Study of recently identified porcine parvoviruses in pig herds of Japan and Thailand

(日本とタイの養豚に感染している新規豚パルボウイルスの研究)

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General introduction

For the past 15 years, the pig industries around the world have been suffering from many diseases caused by combined infections. However, a few years ago, vaccines for the porcine circovirus type 2 (PCV2) have had a great impact and reduced most of these complex diseases, and thereby, it is now recognized that PCV2 is the root cause of these diseases. PCV2 is widely distributed in most pig herds of the pig-producing countries. PCV2 alone does not induce severe symptoms, but cofactors, such as other infections by viruses and Mycoplasma or immunostimulation, augment the severity of a variety of symptoms (Opriessnig & Langohr, 2013).

The complex diseases associated with the PCV2 infection is now called porcine circovirus associated disease (PCVAD) or porcine circoviral disease (PCVD), the terms used in North America or in Europe, respectively. PCVAD includes the diseases formally named postweaning multisystemic wasting syndrome (PMWS) and porcine dermatitis and nephropathy syndrome (PDNS). PCV2 infection is also considered to induce secondary infections with bacteria and viruses, so histopathological lesions of PCVAD are complex and observed in multiple organs such as the lymphoid tissues, lung, skin, kidney, reproductive tissue, brain, or blood vessels, i.e., the so-called "multisystemic" lesions (Ellis, 2014; Opriessnig & Langohr, 2013). Since PCV2 and possible cofactors for PCVAD (Ellis *et al.*, 2000) are common in the pig populations, other illnesses associated with PCV2 infection may not be fully understood.

This thesis describes my study characterizing several newly identified porcine parvoviruses. The initially identified parvovirus in pigs is porcine parvoviruses (PPV), reported in the 1960s (Cartwright & Huck, 1967). PPV is one of the possible cofactors for PCVAD because the severe PCVAD was induced by co-infection with PCV2, PPV, porcine reproductive and respiratory syndrome virus (PRRSV), etc (Allan *et al.*, 1999) (Kennedy *et al.*, 2000) (Ellis *et al.*, 2000) (Kim *et al.*, 2003). PPV mainly causes reproductive failure in naïve dams, and manifestation characterized by stillbirth,

mummification, embryonic death and infertility. In contrast, PPV infection of adult pig causes only a subclinical or mild disease. PPV is now enzootic in most pig herds, and the disease is typically controlled by vaccination and sometimes by natural infection. Infected animals excrete PPV in their feces and fluids which will be oronasally transmitted to the next susceptible animal. Because parvovirus is generally known to be quite resistant to environmental degradation and many disinfectants, it is difficult to completely eliminate the virus from contaminated facilities.

Besides PPV, several other parvoviruses or their genomes have been recently identified in pigs. The existence of these newly identified porcine parvoviruses was reported in several countries, but their association with diseases is still not known. However, as it is suspected that there must be many multifactorial or idiopathic disorders in pig herds, these new parvoviruses should be further characterized.

Parvoviruses are non-enveloped, isometric viruses with a linear single stranded DNA of approximately 4–6 kb in size. Parvoviruses are isolated from a variety of animal species. Viruses infecting vertebrates and arthropods are classified to the subfamilies *Parvovirinae* and *Densovirinae*. The subfamily *Parvovirinae* used to be divided into 5 genera, but in the past decade, new molecular technologies lead to the discovery of many new parvovirus members some of which cannot be classified into the old taxonomy. Therefore, an updated taxonomy of the family *Parvoviridae* has just been recently proposed (Cotmore *et al.*, 2014). According to the proposed taxonomy, the subfamily *Parvovirinae* is divided into 8 genera, i.e., *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* and *Tetraparvovirus* (Cotmore *et al.*, 2014). There are several important parvoviruses in the subfamily *Parvovirinae* which cause severe animal diseases, such as goose and duck parvovirus of the genus *Dependoparvovirus,* bovine parvovirus and canine minute virus of the genus *Bocaparvovirus*, PPV, feline panleukopenia virus and canine parvovirus of the genus

Protoparvovirus, and Aleutian mink disease virus of the genus *Amdoparvovirus* (Parrish, 2010) (Cotmore *et al.*, 2014). These parvoviruses appear to share common biological properties such as resistance to antiseptic substances and extreme environmental conditions like heat and pH. They also have common biological properties in their DNA replication; they require the mitotic S phase of cells to replicate their DNA genome. The cell or tissue tropism of the virus is considered to be directly related to the viral pathogenesis in animals, so the viral property, requiring dividing cells to replicate, appears to be one of factors determining the pathogenesis in parvovirus-infected animals. For example, a variety of developing cells in the fetus and continuously dividing cells in older animals like lymphocytes, hemopoietic precursors and progenitor cells of the intestinal mucosa are the targets of PPV.

I stated to study the porcine parvovirus 2 (PPV2) genomes isolated from Japanese pigs, because, since its first identification of PPV2 in Myanmar in 2001 (Hijikata *et al.*, 2001), there was no report about the infection status of Japanese pig farms. The results of my study will be described in Chapter I of this thesis. I then extended such a study to several other porcine parvoviruses which have been recently identified but not well studied in regard to the epidemiology, biological properties and pathogenesis. Other newly identified porcine parvoviruses which I have been studying in my doctoral course are PPV3 (hokovirus) (Lau *et al.*, 2008), PPV4 (Cheung *et al.*, 2010), PPV5 (Xiao *et al.*, 2013b), and PBo-likeV (Blomstrom *et al.*, 2009). The table shows the relationship between the parvoviruses I studied and the new taxonomy of the family *Parvoviridae* (Cotmore *et al.*, 2014). I epidemiologically characterized the pig populations of Thai and Japanese herds, which will be described in Chapters II and III.

The results of my study on Japanese PPV2 have been published in Microbiology and Immunology. The studies of the newly identified parvoviruses detected in Thailand and Japan have been or will be separately submitted.

Table (General introduction). The parvoviruses studied in the thesis and the new taxonomy of the family *Parvoviridae*.

Genus Species	Virus or virus variants	Abbreviation	Former name
Amdoparvovirus			
Carnivore amdoparvovirus 1	Aleutian mink disease virus	AMDV	
Carnivore amdoparvovirus 2	gray fox amdovirus	GFAV	
Aveparvovirus			
Galliform aveparvovirus 1	chicken parvovirus	ChPV	
	turkey parvovirus	TuPV	
Bocaparvovirus			
Carnivore bocaparvovirus 1	canine minute virus	CnMV	
Carnivore bocaparvovirus 2	canine bocavirus 1	CBoV	
Carnivore bocaparvovirus 3	feline bocavirus	FBoV	
Pinniped bocaparvovirus 1	California sea lion bocavirus 1	CslBoV1	
	California sea lion bocavirus 2	CslBoV2	
Pinniped bocaparvovirus 2	California sea lion bocavirus 3	CslBoV3	
Primate bocaparvovirus 1	human bocavirus 1	HBoV1	
	human bocavirus 3	HBoV3	
	gorilla bocavirus	GBoV	
Primate bocaparvovirus 2	human bocavirus 2a	HBoV2a	
	human bocavirus 2b	HBoV2b	
	human bocavirus 2c	HBoV2c	
	human bocavirus 4	HBoV4	
Ungulate bocaparvovirus 1	bovine parvovirus	BPV	
Ungulate bocaparvovirus 2	porcine bocavirus 1	PBoV1	
	porcine bocavirus 2	PBoV2	
	porcine bocavirus 6	PBoV6	
Ungulate bocaparvovirus 3	porcine bocavirus 5	PBoV5	
Bocaparvovirus			
Ungulate bocaparvovirus 4	porcine bocavirus 7	PBoV7	PBo-
Ungulate bocaparvovirus 5	porcine bocavirus 3	PBoV3	
	porcine bocavirus 4-1	PBoV4-1	
	porcine bocavirus 4-2	PBoV4-2	
Copiparvovirus			
Ungulate copiparvovirus 1	bovine parvovirus 2	BPV2	
Ungulate copiparvovirus 2	porcine parvovirus 4	PPV4	PPV4
Dependoparvovirus			
Adeno-associated	adeno-associated virus-1	AAV1	
dependoparvovirus A	adeno-associated virus-2	AAV2	
	adeno-associated virus-3	AAV3	
	adeno-associated virus-4	AAV4	
	adeno-associated virus-6	AAV6	
	adeno-associated virus-7	AAV7	
	adeno-associated virus-8	AAV8	
	adeno-associated virus-9	AAV9	
	adeno-associated virus-10	AAV10	
	adeno-associated virus-11	AAV11	
	adeno-associated virus-12	AAV12	
	adeno-associated virus-13	AAV13	
	adeno-associated virus-S17	AAVS17	
Adeno-associated	adeno-associated virus-5	AAV5	
dependoparvovirus B	bovine adeno-associated virus	BAAV	
	caprine adeno-associated virus	CapAAV	
Anseriform	duck parvovirus	DPV	
dependoparvovirus 1	goose parvovirus-PT	GPV2	
	goose parvovirus	GPV	
Avian dependoparvovirus 1	avian adeno-associated virus	AAAV	
Chiropteran	bat adeno-associated virus	BtAAV	
Pinniped dependoparvovirus	California sea lion adeno-	CslAAV	
Squamate dependoparvovirus	snake adeno-associated virus	SAAV	

