



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.



EXPERIMENTALLY INDUCED DISEASE

Experimental Teschovirus Encephalomyelitis in Gnotobiotic Pigs

M. Yamada^{*}, A. Miyazaki^{*}, Y. Yamamoto^{*}, K. Nakamura^{*}, M. Ito[†],
H. Tsunemitsu^{*} and M. Narita^{*}

^{*} National Institute of Animal Health, Tsukuba, Ibaraki 305-0856 and [†] Tokachi Livestock Hygiene Service Center, Obihiro, Hokkaido 089-1182, Japan

Summary

A central nervous system (CNS) disorder characterized by non-suppurative encephalomyelitis with neurological signs was induced experimentally in gnotobiotic pigs by intravenous and oral or intranasal inoculation of the porcine teschovirus (PTV) Toyama 2002 strain isolated from breeding pigs in Japan. Lesions consisting of perivascular cuffing of mononuclear cells, focal gliosis, neuronal necrosis and neuronophagia were observed in the brainstem, cerebellum and spinal cord. Non-suppurative ganglionitis in the spinal ganglion and neuritis in the spinal root were also observed. Regardless of the route of inoculation, all pigs infected experimentally with PTV showed a similar distribution of CNS lesions. Histological lesions in the CNS caused by oral or intranasal inoculation of the virus were mild compared with those induced by intravenous infection. Immunohistochemically, the distribution of PTV antigens corresponded closely with the distribution of brain lesions. PTV particles were detected via electron microscopy in the cytoplasm of nerve cells and the endothelial cells of blood vessels in the spinal cord of inoculated pigs. Polymerase chain reaction analysis demonstrated the presence of PTV RNA in the CNS, tonsils and large intestines of 21 of the 22 pigs inoculated. Direct CNS invasion via the blood vessels appears to be a major route of infection for PTV. The gnotobiotic pig provides a useful model for further study of PTV pathogenesis.

© 2013 Elsevier Ltd. All rights reserved.

Keywords: experimental infection; gnotobiotic pig; non-suppurative encephalomyelitis; teschovirus

Introduction

Teschovirus encephalomyelitis (previously known as enterovirus encephalomyelitis) is caused by specific serotypes of the porcine teschovirus (PTV), a member of the family Picornaviridae. The disease is considered to be of socioeconomic importance (Knowles, 2008). Originally called Teschen disease, this swine disease was recognized as a particularly virulent, highly fatal, non-suppurative form of encephalomyelitis (Trefny, 1930). A milder form of the disease with almost no mortality was identified in Wales and named Talfan disease, while in Denmark, the disease is known as poliomyelitis suum (Knowles, 2008). Other pathogenic PTV strains were subsequently also defined as causal agents of polioencephalomyelitis (Knowles,

2008). Infection with PTV is most often asymptomatic and PTV is still frequently isolated from the faeces, tonsils and other non-neural organs of apparently unaffected pigs (La Rosa *et al.*, 2007). However, PTV has also been isolated from pigs displaying a wide variety of clinical signs (Knowles, 2006), and no study has yet clarified how PTV infection leads to lesions in pigs.

Transmission of PTV is most often by the faecal–oral route (Knowles, 2006), with initial replication known to occur in the tonsils and intestinal tract (Long, 1985). Viraemia follows regularly in infections with virulent PTV serotype 1 (PTV-1) strains, resulting in infection of the central nervous system (CNS) (Holman *et al.*, 1966). However, the detailed mechanisms by which this infection leads to the development of encephalitis are still unclear. For example, while CNS disorders could not be induced

Correspondence to: M. Yamada (e-mail: oomae@affrc.go.jp).

in specific pathogen free (SPF) pigs by either intraoral or intranasal inoculation of the PTV Toyama 2002 strain, intravenous inoculation proved successful, resulting in CNS disorders characterized by non-suppurative encephalomyelitis with neurological signs (Yamada *et al.*, 2009a). However, the resulting histological lesions were much milder than those in pigs infected naturally. Conversely, a hysterectomy study has shown that CNS disorders can indeed be induced via intranasal or oral inoculation, provided the inoculum comprises the Talfan or Konratice strains of the Teschen virus in gnotobiotic pigs (Edington *et al.*, 1972). The aim of the present study was to clarify how PTV infection leads to lesions in pigs.

Materials and Methods

Virus

The PTV Toyama 2002 strain was isolated from the cerebellum of pigs with encephalomyelitis and clinical signs of neurological disease in Japan in 2002 (Yamada *et al.*, 2004). This virus strain was identified as PTV-1 by a virus neutralization test using standard antisera specific for PTV-1-11 (Miyamoto *et al.*, unpublished) and by a molecular strategy that encompassed polymerase chain reaction (PCR) amplification of the gene encoding the dominant neutralizing antigenic site of PTV followed by phylogenetic analyses of the amplicons (Kaku *et al.*, 2007). This viral strain was passaged five times in porcine kidney cell line (CPK) cultures. Infected Eagle's minimal essential medium (MEM) culture fluid (Nissui Corporation, Tokyo, Japan) was used to inoculate the pigs (Yamada *et al.*, 2009a).

Animals

Twenty-six pigs were obtained by hysterectomy from three sows and reared under germ-free conditions in plastic isolators. Blood samples and faeces were collected before the experiments from all pigs at the age of 2 days, and reverse-transcriptase PCR (RT-PCR) analysis of these samples and a virus neutralization test using the blood samples confirmed that the animals were free of PTV.

Experimental Design

Three-day-old pigs were anaesthetized with ketamine hydrochloride (30 mg/kg, intramuscularly) and xylazine (2.0 mg intramuscularly) before inoculation with PTV.

Six pigs (numbers 1–6) were inoculated intravenously with a syringe containing 3 ml of 10^6 tissue cul-

ture infectious dose (TCID)₅₀/ml of the PTV Toyama 2002 strain; eight pigs (numbers 7–14) were inoculated orally with 3 ml of 10^6 TCID₅₀/ml; and eight pigs (numbers 15–22) were inoculated intranasally with 3 ml of 10^6 TCID₅₀/ml. Negative controls were animals 23 and 24, which were inoculated intravenously with 3 ml of uninfected Eagle's MEM culture medium, and pigs 25 and 26, which were inoculated intranasally with 3 ml of uninfected culture medium.

