ELSEVIER

Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Quantitation, Biological and Physicochemical Properties of Cell Culture-adapted Porcine Epidemic Diarrhea Coronavirus (PEDV)

MARTIN HOFMANN and ROBERT WYLER*

Institute of Virology, University of Zurich, Winterhurerstrasse 266a, CH-8057 Zurich (Switzerland)

ABSTRACT

Hofmann, M. and Wyler, R., 1989. Quantitation, biological and physicochemical properties of cell culture-adapted porcine epidemic diarrhea coronavirus (PEDV). *Vet. Microbiol.*, 20: 131–142.

The porcine epidemic coronavirus (PEDV), tentatively classified as a coronavirus, was adapted to Vero cells and a plaque test developed for infectivity titration, allowing us to test the biological and biophysical properties of the virus. Growth kinetics showed peak titers of $10^{5.5}$ plaque-forming units ml^{-1} 15 h after infection. Filtration experiments and electron microscopy revealed a particle diameter between 100 and 200 nm. The buoyant density of the virus was 1.18. The particle lost its infectivity on treatment with lipid solvents. Virus replication could not be inhibited by 5-iodo-2'-deoxyuridine. PEDV was moderately stable at 50°C, but heat sensitivity was not altered by divalent cations. At 4°C, the virus was stable between pH 5.0 and 9.0, but at 37°C stability was restricted to the pH range 6.5–7.5. Viral infectivity was not impaired by ultrasonication or by multiple freezing and thawing. PEDV was not neutralized by transmissible gastroenteritis virus antiserum. On the basis of the tests carried out, PEDV is a pleomorphic, enveloped RNA virus with a particle diameter of ∼ 150 nm and a buoyant density of 1.18. Infectivity depends on the presence of trypsin, and infected cells show a tendency to fuse and to form syncytia. All of these properties, as well as its physicochemical characteristics, allow PEDV to be classified as a coronavirus.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhea virus (PEDV), the first a true and the second a probable member of the coronavirus family, cause severe diarrhea in pigs (Pensaert and Debouck, 1978; Debouck and Pensaert, 1980; Debouck et al., 1981; Pensaert, 1981; Witte et al., 1981; Kielstein and Wohlfarth, 1987). The respective infections cannot be dif-

---

*Author to whom correspondence should be addressed

ferentiated clinically (Wood, 1980) and for an etiological diagnosis it is necessary to isolate and characterize the corresponding virus.

TGEV has successfully been adapted to various cell culture systems and therefore has been fully characterized as a typical coronavirus (Witte and Easterday, 1967; Witte, 1971; Pocock and Garwes, 1975; Garwes, 1982; Jiménez et al., 1986; Komaniwa et al., 1986). Due to electron microscopic evidence, PEDV has been deemed to be a coronavirus replicating in the cytoplasma of enterocytes of the small intestine of pigs (Pensaert, 1981; Witte et al., 1981). Propagation of PEDV in Vero cells has recently become possible (Hofmann and Wyler, 1988).

The present report describes a method for the quantitation of PEDV using a novel, simplified plaque test for titration and gives an account of the physicochemical characteristics of PEDV.

MATERIALS AND METHODS

Cells and media

The continuous Vero cell line (ATCC) was used for adaptation and propagation of PEDV. Cells were grown in Eagle's minimal essential medium (EMEM) (Amimed, Basle, Switzerland), buffered with 30 mM Hepes, supplemented with 10% heat-inactivated fetal calf serum (FCS) (North American Biologicals, Miami, FL, U.S.A.), 0.3% (w/v) tryptose phosphate broth (Difco Laboratories, Detroit, MI, U.S.A.) and antibiotics (100 IE penicillin and 100 μg streptomycin ml^{-1}). Medium for virus replication (VM) consisted of EMEM with the same supplements plus 10 μg ml^{-1} trypsin (1:250, Difco Laboratories, Detroit, MI, U.S.A.), but containing no FCS.

