CELL CULTURE PROPAGATION OF BOVINE CORONAVIRUS

Linda J. Saif, Robert A. Heckert, Kathy L. Miller, and Mohamed M. Tarek

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio 44691, Ohio; and Department of Parasitology and Microbiology (M. M. T.), Faculty of Medicine, Zagazig University, Zagazig, Egypt

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 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 polyadenylated 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
Incubator, 32°F, National Appliance
Incubator, CO₂, model 2220, Queue Systems
Waterbath, no. 66648, Precision Scientific
Laminar flow hood, Edgegard, Baker Company
Wheaton Roller Culture Apparatus, Wheaton
Seitz filter, Republic
Millipore Filter, Millipore

B. Chemicals

Mycostatin (Nystatin) 500 000 U, Squibb
Penicillin G potassium NDC 0995-0510-95, Pfizer
Dihydrostreptomycin sulfate 100405, NB Co.
MEM medium, no. 410-1500, GIBCO
NaHCO₃, no. S-233, Fisher Scientific
HCL, no. HX0603-3, Matheson Coleman & Bell
NaHCO₃, no. S-233, Fisher Scientific
HCL, no. S-271
KCL, no. P-217
MgSO₄ · 7H₂O, no. M-63
CaCl₂, no. C-614
Na,HPO₄ · 7H₂O, no. S-373

©1988 Tissue Culture Association, Inc.
KH₂PO₄, no. 73351, Merck
Chloroform, no. C-29813
Dextrose, Anhydrous, Granular, no. DV145 CB361
Disodium salt of ethylenediamine tetraacetate acid
(Versene) no. S-368
Trypsin (1:250) no. 840-72501M
Neutral Red, no. B360
Phenol red sodium salt, no. P-391
Pancreatin 4X NF, 2.5% {10)<)1~
NaH₂PO₄·H₂O, no. S-368
Agar Noble, no. 0142-01, Difco
Supplies
Tissue culture cluster, no. 3506, Costar
Roller Bottle™
Fetal Bovine Serum (FBS ~), Qualified, 200-2640 AJ
Kidney (MDBK), Salisbury
D. Cells
Madin-Darby Bovine Kidney (MDBK), Salisbury Laboratories

III. PROCEDURE

A. Preparation of solutions

1. Antibiotic stock solution (100X)

   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.

2. 0.01 M Phosphate buffered saline (PBS) (0.14 M NaCl, pH 7.4)

   a. Dissolve the following up to 2 liter in 2X distilled water DH₂O.
   b. Sterilize by autoclaving 15 min, slow exhaust

3. Pancreatin 4X N/F

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

4. Sodium bicarbonate (NaHCO₃) solution.

   a. 7% Solution is routinely used in our laboratory.
   i. Add 70 g NaHCO₃ to a 2000-ml Erlenmeyer flask.
   ii. Add 930 ml of distilled water (DH₂O).
   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 CO₂. 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
      ii. Dissolve the following up to 2 liter in 2X distilled water DH₂O.
      iii. Combine these two solutions in a 1000-ml graduate cylinder, and make up to 1000 ml with DH₂O.
      iv. Add 2 ml of chloroform as a preservative.
      v. 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:
      ii. Dissolve in approximately 800 ml of DH₂O.
      iii. Dissolve 2.8 gms of CaCl₂ in approximately 100 ml DH₂O.
      iv. Combine these two solutions in a 1000-ml graduate cylinder, and make up to 1000 ml with DH₂O.
      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.

   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 DH20
- Plus about 0.6 ml of 1N 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 1N 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 NaHCO3 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
NaHCO3 0.58 g
Trypsin (1:250) 0.5 g
porcine parvovirus tested
Versene 0.2 g
Dextrose 1.0 g
DH20 1000 ml
0.20-μm Seitz filter to sterilize, and store frozen.