Genus Species	Virus or virus variants	Abbreviation	Former name
Erythroparvovirus			
Primate erythroparvovirus 1	human parvovirus B19-Au	B19V-Au	
	human parvovirus B19-J35	B19V-J35	
	human parvovirus B19-Wi	B19V-Wi	
	human parvovirus B19-A6	B19V-A6	
	human parvovirus B19-Lali	B19V-Lali	
	human parvovirus B19-V9	B19V-V9	
	human parvovirus B19-D91	B19-D91	
Primate erythroparvovirus 2	simian parvovirus	SPV	
Primate erythroparvovirus 3	rhesus macaque parvovirus	RhMPV	
Primate erythroparvovirus 4	pig-tailed macaque parvovirus	PtMPV	
Rodent erythroparvovirus 1	chipmunk parvovirus	ChpPV	
Ungulate erythroparvovirus 1	bovine parvovirus 3	BPV3	
Protoparvovirus			
Carnivore protoparvovirus 1	feline parvovirus	FPV	
	canine parvovirus	CPV	
	mink enteritis virus	MEV	
	racoon parvovirus	RaPV	
Primate protoparvovirus 1	bufavirus 1a	BuPV1a	
	bufavirus 1b	BuPV1b	
	bufavirus 2	BuPV2	
Rodent protoparvovirus 1	H-1 parvovirus	H1	
	Kilham rat virus	KRV	
	LuIII virus	LuIII	
	minute virus of mice (prototype)	MVMp	
	minute virus of mice	MVMi	
	minute virus of mice (Missouri)	MVMm	
	minute virus of mice (Cutter)	MVMc	
	mouse parvovirus 1	MPV1	
	mouse parvovirus 2	MPV2	
	mouse parvovirus 3	MPV3	
	mouse parvovirus 4	MPV4	
	mouse parvovirus 5	MPV5	
	hamster parvovirus	HaPV	
	tumor virus X	TVX	
	rat minute virus 1	RMV1	
Rodent protoparvovirus 2	rat parvovirus 1	RPV1	
Ungulate protoparvovirus 1	porcine parvovirus Kresse	PPV-Kr	PPV
	porcine parvovirus NADL-2	PPV-	
Tetraparvovirus			
Chiropteran tetraparvovirus 1	Eidolon helvum (bat) parvovirus	Ba-	
Primate tetraparvovirus 1	human parvovirus 4 G1	PARV4G1	
	human parv4 G2	PARV4G2	
	human parv4 G3	PARV4G3	
	chimpanzee parv4	Ch-	
Ungulate tetraparvovirus 1	bovine hokovirus 1	B-PARV4-	
	bovine hokovirus 2	B-PARV4-	
Ungulate tetraparvovirus 2	porcine hokovirus	P-PARV4	PPV3
Ungulate tetraparvovirus 3	porcine Cn virus	CnP-	PPV2
Ungulate tetraparvovirus 4	ovine hokovirus	O-PARV4	

Chapter I

Coexistence of multiple strains of porcine parvovirus 2 in Japanese pig farms.

Summary

The porcine parvovirus 2 (PPV2) genome was first identified in 2001 in Myanmar. Recently, the PPV2 genome has been found in several other countries. In this study, the prevalence of PPV2 in Japanese domestic pigs was investigated and found to be 58% (69/120) in healthy domestic pigs and 100% (69/69) in sick domestic pigs. Sequencing and phylogenetic analysis of the PCR products of the VP1 gene and an almost full length PPV2 clone indicated that diverged PPV2 strains exist in Japan. Clearly distinct strains of PPV2 were detected in 7 of the 10 pig farms.

Introduction

The genome of PPV2, a member of the parvovirus family, was first detected in specimens from domestic pigs in Myanmar in 2001 (Hijikata *et al.*, 2001). The genome was subsequently reported from China (Wang *et al.*, 2010), Hungary (Csagola *et al.*, 2012), the USA (Xiao *et al.*, 2013a) and Germany (Streck *et al.*, 2013). The initially identified PPV causes reproductive failure in sows; however, the clinical disease associated with PPV2 is unclear. Parvoviruses are non-enveloped, isometric viruses with a linear single stranded DNA approximately 4–6 kb in size. Parvoviruses have been isolated from a variety of animal species. Viruses infecting vertebrates and arthropods are classified into the subfamilies *Parvovirinae* and *Densovirinae*. The subfamily Parvovirinae is further divided into five genera; that is, *Dependovirus*, *Bocavirus*, *Erythrovirus*, *Parvovirus* and *Amdovirus* (Cotmore *et al.*, 2014) (International Committee on Taxonomy of Viruses, 2013). Parvoviruses infecting pigs include PPV and PPV2; recently several other porcine parvoviruses have been discovered, such as hokovirus (PPV3) (Lau *et al.*, 2008), PPV4 (Cheung *et al.*, 2010), PPV5 (Xiao *et al.*, 2013b) and porcine bocavirus (Blomstrom *et al.*, 2009). Although the taxonomic classification of these new porcine parvoviruses has not been established, pigs appear to be infected with a variety of parvoviruses.

Because PPV2 infecting the Japanese pig populations had not yet been characterized, we have studied the prevalence and diversity of PPV2, using samples from different sources. This is the first report of the epidemiological and genetic characterization of PPV2 genome detected in Japan.

Materials and Methods

Sample collection

Tonsil samples of 120 domestic pigs were obtained in 2010 from a slaughterhouse. All pigs were around 6 months of age, and fewer than 10 pigs were from any one farm. Sixty-nine other tonsil samples from domestic pigs were also assessed; these had been sent to a livestock hygiene service center for diagnostic autopsy, various disorders being suspected, and were aged from 8 to about 900 days (except for four animals with no age records).

Viral nucleic acid purification

For the 120 samples from healthy pig, about 0.1 g of the tonsils was suspended in 0.9 mL of minimum essential medium containing L-glutamine (Nissui, Tokyo, Japan) or saline and homogenized in 1.5 mL tubes with two Φ5 zirconia beads using a Micro Smash machine (Tomy Seiko, Tokyo, Japan). The homogenates were centrifuged at 15,000 g for 15 min., and aliquots of the supernatant stored at -80°C.

Viral DNA and RNA were isolated from solutions of the 1% tonsil homogenate or 1% blood/serum samples using a DNA/RNA purification machine, Magtration System 6GC (Precision System Science, Chiba, Japan) and a solution kit, GC series Magtration-MagaZorb RNA Common Kit (Precision System Science). Both viral RNA and DNA were successfully isolated from the various virus stocks by this procedure. Nucleic acids isolated from tissue or blood/serum samples were reverse-transcribed with Superscript II reverse transcriptase and primers of random hexamers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). These samples were used for the various PCRs to detect the genomes of both DNA and RNA viruses.

For the sick domestic pigs, a 10% solution of homogenized tonsil was centrifuged and viral DNA isolated from the supernatant with a QIAamp DNA Mini Kit (Qiagen, Gaithersburg, MD, USA).

Detection of viral genome by PCR.

The PPV2 genome was amplified by PCR using Quick Taq HS DyeMix (Toyobo, Osaka, Japan) including Taq polymerase with primers Q1 (5′-GCGCATTCGCCAAACTAGCTC-3) and Q2 (5-GTTTGCCCTTAATGTCGATCC-3′) or CnvirusF (5'-TTACGAGTTTCCCAGTCTCG-3) and Q17 (5-CCAGATATCGTCCTCGTA-3). Q1, Q2, and Q17 primers have previously been described (Hijikata *et al.*, 2001). To determine the prevalence of PPV2 in the pig population, Q1/Q2 primers were used: these amplify the 186 bp DNA of the NS1 gene. CnvirusF/Q17 primers, which amplify the 689 bp DNA of the VP1 gene, were used for direct sequencing of the PCR product and studying sequence variations. The amount of temperate DNA used in one polymerase reaction was estimated to be derived from about 2.5 ng of tissue. The PCR consisted of an initial enzyme activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., extension at 72°C for 30 sec. with the Q1/Q2 primers or for 1.5 min with CnvirusF/Q17 primers, and a final extension at 72°C for 7 min.

Cloning and sequencing of PPV2 genome and phylogenetic analyses.

Almost the full genome of PPV2 was amplified by PCR with Takara Ex Taq (Takara Bio, Shiga, Japan) and the primer RRy (5′-CCGACAGGATAAGTGTCGAG-3′), which was expected to anneal both ends of the PPV2 genome. The PCR products were purified and cloned into pGEM-T. Recombinant plasmids were propagated in 5DHα and purified by using a Wizard Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). The sequence data and phylogenetic tree were compiled and analyzed using MEGA 5 (Tamura *et al.*, 2011) and Genetyx (Genetyx, Tokyo, Japan).

The complete sequence of the near-full genome clone, JPT68, and the partial sequences of the PCR products with the CnvirusF/Q17 primers have

been deposited in DDBJ under accession numbers AB916464 and AB915834–AB915863.

Results

Prevalence of PPV2 in domestic pigs

To determine the prevalence of PPV2, we analyzed two groups of samples using the PPV2-specific PCR primers Q1 and Q2 (Hijikata *et al.*, 2001). The first group of samples was tonsils collected in a slaughterhouse from domestic pigs aged about 6 months. Of these 120 samples, 69 (58%) were positive for the expected PPV2 band (186 bp) (Table I-1). The second group of samples was tonsils of unhealthy domestic pigs aged mostly from 8 to 900 days. A hundred percent (69/69) of the samples was positive. These prevalences suggest that PPV2 has been widely spread in domestic pigs in Japan.

Characterization of a cloned PPV2 genome

We cloned a near complete genome of PPV2, termed JPT68, from the tonsil of a healthy domestic pig. The clone was 5233 bp long with two ORFs, namely ORF1 encoding 661 aa of NS1 and ORF2 encoding 1032 aa of VP1. Currently, six near full genome sequences of PPV2 have been reported; one isolated from Myanmar (Hijikata *et al.*, 2001), three from China (Wang *et al.*, 2010) and two from the USA (Xiao *et al.*, 2013a). Comparing them with other full-length PPV2 sequences, JPT68 showed 95.5–97.7% nucleotide identity with the total genomes of other PPV2s. In the two ORFs, the nucleotide and amino acid identities with JPT68 were 94.3–97.5% and 95.2–97.4%, respectively, in NS1 and 93.9–96.7% and 95.1–97.6%, respectively, in VP1, (Table I-2).

There were size variations in both the proposed NS1 and VP1, probably attributable to minor nucleotide differences (Table I-2). In NS1, six PPV2s had 661 aa whereas one Chinese strain GU938300 (GU938300) had 679 aa, probably because the GU938300 strain had one base change from ATA to ATG at 52 nt upstream of the proposed initiation codon of the other strains, generating a longer ORF. In VP1, six PPV2s had 1032 aa whereas the Myanmar strain AB076669 had 981 aa. The shorter ORF2 of the AB076669

strain had possibly resulted from one base deletion; the genome of AB076669 had the "ATG" at a position equivalent to the initiation codon of the other strains' ORF2; however, at 136 nt downstream there was one base deletion leading to a frame shift: we propose the next downstream "ATG" as the initiation codon of the shorter ORF2 of this strain.

