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Propagation of Hemagglutinating Encephalomyelitis Virus in Porcine Cell Cultures

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With 4 figures and 2 tables

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Introduction

Hemagglutinating encephalomyelitis virus (HEV) is a coronavirus (6, 10) that causes vomiting and wasting (4) or encephalomyelitis (1) in young pigs. The virus was first isolated in primary pig kidney cells by GREIG et al. (9), who described a cytopathic effect characterized by the appearance of multinucleated cells. The hemadsorption and hemagglutination tests were also used for the demonstration of viral growth. With the immunofluorescence test, HEV was shown to propagate in several other porcine cell cultures such as adult thyroid gland, embryonic lung, testicle cell line, PK-15 cell line (16), IBRS₂ cell line (5) and SK cell line (14). Non-porcine cell cultures were shown to have little susceptibility for growth of HEV (5, 16).

It was the purpose of the present study to determine which is the most sensitive cell culture system for the isolation of HEV and the optimal test for the demonstration of viral growth. Since non-porcine cell cultures have been shown to support virus growth poorly, only porcine cell cultures were selected for this study: primary pig kidney cells, primary pig testicle cells, secondary pig thyroid cells and the cell lines PK-15 (pig kidney), SK-6 (swine kidney) and ST (swine testicle).

A viral growth curve was made in the different cell cultures using the following criteria: cytopathic effect, immunofluorescence, hemadsorption and hemagglutination. Also, the optimal time to use the different criteria in relation to the virus concentration inoculated was determined. Finally, using the optimal detecting system, attempts were made to determine whether repeated virus quantitation trials gave reproducible results and whether a blind passage is necessary.

Material and Methods

Virus

Two stocks of HEV were used. A tissue culture stock was prepared from the 11th passage on primary pig kidney (PPK) cells of VW 572 virus, a Belgian isolate of HEV

(15). The supernatant of the infected culture was centrifuged for 20 min. at 2,000 g. and stored in 1 ml. portions at -70°C . The stock had a titer of approximately 10^7 50% tissue culture infective doses (TCID₅₀) per ml. when titrated on secondary pig thyroid cells.

A lung homogenate stock was prepared in 3 gnotobiotic piglets which were inoculated at the age of 3 days with the tissue culture stock mentioned above. The piglets were killed 3 days after inoculation and their lungs were ground with Ten Broecks homogenizers to a 20% (w/v) suspension in phosphate buffered saline (PBS). After low speed centrifugation, the infective supernatant fluid was also stored in 1 ml. portions at -70°C .

Cell cultures

Primary pig kidney (PPK) cells were prepared from kidneys taken from 3 to 5 week old pigs, according to standard procedures (13). Cells were grown in Hanks' lactalbumin medium (HLA) consisting of Hanks' balanced salt solution (BSS), with 0.5% lactalbumin hydrolysate (LAH) and 10% calf serum (CS). Monolayers usually became fully sheeted after 4 days and were then inoculated. After inoculation, cells were maintained with minimum Eagle's medium (MEM) containing 0.25% LAH and 5% bovine fetal serum (BFS).

Primary pig testicle (PPT) cells were prepared from testicles of 3 to 5 week old pigs. The cells were grown in the same medium as used for PPK cells, but the medium was replaced after 2 days. At the time they were inoculated, the fully sheeted monolayers were 5 days old. The maintenance medium consisted of MEM supplemented with 0.25% LAH, 5% BFS and 1% pyruvate.

Primary and secondary pig thyroid (SPTh) cells were prepared from the thyroid glands of fattening pigs. The primary cells were seeded into MEM containing 0.5% LAH, 10% CS and 0.11% NaHCO₃. Prescription bottles (120 cm² growth surface) were inoculated with 200,000 cells per ml. and put in a 5 per cent CO₂ incubator. The cells were refed after 4 to 6 days when they became confluent. The monolayers were trypsinized 1 day later into secondary cells, using the same medium, the same cell concentration and the CO₂ incubator as described for the primary pig thyroid cells. The SPTh cells were refed after 3 days and inoculated after 5 days. After the inoculation, the cells were fed as described for the PPK cells but were incubated without CO₂.

