Research in Veterinary Science 1990, 48, 165–169

Sites of replication of a porcine respiratory coronavirus related to transmissible gastroenteritis virus

E. COX, J. HOOYBERGHS, M. B. PENSAERT, *Laboratory of Veterinary Virology and Immunology, Casinoplein 24, B-9000 Ghent, Belgium*

A porcine respiratory coronavirus (PRCV) was inoculated by aerosol into nine hysterectomy-derived and colostrum-deprived pigs at the age of one week. They were killed at different times after inoculation and tissues were sampled for virus isolation and immunofluorescence. Results indicate that virus replicated to high titres in the respiratory tract. Replication mainly occurred in alveolar cells but also in epithelial cells of nasal mucosa, trachea, bronchi, bronchioli, in alveolar macrophages and in tonsils. After primary replication in the respiratory tract, viraemia occurred. Virus also reached the gastrointestinal tract after swallowing. Subsequently, PRCV was observed to replicate in the ileum. The infection spread within a few days from the ileum to the duodenum. Replication in the small intestine remained limited to a few cells located in or underneath the epithelial layer of villi and, or, crypts. The cell type could not be identified. Virus was isolated from mesenteric lymph nodes in all pigs, but immunofluorescence was not observed. Results show that small changes in molecular structure between transmissible gastroenteritis virus and PRCV resulted in important changes in host cell tropism.

TRANSMISSIBLE gastroenteritis (TGE) is a viral disease characterised by watery diarrhoea in swine and caused by a coronavirus called transmissible gastroenteritis virus (TGEV). TGEV was first isolated by Doyle and Hutchings (1946). It was long known as a prototype enteropathogenic virus. It replicates in the cytoplasm of the absorptive epithelial cells on the villi of the small intestine. Virus replication in these cells induces cellular degeneration and villous shortening leading to diarrhoea in swine of all ages. Mortality approaches 90 to 100 per cent in pigs infected during the first week of life (Hooper and Haelterman 1966, 1969, Pensaert et al 1970). TGEV isolates have been reported to have a tropism not only for the intestinal tract but also for the respiratory tract, without causing respiratory disorders (Kemeny et al 1975). Furuuchi et al (1979) showed that TGEV strains lose their enterotropism but can gain tropism for respiratory tract tissue by serial passaging in cell cultures.

The appearance of a porcine coronavirus related to TGEV was observed in 1984 in the swine population in Belgium (Pensaert et al 1986). This virus has, since then, become widespread in several European countries (Brown and Cartwright 1986, Jestin et al 1987, van Nieuwstadt and Pol 1989). It replicates in the respiratory tract and is transmitted aerogenically. Although respiratory distress has been reported upon infection with this porcine respiratory coronavirus (PRCV), it usually causes a subclinical infection. Infection with this virus does not cause diarrhoea (Pensaert et al 1986, Jestin et al 1987, Duret et al 1988).

Recently, the antigenic relationship between the Belgian isolate of PRCV, designated TLM83, and the classical TGEV has been studied (Callebaut et al 1988). Both viruses show a complete cross neutralisation activity and possess common antigenic determinants on each of their structural proteins. Nevertheless, distinct antigenic differences have been demonstrated between both viruses with the use of monoclonal antibodies. Non-neutralising monoclonal antibodies directed against E2 protein epitopes of TGEV do not recognise PRCV. This indicates that these epitopes of the E2 protein of TGEV are modified or absent in PRCV. Since PRCV and TGEV are antigenically closely related, PRCV can be regarded as a TGEV variant.

The purpose of the present study was to find out in what tissues and cells PRCV replicates and to determine the sequence of events of PRCV infection in pigs particularly in comparison with those known to occur with TGEV.

Materials and methods

Virus stock

The TLM83 isolate of PRCV was used in this study (Pensaert et al 1986). The stock for experimental infection represented the first passage in swine testicle cells (McClurkin and Norman 1966). It was derived from a 20 per cent suspension of lung tissue from an experimentally inoculated piglet. The infectivity titre of this stock was $10^{6.7}$ tissue culture infective doses (TCID50) ml^{-1}.

