CULTIVATION TECHNIQUES FOR ANIMAL CORONAVIRUSES: EMPHASIS ON FELINE INFECTIOUS PERITONITIS VIRUS, CANINE CORONAVIRUS, TRANSMISSIBLE GASTROENTERITIS VIRUS, AND PORCINE HEMAGGLUTINATING ENCEPHALOMYELITIS VIRUS

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SUMMARY: Techniques are described for the growth and characterization of some mammalian coronaviruses. Because of the fastidious nature of their growth requirements, most will replicate only in cells derived from the natural host or a closely related species. Fetal cat cells are used to grow FIPV, and porcine cells are used to grow TGEV and HEV. However, CCV will replicate in both feline and canine cells. Although all four of these viruses prefer to replicate in a cell in the stationary phase of growth, FIPV is able to replicate in an actively growing cell. Each virus causes a cytopathic effect in monolayer cell cultures under agar or media 18 to 72 h postinfection. Primary isolation of each virus from field specimens is difficult, although most can usually be isolated after 1 to 3 blind passages in the cell culture.

Key words: enteric cell lines; isolation; growth requirements; coronavirus.

I. INTRODUCTION

The Coronaviridae family of viruses has a worldwide distribution. These viruses cause economically important diseases in man and in domestic and laboratory animals [33]. At the present time the group consists of 14 recognized viruses and 4 or more unclassified isolates. The viruses and their abbreviations are listed in Table 1. These viruses comprise four distinct antigenic groups plus a miscellaneous group [35]. Generally these viruses infect epithelial cells of the respiratory tract [human coronavirus (HCV), infectious bronchitis virus (IBV), rat coronavirus (RCV), porcine respiratory coronavirus (PCV)] and epithelial cells of the gastrointestinal tract [bovine coronavirus (BCV), canine coronavirus (CCV), transmissible gastroenteritis virus (TGEV), turkey coronavirus (TCV), feline enteric coronavirus (FECV), human enteric coronavirus (HECV)]. In addition, feline infectious peritonitis virus (FIPV), mouse hepatitis virus (MHV), rabbit coronavirus (RbCV), and hemagglutinating encephalomyelitis virus (HEV) exhibit other tissue tropisms and alternate pathogenic mechanisms [40]. The feline, murine, and avian coronaviruses will usually cause a mild or inapparent infection in adults, but usually a severe diarrheal disease and often death in neonatal animals [40]. Basic studies on these viruses have been limited because of their fastidious growth in cell culture [14]. Most coronaviruses will grow only in cells derived from the natural host animal or in cells from a closely related species [33,35]. Growth of HCV, HECV, and RbCV in cell culture is difficult, whereas MHV and CCV are capable of growing to high titers in more than one cell line [14]. Within the last few years several members of the coronavirdae family (MHV, IBV, TGEV) have been studied intensively and serve as models for the molecular biology of the group [7,20,32].

II. MATERIALS

A. Equipment

Water jacketed incubator, model 3158, Forma[1]

Centrifuge, model UV, IEC[2]

Laminar flow hood, Labgard model, NuAire[3]

Pipet-aid, tissue culture model, Drummond Scientific[4]

Freezer, biological to $-85°$ C, no. 8339[1]

Freezer, liquid nitrogen, model LR-35-9, Union Carbide[5]

B. Chemicals

Eagle's MEM with Earle's additives, no. 410-1500, GIBCO[6]

Leibovitz L15, no. 430-1300[6]

Sodium pyruvate, no. 320-1360[6]

Lactalbumin hydrolysate, no. 320-1250[6]

Gentamycin sulfate, 50 mg/ml, Schering[7]

Sodium bicarbonate, no. 810-1810[6]

Trypsin (1:250), no. 840-7250[6]

Ethylenediamine tetraacetic acid (EDTA), no. 890-1266[6]

Fetal bovine serum, no. 309-6309[6]

Glucose, no. 820-5023[6]

Potassium chloride, no. P 5405, Sigma[8]