8. Growth medium
a. MEM (prepared without NaHCO3 before filtering)
b. 5% fetal bovine serum (FBS)
c. 2% NaHCO3
d. 1% antibiotics (100X)
e. 0.1% (0.1 N) HCl

9. Diluent no. 5.
a. MEM
b. 1% NaHCO3
c. 1% antibiotics (100X)

10. Agar overlay medium. Measure out 1.6% Noble agar, mix with DH20, and boil to melt for 20 min, then cool to 42°C and add equal volume of 2X MEM plus:
- 3% NaHCO3 (7% stock)
- 1% antibiotics (100X)
- 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 DDH2O.
b. Filter 2X MEM through 0.22-μm Millipore filter
c. Store 2X 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-cm² 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 10⁷ 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 (1X) 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°C. Titer virus in plaque assay. Generally this procedure results in virus titers of 10⁷ to 10⁸ 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
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—MBK 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 MBBK cell monolayers 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% CO₂ 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% CO₂ 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 (Eagle'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 10⁶ to 10⁸, 50% tissue culture infective dose (TCID₅₀/0.1 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 adsorption 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), MBBK, 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 MBBK 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 10⁵ and 10⁶ TCID₅₀/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 MBBK 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 (PKK) 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 10⁶ TCID₅₀/ml, were achieved but no CPE was observed. They also showed that the PKK-adapted virus multiplied in bovine skin cells, bovine lung cells, MBBK 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
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 cell strain of BCV in bovine embryonic lung cells (Eagle's minimal essential medium, 0.22% sodium bicarbonate, 20 mM 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 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. Trypsin treatment of cells might promote attachment of virosomes 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, Vero, 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 MDBC 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.