Pigs 1, 2, 7, 8, 15 and 16 were examined clinically at 6 days post inoculation (dpi). Pigs 3, 4, 9, 10, 17 and 18 were examined at 13 dpi. Pigs 5, 6, 11, 12, 19 and 20 were examined at 20 dpi and pigs 13, 14, 21, 22, 25 and 26 were examined at 28 dpi. After clinical assessment, the pigs were killed by injection of sodium pentobarbital and then subjected to necropsy examination. The experiments were approved by the Animal Ethics Committee of the National Institute of Animal Health, Japan.

Histopathology and Immunohistochemistry

Tissue samples for microscopical examination were collected from each pig from the liver, spleen, kidney, heart, lung, stomach, small and large intestine, lymph node, brain, spinal cord, spinal ganglia, trigeminal ganglia, peripheral ganglia and nerve fibre, tonsil, nasal cavity, eye and skeletal muscle. Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were stained with haematoxylin and eosin (HE).

For immunohistochemistry (IHC), dewaxed sections were processed using the streptavidin–biotin–peroxidase (SAB-PO) method with a HISTFINE SAB-PO kit (Nichirei, Tokyo, Japan) (Yamada *et al.*, 2007). Monoclonal antibody specific for the PTV Talfan strain (No. 9, diluted 1 in 128; NIAH, Tsukuba, Japan) was used as the primary reagent (Kaku *et al.*, 2007; Yamada *et al.*, 2007). Negative controls for each section used serum from a non-immunized mouse instead of the primary antibody. Samples were counterstained with haematoxylin.

Electron Microscopy

Small samples of the spinal cord of all pigs were fixed in 2.5% glutaraldehyde in phosphate buffer and 1% osmium tetroxide, embedded in resin, processed routinely for semithin and ultrathin sectioning and examined using an H-7500 electron microscope (Hitachi, Tokyo, Japan).

Preparation of RNA and RT-PCR

The serum, cerebrum, brainstem, cerebellum, spinal cord, tonsils, liver, lungs, small intestine and large

intestine were collected from both inoculated and control pigs. Total RNA was extracted using TRIzol-LS from 250 µl of serum or 10% tissue homogenate and subjected to reverse-transcriptase (RT) nested PCR (Zell *et al.*, 2000). The 5' untranslated region of the PTV genome was amplified using a Qiagen OneStep RT-PCR Kit (Qiagen, Valencia, California, USA) with the primer pair pev-1a/pev-1b. The RT-PCR products were further amplified using the Takara ExTaq Kit (Takara Bio Inc., Shiga, Japan) and primer pair pev-1c/pev-1d. Amplicons were analysed via 2% agarose gel electrophoresis and visualized under UV light after ethidium bromide staining.

Serology

Serum samples obtained from individual pigs at the time of necropsy examination were subjected to the serum virus neutralization test. All sera were inactivated at 56°C for 30 min and serially diluted two-fold in Eagle's MEM supplemented with 2% fetal bovine serum. PTV Toyama 2002 strain of 100 TCID₅₀/50 µl was mixed with an equal volume of diluted serum, and the mixture was incubated for 1 h at 37°C. Subsequently, 100 µl of each virus-serum mixture was inoculated in microplate wells containing 100 µl of CPK cell suspension (approximately 1×10^5 cells). All tests were conducted in duplicate and virus neutralization titres were defined as the reciprocal of the highest dilution of the serum that completely inhibited the cytopathic effect (CPE) in either of the wells 7 days after inoculation.

Results

Clinical Observation

Clinically, all six pigs inoculated intravenously with PTV (pigs 1–6) exhibited depression from 6 dpi. Animals 1, 3, 5 and 6 further showed neurological signs including locomotor ataxia and flaccid paralysis of either or both the fore- or hindlimbs from 6 dpi. These clinical signs were so severe in pigs 5 and 6 that the animals were humanely destroyed at 20 dpi. Additionally, pigs 3, 4 and 6 had mild to moderate diarrhoea characterized by watery yellow faeces from 10 dpi. Of the pigs inoculated orally or intranasally (animals 7–22), twelve (numbers 9 to 14 and 17–22) showed locomotor ataxia and five (numbers 9, 11, 17, 19 and 20) showed flaccid paralysis of the hindlimbs from 13 dpi (Fig. 1).

However, clinical signs in pigs inoculated orally or intranasally showed no marked progression and these animals showed recovery from 18 to 21 dpi. At 28 dpi, all surviving pigs had recovered and appeared



Fig. 1. Flaccid paralysis of the hindlimbs in pig 9 (inoculated orally) at 13 dpi.

healthy. Pigs 9 and 11 exhibited mild to moderate diarrhoea characterized by watery yellow faeces from 12 dpi, as did pig 19 from 14 dpi. No other clinical signs were observed. The four control pigs (numbers 23–26) showed no clinical abnormalities.

Pathology

No gross lesions were observed in the brains or spinal cords of pigs inoculated with PTV. The large intestine in all pigs exhibiting diarrhoea (numbers 3, 4, 6, 9, 11 and 19) showed oedema and were thin-walled and flaccid, properties significantly different from the intestines of non-infected control pigs. The intestinal contents in the affected animals were fluid (Fig. 2). Consolidation and reddening of the lungs was identified at 13, 20 and 28 dpi in pigs inoculated intranasally. Additionally, these pigs had extensive pneumonic lesions in the hilus, apical and diaphragmatic lobes.

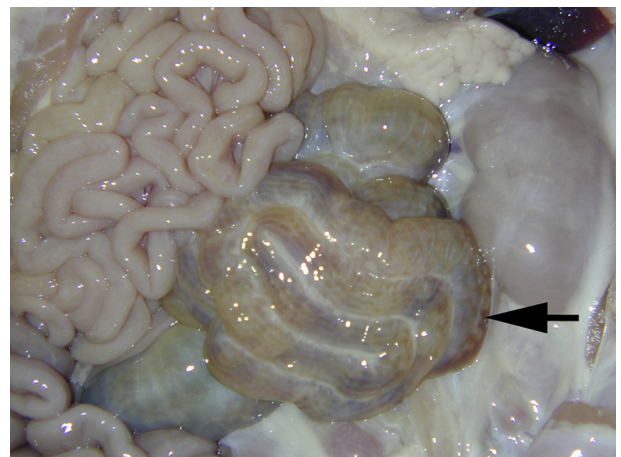


Fig. 2. Gross appearance of the intestine of pig 9 (inoculated orally). The colonic wall is oedematous (arrow), but the small intestine appears normal.

