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BRIEF COMMUNICATIONS

Plaque variations in clinical isolates of bovine coronavirus

Sanjay Kapil, Cindy Chard-Bergstrom, Pam Bolin, Denise Landers

Bovine coronavirus (BCV) causes enterocolitis and respiratory disease[4] in young calves and may cause chronic diarrhea (winter dysentery) in adult cattle.[1] To understand the molecular basis of tissue tropism and virulence, and to study biological differences between clinical isolates, it is important to plaque purify BCV. Plaque assay can also be used to study the effect(s) of antiviral drugs, such as hygromycin B, on BCV replication in vitro. Moreover, there is an urgent need to select better vaccine candidates and develop an effective modified live virus vaccine against BCV, and candidate vaccine isolates must be plaque purified for characterization. Because isolates of BCV are obtained from fecal samples that contain a wide variety of bacteria and viruses, plaque purification is essential. This paper describes the development of a plaque assay to study plaque variation in recent isolates of BCV (1993-1994) and to study the antiviral effects of hygromycin B on BCV in vitro.

Human rectal tumor-18 (HRT-18) cells were plated in 6-mm tissue culture dishes[a] and grown in minimum essential medium (MEM) with 10% fetal calf serum, L-glutamine, and antibiotics (Kapil S, Donnelly C: 1994, Abstr Proc 75th Conf Res Workers Anim Dis, P77). After 3-4 days, confluent monolayers were washed with calcium- and magnesium-free phosphate-buffered saline (CMF-PBS). BCV isolates were obtained from calves with a history of diarrhea. Approximately 250 µl of virus dilutions (10^{-1}-10^{-6}) was added to these plates. For BCV adsorption, the plates were hand-rocked every 5-10 minutes for 1 hour. Monolayers were washed with CMF-PBS and overlaid with 1% agarose in MEM containing trypsin[b] (5 µg/ml) and pancreatin[c] (5 µg/ml). To study the effect of hygromycin B[d] on BCV replication the drug was incorporated in the agarose at concentrations of 0.1 mM, 0.25 mM, 0.5 mM, and 1 mM. Plates were incubated in an inverted position in a humid chamber at 37 C for 3-5 days. To count and measure the plaques, the plates were held against a bright source of light. Since our protocol gave clearly visible plaques, it was not necessary to stain the plates. Viral titers were calculated on the basis of dilutions that gave about 25-30 isolated plaques/well. To confirm the identities of the plaques, the monolayers were fixed with 5 ml of 10% neutral buffered formalin[e] per plate. In preliminary experiments, we compared 100% methanol, 100% ethanol, 5% glutaraldehyde, 100% acetone, and 10% neutral buffered formalin as fixatives and found that buffered formalin gave the best results. After 1 hour, the agarose was removed. Plates were washed once with phosphate-buffered saline to remove residual formalin. Fluorescein-labeled antibovine coronavirus conjugate[f] was added and plates were incubated for an hour at 37 C. The plates were washed 3 times with PBS, coverslips were added and plates were examined by fluorescence microscopy.

The plaque sizes and the viral titers (plaque-forming units [PFU]/ml) are shown in Table 1. HRT-18 cells allowed efficient replication of BCV (10^5-10^6 PFU/ml at passage 4 of the viruses) but HRT-18 cells do not allow replication of all the field isolates of BCV (Kapil S, Donnelly C: 1994, Abst Proc 75th Conf Res Workers Anim Dis, P77). Among the eight clinical isolates of BCV, only one was noncytopathic (WI-9) and did not produce plaques. However, the presence of bovine corona viral antigen was confirmed by a fluorescent antibody test. One cytopathic isolate (WI-10) produced smaller plaques than the other 6 cytopathic isolates. In most samples, plaques first appeared 23 hours after inoculation. Not much is known about the plaque variations in clinical isolates of BCV. There are 3 previous reports[2,3,9] on plaque assay for BCV and only 8 isolates have been studied so far. There is a need to study more isolates of BCV to determine if significant variation exists among isolates and if that variation correlates with behavior in vivo.

