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A Rapid, Quantitative Assay for Titration of Bovine Virus Diarrhoea–Mucosal Disease Virus*

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ABSTRACT

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An end point dilution microtitration assay is described that can be used for the titration of both cytopathic and non-cytopathic isolates of bovine virus diarrhoea–mucosal disease virus. Indirect immunofluorescence is used to detect infected MDBK cells in the wells of Terasaki plates. The virus titre is derived from the number of uninfected wells, using the Poisson distribution. The assay is simple, fast and economical. Titres of cytopathic virus determined by the microtitration assay and standard plaque assay are equivalent.

INTRODUCTION

Bovine virus diarrhoea–mucosal disease virus (BVDV) is the type species of the genus Pestivirus in the family Togaviridae (Westaway et al., 1985). The virus occurs as 2 biotypes, isolates either being cytopathic (c-BVDV) or non-cytopathic (nc-BVDV) in tissue culture. Some intermediate biotypes have also been observed (McClurkin and Coria, 1978).

Cytopathic BVDV can be readily titrated using standard plaque assays, but nc-BVDV presents more of a problem. Several different assays have been developed over the years for titrating nc-BVDV. These have exploited various properties of nc-BVDV: interference with the cytopathogenicity of c-BVDV (Gillespie et al., 1962; Itoh et al., 1983); enhancement of the cytopathogenicity of Newcastle disease virus (NDV) (END assay) (Omori et al., 1967); inhibition of protection of cells by polyriboinosinic acid:polyribocytidylic acid (poly I:C) from vesicular stomatitis virus (VSV) (PINBA assay) infection (Mai-

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sonnave and Rossi, 1982); production of plaques in monolayers stained for several days with neutral red (Straver, 1971); detection of nc-BVDV-infected cells by immunofluorescence (Fernelius, 1964; Frey and Liess, 1971; Rae et al., 1987) or immunoperoxidase techniques (Katz et al., 1987; Hyera et al., 1987).

In the present paper, we describe a rapid, quantitative end point dilution microtitration procedure for the titration of either biotype of BVDV. The assay was adapted from that originally developed by Robb for titrating simian virus 40 (Robb and Martin, 1970) and murine coronaviruses (Robb and Bond, 1979).

MATERIALS AND METHODS

Cells and virus

The BVDV isolates used in this study were the cytopathic isolates Singer, NADL, Oregon C24V, TGAC, ILLC and SSDC, and the non-cytopathic isolates New York-1 (NY-1), TGAN, ILLN and SSDN. All the isolates were the kind gift of Dr. Arlan McClurkin and Dr. Steven Bolin at the National Animal Disease Center, Ames, IA.

MDBK (NBL-1) cells (ATCC CCL 22) were maintained in Ham's F-12/DME (Irvine Scientific) supplemented with 10% equine serum (Hyclone Laboratories, Inc.) (F-12/DME-10).

Preparation of BVDV antiserum

A black angus heifer, seronegative and virus negative for BVDV, was infected with the NY-1 isolate of BVDV by intranasal instillation of 20 ml of infected cell culture fluid, which had a titre of 1.5×10^8 infectious units (IU) of virus ml^{-1} . This stock virus was titrated by the method described in this paper, using hyperimmune serum against the NADL isolate of BVDV, provided by Dr. Steven Bolin. Eight weeks later, the animal was boosted with 4 ml of a 1:1 emulsion of NY-1-infected cell culture fluid and incomplete Freund's adjuvant, administered intramuscularly. Serum was prepared from blood collected 2 weeks later.

End point dilution microtitration assay

The assay was adapted from that described by Robb and Martin (1970). Ten-fold serial dilutions of virus are made in a suspension of 1×10^5 MDBK cells ml^{-1} in Ham's F-12/DME plus 2% equine serum (F-12/DME-2). The suspension is then dispensed into Terasaki plates (Nunc), 10 μl being placed in each well. Alternatively, uninfected cells can be dispensed into the wells and infected the next day with serial dilutions of virus in F-12/DME-2. To prevent dehydration during incubation, 0.5 ml of F-12/DME-2 is distributed around

the inside edge of the plate. The plates are incubated for 72 h at 37°C. The medium is aspirated, the plates rinsed twice with cold Dulbecco's phosphate-buffered saline (PBS), once with cold absolute methanol and flooded with cold absolute methanol. Five minutes later, the methanol is removed and the plates allowed to dry at room temperature. The plates are used immediately or stored at -70°C.