Pigs and experimental procedures

The present study was performed in nine hysterectomy-derived colostrum-deprived pigs which were individually housed in Horsfall-type isolation units. They were inoculated by aerosol with $10^{7.0}$ TCID50 TLM83 in 5 ml phosphate buffered saline (PBS) at six days old. The virus suspension was nebulised with the 'Wright' nebuliser (particle size <8 μm) (Aerosol Products) for 40 minutes with each pig individually.

Six pigs were inoculated in a first experiment (group 1). They were observed daily for clinical signs. One piglet a day was killed on days 1, 2, 3, 4, 5 and 6 after inoculation. A blood sample was collected at the time of euthanasia and processed as further described. Immediately after bleeding the pigs, the following tissues were collected for viral isolation and immunofluorescence: stomach; small intestine; colon; rectum; spleen; liver; kidney; thymus; lymph nodes (mesenteric, bronchial, inguinal, cervical, mandibular and parotid); tonsils; respiratory and olfactory nasal mucosa; trachea and lungs (apical, cardial and diaphragmatic lobes). The small intestine was divided into seven segments, equal in length, designated duodenum, jejunum 1 to 5 and ileum. A piece approximately 1·5 cm long was collected for immunofluorescence. Virus isolation was performed on the remaining parts. Small intestinal, caecal and rectal contents were also collected for virus isolation.

In a second experiment, three pigs were inoculated by aerosol and one killed on each of days 1, 2 and 3 after inoculation (group 2). A post mortem lung washing was performed in these pigs. Furthermore, lung lobes, trachea, tonsils and the same small intestinal segments as described above were collected for immunofluorescence.

Isolation of blood leucocytes and lung washing cells

Blood was collected in heparin from a jugular vein. White blood cells were isolated by sedimentation on dextran, followed by lysis of erythrocytes in ammonium chloride (Gutekunst 1979). After centrifugation (10 minutes at 300 *g*) sedimented cells were resuspended in PBS and smears were made of the white blood cells for examination by immunofluorescence. Cells and plasma were separately investigated by viral isolation.

For lung washing, 50 ml of PBS containing 50 per cent (v/v) Alsever's solution was injected post mortem through a polyethylene catheter placed in the left bronchus. The fluid was immediately aspirated, whereafter the cells were sedimented by centrifugation (10 minutes at 300 *g*). Next, the erythrocytes were lysed with ammonium chloride. Sedimented cells were resuspended in 5 ml Eagle's minimum essential medium whereafter smears were prepared for immunofluorescence.

Viral isolation and titration

All samples collected for viral isolation were immediately frozen at −70°C and kept at this temperature until processed. Viral isolation was performed using 20 per cent suspensions (w/v) in PBS according to standard procedures. Supernatants from homogenates were inoculated into 10 tubes fully sheeted with swine testicle cells. Cells were observed for the occurrence of cytopathogenic effect for seven days. No second passage was made.

Titrations of infectious virus in samples were performed by inoculating 10-fold dilutions of the supernatants into microtitre plates on swine testicle cells.

Immunofluorescence

Tissue pieces collected for examination by immunofluorescence were mounted in a gelatin capsule with methocel (Fluka Chemie AG) and frozen in a liquid alcohol carbon dioxide ice bath. White blood cell smears, lung washing cell smears and frozen sections (8 μm) were fixed in acetone before staining with the direct immunofluorescent method. A hyperimmune serum against TGEV, conjugated with fluorescein isothiocyanate, was used as conjugate following standard procedures. The specificity of fluorescence was determined by the fluorescence inhibition test.

A semiquantitative evaluation of immunofluorescence was made according to the number of fluorescing cells in each section: − = 0 per cent of the epithelial cells fluorescing; + = ⩽5 per cent fluorescing; + + = 6 to 25 per cent; + + + = 26 to 50 per cent; + + + + = >50 per cent.

