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NOTES

Tioman Virus, a Paramyxovirus of Bat Origin, Causes Mild Disease in Pigs and Has a Predilection for Lymphoid Tissues▽

Koon Chu Yaiw,[1]† John Bingham,[2]† Gary Crameri,[2] Bruce Mungall,[2] Alex Hyatt,[2] Meng Yu,[2] Bryan Eaton,[2] Devi Shamala,[1] Lin-Fa Wang,[2] and Kum Thong Wong[1]*

Department of Pathology and Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia[1]; and Australian Animal Health Laboratory, Commonwealth Scientific and Industrial Research Organization Livestock Industries, Geelong, Victoria 3220, Australia[2]

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Disease manifestation, pathology, and tissue tropism following infection with Tioman virus (TioPV), a newly isolated, bat-derived paramyxovirus, was investigated in subcutaneously ($n = 12$) and oronasally ($n = 4$) inoculated pigs. Pigs were either asymptomatic or developed pyrexia, but all of the animals produced neutralizing antibodies. The virus (viral antigen and/or genome) was detected in lymphocytes of the thymus, tonsils, spleen, lymph nodes and Peyer's patches (ileum), tonsillar epithelium, and thymic epithelioreticular cells. Virus was isolated from oral swabs but not from urine. Our findings suggest that the pig could act as an intermediate or amplifying host for TioPV and that oral secretion is a possible means of viral transmission.

Tioman virus (TioPV) was coincidentally discovered in the urine of pteropodid bats found on Tioman Island, Malaysia, in the quest for the natural host of *Nipah virus* (NiV) in 2001 (3). Studies have established TioPV as a new virus in the family *Paramyxoviridae*, subfamily *Paramyxovirinae*, and genus *Rubulavirus* (3, 4).

The close genetic similarity between TioPV (62%) with another paramyxovirus, the Menangle virus (MenPV), which caused disease in pigs, raises the possibility that it could be a potential pathogen. MenPV was isolated from stillborn piglets in Australia in 1997 and was found to be associated with a reduction of the farrowing rate and the number of live piglet births per litter, with abortions, and with congenital abnormalities (15). Two humans in close contact with MenPV-infected pigs had developed neutralizing antibodies and an influenza-like illness, suggesting probable zoonotic potential (2). Interestingly, as for *Hendra virus* and NiV, two recently emerging paramyxoviruses, and MenPV, pteropodid bats are the probable natural hosts of TioPV (4, 15).

We have previously shown that TioPV is capable of infecting and replicating in mouse neurons (22) and human neuroblastoma cells (K. C. Yaiw, A. Hyatt, and K. T. Wong, unpublished data). Moreover, a recent serosurvey showed that 1.8% of Tioman Islanders had neutralizing antibodies against TioPV, indicating probable past infection (21). Bat-to-human transmission is obscure, although direct transmission via ingestion of fruits by humans has been suggested (21). Since MenPV and NiV originate from bats and involve pigs as intermediate or amplifying hosts (9, 14), it is not unreasonable to assume that TioPV could likewise infect pigs. We investigated the susceptibility of pigs to TioPV infection in order to better understand the virus's disease-causing potential, pathology, pathogenesis, viral shedding, and possible transmission routes to humans and other animals.

Susceptibility of pigs to TioPV infection. The original bat urine-derived TioPV was plaque purified twice, further passaged four times in Vero cells (CCL-81; American Type Culture Collection, Manassas, VA) to a titer of approximately 1.5×10^6 50% tissue culture infective doses ($TCID_{50}$)/ml, and used as a virus stock. Virus titration was done as previously described (8). Infection studies were conducted at the Australian Animal Health Laboratory, Geelong, Australia, under bio-

TABLE 1. Neutralizing antibody titers of TioPV-infected pigs during the course of infection

Main study (route of infection) and pig	Time of sacrifice (dpi)	Reciprocal neutralizing antibody titer[a]			
		9 dpi	11 dpi	13 dpi	20 dpi
SS (subcutaneous)					
P61	9	ND[b]	NA[c]	NA	NA
P62	9	320	NA	NA	NA
P66	11	640	640	NA	NA
P70	11	320	320	NA	NA
P64	13	160	ND	320	NA
P68	13	640	160	ND	NA
ON (oronasal)					
P67	20	160	80	160	160
P69	20	320	80	160	160
P72	20	80	320	320	160
P73	20	40	ND	80	320

[a] Sera were collected from 9 dpi onward.
[b] ND, not done.
[c] NA, not applicable.