PK-15 cells were fully sheeted after 3 days when seeded at a concentration of 120,000 cells/ml. The medium consisted of MEM supplemented with 0.5% LAH and 10 per cent CS for growth medium or 0.25% LAH and 5 per cent BFS for maintenance medium.

SK-6 cells (12) and ST-cells* were seeded at a concentration of 150,000 cells/ml. The growth and maintenance medium were the same as for PK-15 cells. Fully sheeted monolayers were present after 3 days and were then used for inoculation with virus.

Virus growth curve

A growth curve of HEV was prepared with the cell culture virus stock inoculated on SPTh, PPK, PK-15, SK-6, PPT and PT cells. For each cell type, 35 tubes were inoculated with a multiplicity of infection of approximately 0.1 and left for 1 h at 22°C . The monolayers were washed three times with PBS to remove non-adsorbed virus and, after addition of medium, the cells were incubated at 37°C . At daily intervals from 1 to 7 days after the inoculation, the monolayers were examined for the occurrence of cytopathic effect. Each day, the nutrient medium of 5 tubes was pooled, centrifuged (700 g. for 10 min.), and collected for titration of infectivity and hemagglutination (HA). The hemadsorption (Hads) test was performed on the remaining monolayers using MEM with 0.1% chicken red blood cells. After incubation for 30 min. at 22°C , the monolayers were washed once with PBS and examined for evidence of Hads. The erythrocytes for the Hads and HA-tests were collected from chickens between four and twelve weeks of age, according to GIRARD et al. (8). Virus growth was detected by immunofluorescence (IF) on coverslips in Leighton tubes which were inoculated using the same virus dilution as described above. Two infected coverslips were fixed daily in cold acetone and stained with a fluorescent

* Provided by Norden Laboratories Inc., Smith Kline Corporation of Philadelphia, Pennsylvania, U. S. A.

antibody conjugate prepared from a hyperimmune HEV antiserum as previously described (3). Control as well as infected cell cultures were counterstained with 0.5 % Evans blue for 5 min.

The virus infectivity of the supernatant fluid of the tubes was assayed using microtiter plates. Serial 10-fold dilutions in amounts of 0.05 ml. were added to 0.1 ml. of cell suspension containing 5×10^4 SPTh cells, using four wells per dilution. The medium in the microtiter plates consisted of MEM supplemented with 10 % BFS, 1 % pyruvate, 1 % yeast extract, 0.25 % LAH and 0.11 % NaHCO_3 . The plates were incubated in CO_2 atmosphere at 37°C for 8 days. Titration end points (TCID_{50}) were calculated according to the method of REED and MUENCH (17).

In order to quantitate virus suspensions by hemagglutination, 0.05 ml. of serial two-fold dilutions of virus were mixed with an equal volume of a 0.5 % suspension of washed chicken red blood cells in PBS. The test was performed at 22°C and reading was done after one hour. Titers were recorded as the reciprocal of the highest dilution showing a distinct pattern of hemagglutination.

Optimal time for use of different criteria

To determine the optimal time at which different criteria could be used when varying virus concentrations were inoculated, serial 10-fold dilutions of the tissue culture stock were inoculated in amounts of 0.1 ml. in 28 tubes with SPTh, PPK, PK-15 or SK-6 cells. The virus was allowed to adsorb for one hour. At 1, 2, 3, 4, 5, 6 and 7 days after the inoculation, four inoculated tubes of each cell type and of each dilution were randomly selected. Monolayers of SPTh and PPK were examined for the presence of CPE and Hads. Supernatant fluid of SPTh, PPK, SK-6 and PK-15 was tested for HA activity. Titration end-points (TCID_{50}) were calculated daily for each criterion for the respective cell type. The HA test was done with the centrifuged (700 g. for 10 min.) supernatant fluid, from which 0.5 ml. was mixed with an equal volume of a 0.5 % suspension of washed chicken erythrocytes in PBS.

