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Molecular characterization and sequence analysis of the 2B region of Aichivirus C strains in Japan and Thailand

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ABSTRACT

Aichivirus C is the third species in the genus *Kobuvirus*, family *Picornaviridae*, and the virus is circulating in pigs worldwide. Aichivirus A in humans and Aichivirus B in cows have been shown to associate with diarrheal diseases, however, the pathogenesis of Aichivirus C has not been demonstrated clearly. In this study, the full genome nucleotide sequence of the Thai strain, CMP06/2007/THA collected from stool sample of a diarrheal piglet was analyzed and identified as a variant type with a 90-nt deletion in the 2B-coding region. In addition, molecular characterization of nucleotide sequences of the 2B-coding region of Aichivirus C strains from six diarrheal and six healthy piglets in Thailand, and four strains from healthy pigs in Japan revealed that all of the strains in this study were variant types. These findings indicate that variant strains of Aichivirus C are circulating in Asian countries such as China, Thailand and Japan, and deletion of tandem repeat of 2B-region is unlikely to associate with the pathogenesis of the virus.

1. Introduction

The family *Picornaviridae* currently consists of 17 genera and contains important viruses that relate to human and animal health. The viruses in the family are small and non-enveloped particles, which have single-stranded, linear non-segmented and positive-sense RNA genomes in the virus particles. *Kobuvirus* is a genus in the family *Picornaviridae*. The genome length of the virus is approximately 8.2–8.3 kb composed of a single ORF encoding a polyprotein which is cleaved into three structural proteins (VP0, VP3, and VP1) and seven nonstructural proteins (2A–2C, 3A–3D). Genus *Kobuvirus* consists of three species, recently renamed Aichivirus A, Aichivirus B, and Aichivirus C (Adams et al., 2013). Aichivirus A, formerly called Aichivirus, was first identified in human stool samples with oyster-associated diarrhea (Yamashita et al., 1991), and at present it includes three types, Aichi virus 1, canine kobuvirus 1 and murine kobuvirus 1. Aichivirus B, formerly known as bovine kobuvirus, was first isolated from cultured cells in 2003 (Yamashita, 2003), and the viruses were detected in stool samples collected from cattle with diarrhea (Khamrin et al., 2008). This species includes two types, namely bovine kobuvirus 1 and sheep kobuvirus 1. Aichivirus C was initially identified in 2008 as porcine kobuvirus in healthy pigs in Hungary (Reuter et al., 2008). Aichivirus C was detected worldwide, including Asia, Europe, and North and South America (Barry et al., 2011; Di Profio et al., 2013; Dufkova et al., 2013; Khamrin et al., 2009, 2010; Park et al., 2010; Ribeiro et al., 2013; Verma et al., 2013; Yu et al., 2009). Recently, the porcine kobuvirus was assigned as species of Aichivirus C, and the third species in the genus *Kobuvirus* (Adams et al., 2013). Porcine kobuvirus 1 is only one type of the species. Initially, three species of kobuviruses were classified based on genetic characterization (Reuter et al., 2009; Yamashita, 2003). The genetic identity of 5′untranslated region (5′UTR) among three species was very low, and the internal ribosomal entry site in the 5′UTR of the porcine kobuvirus 1 was indicated to be distinct from those of three viruses in the Aichivirus A and bovine kobuvirus 1 (U-1 strain) in the Aichivirus B (Reuter et al., 2009; Sweeney et al., 2012). In addition to the genetic diversity, three Aichivirus species were suggested to be held the different antigen type each other (Yamashita, 2003).

The pathogenesis of the Aichivirus C strains is not very clear, as they have been detected in stool samples of both diarrheal and healthy pigs. Recent studies revealed that the virus may play the

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role of a causative agent of acute gastroenteritis in pigs, because in Korea its prevalence in diarrheal pigs was higher than that in healthy ones (Park et al., 2010). On the other hand, virus genomes were detected in the serum samples from the healthy pigs (Reuter et al., 2010). Some studies indicated that the Aichivirus C strain was associated with clinical diarrhea, however, whether the viral infection causes diarrhea in pigs has not been clarified. Variant strains without a 90-nt deletion in the 2B-coding region were found recently in suckling piglets with severe diarrhea (Cao et al., 2012; Fan et al., 2013). On the other hand, a high prevalence of the Aichivirus C strain was found in Hungarian wild boars which indicated that wild boars are important hosts for the virus (Reuter et al., 2013).

At present, the full genome sequences of 13 Aichivirus C strains have been deposited in the GenBank database, with three strains were found in Hungary and the other 10 in China. In this study, the full genome sequence of one Thai strain, CMP06/2007/THA, was initially characterized. The sequence analyses of the 2B region in 13 Thai and four Japanese strains were performed. In addition, detection was made of the Aichivirus C strain in the serum samples of healthy pigs in Thailand.