Histology

Samples for histopathological studies were collected from the lung lobes of all nine pigs, fixed in 10 per cent phosphate buffered formalin and examined by standard histological techniques.

Results

None of the pigs experienced respiratory distress or intestinal disorders.

Viral isolation

Inoculation of the PRCV isolate on the swine testicle cultures induced the formation of typical syncytia between one and four days after inoculation.

The results of the viral isolations and titrations of tissues and samples from pigs of group 1 are presented in Table 1. As can be observed, virus was isolated from the nasal mucosa, trachea, lungs and tonsils in all six pigs. The highest titres were detected in lung tissue (up to $10^{8.3}$ TCID50 g^{-1} lung tissue).

TABLE 1: Results of virus titrations of tissues from six pigs inoculated by aerosol with TLM83 and sequentially killed

	Virus titre*					
Piglet number	1610	1611	1612	1613	1614	1615
Killed DPI	1	2	3	4	5	6
Respiratory tract and tonsils						
Apical lung lobe	3·0	7·7	8·0	8·3	7·5	7·0
Trachea	2·7	3·7	4·5	5·0	3·7	3·5
Nasal mucosa	4·7	6·3	5·5	4·5	4·5	4·5
Tonsils	2·2	3·0	2·7	3·5	3·0	2·2
Digestive tract						
Stomach	3·0	1·9	2·2	4·3	1·3	1·5
Duodenum	1·8	2·2	3·3	3·0	4·0	1·4
Jejunum 1	1·7	1·3	1·3	1·7	3·3	1·3
Jejunum 2	1·4	2·0	1·7	3·6	3·0	Neg
Jejunum 3	1·3	1·9	2·2	3·3	3·7	1·3
Jejunum 4	Neg	1·4	2·2	3·3	3·0	1·3
Jejunum 5	Neg	1·5	2·2	1·8	3·0	2·2
Ileum	1·6	2·2	2·2	1·6	2·2	2·2
Colon	Neg	1·4	1·8	1·6	1·4	Neg
Content:						
Small intestinal	2·2	2·3	2·2	3·7	3·7	Neg
Caecal	Neg	2·0	1·5	2·0	Neg	Neg
Rectal	Neg	1·3	Neg	2·2	2·1	Neg
Lymphoid tissue						
Lymph nodes:						
Bronchial	Neg	1·9	Neg	2·2	Neg	1·4
Mesenteric	1·6	1·8	2·2	2·2	2·2	2·0
Cervical	Neg	Neg	Neg	Neg	Neg	Neg
Inguinal	Neg	Neg	2·2	Neg	Neg	Neg
Maxillary and parotid	Neg	1·8	1·3	1·3	Neg	Neg
Thymus	Neg	1·3	Neg	Neg	Neg	Neg
Spleen	Neg	Neg	1·5	1·3	Neg	Neg
Other						
Liver	Neg	Neg	1·3	NT	1·3	1·3
Kidney	Neg	Neg	Neg	Neg	Neg	Neg
Plasma	Neg	2·3	1·6	3·0	1·5	1·4
Leucocytes	Neg	1·4	Neg	1·9	Neg	Neg

* Virus titre in $\log_{10} g^{-1}$
DPI Days after inoculation
Neg No virus isolated (virus titre $<10^{1·3} g^{-1}$)
NT Not tested

Virus was also isolated from stomach and small intestine in all six pigs. Virus titres in stomach and duodenum were higher than, or in the same range as, titres in caudal small intestinal segments between one and four days after inoculation. Titres increased in small intestinal segments from one to five days after inoculation.

Virus was isolated from colon in four out of six pigs. Virus titres were generally lower than in ileum.

Virus was isolated from mesenteric lymph nodes in all pigs. It was isolated inconsistently and at low titres from other lymphoid tissues and from liver.

