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豬鐵士古病毒地方性感染之血清型分子鑑定

Molecular Serotyping of the Porcine Teschoviruses in Endemically Infected Pigs

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本論文係 郭依婧君（r02644004）在國立臺灣大學獸醫專業學院分子暨比較病理生物學研究所、所完成之碩士學位論文，於民國 104 年 07 月 15 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

豬鐵士古病毒(Porcine Teschovirus, PTV)為 Picornaviridae 科 Teschovirus 屬，直徑約為 25-30nm、球型、正向單股 RNA 病毒。目前 PTV 有 13 種血清型，各年齡豬隻皆有感受性，臨床症狀以引起神經症狀之腦脊髓灰質炎及下痢為主。台灣在 2000 年及 2004 年爆發兩次疫情，現今在豬場呈現普遍的地方性感染。先前檢測出台灣存有五種血清型，分別為 PTV-1、-4、-6、-7、-11，最常見的為 PTV-7 及 PTV-6。本研究目的為利用增幅病毒 VP1 核酸序列進行鑑定台灣目前 PTV 是否有更多血清型的存在。實驗樣材來自於七個豬場，每場採集 3 隻淘汰病弱豬及 1 隻臨床症狀健康豬隻；每頭豬隻收集糞便、迴腸、扁桃、鼠蹊淋巴結、血漿、脾臟、腎臟、膀胱、尿液以及鼻腔拭鏡共 10 個樣材。首先以增幅 5'NTR 高度保留區域檢測 PTV 感染，所有 28 頭豬隻皆為 PTV 陽性(100%, 28/28)，其中又以糞便的陽性率最高(96%, 27/28)，其次為迴腸、扁桃、鼻腔試鏡(93%, 26/28)，血漿(86%, 24/28)，鼠蹊淋巴結及尿液(79%, 22/28)，膀胱(64%, 18/28)，脾臟(57%, 16/28)及腎臟(10%, 10/28)。接著利用本研究新設計之引子增幅病毒 VP1 序列，在 217 個 PTV 陽性樣本中有 63 個成功完成增幅且區分血清型。28 頭豬隻皆至少有一種血清型存在，總共檢測出五種 PTV 血清型，分別為 PTV-2、-4、-5、-6、-10，其中又以 PTV-10 (57%, 36/63) 最為常見，其次為 PTV-2 (14/63, 22%)。其中有 14 頭豬隻有兩種以上血清型存於不同臟器 (14/28, 50%)。本研究再度印證了台灣 PTV 普遍感染的現象，加上先前的結果，證實台灣目前至少存在 8 種血清型，分別為 PTV-1、-2、-4、-5、-6、-7、-10、-11。值得注意的是在分別來自三個豬場的三頭豬隻尿液亦可檢測出 PTV-10，顯示 PTV 可經尿液排出體外，糞尿混合下加速了病毒傳播速度，證實之前的推論。

關鍵字：豬鐵士古病毒、血清型、反轉錄聚合酶連鎖反應

ABSTRACT

Porcine teschoviruses (PTVs) is a non-enveloped, spherical, positive-sense, single-stranded RNA virus, which belong to genus *Teschovirus* within the family *Picornaviridae*. Up to date, PTVs are reclassified into 13 serotypes that are associated with a variety of clinical signs and prominent with polioencephalomyelitis and diarrhea. Two epidemic outbreaks of PTV-1 occurred in Taiwan, one in 2000 and a second in 2004, causing severe economic loss. The enzootic status is now confirmed in swine herds in Taiwan. Previous study has demonstrated at least 5 different serotypes of PTV (PTV-1, -4, -6, -7, -11) existing in Taiwan, and PTV-7 and -6 are the most common serotypes. The aim of this study was to investigate the wider variety of serotypes of PTV by introducing a set of newly designed primer, based on the sequence of VP1 outer capsid gene presented in the feces, plasma, urine, nasal swab, and solid samples in naturally infected piglets. Samples were collected from 7 clinically healthy and 21 culled piglets of 7 different herds. The PTV infections were screened by the published primers to amplify highly conserved 5'NTR region using RT-PCR followed by nested PCR. The PTV detection rate was 100% (28/28) by heads. Out of 217/280 PTV-positive samples the most common detected sample was feces (96%, 27/28), and followed by in decreasing order ileum, tonsil, and nasal swab (93%, 26/28), plasma (86%, 24/28), inguinal LN and urine (both were 79%, 22/28), bladder (64%, 18/28), spleen (57%, 16/28), and kidney (10%, 10/28). VP1 region was successfully amplified, by the newly designed primers, out of 63/217 (29%) pan-PTV nested PCR-positive samples, sequenced, and phylogenetic tree constructed. All 28 piglets had at least one serotype existed, and a total of 5 serotypes of PTV-2, PTV-4, PTV-5, PTV-6, and PTV-10, were identified from 28 piglets. The most common serotype was PTV-10 (36/63, 57%) and the 2nd one was PTV-2 (14/63, 22%). In 14/28 (50%) heads had at least two serotypes

identified in the same animals but in different samples. In 2/7 herds, four PTV serotype co-circulated. In conclusion, this study confirms the enzootic and contaminated status of swine herds, combined with previous study at least 8 serotypes of PTV, namely PTV-1、-2、-4、-5、-6、-7、-10、-11 are identified. In particular PTV-10 is found shed in the urine of 3 piglets from 3 separate herds, consistent with the multiple models of PTV pathogenesis. Urine makes solid feces into semisolid slurry thus enhancing the exposure of piglets to PTV.

Key words: Teschovirus, serotyping, RT-PCR

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Chapter 1 Introduction

Porcine teschoviruses (PTVs) is a non-enveloped, spherical, positive-sense, single-stranded RNA virus, which belong to genus *Teschovirus* within the family *Picornaviridae*. On the basis of (i) cytopathic effect (CPE), (ii) replication properties in various host cell lines, (iii) serological assays, and (iv) sequence data, the original genus *Enterovirus* was divided into three groups: *Teschovirus* (CPE group I, PEV types 1-7 and 11-13), *Sapelovirus* (Porcine Enterovirus A, CPE group II, PEV-8), and Porcine Enterovirus B (CPE group III, PEV-9 and PEV-10) (Kaku et al., 2001; Zell et al., 2001). Up to date, PTVs are reclassified into 13 serotypes that are associated with a variety of clinical signs, including polioencephalomyelitis, enteric disease, female reproductive disorders, and pneumonia (Alexandersen et al., 2012; Boros et al., 2012; Cano-Gomez et al., 2011b).

The virulent PTV-1 strains were associated with polioencephalomyelitis of pigs (Teschen disease), which caused huge losses to the pig breeding industry in Europe during 1930-1950s (Horstmann, 1952). Nowadays, the highly virulent Teschen strains have been replaced by Talfan strains, which include less virulent PTV-1 strains and stains belonging to other serotypes associated with mild encephalomyelitis (Alexandersen et al., 2012, La Rosa et al., 2006).

In the past, two epidemic outbreaks of PTV-1 occurred in Taiwan, one in 2000 and a second in 2004, causing severely economic loss. The isolation rates of PTVs from porcine herds have been increasing since 2004, ranging from 20.59% to 33% by herd, and was the first and second commonly isolated virus from culled pigs (Huang et al., 2009), indicative of the enzootic status. In addition, co-infection with other common swine pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV),

porcine circovirus type 2 (PCV2), and classical swine fever virus (CSFV) are found frequently, revealing the multi-infection status in the field (Chiu et al., 2012).

Previous study has demonstrated at least 5 different serotypes of PTV-1, -4, -6, -7, -11 existing in Taiwan, and PTV-7 is the most common serotypes (Chiu et al., 2012). Moreover, co-existence of two different serotypes in the same animal and co-circulation of several serotypes in a geographic area are common (Chiu et al., 2014, Cano-Gomez et al., 2011b). The aim of this study was to investigate the wider variety of serotypes of PTV by introducing a newly designed primer for molecular serotyping, based on the sequence of VP1 outer capsid gene presented in the feces, plasma, urine, nasal swab, and solid samples in naturally infected piglets.

Chapter 2 Literature Review

2.1 Porcine teschovirus

2.1.1 Taxonomy and Classification

Porcine teschoviruses (PTVs) belong to genus *Teschovirus* within the family *Picornaviridae*. In the past, PTVs was classified as porcine enteroviruses (PEVs). On the basis of (i) cytopathic effect (CPE), (ii) replication properties in various host cell lines, (iii) serological assays, and (iv) sequence data (Knowles et al., 1979), Porcine Teschovirus (CPE group 1, PEV types 1-7 and 11-13) was reclassified as a single species in a new genus, *Teschovirus* (Alexandersen et al., 2012; Kaku et al., 2001; Zell et al., 2001). Recently a PTV-12 was identified in domestic pigs (Cano-Gomez et al., 2011b) and a PTV-13 in wild boars (Boros et al., 2012). Consequently, at least 13 serotypes (PTV-1 to PTV-13) have been identified to date.

2.1.2 Morphology, Genome and Capsid proteins

Porcine teschoviruses (PTVs) is a non-enveloped, spherical RNA virus with a diameter of 25-30 nm. It comprises a single positive-sense RNA with 7.1 kb in length surrounded by an icosahedral capsid. A small, virus-encoded protein VPg is linked to the 5’ terminus. The RNA genome is composed of a long 5’ nontranslated region (NTR) including an internal ribosome entry site (IRES), a single open reading frame (ORF), followed by a shorter 3’ NTR and poly (A) tail. The ORF is translated into a polyprotein which is later processed by virus-encoded proteinases. The polyprotein encoding region consists of P1, P2, and P3 region. The P1 region contains structural polypeptides (VP1, VP2, VP3, and VP4). The P2 and P3 region encode nonstructural polypeptides (Alexandersen et al., 2012).

The virion capsid comprises VP1, VP2, VP3, and VP4. The VP4 is internal and interacts with the RNA molecule protected by the capsid shell (Cano-Gomez et al., 2011b). The VP1, VP2, and VP3 are exposed at the virion surface and would be useful for molecular serotyping (La Rosa et al., 2006).

2.2 Epidemiology

The first report of Teschen disease, a swine polioencephalomyelitis caused by virulent PTV-1 strains, was recognized in Czechoslovakia in 1929, and thousands of outbreaks with large economic losses occurred in Europe during 1930-1950s (Horstmann, 1952). Subsequently, mild forms of the disease were identified as Talfan disease in Wales and in Denmark (Harding et al., 1957). Up to date, teschovirus encephalomyelitis was found in Japan, Latvia, Madagascar, Moldavia, Romania, Russia, Uganda, Ukraine (Anonymous, 2008), USA (Bangari et al., 2010), Canada (Salles et al., 2011), Spain (Cano-Gomez et al., 2013), Brazil (Donin et al., 2014), Haiti (Deng et al., 2012), China (Qiu et al., 2013; Wang et al., 2010) and Taiwan (Chiu et al., 2012). Nowadays, the less virulent Talfan strains have replaced the high virulent Teschen strains.

2.2.1 Epidemiology in Taiwan

The first PTV epidemic resulted in both neurological signs and severe diarrhea was reported in 1975 in Taiwan, and the virus strain identified was PTV-6 by neutralization test (呂榮修 et al., 1981). After 25 years of silence, two epidemic outbreaks of PTV-1 occurred in 2000 and 2004, causing severely economic losses. Neurological signs and diarrhea were the most prominent clinical signs of the infected piglets. The isolation

rates of PTVs from porcine herds have been increasing since 2004, ranging from 20.59% to 33% (Huang et al., 2009), which confirms the enzootic status in Taiwan. In addition, PTVs are found to be frequently co-infected with other common swine pathogens such as porcine PRRSV, PCV2, and CSFV (Chiu et al., 2012). Previous study has demonstrated at least 5 different serotypes of PTV (PTV-1, -4, -6, -7, -11) existing in Taiwan, and PTV-7 is the most common serotype (Chiu et al., 2012).

