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Research paper

Molecular epidemiology and characterization of porcine adenoviruses in pigs with diarrhea in Thailand

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ABSTRACT

Pigs have been assumed as a source of human viral infections. Surveillance of viruses in animals is essential to evaluate the risk to human and animal health and to determine economic impact. A number of studies focused mainly on well- known enteritis viruses such as porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), and porcine group A rotavirus (PRVA), however, little data is available for porcine adenovirus (PAdV). In this study, the presence of PAdV was investigated in fecal samples collected from piglets with and without diarrhea from 31 commercial pig farms in northern Thailand. A total of 781 fecal specimens (516 from diarrheic piglets and 265 from non-diarrheic piglets) were screened for adenovirus using nested-PCR. Initial screening both in diarrheic and non-diarrheic piglets showed the overall prevalence of PAdV infection in piglets at 16.9% (132/781). Co-infection with PRVA was found in 24 out of 132 (18.2%) PAdV positive cases whereas PAdV mono-infection was observed at 81.8% (108/132). The prevalence of PAdV infection in diarrheic piglets (24.2%, 102/516) was significantly higher than those detected in non-diarrheic piglets (2.6%, 7/265). Most of PAdV detected in this study (97%, 128/132) were genotype 3 while the other 4 PAdV positive samples were non identifiable genotype. Phylogenetic analysis revealed that the viruses detected in diarrheic and non-diarrheic piglets displayed a closely related (95.4 to 100%) nucleotide sequence identity. To our knowledge, this is the first report on the epidemiology and molecular characterization of PAdV in piglets in Thailand.

1. Introduction

Adenovirus is one of important pathogens that cause the diseases in both humans and animals. Adenoviruses belong to the family *Adenoviridae*. Adenovirus virions which are non-enveloped, possess spherical icosahedral capsids that are 70–90 nm in diameter and a double-stranded DNA genome of 26–45 kb in length. The viruses in *Adenoviridae* family consist of several members that can infect both humans and wide variety of animals (Echavarria, 2009). The *Adenoviridae* family is divided into four genera, *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*. The *Mastadenovirus* have been known to infect mammals including humans while the *Aviadenovirus* infects avian species. The *Atadenovirus* and *Siadenovirus* infect several host species (Benko and Harrach, 1998; Echavarria, 2009). Pigs have been found to be infected by adenovirus so called porcine adenovirus (PAdV). Currently, there are three PAdV species and five serotypes in the *Mastadenovirus* genus. PAdV serotypes 1 to 3 (PAdV-1 to 3) belong to PAdV species A. Serotype 4 (PAdV-4) belongs to PAdV species B and serotype 5 (PAdV-5) belongs to PAdV species C (ICTV, 2012). The first prototype of PAdV was isolated from the rectal swab of diarrheic pigs in 1964 (Haig et al., 1964). PAdV is transmitted mainly by inhalation and fecal-oral route. Adenovirus infection in pig causes mild illness but usually presents watery diarrhea, dehydration, depression, and vomiting. In some cases, the infection can cause glomerulonephritis, chronic pneumonitis, and leads to death (Abid et al., 1984; Benfield and Richard, 2012; Nietfeld and Leslie-Steen, 1993). There is no specific treatment and no effective vaccine is available against PAdV infection. There is little information about adenovirus-related morbidity and mortality rates. Also, the epidemiological study of PAdV is very limited. Only few documents were reported in the past five decades. Serological studies of

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Table 1
List of primers used for the detection of porcine adenovirus and rotavirus.