Phenol red, no. 870-1160[6]

TABLE 1

CORONAVIRUSES AND THEIR GROWTH IN TISSUE CULTURE

Group[a]	Virus	Tissue culture	References
I.	HCV-229E	1°/2° human embryo cells	Schmidt et al. (34)
	TGEV	2° ST	McClurkin and Norman (22)
	CCV	2° fetal cat	Crandell et al. (9)
		canine A-72	Binn et al. (5)
	FIPV	2° fetal cat	Pedersen and Black (28)
	FECV	2° fetal cat	Pedersen et al. (29)
	PCV	1° pig kidney	Pensaert et al. (31)
II.	HCV-OC43	1°/2° human trachea tissue	Macnaughton and Madge (21)
	MHV	1°/2° mouse embryo	Davis and Macnaughton (10)
	HEV	1°/2° porcine embryo	Andries (2)
	BCV	1°/2° bovine embryo	Mebus et al. (23)
	RbCV	unknown	Small and Woods (37)
III.	IBV	embryonated eggs	Cavanagh (8)
IV.	TCV	embryonated eggs	Adams and Hofstad (1)
Misc	HECV	unknown	Payne et al. (27)
	RCV	1° rat cells	Bhatt et al. (4)
	PCV	embryonated eggs	Hirai et al. (13)
	Foal enteritis coronavirus (FCV)	unknown	Bass and Sharplee (3)

[a]Antigenically cross-reacting viruses: 1° = primary cell cultures: 2° = secondary cell cultures.

C. Supplies

McClurkin swine testes cells (ST) (22) Ames, IA

Crandell feline kidney cells (CRFK) (9), ATCC CCL 94, ATCC[9]

Secondary porcine thyroid cells (SPTh) (2), Ames, IA

Feline infectious peritonitis virus (FIPV) (30), UCD-1, Davis, CA

Canine coronavirus (CCV) (43), UCD-1, Davis, CA

Transmissible gastroenteritis virus (TGEV) (12), Wooster, OH

Hemagglutinating encephalomyelitis virus (HEV) (25), 67N, Ames, IA

Tissue culture flasks, 75 cm², Falcon[10]

Pipettes, plastic[10]

Millex filters, 0.22 μm, Millipore[11]

III. PROCEDURE

A. Preparation of solutions

1. Cell culture

   All procedures should be performed under sterile conditions in a laminar flow hood. Standard sterilization techniques are used for all glassware, instruments, and media.

2. Trypsin-ethylenediamine tetraacetic acid solution (TV)

   a. To a 1-liter volumetric flask, add 8.0 g NaCl, 0.4 g KCl, 1.0 g D-glucose, 0.58 g NaHCO₃, 1.0 g trypsin, 0.4 g EDTA, 0.2 g phenol red, and 1-liter double-glass distilled water (DGDW). Adust pH to 7.0 using 1.0 N HCl or 1.0 N NaOH solution.

   b. Filter through a 0.22-μm filter.

   c. Store in aliquots of 10 to 20 ml frozen at −20° C.

3. Eagle's MEM (EMEM)

   a. In 1 liter DGDW dissolve 1 packet of EMEM dehydrated medium and add, 2.2 g NaHCO₃, 0.1 g sodium pyruvate, and 2.2 g lactalbumin hydrolysate, and adjust pH to 7.0 with 1.0 N HCl or 1.0 N NaOH solution.

   b. Filter through a 0.22-μm filter.

   c. Store in aliquots of 90 and 900 ml at 4° C.

4. Leibovitz (L15)

   a. In 1 liter DGDW dissolve 1 packet of L15 dehydrated medium with L-glutamine. Adjust pH to 7.0 with 1.0 N HCl or 1.0 N NaOH solution.

   b. Filter through a 0.22-μm filter.

   c. Store in aliquots of 90 ml at 4° C.

