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ความชุกและความหลากหลายทางพันธุกรรมของเชื้อ Torque Teno Sus Viruses 1 และ 2 จากสุกรในเขตจังหวัดเชียงใหม่และลำพูน ประเทศไทย

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บทคัดย่อ: เชื้อ Torque teno sus viruses 1 และ 2 (TTSuV1 และ 2) เป็นเชื้อไวรัสในแฟมิลี่ *Anelloviridae* พบรายงานการติดเชื้อในสุกรแต่ยังไม่ทราบพยาธิกำเนิดของโรคอย่างแน่ชัด อย่างไรก็ตามมีรายงานการศึกษาถึงความชุกและความหลากหลายทางพันธุกรรมของเชื้อไวรัสดังกล่าวได้ทั่วโลก ซึ่งเชื้อไวรัส TTSuV1 และ 2 ยังไม่เคยมีรายงานจากสุกรในเขตจังหวัดเชียงใหม่และลำพูน ดังนั้นวัตถุประสงค์ของการศึกษาในครั้งนี้เป็นการตรวจหาความชุกและความหลากหลายทางพันธุกรรมของเชื้อ TTSuV1 และ 2 จากทอนซินของสุกรที่มีสุขภาพปกติจำนวน 100 ตัวอย่างในจังหวัดเชียงใหม่และลำพูนด้วยวิธีปฏิกิริยาลูกโซ่โพลีเมอเรส ผลการตรวจพบว่าความชุกของ TTSuV1, TTSuV2, และการติดเชื้อ TTSuV1 และ 2 สองชนิดร่วมกันเป็นดังนี้ 62% (62/100), 68% (68/100), และ 50% (50/100) ตามลำดับ การตรวจวิเคราะห์ความหลากหลายทางพันธุกรรมด้วยการใช้ความสัมพันธ์เชิงวิวัฒนาการบริเวณ 5' untranslated regions จาก 19 ลำดับนิวคลีโอไทด์ของ TTSuV1 และ 2 ผลการวิเคราะห์พบว่าเชื้อไวรัสทั้งสองชนิดสามารถตรวจพบได้เป็น 2 กลุ่มกล่าวคือ กลุ่มแรกพบว่าไม่มีความสัมพันธ์กับบริเวณที่ศึกษาเนื่องจากมีความแตกต่างของลำดับนิวคลีโอไทด์ของเชื้อไวรัสเป็นจำนวนมาก (ความเหมือนของลำดับนิวคลีโอไทด์จาก TTSuV1 เป็นร้อยละ 85 และความเหมือนของลำดับนิวคลีโอไทด์จาก TTSuV2 เป็นร้อยละ 87) กลุ่มที่สองพบว่าไม่พบความแตกต่างของลำดับนิวคลีโอไทด์ของเชื้อไวรัสที่ได้ทำการศึกษาในครั้งนี้ จากผลการศึกษาในครั้งนั้นแสดงให้เห็นว่าเชื้อไวรัสทั้งสองชนิดอาจพบว่ามีการปรับตัวภายในเขตจังหวัดเชียงใหม่และลำพูน

คำสำคัญ: ความหลากหลายทางพันธุกรรม เชื้อ Torque teno sus viruses *Anelloviridae* สุกร

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Prevalence and Genetic Diversity of Torque Teno Sus Viruses 1 and 2 in Pigs in Chiang Mai and Lamphun, Thailand