V. REFERENCES
1. Akashi, H.; Inaba, Y.; Miura, Y., et al. Properties of a coronavirus isolated from a cow with epizootic diarrhea. Vet. Microbiol. 5:265-276; 1980.
2. Akashi, H.; Inaba, Y.; Miura, Y., et al. Propagation of the Kakegawa strain of bovine coronavirus in suckling mice, rats and hamsters. Arch. Virol. 67:367-370; 1981.
3. Barboi, G. H.; Pirvulescu, M. Le test d'inhibition de l'hémagglutination (IHA) pour le diagnostic de l'infection à coronavirus chez les bovins. Rev. Roum. Med.-Virol. 37:253-256; 1986.
4. Baron, S.; McKerlie, M. L. Broadly active inhibitor of viruses spontaneously produced by many cell types in culture. Infect. Immun. 32:449-453; 1981.
5. Bovignat, C.; Nicolas, J. A. Pneumonie a coronavirus chez les bovins: A propos de deux observations. Rec. Med. Vet. 162:1085-1086; 1986.
6. Bridger, J. C.; Gaul, E. O.; Eggleston, S. I. Replication of an enteric bovine coronavirus in intestinal organ cultures. Arch. Virol. 57:43-51; 1978.
7. Cey-Coats, K. St.; Storz, J. Bovine coronavirus cytopathic expression and plaque formation: host cell and virus strain determine trypsin dependence. 6th Annual Conference of Research Workers in Animal Disease; 1987, November 16-17, Chicago Il., Abstract 83.
8. Dea, S.; Roy, R. S.; Begin, M. E. Bovine coronavirus isolation and cultivation in continuous cell lines. Am. J. Vet. Res. 41:30-38; 1980.
9. Debiaggi, M.; Perduca, M.; Romero, E., et al. Phosphatidyl-serine inhibition of OCS and NCDCV coronavirus infectivity. Microbiologia 8:313-317; 1985.
10. Derest, D.; Croux, C. F.; Sabara, M., et al. Preliminary studies of a bovine coronavirus (BCV) antigen responsible for neutralization. Proceeding, Fourth International Symposium on Neonatal Diarrhea, October 5-5, Saskatoon, Saskatchewan, Canada, Veterinary Infectious Disease Organization, 1984:117-132.
11. Dougherty, D. A.,; Storz, J.; Hajer, L., et al. Morphology and morphogenesis of a coronavirus infecting intestinal epithelial cells of newborn calves. Exp. Mol. Pathol. 25:355-370; 1976.
12. Gerna, G.; Cereda, P. M.; Mellevi, G. S., et al. Antigenic and biological relationships between human coronavirus OC43 and neonatal calf diarrhea coronavirus. J. Gen. Virol. 54:91-102; 1981.
13. Hirano, N.; Sada, Y.; Tuchiya, K., et al. Plaque assay of bovine coronavirus in BEK-1 cells. Jpn. J. Vet. Sci. 47:679-681; 1985.
14. Hughes T. K.; Blaklock J. E.; McKerlie M. L., et al. Cell-produced viral inhibitor: possible mechanism of action and chemical composition. Infect. Immun. 32:454-457; 1981.
15. Inaba, Y.; Sato, K.; Kurogi, H., et al. Replication of bovine coronavirus in cell line BEK-1 culture. Arch. Virol. 50:339-342; 1976.
16. Kaye, H. S.; Yarbrough, W. B.; Reed, C. J. Calf diarrhea coronavirus. Lancet II:509; 1976.
17. L’Haridon, R.; Scherrer, R.; Vautheron, J. F., et al. Adaptation d’un isolé de coronavirus entérique bovine à la culture cellulaire et caractérisation de la souche obtenue. Ann. Rech. Vet. 12:243-251; 1981.
18. Langgäpp, T. J.; Bergeland, M. E.; Reed, D. E. Coronavirus enteritis of young calves: virology and pathologic findings in naturally occurring infections. Am. J. Vet. Res. 40:1476-1478; 1979.
19. Laporte, J.; L’Haridon, R.; Bobuleseco, P. In vitro culture of bovine enteric coronavirus (BEV). In: Blouin J. F., Pease, M. L., Flewett, T. H., et al., compilers. Enterites virales chez l’homme et l’animal; viral Enteritis in humans and animals. Thiverval-Grignon, France: l’IN- SSRM 1979; 90:99-102.
20. Laporte, J.; Bobuleseco, P.; Rossi, F. Une lignee cellulare particulierement sensible a la replication du coronavirus enteritique bovin: les cellules HRT 18. C. R. Acad. Sci. 290:623-626, Serie D. 1980.
21. McNulty, M. S.; Bryson, D. G.; Allan, G. M., et al. Coronavirus infection of the bovine respiratory tract. Vet. Microbiol. 9:425-434; 1984.
22. Mebus, C. A.; Stair, E. L.; Rhodes, M. B., et al. Neonatal calf diarrheas: propagation, attenuation, and characteristics of a coronavirus-like agent. Am. J. Vet. Res. 34:145-150; 1973.
23. Moussa, A. Incurred disc from particles associated with a bovine coronavirus growing in Vero and calf testicle cell cultures. Proceeding, 7th International Congress of Virology, August 9-14, Edmonton, Alberta Canada, Abstract B11.11, 1987:110.
24. Phillips, R. W.; Case, G. L. Altered metabolism, acute shock, and therapeutic response in a calf with severe coronavirus-induced diarrhea. Am. J. Vet. Res. 41:1039-1044; 1980.
25. Reynolds, D. J. Coronavirus replication in the intestinal and respiratory tracts during infection of calves. Ann. Rev. Vet. Med. 14:445-446; 1983.
26. Saiff, L. J.; Redman, D. R.; Moorhead, P. D., et al. Experimentally induced coronavirus infections in calves: viral replication in the respiratory and intestinal tracts. Am. J. Vet. Res. 47:1426-1432; 1986.
27. Sato, K.; Inaba, Y.; Kurogi, H., et al. Physico-chemical properties of calf diarrhea coronavirus. Vet. Microbiol. 2.73-81; 1977.
28. Sharpee, R. L.; Mebus, C. A.; Bass, E. P. Characterization of a calf diarrheal coronavirus. J. Vet. Res. 37:1031-1041; 1976.
29. Siddall, S.; Wege, H.; Ter Meulen, V. The biology of coronaviruses. J. Gen. Virol. 64:761-776; 1983.
30. Suir, E. L.; Rhodes, M. S.; White, R. G., et al. Neonatal calf diarrheas: purification and electron microscopy of a coronavirus-like agent. Am. J. Vet. Res. 33:1147-1156; 1972.
31. Storz, J.; Rott, R.; Kahuzi, G. Enhancement of plaque formation and cell fusion of an enteropathic bovine coronavirus by trypsin treatment. Infect. Immun. 31:1214-1222; 1981.
32. Stott, E. J.; Thomas, L. H.; Bridger, J. C., et al. Replication of a bovine coronavirus in organ cultures of foetal trachea. Vet. Microbiol. 1.65-69; 1976.
33. Sturman, L. S.; Homes, K. V. The molecular biology of coronaviruses. Adv. Virus Res. 28:35-112; 1983.
34. Takahashi, E.; Inaba, Y.; Sato, K., et al. Epizootic diarrhoea of adult cattle associated with a coronavirus-like agent. Vet. Microbiol. 5:151-154; 1980.