Virus was isolated from the plasma samples of pigs killed from two days after inoculation at titres up to 10^3 TCID50 g^{-1}.

Immunofluorescence

Results of the immunofluorescence study are presented in Tables 2 and 3 for groups 1 and 2, respectively.

Viral antigens in the respiratory tract were detected in the cytoplasm of epithelial cells of the nasal mucosa, trachea and in lung tissue.

Fluorescence in lungs was seen in epithelial cells of alveoli, bronchioli and bronchi. The percentage of fluorescent cells in all three epithelia increased from one to three days after inoculation. At this time, generally more than 50 per cent of the alveolar cells were fluorescing, whereas 20 per cent of positive cells were observed in some bronchi and bronchioli. The immunofluorescence in alveoli remained unchanged until day 5 after inoculation, after which it decreased. The immunofluorescence in bronchioli did not change after day 3, but the immunofluorescence in bronchi decreased. At five and six days after inoculation, few cells were still positive in some bronchi, whereas in others no immunofluorescence was seen any more.

Specific fluorescence was detected in 0·2 to 1·6 per cent of cells of the lung washing fluids in all three pigs of group 2. These latter cells had the morphology of alveolar macrophages.

Viral antigens in the tonsils were present in the cytoplasm of a few epithelial cells at the surface or the crypts. Weak fluorescing structures were also seen in the tonsillary lymphoid tissue between the crypts. These structures could not be identified.

Viral antigens could be detected in a few cells in the ileum starting on day 2 after inoculation in both experiments (Tables 2 and 3). At this time, fluorescent cells were scarce and were detected only after careful examination of several sections. The fluorescent cells were generally located in, but sometimes also underneath, the enterocytes of villi and, or, crypts. The cell type could not be identified. The infection in the small intestine gradually spread from caudal to cranial and on day 4 and 5 after inoculation all small intestinal segments were positive. The number of fluorescent epithelial cells per section increased to reach a maximum of four cells in some ileal sections on day 4 post inoculation, whereafter it decreased again. Immunofluorescing cells could not be observed in the dome epithelium above the Peyer's patches or inside the Peyer's patches.

Immunofluorescence was not detected in white blood cell smears, stomach, colon, lymph nodes, spleen and liver.

Gross lesions and histopathology

Pneumonia was observed in the lungs of seven of the nine inoculated pigs at necropsy. In three pigs killed two, three and three days after inoculation, only one or two small lobular lesions were observed. These lesions became more pronounced in apical and cardiac lobes of the pigs killed four and five days after

TABLE 2: Results of immunofluorescence of tissues from pigs inoculated by aerosol with TLM83

	Immunofluorescence*					
Piglet number	1610	1611	1612	1613	1614	1615
Killed DPI	1	2	3	4	5	6
Respiratory tract and tonsils						
Lung lobe:						
Apical	+	+ + + +	+ + + +	+ + + +	+ +	+ +
Cardial	+	+ +	+ + + +	+ + + +	+ + + +	+
Diaphragmatic	—	+	+ + +	+ + +	+ + +	+ +
Trachea	—	+	—	+	+	+
Nasal mucosa:						
Respiratory	—	+ +	—	+ + +	+	+
Olfactory	+	+ + +	+	+ +	+	+
Tonsils	—	+	+	+	—	—
Digestive tract						
Stomach	—	—	—	—	—	—
Duodenum	—	—	NT	+	+	—
Jejunum 1	—	—	—	+	+	—
Jejunum 2	—	—	—	+	+	—
Jejunum 3	—	—	—	+	+	—
Jejunum 4	—	—	+	+	+	—
Jejunum 5	—	—	+	+	+	—
Ileum	—	+	+	+	+	+
Colon	—	—	—	—	—	—
Lymphoid tissue						
Lymph nodes:						
Bronchial	—	—	—	—	—	—
Mesenteric	—	—	—	—	—	—
Inguinal	—	—	—	—	—	—
Spleen	—	—	—	—	—	—
Other						
Liver	—	—	—	—	—	—
Leucocytes	—	—	—	—	—	—