Diversity of PPV2 genome in domestic pigs in Japan

Comparison of the nucleotide sequence among the PPV2 clones suggested that differences in the nucleotide and amino acid sequences were widely distributed in both NS1 and VP1; however, the VP1 region appeared slightly more variable than the NS1 region. Although we had used the PCR primers Q1 and Q2 to amplify a short NS1 region to determine the prevalence of PPV2 (Table I-1), we used another PCR primer set, CnvirusF and Q17, to amplify a relatively variable VP1 region (689 bp, nucleotide positions from 4102 to 4790 of JPT68) and analyze the nucleotide sequence. We directly sequenced the PCR products and subjected a 330 bp fragment to phylogenetic analysis.

We observed considerable sequence variation among the Japanese PPV2s. When sequences with less than a 2% nucleotide difference were considered as the same clade in this analysis, seven phylogenetic clades (Clades A to G) were identified from 21 healthy pig samples from13 farms (farms a to m) of prefecture A and 20 sick pig samples from 9 farms (farms n to v) of prefecture B (Fig. I-1 and Table I-3). Some of the Japanese PPV2s were closely related to the Myanmar (clade F), Chinese (clade C), USA (clades D, E and F) or Romanian (clades E) and Croatia (clade G) PPV2s, whereas clades A and B seemed unique to Japan since no PPV2 with less than a 7% nucleotide difference has been submitted to the GenBank database (Fig. I-1).

We found Clades B and E in both prefectures, whereas clades A, C, D and F and clade G were restricted to prefecture A and B, respectively (Table I-3). Clade A appeared to be the most common in prefecture A because 12 of 21 analyzed samples belonged to this clade and 11 of the 13 farms had this clade

of virus. In prefecture B, clades E and G were common; 9 and 8, respectively, of 20 sequenced samples belonging to these clades. Based on evaluation of each farm, we concluded that there were multiple clades of viruses on some farms. We analyzed more than two samples from ten farms; among these seven farms (farms a, f, h, i, l, m and o) had PPV2s of separate clades with nucleotide differences of 3.7%, 4.1%, 5.4%, 5.4/6.0/4.4%, 9.8%, 6.4% and 8.4/3.7/9.6%, respectively. Only farms j, q and v had two or five identical PPV2s.

Thus phylogenetic analysis of the 41 samples from two prefectures indicated that diverged PPV2 strains exist in Japan and different PPV2 strains coexisted in 7 of the 10 farms from which we analyzed more than two PPV2 samples.

Discussion

This paper describes the prevalence and characterization of the PPV2 genome detected in two prefectures of Japan. The prevalence was high; 58% in tonsil samples of healthy pigs in prefecture A and 100% in tonsil samples of sick pigs in prefecture B (Table I-1). In other countries, the prevalences appear to slightly vary; 6% (25/392) in various pig samples in Hungary (Csagola *et al.*, 2012), 10% (8/86) in the sera of Myanmar pigs (Hijikata *et al.*, 2001), 9% (26/296) in the sera of Chinese domestic pigs (Wang *et al.*, 2010), 21% (100/483) in the lungs of USA domestic pigs (Xiao *et al.*, 2013a) and 78% (78/100) in the tonsils of German domestic pigs (Streck *et al.*, 2013). Japan and Germany appear to have higher prevalences, which could be related to possible tissue tropism of PPV2 because both studies used tonsils as source of temperate DNA for the PCR assay. Tonsils are reportedly better than hearts for detecting the PPV2 genome (Streck *et al.*, 2013). However, regional differences in prevalence may represent either the actual percentages of PPV2-positive animals or technical differences, such as the PCR primers or analyzed organs, that is, tonsil versus serum. Collectively, our study confirmed previous reports that PPV2 has already spread around the world (Wang *et al.*, 2010) (Xiao *et al.*, 2013a) (Streck *et al.*, 2013) (Csagola *et al.*, 2012).

Phylogenetic analysis showed a considerable sequence variation among the Japanese PPV2s, and some of them being highly similar to the Myanmar, Chinese, USA or Romanian PPV2s (Fig. I-1). Of seven phylogenetic clades identified in PPV2s of two prefectures, only clades A and B appeared to be unique to Japan (Fig. I-1). Some clades were common to the two prefectures (clades B and E) whereas some clade(s) predominated in one or other prefecture (clade A in prefecture A and clades E and G in prefecture B) (Table I-3). However, we speculate that most PPV2 strains have recently spread worldwide.

From the perspective of the farm level, we isolated multiple clades of PPV2 from a single farm. We sequenced more than two samples from ten farms.

Seven of these 10 farms had clearly distinct clades of PPV2s with 3.7–9.8% nucleotide differences (Table I-3). Such large nucleotide differences cannot be attributed to an error during PCR amplification or rapid genetic change within a farm. Rather, we speculate that multiple strains of PPV2 have invaded and persisted in these pig farms.

It is not yet known whether PPV2 is associated with a clinical disease. All 69 sick pigs analyzed were positive for PPV2. Interestingly, this prevalence is higher than that of the 120 healthy pigs studied (58%) (Table I-1). About half the sick pigs showed the main clinical sign of poor growth, this poor growth being significantly associated with the presence of PCV2 DNA ($\chi 2 = 13.0$, $P < 0.001$). PCV2 is found in most pig-producing countries worldwide and is thought to be the main causative agent of porcine circovirus associated disease, the clinical signs of which include wasting and poor growth. Therefore, the demonstrated association between poor growth and PCV2 infection is reasonable. Because our sample groups of healthy and sick pigs were not initially matched by age, farm or sanitary conditions, more systemic studies are needed to elucidate whether PPV2 plays a role in any porcine diseases.

We examined the possibility of a relationship between PPV2 clade in samples from the sick pigs and clinical signs. The PPV2 sequences from the 20 sick pigs belonged to one of three clades (clades B, E or G) (Fig I-1 and Table I-3). Fifteen of the 20 pigs exhibited the main clinical sign of poor growth, 17 of them were positive for PCV2 DNA. Poor growth was found in each of the three clades, thus none of them seem to be directly associated with poor growth. Thus, analysis of our samples did not identify an association between PPV2 infection and poor growth.

Parvovirus, a single-stranded DNA virus, is known as a rapidly evolving virus with high sequence diversity, rearrangement and recombination, which can occasionally generate emerging viruses (Shackelton *et al.*, 2005) (Hoelzer *et al.*, 2008b) (Adlhoch *et al.*, 2012). Viral recombination results from coinfection of an animal with genetically distinct viral strains. Multiple

infection and recombination in parvovirus has been reported in pigs with porcine bocavirus 3/4 (Lau *et al.*, 2011), in dogs and cats with canine parvovirus (Hoelzer *et al.*, 2008a), in humans with human bocavirus 4 (Kapoor *et al.*, 2010) and in pigs with porcine bocavirus 4 (Lau *et al.*, 2011). Although we sequenced only one PCR product of the PPV2 genome for one pig, we suspect high frequencies of coinfection with different strains of PPV2 because of the high prevalence in Japanese pig farms.

Table I-1 Detection of PPV2 genome in two sample groups.

Animal, health status and age	Sample	% Prevalence
Healthly domestic pigs		
about 6 months	Tonsil	58% (69/120)
Sick domestic pigs		
8-30 days	Tonsil	100% (8/8)
31-90 days	Tonsil	100% (35/35)
91-180 days	Tonsil	100% (20/20)
900 days	Tonsil	100% (2/2)
unknown	Tonsil	100% (4/4)
	[Sub total	100%(69/69)]

PPV2 genome was detected by PCR using Q1/Q2 primers which amplify a 186 bp fragment of NS1 gene.

Table I-2. Comparison of ORF1 (NS1) and ORF2 (VP1) of JPT68 with other full-length PPV2 genomes.

PPV2 sequence	Country of collection	ORF1 (NS1)				ORF2 (VP1)				Reference
		Nucleotide		Amino acid		Nucleotide		Amino acid		
		Length	% identity with JPT68	Length	% Identity with JP68	Length	% identity with JPT68	Length	% identity with JPT68	
JPT68 (AB916464)	Japan	1986	100	661	100	3099	100	1032	100	This paper
US_153 (JX101461)	USA	1986	97.5	661	97.4	3099	96.4	1032	97.1	(Xiao *et al.*, 2013)
US_523 (JX101462)	USA	1986	97.5	661	97.3	3099	96.7	1032	96.8	(Xiao *et al.*, 2013)
CnPPV_JW8 (GU938299)	China	1986	97	661	97.3	3099	96.7	1032	97.6	(Wang *et al.*, 2010)
CnPPV_JH13 (GU938300)	China	2040	95.1	679	95.8	3099	94.8	1032	95.1	(Wang *et al.*, 2010)
CnPPV_YH14 (GU938301)	China	1986	94.3	661	95.2	3099	95.3	1032	95.3	(Wang *et al.*, 2010)
H-1 (AB076669)	Myanmer	1986	95.6	661	95.9	2946	93.9	981	96.2	(Hijikata *et al.*, 2001)

Table I-3. Phylogenetic clade and number of samples isolated from different farms.

	Farm																					
	Prefecture A													Prefecture B								
"Clade"	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	Total
A	1		1	1		1	1	1	1	2	1	1	1										12
B						1									2				1				4
C	1				1																		2
D								1	1														2
E		1							1				1	1	4					1	1	2	12
F												1											1
G															1	1	5	1					8
Total	2	1	1	1	1	2	1	2	3	2	1	2	2	1	7	1	5	1	1	1	1	2	41

The phylogenetic clades are indicated in Fig.I-1. Farms a to m and Farms n to v are in Prefectures A and B, respectively.

