about 5 to 10% of the cells when fixed at 30 h postinfection (4). Staining is localized in intranuclear areas, with light background staining of the cytoplasm. Infected Bu and EBTr cells were compared to BEK cells by fluorescent antibody techniques to determine if comparable numbers of cells would stain positively and to compare stain intensity which may be related to the relative amounts of viral antigen produced. Figure 1 illustrates the similar staining patterns that were observed in both Bu (Fig. 1A) and EBTr cells (Fig. 1B) at 30 h postinfection. Early-passage Bu cells showed staining in 5 to 10% of the cells. Staining was observed to be localized in the nucleus, with only faint background staining in the cytoplasm. Early-passage EBTr cells also exhibited intranuclear staining. Fluorescent staining occurred in 5 to 10% of the cells, with similar staining intensities as found in BEK and Bu cells. Thus, comparable numbers of positive cells, as well as comparable stain intensities, were found in all three cell types. EBTr and Bu cells can thus be employed for fluorescent antibody studies using this virus.

BPV has been found, in these studies, to infect two new cell strains: buffalo lung fibroblasts and embryonic bovine tracheal fibroblasts. Both cell strains exhibit normal BPV CPE with destruction of the cell sheet within 72 to 96 h. Both cell strains are suitable for BPV titrations. The plaque assay techniques titered the virus with more consistent and reproducible results than did the TCID₅₀ assay. This finding is consistent with the known property of BPV to

![Fig. 1. Fluorescent-antibody staining of BPV virus-infected Bu and EBTr cells. Bu (A) and EBTr (B) cells showed positive nuclear staining when stained with whole-virion antiserum. When infected Bu, EBTr, and BEK cells were stained under identical conditions, the stain intensities were similar to that previously reported for BEK cells (reference 4).](http://iai.asm.org/)
replicate optimally in cell cultures that are in
the exponential replication phase of their nor-
mal growth curve. The two cell strains exhibit
equal sensitivity in titering the virus by the
plaque assay technique.

Both cell strains can be used to produce quan-
titative amounts of infectious virus. Such
amounts of virus have been produced in this lab
and used for morphological and structural stud-
ies of virion polypeptides (unpublished data).
More CF antigen is produced when the cells are
infected at a low MOI. This, however, does not
correlate directly with the amount of infectious
virus produced. Approximately equal
amounts of infectious virus are produced in Bu
cells when infected at both high and low multipli-
cities. Late-passage Bu appear to produce only
slightly less infectious virus. EBTr cells pro-
duce slightly more infectious virus when in-
fected at a low MOI. Late-passage EBTr cells
produce less virus as the line begins to degener-
ate. Both cell strains should be considered suit-
able for producing BPV. However, buffalo cells
appear to produce slightly more infectious virus
than do EBTr cells and are available at a much
lower passage level, thus appearing to be the
cell strain of choice for virus production.

We thank Alicia Bodily and Phyllis Taylor for their
expert technical assistance.

This work was supported by Public Health Service
grant AI-11325 from the National Institute of Allergy and
Infectious Diseases.

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