5. Cell culture and virus growth media

   a. To 90 ml of EMEM add 10 ml of heat-inactivated (56° C for 30 min) fetal bovine serum (FBS); use for the cultivation of swine testis (ST) and secondary porcine thyroid (SPTh) cells.

   b. To 100 ml of EMEM add 1.0 ml of gentamycin; use for the growth of TGEV and HEV.

   c. Combine 50 ml of EMEM and 50 ml of L15 medium, add 15 ml of heat-inactivated FBS and 1.0 ml of gentamycin; use for the cultivation of CRFK cells.

   d. Combine 50 ml of EMEM and 50 ml of L15 medium, add 2 ml of heat-inactivated FBS and 1.0 ml of gentamycin; use for the growth of FIPV and CCV.

C. Preparation of cell cultures

1. Cell passage

   a. Aspirate the medium off 3- to 5-d-old confluent cultures of ST or SPTh cells and add 3 ml of TV solution to each 75-cm² flask. Incubate the cells
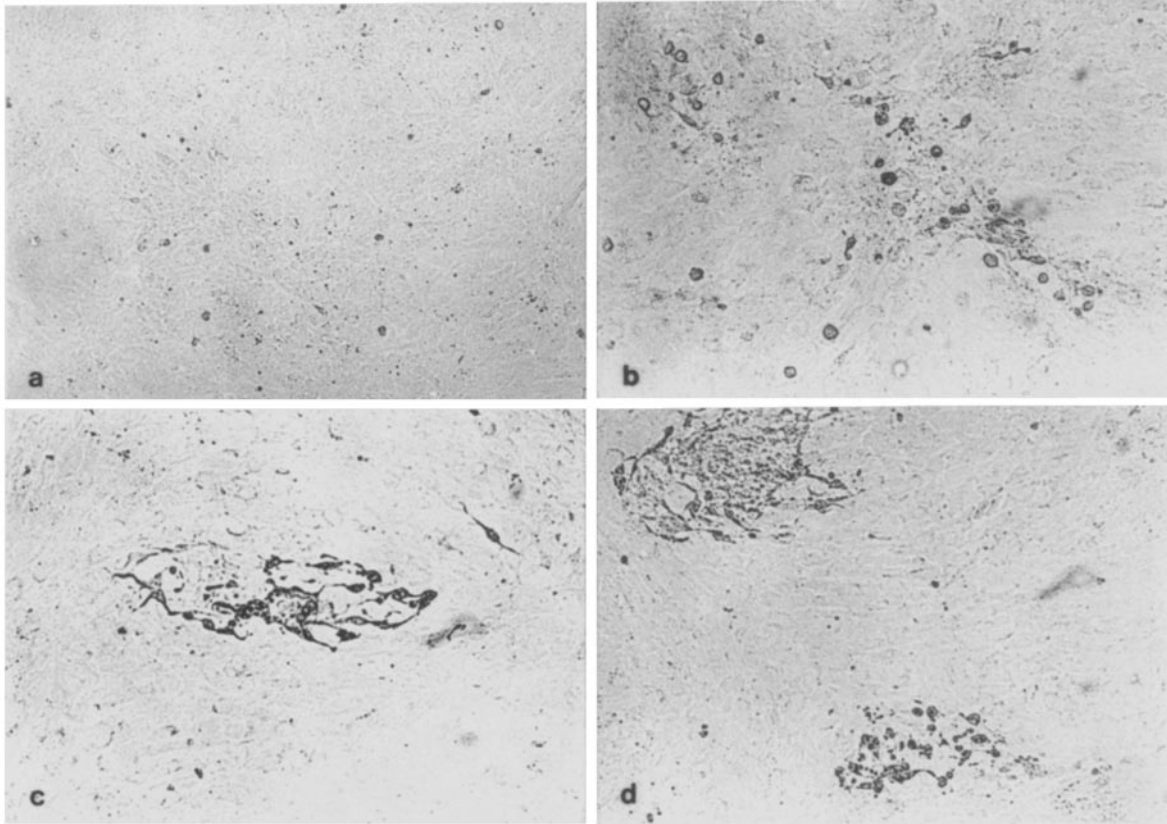
FIG. 1. *a*, Uninfected 5-d-old ST cells. ×70. *b*, Uninfected 5-d-old SPTh cells. ×120. *c*, TGEV-infected ST cells 18 h PI. ×70. *d*, HEV-infected SPTh cells 24 h PI. ×120. (Fig. 2 *b* and *d* courtesy of W. L. Mengeling, Ames, Iowa.)