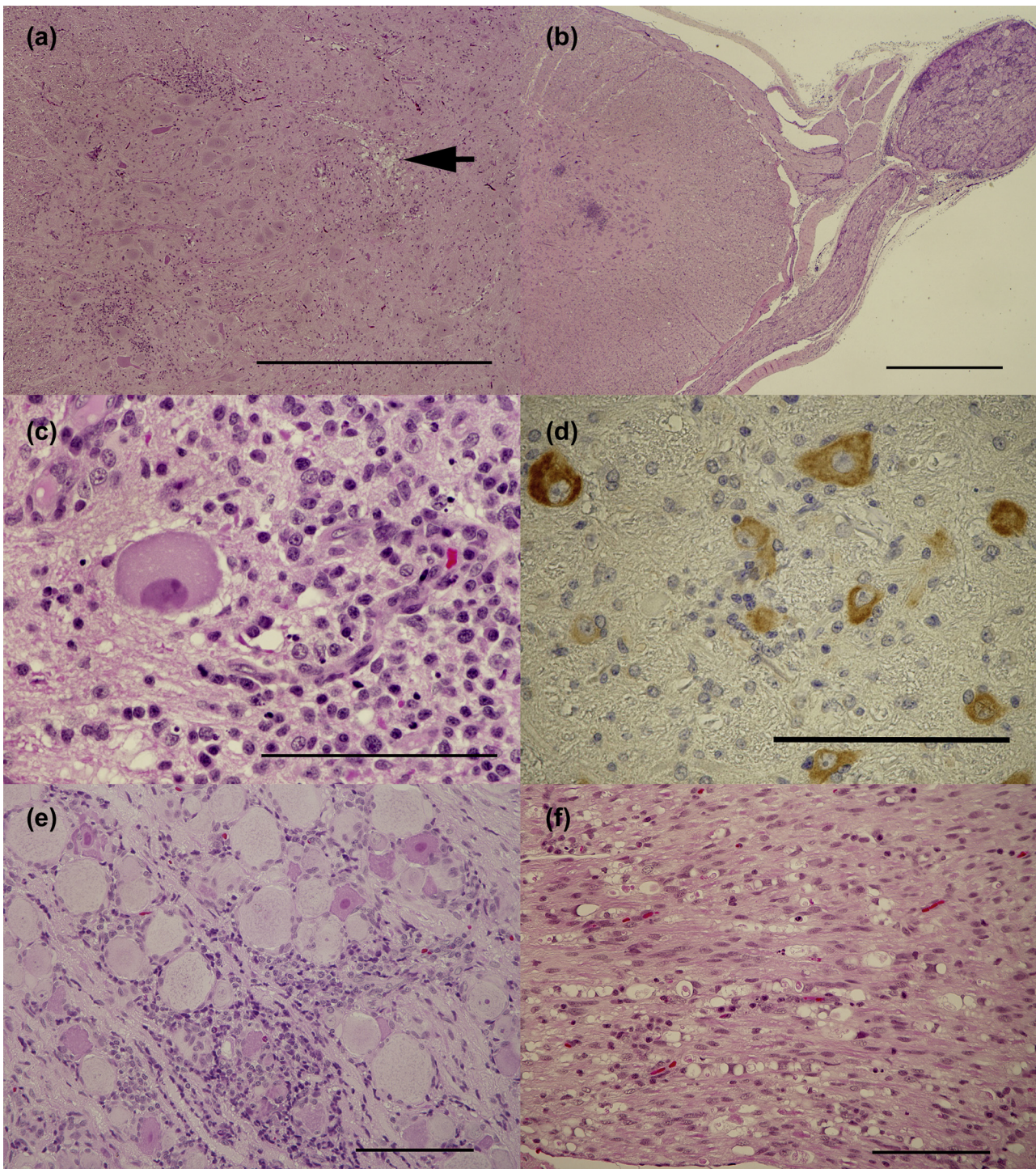


Fig. 3. Histological lesions and IHC of the nervous system of PTV-infected pigs. (a) Ventral horn of the spinal cord of pig 3 (inoculated intravenously) at 13 dpi. Infiltration of mononuclear cells and focal malacia are observed (arrow). HE. Bar, 2 mm. (b) The spinal cord of pig 17 (inoculated intranasally) at 13 dpi. The lesion is seen in the ventral horn of the spinal cord as glial nodules and in the spinal root and dorsal root ganglia as infiltration of mononuclear cells. HE. Bar, 2 mm. (c) Ventral horn of the spinal cord of pig 6 (inoculated intravenously) at 20 dpi. There is central chromatolysis. HE. Bar, 200 μ m. (d) Pons of pig 3 at 13 dpi. PTV antigen is seen in the cytoplasm of nerve cells. IHC. Bar, 200 μ m. (e) Dorsal root ganglia of pig 18 (inoculated intranasally) at 13 dpi. There is infiltration of mononuclear cells and degenerate ganglion cells with satellitosis are present. HE. Bar, 200 μ m. (f) The spinal root of pig 6 (inoculated intravenously) at 20 dpi. There are vacuolar and necrotic changes in the nerve fibre with mild infiltration of mononuclear cells. HE. Bar, 200 μ m.