Reproducibility of virus quantitation

To examine the reproducibility of infectivity titrations, not only the cell culture adapted stock but also the virus-containing lung homogenate was titrated repeatedly. The test was performed on SPTh, PPK, PK-15 and SK-6 monolayers. During each of the 9 trials, all cell types were inoculated with the same dilution series for each stock. The titration end-points were calculated on the seventh day after inoculation, using the HA test.

Effect of a blind passage

Six infectivity titrations were made on PPK cells, using a series of different dilutions of the tissue culture stock each time. Each tube in which no hemagglutinating activity was detected after 7 days of incubation was subjected to two cycles of freezing and thawing and was individually centrifuged for 10 min. at 700 g. The supernatant was inoculated onto a fresh monolayer of PPK cells. Presence of virus was again examined using the HA test 7 days after the inoculation.

Results

Description of the criteria

Figs. 1 to 4 show the virus growth curves based on the following criteria:

Cytopathic effect consisted of the formation of syncytia which were easily visible in vivo with an inverted microscope. Syncytia were small in PPK cells but could be very large in SPTh cells. Soon after their appearance, the syncytia degenerated and holes, surrounded by opaque irregularly shaped

syncytial debris, were formed in the monolayer. After the release of this syncytial debris from the monolayer, part of it was conserved into balloon-like structures. These structures soon represented the only evidence of viral induced cell degeneration, since the formation of new syncytia had stopped. Recording of the CPE in the growth curves was performed using indices 1 to 5, which stand for approximately 10, 20, 30, 40 syncytia per tube or syncytia in the entire monolayer, respectively. The presence of balloon-like structures was not taken into account.

Hemadsorption, in the early stages of infection, was characterized by the presence of attached red blood cells in groups of 100 (PPK cells) up to 500 (SPTh cells). Later, the hemadsorption was more diffuse and large groups of red blood cells were no longer seen. Recording was performed using the indices 1 to 5 which stand for approximately 150, 300, 450, 700 and 2000 adsorbed erythrocytes, respectively, per field, using a 10 x objective and counting about 10 fields.

Recording of cytoplasmic *fluorescence* was performed using the indices 1 to 5 which stand for a few cells, a few small foci, a few large foci, many large foci or almost all the cells positive, respectively. In SPTh and PPK cells, the decrease in fluorescence as shown in Figs. 1 and 2 was mainly due to degeneration and loss of infected cells.

Recording of the *infectivity* and *hemagglutination* titers was also done using indices 1 to 5, which stand for 10^1 to 10^5 TCID₅₀ and 2, 4, 8, 16, 32 HA units per 0.05 ml. of supernatant fluid, respectively.

Virus growth curves

The following pattern of virus growth in different cell types (Fig. 1 to 4) was observed:

SPTh cells: CPE, Hads, HA, IF and infective virus were all present at 24 hours post inoculation. CPE and IF peaks were already reached at that time. The highest quantities of infective virus were found at post inoculation days (PID) 1 and 2. Peaks of Hads and HA were reached at PID 2. At PID 3, all syncytia had disappeared and about 70 % of the monolayer was destroyed by CPE. The HA activity remained high up to the end of the experiment.

PPK cells: the peaks of IF, CPE, Hads, infective virus and HA were reached on PID 1, 1, 2, 2 and 3, respectively. At PID 4, all syncytia had disappeared and about 40 % of the monolayer was destroyed by CPE. The HA activity remained high up to the end of the experiment.

PK-15 cells: peaks of IF, infective virus and HA were reached at PID 3, 5 and 5, respectively. CPE was not clear in unstained cultures, although hematoxylin eosin staining revealed that a few small giant cells were present on PID 3. Hemadsorption did occur, but to such a low degree that it could not be taken into account.

SK-6 cells: HA, Hads or CPE were not observed in this cell line. Infectious virus was, however, produced with a peak on PID 5 to 6. Small foci of fluorescence were found with a peak on PID 3.

PPT and ST cells: no HA, Hads or CPE were observed in these cells. No fluorescence was found in ST cells. A few PPT cells were positive by immunofluorescence at PID 4 only. The presence of infectious virus was not examined. Both PPT and ST cells were excluded from the further experiments because it was clear from these first studies that their sensitivity for HEV was very low.