Viruses

The German isolate V215/78 of PEDV (supplied by Prof. K.H. Witte, Arnsberg, F.R.G.) was adapted to Vero cell culture as described previously (Hofmann and Wyler, 1988) and serially passaged in our laboratory. For the present experiments, a stock of PEDV was used at its 26th Vero cell passage [$10^{5.5}$ plaque-forming units (PFU) ml^{-1}].

Pseudorabies virus (PRV), strain ER 217 (supplied by the Eidgenössisches Vakzine-Institut, Basle, Switzerland) served as control in nucleic acid determination studies and was used at its second Vero cell passage.

Serial virus propagation

PEDV-containing cell culture fluid from the preceeding passage was clarified by low-speed centrifugation (1000$\times$$g$ for 10 min) and diluted 1:10 in VM.

Confluent monolayers of Vero cells in 150-cm^2 cell culture flasks (Corning Glassworks, Corning, NY, U.S.A.) were washed twice with VM to remove residual growth medium. The cells were then inoculated with 5 ml of a diluted viral inoculum. The virus was adsorbed for 2 h at room temperature in the dark by slowly moving the flasks on a orbital shaker (0.5 r.p.m.) After adsorption, 35 ml of VM was added to each flask without removing the primary inoculum. The infected cells were subsequently incubated at 37°C until the cell layer showed a 100% cytopathic effect. Cell cultures serving as controls were treated in the same way, but were mock inoculated with 5 ml VM instead of the viral inoculum.

Electron microscopy

Samples of 50 μl each were placed on parlodion-coated grids for 5 min. After adsorption, grids were negatively stained with 2% phosphotungstic acid, pH 6.6, and subsequently examined using a Philips EM201 electron microscope.

Immunofluorescence studies

PEDV-containing cells were identified by fluorescent antibody staining. Infected monolayers, grown in slide flasks (Nunc, Denmark) were fixed 22 h after inoculation with 100% ethanol for 20 min at 4°C. Then they were incubated with an anti-PEDV–FITC conjugate (provided by Prof. M. Pensaert, Ghent, Belgium) for 1 h at 37°C. After three washings in phosphate-buffered saline (PBS, 0.15 M NaCl, 50 mM phosphate buffer, pH 7.4) the prepared samples were embedded in buffered glycerol and examined with a Leitz Ortholux ultraviolet microscope (Leitz, Wetzlar, F.R.G.).

Plaque test for PEDV infectivity titration

Cells for virus titration were grown in 24-well tissue culture plates (Multiwell Falcon 3047, Becton Dickinson Labware, Oxnard, CA, U.S.A.). Forty-eight hours after seeding, confluent monolayers were washed twice with VM prior to inoculation. Serial 10-fold dilutions of PEDV were made in VM and 0.2-ml portions added in duplicate wells. Adsorption was carried out as described for flasks, but the orbital shaker speed was increased to 1 r.p.m. After adsorption, the viral inocula were removed by aspiration and the cell cultures washed once with VM. Then 0.5 ml VM was added to each well and cultures were incubated in a humid chamber at 37°C for 4 h. Another 0.5 ml of VM, supplemented with 4% FCS for blocking the action of trypsin, was added to each well and cells were incubated for 48 h at 37°C. Cells were fixed by adding 1 ml of 4% formaldehyde to each well without removing VM. After 1 h, the supernatants were discharged and the monolayers stained for 10 min with 0.5%

(w/v) cristal violet in deionized water. After washing away the excess stain, plates were air dried and plaques counted microscopically. For the determination of PEDV infectivity titers, wells containing 20–200 plaques were counted and titers expressed as PFU ml^{-1}.