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Abstract: Torque teno sus viruses 1 and 2 (TTSuV1 and 2) belong to the family *Anelloviridae,* which is known to commonly infect pigs; however, their pathogenesis is unclear. Studies of TTSuV1 and TTSuV2 in pigs worldwide, especially with regard to their prevalence and genetic diversity, have been conducted; however, the prevalence and genetic diversity of both viruses have not been studied in Chiang Mai and Lamphun, Thailand. Therefore, this study aimed to investigate both viruses in terms of their prevalence and genetic diversity using polymerase chain reaction with specific primers in the tonsils of 100 apparently clinically healthy pigs that are being raised in this region. Overall, the prevalence rates of TTSuV1, TTSuV2, and both in combination, in the pigs were 62% (62/100), 68% (68/100), and 50% (50/100), respectively. Phylogenetic analysis of 5' untranslated regions of 19 nucleotide sequences of both viruses was conducted. Both viruses were separated into 2 groups. The first group displayed a lack of geographic clustering because the nucleotide sequences in these groups showed extreme differences (TTSuV1 minimum nucleotide sequence identity was recorded at 87% and TTSuV1 minimum nucleotide sequence identity was recorded at 85%). The second group revealed the identical nucleotide sequences as no differences were observed in terms of the nucleotide sequences among the specimens. Our data indicate that the nucleotide sequences of TTSuV1s and s that were detected in Chiang Mai and Lamphun, Thailand, likely were adapted from their genome.

Keywords: Genetic diversity, Torque teno sus viruses, *Anelloviridae*, Pigs

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Introduction

Torque teno sus viruses include two distinct species, namely TTSuV1 and TTSuV2. TTSuV1 is a member of the genus *Alphatorquevirus*, whereas TTSuV2 is a member of the *genus Kappatorquevirus;* both of these genera belong to the family *Anelloviridae* (Hino and Miyata, 2007; Taira et al., 2009). Torque teno virus (TTV) was first discovered in a patient suffering from hepatitis. Since that time, it has been reported in many species of mammals. It has not only been identified in domestic animals, such as pigs, dogs, cats, cattle, and sheep, but also in wildlife, including wild boars, tupaias, sea lions, and primates (Meng, 2012; Okamoto, 2009). The genome of anellovirus is negative sense and single-stranded DNA with a genome size ranging from 2 to 3.9 kb (Spandole et al., 2015). This viral genome consists of at least three regions: the 5′ untranslated region (5′UTR), open reading frame 1 (ORF1), and open reading frame 2 (ORF2) (Ninomiya et al., 2007). Although a transcription profile of TTV showed that it has at least three mRNAs, the mechanism of viral replication is unclear (Kamahora et al., 2000). The prominent 5′UTR is composed of a conserved region and a region exhibiting variations that allowing for identification of the viral nucleotide sequences of TTSuV1 and TTSuV2 (Kekarainen et al., 2006; Taira et al., 2009; Zhai et al., 2013).

Although the pathogenic significance of these viruses is currently unclear, TTSuV1 and TTSuV2 genomes have been detected in pigs that have been diagnosed with clinical diseases, namely, porcine circovirus associated disease (Kekarainen et al., 2006; Taira et al., 2009) and respiratory disease (Gimenez-Lirola et al., 2014; Lee et al., 2015). However, TTSuV1 and TTSuV2 genomes have also been detected in apparently clinically healthy pigs. Moreover, TTSuV1 and TTSuV2 have been detected in many tissues and samples, including the semen, lungs, kidneys, liver, intestine, bone marrow, lymph nodes, and tonsils of aborted fetuses (Bigarre et al., 2005; Kekarainen et al., 2007; Lee et al., 2010; Martelli et al., 2006; Novosel et al., 2012; Taira et al., 2009). The total prevalence rates of TTSuV1 and TTSuV2 in pigs have been reported at multiple levels, including 52.2% in the USA (Xiao et al., 2012), 46.0% in Canada (McKeown et al., 2004), 93.0% in France (Bigarre et al., 2005), 40.1% in Italy (Martelli et al., 2006), 97.0% in Spain (Kekarainen et al., 2006), 85.0% in South Korea (Lee et al., 2010), 78.9% in China (Zhai et al., 2013), and 40.0% in Thailand (McKeown et al., 2004). The genetic diversity, especially of the 5′UTR of both viruses, revealed heterogeneity and a lack of geographical clustering (McKeown et

al., 2004). Two previous studies on Thai TTSuV1s and TTSuV2s (Cortey et al., 2012; McKeown et al., 2004) reported a lack of geographic clustering. However, information on both viruses has been insufficient in allowing us to understand the genetic diversity of the TTSuV1 and TTSuV2 viruses that are circulating in Chiang Mai and Lamphun, Thailand. Therefore, this study was established to investigate the prevalence of the viruses, and to characterize the 5′ untranslated region of TTSuV1 and TTSuV2 in pigs located in Chiang Mai and Lamphun Provinces.

