CELL CULTURE PROPAGATION OF BOVINE CORONAVIRUS

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SUMMARY: Although most field strains of bovine coronavirus (BCV) grow poorly in cell culture and fail to produce cytopathic effects (CPE) until after blind passage, primary calf kidney (PCK) and Vero cells have permitted primary isolation of virus. Cell culture-adapted strains of BCV replicate in PCK, bovine embryonic lung, bovine fetal thyroid, bovine fetal brain, bovine skin cells, ovine fetal kidney cells, and the cell lines pig kidney K3 and 15, Vero, human embryonic lung fibroblasts, HRT-18, MDBK and BEK-1, with trypsin useful for enhancing replication. Organ culture as well as suckling mouse, rat, and hamster brains also support the growth of cell culture-adapted BCV strains. Viral growth is most commonly detected by CPE, immunofluorescence, hemagglutination, and hemadsorption assays or electron microscopy of supernatants from infected cells. In this report, the optimal conditions for the growth and plaque assay of the NCDV strain of BCV in MDBK cells are described.

Key words: bovine coronavirus; cell culture; propagation.

I. INTRODUCTION

The coronaviruses were first recognized and morphologically defined as a group by Tyrrell et al., (39–41) but have have been known by other names for almost 5 decades (33). The Coronaviridae is a monogeneric family comprising 11 viruses that infect vertebrates (29).

Stair et al. (30) first described the isolation and partial characterization of a coronavirus-like agent from calves with diarrhea. The viral particles were 107 to 160 nm, polymorphic, and had an 11-nm petal-shaped fringe. Sharpee et al. (28) further characterized this coronavirus-like agent and showed that it possessed all six major properties common to members of the Coronaviridae family. The virion is composed of four proteins and possesses a lipid bilayer. The genome consists of a single-stranded polyadeylated RNA which is infectious and of positive polarity.

Bovine coronavirus (BCV) causes severe diarrhea in calves 3 to 30-d old which can result in death due to dehydration and acidosis (18,24,30). In addition to enteric replication of BCV, several workers have reported respiratory replication and clinical signs of upper respiratory infection. Thomas et al. (37) first reported the isolation of coronavirus from the respiratory tract of several calves showing respiratory illness. Since then other workers have detected bovine coronavirus upper respiratory infections, both in experimental and field situations (5,21,25,26).

In this report, the optimal cell culture conditions for the growth and plaque assay of the NCDV (Mebus) strain of BCV in MDBK cells are described. Also included is a review of the literature pertaining to the cultivation of BCVs in cell and organ cultures.

II. MATERIALS

A. Equipment
- Vortex Mixer, model K-550-G, Scientific Industries[1]
- Incubator, 32 ft^3, National Appliance[2]
- Incubator, CO_2, model 2220, Queue Systems[3]
- Waterbath, no. 66648, Precision Scientific[4]
- Laminar flow hood, Edgegard, Baker Company[5]
- Wheaton Roller Culture Apparatus, Wheaton[6]
- Seitz filter, Republic[7]
- Millipore Filter, Millipore[8]

B. Chemicals
- Mycostatin (Nystatin) 500 000 U, Squibb[9]
- Penicillin G potassium NDC 0995-0510-95, Pfizer[10]
- Dihydrostreptomycin sulfate 100405, NB Co.[11]
- MEM medium, no. 410-1500, GIBCO[12]
- $NaHCO_3$, no. S-233, Fisher Scientific[13]
- HCL, HX0603-3, Matheson Coleman & Bell[14]
- NaCl, no. S-271[13]
- KCL, no. P-217[13]
- $MgSO_4 \cdot 7H_2O$, no. M-63[13]
- $CaCl_2$, no. C-614[13]
- $Na_2HPO_4 \cdot 7H_2O$, no. S-373[13]