2. Materials and methods

2.1. Full genome sequence of the Aichivirus C strain

In a previous study, 127 (97.0%) of 131 stool samples were found to be positive for the Aichivirus C strain collected between 2006 and 2008 from diarrheal piglets in northern Thai farms (Okitsu et al., 2012). In this study, one Aichivirus C strain (CMP06/2007/THA) was selected, and its full genome sequence was determined. The sequencing-specific primers were designed and modified from the previous report (Reuter et al., 2009) (Supplement 1).

2.2. Analysis of the 2B-coding region of Aichivirus C strains

A total of 52 stool samples were collected during 2010. Samples were from healthy piglets in 24 farms, in northern Thailand, age from neonate to four weeks old. The presence of Aichivirus C strain was determined by method previously reported (Okitsu et al., 2012). For the nucleotide sequence analysis of the 2B region of the Aichivirus C strain, this study selected six positive strains from the healthy piglets, six from the above mentioned diarrheal piglets in Thailand (collected between 2006 and 2008), and four from healthy Japanese pigs (collected between July and December, 2009) (Khamrin et al., 2010; Okitsu et al., 2012).

2.3. Detection of Aichivirus C strains in serum samples

A total of 376 serum samples were collected between 2003 and 2008 from healthy pigs in 13 northern Thai farms for detecting the Aichivirus C strains in porcine serum samples. The age distribution of the pig ranged from one to 8 months. Detection of the Aichivirus C strain in the serum samples was conducted by amplifying of the 3D region (Reuter et al., 2009). The negative samples of the first polymerase chain reaction (PCR) assay were further amplified by a semi-nested PCR assay, with the reverse primer of a sequence (PK7496R) (Supplement 1). Existence of the virus in some samples was confirmed by sequencing the PCR product.

2.4. Amplification and characterization of the 2B region

Amplification of the 2B region was performed using newly designed primers. A forward primer (CMP06/07-3634F) was designed by comparing previously known sequences with the sequencing results of the CMP06/2007/THA strain, and a reverse primer (mS-1-5184R) was designed by comparing eight known sequences of the Aichivirus C strains (Supplement 1). The PCR conditions were as follows: 98 °C for 3 min, 40 cycles at 98 °C for 10 s, 60 °C for 15 s, and 68 °C for 1 min 30 s, and a final extension at 68 °C for 5 min. PrimeStar®GXL DNA polymerase (Takara Bio Inc., Japan) was used as the PCR enzyme. The PCR product size was 1550 bp, which included partial VP1, full 2A and 2B and partial 2C sequences. The PCR amplicons of the products were subjected to direct sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Forster City, CA) on an automated Sequencer (ABI 3730x1: Applied Biosystems, Forster City, CA). The nucleotide sequences of the 2B region were compared with those of the Aichivirus C strains available in the NCBI Genbank database by using the BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence analyses were based on the maximum likelihood method using MEGA 5.2 (Tamura et al., 2011).

Nucleotide sequences of the full genome, CMP06/2007/THA and the 2B region of the Aichivirus C strains in this study, were deposited in the GenBank database. The accession numbers are AB624490 to AB624493, AB624498 to AB624500, AB624502, AB624504, and AB904925 to AB904932.

3. Results

3.1. Detection of the Aichivirus C strain in the stool samples of healthy piglets and serum samples of healthy pigs in Thailand

Forty-nine of 52 (94%) stool samples collected in 2010 from healthy piglets in Thailand, were positive for the Aichivirus C strain. Six strains from the positive samples were selected randomly and used for further analyses of the 2B region. In addition, 72 samples (19%) of 376 serum samples collected between 2003 and 2008 from healthy pigs in Thailand, were positive for the Aichivirus C strain (Table 1). Thirty-two serum samples in four-weeks old healthy piglets were all negative, but the serum from 11% of piglets aged six to eight weeks old were positive.

3.2. Full genome sequence of the CMP06/2007/THA strain

The full genome sequence analysis shows that the CMP06/2007/THA strain shares between 86.1% and 88.5% of nucleotide identities with the 13 reference strains of Aichivirus C available in the Genbank database, and as well as between 92.2% and 96.1% of amino acid identities (Table 2). The strain is most closely related to the WUH1/2011/CHN and GS-2/2012/CHN strains in China. The phylogenetic analysis indicated that the CMP06/2007/THA strain is also related mostly to the WUH1/2011/CHN strain (Fig. 1). Full genome sequence alignment of the CMP06/2007/

Table 1
Prevalence of Aichivirus C in the serum samples of healthy pigs, Thailand.

Age	Numbers of tested	Positive numbers	Prevalence (%)
4 weeks	32	0	0
6–8 weeks	100	11	11
10–12 weeks	45	6	13
14–16 weeks	30	13	43
18–20 weeks	18	2	11
24 weeks	15	7	47
Gilt	136	33	24
Total	376	72	19

Gilt means young female swine, and the age of gilt is about 7–8 months.

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