Fig. I-1 Phylogenetic tree of PPV2

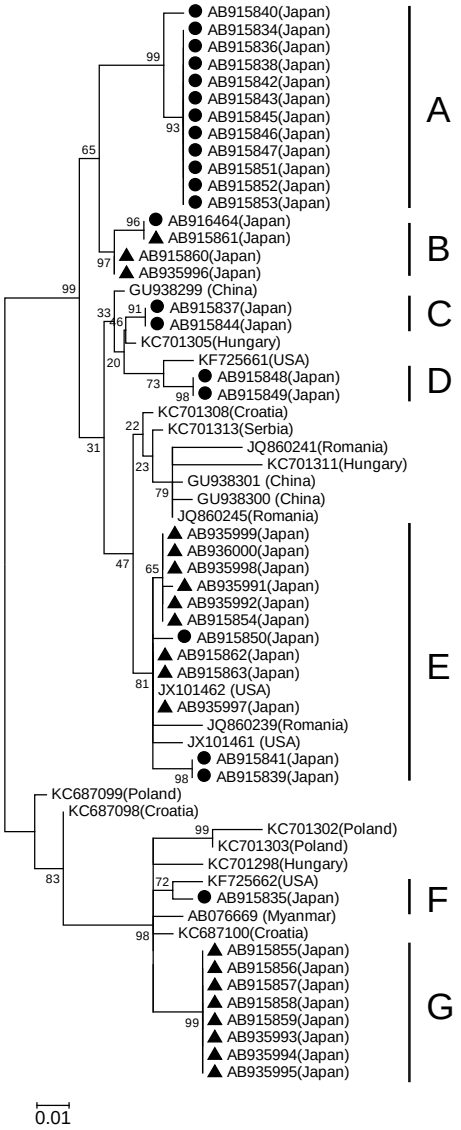


Fig. I-1. Phylogenetic analysis of a 330 bp fragment of PPV2 VP1 gene. A phylogenetic tree was constructed by the maximum-likelihood method of pairwise nucleotides based on alignments generated by Clustal W implemented in MEGA5. Japanese PPV2s isolated from healthy pigs in prefecture A are indicated as ● and from sick pigs in prefecture B as ▲. The clades, designated A to G, were grouped when the nucleotide difference was less than 2%. Sequence data were obtained from the Genbank database; the parentheses indicated the country in which they were isolated.

Chapter II

Prevalence and genomic characterization of porcine parvoviruses in Thailand.

Summary

Porcine parvovirus (PPV) causes reproductive failure in sows and is spreading worldwide. Many other types of porcine parvoviruses have been recently identified in pig herds. We studied the prevalence of 5 porcine parvoviruses in Chiangmai area of Thailand. The prevalence was 53% (42/80) for PPV (PPV-Kr or -NADL2 as the new abbreviation), 83% (66/80) for PPV2 (CnP-PARV4), 73% (58/80) for PPV3 (P-PARV4), 44% (41/80) for PPV4 (PPV4) and 18% (23/80) for PBo-likeV (PBoV7). We performed phylogenetic analyses for PPV2 and PPV3, indicating the existence of two and one clade(s) of viruses, respectively. Over 60% of animals carried more than 3 of the 5 viruses.

Introduction

Porcine parvovirus (PPV) is the causative agent of reproductive failure in sows. The infection of seronegative pregnant sows results in infection of the embryo and fetus which causes embryonic death, stillbirths, mummification without severe clinical signs in the mother. PPV was initially identified in the 1960s and is now endemic in most pig-producing countries throughout the world. Genomes of several new members of porcine parvoviruses have recently been identified, including PPV2 (Hijikata *et al.*, 2001), PPV3 (Lau *et al.*, 2008), PPV4 (Cheung *et al.*, 2010), PPV5 (Xiao *et al.*, 2013b) and porcine bocavirus-like virus (PBo-likeV) (Blomstrom *et al.*, 2009). Diseases associated with these new parvoviruses have not been well characterized.

The family *Parvovirinae* includes two subfamilies, *Parvovirinae* and *Densovirinae* infecting vertebrates and invertebrates, respectively. The subfamily *Parvovirinae* is further divided into 8 genera, *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus, Tetraparvovirus* (Cotmore *et al.*, 2014). Parvoviruses are non-enveloped, isometric viruses with a linear single stranded DNA of approximately 4–6 kb in size. An increasing number of new parvoviruses has been found in pigs, i.e., the pig appears to be co-infected by multiple groups of parvoviruses.

The detection and genetic characterization of the new porcine parvoviruses have been done in several countries since their discovery, and some of the viruses show variation in their geographical distribution. Since Thailand has not been investigated except for the seroprevalence of PPV (Tummaruk & Tantilertcharoen, 2012), we investigated the prevalence of the five porcine parvoviruses, PPV, PPV2, PPV3, PPV4 and PBo-likeV, using the tissue samples collected in the Chiangmai area of Thailand and characterized the genetic diversity of PPV2 and PPV3.

Since many parvovirus genomes have been reported in the past decade and named differently (Xiao *et al.*, 2013c), the updated taxonomy of the family *Parvoviridae* has recently been proposed (Cotmore *et al.*, 2014). According,

the 5 viruses we describe here are classified into the same subfamily *Parvovirinae*, but with different genus or different abbreviation. PPV is abbreviated as PPV-Kr or PPV-NADL2 and classified in genus *Protoparvovirus*, species *Ungulate protoparvovirus 1*, PPV2 as CnP-PARV4 in genus *Tetraparvovirus*, species *Ungulate tetraparvovirus 3*, PPV3 as P-PARV4 in genus *Tetraparvovirus*, species *Ungulate tetraparvovirus 2*, PPV4 as PPV4 in genus *Copiparvovirus,* species *Ungulate copiparvovirus 2*, PBo-likeV as PBoV7 in genus *Bocaparvovirus*, species *Ungulate bocaparvovirus 4*. However, we use the previous abbreviations in this paper to avoid confusion.

Materials and Methods

Sample collection and Viral DNA extraction

Eighty tonsil samples of pig were collected at a slaughterhouse in the Chiangmai area of Thailand in 2011. All of the pigs were around 6 months of age and had been farmed in the Chiangmai and Lampung provinces. About 0.1 g of a tonsil was suspended in 0.9 ml of saline and homogenized in a 1.5 ml tube with two Φ5 zirconia beads using Mini-Beadbeater-1 (Bipspec, USA). The homogenates were centrifuged at 3000xg for 5 min., and aliquots of the supernatant were stored at -20°C. The viral DNA and RNA were isolated from solutions of the 1% tonsil homogenate by using the QIAamp RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

Detection of porcine parvoviruses genomes by PCR

In order to determine the prevalence of porcine parvoviruses we carried out PCR in a separate reaction with each pair of primers. The PCR primers used for the detection of PPV, PPV2, PPV3, PPV4 and PBo-likeV are listed in Table II-1. The PCR was performed with the Quick TaqTM HS DyeMix (Toyobo, Japan) including the Taq polymerase. The PCR cycles consisted of an initial enzyme activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., extension at 72°C for 30 sec. for 1.5 min. and a final extension at 72°C for 7 min.

Phylogenetic analysis

For the phylogenetic analysis of PPV2 and PPV3, we directly sequenced the PCR products of the viral VP regions different from those of the conventional PCRs used to detect short common viral regions (Tab II-1), because of the relatively higher divergences. For PPV2, the 688 bp DNA of the VP1 gene (nucleotide positions from 4103 to 4790 of JPT68 strain AB916464) was amplified with the primer pairs, CnvirusF (5'-TTACGAGTTTCCCAGTCTCG-3') (Prayuth) and Q17

(5'-CCAGATATCGTCCTCGTA-3') (Hijikata *et al.*, 2001), and after direct sequencing with the two PCR primers, the internal overlapped 330 bp sequence was subjected to phylogenetic analysis.

For PPV3, the 622 bp of the VP1 gene was analyzed after amplifying the 713 bp of the VP1 (nucleotide positions from 3359 to 4072 of the stain AB916464) with the primers PPV3 P7F (3'-GGGGCACTCATTTCTCTGAT-5') and PPV3 P7R (3'-CTGGCCTTTTCCACTTAGGA-5') (Streck *et al.*, 2013) and sequencing with both primers and the two internal primers PPV3 7F2 (3'-GGAGAATAATGTTCTTCCTC-5') and PPV3 7R2 (3'-TCGTACTCATCAAGCAGCTG-5').

The sequence data and phylogenetic tree were compiled and analyzed using MEGA 5.1 (Tamura *et al.*, 2011) and Genetyx (Genetyx Co., Japan). The phylogenetic trees were generated by the maximum likelihood method.

The partial sequences of the PCR products of PPV2 and PPV3 have been deposited in DDBJ under accession numbers OOOO-OOOO and OOOO-OOOO.

Results

Prevalence of the five porcine parvoviruses

The prevalence of the five porcine parvoviruses, PPV, PPV2, PPV3, PPV4 and porcine parvovirus was examined using 80 tonsil samples of pig collected at a slaughterhouse in the Chiangmai area of Thailand. The viral genomes were detected by PCR with the specific primers (Table II-1). The prevalences of PPV, PPV2, PPV3, PPV4 and PBo-likeV were 53% (42/80), 83% (66/80), 73% (58/80), 44% (41/80) and 18% (23/80), respectively (Table II-2). The results suggested that the five porcine parvoviruses were distributed at high frequencies in the pigs in this area.

Of the 5 viruses we examined, more than 3 viruses were found in 61% (49/80) of the animals, more than 4 viruses in 28% (22/80) of the animals, and all viruses in 5% (4/80) of the animals. To determine whether the detection of the five viral genomes was random or correlated, we tested it by the chi-square test. Significant associations of the detection were found between PPV and PPV3 ($\chi 2 = 5.20$, $P<0.02$) and between PPV2 and PBo-likeV ($\chi 2 = 4.24$, $P<0.04$) (Table II-3), indicating that the two pairs of viruses were coincidentally detected in this analysis.

Genetic diversity of PPV2 and PPV3.

PPV2

Genetic diversity of the 20 PPV2s detected in the Chiangmai area was investigated by phylogenetic analysis with the 330 bp PCR product of the VP1 region as previously described (Saekhow et al 2014). In the phylogenetic tree of 107 PPV2 sequences including the 20 Thai sequences and the 53 sequences currently deposited in the databank from various countries, two roughly separated clades, tentatively called clades I and II, were found (Fig. II-1). The Thai samples belonged to either clade, 12 samples of clade I and 8 samples of clade II, and their maximum nucleotide difference was 11.2% overall, and 1.5% within clade I and 2.8% within the clade II. In both clades, other PPV2 sequences, which were closely related

to the Thai samples, have been detected from China, Japan, Europe and USA (Fig. II-1). The phylogenetic analysis with amino acid sequence showed similar results. The results suggested that the two clades of PPV2 existed in the Chiangmai area and the Thai PPV2s of both clades were closely related to those of other countries.