One-step growth curve

For the determination of PEDV growth characteristics, cells were seeded in 16 25-cm^2 flasks and inoculated as described for serial propagation, except that the inoculum consisted of undiluted stock virus. After adsorption, monolayers were washed twice with VM to remove residual unadsorbed virus, then 5 ml of VM were added to the flasks and the cell cultures incubated at 37°C for 0, 2, 4, 6, 8, 10, 12, 15, 18, 21, 24, 27, 31, 36 or 48 h. At the end of the incubation periods, VM was removed, clarified by low-speed centrifugation and stored at 4°C for titration of extracellular PEDV. The pelleted cells were resuspended in 5 ml fresh VM. Cells still adhering to the bottom of the flask were washed twice with VM, scraped off with a rubber policeman, pooled with the resuspended cells, then frozen and thawed three times to release intracellular virus particles. The ensuing suspension was clarified by centrifugation and titrated using the plaque test (see above).

Nucleic acid determination

The type of viral nucleic acid was determined by adding 50 or 200 $\mu g\ ml^{-1}$ 5-iodo-2′-deoxyuridine (IUDR) to the VM. Vero cells were infected with 10 000 PFU ml^{-1} PEDV or 10 000 $TCID_{50}\ ml^{-1}$ PRV, respectively. After virus adsorption, cultures were washed once and VM containing no IUDR, 50 $\mu g\ ml^{-1}$ IUDR or 200 $\mu g\ ml^{-1}$ IUDR was added to the cultures. Uninfected cultures were treated in the same manner with or without IUDR. The cultures were incubated at 37°C for 24 h after infection. Infectivity titers of each sample were determined after cells had been subjected to freezing and thawing three times, and subsequently centrifuged at low speed.

Filtration

A 2-ml sample of stock virus suspension was diluted in 20 ml of VM and filtered through a series of membrane filters of 450-, 200-, 100- and 50-nm pore diameter (Schleicher und Schüll AG, Feldbach, Switzerland), after which the filtrates were titrated.

Sensitivity to lipid solvents

The sensitivity of PEDV to diethyl ether and chloroform was tested according to the procedure described elsewhere (Akashi et al., 1980). Briefly, the

virus was mixed with the lipid solvent, vigorously shaken and left for 5 min at room temperature. The samples were then centrifuged at 10 000×g min and the resulting aqueous phases immediately titrated.

Heat inactivation

The thermostability of PEDV was examined by two different methods. First, 1-ml samples of undiluted virus were incubated in a water bath at 50 ± 0.1°C for 5, 10, 15, 30, 60, 120 or 180 min (all samples were kept on ice before and after heating) and subsequently titrated. An untreated virus control was stored at 4°C during the whole experiment.

Second, 1-ml portions of undiluted virus were incubated for 30 min at 50, 60, 70 or 80°C and further treated in the same way as described above.

Heat stabilization by divalent cations

A 0.5-ml sample of stock virus suspension was mixed with the same volume of VM, distilled water, or 2 M $MgCl_2$ solution (prepared in distilled water). The mixtures were incubated in a water bath at 50°C for 30 min. After heating, mixtures were recooled to 4°C and assayed for infectivity.

pH-dependent thermostability

Stock virus was diluted 1:10 in VM. Volumes of 10 ml of diluted virus were adjusted to pH, 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, 9, 10 or 11 by the addition of 2 N HCl or 2 N NaOH. After pH change, 5 ml of each sample were kept at 4°C for 6 h. The residual 5 ml were incubated in a water bath at 37°C for 6 h. After this time, samples were recooled to 4°C and the pH of each readjusted to 7.2–7.4. Thereafter, each sample was assayed for residual PEDV infectivity.

Resistance to ultrasonication and freezing and thawing

PEDV at its 50th passage in Vero cells was tested for resistance to multiple freezing and thawing cycles. A 1-ml sample of undiluted virus was first frozen to −70°C then warmed to 37°C 10 times, whereas a virus control was stored at 4°C during the experiment. Resistance to ultrasonication was tested by son-icating 10 ml of PEDV diluted 1:10 in VM for 3 min with a Branson sonifier B130 using a power output of 20 W at a frequency of 20 kHz. Overheating was avoided by placing the virus-containing tube in an ice bath and interrupting sonication every minute for 3 min.