2.3 Clinical signs

Although PTV infections are most frequently subclinical, various clinical syndromes including polioencephalomyelitis, reproductive disorders, diarrhea, pneumonia, pericarditis, and myocarditis have been associated with certain strains (Alexandersen et al., 2012).

2.3.1 Polioencephalomyelitis

The severe form of polioencephalomyelitis of high morbidity and high mortality is produced by highly virulent PTV-1 strains that caused Taschen disease. It affects all ages of swine and has been considered of socioeconomic importance. The early signs of the disease include fever, anorexia, listlessness, and locomotors ataxia. Neurological symptoms such as nystagmus and paralysis may be observed in severe cases (Alexandersen et al., 2012).

A milder form of polioencephalomyelitis (Talfan disease, benign enzootic paresis) with relatively low morbidity and low mortality is caused by less virulent PTV-1 strain, PTV-2, -3, -4, -5, -6, -9 and -10 (Alexandersen et al., 2012; Anonymous, 2008; Kaku et al., 2007). It affects mainly young pigs and rarely progress to complete paralysis.

2.3.2 Reproductive diseases

PTVs had been isolated from pigs with reproductive disorders, such as stillbirth, mummified fetus, embryonic death, and infertility. Abortion in association with PTV infection had been confirmed by experimental and field data. However, parvoviruses would also lead to similar diseases and had been substantiated more frequently of early and midgestation disorders (Alexandersen et al., 2012).

2.3.3 Diarrhea

Diarrhea can be produced experimentally by PTVs in piglets that are free of other pathogens. In the field situation, PTVs have frequently been isolated form feces of both normal and diarrheal piglets, and since a variety of other viral and bacterial agents would cause diarrhea, their presence may be coincidental (Alexandersen et al., 2012).

2.3.4 Pneumonia, pericarditis, and myocarditis

Experimentally, PTV-1, -2, and -3 had been shown capable of causing pneumonia, while the role of PTVs as respiratory pathogens is still uncertain. Pericarditis and myocarditis are observed involving with PTV-2 and PTV-3 infection (Alexandersen et al., 2012).

2.4 Pathological changes

No gross lesions can be observed in polioencephalomyelitis with PTV infection. The histopathological changes include neuronal chromatolysis, focal gliosis and lymphocytic cuffing, which are widely distributed in the CNS but are especially present

in the ventral columns of spinal cord, the cerebellar cortex, and the brain stem (Alexandersen et al., 2012; Yamada et al., 2009).

Mild focal gliosis and perivascular cuffing are occasionally found in the brain stem of stillborn or neonatal piglets. No specific gross and histopathological lesions have been associated with intestinal PTV infections (Alexandersen et al., 2012).

2.5 Pathogenesis

Natural infection of Porcine teschoviruses occurs primarily by ingestion of the virus, with primary replication in the tonsil and intestinal tract, especially in large intestine and ileum, and followed by a transient viremia (Alexandersen et al., 2012). Viremia follows by infection with virulent PTV-1 strains, but less with the less virulent strain (Holman et al., 1966). Viral entry into CNS is via hematogeneous route, breaching the blood-brain-barrier (BBB) by infecting endothelial cells and/or retrograde axonal transport (Chiu et al., 2014). Viremia is the major route of direct CNS invasion of PTVs (Yamada et al., 2009). The spinal ganglion and dorsal root are the earliest locations infected by PTVs may be associated with the absence of BBB (Yamada et al., 2009). The capillary endothelial cells and glial cells are not only transient stores but also important locations of viral replication within the CNS (Alexandersen et al., 2012; Holman et al., 1966; Racaniello, 2006). PTVs are shed through feces since the virus can infect enterocytes of duodenum and goblet cells in intestines (Chiu et al., 2014), which causes the high prevalence of PTVs in herds and contamination of water (Jimenez-Clavero et al., 2003). Previous studies also demonstrate that PTVs can infect the glomeruli and epithelium of renal tubules, lead to the speculation that PTV may shed in urine (Chiu et al., 2014) but this speculation remains to be confirmed with numerical data.

2.6 Detection of PTVs by Reverse transcription polymerase chain reaction (RT-PCR) and Nested PCR

Several diagnostic techniques have been established for diagnosing PTVs infection: virus isolation in cell cultures followed by virus neutralization (VN), complement fixation (CF), or indirect fluorescent antibody (IFA) test (Anonymous, 2008). With the availability of genomic sequence data of all PTVs, now it is possible to use RT-PCR and nested PCR to rapidly detect and differentiate the specific gene regions of porcine teschovirus (Alexandersen et al., 2012), and is less laborious than virus isolation in tissue culture. The primer sets for diagnosing PTVs infection were designed to bind to highly conserved 5'NTR region (Zell et al., 2000).

2.7 Serotyping and Molecular serotyping (Genotyping) of PTVs

2.7.1 Serotyping of PTVs

Serotyping of PTVs has been performed to distinguish virulent PTV-1 strains and other serotypes, to analyze the relationship between serotypes and clinical symptoms, and to prevent outbreaks by identifying new serotypes or variants (Kaku et al., 2007). Differentiation of serotypes was traditionally established by virus isolation in cell culture followed by virus neutralization (VN), complement fixation (CF), or indirect fluorescent antibody (IFA) test (Anonymous, 2008; La Rosa et al., 2006).

2.7.2 Molecular serotyping (Genotyping) of PTVs

Compared to the labor- and time-intensive traditional tests of serotyping, RT-PCR and nested RT-PCR assays followed by sequence analysis for genotyping achieve a

more rapid and sensitive detection (Kaku et al., 2001). Several primer pairs were designed for genotyping by amplifying different regions of genome, including the whole P1 region (Zell et al., 2000), VP1 (Cano-Gomez et al., 2011b; La Rosa et al., 2006), VP2 (N terminus and EF loop) (Kaku et al., 2001; Kaku et al., 2007), VP4 (La Rosa et al., 2006), RdRp (RNA-dependent RNA polymerase, 3D region) (Kaku et al., 2001), and 3'NTR regions (Kaku et al., 2001).

For molecular serotyping of PTVs, it would be essential to analyze the exposed areas at the outer capsid proteins including VP1, VP2, and VP3 (Smith & Baker, 1999). VP4 lies internally in close association with the RNA core, and is free from the pressure of neutralizing antibodies (La Rosa et al., 2006). In addition, close to VP4, the N terminus of VP2 is located in the interior of the mature virion, also has seemed to evolve independently of the neutralizing type (Kaku et al., 2001). Very little genetic diversity was found in the highly conserved RdRP region among samples. Due to the lacks of correlation of genotypes and serotypes, the VP2 (N terminus), VP4, RdRp, and 3'NTR are not suitable for PTV typing (La Rosa et al., 2006).

2.7.3 Whole P1 region

The whole P1 region encodes for the capsid proteins, which are the most divergent proteins among the PTV serotypes, and are shown to correlate with serotypes in phylogenetic analyses. However, due to the large size (approximately 2600 nucleotides) of the entire region, it is difficult for rapid serotyping of diagnosis but more suitable in the study of the evolution of PTVs (Kaku et al., 2007; Zell et al., 2000).

2.7.4 VP1 region

The VP1 region, the major surface-accessible protein of the virion, is where the most genetic variability concentrated and encodes the most important serotype specific neutralization epitopes. Good correlation between genotypes and serotypes of this region makes it the best choice for molecular serotyping. Methods for molecular serotyping based on partial sequence of VP1 have been published (La Rosa et al., 2006; Cano-Gomez et al., 2011b).

2.7.5 VP2 region

Previous study has suggested that the puff in the EF loop of VP2 is an immunodominant site through epitope mapping using neutralizing monoclonal antibodies followed by 3-dimensional modeling, and consequently designed a primer pair covering this region. The phylogenetic analysis of the puff sequence has good correlation between molecular and antigenic serotyping of PTVs, enabling a rapid serotyping method or diagnosis (Kaku et al., 2007).

Chapter 3 Materials and Methods

3.1 Sample preparations

3.1.1 Animals

Twenty-eight post-weaning piglets, aged from 4-8 weeks, were collected from 7 herds at 7 different locations in Taiwan. One clinically healthy piglet and three culled piglets were sampled from each herd.

3.1.2 Sampling for RT-PCR

The samples were obtained from piglets immediately after electrical euthanasia. Nasal swab (each immerged in 1 ml DEPC-treated PBS), EDTA-anticoagulated blood (1.5 mg/ml blood), urine (collected by urinary bladder centesis), feces (taken directly from rectum), urinary bladder, kidney, spleen, tonsil, ileum, and inguinal lymph node were obtained during necropsy. The 10 ml blood was obtained from each pig by cardiac puncture, using18G needle. The skin of the puncture site, located between Rt. 4-6th ribs, was sterilized with 70% ethanol before puncture to prevent contamination. During necropsy, the puncture site was confirmed to locate at the lower half of the right ventricle. All of these samples were shipped on ice and stored at -80°C until RNA extraction.

3.2 Sources and cell culture of prototype virus

The serotypes and names of reference strains of Porcine teschovirus are listed in Table 3.1. Porcine teschovirus 1-7 were purchased from the National Veterinary Service Laboratory (NVSL), USA (Chiu et al., 2012). Porcine teschovirus 10 strain was gifted by Dr. Malte Dauber of Federal Research Institute for Animal Health, Germany (Chiu et

al., 2014) (Table 3. 1). The virus was grown in porcine kidney (PK-15) cells maintained in Dulbecco modified Eagle medium and supplemented with 5% fetal bovine serum. The time of harvesting was dependent on the extent of CPE and rounding and floating of cells. The supernatants were harvested in between 2 to 4 days when 80-90% CPE was present.

Table 3.1 References strains used

PTV serotypes	Strain names	GenBank accession nos.
PTV-1	PS34	AF296105
PTV-2	O 3b	Not available
PTV-3	O 2b	AF296088
PTV-4	PS 36	AF296089
PTV-5	F 26	AF296090
PTV-6	PS 37	AF296091
PTV-7	WR2	GQ293237
PTV-10	Vir 460/88	AF296095

3.3 RNA extraction

3.3.1 RNA extraction from solid tissues

Frozen solid tissues weighed approximately 30 mg for urinary bladder, 25 mg for kidney, spleen, tonsil, ileum, and inguinal lymph node were micro-dissected. The tissue was mixed with 600 µl of RTL buffer (Qiagen, Germany) and homogenized by Tissue ruptor® (Qiagen, Germany) with a 7 mm disposable probe in a 2 ml microcentrifuge

tube. Total RNA was extracted using RNeasy® mini kit (Qiagen, Germany) according to the manufacturer's manual. The concentration of RNA was determined by measuring the absorbance at 260 nm and 280 nm in Picodrop™ spectromter (Picodrop P200, Picodrop Limited, Saffron Walden, UK). The A260/A280 ratio of RNA solution ranged from 1.8 to 2.0. The extracted RNA was stored at -80℃ until used.

3.3.2 RNA extraction from fluid samples (diluted feces, urine, plasma, and nasal swab)

The feces were weighed approximately 100 mg, and mixed with 900 μl of diethylpyrocarbonate (DEPC)-treated PBS, followed by centrifugation at 7000 rpm for 10 min at 4℃. The supernatant was filtered through 0.22-μm filter, and used for RNA extraction. The plasma was obtained from the anticoagulated whole blood after centrifugation at 5000 rpm at 4℃ for 15 min.