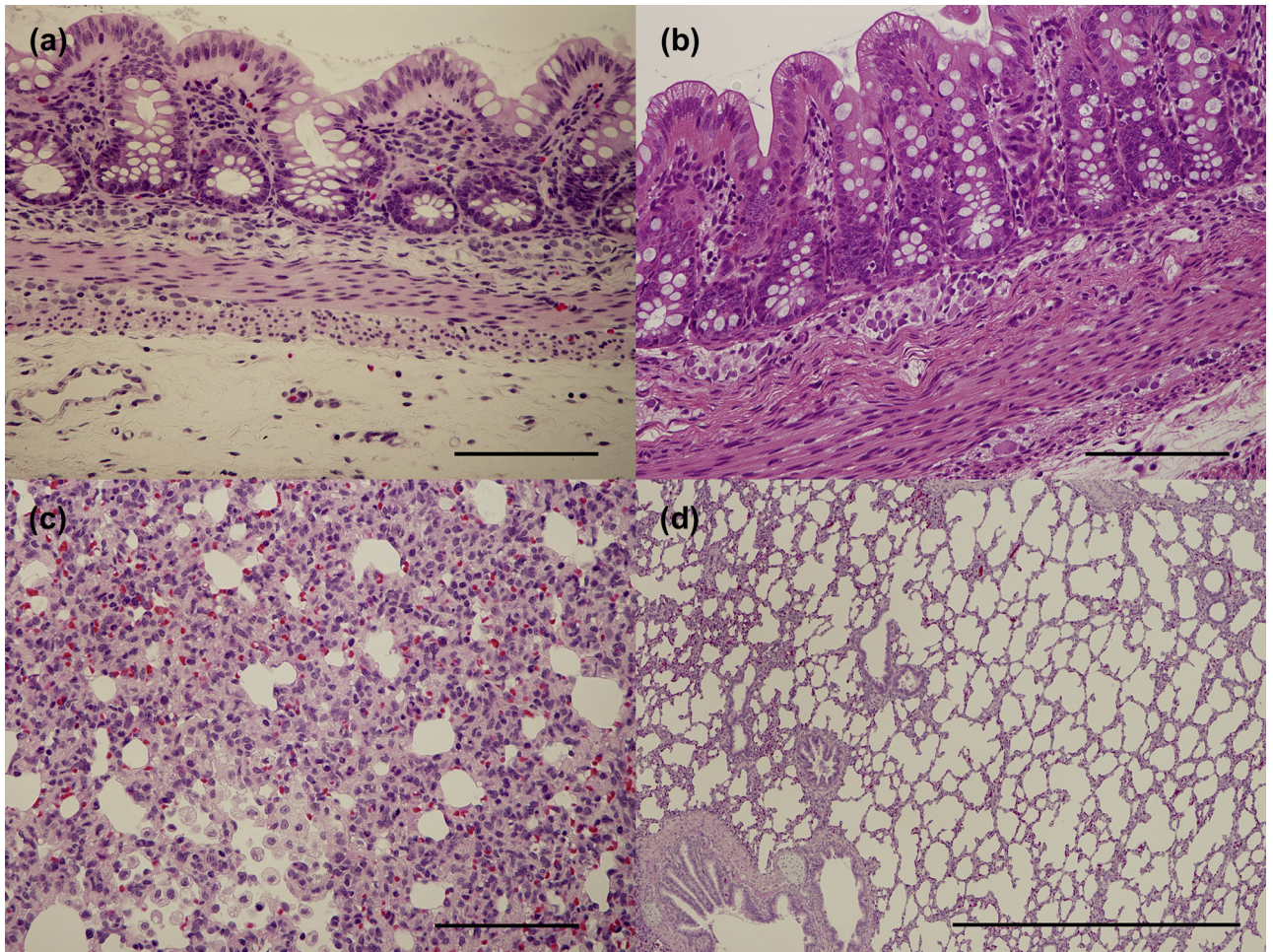


Fig. 4. Histological lesions in the colon and lung of PTV-infected pigs. (a) The colon of pig 9 (inoculated orally) at 13 dpi. There is atrophy of the epithelium and a thin wall. HE. Bar, 200 μ m. (b) The colon of pig 23, an uninfected control. HE. Bar, 200 μ m. (c) The lung of pig 20 (inoculated intranasally) at 20 dpi. The alveolar wall is thickened and the air space is decreased. Macrophage infiltration is seen in the alveolar lumen. HE. Bar, 200 μ m. (d) The lung of pig 11 (inoculated orally) at 20 dpi. No significant changes are observed. HE. Bar, 2 mm.

Microscopically, all inoculated pigs showed non-suppurative encephalomyelitis consisting of perivascular cuffing of mononuclear cells, focal gliosis, neuronal necrosis and a similar distribution of CNS lesions. Lesions were observed in the grey matter of the midbrain, pons, medulla oblongata and spinal cord (Figs. 3a and b). An infiltration of mononuclear cells and degenerate ganglion cells with satellitosis was observed in the spinal ganglia (Fig. 3e) and an infiltration of mononuclear cells with axonal spheroids was also observed in the spinal root (Fig. 3f). The most severe lesions tended to appear in the ventral horn of the spinal cord. No histological changes were observed in the cerebral hemispheres in any pigs inoculated with PTV, regardless of inoculation route. In pigs inoculated intravenously, lesions in the CNS progressed to involve the cerebellum and thalamus and were most severe at 20 dpi. In contrast,

in pigs inoculated orally or intranasally, severity of lesions in the CNS was greatest at 13 dpi but reduced by 20 and 28 dpi. In addition to the infiltrative changes, focal malacia in the ventral horn and vacuolar changes and axonal swelling in the white matter were observed in the spinal cord of pigs inoculated intravenously. In animals 5 and 6, some degenerate nerve cells with central chromatolysis were seen in the spinal cord at 20 dpi (Fig. 3c). Overall, pigs inoculated orally or intranasal with PTV had milder lesions than pigs inoculated intravenously.

In the pigs that exhibited diarrhoea, neither necrotic nor inflammatory lesions were observed in the large intestine, but the epithelium and wall of the large intestine were atrophic (Fig. 4a) and the serosal surface was markedly oedematous, properties that distinguish these pigs from those that were not infected (Fig. 4b). No significant lesions were observed in the small