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KH_2PO_4, no. 73351, Merck[15]
Chloroform, no. C-298[13]
Dextrose, Anhydrous, Granular, no. DV145 CB361[14]
Disodium salt of ethylenediamine tetraacetate acid (Versene) no. S-311[13]
Trypsin (1:250) no. 840-72501M[12]
Neutral Red, no. B360[14]
Phenol red sodium salt, no. P-391[13]
Pancreatin 4× NF, 2.5% (10×)[12]
$NaH_2PO_4 \cdot H_2O$, no. S-368[13]
Agar Noble, no. 0142-01, Difco[16]

C. Supplies
Tissue culture cluster, no. 3506, Costar[17]
Roller Bottles[6]
T-150 cm² Falcon 3028, Becton Dickinson[18]
Fetal Bovine Serum (FBS), Qualified, 200-2640 AJ[12]

D. Cells
Madin-Darby Bovine Kidney (MDBK), Salisbury Laboratories[19]

III. PROCEDURE

A. Preparation of solutions

1. Antibiotic stock solution (100×)

10 vials	1-million U each, penicillin (for injection, stored at room temperature)
10 g	dihydrostreptomycin sulfate (not for drug use) (store 4° C)
5 vials	500 000-U each, mycostatin[9] (store 4° C)

To make 1000 ml of stock solution
a. Dissolve 10 g of dihydrostreptomycin in 100 ml sterile Hanks' balanced salt solution (HBSS), sterilize with 0.20-μm Nalge filter.
b. With a syringe and needle, aspirate 2 to 3 ml of HBSS (of remaining 900 ml HBSS) and use to dissolve the powdered contents of all vials of penicillin and mycostatin. Expel into 1000-ml flask.
c. Combine all ingredients and mix well.
d. Dispense into 2-oz. bottles.
e. Store in a frozen state.

	each cc of above 100× stock solution
Penicillin	10 000 U
Dihydrostreptomycin	10 000 μg
Mycostatin	500 U

2. 0.01 *M* Phosphate buffered saline (PBS) (0.14 *M* NaCl), pH 7.4
a. Dissolve the following up to 2 liter in 2× distilled water DH_2O.

$NaH_2PO_4 \cdot H_2O$	0.78 g
$Na_2PO_4 \cdot 7H_2O$	3.85 g
NaCl	16.40 g

b. Sterilize by autoclaving 15 min, slow exhaust

3. Pancreatin 4× N/F
a. Stock (10×): 25 g pancreatin 4× NF and 8.5 g NaCl/liter
b. Pancreatin (10×) is diluted 1:10 with PBS, pH 7.4, and stored at −20° C as the 1× stock.

4. Sodium bicarbonate ($NaHCO_3$) solution.
a. 7% Solution is routinely used in our laboratory.
i. Add 70 g $NaHCO_3$ to a 2000-ml Erlenmeyer flask.
ii. Add 930 ml of distilled water (DH_2O).
iii. Dissolve. To facilitate or to shorten the time necessary, a magnetic mixer may be used.
iv. Sterilize using S-99 filter.
v. Dispense in sterile 4-oz. bottles. Screw the caps on tight and then tape the caps with masking tape to prevent loss of CO_2. Store at 4° C.

5. Neutral red (NR) solution (1:1000)
a. Weigh out 1.0 g of NR and add to 1000 ml deionized water. Mix well.
b. Dispense into 4-oz. bottles.
c. Autoclave at 15 lb for 20 min, then screw the caps tight and seal with tape.

6. HBSS
This salt solution is commonly employed as one of the basic solutions in the cultivation or maintenance of cell cultures. The method described below for the preparation of this solution is a slight modification of the method described by Weller et al (43).

a. Stock solution A—for 1000 ml
i. Weigh out the following and place in a large, clean flask

NaCl	160 g
KCl	8 g
$MgSO_4 \cdot 7H_2O$	4 g

ii. Dissolve these salts in approximately 800 ml of DH_2O.
iii. Dissolve 2.8 gms of $CaCl_2$ in approximately 100 ml DH_2O.
iv. Combine these two solutions in a 1000-ml graduate cylinder, and make up to 1000 ml with DH_2O.
v. Add 2 ml of chloroform as a preservative.
vi. Place the solution in a suitable container, such as a large bottle; cap and store at 4° C.