and TV solution at room temperature for 5 min. After incubation, pour off the TV solution and return the cells to the 37° C incubator for 2 h. After incubation, resuspend the detached cells from one 75-cm² flask in 100 ml of EMEM culture medium and distribute to five new 75-cm² flasks (20 ml/flask). This gives a seeding ratio of 1:5 (approx. 6 to 8 million cells/flask). After seeding, incubate the flasks at 37° C in a humidified incubator with 97% air:3% CO_2. Confluent monolayers of ST or SPTh cells are obtained within 5 d (Fig. 1 *a*, *b*).

b. Aspirate the medium in one flask of 3- to 5-d-old confluent CRFK cells and replace with 3 ml of TV solution. Incubate the cells and TV solution for 2 min at 20° C. After incubation, pour off the TV solution and return the flask to the 37° C incubator for 30 min. After incubation, resuspend the detached cells in 100 ml of EMEM-L15 culture medium and distribute to five new 75-cm² flasks (20 ml/flask). This gives a seeding ratio of 1:5 (approx. 4 to 5 million cells/flask). Confluent CRFK monolayers are obtained within 3 d (Fig. 2 *a*).

D. Virus growth
  1. Virus
     Original stock viruses are maintained in glass, sealed ampules in liquid nitrogen. Stock working virus is within 10 passes of original source.

Working virus is frozen in EMEM with 20% FBS and maintained at −75% C.

2. Virus growth
  a. Remove one tube of FIPV and one tube of CCV from the −75° C freezer and thaw at 37° C. Remove two 75-cm² flasks of confluent (5-d-old) CRFK cells from the incubator and aspirate the spent medium. Rinse the cells twice with EMEM-L15 virus growth medium. Inoculate one flask with FIPV at a multiplicity of infection (MOI) of 0.05 to 0.5 plaque forming units (pfu)/cell (1 to 5 ml of virus fluids) and inoculate the other flask with CCV at an MOI of 0.1 to 1.0 pfu/cell (1 to 5 ml of virus fluids). Return the flasks to the 37° C incubator for 1 h. After incubation, remove the viral fluids from each flask and replace with 10 ml of EMEM-L15 virus growth medium. Incubate the flask at 37°C until a viral-induced cytopathic effect (CPE) is observed. After CPE appears on approximately 25% of the cell sheet (Fig. 2 *b*, *c*), freeze the flask at −75° C. Under the conditions described above, FIPV will have a titer of approximately 1 to 2 × 10⁵ pfu/ml whereas CCV will have a titer of approximately 5 × 10⁶ pfu/ml.