Primers	Nucleotide sequences (5’-3’)	Nucleotide position	PCR product (bp)	Reference
Porcine adenovirus				
PALF	GATGTCATGGAYAACGTCAAC	20627–20647	612	Maluquer de Motes et al., 2004
PARF	CACGGAGGAGTCRAACTGGATG	21217–21238		
PALN	TACTGCMAGTTYCACATCCAGGT	20711–20733	344	
PARN	GGAATGGAGATGGGCAGGTT	21035–21054		
PoAdV3f	CCTCAACAACCTCATTGATACC	20574–20595	144	Wolf et al., 2010
PoAdV3r	CTTGCAGTAGCGGCCGT	20702–20718		
PoAdV5f	CGGCGCTTCCATCAGAATAG	18130–18149	133	
PoAdV5r	ACGWCAGGTAATCAATAAAGGAC	18241–18263		
Porcine rotavirus				
Beg9	GGCTTTAAAAGAGAGAATTTCCGTCTGG	001-028	1062	Yan et al., 2004
End9	GGTCACATCATACAATTCTAATCTAAG	1036-1062		
VP7-1'	ACTGATCCTGTTGGCCATCCTTT	373-395	395	

PAdV conducted in England and Cuba demonstrated the prevalence of PAdV infection among domestic pigs ranging from 15 to 60% (Darbyshire, 1967; Darbyshire and Pereira, 1964; Elazhary et al., 1985). Other study reported the prevalence of PAdV detected in diarrheic pigs at 30% (Abid et al., 1984). More recent surveillance studies in Spain and Ireland reported that the prevalence of PAdV detected in fecal samples from non-diarrheic pigs were 70% and 25%, respectively (Gunn et al., 2015; Maluquer De Motes et al., 2004). In addition to the occurrence of PAdV in pigs, it has been shown that PAdV was also detected in environmental water in Brazil, New Zealand, and Serbia with the prevalence of 8.3 to 30% (Garcia et al., 2012; Lazic et al., 2015; Wolf et al., 2010). From the literature search, so far, the epidemiological information of PAdV has not been well documented and the role of PAdV infection on the diarrheal disease is unclear. This study aimed to investigate the occurrence and genotype distribution of PAdV infection in piglets with and without diarrhea to elucidate the association of PAdV infection with diarrhea.

2. Materials and methods

2.1. Stool specimen collection

Stool specimens were collected from piglets (under 4 weeks of age) in 31 commercial farms in Chiang Mai and Lamphun provinces, northern Thailand during the period of January 2014 to December 2015. Among these 31 commercial farms, 24 were the small size (50–500 sows) and 7 were the medium size (500–5000 sows) farms. The vaccination program routinely applied for sows and boars twice a year in these farms including the vaccines for swine fever, foot and mouth disease, Aujeszky's disease, mycoplasma hyopneumoniae, and porcine reproductive and respiratory syndrome vaccines. These farms were certified by the Department of Livestock Development, Ministry of Agriculture of Thailand. The stool samples were kept in a − 20 °C freezer until use. Over all, a total of 781 fecal samples were included in this study. Among these samples, 516 were from piglets with a history of diarrhea such as anorexia, vomiting, loose stools, and having dramatic weight loss and 265 samples were from piglets without clinical sign of diarrhea. This study was approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (MIC-2560-04495).

2.2. Viral genomic DNA extraction and polymerase chain reaction (PCR) amplification

Stool samples were prepared as 10% (wt/v) suspension in phosphate buffered saline (pH 7.4) and viral genomic DNA was extracted from 200 μl of the supernatant of sample suspensions using a Geneaid Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan) according to the manufacturer's instruction. Nested-PCR amplification was used for the detection of PAdV in the samples as described previously (Maluquer De Motes et al., 2004). The first round PCR amplification was carried out by using GoTaq DNA polymerase (Promega, USA) with the outer primers PALF and PARF targeting conserved region within the hexon gene of PAdV. The PCR reaction was performed under the following conditions: 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, followed by 72 °C for 7 min. The PALN and PARN primers were used for the second round PCR amplification using the same PCR cycling conditions as described above. The DNA extracted from fecal sample of known PAdV positive case was used as positive control and water was used negative control for the PCR reaction. The obtained PCR product of 344 bp indicated the presence of PAdV in the sample. Then all positive samples were further investigated for PAdV genotypes by using PCR based method with PAdV specific primers for genotypes 3 and 5 (Wolf et al., 2010). It should be noted that the primers specific for genotypes 1, 2, and 4 were not available in the literature. Samples that could not be assigned their genotypes by using PAdV specific primers were further identified by nucleotide sequencing method. In addition, all samples were also screened for PRVA using RT-PCR protocols as described previously (Yan et al., 2004). The sequences of primers used in this study are shown in Table 1.