b. Stock solution B—for 1000 ml
i. Weigh out the following and place in a large clean flask:

$Na_2HPO_4 \cdot 7H_2O$	2.2 g
KH_2PO_4	1.2 g
Dextrose	20.0 g

ii. Dissolve in approximately 800 ml DH_2O.
iii. Add 100 ml of 0.4% phenol red solution. It may be prepared as follows: weigh out 1 g of the water-soluble (sodium salt) phenol red indicator and dissolve in 250 ml DH_2O. Adjust the pH to 7 by addition of either 0.1 *N* HCl or 0.1 *N* NaOH.
iv. Add 2 ml of chloroform to stock solution B as a preservative.
v. Place stock solution B in a 1000-ml graduate cylinder, and make up to 1000 ml with DH_2O.
vi. Place the solution in a suitable container, such as a large bottle, cap and store at 4° C.

c. The complete HBSS (pH adjusted to 6.5)

i. Add the following to a suitable container:
- 1 part stock solution A, 150 ml
- 1 part stock solution B, 150 ml
- 18 parts DH_2O
- Plus about 0.6 ml of 1*N* HCl/3000 ml solution to bring pH to about 6.5. In our laboratory, several thousand milliliter of the solution is prepared and then dispensed in 300-ml amounts in 16 oz bottles or other suitable containers.

ii. Autoclave at 15 lb pressure for 15 min. If a precipitate occurs when autoclaving this can be subsequently prevented by the addition of 0.6 ml of 1 *N* HCl to reduce the pH to about 6.5 before autoclaving. Amount depends on color.

iii. The solution is stored at 4° C.

iv. Before use, the pH of the HBSS is increased to the desired point by the addition of sterile $NaHCO_3$ solution (pH 7.2). In our laboratory a 7% stock solution is used which has been sterilized by filtration through a Seitz filter using positive pressure.

7. Preparation of Alkaline Trypsin Versene (ATV)

NaCl	8.0 g
KCL	0.4 g
$NaHCO_3$	0.58 g
Trypsin (1:250)	0.5 g
porcine parvovirus tested	
Versene	0.2 g
Dextrose	1.0 g
DH_2O	1000 ml

0.20-μm Seitz filter to sterilize, and store frozen.

8. Growth medium
   a. MEM (prepared without $NaHCO_3$ before filtering)
   b. 5% fetal bovine serum (FBS)
   c. 2% $NaHCO_3$
   d. 1% antibiotics (100×)
   e. 0.1% (0.1 *N*) HCl
9. Diluent no. 5.
   a. MEM
   b. 1% $NaHCO_3$
   c. 1% antibiotics (100×)
10. Agar overlay medium. Measure out 1.6% Noble agar, mix with DH_2O, and boil to melt for 20 min, then cool to 42° C and add equal volume of 2× MEM plus:
    3% $NaHCO_3$ (7% stock)
    1% antibiotics (100×)
    0.7% (0.1%) NR
    1% Pancreatin (1:40)
    1% DEAE (100 μg/ml)
11. MEM
    a. Weigh out 9.8 g/liter of MEM powder and dissolve in double (DDH_2O).
    b. Filter MEM through 0.22-μm Millipore filter
    c. Store MEM at 4° C.
12. 2× MEM
    a. Weigh out 19.6 g/liter of MEM powder and dissolve in DDH_2O.
    b. Filter 2× MEM through 0.22-μm Millipore filter
    c. Store 2× MEM at 4° C