To obtain 5- to 10-fold higher titers of FIPV, the virus can be grown in CRFK cells while they are forming a monolayer. To do this, add 1 to 3 ml of FIPV fluid (MOI of 0.05 to 0.5 pfu/cell)
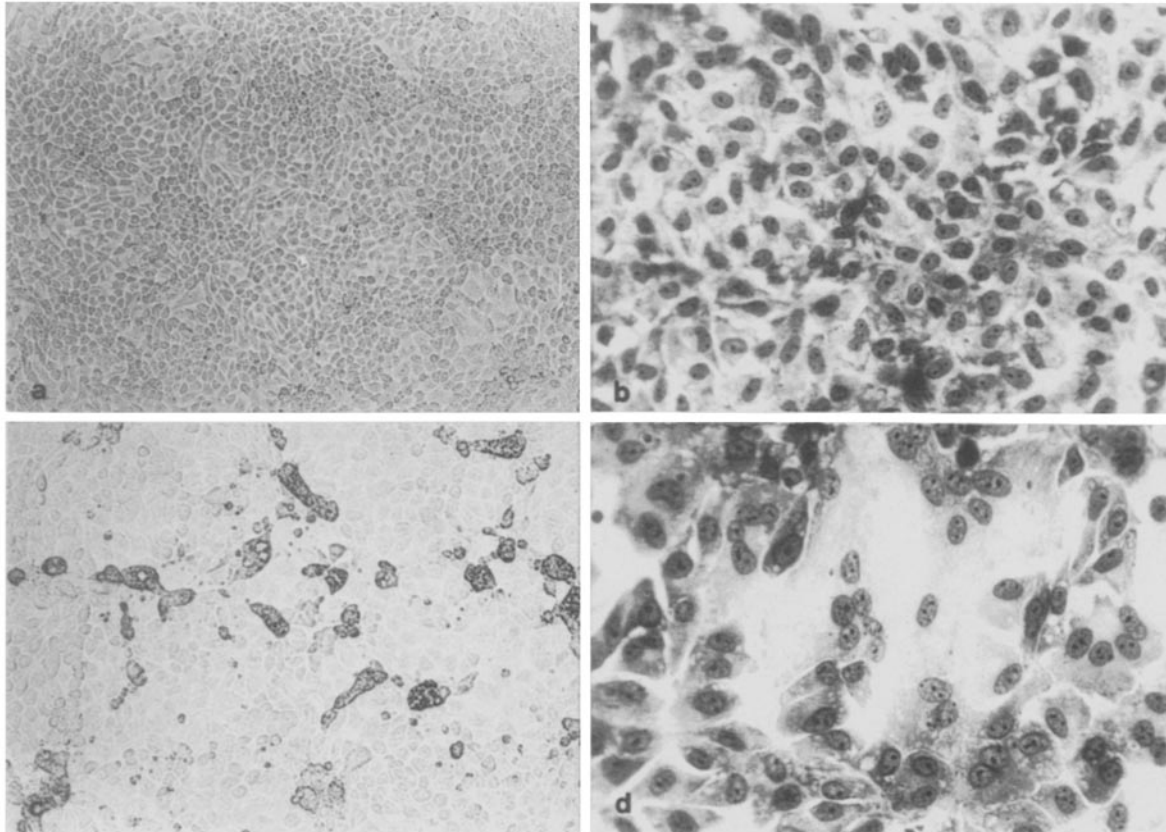
FIG. 2 a, Uninfected 3-d-old CRFK cells. b, FIPV-infected CRFK cells 48 h PI. c, CCV-infected CRFK cells 18 h PI. d, TGEV-infected CRFK cells 30 h PI. ×70.

and trypsin (10 µg/ml) to the cells after seeding. The CRFK cells will establish a confluent monolayer within 3 d, and a viral-induced CPE will be observed 24 h later.

B. Remove one tube of TGEV and one tube of HEV from the −75° C freezer and thaw at 37° C. Remove one flask of 5-d-old ST cells and one flask of 5-d-old SPTh cells from the incubator. Aspirate the spent medium and rinse the cell sheets twice with serum-free EMEM. Inoculate ST flask with TGEV at an MOI of 0.1 to 2.0 pfu/cell (0.1 to 1.0 ml of viral fluids), and inoculate the SPTh flask with HEV at an MOI of 0.1 to 2.0 pfu/cell (0.1 to 1.0 ml of viral fluid). Incubate the flasks at 37° C for 1 h. After incubation, aspirate the inoculum and replace with 10 ml of serum-free EMEM. Return the flasks to the 37° C incubator and observe until approximately 25% of the cell sheet is showing CPE (Fig. 1 c, d). After CPE is observed, freeze each flask at −75° C. When grown under these conditions, TGEV will have a plaque titer of approximately 7×10^7 pfu/ml 18 to 20 h after inoculation, and HEV will have a plaque titer of approximately 3×10^7 pfu/ml 24 to 48 h after inoculation.