* Immunofluorescence was scored: — No immunofluorescence; + ≤ 5 per cent cells fluorescing; + + 6 to 25 per cent; + + + 26 to 50 per cent; + + + + > 50 per cent
DPI Days after inoculation
NT Not tested

TABLE 3: Results of detailed immunofluorescent examination in three pigs inoculated by aerosol with TLM83

	Immunofluorescence*		
Piglet number	1571	1573	1574
Killed DPI	1	2	3
Respiratory tract and tonsils			
Lung lobe:			
Apical	+	+	+
Cardiac	+	+	+ +
Diaphragmatic	+	+	+
Lung washing cells	+	+	+
Trachea	+	+	+
Respiratory nasal mucosa	—	—	—
Tonsils	—	—	—
Digestive tract			
Duodenum	—	—	—
Jejunum 1	—	—	—
Jejunum 2	—	—	—
Jejunum 3	—	—	—
Jejunum 4	—	—	—
Jejunum 5	—	+	—
Ileum	—	+	+

* Immunofluorescence was scored: — No immunofluorescence; + ≤ 5 per cent fluorescing cells; + + 6 to 25 per cent immunofluorescence
DPI Days after inoculation

inoculation and were scattered over the lungs six days after inoculation. All other organs had a normal appearance.

Histological investigation of lung lesions revealed capillary congestion and a diffuse interstitial pneumonia in pigs killed one and two days after the experimental infection. This interstitial pneumonia became more severe three days after inoculation. Besides the pneumonia, an early focal degeneration could be observed in the alveolar interstitium in apical and cardiac lobes on days 4 and 5. These degenerative lesions were also seen in alveolar and bronchiolar epithelium six days after inoculation. The bronchioli and bronchi were partially filled with cellular debris.

Discussion

In earlier studies, preliminary viral isolation attempts were performed on organs or tissues of pigs killed after intranasal inoculation with $10^{3.6}$ TCID50 TLM83. Virus was consistently isolated from respiratory tract tissue and not from the intestine (Pensaert et al 1986). The efficacy of the viral

isolation technique has been improved since then. Swine testis cells inoculated when fully sheeted five days after seeding were found to be most valuable for obtaining optimal results in isolating small amounts of virus. No second cell culture passage was needed. The high sensitivity of the swine testis cell line may explain why in this study virus was isolated in high titres from respiratory tract tissue and even at low titres from the intestinal tract.

The results of previous studies which indicated that respiratory tract tissues served as primary sites of multiplication of PRCV in pigs, were confirmed by the present study (Pensaert et al 1986, Duret et al 1988). Production of large amounts of virus on the respiratory tract surface during several days supports the observation that virus is transmitted by the aerogenic route. This mode of transmission has resulted in a rapid spread of PRCV among the pig population in many countries in Europe. Virus was consistently isolated in high titres from nasal mucosa. This could indicate that diagnosis of PRCV might be made by isolating the virus from nasal swabs taken during infection.

The intestinal infection of TLM83 was totally different from those of the PRCV-related enteropathogenic coronaviruses TGEV (Pensaert et al 1970), canine coronavirus (Keenan et al 1976) and feline enteric coronavirus (Pedersen et al 1981). After oral inoculation, these viruses pass through the stomach and infect the absorptive enterocytes in the cranial and, or, mid small intestine and subsequently cause a contiguous infection of villous enterocytes. Within a few days, most small intestinal villi become infected and diarrhoea occurs. In contrast, TLM83 infected only very few cells at villus or crypt sites in the small intestinal mucosa and spread from the ileum to the duodenum. These results show a striking modification in tropism of PRCV for the intestine itself. It is unlikely that virus is spreading from the ileum to the duodenum through the gut lumen in the opposite direction to peristalsis. Other routes, such as via lymph or blood probably play a role.