For reference strains, 150 μl supernatant was used to extract RNA using Viogene kit (see below)

Two hundred fifty microliter (250 μl) of the fecal supernatant, urine, plasma, and nasal swab was mixed with 750 μl of TRIzol® LS reagent (Invitrogen, USA) or RNA extraction kit (Viogene, Taipei, Taiwan) for RNA extraction. The total RNA was extracted as manufacturer's protocol with 20 μg of glycogen added during RNA precipitation, and diluted in 50 μl of RNase-free water.

The concentration of RNA was determined by measuring the absorbance at 260 nm and 280 nm in Picodrop™ spectromter (Picodrop P200, Picodrop Limited, Saffron Walden, UK) to ensure that the A260/A280 ratio is ranged from 1.8 to 2.0. The extracted RNA was stored at -80℃ until used.

The RNA extracted from field samples used in this study were already processed

by real-time RT-PCR in a previously study (Kuo, 2014), confirming the quality and efficiency of the RNA extraction using internal control G3PDH.

3.4 Primer selection

3.4.1 Porcine teschoviruses

The primer pairs of PTV 1F/1R and PTV 1nF/1nR were designed to amplify the highly conserved 5'NTR region of PTVs (Zell et al., 2000), with expected products sizes of 321 bp and 158 bp for all PTVs.

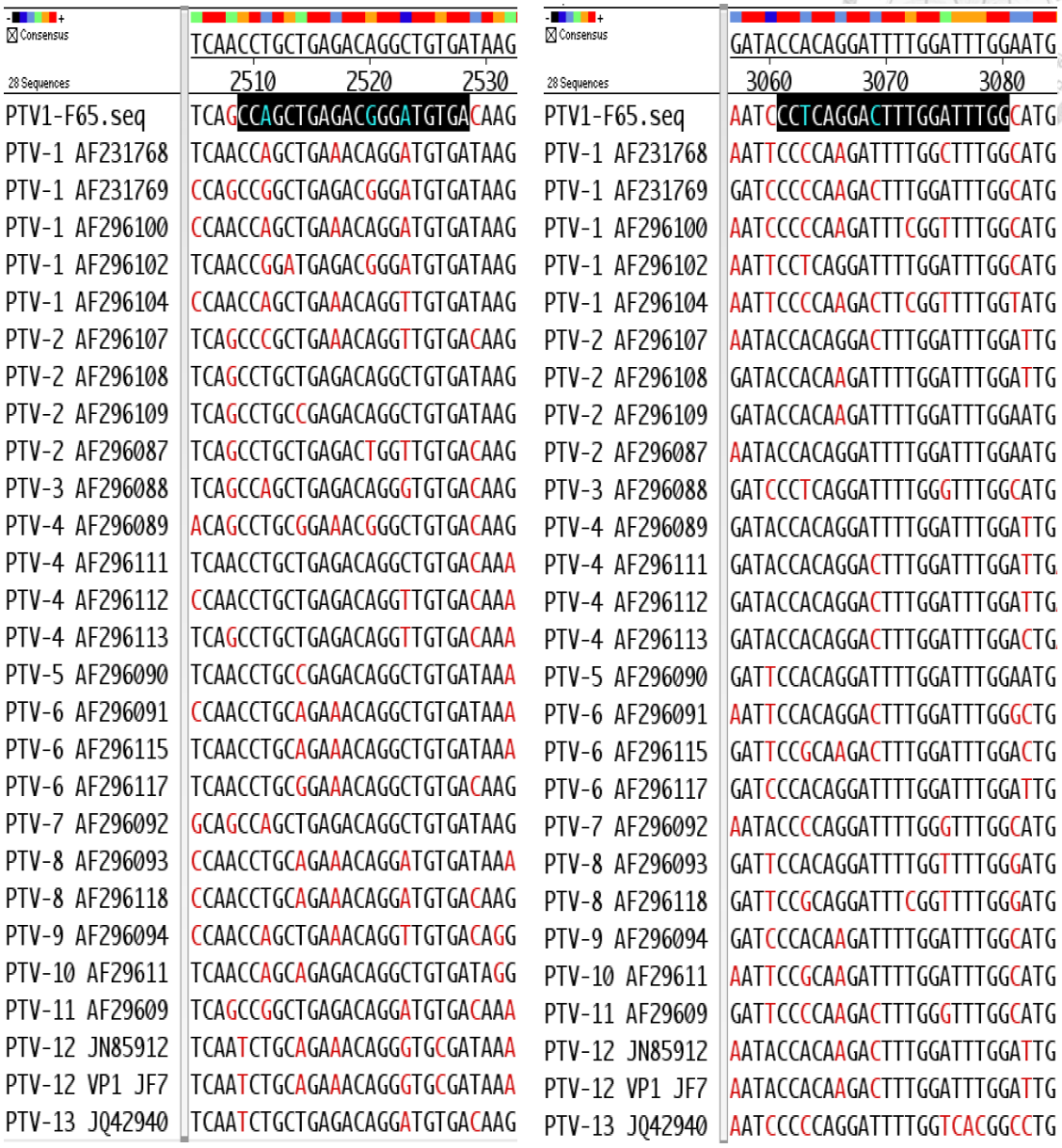
3.4.2 Primers for PTVs molecular serotyping on VP1

For VP1 amplifications, two novel degenerate primer pairs (PTV-SF/SR and PTV-nSF/nSR) (Table 3.2) were designed by DNA star software (Lasergene), based on 28 available PTVs nucleotide sequences representing all PTV serotypes (PTV1-13) in GenBank (accession nos: NC_003985, af231768-69, af296087-89, af296090-94, af296096, af296100, af296102, af296104, af296107-09, af296111-13, af296115, af296117-19, JN859128, JF724001, JQ429405) (Fig. 3. 1, 3. 2). Primer pair PTV-SF/SR was used in a first RT-PCR step and PTV-nSF/nSR was used in the nested PCR step, with the expected product sizes of 571 bp and 348 bp respectively.

Table 3.2 Primers used for RT-PCR and nested PCR for 5'NTR and VP1

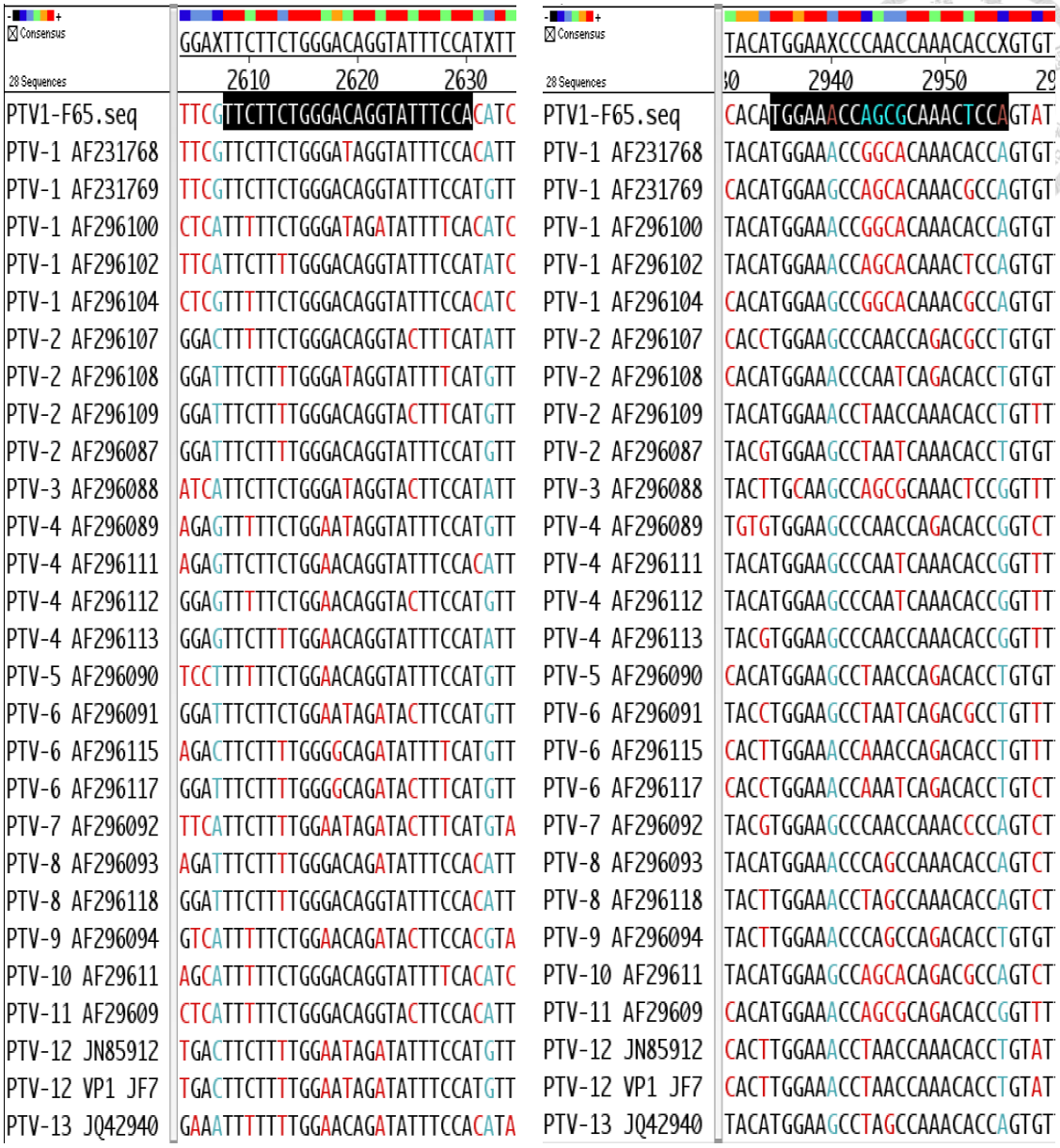
Primer	Sense	Sequence (5'-3')	Product (bp)	Location* (Target)
Pan-PTV 5'NTR (PTV1-11) (Zell et al., 2000)				
PTV-1F	F	AGTTTTGGATTATCTTGTGCCC	321	79-396
PTV-1R	R	CCAGCCGCGACCCTGTCAGGCAGCAC		(5'NTR)
PTV-1nF	F	TGAAAGACCTGCTCTGGCGCGAG	158	178-335
PTV-1nR	R	GCTGGTGGGCCCCAGAGAAATCTC		(5'NTR)
PTV VP1 region (this study)				
PTV-SF	F	CCTGCTGAGACAGGCTGTGA	571	3070-2500
PTV-SR	R	CCTGCTGAGACAGGCTGTGA		(VP1)
PTV-nSF	F	TTCTTCTGGGACAGGTATTTCCA	348	2945-2598
PTV-nSR	R	GGTGTTTGNKTNGGYTTCCA		(VP1)

*Nucleotide numbering according to F65 strain (GenBank acc. nos NC_003985)
*All primers were purchased from Tri-I Biotech, Taipei, Taiwan.



(A) Forward primer (PTV-SF) (B) Reverse primer (PTV-SR)

Fig. 3.1 Alignment of nucleotide sequences of 13 serotypes PTV strains for VP1 RT-PCR primer design.



(A) Forward primer (PTV-nSF) (B) Reverse primer (PTV-nSR)

Fig. 3.2 Alignment of nucleotide sequences of 13 serotypes PTV strains for VP1 nested PCR primer design.