Materials and Methods

Sample collection

Tonsil samples were obtained from 100 apparently cadaveric pigs at approximately 6 months of age from animals raised in Chiang Mai and Lamphun provinces. The status of all pigs in this study were apparently clinically healthy pigs. Sample collection was performed in December, 2016. All tonsil samples were kept at −20 °C until being used.

Detection of TTSuV1 and TTSuV2 genomes by polymerase chain reaction (PCR)

Viral DNA extraction

Tonsil samples were homogenized in 0.9% normal saline using zirconia beads and a Mini-Beadbeater1 (Bipspec, Bartlesville, OK, USA). The samples were then centrifuged at 1,500 rpm for 5 min. These supernatant homogenized tonsils were used for DNA extraction by NucleoSpin® Tissue (Macherey-Nagel, GmbH, Duren, Germany), according to the manufacturer's instructions.

Detection of TTSuV1 and TTSuV2 genomes by PCR

To detect the prevalence of TTSuV1 and TTSuV2, PCR was carried out in separate reactions with each pair of primers. The target of the primers in this study were 5' UTR of both viruses (Kekarainen et al., 2006). The pair of primers in the PCR for TTSuV1 was TTSuV1-F 5′ TACACTTCCGGGTTCAGGAGGCT 3′ and TTSuV1-R 5′ ACTCAGCCATTCGGAACCTCAC 3′, whereas the pair of primers in the PCR for TTsuV2 was TTSuV2-F 5′ AGTTACACATAACCA CCAAACC 3′ and TTSuV2-R 5′ ATTACCGCCTGC CCGATAGGC 3′. Quick Taq HS DyeMix (Toyobo, Osaka, Japan) was performed for PCR amplification with specific primers. The PCR was performed with an initial step of 94 °C for 4 min; followed by 35 cycles of 94 °C for 1 min, 58 °C for 30 s, and 72 °C for 30 s; and a final step of 72 °C for 7 min.

Nucleotide sequencing and phylogenetic analysis

The phylogenetic analyses of TTSuV1 and TTSuV2 were carried out by directly sequencing the PCR products of the viral 5' UTR regions. Sequencing primers, for TTSuV1, the 310 bp DNA of the 5' UTR used with the

primer pairs, TTSuV1-F 5′ TACACTTCCGGGTTC AGGAGGCT 3′ and TTSuV1-R 5′ ACTCAGCCA TTCGGAACCTCAC 3′, while for TTSuV2, the 210 bp DNA of the 5' UTR was used with the primer pairs, TTSuV2-F 5′ AGTTACACATAACCA CCAAACC 3′ and TTSuV2-R 5′ ATTACCGCCTGC CCGATAGGC 3′.

All PCR amplicons were amplified with specific primers used in the genome detection and were purified using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel). To investigate the nucleotide sequences, all purified amplicons were sent to the DNA sequencing service provider 1st Base (Selangor, Darul Ehsan, Malaysia).

Nineteen of neucleotide sequences of TTSuV1 and TTSuV2 were randomly chosen to construct phylogenetic trees. The maximum likelihood method with 1000 bootstraps implemented in MEGA X was performed to draw the phylogenetic trees. (Kumar et al., 2018).

Results

Prevalence of TTSuV1 and TTSuV2

In our study, we examined the prevalence of TTSuV1 and TTSuV2 that were obtained from 100 tonsils of approximately 6-month-old pigs collected from a slaughterhouse by applying PCR using primers for TTSuV1 and TTSuV2. Overall, the prevalence rate of TTSuV1 was recorded at 62% (62/100) and that of TTSuV2 was recorded at 68% (68/100). Moreover, the prevalence of co-infection of TTSuV1 and TTSuV2 was 50% (50/100). The prevalence of only TTSuV1 was 12% (12/100), and the prevalence of only TTSuV2 was 18% (18/100). The data indicates that TTSuV1 and TTSuV2 have spread in Chiang Mai and Lamphun, and the prevalence of co-infection with both viruses is higher than that for either TTsuV1 or TTsuV2 alone.