B. Propagation of the NCDV strain of BCV in MDBK cells
1. Subculture 7-d-old culture of MDBK cells using ATV. Dilute the subcultured cells 1:16 with growth medium.
2. Dispense 200 ml of diluted cells into 900-cm^2 roller bottles and place in a roller culture apparatus at 80 rpm for 24 h at 37° C. After 24 h, turn speed up to 200 rpm. Allow cells to grow 5 to 6 d. MDBK cells can overgrow very quickly causing the cell layer to peel off.
3. At 5 to 6 d of age, rinse the cell layer once with diluent no. 5 and change media with diluent no. 5 warmed to 37° C, 3 h before inoculation.
4. Inoculate each roller bottle with final volume of 30 ml of BCV (initial titer of approximately 10^7 plaque-forming units (pfu/ml) diluted 1:5 in diluent no. 5. Roller bottles are again placed on the roller culture apparatus at 80 rpm for 1 h at 37° C.
5. After 1 h the cell monolayers are rinsed once with diluent no. 5. Place 200 ml diluent no. 5 in each roller bottle and add 480 μl of Pancreatin (1×) diluted 1:40. Place bottles on roller culture apparatus at 200 rpm at 37° C.
6. Allow cytopathic effects (CPE) to develop to 40 to 70% (2 to 3 d), and freeze roller bottles.
7. Thaw roller bottles and centrifuge the virus-cell suspensions at 2000 rpm for 30 min at 10° C.
8. Remove supernatant, dispense, and freeze at −70°. Titer virus in plaque assay. Generally this procedure results in virus titers of 10^7 to 10^8 pfu/ml. An electron micrograph of typical BCV particles (NCDV strain) grown in MDBK cells is shown in Fig. 1.

C. Procedures for determining virus titers and neutralizing

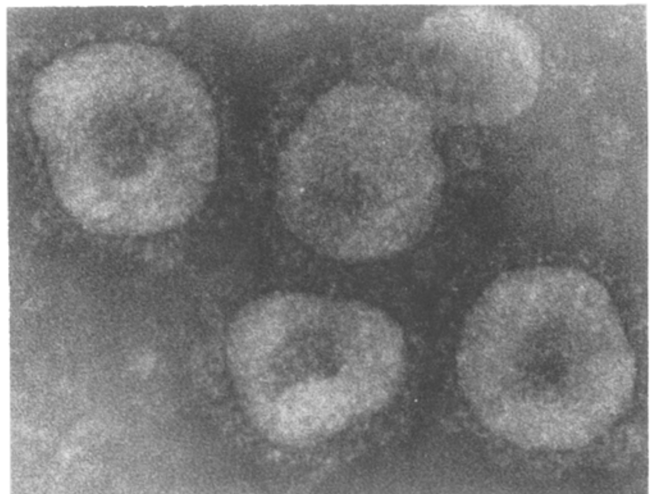

FIG. 1. Electron micrograph of an aggregate of BCV particles (NCDV strain) grown in MDBK cells, and reacted with calf anti-coronavirus serum. ×195 000.

antibodies against BCV using the plaque reduction assay with constant-virus varying serum.

1. Virus—a cell culture-adapted strain of BCV.
2. Cell culture—MDBK continuous cell line. Cells are grown in 6-well Costar plastic plates in growth medium.
3. Make fourfold serial dilutions of heat-inactivated serum samples to be tested for antibodies using diluent no. 5.
4. Add an equal volume of the virus dilution (containing 30 to 50 pfu/0.1 ml) to the serum dilutions and virus control tube containing diluent no. 5 only. Mix well.
5. Place serum-virus mixture in a 37° C incubator for 90 min (neutralization period).
6. Empty fluid from 6-well plates containing MDBK cell monlayers which were rinsed 1× and media (diluent no. 5) changed 3 h before inoculation.
7. Inoculate 0.1 ml/well to duplicate wells.
8. Place plates at 37° C, in 7% CO_2 for 60 min; rotate plates every 15 min.
9. Cool agar overlay medium to 42° C and dispense approximately 3 ml/well.
10. Protect plates from light at room temperature while agar solidifies for 15 to 30 min.
11. Invert plates *gently* with agar side up and place plates in an incubator at 37° C in 7% CO_2 and at least 85% humidity. Plaques will appear within 3 or 4 d.
12. For virus titration, serial 10-fold dilutions of the virus are made in diluent no. 5, and the rest of the test conducted according to steps 6 through 11.
13. The virus titer is determined by counting the number of plaques per well and multiplying the average number of plaques by the final dilution of the virus to give the total pfu per milliliter.
14. The antibody titer is expressed as the reciprocal of the dilution of serum which results in a plaque reduction of 80%, as compared to the virus control.