2.3. Nucleotide sequencing and phylogenetic analysis

The PCR products were purified using a Gel/PCR DNA Fragment Extraction Kit (Geneaid, Taiwan) according to the manufacturer's protocol and direct sequenced using the PALF or PALN forward primers and Big Dye Terminator Cycle Sequencing Kit (Thermo Fisher Scientific, USA), and analyzed on an automated DNA sequencer ABI3100 Applied Biosystems (Applied Biosystems, USA). The nucleotide sequences of the viruses detected in this study were compared with the sequences of corresponding reference virus strains available in the GenBank database to investigate the genetic relationship among them. Phylogenetic tree of the partial hexon gene was constructed by the neighbor-joining method using MEGA6.0 (Tamura et al., 2013) with 1000 bootstrap replicates complemented with BioEdit and Clustal X softwares. Nucleotide sequences of the PAdV strains detected in this study have been deposited in the GenBank database under accession numbers MF770165 to MF770194.

2.4. Statistical analysis

To evaluate the association of PAdV infection and the diarrheal disease in piglets with and without diarrhea, chi-square test was used. Statistical significance was considered at $p < .05$.

Table 2
Prevalence of porcine adenovirus infection and co-infection with porcine rotavirus detected in Thailand during 2014-2015.

Subject	Number of samples tested	PAdV positive samples (%)	PAdV mono-infection (%)	PAdV co-infection with PRVA (%)
Diarrhea	516	125 (24.2)	102 (81.6)	23 (18.4)
Non-diarrhea	265	7 (2.6)	6 (85.7)	1 (14.3)
Total	781	132 (16.9)	108 (81.8)	24 (18.2)

3. Results

3.1. Prevalence of porcine adenovirus infection and co-infection with rotavirus

From a total of 781 fecal samples tested, PAdV was detected in 132 specimens (16.9%). Among the PAdV positive cases, most of them (108/132, 81.8%) were infected solely with PAdV whereas 24 out of 132 (18.2%) were co-infected with PRVA (Table 2). To investigate association of PAdV infection and diarrheal disease, the prevalences of PAdV detected in piglets with and without diarrhea were determined. Of these 781 specimens, 516 were collected from piglets with diarrhea and 265 were collected from non-diarrheic piglets. The prevalence of PAdV infection in diarrheic piglets was much higher than those in piglets without diarrhea with percentage of 24.2 (125/516) vs 2.6, (7/265), respectively and the difference was statistically significant ($p < .0001$) with odds ratio of 11.7 and 95% confidence interval from 5.41 to 25.63. In diarrheic group, the majority (102/125, 81.6%) of PAdV positive samples were mono-infection and 18.4% (23 out of 125) were co-infected with PRVA. Similarly, in non-diarrheic piglets, 85.7% (6 out of 7) of PAdV positive samples were mono-infection and 14.3% (1 out of 7) was co-infected with PRVA.

3.2. Porcine adenovirus genotypes and seasonal distribution

All PAdV positive samples were determined for their genotypes by PCR genotyping based method. It was found that 128 out of 132 (97%) PAdV positive samples were genotype 3. None of genotype 5 was detected. For the other 4 samples that were negative for genotype 3 specific primers, re-amplification of these 4 samples was also unsuccessful so that their genotypes could not be assigned by nucleotide sequencing method. Monthly distribution of PAdV infection throughout two years period of study from January 2014 to December 2015 was investigated (Fig. 1). The data revealed that the patterns of seasonal distribution were variable. The high detection rates of PAdV in 2014 were observed in January (69.8%), February (55.1%), March (53.1%), and May (20%) while in 2015 was observed in May (26.7%) and June (11.9%).