Genetic diversity of TTSuV1 and TTSuV2

The genetic diversity of the nucleotide sequences of TTSuV1 was determined by phylogenetic analysis based on the partial nucleotide sequence of the 5′UTR obtained from 19 Thai TTSuV1s and 63 nucleotide sequences that had been deposited in a databank. The 20 nucleotide sequences of Thai TTSuV1s were allocated into 8 phylogenetic clusters (Fig. 1 and Table 1). The Thai TTSuV1s were genetically diverse, as is shown in the phylogenetic tree, with 87% minimum nucleotide sequence identity. Two groups were clustered together in terms of nucleotide sequence identity when the nucleotide sequence identity was compared with the sequence group of the Thai TTSuV1, and the reference sequence was recorded at 98%. The first group was labeled as group 1 and was made up of Chinese TTSuV1.

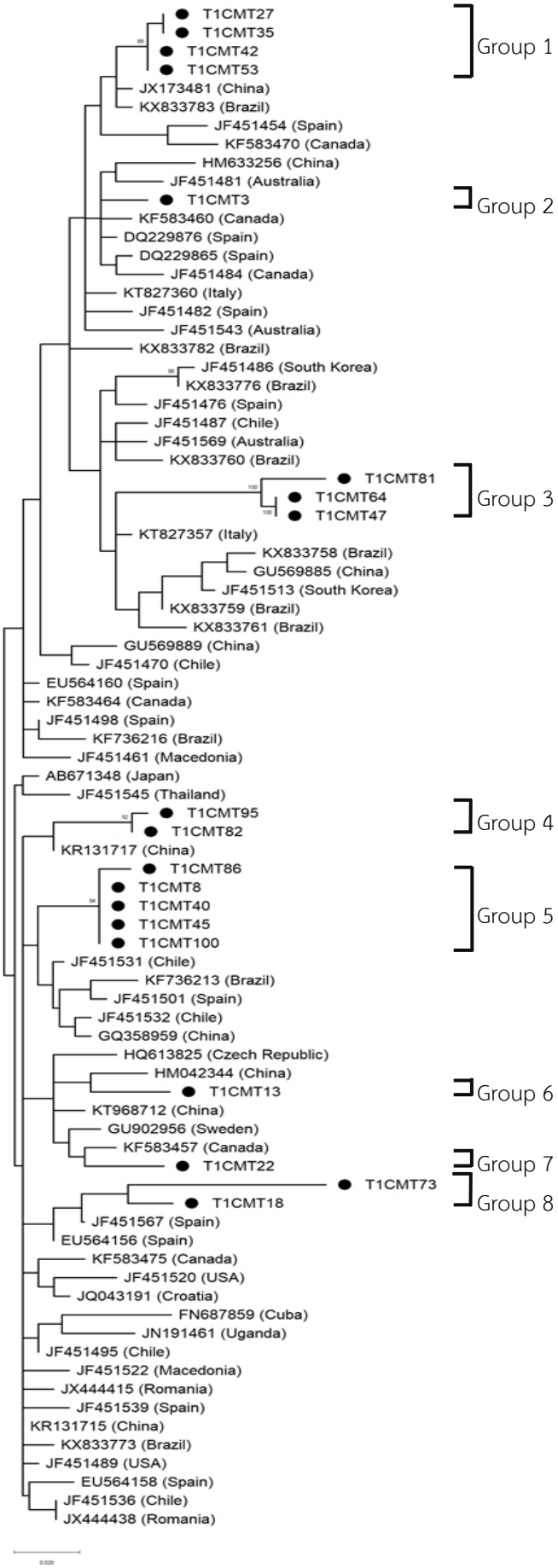

Figure 1 Genetic diversity of Thai TTSuV1s. The phylogenetic tree was created using 310 bp of 19 Thai TTSuV1s with 63 nucleotide sequences deposited in a databank. Provisionally, the 7-nucleotide sequence group was defined by the phylogenetic branch and the % nucleotide sequence identity. The maximum likelihood method with the bootstrap value from 1000 replicates was applied using Mega X software. In this figure, Thai TTSuV1s are represented by black circles.