PPV3

We carried out phylogenetic analysis of the 20 Thai PPV3s together with the 71 PPV3 sequences deposited from around the world (Fig. II-2), based on the 622 bases of the PPV3 VP1 gene. PPV3 was less diverged (divergent?) than PPV2. Among the 91 total PPV3 sequences, only 6 isolates, three Chinese sequences (JQ177081 and JQ177083 and JQ177083) (Pan *et al.*, 2012), two Cameroon sequences (KF225548 and KF225549) (Adlhoch *et al.*, 2013) and one Romanian sequence (JF738352) (Cadar *et al.*, 2011b), appeared to be relatively diverged from the others. All the Thai PPV3s showed one group of closely related sequences with a 3.0% of the maximum nucleotide difference, while the maximum nucleotide difference among the 107 sequences collected from around the world was 6.5%. The Thai PPV3s appeared to be more related to two UK PPV3s (FJ982254 and FJ982251) (Szelei *et al.*, 2010) with 0.8-2.0% nucleotide difference and two Cameroon PPV3s (KF225548 and KF225549)(Adlhoch *et al.*, 2013) with 2.8-4.8% nucleotide difference, but also related to many PPV3s from Europe (Cadar *et al.*, 2011b), Germany (Streck *et al.*, 2013) (Adlhoch *et al.*, 2010), the USA (Xiao *et al.*, 2012) and Hong Kong (Lau *et al.*, 2008) and China (Pan *et al.*, 2012) (Li *et al.*, 2012) (Fig. II-2).

The phylogenetic analysis of the amino acid sequence showed that the PPV3s were very homogenous except for the three Chinese strains (accession numbers JQ177081, JQ177083 and JQ177083) (Pan *et al.*, 2012). The Thai PPV3s showed less than 2.0% amino acid difference.

Discussion

We studied the prevalence of the 5 porcine parvovirus genomes in the pigs of the Chiangmai and Lamphun provinces which is one of the most crowded pig farming areas in Thailand. The parvoviruses we studied were the classical PPV and the newly identified PPV2, PPV3, PPV4 and PBo-likeV. We detected all of them and the prevalence was 53% (42/80) for PPV, 83% (66/80) for PPV2, 73% (58/80) for PPV3, 44% (41/80) for PPV4 and 18% (23/80) for PBo-likeV (Table II-2).

PPV was identified in the 1960s in Europe (Cartwright & Huck, 1967) and is now commonly found in most pig producing countries. Diseases by PPV infection are mostly controlled by vaccination, but it is still one of the most important causes of infectious infertility, together with the porcine reproductive and respiratory syndrome (PRRS). PPV shares its virological and biological properties with other members of the family *Parvoviridae*. Parvoviruses require cellular DNA synthesis to replicate, and thereby the target cell is typically restricted to such cells in infected animals. Another important property is the stability under various environmental conditions, such as pH and temperature, and the resistance against many disinfectants. The latter properties leads to difficulty in eradicating the PPV-associated diseases at the pig farms. The status of PPV infection in Thailand had been reported that the seroprevalence was 86% (137/166) in pigs with a history of reproductive disorders aged 211 to 504 days (Tummaruk & Tantilertcharoen, 2012). The seroprevalence seems to correspond to our data for the prevalence of the PPV genome, i.e., 53% (42/80) (Table II-2). In most pig farms, embryonic and fetal infections are restricted in vaccinated or naturally infected sows, and, after decreasing the long-lasting maternal antibody to PPV, piglets are infected. Around 6-month-old when most animals are slaughtered, a high prevalence of the PPV genome is observed in many countries. Therefore, the prevalences of an antibody to PPV (Tummaruk & Tantilertcharoen, 2012) and PPV genome (Table II-2) in Thailand appears consistent with those of other countries.

The PPV2 genome was first identified in 2001 in Myanmar, and at that time the PPV2 genome was detected from 10% (8/86) of the sera from Myanmar pigs (Hijikata *et al.*, 2001). Since 2010 the prevalence of PPV2 was reported from various countries; 9% (26/296) in the sera of Chinese pigs (Wang *et al.*, 2010), 6% (25/392) in various pig samples from Hungary (Csagola *et al.*, 2012), 21% (100/483) in the lungs of US pigs (Xiao *et al.*, 2013a), 78% (78/100) in the tonsils of German pigs (Streck *et al.*, 2013) and 58% (69/120) in healthy pigs and 100% (69/69) in sick pigs in Japan (Prayuth, 2014). The prevalence of Thai pigs was 83% (66/80) (Table II-2) which appears to be a higher prevalence like Germany and Japan. Interestingly, these three studies used tonsil as the source of the DNA temperate for the PCR. Because the PPV2, PPV3 and PPV4 genomes were detected from the tonsil marginally better than from hearts (Streck *et al.*, 2013), the different prevalences might be related to the organs that are assayed. PPV2 has been detected from feces, blood, lungs and other tissues (Csagola *et al.*, 2012), but the tissue tropism or the infection route of PPV2 is not known.

The phylogenetic analysis with the PPV2 VP1 region indicated that the overall PPV2s were largely classified into 2 clades (Fig. II-1). The Thai pigs had both clades of PPV2 and, because the Thai PPV2s belonged to one or two branches within each clade, we speculated that a limited source of PPV2 of the two clades had spread into the examined population of the Chiangmai area.

The PPV3 genome was detected in 73% (58/80) of the Thai samples (Table II-2). The PPV3 genome was first detected and cloned in 2008 as a viral genome closely related to human parvovirus 4 and initially called porcine hokovirus (Lau *et al.*, 2008). The genome was detected in 44 % (148/333) of the porcine samples (including lymph nodes, liver, serum, nasopharyngeal and fecal samples) from Hong Kong (Lau *et al.*, 2008). The genome has been detected in various countries. The prevalence in pigs was 6% (25/392) in Hungary (Csagola *et al.*, 2012), 5% (113/242) (Pan *et al.*, 2012) and 51% (249/485) (Li *et al.*, 2013) in China, 68% (65/94) in Cameroon (Adlhoch *et al.*,

2013), 12% (60/483) in the USA (Xiao *et al.*, 2012) and 20% (20/100) (Streck *et al.*, 2013) in Germany. The PPV3 genome was also detected in wild boars with the prevalences of 50% (188/372) in Romania (Cadar *et al.*, 2011b) and 33% (51/156) (Adlhoch *et al.*, 2010). Although the prevalences are varied, it is suggested that PPV3 has already been spread worldwide.

All the 20 Thai PPV3s were clustered in the phylogenetic tree as a closely related group with less than a 3.0% nucleotide difference (Fig II-2) in contrast to the clearly distinct two clades in the Thai PPV2s (Fig. II-1). As PPV3 is variable and shows a worldwide distribution, the Thai PPV3s in this area may have recently invaded from a limited source.

The prevalence of the PPV4 genome was 44% (41/80) in the Thai pigs (Table II-2), which is considerably higher than those in other countries, that is, 2% (12/573) in sick pigs and 1% (1/132) in healthy pigs in China (Huang *et al.*, 2010), 6% (25/392) in Hungary (Csagola *et al.*, 2012), 7% (7/100) in Germany (Streck *et al.*, 2013) and 4% (20/483) in the USA (Xiao *et al.*, 2013b).

The PPV4 genome was initially cloned in the USA in 2010 from pig herds suffering from PRRS and being co-infected with many viruses including PCV2 (Cheung *et al.*, 2010). However, it is not clear that any disease is associated with PPV4. Based on our phylogenetic analysis of the 2.5 kb PPV4 VP1 region, PPV4 is less diverged with about a 2.2 % maximum nucleotide difference than those of PPV2 (12%) or PPV3 (7%). PPV4 is unique in the genome organization (Cheung *et al.*, 2010). PPV4 has the ORF3 located between ORF1 and ORF2, like bocaviruses, but ORF3 is distinct from other bocaviruses, and ORF1 and ORF2 are most closely related to bovine parvovirus 2 (BPV2) which is not a bocavirus but a parvovirus (Cheung *et al.*, 2010). More recently, a virus closely related to PPV4, called PPV5, was cloned (Xiao *et al.*, 2013b). PPV5 lacks ORF3 but has ORF1 and ORF2 carrying highest amino acid identities with PPV4. The relationship among PPV4, PPV5, BPV2 and other members of the parvoviruses is complicated, but it may be representative for the complexity

of the family *Parvoviridae*.

Diverged species of the bocaviruses have been identified (Xiao *et al.*, 2013c), and in this study, we examined PBo-likeV, the first bocavirus identified in pigs (Blomstrom *et al.*, 2009) which was also called PBoV (PBoV-SX) (Zeng *et al.*, 2011) or PBoV1 (Zhang *et al.*, 2011) (Shan *et al.*, 2011) and more recently proposed as PBoV7 of the genus *Bocaparvovirus*, the species *Ungulate bocaparvovirus 4* by the taxonomy study group (Cotmore *et al.*, 2014). The PBo-likeV genome was detected from 18% (23/80) of the Thai samples (Table II-2). The genome has been detected from 39% (74/191) of the pigs with respiratory tract symptoms and in 7% (3/41) of healthy pigs in China (Zhai *et al.*, 2010), 63% (215/340) in different areas of China (Shan *et al.*, 2011), 13% (109/842) of wild boars in Romania (Cadar *et al.*, 2011a) and 2% (6/392) in Romania (Csagola *et al.*, 2012). As the detection rate was much higher from weanling piglets with respiratory tract diseases than from healthy pigs (Zhai *et al.*, 2010), PBo-likeV might have a potential to associate with the disease, similar to some of human bocavirus which probably involves a lower respiratory tract disease and gastroenteritis in young children.