The Terasaki plates are rehydrated by rinsing once with PBS. The primary antiserum is diluted 1:12 in PBS containing 1% porcine gelatin (Sigma) and 10 µl dispensed into each well of the Terasaki plate. The plates are incubated for 2 h at 37°C, in a humid atmosphere. After incubation, the plates are rinsed 5 times with PBS containing 0.05% Tween 80 (Sigma). The secondary antiserum, fluorescent rabbit anti-bovine gamma globulins (Antibodies Incorporated), is diluted and dispensed as for the primary antiserum. The plates are incubated for 2 h at 37°C, washed twice with PBS containing 0.05% Tween 80 and twice with distilled water (5 min per wash).

Prior to observing the cells for fluorescence, the wells are filled with PBS. In this laboratory, an Olympus inverted microscope, with an epifluorescence attachment, is used to observe wells for BVDV-specific fluorescence. The entire bottom of a well fits in the field of view of a 10× objective lens, making scoring simple. Epifluorescence, with a water immersion objective lens, can be used for viewing wells with an upright microscope. The virus titre is calculated from the number of uninfected wells using the Poisson distribution.

Plaque assay

Six-well plates are seeded with 4×10^5 MDBK cells per well in F-12/DME-10 and incubated overnight. The next day, the medium is aspirated and 0.2 ml of 10-fold serial dilutions of virus, in F-12/DME-2, are dispensed onto the monolayers. Virus is allowed to adsorb for 1 h with regular agitation. The monolayers are overlaid with 3 ml 0.7% SeaPlaque agarose (FMC) in F-12/DME-2. The agarose is allowed to gel at room temperature and the plates incubated inverted at 37°C. Three to 5 days later, the cells are fixed with 0.5 ml 2% glutaraldehyde, the agarose removed, the monolayers stained with 0.4% crystal violet in 20% ethanol and plaques counted. Alternatively, the cells are stained for at least 6 h with 3 ml 0.01% neutral red in F-12/DME-2 at 37°C in the dark, fixed with 0.5 ml 2% glutaraldehyde, the agarose removed and plaques counted.

RESULTS

Raising of BVDV-specific antiserum

When initially setting up the microtitration assay, bovine hyperimmune serum raised against the NADL isolate of BVDV (the kind gift of Dr. Steven

Bolin), was used. It was found that this worked well for assaying c-BVDV isolates, but the assay of nc-BVDV isolate NY-1 proved more difficult. The level of virus-specific fluorescence observed was often barely above background, increasing the chance of scoring false positives or negatives. Fernelius (1964) indicated that fluorescent antiserum raised against nc-BVDV is better for detecting both biotypes than that raised against c-BVDV, therefore it was decided that antiserum raised against NY-1 would be more suitable for the assay.

The stock animals of the Veterinary Research Laboratory were screened to identify cattle seronegative for BVDV, using a plaque reduction assay. These animals were then screened for persistent BVDV infection by co-cultivation of peripheral blood leucocytes with MDBK cells. The MDBK cells were subsequently assayed for the presence of BVDV by indirect immunofluorescence. Three seronegative and BVDV-free animals were identified. One was selected and immunised as described in Materials and methods.

The specificity of the antiserum for BVDV was checked by comparing immunofluorescence with BVDV-infected and uninfected MDBK cells. With un-

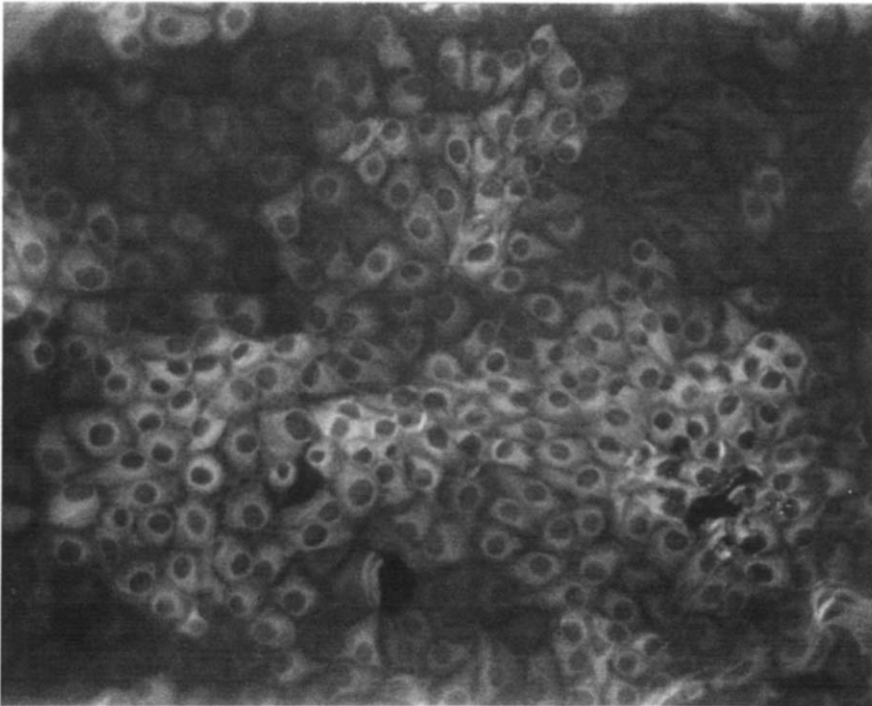


Fig. 1. Focus of infection of the ILLC isolate of BVDV on MDBK cells, in a well of a 72-well Terasaki plate. The cells were fixed 72 h p.i. Virus-infected cells were detected by indirect immunofluorescence, as described in the text. $\times 150$.