* Corresponding author. Mailing address: Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. Phone: 60-3-7967 5762. Fax: 60-3-7955 6845. E-mail: wongkt@um.edu.my.

† K.C.Y. and J.B. contributed equally to this work.

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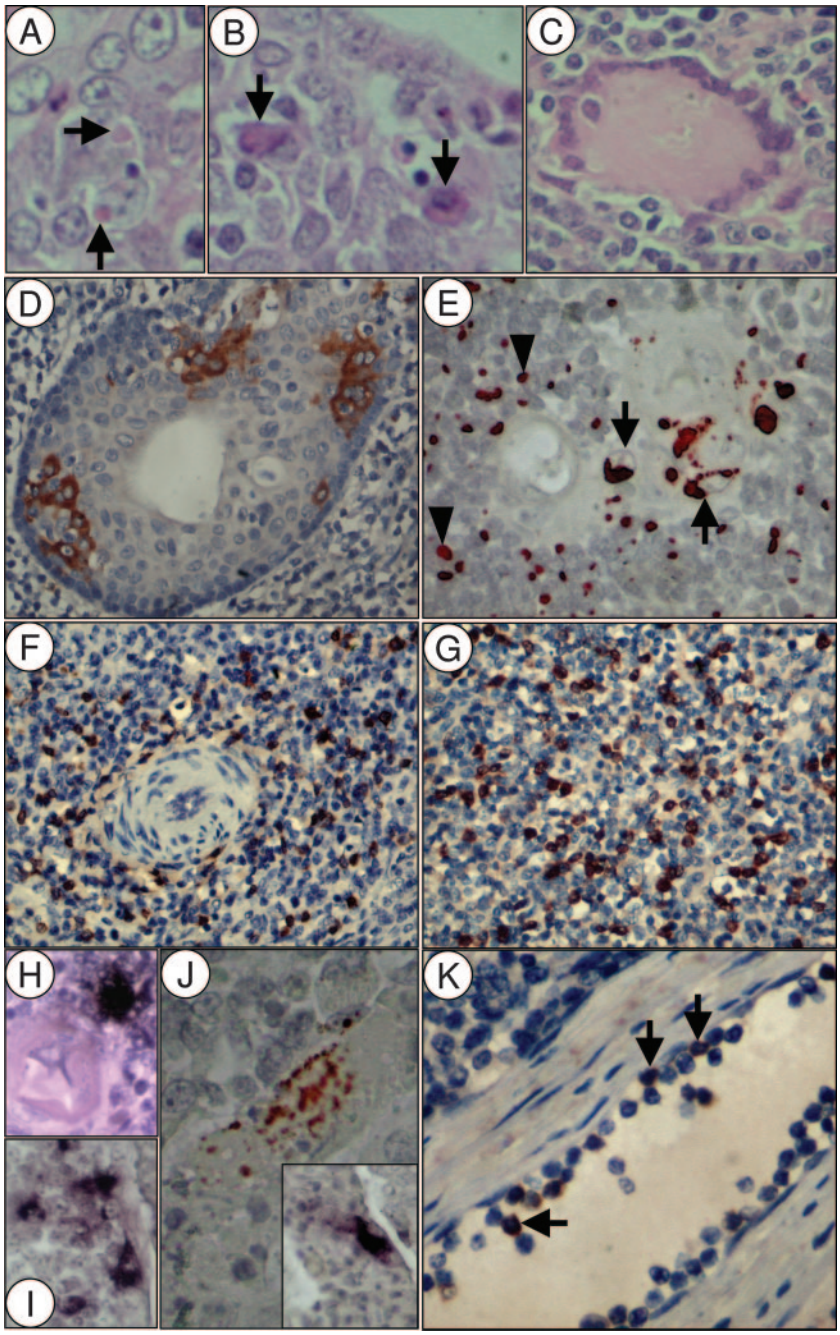