3.5 Reverse transcription polymerase chain reaction (RT-PCR) for 5'NTR (pan-PTV) and VP1

The RT-PCR reaction was performed using 2720 thermal cycler (Applied Biosystem, Foster City, CA, USA). Reaction tubes contained 2 μl of template RNA, 1 μl of 10 μM forward primer, 1 μl of 10 μM reverse primer, 0.5 μl of 10 mM dNTP mix (Promega, Madison, WI, USA), 0.5 μl of Pro Taq Plus DNA polymerase (Protech, Taipei, Taiwan), 0.5 μl of M-MuLV Reverse Transcriptase (Protech), 2 μl of 10X Taq buffer (Protech), 0.1 μl of RNAse inhibitor (Protech) and filled with 12.4 μl of diethylpyrocarbonate (DEPC)-treated double distilled water (DDW) to a total volume of 20 μl. The reverse transcription and polymerase chain reactions were performed sequentially in one step, consisting of a first reverse transcription at 42℃ for 30 min, and 5 min at 95℃ to inactivate RTase, followed by PCR cycling according the following conditions:

For 5'NTR (Zell et al., 2000): initial denaturation at 94℃ for 5 min, 35 cycles of denaturation at 95℃ for 50 sec, annealing at 55℃ for 1 min, extension at 72℃ for 1 min, and final elongation at 72℃ for 5 min.

For VP1 (this study): initial denaturation at 94℃ for 5 min, 35 cycles of denaturation at 95℃ for 30 sec, annealing at 52℃ for 30 sec, extension at 72℃ for 30 sec, and final elongation at 72℃ for 7 min.

3.6 Nested PCR for PTV 5'NTR and VP1

Nested PCR was preformed in reaction containing 1 μl RT-PCR product, 1 μl forward and reverse primers (10 μM of each), 0.5 μl of 10 mM dNTP mix (Promega),

0.5 μl of Pro Taq Plus DNA polymerase (Protech), 2 μl of 10X Taq buffer (Protech), and 14 μl DEPC-treated DDW to a total volume of 20 μl.

For 5'NTR (Zell et al., 2000): The cycling conditions were initial denaturation at 94℃ for 5 min, 35 cycles of denaturation at 95℃ for 50 sec, annealing at 60℃ for 1 min, extension at 72℃ for 1 min, and a final elongation at 72℃ for 5 min.

For VP1 (this study): The cycling conditions were initial denaturation at 94℃ for 5 min, 35 cycles of denaturation at 95℃ for 50 sec, annealing at 55℃ for 1 min, extension at 72℃ for 1 min, and a final elongation at 72℃ for 5 min.

3.7 Gel electrophoresis

The resulting amplified DNAs were electrophoresed in 2% AGAROSE I™ agarose gel (AMRESCO, Solon, Ohio, USA) stained with O' SAFE Red 6X Loading Dye (the Ethidium Bromide replacement) (Omics Bio, Taipei, Taiwan), before visualized under UV light and photographed. A marker of known molecular weight (100 bp DNA ladder; Omics bio) and negative control (DEPC-treated DDW) were run on the same electrophoresis gel to confirm the validity of the results.

3.7.1 Gene sequencing and data analysis for VP1

Amplified DNA products of correct size were purified from the gel using Gel Advanced™ DNA extraction kit (Viogene, Taipei, Taiwan), and then sequenced in ABI Prism model 3730 (Applied Biosystem at Tri-I Biotech, Taipei, Taiwan), using the same forward primer (PTV-nSF) of the nested PCR.

The obtained VP1 nucleotide sequences were assembled by SeqMan program (DNASTAR), and consensus sequences compared with nucleotide sequences in the

database using BLAST algorithm and assigned serotype having the highest identity score with the sequences inquired. Multiple alignments of the sequences obtained in this study and those from the Genbank were conducted by CLUSTAL W program, and subsequent phylogenetic analyses were performed by neighbor-joining method based on the matrix of the distances. The phylogenetic tree was drawn with MEGA 6 software package.

Chapter 4 Results

4.1 Sample collection

Twenty-eight post-weaned pigs, 7 clinically healthy (per owner) and 21 culled, were collected from 7 herds. Ages of the pigs ranged from 5 to 8 weeks old, and their average body weight was 6.1±1.6 kg. These 7 herds were located in Linkou (林口), Guanmiao (關廟), Minsyong (民雄), Sikou (溪口), Taian (泰安), Mailiao (麥寮, 2 herds). The culled piglets showed weakness, respiratory symptoms, emaciation, or poor feed conversion. This set of samples was the same as those in Kuo (2014).

4.2 Screening for PTVs infection in endemic infected pigs

A set of 10 samples of each pig were selected for PTVs detection and arranged according to the fecal-oral pathogenesis model, including feces, ileum, tonsil, inguinal lymph node (abbr. Ig LN), plasma, spleen, kidney, bladder, urine, nasal swab (abbr. nasal).

4.2.1 PTVs detection by RT-PCR followed by nested PCR

The PTVs infection of field samples was detected by using two pairs of primers (PTV 1F/1R and PTV 1nF/1nR) with expected product sizes of 321 bp (PTV 1F/1R) in RT-PCR and 158 bp (PTV 1nF/1nR) in nested PCR. The results of PTVs detection are listed in Appendix 1, summarized in Table 4.1, and in Fig. 4.1.

By RT-PCR, the PTV detection rate by heads was 4% (1/28) and by nested PCR 100% (28/28). Only one fecal sample (CY27) was positive in RT-PCR stage. In nested PCR, the most common detected sample was feces (96%, 27/28), and followed in

decreasing order ileum, tonsil, and nasal swab (93%, 26/28), plasma (86%, 24/28), inguinal LN and urine (both were 79%, 22/28), bladder (64%, 18/28), spleen (57%, 16/28), and kidney (36%, 10/28).

4.3 Molecular serotyping of PTVs based on VP1 sequence

4.3.1 VP1 amplification of reference strains

To demonstrate the feasibility of the primers designed in this study (PTV SF/SR and PTV nSF/nSR), reference strains of 8 different serotypes (PTV-1-7, -10) were assayed (Tables 3.1-3.2). The two pairs of primers had successfully amplified the reference strains of 7 serotypes: PTV-1, -2, -3, -4, -5, -6 and -10 with the expected product size as 571 bp and 347 bp in RT-PCR and nested PCR (Fig. 4.2). The validity of the results was confirmed by the gene sequencing followed by BLAST analysis of the products.

4.3.2 Genotyping of VP1 region in samples

All the samples (n = 280) were subjected to amplification with VP1 specific primer set PTV SF/SR and PTV nSF/nSR, and out of 217 pan-PTV nested PCR-positive samples (Table 4.2). In 83 samples revealed a band of expected size amplified (Fig. 4.3, Table 4.2a), in which only 63 (63/217, 29%) of them were successfully sequenced, serotyped, and phylogenetic tree constructed (Fig. 4.4). The phylogenetic tree is constructed with field samples, reference strains, and the 27 strain sequences obtained from GenBank (Fig. 4.4). In the phylogenetic tree, the strains of the same serotype cluster together, indicating this targeted partial VP1 region is monophyletic with respect to the serotype.

No field samples was detected positive by RT-PCR, and all were detected positive until the nested PCR was performed, and none of the pan-PTV nested PCR-negative samples had positive results on VP1 amplification (Appendix 2). All 28 piglets had at least one serotype identified. A total of five different serotypes (PTV- 2, -4, -5, -6, -10) were identified from the field samples (Table. 4.2, Appendix 2), and the most common serotype was PTV-10 (36 samples, 36/63, 57%), the 2nd common serotype is PTV-2 (14 samples, 14/63, 22%), followed by PTV-6 (8 samples, 8/63, 13%), PTV-4 (4 samples, 4/63, 6%), and PTV-5 (1 sample, 1/63, 2%) is the least common one.

In 14 heads (14/28, 50%) had at least two serotypes identified in different samples of the same animals (Table 4.3): TY2: PTV-10 in feces and PTV-6 in nasal; TY5: PTV-6 in feces and PTV-10 in nasal; TY6: PTV-6 in feces and ileum, PTV-10 in bladder; KM1: PTV-10 in feces and ileum, PTV-4 in tonsil; KM2: PTV-2 in ileum, PTV-4 in tonsil, PTV-10 in Ig LN and nasal; KM6: PTV-10 in feces, Ig LN, and urine, PTV-2 in ileum and tonsil, PTV-6 in bladder; CY1: PTV-10 in ileum and plasma and nasal, PTV-2 in Ig LN; CY2: PTV-6 in feces, PTV-10 in tonsil; CY9: PTV-6 in ileum and PTV-2 in Ig LN; CY14: PTV- 10 in feces and plasma, PTV-2 in spleen, PTV-10 in nasal; CY15: PTV-2 in ileum and Ig LN, PTV-10 in nasal; CY21: PTV-10 in feces and plasma and urine, PTV-2 in Ig LN; CY23: PTV-5 in feces, PTV-10 in ileum; CY29: PTV-6 in feces, PTV-10 in ileum (Table 4.3).

Fecal sample had the highest VP1 amplification rate (17/27, 63%), followed by ileum (14/26, 54%), Ig LN (8/22, 36%), and tonsil (7/26, 27%). In particular, 3 urinary samples (KM6, CY13, and CY21) (Table 4.3, Appendix 2) were successfully typed as PTV-10.

Chapter 5 Discussion

Instead of using traditional tests such as virus neutralization assay for serotyping of PTVs, molecular methods including RT-PCR and nested PCR now provide an alternative to more rapid serotyping. Previous study had demonstrated the existence of at least 5 different serotypes of PTV-1, PTV-4, PTV-6, PTV-7, and PTV-11 in Taiwan (Chiu et al., 2012). The aim of this study was to investigate the possibility of a wider variety of PTV serotypes in Taiwan by introducing two newly designed primers for molecular serotyping, based on the partial sequence of VP1 outer capsid gene, where the most genetic variability is concentrated and contains important neutralization sites.

The pan-PTV primer pairs, PTV-1F/1R and PTV-1nF/1nR, previously designed for amplifying a 158 bp fragment of 5'NTR region (Zell et al., 2000) were used to screen for PTV infection by RT-PCR followed by nested PCR. The detection rate was 4% in RT-PCR (by heads) and rose to 100% (by heads) in nested PCR, confirming the ubiquity of PTVs, which were found most frequently in feces, ileum, tonsil, nasal swab, plasma, Ig LN, and urine (> 79%), and relatively less common (< 65%) in the visceral organs including urinary bladder, spleen, and kidney (Table. 4.1, Appendix 1). The highest detection rates of fecal samples and intestine indicate the highly contaminated environment of PTVs.

For screening PTVs infection using pan-PTV 5'NTR, only 1/280 sample (feces of CY27) was positive in the RT-PCR stage (Table 4.1; Figure not shown). All solid samples, including ileum, Ig LN, tonsil, urine bladder, and kidney, revealed multiple faint bands of incorrect sizes (Fig. 4.1A, lanes 5 and 7), in which correct sequences constituted a minor portion of the whole products, suggesting the primers may have cross reacted with other genomes present in the field samples causing false-negative results.