Fig. 1. Growth curve of VW 572 virus in secondary pig thyroid cells. HA = hemagglutination; Hads = hemadsorption; IF = immunofluorescence; CPE = cytopathic effect. For the explanation of the indices see Results

Fig. 2. Growth curve of VW 572 virus in primary pig kidney cells. For the explanation of symbols and indices see Fig. 1

Fig. 3. Growth curve of VW 572 virus in Pk-15 cells. For the explanation of symbols and indices see Fig. 1

Fig. 4. Growth curve of VW 572 virus in SK-6 cells. For the explanation of symbols and indices see Fig. 1

Optimal time for use of different criteria

The period during which maximal infectivity titers were recorded with a given criterion was considered as the optimal period to apply this criterion. The titers calculated daily using the different criteria are presented in Table 1. It can be seen that maximal titers on SPTh cells were obtained when reading was done on the basis of the CPE between PID 2 and 4, on the basis of Hads between PID 2 and 6, or on the basis of HA between PID 2 and 7. Using PPK cells, reading the CPE between PID 2 and 5 gave about the same results as reading by Hads between PID 2 and 6 or reading by HA between PID 3 and 7. The HA test applied on PID 7 appeared to be optimal for both PK-15 and SK-6 cells.

Table 1

Infectivity titers of HEV strain VW 572 in different cell cultures calculated daily using different criteria

Cell type	Reading based on	Infectivity titer in log ₁₀ per 0.1 ml at						
		PID 1	PID 2	PID 3	PID 4	PID 5	PID 6	PID 7
SPTh	CPE	3.5	4.6	4.5	4.5	0	0	0
	Hads	4.3	4.6	4.5	4.5	4.5	4.6	0
	HA	2.6	4.5	4.5	4.5	4.5	4.6	4.5
PPK	CPE	3.5	5.3	5.6	5	5.5	0	0
	Hads	3.6	5.6	5.6	5	5.5	6	0
	HA	2.5	4.6	5.6	5.3	5.5	6	5.6
PK-15	HA	≤0.5	1.6	2	2.5	3	2.6	4.2
SK-6	HA	≤0.5	≤0.5	≤0.5	≤0.5	1.5	2	3.5

SPTh = secondary pig thyroid; PPK = primary pig kidney

CPE = cytopathic effect; Hads = hemadsorption; HA = hemagglutination

PID = post inoculation day

Reproducibility of virus quantitation

The infectivity titers of the tissue culture stock and the lung homogenate stock obtained in nine separate trials are represented in Table 2. The geometric mean titers (GMT) of these titrations showed that SPTh and PPK cells were more susceptible to VW 572 than PK-15 and SK-6 cells. SPTh cells were slightly more sensitive than PPK cells and PK-15 cells somewhat better than SK-6 cells. A wide variation in infectivity titers (maximum 1.9 log₁₀) was observed within the same system during different trials.

Table 2

Comparative infectivity titrations of HEV strain VW 572 in different cell types

Virus stock	Cell type	Infectivity titer in log ₁₀ per 0.1 ml (9 trials)									GMT	MV
TCS	SPTh	6.5	6.2	7.2	6.2	5.5	6.0	6.2	NT	NT	6.3	1.7
TCS	PPK	5.5	4.7	6.2	6.2	5.0	5.5	6.3	5.8	5.6	5.6	1.6
TCS	PK-15	4.0	3.6	4.1	4.4	3.8	3.5	NT	4.5	3.5	3.9	1.0
TCS	SK-6	NT	3.2	2.6	3.2	NT	2.3	NT	4.0	2.5	3.0	1.7
LHS	SPTh	3.5	3.8	4.5	4.6	3.5	3.5	4.0	NT	NT	3.9	1.1
LHS	PPK	3.3	4.0	4.0	4.5	2.6	3.8	4.0	3.2	3.5	3.7	1.9
LHS	PK-15	2.5	2.8	2.0	2.5	1.5	NT	NT	2.2	1.5	2.1	1.3
LHS	SK-6	NT	1.5	2.3	NT	NT	NT	NT	1.6	0.6	1.6	1.7