IV. DISCUSSION

To study the pathogenesis and molecular biology of coronaviruses, it is beneficial to identify cell lines in which the viruses will grow to titers greater than 5×10^5 pfu/ml, thus providing sufficient viral mass for molecular studies. At the present time, most of the animal coronaviruses have been adapted to cell culture (14,33,35). The methods presented in this report are easy to reproduce and could be adapted by laboratories worldwide for in vitro growth of FIPV, CCV, HEV, and TGEV. Such procedures have been used to grow these viruses to titers ranging from about 1×10^6 pfu/ml for FIPV (43) to 1×10^9 pfu/ml for TGEV (16).

Although most coronaviruses can now be grown in cell culture, their primary isolation from field specimens is still difficult (14). No one isolation technique or method can be used for the entire group. Probably the most successful isolation method is to blindly passage 0.2-µm-filtered fluids 1 to 3 times in a host organ cell line known to support growth of suspected virus, until a CPE is observed. The use of monospecific fluorescent antibodies is recommended to follow in vitro growth of the virus and to confirm virus identity. Several techniques have been used to enhance or improve the chances of isolating these viruses. Some of these techniques include addition of pancreatin (0.5%), trypsin (10 µg/ml), or DEAE-dextran to cell culture media at the time of initial inoculation, maintaining a slightly acidic pH in the culture media, the use of primary cell cultures instead of secondary cultures, and incorporation of high titer hyperimmune antisera in the culture media to suppress the growth of contaminating viruses.

Coronavirus-induced CPE is dependent on the virus, cell line, and isolate. The CPE ranges from an inap-

parent infection in persistently infected cell lines to nearly complete cell disruption with several cell culture-adapted viruses (14,42). In a typical coronavirus-infected cell culture the first sign of CPE will be the appearance of granular and refractile cells, followed by formation of enlarged rounded cells, ballooned cells, and finally the detachment of infected cells from the culture flask (19). When grown under the conditions described, optimal viral titers are usually obtained when approximately 25% of the cell sheet is observed with CPE.

Both FIPV and CCV induce a similar CPE in CRFK cells. Thirty-six to 48 h postinfection (PI), FIPV-infected CRFK cells are granular and refractile. Over the next 24 h small multinucleated (8 to 10 nuclei) cells are formed by fusion and then they detach from the flask (9). The CPE is focal and under 0.5% agar-MEM-2% FBS, distinct plaques up to 1 mm in diameter form in the cell sheet in 48 to 72 h. Initial CPE in CCV-infected CRFK appears within 12 h and consist of granular, refractile, and amorphorus multinucleated (greater than 10 nuclei) cells. During the next 12 to 24 h the CPE will spread over the entire sheet producing numerous ballooned cells and finally infected cells detaching from the flask. Under 0.5% agar-MEM, CCV will form distinct plaques 1.5 mm diameter within 48 h (5).

Initial CPE produced by attenuated and virulent TGEV in ST cells is similar to that observed with FIPV and CCV in CRFK cells, whereas CPE produced by HEV in SPTh cells is slightly different. Sixteen to 18 h PI, TGEV infected ST cells are granular, refractile, and greatly enlarged. Over the next 12 to 16 h the infection will spread over the cell sheet, and with advancing infection the round cells become ballooned and detach from the flask. Under 0.5% agar-MEM, attenuated TGEV produces clear uniform plaques up to 3 mm in diameter within 48 h, whereas the virulent virus produces diffuse, irregular plaques approximately 0.5 to 1 mm in diameter within 48 h (19,42). In 24 to 48 h PI, HEV infected SPTh cells the CPE will show small areas of syncytia, which are easily visible with an inverted microscope. Over the next 24 h the syncytia degenerates, producing syncytial debris in the culture medium and clear holes with an opaque irregular shape in the cell sheet (2). After disruption of the syncytia, ballooned structures appear and the formation of new syncytia is seldom observed. A hemadsorption plaque assay has been developed for titration of infectivity of HEV (24).