Isopycnic density gradient centrifugation

Crude PEDV from infective culture fluid, from which cell debris had been removed by low-speed centrifugation at $4000 \times g$ for 20 min at $4°C$, was pelleted by centrifugation at $85\ 000 \times g$ for 2 h at $4°C$, the resulting pellet resuspended in 1% of the original volume of TNE buffer (0.05 M Tris–HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA) and layered on top of a 10–50% (w/w) continuous sucrose gradient prepared in TNE buffer. The gradient was then centrifuged for 16 h at $85\ 000 \times g$ and $4°C$. The virus-containing fractions (detected by electron microscopy) were pooled and sucrose density was measured with a density meter (DMA 35, Anton Paar, Graz, Austria).

Plaque inhibition test

Two sera from pigs known to contain antibodies to PEDV and one to TGEV were diluted in VM. Each dilution was mixed with 10 000 PFU ml^{-1} of PEDV and incubated overnight at $4°C$. The mixtures were then inoculated onto confluent Vero cell cultures. The plaque inhibition test was further carried out in the same way as described for the plaque test for PEDV infectivity titration, and the antibody titer finally expressed as the reciprocal of the highest serum dilution leading to at least an 80% reduction of the plaque number.

RESULTS

Growth characteristics of cell culture-adapted PEDV

Infected Vero cell cultures showed PEDV-characteristic alterations, as described earlier (Hofmann and Wyler, 1988). Briefly, signs of cytopathic effect (CPE) (characterized by single cells losing their individual demarcation and forming syncytia by fusing with other cells at their periphery) were first seen 9 h after adsorption. Numerous nuclei accumulated in the center of these plaque-like lesions. Within 18 h after adsorption, all the cells were completely fused. Later, nuclei became pycnotic and the whole monolayer thereafter detached from the bottom of the flask. Mock-inoculated control cultures showed no CPE.

Electron microscopic examination of the supernatants of infected cells regularly showed numerous typical coronavirus particles.

Infectivity titration of PEDV

When cell cultures were fixed 48 h after infection, all plaques had diameters of 0.3–0.5 mm and had to be counted microscopically.

The number of plaques in corresponding duplicate wells differed by $<30\%$

if tested in the same assay. When the same PEDV sample was titrated in different assays, results never differed by $> 0.3 \log_{10}$ (data not shown).

Cell culture-grown PEDV always reached titers between $10^{5.3}$ and $10^{6.5}$ PFU ml^{-1}. For most of the characterization experiments described in this paper, the same PEDV stock suspension (Passage 26 on Vero cells) with a titer of $10^{5.5}$ PFU ml^{-1} was used.

Properties of cell culture-adapted PEDV

The replication kinetics are illustrated in Fig. 1. Six hours after adsorption, the amount of cell-bounded PEDV began to increase and reached a peak after 15 h. Release of infectious virus into the medium started 2 h later and the titer was as high as that of the intracellular virus after 10 h.
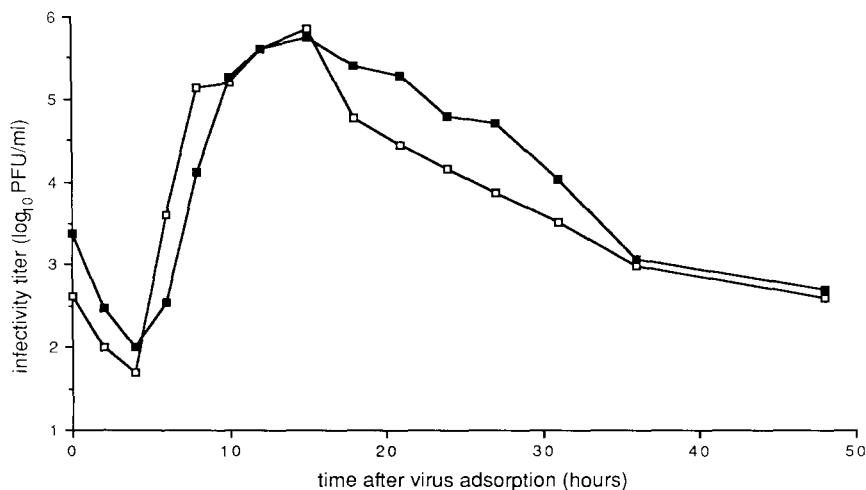

Fig. 1. One-step growth curve of PEDV in Vero cells. □, cell-bound virus; ■, virus in the supernatant.