IV. DISCUSSION

A. Cell and organ culture propagation of BCV

Mebus et al. (22) in 1973 first demonstrated that a coronavirus-like agent isolated from a calf with diarrhea could be adapted to grow in cell culture. The agent was first isolated in primary fetal bovine kidney cells (Earle's balanced salt solution, 0.5% lactalbumin hydrolysate, 10% adult bovine serum, antibiotics), then adapted to secondary fetal bovine kidney cells (Earle's 199, 0.5% lactalbumin hydrolysate, 10% adult bovine serum, antibiotics). The virus produced CPE and replicated in these cells as indicated by staining with fluorescein-labeled rabbit gamma globulin against the virus. The type of CPE observed after 24 passages was a rounding and loss of adherence of cells with formation of syncytia. Takahashi et al. (34) also used primary bovine kidney cells for the isolation of the Kakegawa strain of BCV (a field strain from the feces of a cow with epizootic diarrhea). CPE, characterized by syncytia and granularity of cells and positive immunofluorescence, became noticeable by Passage 8.

Inaba et al. (15) and Sato et al. (27) utilized the continuous cell line, BEK-1 (Eagles's minimum essential medium, 10% tryptose phosphate broth, 10% bovine serum, antibiotics) derived from bovine embryonic kidney to grow the Mebus (cell culture-adapted, NCDV) strain of BCV. CPE first appeared at 3 d as cell rounding, which progressed to cell fusion, monolayer disintegration, and sloughing. After further passages viral yield ranged from 10^4 to 10^5, 50% tissue culture infective dose ($TCID_{50}$)/0.1 ml. CPE could be specifically inhibited by antiserum to BCV and infected cells could be detected by immunofluorescent staining. Akashi et al. (1) then utilized this cell line to characterize the Kakegawa isolate of BCV, which had previously been adapted to primary bovine kidney cells. Hirano et al. (13) showed that the BEK-1 cell line could be used in a plaque assay for the Kakegawa strain of BCV. After a 90-min absorption time and a 48-h incubation period, clear plaques were visible without staining with neutral red.

Dea et al. (8) demonstrated that African green monkey kidney (Vero), MDBK, and porcine kidney-15 (PK-15) continuous cell lines (Eagle's minimal essential medium, Earle's salt, 0.584 g of glutamine/liter, with 10, 5, and 3%, respectively, heat-inactivated FBS without gamma-globulin) were satisfactory for the isolation and multiplication of the Mebus strain of BCV. Several field isolates were also cultivated in Vero cells and then adapted to MDBK and primary bovine fetal kidney cells. CPE and positive immunofluorescent staining were observed in all cell lines. CPE consisted of cell rounding, detachment, and complete monolayer destruction after 96 h. Viral yield varied between 10^6 and 10^7 $TCID_{50}$/ml. Cells tested in which the virus did not produce CPE were embryonic bovine derma (EBD), embryonic bovine trachea (EBTr), human epitheloid carcinoma (HeLa), human epidermoid carcinoma (Hep-2), African green monkey (BSC-1), Madin Darby Canine Kidney (MDCK), embryonic lamb kidney (LK), and mouse L cells. The MDBK cell line has also been useful for plaque and plaque-reduction assays. After a 1-h adsorption time, use of an overlay with agarose and a 3-to-4-d incubation period, plaques were visible after staining with neutral red (10).

Laporte et al. (19,20) and L'Haridon et al. (17) also reported the adaptation of a wild type BCV (G110 strain) from France to primary calf kidney (PCK) cells (Earle's medium, 0.5% lactalbumine, 0.1% yeast extract, 6% FBS). There was no CPE and specific fluorescence appeared late, 8 to 12 d postinfection. After 12 passages in an established human rectal adenocarcinoma cell line (HRT-18) (RPMI 1640, 20% FBS), high viral yields, in excess of 10^7 $TCID_{50}$/ml, were achieved but no CPE was observed. They also showed that the PCK-adapted virus multiplied in bovine skin cells, bovine lung cells, MDBK cells, and K3 pig kidney cell lines. There was no virus growth in other pig kidney lines, baby hamster kidney (BHK21), Chinese hamster ovary (CHO), or MDCK cell lines. The HRT-18 cell line was also useful for titration of the cell culture-adapted G110 and F15 strains of BCV in a

plaque assay. Plaques appeared within 2 to 3 d as opalescent areas, which remained colourless after neutral red or crystal violet staining. (42)