Our study showed that multiple parvoviruses persistently infect to pigs of the populations in the Chiangmai area, the situation largely similar to other pig-producing countries. Over 60% of 80 pigs were infected with more than 3 viruses out of the 5 tested viruses (data not shown). Some of the parvoviruses were coincidentally detected, such as PPV/PPV3 and PPV2/PBo-likeV (Table II-3). The reasons are not known, but among the many possibilities we speculate that some virus infections might induce an increased susceptibility of the host to other virus infections, for example, by immunosuppression or activation of cellular DNA replication which is suitable for replication of parvoviruses.

Table II-1. List of PCR primers used in this study for detection of porcine parvoviruses.

Virus	Primers	Size of PCR product (bp)	Reference
PPV	mPPVF:5'-CACAGAAGCAACAGCAATTAGG-3′	203	(Ogawa *et al.*, 2009)
	mPPVR:5'-CTAGCTCTTGTGAAGATGTGG-3'		
PPV2	Q1 F: 5'-GCGCATTCGCCAAACTAGCTC-3'	199	(Hijikata *et al.*, 2001)
	Q2 R:5'-GTTTGCCCTTAATG CGATCC-3'		
PPV3	PPV3 F:5'-GTGGCAGTGATATTGCATCG-3'	247	(Streck *et al.*, 2013)
	PPV3 R:5'-TGGCAGTCATTGAATGGAAA-3'		
PPV4	PPV4 F:5-ACAAGGTGGAGGAACGTTTG-3’	239	(Streck *et al.*, 2013)
	PPV4 R: 5'-TTCCATGAGGGAGAGGATTG-3’		
PBo-likeV	SbocaF: 5'-GGGCGAGAACATTGAAGAGGT-3'	499	(Zhai *et al.*, 2010)
	SbocaR:5'-TTGTGAGTATGGGTATTGGTG-3'		

Table II-2. Prevalences of the five porcine parvoviruses in Chiangmai of Thailand.

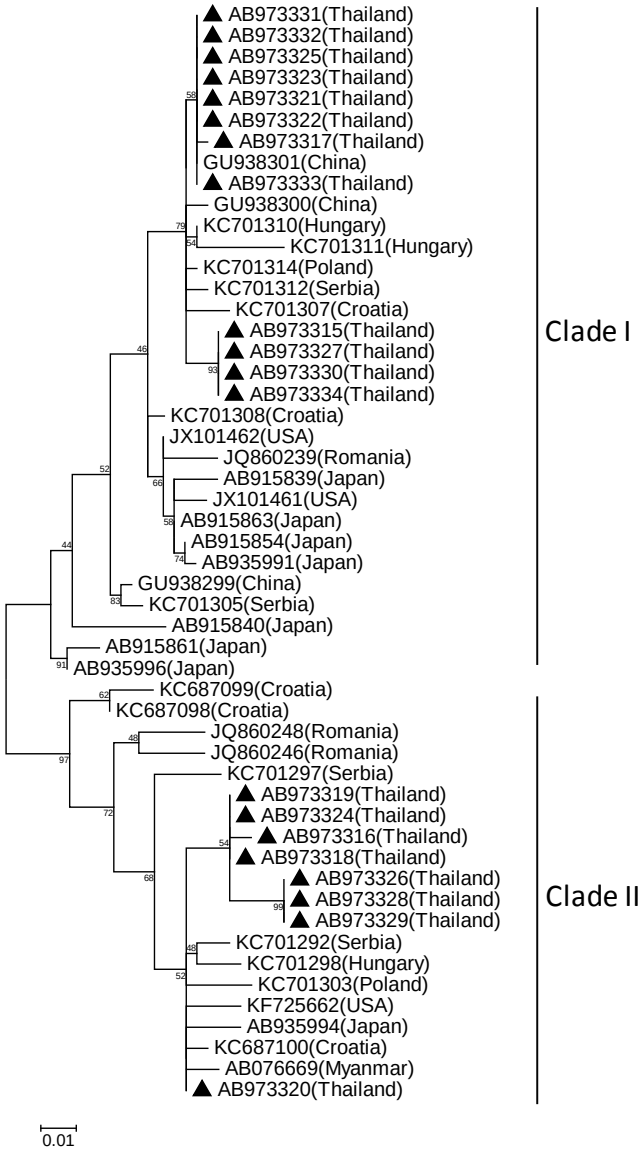
Virus	Prevalence
PPV	53% (42/80)
PPV2	83% (66/80)
PPV3	73% (58/80)
PPV4	44% (41/80)
PBo-likeV	18% (23/80)

Table II-3. Chi-squared analysis of coincidental detection among the five porcine parvoviruses.

Relationship between two viruses		Number of pigs				χ2 value	P value
		+/+	+/-	-/+	-/-		
PPV /	PPV2	34	32	8	6	0.15	0.702
	PPV3	35	23	7	15	5.2	0.023* [a]
	PPV4	25	16	17	22	2.42	0.120
	PBo-likeV	9	7	33	31	0.11	0.737
PPV2 /	PPV3	50	8	16	6	2.01	0.157
	PPV4	34	7	32	7	0.01	0.918
	PBo-likeV	16	0	50	14	4.24	0.039*
PPV3 /	PPV4	50	8	16	6	2.01	0.157
	PBo-likeV	9	7	49	15	2.65	0.104
PPV4 /	PBo-likeV	6	10	35	29	1.51	0.219

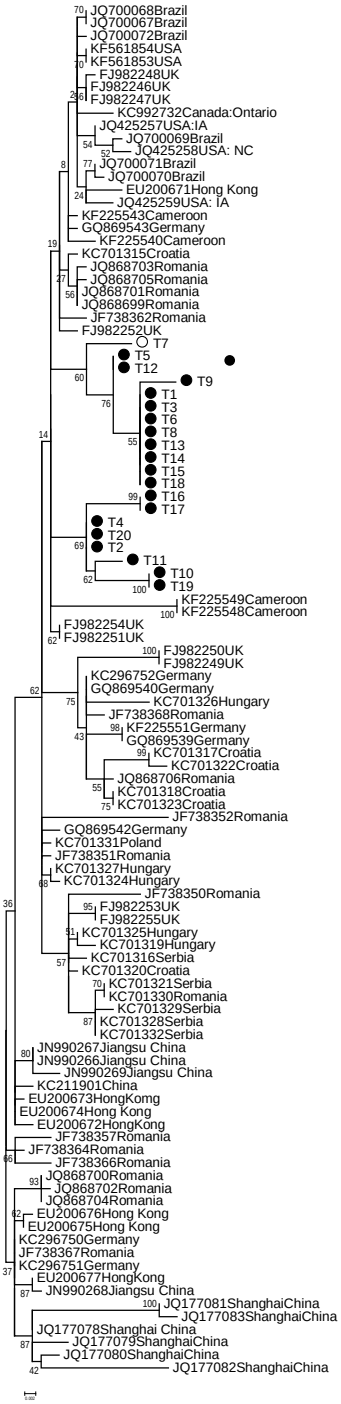
a, *: significant (0.05>p>0.01). Others without asterisk mean not significant (p>0.05).

Fig II-1. Genetic Diversity of PPV2



Legend of Fig I-1. The phylogenetic tree was constructed with 20 Thai PPV2s, 20 Japanese PPV2s and 46 reference strains deposited in GenBank. Thai PPV2 is indicated as closed triangle.

Fig II-2. Genetic diversity of PPV3



Legend of Fig II-2. The phylogenetic tree was constructed with the 20 Thai PPV3s and the 87 reference strains of other countries deposited in GenBank. Thai PPV3s are indicated as closed circles.

Chapter III

Coincidental detection of Porcine parvoviruses and porcine circovirus 2 persistently infecting pigs in Japan

Summary

The infection status of 14 viruses in 120 pigs was investigated based on tonsil specimens collected from a slaughterhouse. Only four species of porcine parvovirus (PPV) and porcine circovirus 2 (PCV2) were detected at high frequencies; 67% (80/120) for PPV (PPV-Kr or -NADL2 as the new abbreviation), 39% (47/120) for PPV3 (P-PARV4), 33% (32/120) for PPV4 (PPV4), 55% (66/120) for PBo-likeV (PBoV7) and 80% (96/120) for PCV2. A phylogenetic analysis of PPV3 suggested that the Japanese PPV3s showed a slight variation, and possibly, there were farms harboring homogeneous or heterogeneous PPV3s. Statistical analyses indicated that the detection of PCV2 was significantly coincidental with each detection of PPV, PPV2 and PPV4, and PPV and PPV4 were also coincidentally detected. The concurrent infection with PCV2 and porcine parvoviruses in the subclinically infected pigs may relate to the clinical manifestations of the PCV-associated disease for which the PCV2 infection is thought to be the primary cause.

Introduction

A number of new parvoviruses has been identified during the past 15 years and given various manes, and thereby the updated taxonomy of the family *Parvoviridae* was proposed in 2014 (Cotmore *et al.*, 2014). The classical porcine parvovirus (PPV), which was first identified in the 1960s (Cartwright & Huck, 1967) and now present worldwide, causes embryonic death, stillbirths and mummification when embryos or fetuses in seronegative dams are infected. The newly identified porcine parvoviruses have been detected in various areas of the world, but its relationship with any other diseases remains obscure. PPV is thought to be one of the cofactors for porcine circovirus associated disease (PCVAD) whose main etiologic agent is porcine circovirus 2 (PCV2) (Allan *et al.*, 1999) (Ellis *et al.*, 1999) (Kim *et al.*, 2003). PCV2 infection alone does not a cause clinical disease, but concurrent viral or bacterial infections may augment the severity of PCVAD possibly through stimulating the PCV2 replication or suppressing the PCV2 clearance by altered cytokine regulation (Darwich & Mateu, 2012; Ellis, 2014; Opriessnig & Halbur, 2012).

During our screening for known viral genomes and newly identified porcine parvovirus genomes in specimens of apparently healthy pigs collected in a slaughterhouse, we found that the genomes of PCV2 and the classical and new porcine parvoviruses were coincidentally detected. This chapter describes the infection status of the 5 porcine parvoviruses; PPV (Cartwright & Huck, 1967), PPV2 (Hijikata *et al.*, 2001), PPV3 (Lau *et al.*, 2008), PPV4 (Cheung *et al.*, 2010) and porcine bocavirus-like virus (PBo-likeV) (Blomstrom *et al.*, 2009). According to the updated proposal of the taxonomy of the family *Parvoviridae* published this year (Cotmore *et al.*, 2014), most of the virus names have been changed as indicated in Table III-1. However, we use the previous abbreviations in this paper to avoid confusion.