FIG. 1. Histology, IHC, and ISH of TioPV infection of pigs. (A) Intracytoplasmic TioPV inclusions within the epithelial cells of an infected pig tonsil (arrows). Hematoxylin and eosin, 40× objective. (B) Intranuclear inclusion in a TioPV-infected pig tonsil (arrows). Hematoxylin and eosin, 40× objective. (C) A Warthin-Finkeldey-type giant cell in the medulla of the thymus. Hematoxylin and eosin, 40× objective. (D) TioPV antigens in epithelial cells of a tonsillar crypt. Rabbit anti-TioPV antibody, chromogen 3,3′-diaminobenzidine, brown, counterstained with Harris's hematoxylin, 20× objective. (E) TioPV antigens in the epithelioreticular cells (arrows) of the medulla and within lymphocytes (arrowheads) of the thymus. Pig anti-TioPV antibody, chromogen 3-amino-9-ethylcarbazole, red, lightly counterstained with Mayer's hematoxylin, 40× objective. (F) Viral antigens in lymphocytes surrounding the periarterial lymphatic sheath of the spleen. Rabbit anti-TioPV antibody, chromogen 3,3′-diaminobenzidine, brown, counterstained with Harris's hematoxylin, 20× objective. (G) Viral antigens in the parafollicle of the lymph node. Rabbit anti-TioPV antibody, chromogen 3,3′-diaminobenzidine, brown, counterstained with Harris's hematoxylin, 20× objective. (H) ISH of TioPV in the thymus showing the viral genome in an epithelioreticular cell adjacent to Hassall's corpuscle. Chromogen nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, dark blue, 40× objective. (I) TioPV genome detected in epithelial cells lining a tonsillar crypt by ISH. Chromogen nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, dark blue, 40× objective. (J) Viral antigens in cell debris within a tonsillar crypt. Inset, ISH of TioPV in the cell debris within the crypt. Pig anti-TioPV antibody, chromogen 3-amino-9-ethylcarbazole, red, lightly counterstained with Mayer's hematoxylin, 40× objective; inset, chromogen nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, dark blue, 40× objective. (K) IHC positivity in circulating leukocytes (arrows). Rabbit anti-TioPV antibody, chromogen 3,3′-diaminobenzidine, brown, counterstained with Harris's hematoxylin, 40× objective.

TABLE 2. Viral antigens[a] in lymphoid tissues of, oral virus isolation from, and urine real-time PCR of subcutaneously TioPV-infected pigs

Animal	Time of sacrifice (dpi)	Thymus[b]	Tonsils[c]	Spleen[d]	Lymph nodes				Peyer's patches (ileum)	Virus isolation from oral swab	Real-time PCR[f] on urine
					Prescapular[e]	Submandibular[e]	Mediastinal[e]	Mesenteric[e]			
P74	2	+	+	−	−	+	−	−	−	−	−
P76	2	ND[g]	−	−	−	−	−	+	−	−	−
P63	4	++	++	++	+++	++	+++	+++	+	−	−
P65	4	ND	+++	++	+++	+++	+++	++	+	−	−
P71	6	+++	+++	+	++	++	++	++	+	−	−
P75	6	+++	+	++	++	++	+	++	++	−	−
P61	9	+	+/−	−	+/−	+/−	+	+/−	+/−	+[h]	−
P62	9	+	+/−	−	+/−	−	+/−	−	−	−	−
P66	11	+	+/−	+/−	+	−	++	+	+	+[i]	−
P70	11	ND	+/−	ND	+	+	+/−	+/−	+/−	−	−
P64	13	−	−	−	+/−	+/−	+/−	+/−	−	−	−
P68	13	+	+/−	−	+/−	−	+/−	−	−	−	−