Table 1 Sequence group of Thai TTSuV1s and sequence identity with reference sequences.

Sequence groups	Thai TTSuV1s	Reference sequence	Maximum nucleotide sequence identity (%) *
1	T1CMT27, T1CMT35, T1CMT42, T1CMT53	JX734481 (China)	98%
2	T1CMT3	DQ229876 (Spain)	98%
3	T1CMT81, T1CMT64, T1CMT47	KT827357 (Italy)	95%
4	T1CMT95, T1CMT82	KR131717 (China)	97%
5	T1CMT86, T1CMT8, T1CMT40, T1CMT45, T1CMT100	JF451531 (Chile)	97%
6	T1CMT13	HM042344 (China)	95%
7	T1CMT22	KF583457 (Canada)	96%
8	T1CMT73, T1CMT18	JF451567 (Spain)	93%

*Maximum nucleotide sequence identity was defined by the phylogenetic branch (Fig. 1) and the % nucleotide sequence identity.

Sequence group 2 was made up of Thai TTSuV1 and Spanish TTSuV1. In addition, the minimum sequence identity between the sequence group of group 8 and the Spanish TTSuV1 was recorded at 93%.

With regard to TTSuV2, 19 Thai TTSuV2s and 56 nucleotide sequences of the partial nucleotide sequences of the 5′UTR that were deposited in a databank were used to investigate genetic diversity. Of these, the Thai TTSuV2s displayed either high or low genetic diversity as is demonstrated in the phylogenetic tree, with 85% minimum nucleotide sequence identity. There were 11 phylogenetic clusters (Fig. 2 and Table 2) on the phylogenetic tree. The terms of nucleotide sequence identity were the same as the terms of those that were mentioned above. There were two clustered groups, including that which was closest in nucleotide sequence identity when comparing the nucleotide sequence identity between the sequence group of the Thai TTSuV2 and the reference sequence which the sequence identity was recorded at 100%. Moreover, the minimum sequence identity between the sequence group of 11 and the Spanish TTSuV2 was recorded at 91%.

Discussion

TTSuV1 and TTSuV2 are viruses that have been reported in both domestic pigs and wild boars. To understand the distribution of TTSuV1 and TTSuV2 among