For molecular serotyping two published primers (La Rosa et al., 2006 in Chiu et al., 2012) were initially used to amplify partial VP1 region and serotyping with success rate of 19.3% as compared to nested PCR (Zell et al., 2000 in Chiu et al., 2012). Multiple bands were also detected with one band of expected size (data not shown), however, sequencing results revealed a 86% similarity with wild boar mitochondrion genome, suggesting the primers may have a higher affinity for swine DNA and thus causing false negative results. Therefore, in this study, two new primer pairs PTV-SF/SR and PTV-nSF/nSR, targeted on partial VP1 region, were designed (Table. 3.2). The inner primer pair PTV-nSF/nSR used in nested PCR was expected to solve detection problems of field samples containing low copy numbers of targets against a high background of tissue DNA and inhibitors of DNA polymerase (Sachse, 2003). This set of primer had increased the VP1 amplification rate to 29% (Tables 4.2-4.3) and can detect at least 7 serotypes of PTV-1, 2, 3, 4, 5, 6, and -10 from reference strains (Table 3.1), for which the results were validated by VP1 amplification, sequencing and phylogenetically consistent with their respective serotypes. Although not clear from Fig. 4.2, this new set of primer may have preferentially amplified VP1 of PTV-10 (Tables 4.2-4.3) over other PTV serotypes, because similar preferential amplification on certain PTV serotypes was observed in qRT-PCR targeting 5'NTR (Chiu et al., 2014). Although there was a band of expected size amplified from PTV-7 reference strain (Table 3.1), the sequencing result revealed it to be PTV-6 for unknown reason (data not shown). Apparently the new primer designed in this study cannot differentiate PTV-7 from PTV-6. On the other hand the LaRosa primer (2006, in Chiu et al., 2012) has the ability to distinguish them from each other.

All 28 piglets had at least one serotype existed, and a total of five serotypes PTV-2, PTV-4, PTV-5, PTV-6, and PTV-10, were identified (Table. 4.2, Appendix 2). The most

frequently detected serotype was PTV-10 (57%) and PTV-2 (22%). Fourteen out of 28 (14/28, 50%) piglets had more than one serotype co-existed in different samples of the same animals (two serotypes co-existed in 12 pigs and three serotypes in 2 pigs) (Table 4.3), further strengthening the multiple models of PTV pathogenesis (Chiu et al., 2014) in which different PTVs were not mutually exclusive. All 7 herds had at least two different serotypes identified, and in 2/7 herds had four serotypes co-circulated. Visual inspection revealed no correlation between the serotypes and geographical locations of the sampled herds. Further the number of serotyped samples was insufficient for statistical analysis.

In particular, PTV-10 was detected in urine of 3 piglets from 3 separate herds. Although it was suspected that this nested PCR for VP1 may preferentially amplify that of PTV-10, the results were likely authentic. First, pipette carryover was avoided by using filtered tip and additional measures such as decontaminating the pipettes and desktop with DNA-ExitusPlus^{TM} (AppliChem, Germany). Second, reference strain PTV-10 was used only in the optimization of primer designed stage, whereas in VP1 amplification from field samples only negative control was employed thus ruling out reagent contamination. Note that positive control in Fig. 4.3 was later incorporated for comparison in gel electrophoresis. Third, during experiment executions the sequential order of pipetting was consistent by which tracing carryover from upstream to downstream sample was possible. Fourth, in 1/295 nucleotide (0.0034%) at position 2688 of CY13 was different from that of PTV-10 reference strain Vir 460/88. This PTV-10 Vir 460/88 had a 0.0017% (12/7009 nucleotide) background difference from the PTV-10 GenBank accession number AF296119 (Zell et al., 2001). The 0.0034% difference was above the background of 0.0017%. In this specific 295 nucleotide region, there was difference in 1 nucleotide (0.0034%) at position 2673. Fifth, alignment of all

PTV-10 sequences in this study, in sequential order of experiment execution, does not implicate any carryover between samples (Appendix 3).

The finding of PTV-10 and PTV-6 in nasal swab of 6 piglets (Table 4.3) is also likely authentic. Nasal swabs would be most likely contaminated by the feces due to the digging behavior of pigs. In additional to cleaning up the nose before swabbing, all 5 piglets with serotypes identified in the nasal and feces were different (Table 4.3), ruling out that those PTVs in nasal were not contaminated from the feces. However, the significance of finding PTV in nasal is not clear regarding the pathogenesis, it could be either in entry or in exit, but it does substantiate the role of intranasal infection in the multiple models (Chiu et al., 2014).

In the previous study, 5 serotypes PTV-1, PTV-4, PTV-6, PTV-7, and PTV-11 were identified in Taiwan and the most prevalent serotypes were PTV-7 and PTV-6 (Chiu et al., 2012). This study identified the existence of 3 additional serotypes: PTV-2, PTV-5, and PTV-10, indicating the high diversity existing among PTVs in swine herds. Three previously detected serotypes PTV-1, PTV-7, and PTV-11, were not identified in this study, likely because the use of different set of samples and primers (Chiu et al., 2012). Due to the limited detection ability of the primers designed in this study on only 7 reference strains (Table 3.1), the presence of a wider variety of serotypes is highly possible.

Despite the use of newly designed primers in this study, the 29% VP1 amplification rates was still unsatisfactory and may be due to (1) the limitation of the primers used in this study (only seven serotypes have been tested, Table 3.1); (2) high genetic variation in VP1 region, interfering the efficiency of primer annealing; (3) the existing of co-purified PCR-inhibitory substance in field samples, such as bile salts and complex polysaccharides in feces, heme in blood, and urea in urine, would reduce or

block the amplification capacity of PCR (Radstrom et al., 2003); (4) the viral load is too low to be detected (compared with the isolations from cell culture).

As expected from the fecal-oral model of pathogenesis, higher success rates were detected in fecal samples, ileum, Ig LN, and tonsil, which were also the samples having higher viral loads processed in qRT-PCR (Kuo, 2014). This finding is consistent with the results of a previous study (Chiu et al., 2012) and others using isolates from fecal samples (Buitrago et al., 2010, La Rosa et al., 2006, Sozzi et al., 2010). In addition, feces is also the sample containing the most diverse serotypes: four serotypes PTV- 2, 5, 6, 10 were identified (Table 4.2).

The same set of samples was also processed by real-time RT-PCR (qRT-PCR) to determine the viral loads (Kuo, 2014). Both targeting on the 5'NTR, qRT-PCR (Kuo, 2014) is more sensitive than nested PCR (Table. 4.4, Appendix 2). However, there were instances in which 10 positive samples by nested PCR but negative by qRT-PCR, and three of them (KM2 tonsil, CY9 Ig LN, and CY14 spleen) were successfully typed as PTV-4, PTV-2, and PTV-2 respectively (Appendix 2).

The detection rate of PTV in urine by nested PCR (79%, by heads, Table 4.1) was not as high as in qRT-PCR (100%, by heads, Kuo, 2014, Appendix 2). These detection rates by 5'NTR, together with the identification of PTV-10 in 3 piglets of 3 separate herds (Table 4.3), further substantiate the urinary shedding of PTV in the naturally infected setting. It is likely that liquid urine making solid feces into semisolid slurry and thus enhancing the exposure of piglets to PTVs.

In conclusion, this study further confirmed the enzootic and highly contaminated status of PTVs in swine herds, and revealed 3 additional serotypes PTV-2, -5, and -10 that were not identified before. In all herds, at least two serotypes co-existed and in 2/7 herds had four serotypes co-circulated. In particular, the presence of PTV-10 in urine

of 3 piglets of 3 separate herds demonstrated a possibility of enhanced exposure to PTV by contacting semisolid slurry instead of solid feces. The significance of presence of PTVs in nasal swabs is still unclear with regard to pathogenesis.

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primer sets. J. Virol. Methods 88, 205-218.

FIGURES

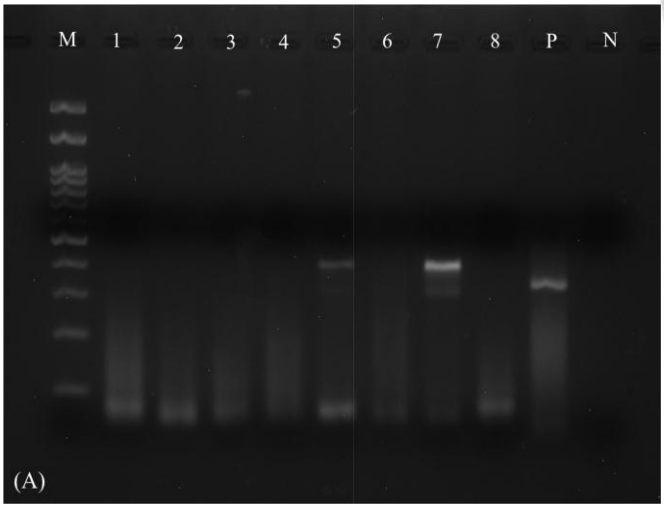


Figure 4.1A Amplification of pan-PTV 5'NTR specific products by RT-PCR from field samples. The expected product size is 321 bp, which constitutes a minor portion against a high background of undesirable products likely from tissue RNA (lanes 5 and 7).

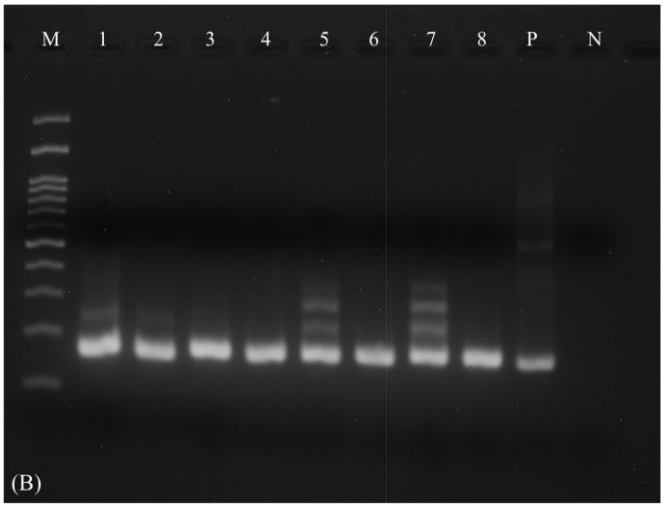


Figure 4.1B Amplification of pan-PTV 5'NTR specific products by nested PCR from field samples. The expected product size is 158 bp, and the specific product now constitutes a major portion of the products.

Lane 1: KM2 Feces
Lane 2: KM5 Feces
Lane 3: CY15 Feces
Lane 4: CY17 Feces
Lane 5: CY19 Feces
Lane 6: CY21 Feces
Lane 7: CY23 Feces
Lane 8: CY24 Feces
P: Positive control PTV-10
N: Negative control

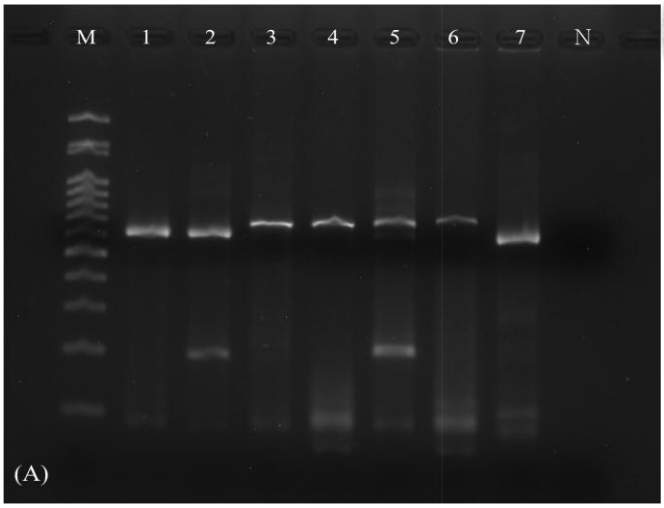


Figure 4.2A Amplification of VP1 region for genotyping by RT-PCR from reference strains (Table 3.1). The specific product size is 571 bp. The variable degree of band shift is suspected to be related to safe DNA dye (O' SAFE Red 6X Loading Dye) used.