Table 1
Markers of PTV infection in gnotobiotic pigs

Pig no.	Route	dpi	Nervous signs	Diarrhoea	Encephalomyelitis	IHC in CNS lesions	Virus particles in the spinal cord by EM	Virus neutralization titre	RT-PCR										
									Serum	Cerebrum	Brain-stem	Cerebellum	Spinal cord	Tonsil	Liver	Lung	Small intestine	Large intestine	
1	IV	6	+	-	+	+	-	2	+	++	++	++	++	++	++	++	++	++	++
2		6	-	-	+	+	-	2	+	++	++	++	++	++	++	++	++	++	++
3		13	+	+	+	+	+	16	++	++	++	++	++	++	++	++	++	++	++
4		13	-	+	+	+	+	32	++	++	++	++	++	++	++	++	++	++	++
5		20	+	-	+	+	+	256	-	++	++	++	++	++	++	+	++	++	++
6		20	+	+	+	+	+	128	+	++	++	++	++	++	++	++	++	++	++
7	O	6	-	-	+	+	-	4	+	++	++	++	++	++	++	++	++	++	++
8		6	-	-	+	+	-	2	+	++	++	++	++	++	++	++	++	++	++
9		13	+	+	+	+	+	32	+	++	++	++	++	++	++	+	++	++	++
10		13	+	-	+	+	+	128	+	++	++	++	++	++	++	-	++	++	++
11		20	+, R	+	+	+	+	256	+	++	++	++	++	++	++	-	++	++	++
12		20	+, R	-	+	+	+	256	-	++	++	++	++	++	++	-	++	++	++
13		28	+, R	-	+	-	-	1,024	-	+	++	++	++	++	++	-	+	-	++
14		28	+, R	-	+	-	-	1,024	-	-	-	-	-	-	-	-	-	-	-
15	IN	6	-	-	+	+	-	2	+	++	++	++	++	++	++	++	++	++	++
16		6	-	-	+	+	-	2	+	++	++	++	++	++	++	++	++	++	++
17		13	+	-	+	+	+	128	+	++	++	++	++	++	++	+	++	++	++
18		13	+	-	+	+	+	64	+	++	++	++	++	++	++	+	++	++	++
19		20	+, R	+	+	+	+	256	+	++	++	++	++	++	++	-	++	++	++
20		20	+, R	-	+	+	+	256	+	++	++	++	++	++	++	-	++	++	++
21		28	+, R	-	+	-	-	1,024	-	-	++	+	++	++	-	++	-	-	++
22		28	+, R	-	+	-	-	512	-	++	++	++	++	++	++	-	-	-	+
23	Control	20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24		20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25		28	-	-	-	-	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
26		28	-	-	-	-	ND	-	-	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

IV, intravenous; O, oral; IN, intranasal; dpi, days post infection; R, recovery; ND, not done; IHC, immunohistochemistry; CNS, central nervous system; EM, electron microscopy; RT-PCR, reverse-transcriptase polymerase chain reaction; +, present; -, absent.

For RT-PCR data ++, positive for first PCR; +, positive for nested PCR; -, negative.

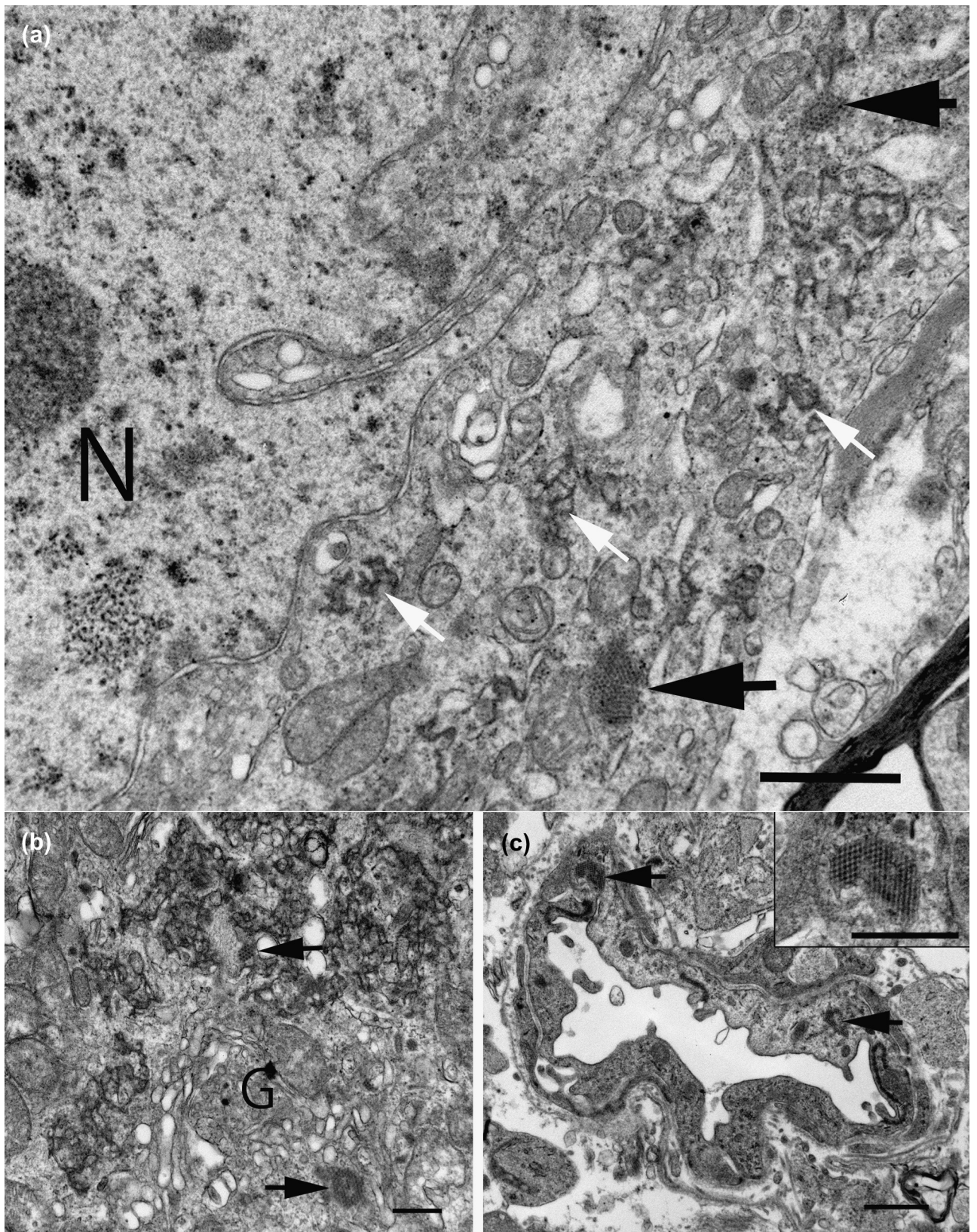


Fig. 5. Electron micrograph of the spinal cord from pigs inoculated intravenously at 20 dpi. (a) Crystalline arrayed non-enveloped virus particles (black arrows) are seen in the cytoplasm of a nerve cell in the ventral horn of pig 5. The structure of the rough endoplasmic reticulum in the nerve cells is destroyed (white arrows). N, nucleus. TEM. Bar, 1 μ m. (b) Virus particles (black arrows) are seen in

intestine of any pigs. Some degree of interstitial pneumonia with macrophage infiltration was seen in pigs inoculated intranasally with PTV at 13 (Fig. 4c), 20 and 28 dpi; however, no lung lesions were observed in pigs inoculated orally or intravenously (Fig. 4d). No histological changes were observed in any other organs in inoculated pigs and control pigs (numbers 23–26) showed no lesions whatsoever.