The CRFK cell line has been used for the primary isolation and growth of FIPV. Isolation of FIPV from clinical specimens is very difficult using cell lines. However, virus can frequently be recovered if CRFK cells are inoculated and blindly passaged 1 to 3 times in the presence of trypsin (43). The incorporation of trypsin into the FIPV growth medium enhances both the isolation and growth of FIPV. The reason for this enhancement is unknown, but a similar observation has been recorded for BCV, IBV, and MHV (39,40). In addition to CRFK cells, a fetal cat whole fibroblast (FCWF) cell line will support the growth of FIPV, as well as FECV, CCV, and TGEV (28,43), and com-

parison of antigenic and serologic relatedness of the enteric coronaviruses was done in this cell line (43). One to three cell culture passages of virulent FIPV in CRFK or FCWF cells do not destroy its virulence for susceptible cats. A vaccine produced with a CRFK cell culture attenuated strain was unable to protect cats against the original virulent strain, although vaccinated cats do develop a neutralizing antibody response (27). Using cDNA clones of cell-culture-adapted FIPV, the complete nucleotide sequence of the FIPV peplomer protein has been determined (15).

The CRFK and A-72 (5) cell lines can be used for the isolation and propagation of CCV. This virus will grow to a titer of 1×10^7 pfu/ml or higher in either cell line. In addition, CCV can be adapted to grow in FCWF and ST cells. However, neither CCV nor FIPV will grow on primary isolation in ST cells, and this property may allow one to biologically differentiate these antigenically related coronaviruses. A single passage of CCV in CRFK cells does not decrease the infectivity for the natural host; however, prolonged serial passage in any cell line may result in an attenuated virus that grows well in cell culture but is avirulent for dogs. A cell-culture-adapted CCV vaccine has been prepared and provides dogs vaccinated either i.m. or s.c. with neutralizing antibodies that are protective against a virulent CCV challenge (11).

The SPTh cell line can be used for the primary isolation of virus from clinical specimens and for the in vitro growth of HEV (2). Although virus grown in cell culture is still infective for pigs, it is less virulent than field strains (W. L. Mengeling, Ames, IA, personal communication). No vaccine is currently available for this virus.

The ST cell line has been used for more than 20 yr to grow TGEV (22). This cell line can be used for primary isolation of virus from clinical specimens and in vitro growth of TGEV (10). Virus from clinically positive animals will usually have a titer near 1×10^5 pfu/ml, and after 1 or 2 passages in ST cells it may have a titer of 1 to 2×10^7 pfu/ml. Passage of the virus in serum-free media in 5-d-old ST cells at an MOI of 0.5 produces a maximum virus titer 18 to 24 h postinoculation. Sub-passage of the virus under the same conditions in ST cells that are less than 4 d old or more than 7 d old will produce a lower virus titer (38). Cell-culture-adapted TGEV is still infective for pigs after 150 passages in ST cells, but pig virulence is usually reduced after only 10 to 15 passages in ST cells. Three tissue-culture-adapted, modified-live TGEV vaccine strains are sold for use against virulent TGEV in pregnant swine. Two of the vaccine viruses are grown in porcine cell lines, and the source of the third vaccine virus is unknown. Although the safety and ability of these vaccines to elicit virus-neutralizing antibodies in pregnant swine has been proven, their efficacy has been questioned (6,26). The complete nucleotide sequence of the three major structural proteins of TGEV has been determined on cDNA clones of attenuated virus grown in ST cell culture (15,17,18,32).

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[1] Forma Scientific, Marietta, OH
[2] International Equipment Co., Boston, MA
[3] NuAire, Inc., Plymouth, MI
[4] Drummond Scientific Co., Broomall, PA
[5] Union Carbide, Linde Division, New York, NY
[6] GIBCO Laboratories, Grand Island, NY

[7] Schering Corp., Kenilworth, NJ
[8] Sigma Chemical Co., St. Louis, MO
[9] American Type Culture Collection, Rockville, MD
[10] Falcon Division, Beckton Dickinson and Co., Orangeburg, NY
[11] Millipore Corp., Bedford, MA