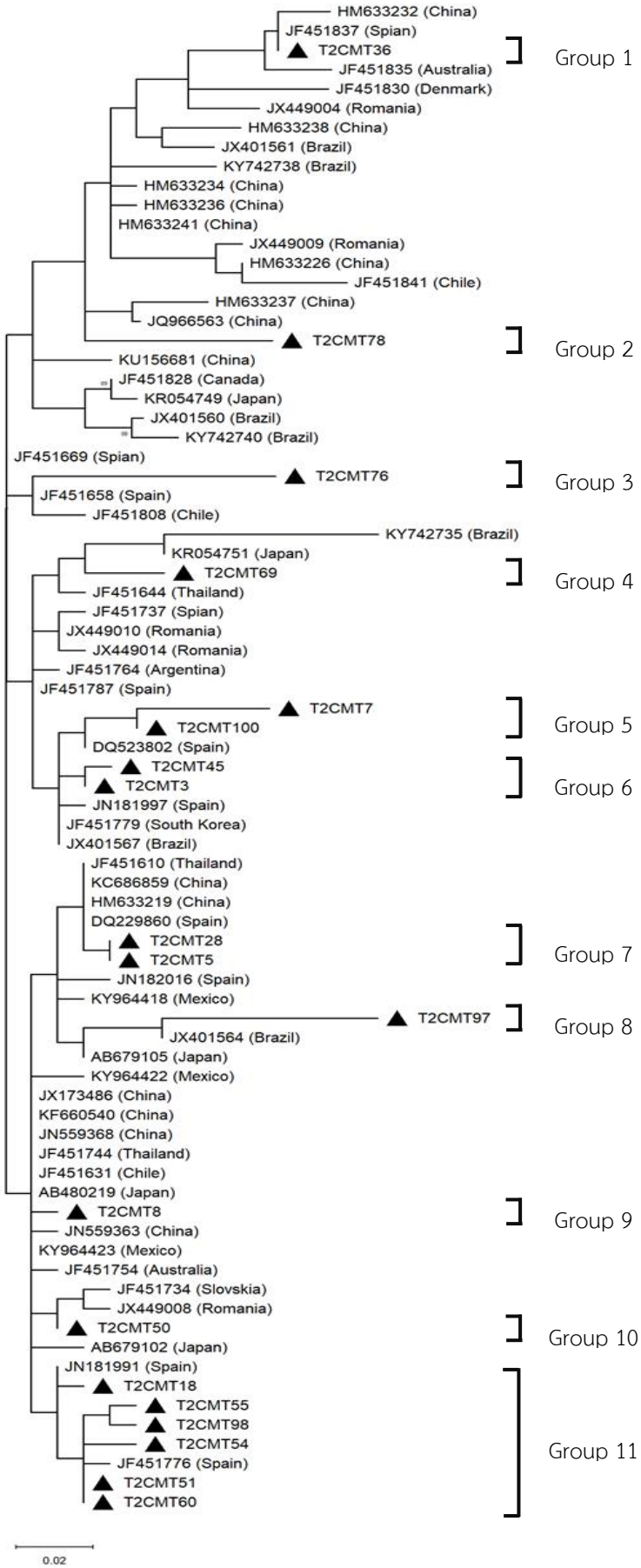

Figure 2. Genetic diversity of Thai TTSuV2s. Tentatively, the 11-nucleotide sequence group is based on 210 bp of 19 Thai TTSuV2s with 56 nucleotide sequences that were deposited in a databank and used to draw the phylogenetic tree. The method for construction of the phylogenetic tree and cluster are described in Figure 1. Thai TTSuV2s are represented by black triangles.

Table 2 Sequence group of Thai TTSuV2s and the sequence identity with reference sequences.

Sequence group	Thai TTSuV2s	Reference	Maximum nucleotide sequence identity (%)
1	T2CMT36	JF451837 (Spain)	100
2	T2CMT78	JQ966563 (China)	94
3	T2CMT76	JF451658 (Spain)	95
4	T2CMT69	KR054751 (Japan)	94
5	T2CMT7, T2CMT100	DQ523802 (Spain)	94
6	T2CMT3, T2CMT45	JN181997 (Spain)	98
7	T2CMT5, T2CMT28	JF451610 (Thailand)	100
8	T2CMT97	JX401564 (Brazil)	94
9	T2CMT8	AB480219 (Japan)	99
10	T2CMT50	JX449008 (Romania)	99
11	T2CMT18, T2CMT55, T2CMT98, T2CMT54, T2CMT51, T2CMT60	JN181991 (Spain)	91

*Maximum nucleotide sequence identity was defined by the phylogenetic branch (Fig. 2) and the % nucleotide sequence identity.

pigs in Chiang Mai and Lamphun, Thailand, we examined the prevalence of both viruses by PCR using species-specific primers and characterized the genetic diversity of their 5′UTR. In this study, the prevalence rates of TSuV1, TTsuV2, and their combined incidences of infection, were 62% (62/100), 68% (68/100), and 50% (50/100), respectively. The higher prevalence of TTSuV2 compared with that of TTSuV1 infection corresponds with previous reports from Italy (Martelli et al., 2006) and Spain (Kekarainen et al., 2006), among others. However, it contrasts with studies from China (Zhai et al., 2013) and Uruguay (Ramos et al., 2018), where the opposite trend was observed. Variations in prevalence was also reported among different geographic regions.