Immunohistochemistry

PTV antigen was mainly detected in the nervous system tissue of pigs inoculated with PTV (Table 1). The distribution of the antigen corresponded closely to the distribution of the CNS lesions. In all pigs inoculated intravenously (numbers 1–6), PTV antigen was detected in the cytoplasm of large nerve cells (Fig. 3d), in the glial cells that formed glial nodules in the grey matter of the brainstem, and in the ventral horn of the spinal cord and ganglion cells in the spinal ganglia. Some endothelial cells in these lesions were also labelled. Antigen was also detected in some nerve fibres of the dorsal root of the spinal ganglion. In pigs inoculated orally or intranasally, PTV antigen was detected in CNS lesions at 6, 13 and 20 dpi, but not at 28 dpi. No PTV antigen was detected in the cerebral hemisphere of any inoculated pigs.

In all other examined organs of pigs inoculated intravenously (pigs 1–6), PTV antigen was detected in the epithelial cells of the tonsils in pigs 1 and 2 at 6 dpi. PTV antigen was also detected in the cytoplasm of the majority of bronchiolar epithelial cells in the lung, the cytoplasm of some hepatocytes, the cytoplasm of some epithelial cells in the tonsils, and the myenteric nerve plexus in the small and large intestine in pigs 3–6 at 13 and 20 dpi. In pigs inoculated orally with PTV (animals 7–14), PTV antigen was detected in the tonsils of pigs 7–10 at 6 and 13 dpi. In the pigs inoculated intranasally (animals 15–22), PTV antigen was detected in the tonsils of pigs 15–18 at 6 and 13 dpi and in bronchiolar epithelial cells in the lungs of pigs 15–20 at 6, 13 and 20 dpi. PTV antigen was also observed in the lung lesions of pigs 17–20 at 13 and 20 dpi. No PTV antigen was observed in any other examined organs in any pigs, including the intestinal epithelium in the small and large intestine. No PTV antigen was detected in any control pigs (numbers 23–26).

Electron Microscopy

Crystalline arrayed non-enveloped virus particles, 25–30 nm in diameter, were detected in the cytoplasm of the nerve cells in the spinal cord of pigs 5 and 6 at 20 dpi and the structure of the rough endoplasmic reticulum in these nerve cells was completely destroyed (Figs. 5a, b). Similar virus particles were also observed in the cytoplasm of the endothelial cells of blood vessels (Fig. 5c) in the spinal cord of pigs 3 and 4 at 13 dpi and pigs 5 and 6 at 20 dpi. In pigs inoculated orally and intranasally, crystalline arrayed non-enveloped virus particles were seen in the cytoplasm of the endothelial cells of the blood vessels in the spinal cord at 13 dpi (pigs 9, 10, 17 and 18) and at 20 dpi (pigs 11, 12, 19 and 20), which was the same as in pigs inoculated intravenously; however, such virus particles were very rare in nerve cells. By 28 dpi, no virus particles were detected in any pigs inoculated with PTV.

Detection of PTV RNA

RT-PCR findings are summarized in Table 1. PTV RNA was detected in the cerebrum, brainstem, cerebellum, spinal cord, tonsils and large intestine of all pigs inoculated with PTV except for pig 14. In pigs inoculated intravenously, PTV RNA was detected in sera and all examined organs except for the serum of pig 5, which was killed at 20 dpi. The PTV gene was detected in the liver of pigs inoculated intravenously (but not orally or intranasally) at 20 dpi. At 28 dpi, no PTV genes were detected in the serum, liver or small intestine of pigs inoculated orally or intranasally. Findings were negative for all examined samples from control pigs.

Serology

The results of PTV neutralization tests are presented in Table 1. Although PTV-neutralizing antibodies were not detected in any pigs before PTV inoculation, all pigs seroconverted against PTV after inoculation. High titres were detected from 13 dpi, at which point the titres in pigs inoculated intravenously fell slightly below those in pigs inoculated orally or intranasally. No antibodies were detected in the four control animals (pigs 23–26).

the cytoplasm of a nerve cell in the ventral horn of pig 6. The structure of the rough endoplasmic reticulum is destroyed, but other organelles including the mitochondria and Golgi apparatus (G) are intact. TEM. Bar, 500 nm. (c) Virus particles (black arrows) in the cytoplasm of an endothelial cell in the ventral horn of pig 5. TEM. Bar, 1 μ m. Inset: high magnification of virus particles shown in (c). TEM. Bar, 500 nm.

Discussion

A CNS disorder characterized by non-suppurative encephalomyelitis with neurological signs was induced experimentally in gnotobiotic pigs by intravenous, oral and intranasal infection with the PTV-1 Toyama 2002 strain. PTV-1 infection was confirmed by the virus neutralization test in all inoculated pigs and infection in the CNS was further confirmed via detection of PTV RNA using RT-nested PCR in all pigs inoculated with PTV except for animal 14. Histological lesions in the CNS induced by oral or intranasal inoculation were mild compared with those in pigs inoculated intravenously. Oral and intranasal inoculations led to neurological signs of infection at 13 to 15 dpi. These signs were not progressive and disappeared within 3 or 4 days. There was a decreasing severity and range of histological lesions in the CNS at 20 and 28 dpi, presumably because of the increased serum antibody titre. In contrast, pigs inoculated intravenously showed a progressive neurological disorder and severe histological CNS lesions at 20 dpi despite an increase in serum antibody titre, possibly due to the host defence mechanism (i.e. the serum antibody titre) being too slow to prevent CNS lesions caused by PTV on induction of severe viraemia. The above findings underscore the potential importance of development of viraemia in the passage of the PTV to the CNS for neural infection in pigs. PCR analysis showed that PTV RNA was detected for shorter periods in the liver of pigs inoculated orally or intranasally than intravenously, suggesting that the infection route may affect how the serum antibody titre initiates host defence on primary infection of PTV and development of viraemia.