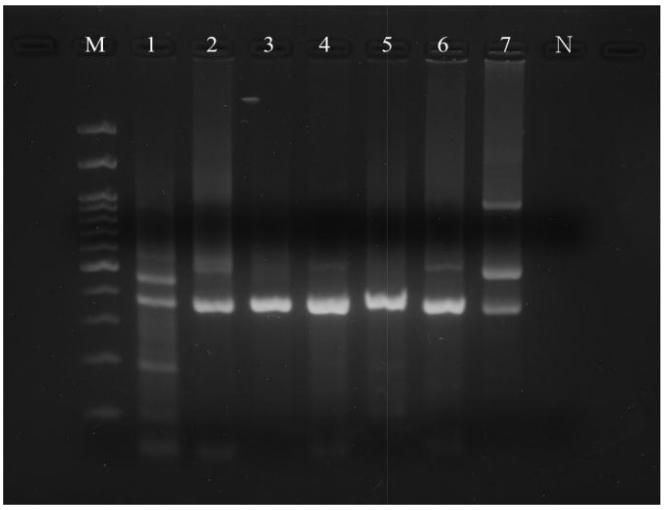


Figure 4.2B Amplification of VP1 region for genotyping by nested PCR from reference strains (Table 3.1). The specific product size is 347 bp. Judgeing from the band intensity in lane 7, this primer did not seem to preferentially amplify PTV-10.

Lane 1: PTV-1
Lane 2: PTV-2
Lane 3: PTV-3
Lane 4: PTV-4
Lane 5: PTV-5
Lane 6: PTV-6
Lane 7: PTV-10
N: Negative control

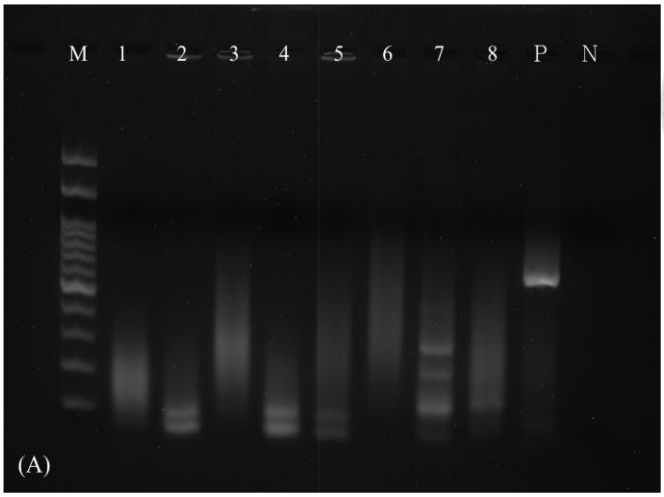


Figure 4.3A Amplification of VP1 region for genotyping by nested PCR from field samples. The specific product size is 571 bp. Note that positive control (P) PTV-10 was not included in the nested PCR for VP1 amplification for field sample, and was incorporated for comparison in gel electrophoresis.

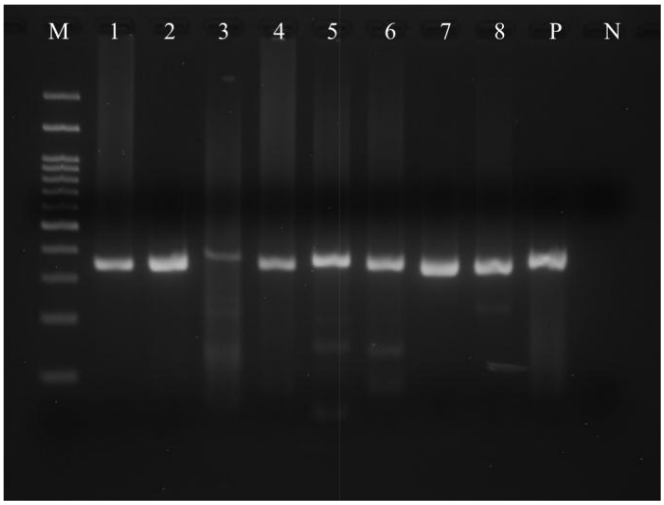


Figure 4.3B Amplification of VP1 region for genotyping by nested PCR from field samples. The specific product size is 347 bp.

Lane 1: KM2 Feces
Lane 2: KM5 Feces
Lane 3: CY15 Feces
Lane 4: CY17 Feces
Lane 5: CY19 Feces
Lane 6: CY21 Feces
Lane 7: CY23 Feces
Lane 8: CY24 Feces
P: Positive control PTV-10
N: Negative control

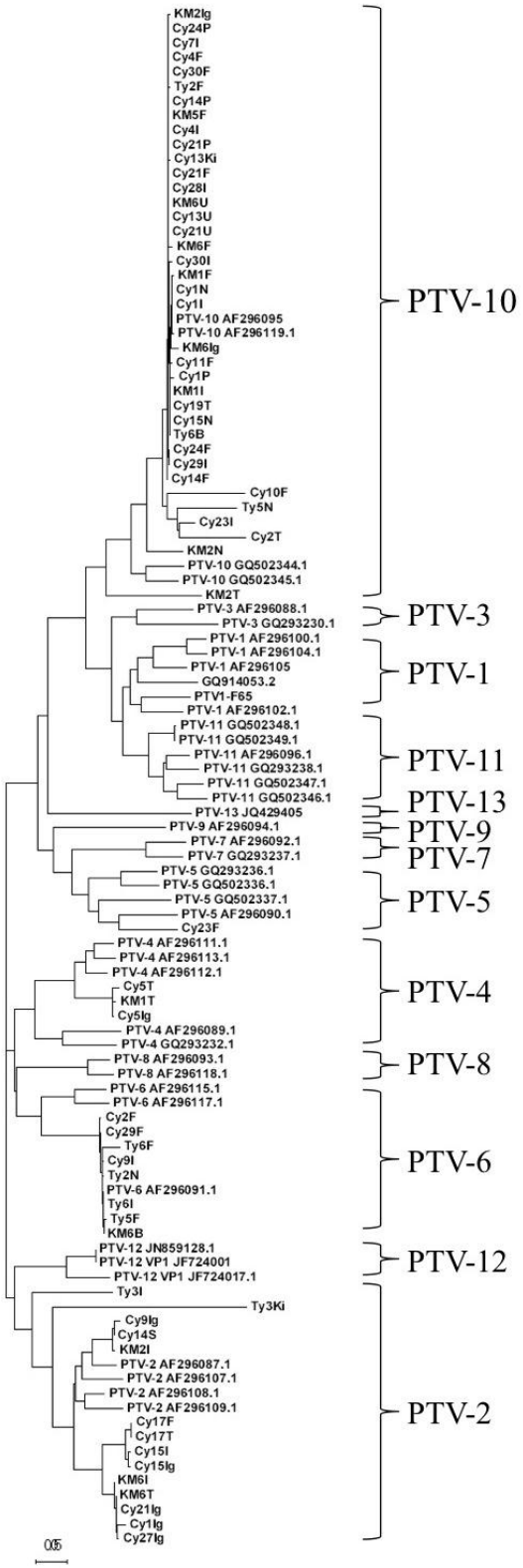


Fig. 4.4 Phylogenetic tree of partial VP1 nucleotide sequences constructed by the neighbor-joining method. The PTVs from field samples of this study cluster in PTV-2, PTV-4, PTV-5, PTV-6, and PTV-10.

TABLES

Table 4.1 The PTV detection rates (by heads) for 5'NTR in different samples.

	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
RT-PCR	4% (1/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)	0% (0/28)
Nested-PCR	96% (27/28)	93% (26/28)	93% (26/28)	79% (22/28)	86% (24/28)	57% (16/28)	36% (10/28)	64% (18/28)	79% (22/28)	93% (26/28)

Note: refer also to Appendix 1.

Table 4.2 Successful VP1 amplification rates and the serotypes identification rates in different samples

	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
Percentage	63% (17/27)	54% (14/26)	27% (7/26)	36% (8/22)	17% (4/24)	6% (1/16)	20% (2/10)	11% (2/18)	14% (3/22)	19% (5/26)
Serotype	PTV-2, PTV-5, PTV-6, PTV-10	PTV-2, PTV-6, PTV-10	PTV-2, PTV-4, PTV-10	PTV-2, PTV-4, PTV-10	PTV-10	PTV-2	PTV-2, PTV-10	PTV-6, PTV-10	PTV-10	PTV-6, PTV-10

Note: refer also to Appendix 2

Table 4.3 Different serotypes co-existed in the same animals but in different samples.

Piglet ID	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08Bladder	09 Urine	10 Nasal
TY5	PTV-6									PTV-10
TY6	PTV-6	PTV-6						PTV-10		
TY2	PTV-10									PTV-6
KM2		PTV-2	PTV-4	PTV-10						PTV-10
KM6	PTV-10	PTV-2	PTV-2	PTV-10				PTV-6	PTV-10	
KM1	PTV-10	PTV-10	PTV-4							
CY2	PTV-6		PTV-10							
CY1		PTV-10		PTV-2	PTV-10					PTV-10
CY9		PTV-6		PTV-2						
CY14	PTV-10				PTV-10	PTV-2				
CY15		PTV-2		PTV-2						PTV-10
CY21	PTV-10			PTV-2	PTV-10				PTV-10	
CY23	PTV-5	PTV-10								

CY29	PTV-6 PTV-10

Note: refer also to Appendix 2. All 28 piglets had at least one serotype identified, and 14/28 pigs had more than one serotype co-existed in different samples of the same animal.

Note: This experiment was executed in sequential order (appendix 2)(by samples, feces, pig 1 → 28; then ileum pig 1 → 28 ….). This RNA was 3rd time thawed from RNA extracted for qRT-PCR).

Table 4.4 Comparison of real-time RT-PCR, pan-PTV 5'NTR nested PCR, and Nested PCR on VP1

	Head	Positive Samples	Note
Real-time RT-PCR (5' NTR) (Kuo, 2014)	100% (28/28)	80% (224/280)	17/224 samples were negative in pan-PTC nested PCR (row 2)
Pan-PTV nested PCR (5' NTR) (Zell et al., 2000)	100% (28/28)	77.5% (217/280)	10/217 samples were negative in real-time RT-PCR (row 1)
Nested PCR (VP1) (This study)	100% (28/28)	22.5% (63/280) 29% (63/217)	All 63 samples were pan-PTV nested PCR positive (row 2). 3 real-time RT-PCR negative sample (row 1) was successfully VP1 amplified and serotype identified.

Note: refer to Appendix 2 for raw data.

APPENDICES

Appendix 1 The detection results of the pan-PTV 5'NTR RT-PCR and nested PCR in different samples

Herds	Healthy	Pig No.	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
1	C	TY3	-/+	-/+	-/+	-/-	-/+	-/-	-/+	-/+	-/+	-/+
1	C	TY5	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+
1	C	TY6	-/+	-/+	-/-	-/-	-/+	-/-	-/-	-/+	-/+	-/+
1	H	TY2	-/+	-/+	-/+	-/+	-/-	-/+	-/-	-/-	-/+	-/+
2	C	KM2	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/+	-/+	-/+
2	C	KM5	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/-	-/+
2	C	KM6	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/+	-/+	-/+
2	H	KM1	-/+	-/+	-/+	-/+	-/-	-/+	-/-	-/-	-/+	-/-
3	C	CY2	-/+	-/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
3	C	CY4	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/+
3	C	CY5	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+
3	H	CY1	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/+	-/+
4	C	CY9	-/+	-/+	-/-	-/+	-/+	-/+	-/-	-/-	-/+	-/+
4	C	CY10	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+	-/+
4	C	CY11	-/+	-/+	-/+	-/+	-/-	-/-	-/+	-/+	-/-	-/+
4	H	CY7	-/+	-/+	-/+	-/-	-/+	-/-	-/-	-/-	-/-	-/+
5	C	CY14	-/+	-/+	-/+	-/-	-/+	-/+	-/-	-/-	-/-	-/+
5	C	CY15	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/-	-/+
5	C	CY17	-/+	-/+	-/+	-/-	-/+	-/-	-/+	-/+	-/+	-/-
5	H	CY13	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+

Herds	Healthy	Pig No.	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
6	C	CY21	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+	-/+
6	C	CY23	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
6	C	CY24	-/+	-/+	-/+	-/+	-/+	-/+	-/-	-/+	-/+	-/+
6	H	CY19	-/+	-/-	-/+	-/-	-/+	-/+	-/-	-/+	-/+	-/+
7	C	CY27	+/-	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
7	C	CY29	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/-	-/+	-/+
7	C	CY30	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+
7	H	CY28	-/+	-/+	-/+	-/+	-/+	-/-	-/-	-/+	-/+	-/+
	Detection number		27	26	26	22	24	16	10	18	22	26
	Positive rate		96%	93%	93%	79%	86%	57%	36%	64%	79%	93%

Note 1: “ C ” indicates culled piglets, “ H ” indicates healthy piglets.