TABLE 1

Nucleic acid determination of PEDV by the addition of IUDR

Virus	Infectivity titer[1]			Change	
	No IUDR (a)	50 μg ml^{-1} IUDR (b)	200 μg/ml^{-1} IUDR (c)	$a-b$	$a-c$
PEDV	6.7	6.6	5.9	0.1	0.8
PRV	8.0	5.0	4.6	3.0	3.4

[1]Viral titers expressed as log PFU ml^{-1} for PEDV and log $TCID_{50}$ ml^{-1} for PRV, respectively.

The replication of PEDV was not affected significantly by the presence of 50 or 200 μg IUDR ml^{-1} VM (Table 1). On the contrary, the multiplication of PRV, a DNA-containing herpesvirus, was markedly inhibited, depending on the concentration of IUDR in the VM.

PEDV passed membrane filters with a pore diameter of 450 and 200 nm, but was completely retained by one with 100- and 50-nm pore size.

When treated with ether or chloroform for 5 min, PEDV was completely inactivated in both experiments.
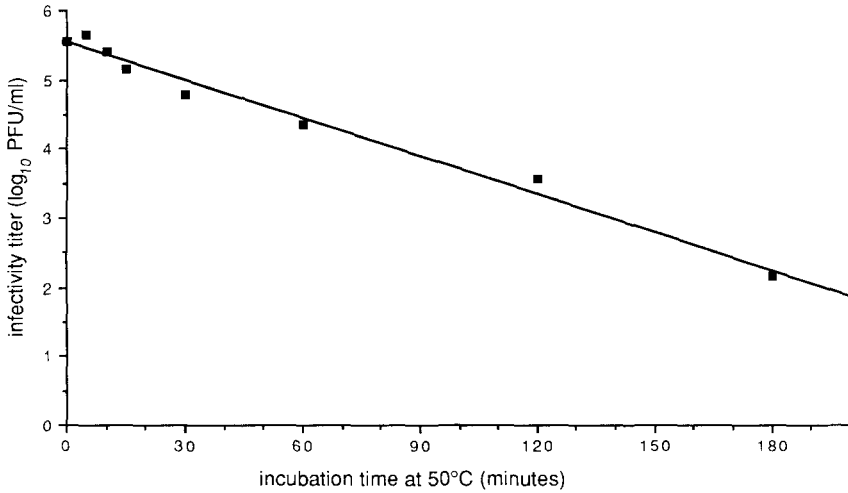

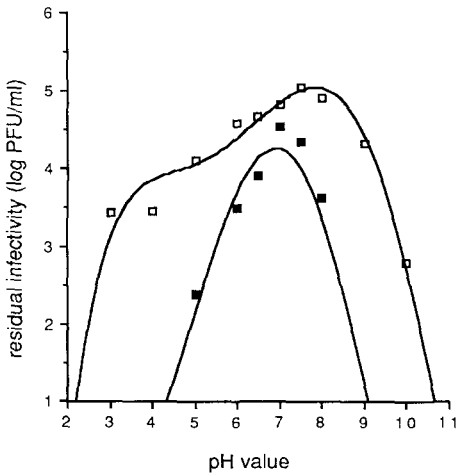Fig. 2. Thermoinactivation kinetics of PEDV at 50°C.

Fig. 3. Thermostability of PEDV at different pH values, when incubated at 50°C for 30 min. □, residual infectivity at 4°C; ■ residual infectivity at 37°C.