Electron microscopy detected virus particles in the cytoplasm of nerve cells in the spinal cord of pigs inoculated with PTV. The particles within these specimens corresponded in size, shape and substructure to mature particles of the swine polioencephalomyelitis virus (Koestner *et al.*, 1966). Virus particles have been reported in capillary endothelial cells, astrocyte footpads and glial processes in proximity to ganglion cell borders in germ-free pigs inoculated orally with the enterovirus o3b strain (now identified as PTV) at 14 dpi (Koestner *et al.*, 1966). However, the present report is the first to identify virus particles in the neurons of pigs infected with PTV. The ultrastructural lesion of organelles in nerve cells in which viral particles were detected was selective for the rough endoplasmic reticulum, and other organelles, including the mitochondria, remained intact. This selective injury of the rough endoplasmic reticulum may correlate with the central chromatolysis of nerve cells observed in HE-stained sections.

Electron microscopy at 13 dpi found virus particles only in the cytoplasm of endothelial cells of the blood vessels in the spinal cord of all pigs infected with PTV, regardless of inoculation route. These results suggest that the PTV Toyama 2002 strain may initially invade the endothelial cells of blood vessels in the CNS due to viraemia. In the CNS, vascular endothelial cells organize the blood–brain barrier (BBB), which is sealed together at its edges by tight junctions to block viral infection of the CNS and prevent the free transport of pathogens between the blood stream and parenchyma of the CNS (Spindler and Hsu, 2012). PTV may pass through the BBB, however, by infecting endothelial cells. The Japanese encephalitis virus (JEV) reportedly enters the CNS via a haematogenous route (Joo and Chu, 1999) and may also enter the brain via the olfactory pathway based on the distribution of lesions and viral antigens following intranasal and intravenous inoculations (Yamada *et al.*, 2009b). Some viruses, such as Aujeszky's disease herpesvirus (ADV) (Narita *et al.*, 1991) and highly pathogenic avian influenza H5N1 virus (Yamada *et al.*, 2012), also show multiple routes of invasion into the CNS. However, our present findings indicate that PTV does not enter the brain via the olfactory pathway, since the distribution of the lesions and viral antigen did not differ between pigs inoculated intranasally and intravenously.

The present findings also indicate that direct CNS invasion via the blood vessels is a major route of infection for PTV, as suggested previously (Holman *et al.*, 1966). We previously described that the earliest lesions in the CNS of piglets infected with the PTV Toyama 2002 strain occur in the spinal ganglion and spinal cord and speculated that the initial infection of the spinal ganglion may reflect the absence of a BBB at this location, thereby allowing the PTV to spread to the spinal cord by passing through the nerve fibres of the dorsal root of the ganglion via retrograde axonal transport (Yamada *et al.*, 2009a). Similarly, the poliovirus (Ohka *et al.*, 1998, 2004; Mueller *et al.*, 2005) and enterovirus 71 (Chen *et al.*, 2007), which like PTV are members of the Picornaviridae family, have been reported to enter the CNS from the blood across the BBB or be transmitted to the CNS through peripheral nerves via retrograde axonal transport. Immunohistochemical findings in the present work indicated that PTV enters the CNS by similar retrograde axonal transport or via haematogenous spread. However, we did not detect virus particles in the axons, arguing against retrograde axonal transport of PTV. Further studies are necessary to clarify the pathogenesis of the PTV Toyama 2002 strain and the detailed mechanisms of its CNS entry.

and neurological infections, which lead to encephalitis in pigs.

Pigs suffering from enteroviral encephalomyelitis brought on naturally or experimentally generally exhibit a similar distribution of CNS lesions, which mainly affect the brainstem, cerebellum and spinal cord, except for Teschen disease, which additionally affects the cerebrum (Trefny, 1930; Manuelidis *et al.*, 1954; Harding *et al.*, 1957; Long *et al.*, 1966; Jubb and Huxtable, 1993). In natural infections, CNS lesions are distributed in the brainstem, cerebellum, spinal cord and spinal ganglion, but the most severe lesions are in the spinal cord (Yamada *et al.*, 2004). In the present study, all pigs infected experimentally with PTV showed a similar distribution of CNS lesions, regardless of the route of inoculation. This distribution of CNS lesions may be one of the important findings for differential diagnosis of PTV encephalomyelitis from other non-suppurative encephalomyelitis in pigs.

Interstitial pneumonia was observed in pigs inoculated intranasally with PTV at 13, 20 and 28 dpi. These pigs, however, did not show clinical signs of respiratory disease. No lung lesions were noted in pigs inoculated intravenously or orally or in control pigs. The lung lesions caused by PTV may develop in association with a primary infectious site of PTV. For example, the experimental intranasal inoculation of PTV may lead to lung infection (Meyer *et al.*, 1966). However, while clinical signs of respiratory disease have been reported to be inducible by exposure to an aerosol of PTV (Pospisil *et al.*, 1971), more recent evidence has argued that aerosol exposure alone is an unlikely cause (Knowles, 2006). Our results suggest that the PTV Toyama 2002 strain may produce subclinical pneumonia in pigs by intranasal inoculation.

Diarrhoea was noted in six of the 22 pigs infected with PTV in this study and may have been caused by lesions in the large intestine, although no evidence could definitively show that such lesions were caused by the experimental inoculation. While PCR analysis detected PTV in the small and large intestines, lesions were only seen in the latter. PTVs have frequently been isolated from the faeces of piglets with diarrhoea, but since they can be readily isolated from normal piglets and since diarrhoea can be caused by a variety of other viral and bacterial agents, the relationship has remained correlative and not causal (Knowles, 2006). PTVs may be considerably less important enteric pathogens than rotaviruses or coronaviruses (Knowles, 2006). On the other hand, diarrhoea has been produced experimentally by PTVs in piglets believed to be free of other pathogens and the role of PTVs as enteric pathogens is uncertain (Knowles,

2006). Although the PTV Toyama 2002 strain may have less enteric virulence than neurological virulence, it may have the potential to produce diarrhoea in some pigs. In addition, it would seem that the clinical manifestation of Teschovirus encephalomyelitis in the field is dependent on other, so far unidentified, factors. Further experimental studies in gnotobiotic pigs are necessary to clarify the pathogenesis of PTVs using other strains.

A CNS disorder was successfully induced here via either oral or intranasal inoculation of the PTV Toyama 2002 strain. PCR analysis confirmed the distribution of PTV in the organs of infected pigs, demonstrating the value of the gnotobiotic pig as a model for the study of PTV pathogenesis.

Acknowledgements

We thank Mr. M. Kobayashi and Ms. M. Shimada for preparing the histopathological sections.