Because the mode of transmission of these viruses is unclear, especially with regard to viral shedding, there have been considerable variations in the reported prevalence. Specifically, the samples used as sources of the viral genome have varied among studies, and this includes samples obtained from serum, semen, lungs, kidneys, liver, intestine, bone marrow, and lymph nodes (Bigarre et al., 2005; Kekarainen et al.,

2007; Lee et al., 2010; Martelli et al., 2006; Novosel et al., 2012). In our study, we detected viral genomes in 6-month-old pig tonsils; our data suggests that the viral replication process in tonsils might involve the shedding of virions via oral and nasal secretions. In addition, viral genomes have been detected in the tonsils of stillborn piglets (Taira et al., 2009), and the pattern of viral infection is thought to be persistent (Aramouni et al., 2010). Moreover, the viral genome can be detected in nasal and fecal samples of 1-week-old piglets, and the prevalence in older pigs (>60 days) is higher than in younger ones (<60 days) (Zhai et al., 2013). As a result, the probable reasons for the presence of TTSuV1 and TTSuV2 in 6-month-old pigs were likely the direct contact that occurs in pens, which can promote transference via nasal secretions or feces and persistent infection.

In an attempt to understand the genetic diversity of TTSuV1 and TTSuV2, we have characterized the nucleotide sequence of the 5′UTR by phylogenetic analysis. The 5′UTR generally comprises a highly conserved sequence that viruses use as both promoter and enhancer elements in viral replications. However, the 5′UTR can also display high genetic diversity, making it useful for molecular epidemiological studies (Kekarainen et al., 2006; Taira et al., 2009; Zhai et al., 2013). In this study, we classified the sequence groups of Thai TTSuV1s and TTSuV2s in order to understand the genetic diversity in these taxa. Previous studies of 5′UTR of TTSuV1 and TTSuV2 have demonstrated a lack of geographic clustering, with the nucleotide sequences of TTSuV1 and TTSuV2 in the same area exhibiting great differences. Our data obtained from the phylogenetic analysis of the 5′UTR of Thai TTSuV1s indicated that they could be classified into 8 groups (Fig. 1 and Table 1). Of these, the Thai TTSuV1 sequence groups 2, 4, 6, 7, and 8 revealed a lack of geographic clustering because the nucleotide sequences in these groups displayed great differences. However, the Thai TTSuV1 sequence groups 1, 3, and 5 showed the identical nucleotide sequence, which was inconsistent with the previous study. In contrast, the phylogenetic analysis based on the 5′UTR of Thai TTSuV2s enabled them to be classified into 11 groups **(**Fig**.** 2 and Table 2**).** Of these, the 9 Thai TTSuV2 sequence groups, including groups 1–6 and 8–10, corresponded to a lack of geographic clustering, while the Thai TTSuV2 groups 7 and 11 revealed an identical nucleotide sequence that was also inconsistent with the findings of previous studies. TTSuV1 and TTSuV2 might have originated elsewhere and have arrived due to intercontinental transportation, which could

explain the lack of geographic clustering, which is in contrast with previous studies (Cortey et al., 2012; McKeown et al., 2004). Our results also revealed an identical nucleotide sequence of TTSuV1 and TTSuV2. This could have been an adaptation of the virus in an epidemic area via mutation and natural selection, which may have led to the domination of this nucleotide sequence in this area. Notably, this finding has been reported elsewhere with regard to the ungulate circulating strains of tetraparvovirus3 in Chiang Mai (Saekhow and Ikeda, 2015).

This is a preliminary study on the prevalence and genetic diversity of TTSuV1s and TTSuV2s in the Chiang Mai and Lamphun areas of Thailand. The pathogenesis of TTSuV1s and TTSuV2s is not well understood, especially in terms of the role of the pathogenesis of TTSuV1s and TTSuV2s during coinfection with other pathogens among clinically diseased pigs. Clinically diseased pigs, e.g. those affected by PCAVD, have also been found to be infected with PCV2 together with porcine parvoviruses, swine influenza, and TTSuV1s and TTSuV2s (Nieto et al., 2013; Novosel et al., 2012). Recent studies on TTSV1 and TTSV2 have found that many pigs were coinfected with emerging viruses such as PCV3 (Zheng et al., 2018). In addition, TTSuV1s and TTSuV2s have been shown to potentially be zoonotic (Karuppannan and Opriessnig, 2018). Consequently, surveillance for TTSuV1s and TTSuV2s should be continued.

Conflict of Interest

The authors declare that they hold no conflicts of interest.

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