PEDV totally lost its infectivity when heated $\geq 60°C$ for 30 min, whereas its titer remained relatively stable when exposed to $50°C$ for the same time (reduction of $0.4 \log_{10}$ PFU ml^{-1}, compared with control).

Thermoinactivation kinetics at $50°C$ are demonstrated in Fig. 2. Viral infectivity decreased steadily for $1.1 \log_{10} h^{-1}$ and was reduced to 0.05% of the original value after 3 h of heating, again indicating that PEDV is moderately stable at $50°C$.

When PEDV was heated to $50°C$ for 30 min after having been diluted in distilled water or fresh VM, respectively, no significant difference in heat inactivation rates was observed. However, when diluted in $MgCl_2$ solution at a final concentration of 1 M, an ~ 100-fold virus inactivation took place.

PEDV was incubated at various pH values for 6 h at 37 or $4°C$. The virus remained relatively stable at $4°C$ over a pH range from 5 to 9, whereas at $37°C$ it retained its infectivity only between pH 6 and 8, and was completely inactivated $<pH 4$ and $>pH 9$ (Fig. 3).

The infectivity of PEDV was not significantly reduced either by freezing and thawing 10 times or by ultrasonication for 3 min.

On three independent centrifugations in a linear sucrose density gradient, PEDV samples pelleted previously by high-speed centrifugation had densities of 1.176, 1.182 and 1.181 in the pooled virus-containing fractions.

PEDV was neutralized in the plaque inhibition test by sera known to contain antibodies to PEDV. The two sera tested had neutralizing titers of 119 and 315. The TGEV antibody-containing serum did not neutralize PEDV even in its lowest dilution (1:4), although it had a high neutralizing effect when tested in the homologous system.

DISCUSSION

The multiplication of PEDV in Vero cells with the aid of trypsin, leading to characteristic cytopathic changes, is a useful method for producing high amounts of virus and also represents a sensitive and effective assay system for PEDV infectivity. During subsequent passage in Vero cells, PEDV was readily adaptable to this cell line and could be serially passaged >50 times without changing its replication pattern.

PEDV does not produce infective virus particles in Vero cells as long as the action of trypsin is inhibited by FCS. Because the primarily produced virions in the plaque-like syncytia are not activated, they are not able to induce secondary plaques. The plaque test described can therefore be used for the quantification of infective virus particles. Additionally, it could be shown that each infectious virus particle adsorbed to the cell layer produces one plaque (data not shown). Reproducibility and accuracy of the plaque test were good. The infectivity titers were in the same range as described for other coronaviruses

(Robb and Bond, 1979b; Schmidt et al., 1979; Vautherot, 1981; Hirano et al., 1985).

Results of IUDR, filtration and inactivation experiments with ether and chloroform confirm that we are dealing with an enveloped RNA virus. As with other coronaviruses, PEDV is rapidly and totally inactivated by heating for 30 min to $60°C$ (Dea et al., 1980a,b; Daniel and Talbot, 1987). PEDV showed a certain resistance to acid pH values, although optimal pH values for virus stability were higher than for other enteropathogenic coronaviruses (Pocock and Garwes, 1975; Sturman and Holmes, 1983).

In contrast to most of the other known coronaviruses but in agreement with the findings concerning the bluecomb coronavirus of turkeys (Deshmukh and Pomeroy, 1974), PEDV thermostability decreased in the presence of 1 M $MgCl_2$ instead of increasing. Surprisingly, PEDV was not inactivated significantly by the addition of distilled water, therefore the virus may be resistant to hypotonic conditions.

PEDV infectivity was not significantly reduced either by ultrasonication or by multiple freezing and thawing. This may be due to the disintegration of virus clumps, thus compensating for the number of virus particles which were inactivated during mechanical treatment.

Isopycnic ultracentrifugation in a linear sucrose gradient resulted in only one distinct virus band, which contained numerous morphologically intact virus particles when examined by electron microscopy. This band had a low buoyant density of 1.18 in sucrose, a value that is typical for coronaviruses (Tyrrell et al., 1978; Siddell et al., 1983). Ultracentrifugation under the described conditions is also a useful method to purify PEDV from host cell fragments.