infected cells, a 1:12 dilution of the antiserum resulted in the elimination of non-specific fluorescence, while BVDV-infected cells were readily detected, as shown in Fig. 1. The cells infected with BVDV have a distinctive fluorescence that is confined to their cytoplasm. Uninfected cells either do not fluoresce or have a low level of fluorescence of the entire cell.

Optimal incubation period for assay

BVDV-specific fluorescence can be detected as early as 12 h post-infection (p.i.) in MDBK cells (data not shown). With care, plates can be scored after 24 h, but the accuracy of the microtitration assay is enhanced by incubating the cells for 72 h. This allows the virus to propagate, amplifying the viral antigens in infected wells. This increases the rate at which plates can be scored under the microscope and reduces errors due to scoring false-negative wells. It is better to score the plates containing higher virus dilutions first. These will probably have uninfected wells, so the level of non-specific fluorescence can be assessed, allowing for more accurate scoring.

Calculation of virus titre

In this laboratory, the virus titre is calculated using a simple Turbo Pascal 4.0 (Borland) computer program. A compiled version of the program in PC-DOS format may be obtained by sending a formatted 5.25-inch disk to the authors. The virus titre is calculated from the Poisson distribution using the following equation:

$$P(0) = e^{-m}$$

where $P(0)$ is the number of uninfected wells and m is the multiplicity of infection per well (IU per 10 μ l). As an example of how the titre is calculated, the titre of the Singer isolate in Table 1 was determined as follows. Twenty-four of 36 wells were uninfected at a dilution of 10^{-6} ; this gives a value of $P(0)$ of 0.67 (24/36), so m equals 0.41 [$-\ln P(0)$]. To obtain the titre per millilitre of the original virus stock, m is multiplied by $100 \times$ reciprocal of the dilution, in this case 10^6 . The result is a titre of 4.1×10^7 IU ml $^{-1}$.

Large statistical errors occur when titres are calculated from plates where either very few or most of the wells are infected (Robb and Martin, 1970). The accuracy of the assay is also dependent on the number of wells used per dilution. In this laboratory, half the wells of a Terasaki plate, 30 or 36 wells depending on whether it is a 60- or 72-well plate, are generally used per dilution.

TABLE 1

Comparison of virus titres determined by plaque assay and end point dilution microtitration assay

Isolate	Virus titre	
	Plaque assay (PFU ml ⁻¹)	Microtitration assay (IU ml ⁻¹)
Singer	4.0 × 10 ⁷	4.1 × 10 ⁷
NADL	7.1 × 10 ⁶	6.9 × 10 ⁶
Oregon C24V	5.3 × 10 ⁷	5.4 × 10 ⁷
New York-1	— ^a	2.5 × 10 ⁷
TGAC	5.6 × 10 ⁵	4.9 × 10 ⁵
TGAN	— ^a	3.6 × 10 ⁶
ILLC	2.2 × 10 ⁶	3.6 × 10 ⁶
ILLN	— ^a	4.9 × 10 ⁶
SSDC	7.9 × 10 ⁶	9.4 × 10 ⁶
SSDN ^b	6.0 × 10 ⁵	1.0 × 10 ⁶

^aPlaques not detected; non-cytopathic.

^bThe detection of plaques of the nc-BVDV SSDN isolate indicate that this putatively non-cytopathic isolate is contaminated with c-BVDV. This is not surprising as this isolate, and the c-BVDV SSOC isolate, were recovered from an animal suffering from chronic BVD (S. Bolin, personal communication, 1987). It appears that the 2 biotypes have not been totally separated from each other in the non-cytopathic virus stock, hence the plaque formation.