Tektoff et al. (35,36) also proved that HRT-18 (RPMI 1640, 20% FBS), Vero (Medium 199, 5% FBS), and MDBK (Eagle-Earle medium, 5% FBS, 0.25 g/liter casein hydrolysate) cell lines were permissive to cell culture-adapted strains of BCV. Infectivity and viral morphogenesis were followed by transmission electron microscopy, hemadsorption and scanning electron microscopy, immunofluorescence, and hemagglutination (HA) assays.

The Mebus cell culture-adapted strain of BCV has also been propagated in the MA-321 strain of human embryonic lung fibroblasts (Eagle's minimum essential medium, 10% FBS). HA titers with rat or chicken erythocytes reached a peak at 72 h and remained stable until 144 h postinfection. At 4 to 5 d CPE appeared as refractile, oval or rounded cells, with a cytoplasm rich in microvacuoles. Complete degeneration of cell monolayers was observed 6 to 10 d postinfection (12).

A porcine renal cell culture-adapted strain of BCV (SC-1) was grown in Passages 2 to 5 of primary fetal (3 to 4 mo. gestation) ovine renal cells (Eagle's minimum essential medium, 10 to 15% FBS). CPE was observed at 48 to 72 h postinoculation as formation of syncytia. Virus replication was detected by hemadsorption of rat erythrocytes and cell culture supernatant HA titers of 1/64 to 1/128 (3). BCV has also been shown to replicate in calf testicle cells (23), and D2 bovine fetal spleen cells (7).

Organ culture of BCV has been achieved in two organ systems. Stott et al. (32) showed that the Mebus cell culture-adapted strain would produce hemagglutinating activity and replicated in 5- to 6-mo. gestation fetal bovine tracheal organ culture (Eagle's basal medium, 0.14% sodium bicarbonate, 0.09% bovine plasma albumin, 5% tryptose phosphate broth, antibiotics, HEPES buffer, pH 7.2). Hemagglutinin titers increased with viral passage. Positive immunofluorescence was first observed at 7 d and peaked at 21 d post-inoculation, and electron microscopic examination of culture fluids revealed typical coronavirus particles. No gross or histologic damage to the cilia was visible.

Bridger et al. (6) also demonstrated hemagglutinating activity and replication of a British isolate (passaged in tracheal organ culture) of BCV in 4.5- to 6-mo. gestation fetal bovine intestinal organ cultures. Hemagglutinating activity peaked at 24 to 48 h postinoculation, whereas immunofluorescence, detected solely in the columnar epithelial cells, was maximal at 24 h. At 2 to 4 d postinoculation the columnar epithelium lost its normal morphology, becoming irregular and disorganized. Typical coronavirus particles were seen in extracellular fluids harvested 18 to 96 h postinoculation. Electron microscopic examination of inoculated cultures revealed evidence of viral replication and budding.

B. Enhancement of in vitro replication

Conditions for enhancing growth of cell culture-adapted strains of BCV in certain cell culture systems have been described. Dea et al. (8) showed that the factors important in increasing yield and appearance of CPE in Vero cells were a) a slightly acidic inoculum (pH 6.5 to 7.0); b) growth in a basic medium (pH 8.0 to 8.5); c) exposure to hypertonic medium; d) polycation DEAE-dextran (25 μg) treatment of cells; e) washing of cells with medium containing trypsin (5 μg/ml); and f) incubation of cells with dactinomycin (0.01 to 0.05 μg).