Note 2: “ + ” indicates positive detection and “ - ” indicates negative detection by either PCR reactions. The results are presented as RT/nested PCR.

Note 3: Detection number and Positive rate are calculated by the result of nested PCR.

Note 4: refer also to Table 4.1.

Appendix 2. The comparison of results on real-time RT-PCR, pan-PTV 5'NTR nested PCR, and nested PCR for VP1 serotyping

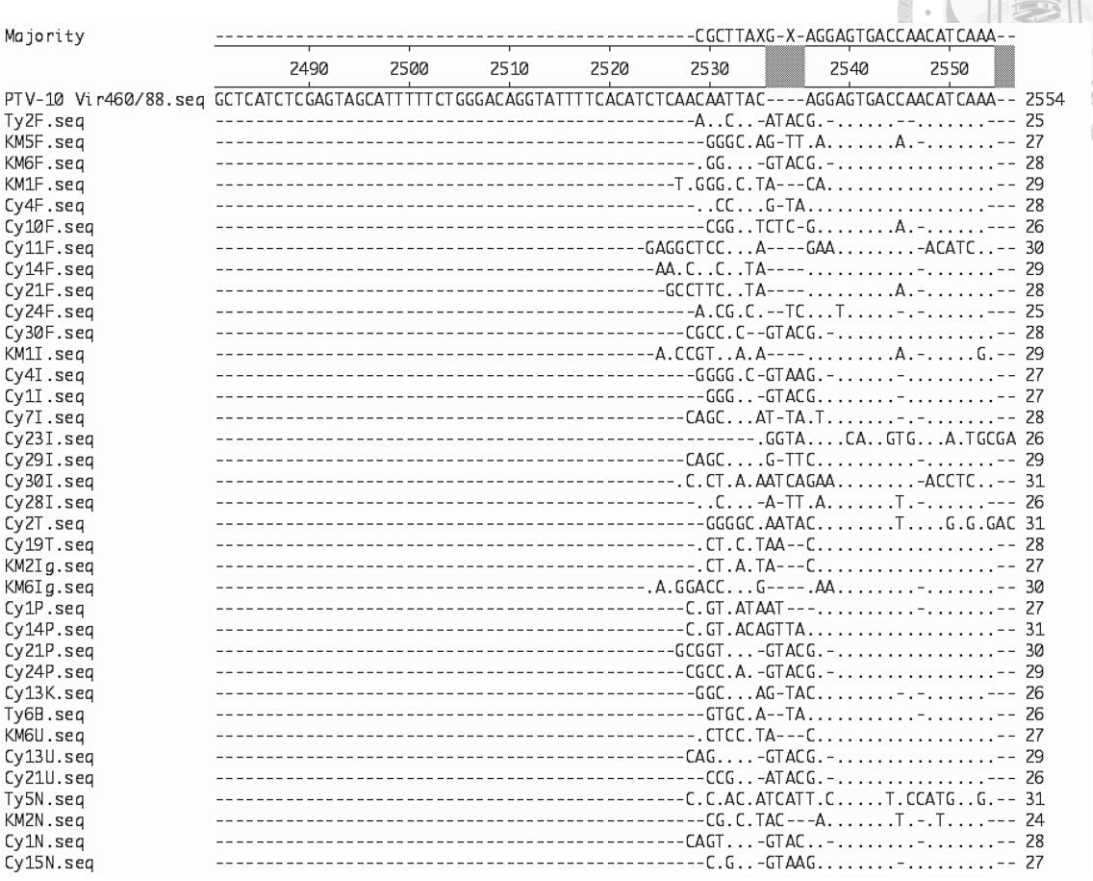
Herds	Healthy	Pigs	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
1	C	TY3	+/+	+/+/-2	+/+	-/-	+/+	-/-	+/+/-2	+/+	+/+	+/+
1	C	TY5	+/+/-6	+/+	+/+	+/+	+/+	+/+	-/-	+/+	+/+	+/+/-10
1	C	TY6	+/+/-6	+/+/-6	-/-	-/-	+/+	-/-	-/-	+/+/-10	+/+	+/+
1	H	TY2	+/+/-10	+/+	+/+	+/+	+/-	-/+	-/-	-/-	+/+	+/+/-6
2	C	KM2	+/+	+/+/-2	-/+/-4	+/+/-10	+/+	-/-	-/-	-/+	+/+	+/+/-10
2	C	KM5	+/+/-10	+/+	+/+	+/+	+/+	-/-	-/-	-/-	+/-	+/+
2	C	KM6	+/+/-10	+/+/-2	+/+/-2	+/+/-10	+/-	-/-	-/-	+/+/-6	+/+/-10	+/+
2	H	KM1	+/+/-10	+/+/-10	+/+/-4	+/+	+/-	+/+	-/-	+/-	+/+	+/-
3	C	CY2	+/+/-6	+/-	+/+/-10	+/+	+/+	+/+	+/+	+/+	+/+	+/+
3	C	CY4	+/+/-10	+/+/-10	+/+	+/+	+/+	+/+	-/-	-/-	+/-	+/+
3	C	CY5	+/+	+/+	+/+/-4	+/+/-4	+/+	+/+	-/-	+/+	+/+	+/+
3	H	CY1	+/+	+/+/-10	+/+	+/+/-2	+/+/-10	+/+	-/-	-/-	+/+	+/+/-10
4	C	CY9	+/+	+/+/-6	-/-	-/+/-2	+/+	-/+	-/-	-/-	+/+	+/+
4	C	CY10	+/+/-10	+/+	+/+	+/+	+/+	-/-	+/+	+/+	+/+	+/+
4	C	CY11	+/+/-10	+/+	+/+	+/+	+/-	+/-	+/+	+/+	+/-	+/+
4	H	CY7	+/+	+/+/-10	+/+	-/-	+/+	-/-	-/-	-/-	+/-	+/+
5	C	CY14	+/+/-10	+/+	+/+	-/-	+/+/-10	-/+/-2	-/-	-/-	+/-	+/+
5	C	CY15	+/+	+/+/-2	-/+	+/+/-2	+/+	+/+	-/-	+/+	+/-	+/+/-10
5	C	CY17	+/+/-2	+/+	+/+/-2	-/-	+/+	-/-	+/+	+/+	+/+	+/-
5	H	CY13	+/+	+/+	+/+	+/+	+/+	+/+	+/+/-10	-/-	+/+/-10	+/+
6	C	CY21	+/+/-10	+/+	+/+	+/+/-2	+/+/-10	-/-	+/+	+/+	+/+/-10	+/+
6	C	CY23	+/+/-5	+/+/-10	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+

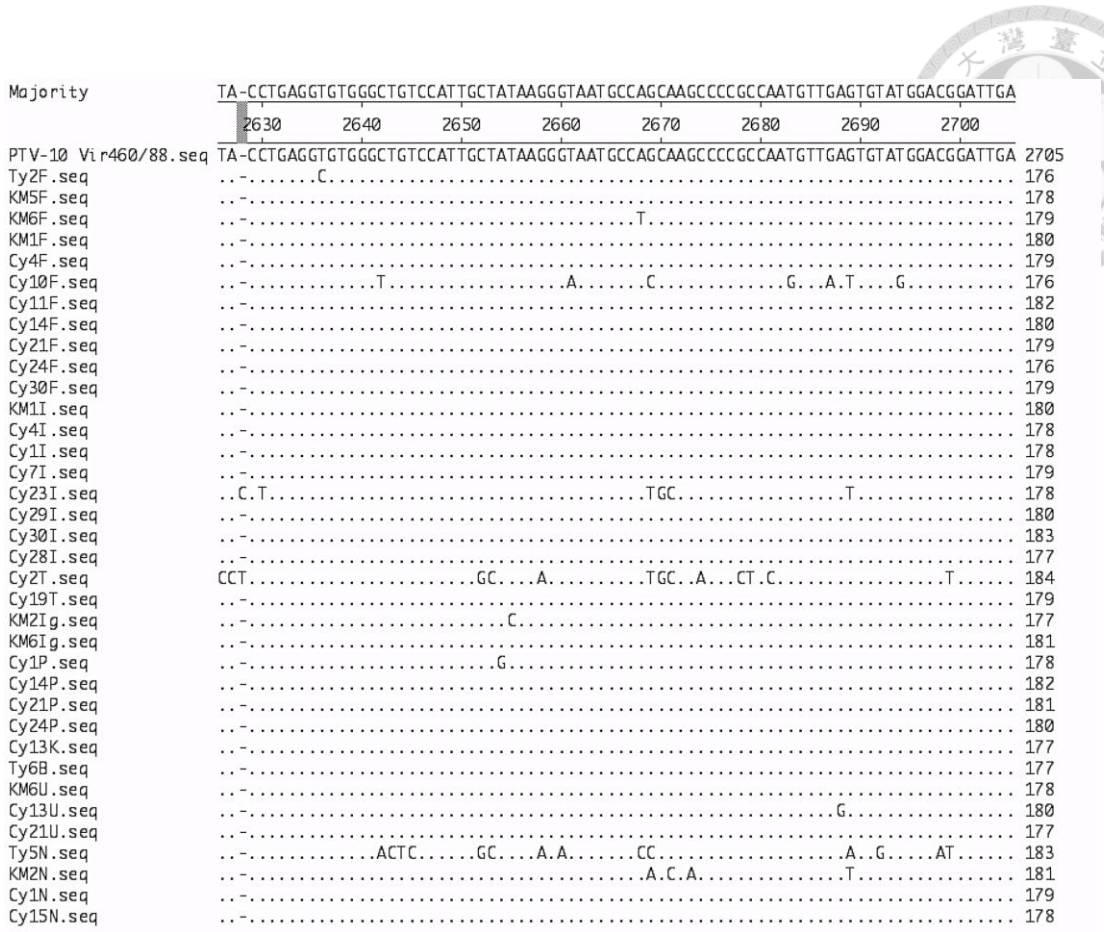
Herds	Healthy	Pigs	01 Feces	02 Ileum	03 Tonsil	04 Ig LN	05 Plasma	06 Spleen	07 Kidney	08 Bladder	09 Urine	10 Nasal
6	C	CY24	+/+/-10	+/+	+/+	+/+	+/+/-10	+/+	-/-	+/+	+/+	+/+
6	H	CY19	+/+	-/-	+/+/-10	-/-	+/+	+/+	+/-	+/+	+/+	+/+
7	C	CY27	+/-	+/+	+/+	+/+/-2	+/+	-/+	+/+	+/+	+/+	+/+
7	C	CY29	+/+/-6	+/+/-10	-/+	+/+	+/+	-/-	-/-	-/-	+/+	+/+
7	C	CY30	+/+/-10	+/+/-10	+/+	+/+	+/+	-/+	+/+	+/+	+/+	+/+
7	H	CY28	+/+	+/+/-10	+/+	+/+	+/+	-/-	-/-	+/+	+/+	+/+
	Serotype identification rate (number of VP1 positive / number of 5'NTR positive)		63% (17/27)	54% (14/26)	27% (7/26)	36% (8/22)	17% (4/24)	6% (1/16)	20% (2/10)	11% (2/18)	14% (3/22)	19% (5/26)