35. Tektoff, J.; Cauvergne, M.; Durafour, M., et al. Propagation of bovine coronavirus on Vero cell line: electron microscopic studies. In: International Symposium on Enteric Infections in Man and Animals: Standardization of Immunological Procedures. Dublin Ireland Develop. Biol. Standard. 53:299-310; 1982. Basel: S. Karger; 1983.

36. Tektoff, J.; Dauvergne, M.; Durafour, M., et al. Propagation of bovine enteric coronavirus in three cell systems: electron microscopic studies. Proceeding of the Fourth International Symposium on Neonatal Diarrhea. Veterinary Infectious Disease Organization; 1984:134-153.

37. Thomas, L. H.; Gourlay, R. N.; Stott, E. J., et al. A search for new microorganisms in calf pneumonia by the inoculation of gnotobiotic calves. Res. Vet. Sci. 33:170-182; 1982.

38. Toth, T. E. Trypsin-enhanced replication of neonatal calf diarrhea coronavirus in bovine embryonic lung cells. Am. J. Vet. Res. 43:967-972; 1982.

39. Tyrrell, D. A. J.; Almeida, J. D.; Berry, D. M.; Coronaviruses. Nature 220:650; 1968.

40. Tyrrell, D. A. J.; Almeida, J. D.; Cunningham, C. H., et al. Coronaviridae. Intervirology 5:76-82; 1975.

41. Tyrrell, D. A. J.; Alexander, J. D.; Almeida, J. D., et al. Coronaviridae: second report. Intervirology 10:321-328; 1978.

42. Vautherot, J. Plaque assay for titration of bovine enteric coronavirus. J. Gen. Virol. 56:451-455; 1981.

43. Weller, T. H.; Enders, J. F.; Robbins, F. C., et al. Studies on the cultivation of poliomyelitis viruses in tissue culture. I. The propagation of poliomyelitis viruses in suspended cell culture of various human tissues. J. Immunol. 69:645-671; 1952.

1 Scientific Industries, Inc., Bohemic, NY
2 National Appliance Co., Portland, OR
3 Queue Systems, Parkersburg, WV
4 Precision Scientific Co., Chicago, IL
5 Baker Co., Inc., Sanford, MA
6 Wheaton, Millville, NJ
7 Republic Seitz Filter Corp., Milford, CT
8 Millipore, Bedford, MA
9 Squibb & Sons, Inc., Princeton, NJ
10 Pfizer, Inc., New York, NY

11 NB Co. Biochemicals, Cleveland, OH
12 GIBCO, Inc., Grand Island, NY
13 Fisher Scientific Co., Fairlawn, NJ
14 Matheson Coleman and Bell, Los Angeles, CA
15 Merck & Co., Inc., Rahway, NJ
16 DIFCO Laboratories, Detroit, MI
17 Costar, Cambridge, MA
18 Falcon, Becton Dickinson Co., Oxnard, CA
19 Salisbury Laboratories, Charles City, IA