Various factors affect plaque assay. BCV plaque formation is dependent on the presence of trypsin in the overlay medium.[8] In the absence of trypsin, we did not observe any plaques in our study. By using cells at different days after plating, we found that plaque morphology also depends on the age of the HRT-18 cells after plating. A time interval of 4 days after plating the cells was found to be optimal in our studies. Plaque morphology also depends on BCV strain and host ce11.[2] Based on this study, plaque variations occur in the clinical isolates of BCV. Correlation of plaque morphology with virulence and site of replication in calves needs to be determined. On the basis of preliminary studies using reverse transcriptase polymerase chain reaction (RT-PCR), plaque-forming ability and cytopathogenicity may correlate with hemagglutinin esterase gene sequences (Kapil and Muenzenberger, unpublished observations). The plaque assay described above was found to be reproducible and easy to perform.

The plaque assay was used to study the effect of hygromycin B on BCV replication. After hygromycin B treatment, plaque numbers were lower than in untreated controls. Thus,

From the Department of Veterinary Diagnostic Investigation, College of Veterinary Medicine, Manhattan, KS 66506 (Kapil, Chard-Bergstrom, Landers), and the Wisconsin Animal Health Laboratory, Madison, WI 53705 (Bolin).

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Table 1. Plaque sizes in clinical isolates of bovine coronavirus.

Isolates*	Viral titer† (PFU/ml)	Plaque shape	Mean plaque size at 48 hr post-inoculation (mm)	Mean plaque size at 72 hr post-inoculation (mm)	Susceptibility to hygromycin B‡
WI-1.PB	1×10^5	circular	2–3	2.5–6	+
WI-1.SK	4×10^4	circular	1.5–3	3–5	§
WI-2	8.7×10^6	circular	1.5–3	3–5	+
WI-9	no plaques, noncytopathic	circular	...	...	+
WI-10	1.2×10^5	circular	0.5–1.5	ND‖	+
WI-11	1.2×10^4	circular	1–2	3.5–5	+
WI-17	2.4×10^6	fuzzy edges	1–2.5	ND	+
WI-18	2.4×10^5	circular	2–2.5	3–6	§

* All the clinical isolates of BCV from Wisconsin (WI) calves were used at passage 4. All of these isolates were isolated and propagated in human rectal tumor-18 (HRT-18) cell line. All samples were submitted during 1993-1994 to the Wisconsin Animal Health Laboratory, Madison, from calves with a history of diarrhea. All these clinical isolates were negative for rota, BVD, parvo, and reoviruses by direct fluorescent antibody tests.

† Titers of the viruses were calculated at passage 4 in HRT-18 cell line.

‡ Susceptibility to hygromycin B was evaluated by a plaque assay. The identities of plaques were confirmed by a fluorescent antibody test; + = susceptible. In subsequent studies in our laboratory, we found that hygromycin at 0.5 mM concentration and above was effective. (Zhang Z, Kapil S, unpublished data.)

§ Effect(s) of hygromycin B on BCV replication was quantitated by a hemagglutination test using 1% mouse erythrocytes. After treatment with hygromycin B (0.5 mM), there was a 128-fold decrease in hemagglutinating activity of WI-1.SK and a 16-fold decrease in hemagglutinating activity of WI-18.

‖ Not done.

hygromycin B inhibits replication of BCV in vitro. Hygromycin B, an aminoglycoside, prevents murine hepatitis virus (a coronavirus) transcription in vitro and in vivo[6] by inhibiting the corona viral RNA synthesis.[7] Because hygromycin B inhibits BCV replication at low doses, it may be useful in the treatment of chronic BCV shedders[5] that are a contact source of the virus for other calves. Hygromycin B has not been approved for use in calves in the USA.

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Sources and manufacturers

a. Falcon, Becton Dickinson Labware, Lincoln Park, NJ.
b. Trypsin (1:250), Grand Island Biological Company, Grand Island, NY.
c. Pancreatin (P-3292), Sigma, St. Louis, MO.
d. Hygromycin B (H-9773), Sigma, St. Louis, MO.
e. Buffered Formalde-Fresh (SF 93-4) Fisher Scientific, Fair Lawn, NJ.
f. National Veterinary Services Laboratory, Ames, IA.

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