3.3. Phylogenetic analysis

To investigate the genetic relationship between PAdV detected in this study and reference strains, the partial sequence of hexon gene of PAdV were sequenced and analyzed. Since 128 out of 132 PAdV positive samples detected in this study were genotype 3, we selected 30 representative PAdV strains (25 from diarrheic and 5 from non-diarrheic piglets) for further analysis. One PAdV strain was randomly selected from each farm except for one farm where there was no PAdV positive case. Phylogenetic analysis shown in Fig. 2 revealed that all of 30 PAdV strains from both diarrheic and non-diarrheic piglets formed a major cluster together with PAdV-3 reference strains reported from several countries in Europe such as Spain (AY288806, AY288810, AY288813), Germany (KP274050, KP274051, KP274052), and Ireland (KJ923328), and sharing 94.1 to 99.1% and 91.2 to 100% nucleotide and amino acid identities, respectively to those of reference sequences. Furthermore, the nucleotide and amino acid sequences of PAdV strains detected in piglets with diarrhea and non-diarrhea were closely related to each other (95.4 to 100% and 95.0 to 100% of nucleotide and amino acid sequences identities, respectively). In addition, the viruses detected in diarrheic piglets shared 95.4 to 100% nucleotide and 93.7 to 100% amino acid sequences identity among themselves. Similarly, high nucleotide and amino acid sequence identities (96.6 to 100% and 96.2 to 100%, respectively) were also observed among the viruses detected in non-diarrheic piglets.

4. Discussion

Porcine viral diarrhea is a major problem of pig farming industry and can result in significant economic losses. Among those viruses that cause diarrhea in pigs, PEDV, TGEV, and PRVA were the most common causative agents (Pensaert and Martelli, 2016; Vlasova et al., 2017; Zhai et al., 2016; Zhang et al., 2013). In addition to these viral agents,

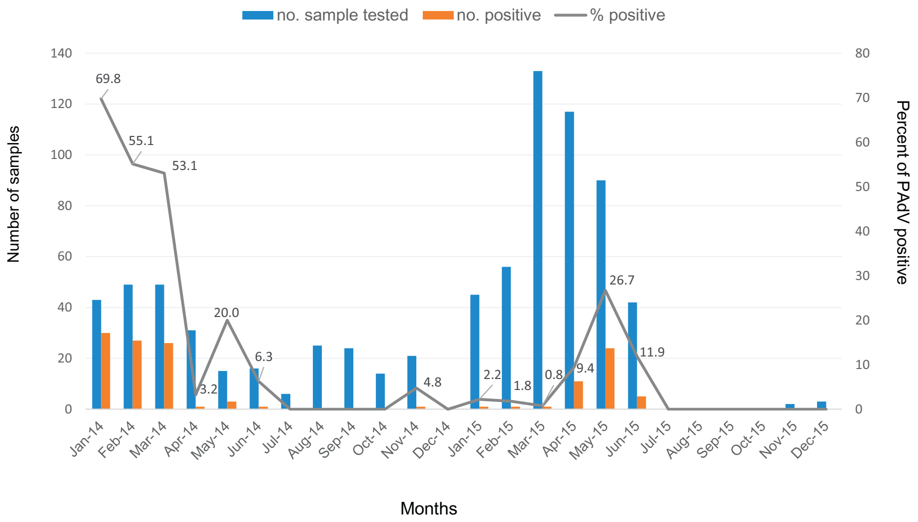

Fig. 1. Seasonal distribution of porcine adenovirus detected between 2014 and 2015.