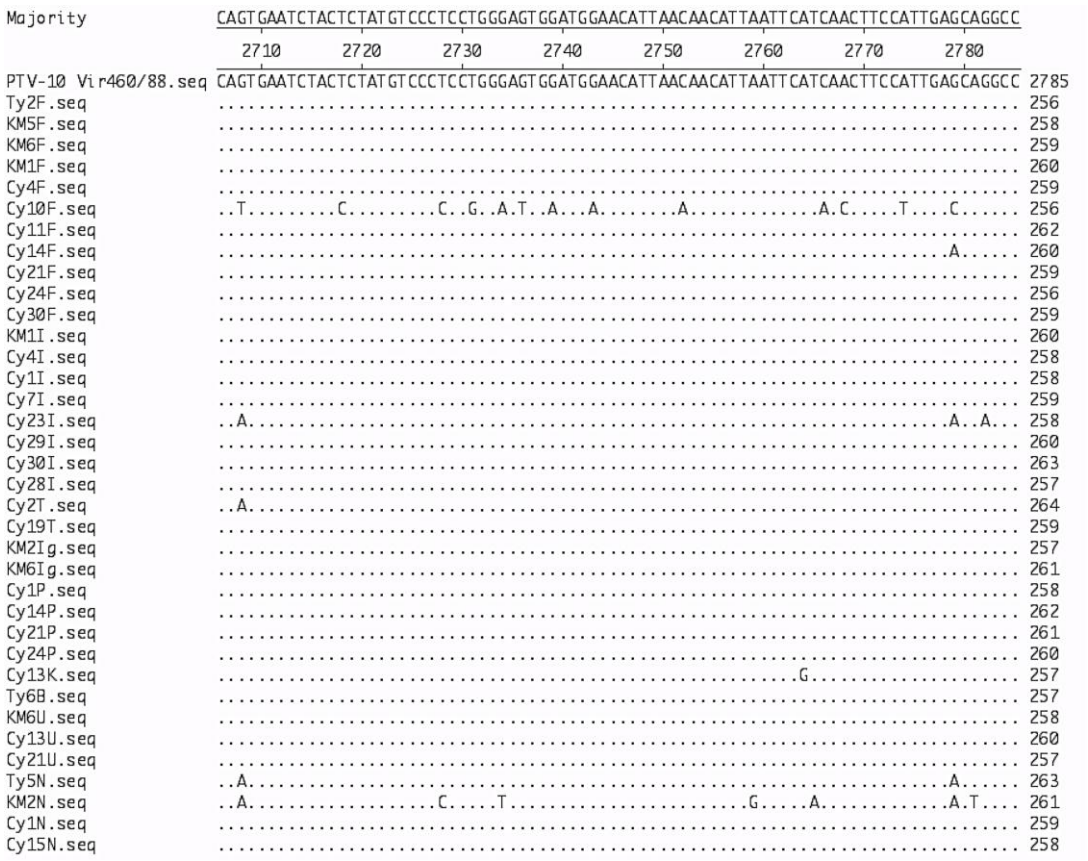
Note 1: Herds with four serotypes co-circulated are boxed. " C " indicates culled piglets, " H " indicates healthy piglets.

Note 2: The results are presented as qRT-PCR for 5'NTR/pan-PTV nested PCR for 5'NTR/identified serotype on VP1. Refer also to Tables 4.2, 4.3, 4.4. Results disagreed between qRT-PCR and pan-PTV nested PCR are masked. Those with identified serotypes are underlined. Those that are negative in qRT-PCR but with positive VP1 amplification and serotype identified are also boxed.

Appendix 3. Alignment of PTV-10 reference strain and all PTV-10 sequences in this study




Majority TA-CCTGAGGTGTGGGCTGTCCATTGCTATAAGGGTAATGCCAGCAAGCCCCGCCAATGTTGAGTGTATGGACGGATTGA
2630 2640 2650 2660 2670 2680 2690 2700
PTV-10 Vir460/88.seq TA-CCTGAGGTGTGGGCTGTCCATTGCTATAAGGGTAATGCCAGCAAGCCCCGCCAATGTTGAGTGTATGGACGGATTGA 2705
Ty2F.seq ..-.......C..... 176
KM5F.seq ..-..... 178
KM6F.seq ..-.....T..... 179
KM1F.seq ..-..... 180
Cy4F.seq ..-..... 179
Cy10F.seq ..-.....T.....A.....C.....G...A.T....G..... 176
Cy11F.seq ..-..... 182
Cy14F.seq ..-..... 180
Cy21F.seq ..-..... 179
Cy24F.seq ..-..... 176
Cy30F.seq ..-..... 179
KM1I.seq ..-..... 180
Cy4I.seq ..-..... 178
Cy1I.seq ..-..... 178
Cy7I.seq ..-..... 179
Cy23I.seq ..C.T.....TGC.....T..... 178
Cy29I.seq ..-..... 180
Cy30I.seq ..-..... 183
Cy28I.seq ..-..... 177
Cy2T.seq CCT.....GC....A.....TGC..A...CT.C.....T..... 184
Cy19T.seq ..-..... 179
KM2Ig.seq ..-.....C..... 177
KM6Ig.seq ..-..... 181
Cy1P.seq ..-.....G..... 178
Cy14P.seq ..-..... 182
Cy21P.seq ..-..... 181
Cy24P.seq ..-..... 180
Cy13K.seq ..-..... 177
Ty6B.seq ..-..... 177
KM6U.seq ..-..... 178
Cy13U.seq ..-.....G..... 180
Cy21U.seq ..-..... 177
Ty5N.seq ..-.....ACTC.....GC....A.A.....CC.....A..G.....AT..... 183
KM2N.seq ..-.....A.C.A.....T..... 181
Cy1N.seq ..-..... 179
Cy15N.seq ..-..... 178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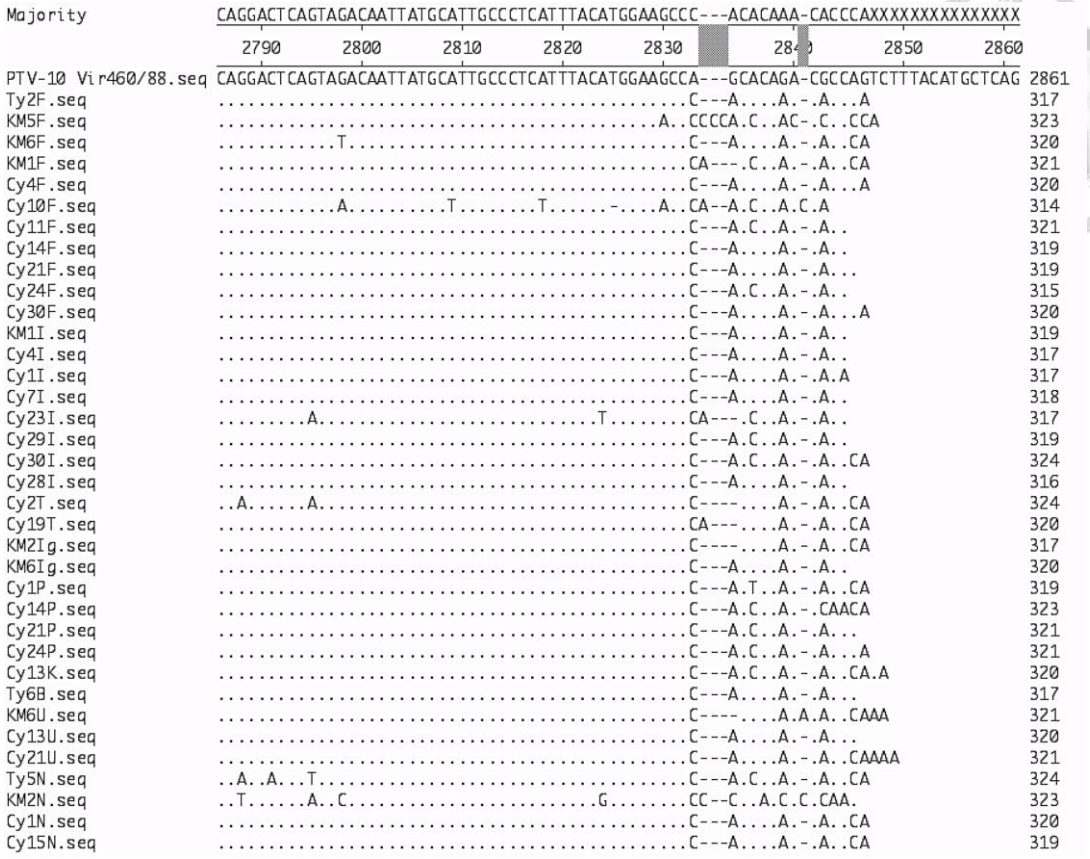
2710 2720 2730 2740 2750 2760 2770 2780
PTV-10 Vir460/88.seq CAGTGAATCTACTCTATGTCCCTCCTGGGAGTGGATGGAACATTAACAACATTAATTCATCAACTTCCATTGAGCAGGCC 2785
Ty2F.seq 256
KM5F.seq 258
KM6F.seq 259
KM1F.seq 260
Cy4F.seq 259
Cy10F.seq ..T.....C.....C..G..A.T..A...A.....A.....A.C.....T....C..... 256
Cy11F.seq 262
Cy14F.seq .....A..... 260
Cy21F.seq 259
Cy24F.seq 256
Cy30F.seq 259
KM1I.seq 260
Cy4I.seq 258
Cy1I.seq 258
Cy7I.seq 259
Cy23I.seq ..A.....A..A... 258
Cy29I.seq 260
Cy30I.seq 263
Cy28I.seq 257
Cy2T.seq ..A..... 264
Cy19T.seq 259
KM2Ig.seq 257
KM6Ig.seq 261
Cy1P.seq 258
Cy14P.seq 262
Cy21P.seq 261
Cy24P.seq 260
Cy13K.seq .....G..... 257
Ty6B.seq 257
KM6U.seq 258
Cy13U.seq 260
Cy21U.seq 257
Ty5N.seq ..A.....A..... 263
KM2N.seq ..A.....C.....T.....G.....A.....A.T.... 261
Cy1N.seq 259
Cy15N.seq 258


Majority CAGGACTCAGTAGACAATTATGCATTGCCCTCATTTACATGGAAGCCC---ACACAAA-CACCCAXXXXXXXXXXXXXXX
2790 2800 2810 2820 2830 2850 2860
PTV-10 Vir460/88.seq CAGGACTCAGTAGACAATTATGCATTGCCCTCATTTACATGGAAGCCA---GCACAGA-CGCCAGTCTTTACATGCTCAG 2861
Ty2F.seq C---A....A.-.A...A 317
KM5F.seq A..CCCCA.C..AC-.C..CCA 323
KM6F.seq C---A....A.-.A..CA 320
KM1F.seq CA---.C..A.-.A..CA 321
Cy4F.seq C---A....A.-.A...A 320
Cy10F.seq A.....T.....T.....-....A..CA--A.C..A.C.A 314
Cy11F.seq C---A.C..A.-.A.. 321
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