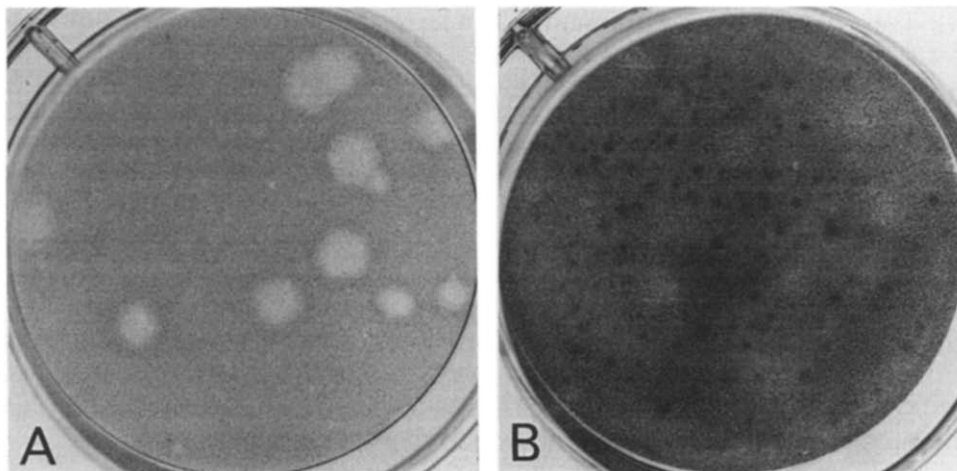


Fig. 2. Plaques of the Oregon C24V isolate of BVDV: comparison of staining with (A) neutral red and (B) crystal violet. A plaque assay was performed in duplicate 6-well plates, as described in the text. The plates were stained after 4 days, the neutral red staining being for 9 h. The illustration shows the wells inoculated with the 10⁻⁶ dilution of virus.

Comparison with plaque assay

To assess the accuracy of the microtitration assay as compared with a plaque assay, 10 isolates of BVDV (6 c-BVDV and 4 nc-BVDV) were titrated using the 2 methods. It has been observed with the Oregon C24V isolate that plaques are readily detected using neutral red, but not crystal violet, staining (Fig. 2). For this reason, plaque assays were carried out in duplicate plates. After 4 days, one plate was stained with neutral red and the other with crystal violet. The titre was taken as the average of the duplicate plates, except for Oregon C24V, which could only be determined from the plate stained with neutral red.

The titres determined by the individual assays are in very close agreement (Table 1). Although the data presented are from a single experiment, similar results have been consistently produced. The detection of plaques of the nc-BVDV SSDN isolate indicate that this putatively non-cytopathic isolate is contaminated with c-BVDV. This is not surprising as this isolate, and the c-BVDV SSDC isolate, were recovered from an animal suffering from chronic BVD (S. Bolin, personal communication, 1987). It appears that the 2 biotypes have not been totally separated from each other in the non-cytopathic virus stock, hence the plaque formation.

DISCUSSION

There are several advantages to the end point dilution microtitration assay described over other BVDV assay methods, especially those for nc-BVDV. A major consideration is that the assay can be used for the titration of either biotype of BVDV. As results of comparisons between plaque and microtitration assays of c-BVDV isolates give equivalent results, it is reasonable to assume that titres of nc-BVDV isolates, determined using the microtitration assay, are equally accurate. The assay has also been adapted for measuring serum neutralisation titres and for the biological cloning of BVDV isolates. If desired, it could also be adapted to an immunoenzymic mode of detection (Katz et al., 1987; Hyera et al., 1987; Howard et al., 1987; Justewicz et al., 1987).

The microtitration assay has certain advantages over the standard plaque assays for c-BVDV isolates. It takes less time to set up, requires less in terms of materials, takes up less space in incubators and can be read after 3 days or, with care, less than 2 days.

For nc-BVDV isolates, the microtitration assay has distinct advantages over other assay methods. The assay is a direct assay of infectious BVDV, whereas most of the other assays are indirect. Compared with these assays, the microtitration assay is much simpler, faster and less prone to error. It does not require a challenge virus, unlike the interference assay (Gillespie et al., 1962) and the reverse plaque assay (Itoh et al., 1983), which both require a c-BVDV challenge virus. The interference assay also suffers from ill-defined end points,

leading to statistical inaccuracies. The PINBA (Maisonave and Rossi, 1982) and END (Omori et al., 1967) assays require challenge with VSV and NDV, respectively. The PINBA assay varies in accuracy between cell lines (Rossi and Kiesel, 1983), some cells infected with nc-BVDV being resistant to challenge with VSV. The END assay has been shown to be less sensitive than other assays for nc-BVDV (Itoh et al., 1983).

Compared with the few direct assays for nc-BVDV, the microtitration assay is statistically more accurate, due to the larger number of replicates, than the previously described end point dilution assays (Fernelius, 1964; Frey and Liess, 1971; Hyera et al., 1987; Katz et al., 1987). In this laboratory, nc-BVDV plaques have occasionally been observed after neutral red staining, as originally described by Straver (1971). The inconsistency of plaque formation and the time required in staining make this method less than ideal.

The only problems encountered with the microtitration assay described are due to the lack of specificity or low titre of the primary anti-BVDV antiserum used. The use of antiserum raised against the nc-BVDV NY-1 isolate alleviated these problems in the present study.

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