TCS = tissue culture stock; LHS = lung homogenate stock; NT = not tested

GMT = geometric mean titer; MV = maximal variation

SPTh = secondary pig thyroid; PPK = primary pig kidney; SK-6 = swine kidney cell line

PK-15 = pig kidney cell line

Effect of a blind passage

The six infectivity titers obtained by titration of the tissue culture stock on PPK cells were 10^{4.4}, 10^{5.4}, 10^{5.4}, 10^{5.6} and 10^{6.2} TCID₅₀. After one blind passage the titers obtained were 10^{4.5}, 10^{5.5}, 10^{5.6}, 10^{5.6} and 10^{6.4}, respectively.

Discussion

Based on the results of the present study, it can be concluded that of all the cell cultures examined, SPTh and PPK cells were the most susceptible for cultivation and quantitation of VW 572 virus. The other cell types were

either refractory (ST cells), or allowed viral growth only to such a slight extent (PPT, SK-6 and PK-15 cells) that they cannot be considered for practical use.

In SPTh and PPK cells, all the criteria tested (IF, CPE, Hads, HA) were useful to demonstrate viral replication. When the different criteria were correlated, it was observed that in SPTh cells peaks of all 4 criteria were reached at 48 h post inoculation. The amount of IF, Hads and CPE decreased quickly, while the HA titer remained high until 7 days post inoculation. In PPK cells a similar correlation was observed except that the peaks had a tendency to appear somewhat later and, especially for CPE, reached lower levels. Hemagglutinating activity in the supernatant fluid was the last criterion to become positive and persisted longest in these cells also.

The daily calculation of the infectivity titers revealed that CPE, Hads and HA became positive even in the tubes which were inoculated with the highest virus dilutions. Although CPE and Hads may be more sensitive to detect the beginning of viral growth, virus replication was finally high enough for HA to become positive, even in tubes inoculated with a low virus concentration. Of the three tested criteria, HA was finally preferred because it is simple to perform and remains clearly positive for several days.

The comparative infectivity titrations provided further evidence that secondary thyroid and primary kidney cells were more susceptible than the established cell lines. Comparable results were obtained by PIRTLE (16), who made one titration of HEV in SPTh cells, embryonic porcine kidney cells and PK-15 cells, using the IF test after 48 hours of inoculation as criterion.

In the present studies, the repeated quantitation trials revealed an unacceptable variation in the titration end-points (Table 2). Similar difficulties in which considerable variation was encountered have also been described for two other coronaviruses, infectious bronchitis virus (7) and transmissible gastroenteritis virus (11).

It was not clear whether this variation was related to the condition of the cells at the time of inoculation, to dilution faults or to the presence of aggregates in the virus stock. However, we have observed several times that PPK cells are reliable for virus isolation trials only when the cells were used during rapid growth. If the cells were inoculated when fully sheeted 4 days after planting, the results of virus isolation were much more reproducible than when fully sheeted cells were used after a slow start. If no attention is given to this point, the results of virus isolation in PPK cells varied from week to week.

The final infectivity titer of the virus stocks titrated on PPK cells was not significantly increased after carrying out a blind passage. However, in virus isolation trials starting from diagnostic specimens, such a blind passage is recommended since tissue homogenates from pigs infected with VW 572 often contain very small amounts of infectious virus (2).

We conclude that the optimal procedure for isolation of HEV from clinical specimens is preferably to inoculate the material on SPTh or PPK cells and to make a blind passage if the HA test at PID 7 is negative. Hemagglutination inhibition or seroneutralisation tests using specific antisera should then be performed for further virus identification.