PEDV was neutralized by both PEDV antibody-containing pig sera when assayed with the plaque inhibition test. TGEV antibody-containing serum, on the other hand, had no neutralizing effect. The plaque inhibition test therefore is well adapted as a neutralization test.

The results of the present biological and physicochemical studies show that PEDV is probably a member of the coronavirus family (Mebus et al., 1973; Hierholzer, 1976; Robb and Bond, 1979a; Caul and Egglestone, 1982; Wege et al., 1982).

Because PEDV is not serologically related to other members of the family coronaviridae (Pedersen et al., 1978; Pensaert et al., 1981), characterization of structural proteins and the genome of PEDV is needed for a definite classification of this virus.

REFERENCES

Akashi, H., Inaba, Y., Miura, Y., Tokuhisa, S., Sato, K. and Satoda, K., 1980. Properties of a coronavirus isolated from a cow with epizootic diarrhea. Vet. Microbiol., 5: 265-276.

Caul, E.O. and Egglestone, S.I., 1982. Coronaviruses in humans, In: D.A.J. Tyrrell and A.Z. Kapikian (Editors), Virus Infections of the Gastrointestinal Tract. Marcel Dekker, New York Basel, pp. 179-193.

Daniel, C. and Talbot, P.J., 1987. Physico-chemical properties of murine hepatitis virus, strain A59. Arch. Virol., 96:241-248.

Dea, S., Roy, R.S. and Begin, M.E., 1980a. Physicochemical and biological properties of neonatal calf diarrhea coronaviruses isolated in Quebec and comparison with the Nebraska calf coronavirus. Am. J. Vet. Res., 41: 23-29.

Dea, S., Roy, R.S. and Begin, M.E., 1980b. Bovine coronavirus isolation and cultivation in continuous cell lines. Am. J. Vet. Res., 41: 30-38.

Debouck, P. and Pensaert, M., 1980. Experimental infection of pigs with a new porcine enteric coronavirus, CV 777. Am. J. Vet. Res., 41: 219-223.

Debouck, P. Pensaert, M. and Coussement, W., 1981. The pathogenesis of an enteric infection in pigs, experimentally induced by the coronavirus-like agent, CV 777. Vet. Microbiol., 6: 157-165.

Deshmukh, D.R. and Pomeroy, B.S., 1974. Physicochemical characterization of a bluecomb coronavirus of turkeys. Am. J. Vet. Res., 35: 1549-1552.

Garwes, D.J., 1982. Coronaviruses in animals. In: D.A.J. Tyrrell and A.Z. Kapikian (Editors), Virus infections of the Gastrointestinal Tract. Marcel Dekker, New York, Basel, pp. 315-359.

Hierholzer, J.C., 1976. Purification and biophysical properties of human coronavirus 229E. Virology, 75: 155-165.

Hirano, N., Ono, K., Sada, Y., Inoue, A., Murakami, T. and Takamuru, H., 1985. Replication of rat coronavirus in a rat cell line, LBC. Arch. Virol., 85: 301-304.

Hofmann, M. and Wyler, R., 1988. Propagation of the virus of porcine epidemic diarrhea (PED) in cell culture. J. Clin. Microbiol., 26: 2235-2239.

Jiménez, G., Correa, I., Melgosa, M.P., Bullido, M.J. and Enjuanes, L., 1986. Critical epitopes in transmissible gastroenteritis virus neutralization. J. Virol., 60: 131-139.

Kielstein, P. and Wohlfarth, E. (Editors), 1987. Schweinekrankheiten. Ferdinand Enke, Stuttgart, pp. 307-312.

Komaniwa, H., Makabe, T., Fukusho, A. and Shimizu, Y., 1986. Isolation of transmissible gastroenteritis virus from feces of diarrheic pigs in roller culture of CPK cells in the presence of trypsin. Jpn. J. Vet. Sci., 48: 1245-1248.