Storz et al. (31) also showed the importance of trypsin in the replication and cytopathogenicity of the cell culture-adapted L9 strain of BCV. Trypsin treatment (10 μg/ml) accelerated CPE and size of plaques, facilitated cell fusion, improved the amount of cell-released hemagglutinin, and increased the infectivity yields in bovine fetal thyroid (BFTy) and bovine fetal brain cells (BFB) (Eagle minimum essential medium, antibiotics, 10% heat-inactivated fetal calf or lamb serum). When virus was pretreated with trypsin or trypsin was present only during viral adsorption, plaque enhancement was not observed.

Trypsin was also important for replication of the Mebus strain of BCV in bovine embryonic lung cells (Eagle's minimal essential medium, 0.22% sodium bicarbonate, 20 m*M* HEPES buffer, 0.01% pyruvic acid, 0.029% L-glutamine, 10% FBS, antibiotics) (38). Toth showed that increased amounts of trypsin (1,5,or 10 μg/ml) increased the susceptibility of bovine embryonic lung cells to BCV a millionfold.

Hirano et al. (13) found that when trypsin, at a final concentration of 5 μg/ml, was added to both the viral diluent and the overlay medium, plaques increased in size and numbers on BEK-1 cells infected with the Kakegawa strain of BCV. This did not occur if 1% trypsin was added to either the viral diluent or the overlay medium alone.

Cyr-Coats and Storz (7) demonstrated that the cell-adapted BCV L9 strain could be induced to replicate noncytopathically in D2 bovine fetal spleen cells only in the presence of trypsin. The bovine fetal spleen cells were however nonpermissive for all wild-type BCV, even in the presence of trypsin.

The mechanism(s) whereby trypsin enhances coronavirus replication is not yet fully understood. Tryspin treatment of cells might promote attachment of virions by uncovering otherwise unavailable receptor sites. Trypsin treatment of virus might modify configurations of protein molecules in the virus envelope to render them more compatible with cellular receptor sites. Alternatively, trypsin may destroy a broadly active viral inhibitor produced by cells in culture, thereby allowing multiple rounds of viral replication (4,9,14,38).

C. Animal inoculation

Bovine coronavirus can also be propagated by animal inoculation. This may be a useful, if not necessary, method of producing large pools of field BCV strains which may not otherwise propagate well in cell culture systems. The LY-138 strain of BCV, first isolated in

1965, was maintained by oral inoculation of conventional, colostrum-deprived calves for many years by this method (11).

Akashi et al. (2) serially passaged the Kakegawa strain of BCV in suckling mice, rats, and hamsters by inoculation with brain emulsions from infected laboratory animals. The Kakegawa strain, at Passage 10 in primary bovine kidney cell cultures, was inoculated intracerebrally and subcutaneously into the laboratory animals. Infected animals showed nervous symptoms, and died. The recovered virus could be clearly differentiated from mouse hepatitis virus by cross-neutralization tests.

The Mebus strain of BCV was also adapted to suckling mouse brain by Kaye et al. (16) and later by Gerna et al. (12), facilitating comparative serologic testing with human coronavirus strains.

D. Final remarks

Several cell types are permissive for the replication of cell culture-adapted strains of BCV and several cell lines allow the virus to be grown to high titer. Trypsin is useful for enhancing replication of cell culture-adapted strains of BCV. Field isolates of BCV have been propagated in primary bovine kidney and Vero cells. These cell types may be useful for virus isolation or virus quantitation, or both, from diagnostic samples. Several methods have been established for determination of BCV yields from cell or organ culture. These include enumeration of viral particles in culture supernatants by electron microscopy; determination of $TCID_{50}$ on BEK-1, Vero, MDBK, PK-15, BEL, and HRT-18 cell lines; HA and hemadsorption assays with rat and chicken erythrocytes; direct or indirect immunofluorescent assays; and plaque assays on BEK-1, MDBK, BFT, BFTy, and HRT-18 cells.

In our laboratory we have successfully propagated NCDV BCV to high titers (10^7 pfu/ml) in roller bottles of MDBK cells using pancreatin in the maintenance medium. We have also developed a plaque assay for BCV in these cells by adding pancreatin and DEAE to the agar overlay medium.

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