Summary

This study reports some cultural characteristics of the VW 572 strain of hemagglutinating encephalomyelitis virus (HEV) in primary pig kidney (PPK) cells, primary pig testicle (PPT) cells, secondary pig thyroid (SPTh)

cells and the cell lines PK-15 (pig kidney), SK-6 (swine kidney) and ST (swine testicle). A growth curve, based on cytopathic effect (CPE), immunofluorescence (IF), hemadsorption (Hads) and hemagglutination (HA), showed that SPTh and PPK cells were most susceptible for cultivation and quantitation of the virus. For the detection of replication in tubes inoculated with small amounts of virus, CPE, Hads and HA appeared to be useful and sensitive criteria. Repeated virus quantitation trials revealed a high variation in titration end-points, even in the most susceptible cell types. The optimal procedure for the isolation of HEV from clinical specimens is preferably to inoculate the material on SPTh or PPK cells and to make a blind passage if the HA test at 7 days post inoculation is negative.

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Zusammenfassung

Züchtung von Hemagglutinating Encephalomyelitis Virus (HEV) in porcinen Zellkulturen

Kulturelle Eigenschaften des HEV-Stammes VW 572 in primären Schweinenierenzellen (PPK), primären Schweinehodenzellen (PPT), sekundären Schweineschilddrüsenzellen (SPTh) und in den Zelllinien PK-15 (Schweinenieren), SK-6 (Schweinenieren) und ST (Schweinehoden) werden beschrieben. Vermehrungskurven, die mit Hilfe des cytopathischen Effektes (CPE), der Immunofluoreszenz (IF), der HämadSORption (Hads) und der Hämagglutination als Kriterien für die Virusreplikation erstellt wurden, zeigten, daß SPTh und PPK-Zellen am empfindlichsten für die Züchtung und Titration des Virus sind. Zum Nachweis der Virusvermehrung in Röhrchen, die mit kleinen Virusmengen beimpft wurden, waren CPE, Hads und HA empfindliche und brauchbare Kriterien.

Wiederholte Virustitrationen zeigten eine hohe Variation der Titerendpunkte auch in hochempfindlichen Zellkulturen. Die optimale Methode für die Isolierung von HEV von klinischem Material ist die Verimpfung auf SPTh oder PPK mit anschließender Blindpassage, wenn der HA-Test sieben Tage p. inf. negativ ist.

Résumé

Culture du virus hémagglutinant de l'encéphalomyélite (HEV) dans des cultures de cellules de porc

On décrit les propriétés de culture de la souche HEV VW 572 dans des cellules primaires de reins de porcs (PPK), dans des cellules primaires de testicules de porcs (PPT), dans des cellules secondaires de glandes thyroïdes de porcs (SPTh) et dans les lignées cellulaires PK-15 (reins de porcs), SK-6 (reins de porcs) et ST (testicules de porcs). Les courbes de multiplication établies avec l'effet cytopathique (CPE), l'immunofluorescence (IF), l'hémasorption (Hads) et l'hémagglutination (HA) comme critères pour la réplique du virus ont montré que les cellules SPTh et PPK étaient les plus sensibles pour la culture et la titration du virus. CPE, Hads et HA furent les critères sen-

sibles et utilisables pour la mise en évidence de la multiplication virale en tubes inoculés avec de petites quantités de virus.

Des titrations du virus répétées ont montré une forte variation du titre final également dans les cultures cellulaires hautement réceptrices. La méthode optimale pour l'isolement de HEV à partir d'un matériel clinique est l'inoculation sur SPTh ou PPK avec passages à l'aveugle si le test HA est négatif 7 jours après l'infection.

Resumen

Propagación del virus hemoaglutinante de la encefalomiелitis (HEV) en los cultivos de células de cerdos

Se describen las propiedades culturales de la estirpe HEV VW 572 en células renales primarias de cerdo (PPK), células testiculares primarias de cerdo, células tiroideas secundarias de cerdo (SPTh) y en las líneas celulares PK-15 (riñones de cerdo), SK-6 (riñones porcinos) y ST (testículos de cerdo). Las curvas de multiplicación, las cuales se establecieron con ayuda del efecto citopático (CPE), la inmunofluorescencia (IF), la hemoadsorción (Hads) y la hemoaglutinación como criterios para la replicación virósica, mostraban que las células SPTh y PPK son las más sensibles para el cultivo y titulación del virus. Para la puesta en evidencia de la multiplicación virósica en tubitos, los cuales se inocularon con cantidades pequeñas de virus, eran CPE, Hads y HA criterios sensibles y útiles.