Materials and methods

Sample collection

Tonsil specimens of 120 healthy pigs were described in Chapter I.

Viral nucleic acid purification

The procedures of viral DNA/RNA purification and reverse-transcription were described in Chapter I.

Detection of viral genome by PCR

Two multiplex PCRs for 3 DNA viruses (porcine circovirus type 2 (PCV2), suid herpesvirus 1 and porcine parvovirus (PPV)) and 6 RNA viruses (porcine reproductive and respiratory syndrome virus (PRRSV), Japanese encephalitis virus, porcine rotavirus A (PoRV-A), porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV) and Getah virus) were performed in separate tubes according to the published method (Ogawa *et al.*, 2009). Other PCR primer pairs included; NP1200 and NP1529 for swine influenza virus (Lee *et al.*, 2001), HE5-1 and HE5-4m for hepatitis E virus (Takahashi *et al.*, 2001), Q1 F and Q2 R for porcine parvovirus 2 (Hijikata *et al.*, 2001), PPV3 F and PPV3 R for porcine parvovirus 3 (Streck *et al.*, 2013), PPV4 F and PPV4 R for porcine parvovirus 4 (Streck *et al.*, 2013), and SbocaF and SbocaR for PBo-likeV (Zhai *et al.*, 2010).

Viral genomes were amplified by PCR using Quick Taq HS DyeMix (Toyobo, Osaka, Japan) including Taq polymerase with primers specific for the viruses. The PCR consisted of an initial enzyme activation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec., annealing at 55°C for 30 sec., extension at 72°C for 30 sec. and a final extension at 72°C for 7 min.

Results

Prevalence of porcine parvoviruses and porcine circovirus 2 in 120 Japanese pigs.

We previously analyzed the prevalence of the PPV2 genomes in the tonsil specimens from 120 pigs (Saekhow *et al.*, 2014). With the same specimens, we extended such a screening for 14 other viral genomes listed in Table III-1. Five of the 14 viral genomes were detected; four were the members of the family *Parvoviridae* and another one was PCV2. The prevalences were 67% (80/120) for PPV, 39% (47/120) for PPV3, 33% (39/120) for PPV4, 55% (66/120) for PBo-likeV and 80% (96/120) for PCV2 (Table III-1), in addition to 58% for PPV2 (Saekhow *et al.*, 2014). Multiple viral genomes were detected from the individual pigs, and thereby 53 % of the pigs were positive for more than 3 of the 5 examined porcine parvoviruses. Among the 22 tested farms of which the sample number per farm ranged from 1 to 10, 7 of 8 farms with larger sample numbers (8 to 10 samples per farm) were positive for all five parvoviruses. The results suggested that a high proportion of these pigs were co-infected with the five parvoviruses and PCV2.

We tested the possibility that the detections of these highly prevalent viruses were random or coincidental. The chi square analyses indicated that PCV2 was coincidentally detected with PPV ($\chi 2=5.86$, $p<0.02$), PPV2 ($\chi 2=4.91$, $p<0.03$) or PPV3 ($\chi 2=4.23$, $p<0.04$) and that PPV and PPV4 were also coincidentally detected ($\chi 2=6.15$, $p<0.02$) (Table III-2).

Nucleotide sequence diversity of PPV3.

In order to know the genetic diversity of the Japanese PPV3s, a phylogenetic analysis based on the 622 bases of VP gene was performed using 20 Japanese samples, 5 samples each from 4 farms, and 87 reference sequences from around the world. The Japanese PPV3s were slightly diverged in the phylogenetic tree with 1.6% (10/622 bases) of the maximum nucleotide difference and closely related to other PPV3s detected in Europe, North America, South America and Hong Kong (Fig. III-1).

To characterize the variation in the nucleotide sequence among the farms or within a farm, the 20 Japanese PPV3 sequences were tentatively separated into 6 sequence groups based on the phylogenetic branch and % nucleotide difference (Figs. III-1 and -2, and Table III-3). The sequence group 1 was a major one to which 10 of the 20 sequences belonged. Farms C and D appeared homogeneous, having 4 and 5 sequences of the sequence group 1 respectively, while farms A and B had relatively heterogeneous PPV3 sequences. Farm A had the sequence groups 2 and 4 with a 1.3% nucleotide difference (8/622 bases), and farm B had the sequence groups 1, 3 and 5 with 0.6 - 1.4% nucleotide differences (4-9/622 bases) (Table III-3).

Discussion

The present study, together with our previous study (Saekhow *et al.*, 2014), examined the tonsil specimens of 120 apparently healthy pigs for the screening of 15 viruses which can infect pigs. Only the five porcine parvoviruses, i.e., PPV, PPV2, PPV3, PPV4 and PBo-likeV, and PCV2 were detected, and their prevalences were quite high, ranging from 33% to 80% (Table III-1) (Saekhow *et al.*, 2014). The high prevalences of the classical PPV and PCV2 at the age of about 6 months are common as in most pig-producing countries, whereas this paper is the first report characterizing the prevalences of PPV3, PPV4 and PBo-likeV in Japanese pigs.

The PPV3 DNA was detected in 39% of the 120 Japanese pigs (Table III-1). Since the first report (Lau *et al.*, 2008), the prevalence has been reported in several countries and appears to widely vary from lower frequencies (6-20%) like Hungary (Csagola *et al.*, 2012), China (Pan *et al.*, 2012), the USA (Xiao *et al.*, 2012) and Germany (Streck *et al.*, 2013) to higher frequencies (44-73%) like Hong Kong (Lau *et al.*, 2008), China (Li *et al.*, 2013) and Thailand (Saekhow *et al.*, 2014). The Asian countries may have slightly higher prevalences, although the reason is unknown.

The phylogenetic analysis of PPV3 suggested that, compared to the variation of 87 sequences deposited from around the world, the 20 Japanese

sequences showed less variation and belonged to limited branches (Fig. III-1). In the 4 farms we analyzed, 2 farms appeared to have heterogeneous PPV3s (Table III-3 and Fig. III-3). Although the variations within the farms were not high, this raises the possibility that the observed variation within a farm resulted from multiple invasions of different strains rather than natural mutations within a farm after the invasion of a strain. If so, multiple infection of a pig might be possible when viewed from an epidemiological standpoint.

The prevalence of the PPV4 genome was 33% in this study which is comparable to the prevalence (44%) in Thailand (Saekhow *et al.*, 2014), but rather higher than those of several other countries, that is, 1% in China (Huang *et al.*, 2010), 6% in Hungary (Csagola *et al.*, 2012), 7% in Germany (Streck *et al.*, 2013) and 4% in the USA (Xiao *et al.*, 2013b). The reasons for the difference are unknown.

PBo-likeV (Blomstrom *et al.*, 2009), which was also called PBoV (PBoV-SX) (Zeng *et al.*, 2011) or PBoV1 (Zhang *et al.*, 2011) (Shan *et al.*, 2011), is one of several porcine bocaviruses which have been recently discovered (Xiao *et al.*, 2013c). The prevalence of PBo-likeV was 55% in our study (Table III-1), in contrast to 18% in Thailand (Saekhow *et al.*, 2014), 7% in China (Zhai *et al.*, 2010), 63% in different areas of China (Shan *et al.*, 2011), 2% in Romania (Csagola *et al.*, 2012) and 13% in the wild boars of Romania (Cadar *et al.*, 2011a). The PBo-likeV infection was supposed to be associated with respiratory tract diseases in pigs due to the remarkable difference in the prevalences between sick (39% (74/191)) and healthy (7% (3/41)) pigs (Zhai *et al.*, 2010).

The PCV2 genome was detected at a high frequency (80%) (Table III-1) which is common among the pig-producing countries. Interestingly, PCV2 was coincidentally detected along with PPV, PPV2 or PPV3, and they were weak but statistically significant associations (Table III-2). PCV2 is recognized as a causative agent of PCVAD. The clinical features of PCVAD or formerly named postweaning multisystemic wasting syndrome (PMWS)

caused by PCV2 are systemic including enlargement of the lymph nodes, progressive loss of body weight or wasting combined with difficulty in breathing, diarrhea, pale skin, and jaundice (Opriessnig & Langohr, 2013) (Ellis, 2014). The histopathologic changes in the affected lymphoid tissues are a severe lymphoid depletion, a diffuse infiltration of histolytic cells and various inflammatory lesions. The pathogenesis of PCVAD or PCV2-induced diseases is complex, probably involving PCV2 infection and cofactors such as other infections and altered cytokine or immune responses (Darwich & Mateu, 2012). Particularly, concurrent infection of PCV2-infected pigs by viruses (PPV, PRRSV, Torque teno sus virus, etc.), bacteria (*Mycoplasma hyopneumoniae, Lawsonia intracellularis* and *Salmonella spp*) or parasites may not be only a secondary infection after PCV2-induced depletion of lymphocytes but could be important for the disease manifestation (Opriessnig & Halbur, 2012). The experimental inoculation with PCV2 and PPV, but not PCV2 alone, could reproduce lesions similar to those of the field cases of PMWS (Allan *et al.*, 1999) (Ellis *et al.*, 1999) (Kim *et al.*, 2003). The mechanism for the synergetic effect of coinfection was proposed that the coinfection may promote the PCV2 infection by stimulating immune cells and providing target cells for PCV2 replication or suppressing PCV2 clearance by alteration of the cytokine production and profiles (Allan *et al.*, 1999) (Opriessnig & Halbur, 2012).

Our study demonstrated that, even in the field samples which were taken from apparently healthy pigs at a slaughterhouse, PCV2 was concurrently detectable along with PPV, PPV2 and PPV3, in addition to the concurrent detection of PPV and PPV4 (Table III-2). PCV2 and parvoviruses are both DNA viruses that require actively dividing cells in order to replicate. Therefore, the mechanisms proposed for the enhanced PCV2 replication leading to severe PCVAD (Allan *et al.*, 1999) (Opriessnig & Halbur, 2012) could be also applied to the subclinical pigs which are co-infected by PCV2 and various parvoviruses.