Mebus, C.A., Stair, E.L., Rhodes, M.B. and Twiehaus, M.J., 1973. Neonatal calf diarrhea: Propagation, attenuation, and characteristics of a coronavirus-like agent. Am. J. Vet. Res., 34: 145-150.

Pedersen, N.C., Ward, J. and Mengeling, W.L., 1978. Antigenic relationship of the feline infectious peritonitis virus to coronaviruses of other species. Arch. Virol., 58: 45-53.

Pensaert, M.B., 1981. Porcine epidemic diarrhea., In: H.W. Dunne and A.D. Leman (Editors), Diseases of Swine, 5th Edn. Iowa State University Press, Ames, IA, pp. 344-346.

Pensaert, M.B. and Debouck, P., 1978. A new coronavirus-like agent associated with diarrhea in swine. Arch. Virol., 58: 243-247.

Pensaert, M.B., Debouck, P. and Reynolds, D.J., 1981. An immunoelectron microscopic and immunofluorescent study on the antigenic relationship between the coronavirus-like agent, CV 777, and several coronaviruses. Arch. Virol., 68: 45-52.

Pocock, D.H. and Garwes, D.J., 1975. The influence of pH on the growth and stability of transmissible gastroenteritis virus in vitro. Arch. Virol., 49: 239-247.

Robb, J.A. and Bond, C.W., 1979a. Coronaviridae. In: H. Fraenkel-Conrad and R.R. Wagner (Editors), Comprehensive Virology. Vol. 14. Plenum press, New York/London, pp. 193–247.

Robb, J.A. and Bond, C.W., 1979b. Pathogenic murine coronavirus. I. Characterization of biological behavior in vitro and virus-specific intracellular RNA of strongly neurotropic JHMV and weakly neurotropic A59V viruses. Virology, 94: 352–370.

Schmidt, O.W., Cooney, M.K. and Kenny, G.E., 1979. Plaque assay and improved yield of human coronaviruses in a human rhabdomyosarcoma cell line. J. Clin. Microbiol., 9: 722–728.

Siddell, S., Wege, H. and Ter Meulen, V., 1983. The biology of coronaviruses. J. Gen. Virol., 64: 761–776.

Sturman, L.S. and Holmes, K.V., 1983. The molecular biology of coronaviruses, In:M.A. Lauffer and K. Maramorosch (Editors), Advances in Virus Research. Vol. 28. Academic, New York/London, pp. 35–112.

Tyrrell, D.A.J., Alexander, D., Almeida, J.D., Cunningham, C.H., Easterday, B.C., Garwes, D.J. Hierholzer, J.C., Kapikian, A., Macnaughton, M.R. and McIntosh, K., 1978. Coronaviridae: Second report. Intervirology, 10: 321–328.

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

Wege, H., Siddell, St. and Ter Meulen, V., 1982. The biology and pathogenesis of coronaviruses, In: M. Cooper, W. Henle, P.H. Hofschneider, H. Koprowski, F. Melchers, R. Rott, H.G. Schweiger, P.K. Vogt, R. Zinkernagel (Editors), Current Topics in Microbiology and Immunology, Vol. 99. Springer, Berlin/Heidelberg/New York, pp. 165–200.

Witte, K.H., 1971. Isolation of the virus of transmissible gastroenteritis (TGE) from naturally infected piglets in cell culture. Zentralbl. Veterinaermed. Reihe B, 18: 770–778.

Witte, K.H. and Easterday, B.C., 1967. Isolation and propagation of the virus of transmissible gastroenteritis of pigs in various pig cell cultures. Arch. Virol., 20: 327–350.

Witte, K.H., Prager, D., Ernst, H. and Nienhoff, H., 1981. Die Epizootische Virusdiarrhoe (EVD). Tieraerztl. Umsch., 36: 235–250.

Wood, E.N., 1980. Transmissible gastroenteritis and epidemic diarhoea of pigs. Br. Vet. J., 135: 305–314.