It was not clear from the results of this study how virus reached the ileum. Two possible routes may be postulated: either by ingestion of virus produced in the respiratory tract or by viraemia. Isolation of virus from the stomach in all pigs and from duodenum and jejunum in pigs killed at days 1 and 2 after inoculation in the absence of fluorescence indicated that infectious virus was swallowed and reached the cranial intestinal segments.

The consistent isolation of virus from the plasma of all pigs killed between two and six days after inoculation indicated that viraemia may play a role in the spread of TLM83 from the respiratory tract to the gut. That viraemia occurred was substantiated by the ability to isolate virus from lymph nodes and abdominal organs (liver and spleen) in some pigs in the absence of immunofluorescence in these tissues.

In conclusion, aerosol inoculation of pigs with the TGEV-related PRCV caused an infection of the respiratory tract followed by viraemia and ingestion of virus. Subsequently, replication of PRCV was observed in a few unidentified small intestinal cells. The small changes in molecular structure between TGEV and PRCV have caused important changes in host cell tropism.

Acknowledgements

These studies were supported by the Institute for the Encouragement of Research in Industry and Agriculture (IWONL), Brussels, Belgium. We thank Dr H. Thoonen and Professor J. Hoorens (Laboratory of Pathology, Ghent) for carrying out the histopathological studies. The excellent technical assistance of Mrs L. Sys and Mrs M. Carpentier is gratefully acknowledged.

References

BROWN, I. & CARTWRIGHT, S. (1986) *Veterinary Record* **119**, 282–283

CALLEBAUT, P., CORREA, I., PENSAERT, M., JIMÉNEZ, G. & ENJUANES, L. (1988) *Journal of General Virology* **69**, 1725–1730

DOYLE, L. P. & HUTCHINGS, L. M. (1946) *Journal of the American Veterinary Medical Association* **108**, 257–259

DURET, C., BRUN, A., GUILMOTO, H. & DAUVERGNE, M. (1988) *Recueil de Médecin Vétérinaire* **164**, 221–226

FURUUCHI, S., SHIMIZU, Y. & KUMAGAI, T. (1979) *Veterinary Microbiology* **3**, 169–178

GUTEKUNST, D. E. (1979) *American Journal of Veterinary Research* **40**, 66–68

HOOPER, B. E. & HAELTERMAN, E. O. (1966) *Journal of the American Veterinary Medical Association* **149**, 1580–1586

HOOPER, B. E. & HAELTERMAN, E. O. (1969) *Canadian Journal of Comparative Medicine* **33**, 29–36

JESTIN, A., LE FORBAN, Y., VANNIER, P., MADDEC, F. & GOURREAU, J. (1987) *Receuil de Médecin Vétérinaire* **163**, 567–571

KEENAN, K. P., JERVIS, H. R., MARCHWIKI, R. H. & BURN, L. N. (1976) *American Journal of Veterinary Research* **37**, 247–256

KEMENY, L. J., WILTSEY, V. L. & RILEY, J. L. (1975) *Cornell Veterinarian* **65**, 352–362

McCLURKIN, A. W. & NORMAN, J. O. (1966) *Canadian Journal of Comparative Medecine* **30**, 190–198

PEDERSEN, N. C., BOYLE, J. F., FLOYD, K., FUDGE, A. & BAKER, J. (1981) *American Journal of Veterinary Research* **42**, 368–377

PENSAERT, M., CALLEBAUT, P. & VERGOTE, J. (1986) *Veterinary Quarterly* **8**, 257–261

PENSAERT, M. B., HAELTERMAN, E. O. & BURNSTEIN, T. (1970) *Archive für Gesamte Virusforschung* **31**, 321–334

VAN NIEUWSTADT, A. P. & POL, J. M. A. (1989) *Veterinary Record* **124**, 43–44

Received May 8, 1989
Accepted June 19, 1989