Table III-1. Prevalence of 15 virus genomes in the 120 tonsil specimens of pigs.

Virus	Abbreviation	Prevalence
porcine parvovirus (porcine parvovirus)*	PPV	67% (80/120)
porcine parvovirus 2 (porcine Cn virus)*	PPV2 (CnP-PARV4)*	58% (69/120)
porcine parvovirus 3 (porcine hokovirus)*	PPV3 (P-PARV4)*	39% (47/120)
porcine parvovirus 4 (porcine parvovirus 4)*	PPV4 (PPV4)*	33% (39/120)
porcine boca-like virus (porcine bocavirus 7)*	PBo-likeV (PBoV7)*	55% (66/120)
porcine circovirus 2	PCV2	80% (96/120)
suid herpesvirus 1	SuHV1	0% (0/120)
hepatitis E virus	HEV	0% (0/120)
swine influenza virus	SIV	0% (0/120)
porcine reproductive and respiratory syndrome virus	PRRSV	0% (0/120)
Japanese encephalitis virus	JEV	0% (0/120)
porcine epidemic diarrhea virus	PEDV	0% (0/120)
porcine rotavirus A	PoRV - A	0% (0/120)
transmissible gastroenteritis virus	TGEV	0% (0/120)
Getah virus	GETV	0% (0/120)

* New names recently proposed for the family Parvoviridae (Cotmore *et al.*, 2014).

The prevalence of PPV2 was previously described (Saekhow *et al.*, 2014), but for convenience, the data was included in this table.

Table III-2. Coincidental isolation among 4 parvoviruses and PCV2.

Relationship between two viruses		Number of pigs				$\chi 2$ value	P value	Significance
		+/+	-/+	+/-	-/-			
PPV /	PPV2	47	22	33	18	0.153	0.695	
	PPV3	33	14	47	26	0.437	0.508	
	PPV4	32	7	48	33	6.154	0.013	*
	PBo-likeV	46	20	34	20	0.606	0.436	
	PCV2	69	27	11	13	5.859	0.015	*
PPV2 /	PPV3	28	19	41	32	0.136	0.712	
	PPV4	25	14	44	37	1.031	0.310	
	PBo-likeV	39	27	30	24	0.152	0.697	
	PCV2	60	36	9	15	4.910	0.027	*
PPV3 /	PPV4	15	24	32	49	0.012	0.913	
	PBo-likeV	28	38	19	35	0.653	0.419	
	PCV2	42	54	5	19	4.232	0.040	*
PPV4 /	PBo-likeV	26	40	13	41	3.177	0.075	
	PCV2	32	64	7	17	0.152	0.697	
PBo-likeV	PCV2	53	43	13	11	0.008	0.927	

a, *: significant ($0.05>p>0.01$). Others without asterisk mean not significant ($p>0.05$).

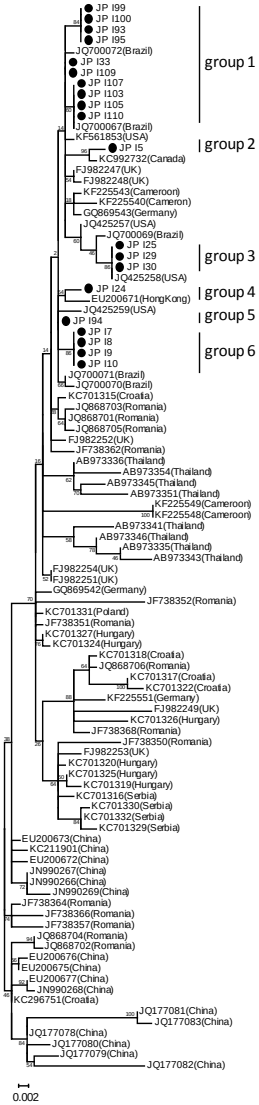
Table III-3. Nucleotide sequence diversity of PPV3 within 4 farms.

	"sequence group"						Nucleotide difference
Farm	1	2	3	4	5	6	
A		JP I5				JPI7 JPI8 JPI9 JPI10	Sequence groups 2 vs 4: 1.3% (8/622)
B	JP I33		JP I25 JP I29 JP I30	JP I24			Sequence groups 1 vs 3: 0.8% (5/622) Sequence groups 1 vs 4: 0.6% (4/622) Sequence groups 3 vs 4: 1.4% (9/622)
C	JP I93 JP I95 JP I99 JP I100				JP I94		Sequence groups 1 vs 5: 0.5% (3/622)
D	JP I103 JP I105 JP I107 JP I110 (JPI 109)*						

PCR products were directly sequenced and the sequence data of the 622 bases of the PPV3 VP gene were subjected to a phylogenetic analysis. The “sequence group” in this analysis was defined by the phylogenetic branch (Fig. III-1) and % nucleotide difference.

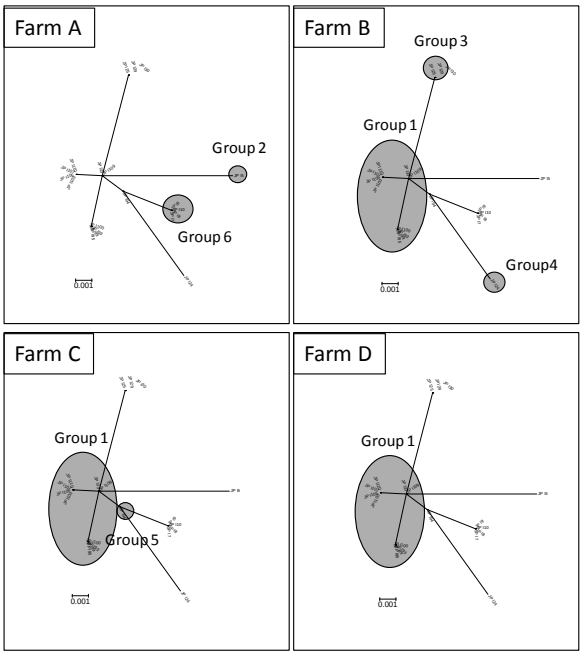
*, the JPI 109 sequence was slightly different (0.2% (1/622)) from the other 4 sequences at the same farm.

Fig. III-1. Phylogenetic tree of PPV3s including 20 Japanese sequences.



The phylogenetic tree was constructed, based on the 622 bases of the PPV3 VP gene, with the 20 Japanese PPV3s and 87 PPV3s currently deposited in the data bank. For the Japanese sequences, the 6 tentative sequence groups (Sequence groups 1-6) were defined by phylogenetic branch and % nucleotide difference, i.e., <0.5% (3/622) within each sequence group. The relationship between the farm and the sequence group of the detected PPV3 sequences is indicated in Table III-3.

Fig. III-2. Phylogenetic tree of 20 PPV3 Japanese sequences and phylogenetic sequence groups of the PPV3 sequences detected from the 4 farms.



The phylogenetic tree was constructed with the 20 Japanese PPV3s detected from the 4 farms based on the 622 bases of the PPV3 VP gene. The 6 sequence groups were tentatively defined by the phylogenetic branch and % nucleotide difference, i.e., <0.5% (3/622) within each sequence group. The 6 sequence groups detected from the 4 pig farms are indicated. The relationship among the sequence data, the sequence group and the farm is indicated in Table III-3.

General Summary

I have been studying porcine parvoviruses during the PhD course. The initially identified parvovirus in pigs is porcine parvoviruses (PPV), which was reported in the 1960s and is now endemic in most pig-producing countries. PPV mainly causes reproductive failure in naïve dams, and manifestation characterized by stillbirth, mummification, embryonic death and infertility. In contrast, PPV infection of adult pig causes only a subclinical or mild disease.

Besides the classical PPV, several other parvoviruses or their genomes have been recently identified in pigs. These newly identified porcine parvoviruses have not been well characterized. I therefore investigated several porcine parvoviruses infecting pig herds of Japan and Thailand by isolating and characterizing viral DNAs.

These parvoviruses appear to share common biological properties such as resistance to antiseptic substances and extreme environmental conditions like heat and pH. They also have common biological properties in their DNA replication; they require the mitotic S phase of cells to replicate their DNA genome. These properties are related both to difficulty in eradicating the PPV-associated diseases at the pig farms and to its pathogenesis.

In the chapter I, I describe my study of the characterization of porcine parvovirus 2 (PPV2) detected in Japanese pig herds. PPV2 genome was first detected in Myanmar in 2001. The genome was subsequently reported from several countries. The prevalence of Japan was 58% in healthy pigs. I cloned a near complete genome of PPV2 from a healthy pig. I sequenced a region of 41 PPV2s and compared them with those of other countries using the phylogenetic analysis. The analysis showed that diverged PPV2 strains exist in Japan and 7 of the 10 pig farms carried clearly distinct strains of PPV2. Circulating multiple strains within a farm may be a risk for generating emerging virus as reported in other parvoviruses.

In the chapter II, I describe my study of the five newly identified porcine parvoviruses detected in the Chiangmai area of Thailand. I examined the 80

tonsil samples, and the prevalence of the five porcine parvoviruses (PPV、PPV2、PPV3、PPV4、PBo-likeV) were 23～73%. The phylogenetic analyses for PPV2 and PPV3 indicated the existence of two and one clade(s) of viruses, respectively, suggesting an invasion from a limited source for each virus.

In the chapter III, I describe the characterization of the infection status of the four porcine parvoviruses (PPV, PPV3, PPV4, and PBo-likeV) and PCV2 which is a causative agent of PCVAD. The prevalences of these viruses in 120 apparently healthy pigs aged about 6 months were 33～80%, and the detection of PCV2 was significantly coincidental with each detection of PPV, PPV2 and PPV3, and PPV and PPV4 were also coincidentally detected. The exact reason for the coincidental detection remains unknown, but the coinfection we observed in the field samples of the subclinical pigs may relate to the mechanisms leading to severe PCVAD in which the coinfection with PCV2 and other viral and bacterial agents may promote the PCV2 infection by stimulating immune cells and providing target cells for PCV2 replication or suppressing PCV2 clearance by alteration of the cytokine production and profiles.

Finally, this study shows the infection status of newly identified porcine parvoviruses in pig herds of Japan and Thailand. Since these viruses are not known regarding the association with any disease, our investigation will provide useful information for further studies.

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