[a] Viral antigen was semiquantitated with rabbit anti-TioPV antiserum as the primary antibody. Symbols: +/−, equivocal staining; −, negative; +, positive; ++, moderately positive; +++, strongly positive.
[b] Detected in epithelioreticular cells in the vicinity of Hasall's bodies, thymocytes, and syncytia.
[c] Found in lymphocytes and in tonsillar epithelium and crypts.
[d] In lymphocytes surrounding the periarterial lymphatic sheath, the marginal area of the splenic nodule (white pulp), and splenic cords of the red pulp.
[e] In lymphocytes (mantle/marginal zone of the lymph node), parafollicle, medullary cord, surrounding medullary sinus, paracortex, and circulating lymphocytes.
[f] In-house TioPV TaqMan real-time PCR with forward primer 5′-GTT CGA GCT CTT TAC CTT AGA GCA A-3′ (900 nM), reverse primer 5′-CAC ATC ATA TCA TGA CGT GTT TGA G-3′ (900 nM), and probe 5-carboxyfluorescein–CTG ACT GTT TAA GAT ACA AAC–molecular-groove-binding nonfluorescent quencher (250 nM) targeting the TioPV nucleoprotein-encoding gene.
[g] ND, not done.
[h] 1.30×10^5 $TCID_{50}$/ml at 6 dpi.
[i] 2.30×10^5 $TCID_{50}$/ml at 6 dpi.

safety level 3 containment as previously described (10) after obtaining ethical clearance. A pilot study was conducted with six 6-week-old piglets before the main study to assess susceptibility to infection and to generate antisera against TioPV. This was confirmed when pigs developed pyrexia and all seroconverted with serum neutralizing test titers of 160 to 320. The serum neutralizing test was performed as described previously (10).

The main study consisted of serial-sacrifice (SS) and oronasal (ON) trials. The SS trial was conducted with 12 piglets, each given 5×10^4 $TCID_{50}$ of TioPV subcutaneously, with 2 pigs serially sacrificed every 2 to 3 days postinfection (dpi) (i.e., 2, 4, 6, 9, 11, and 13 dpi). The ON trial consisted of four piglets each given 1×10^6 $TCID_{50}$ oronasally to determine infection susceptibility, and all were sacrificed at 20 dpi. From all animals, whole blood, serum, and nasal, oral, rectal, and prepucial swabs were taken before and after inoculation. Clinical signs, temperature, and weight changes were monitored daily. At sacrifice, tissues were processed accordingly for histopathology, immunohistochemistry (IHC), in situ hybridization (ISH), electron microscopy, virus isolation, and real-time PCR as described previously (7, 10, 11, 22). For IHC, the primary antibodies used were pig anti-TioPV antiserum (1:50) and rabbit anti-TioPV (1:750) (22).

In the main study, apart from pyrexia, no other clinical signs were observed. Two pigs (16.7%) from the SS trial and all pigs from the ON group had pyrexia (up to 40.3°C) between 4 and 9 dpi. All pigs developed neutralizing antibody titers ranging from 1:40 to 1:640 (Table 1).

Macroscopically, tongue and tonsillar ulcerations were noted in four pigs (two each in the SS and ON groups). Mild submandibular lymph node swelling was observed from 4 to 6 dpi.

Histopathology and virus detection in tissues. In the SS trial, infiltration of inflammatory cells (neutrophils, eosinophils, and mononuclear cells) was noted in the tonsils, thymus, and spleen. Intracytoplasmic and intranuclear viral inclusions were detected in viable and necrotic epithelial cells lining tonsillar crypts (Fig. 1A and B) and in lymphocytes. Lymph nodes showed subcapsular and sinus histiocytosis and reactive follicular hyperplasia. Interestingly, Warthin-Finkeldey giant cells, cells typically found in morbillivirus infections such as measles (23), were seen at 6 dpi in the medulla of the thymus (Fig. 1C). Warthin-Finkeldey giant cells have not been reported previously in MenPV and other rubulavirus infections.