Titulaciones repetidas de virus mostraban una variación elevada de los puntos finales de títulos incluso en cultivos celulares harto receptibles. El método óptimo para el aislamiento de HEV a partir de material clínico consiste en la inoculación a SPTh o PPK con pase ciego inmediato si la prueba HA es negativa 7 días después de la infección.

References

1. ALEXANDER, T. J. L., 1962: Viral encephalomyelitis of swine in Ontario — Experimental and natural transmission. *Am. J. vet. Res.* 23, 756—762.
2. ANDRIES, K., M. PENSART, and P. CALLEBAUT, 1978: Pathogenicity of hemagglutinating encephalomyelitis (vomiting and wasting disease) virus of pigs, using different routes of inoculation. *Zbl. Vet. Med. B* 25, 461—468.
3. ANDRIES, K., and M. B. PENSART: Virus isolation and immunofluorescence in different organs of pigs infected with hemagglutinating encephalomyelitis virus. *Am. J. vet. Res.*, in press.
4. CARTWRIGHT, SHEILA F., M. LUCAS, J. P. CAVILL, A. F. GUSH, and T. BLANDFORD, 1969: Vomiting and wasting disease of piglets. *Vet. Rec.* 84, 175—176.
5. CHAPPUIS, G., J. TEKTOFF, et Y. TURDU, 1975: Isolement en France et identification de la maladie du vomissement et du dépérissement des porcelets (corona-like virus). *Rec. Méd. Vét.* 151, 557—566.
6. CLARKE, J. K., and J. B. MCFERRAN, 1971: An electron microscopic study of haemagglutinating encephalomyelitis virus of pigs. *J. Gen. Virol.* 13, 339—344.
7. COWEN, B. S., and S. B. HITCHNER, 1975: pH stability studies with avian infectious bronchitis virus (corona-virus) strains. *J. Virol.* 15, 430—432.
8. GIRARD, A., A. S. GREIG, and D. MITCHELL, 1964: Encephalomyelitis of swine caused by a hemagglutinating virus. III. Serological studies. *Res. Vet. Sci.* 5, 294—302.
9. GREIG, A. S., D. MITCHELL, A. H. CORNER, G. L. BANNISTER, E. B. MEADS, and R. J. JULIAN, 1962: A hemagglutinating virus producing encephalomyelitis in baby pigs. *Can. J. Comp. Med.* 26, 49—56.
10. GREIG, A. S., C. M. JOHNSON, and A. M. P. BOUILLANT, 1971: Encephalomyelitis of swine caused by a hemagglutinating virus. VI. Morphology of the virus. *Res. Vet. Sci.* 12, 305—307.
11. HESS, R. G., and P. A. BACHMANN, 1976: In vitro differentiation and pH sensitivity of field and cell culture attenuated strains of transmissible gastroenteritis virus. *Inf. & Imm.* 13, 1642—1646.

12. KASZA, L., J. A. SHADDUCK, and G. J. CRISTOFINIS, 1972: Establishment, viral susceptibility and biological characteristics of a swine kidney cell line SK-6. *Res. Vet. Sci.* 13, 46—51.
13. LENETTE, E. H., and N. J. SCHMIDT, 1969: Diagnostic procedures for viral and rickettsial diseases, 4th Ed., p. 90. Amer. publ. Hlth. Ass.
14. LUCAS, MARGARET H., and P. NAPHTHINE, 1971: Fluorescent antibody technique in the study of three porcine viruses transmissible gastroenteritis virus, vomiting and wasting virus and the parvovirus 59 E / 63. *J. Comp. Path* 81, 111—117.
15. PENSART, M. B., and P. E. CALLEBAUT, 1974: Characteristics of a coronavirus causing vomition and wasting in pigs. *Arch. ges. Virusforsch.* 44, 35—50.
16. PIRTLE, E. C., 1974: Titration of two porcine respiratory viruses in mammalian cell cultures by direct fluorescent antibody staining. *Am. J. Vet. Res.* 35, 249—250.
17. REED, L. J., and H. MUENCH, 1938: A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* 27, 493—497.

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