References

- Chen CS, Yao YC, Lin SC, Lee YP, Wang YF *et al.* (2007) Retrograde axonal transport: a major transmission route of enterovirus 71 in mice. *Journal of Virology*, **81**, 8996–9003.
- Edington N, Christofinis GJ, Betts AO (1972) Pathogenicity of Talfan and Konratice strains of teschen virus in gnotobiotic pigs. *Journal of Comparative Pathology*, **82**, 393–399.
- Harding JDJ, Done JT, Kershaw GF (1957) A transmissible polio-encephalomyelitis of pigs (Talfan disease). *Veterinary Record*, **69**, 824–832.
- Holman JE, Koestner A, Kasza L (1966) Histopathogenesis of porcine polioencephalomyelitis in the germ free pig. *Veterinary Pathology*, **3**, 633–651.
- Joo HS, Chu RM (1999) Japanese B encephalitis. In: *Diseases of Swine*, 8th Edit., BE Straw, S D'Allaire, WL Mengeling, DJ Taylor, Eds., Iowa State University Press, Ames, pp. 173–178.
- Jubb KVF, Huxtable CR (1993) The nervous system. In: *Pathology of Domestic Animals*, 4th Edit., Vol. 1, KVF Jubb, PC Kennedy, N Palmer, Eds., Academic Press, San Diego, pp. 267–439.
- Kaku Y, Murakami Y, Sarai A, Wang Y, Ohashi S *et al.* (2007) Antigenic properties of porcine teschovirus 1 (PTV-1) Talfan strain and molecular strategy for serotyping of PTVs. *Archives of Virology*, **152**, 929–940.
- Knowles NJ (2006) Porcine enteric picornaviruses. In: *Diseases of Swine*, 9th Edit., BE Straw, JJ Zimmerman, S D'Allaire, DJ Taylor, Eds., Blackwell Publishing, Iowa, pp. 337–345.
- Knowles NJ (2008) Teschovirus encephalomyelitis (previously enterovirus encephalomyelitis or Teschen/Talfan disease). In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, 6th Edit., Office International des Epizooties, Paris, pp. 1146–1152.

- Koestner A, Kasza L, Holman JE (1966) Electron microscopic evaluation of the pathogenesis of porcine polioencephalomyelitis. *American Journal of Pathology*, **49**, 325–337.
- La Rosa G, Muscillo M, Di Grazia A, Fontana S, Iaconelli M *et al.* (2007) Validation of RT-PCR assays for molecular characterization of porcine teschoviruses and enteroviruses. *Journal of Veterinary Medicine B*, **53**, 257–265.
- Long JF (1985) Pathogenesis of porcine polioencephalomyelitis. In: *Comparative Pathology of Viral Diseases*, Vol. 1, RA Olsen, S Krakowka, JR Blakeslee, Eds., CRC Press Inc., Florida, pp. 179–197.
- Long JF, Koestner A, Kasza L (1966) Infectivity of three porcine polioencephalomyelitis viruses for germ-free and pathogen-free pigs. *American Journal of Veterinary Research*, **27**, 274–279.
- Manuelidis EE, Sprinz H, Horstmann DM (1954) Pathology of Teschen disease (virus encephalomyelitis of swine). *American Journal of Pathology*, **30**, 567–597.
- Meyer RC, Wood GT, Simon J (1966) Pneumonitis in an enterovirus infection in swine. *Journal of Comparative Pathology*, **76**, 397–405.
- Mueller S, Wimmer E, Cello J (2005) Poliovirus and poliomyelitis: a tale of guts, brains, and an accidental event. *Virus Research*, **111**, 175–193.
- Narita M, Imada T, Haritani M (1991) Immunohistochemical demonstration of spread of Aujeszky's disease virus via the olfactory pathway in HPCD pigs. *Journal of Comparative Pathology*, **105**, 141–145.
- Ohka S, Matsuda N, Tohyama K, Oda T, Morikawa M *et al.* (2004) Receptor (CD155)-dependent endocytosis of poliovirus and retrograde axonal transport of the endosome. *Journal of Virology*, **78**, 7186–7198.
- Ohka S, Yang WX, Terada E, Iwasaki K, Nomoto A (1998) Retrograde transport of intact poliovirus through the axon via the fast transport system. *Virology*, **250**, 67–75.
- Pospisil Z, Gois M, Veznikova D, Cerny. (1971) The pathogenesis of experimental infection of gnotobiotic piglets with enterovirus strain Kr 69TK. *Acta Veterinaria Brno*, **40**(Suppl. 2), 43–46.
- Spindler KR, Hsu TH (2012) Viral disruption of the blood-brain barrier. *Trends in Microbiology*, **20**, 282–290.
- Trefny L (1930) Massive illness of swine in Teschen area. *Žverolekarsky Obzor*, **23**, 235–236.
- Yamada M, Bingham J, Payne J, Rookes J, Lowther S *et al.* (2012) Multiple routes of invasion of wild-type Clade 1 highly pathogenic avian influenza H5N1 virus into the central nervous system (CNS) after intranasal exposure in ferrets. *Acta Neuropathologica*, **124**, 505–516.
- Yamada M, Kaku Y, Nakamura K, Yoshii M, Yamamoto Y *et al.* (2007) Immunohistochemical detection of porcine teschovirus antigen in the formalin-fixed paraffin-embedded specimens from pigs experimentally infected with porcine teschovirus. *Journal of Veterinary Medicine A*, **54**, 571–574.
- Yamada M, Kozakura R, Ikegami R, Nakamura K, Kaku Y *et al.* (2004) Enterovirus encephalomyelitis of pigs caused by porcine teschovirus in Japan. *Veterinary Record*, **155**, 304–306.
- Yamada M, Kozakura R, Nakamura K, Yamamoto Y, Yoshii M *et al.* (2009a) Pathological changes in pigs experimentally infected with porcine teschovirus. *Journal of Comparative Pathology*, **141**, 223–228.
- Yamada M, Nakamura K, Yoshii M, Kaku Y, Narita M (2009b) Brain lesions induced by experimental intranasal infection of Japanese encephalitis virus in piglets. *Journal of Comparative Pathology*, **141**, 156–162.
- Zell R, Krumbholz A, Henke A, Birch-Hirschfeld E, Stelzner A *et al.* (2000) Detection of porcine enteroviruses by nRT-PCR: differentiation of CPE groups I–III with specific primer sets. *Journal of Virological Methods*, **88**, 205–218.

[Received, June 13th, 2013]
 [Accepted, August 29th, 2013]