Viral antigens could be seen as early as 2 dpi in the thymus, tonsils, and lymph nodes (Table 2). Intense and maximum IHC staining was detected from 4 to 6 dpi in the tonsillar epithelium and in thymic epithelioreticular cells and lymphocytes in the tonsils, thymus, spleen, lymph nodes (prescapular, submandibular, mediastinal, and mesenteric), and Peyer's patches (ileum) (Fig. 1D to G). ISH staining demonstrated the presence of viral genome in these same cells (Fig. 1H and I). IHC positivity in tonsillar crypt cell debris was confirmed by ISH (Fig. 1J). This coincided with virus isolation at 6 dpi (see below). Electron microscopy revealed paramyxovirus-like budding viruses and viral inclusions in lymph nodes (Yaiw and Hyatt, unpublished). All other tissues were negative by IHC and ISH in the SS group. Appropriate positive and negative controls included in the IHC and ISH assays yielded stains as expected.

Assuming that infection of various organs followed viremia, it is likely that circulating infected leukocytes played a role, as viral antigens were positive in these cells (Fig. 1K). Circulating leukocytes in viral dissemination have been reported in mumps and measles virus infections (13, 19).

Our findings suggested that TioPV is lymphotropic, as was

reported for other rubulaviruses e.g., mumps virus (6) and morbilliviruses, e.g., rinderpest virus (RPV) (16, 18), measles virus (23), and phocine distemper virus (17). Interestingly, unlike mumps virus, TioPV does not appear to involve the kidneys or the central nervous system but appears to be more similar to RPV in its target organs (12). Whether TioPV-associated lymphotropism alters cell-mediated immunity or causes immunosuppression, as documented for measles virus and RPV (5, 23), remains to be investigated.

In the ON group, tissues obtained at 20 dpi showed subtle IHC and ISH positivity only in the tonsils of two pigs; other tissues were negative (data not shown).

Virus shedding. Virus was reisolated from oral swabs of two pigs (Table 2) in the SS group and one pig (P69; 7.10×10^4 $TCID_{50}$/ml) in the ON group at 6 dpi only. All other swabs from both groups were negative for virus isolation. This suggests that virus shedding occurred mainly at about 6 dpi (possibly 5 to 8 dpi) and that oral secretions might be one potential route of transmission to humans or uninfected pigs. Further pig-to-pig transmission studies are needed to address the latter possibility. We postulate that virus was shed into the oral cavity following infection of the tonsillar epithelium since salivary gland tissues were negative by IHC and ISH (data not shown). All urine samples collected from animals in both groups were negative by real-time PCR, suggesting that viral transmission via urine is unlikely (Table 2). Since pigs could be oronasally infected, this could be a potential route for bat-to-pig transmission, similar to MenPV and NiV (15, 20).

MenPV was readily recovered from nasopharyngeal secretions, feces, and urine following intranasal inoculation of weaned pigs, and the infection appeared to be systemic but mild (1). Unfortunately, no histopathogical studies of these animals were done and thus comparison with our findings is impossible. In congenitally infected pig fetuses, MenPV infection seems to involve the central nervous system, lungs, and heart (9).

In conclusion, TioPV is capable of infecting and replicating in pigs and the main cellular targets are lymphocytes, thymic epithelioreticular cells, and the tonsillar epithelium. Hence, the pig could act as an intermediate or amplifying host for human transmission, as has happened in MenPV and NiV outbreaks. In human NiV outbreaks, pigs played a critical role in transmitting the disease to pig handlers by direct contact (14). As our findings suggest, unsuspected mild TioPV infection could occur in naturally infected pigs and this could facilitate viral transmission to humans via contact with oral secretions. Finally, the diagnostic assays and reagents generated by this study should be useful in detecting diseased humans and animals in possible future outbreaks of TioPV infection.

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