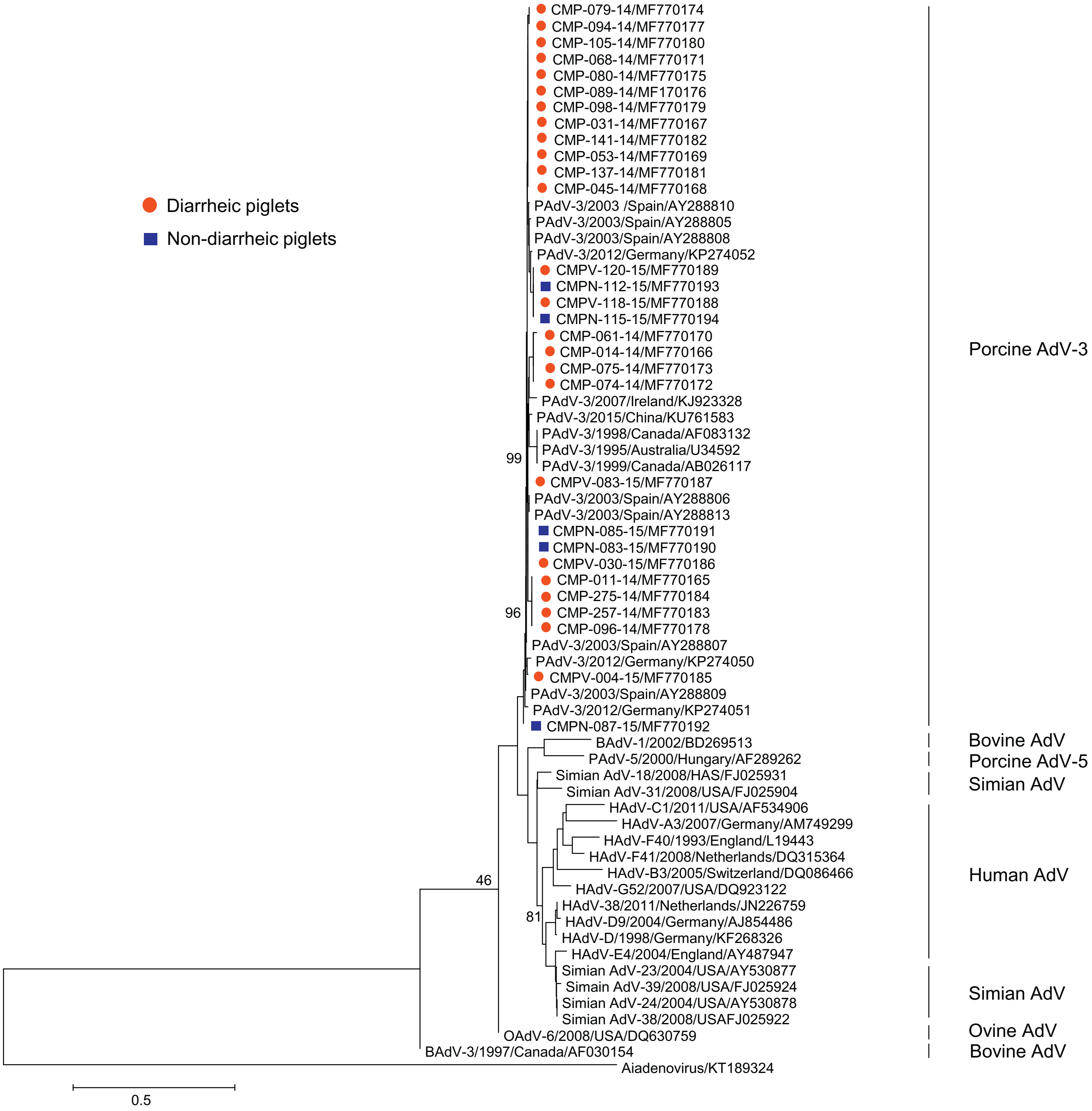

Fig. 2. Phylogenetic analysis of porcine adenovirus detected in piglets with and without diarrhea.

PAdV is endemic in most conventional swine herds throughout the world. This study investigated the prevalence of PAdV infection in piglets with and without diarrhea in several commercial farms in northern Thailand. In the present study, the prevalence of PAdV infection in non-diarrheic piglets is 2.6% which is similar to the report conducted in healthy piglets in Spain (3%) (Buitrago et al., 2010) but differs from the study conducted in Ireland (25%) (Gunn et al., 2015). Epidemiological data and the role of PAdV in association with the diarrheal disease are very limited. The only available information on the rates of PAdV infection in diarrheic pigs has been documented long time ago with the prevalence rate of 2% (Coussement et al., 1981; Nagy et al., 1996) to 30% (Abid et al., 1984). In this study, the prevalence of PAdV infection in piglets with diarrhea is 24.2% which is significantly higher than those in non-diarrheic piglets, suggesting the association between PAdV infection and diarrheal disease. PRVA has been implicated as an important causative agent for gastroenteritis in piglets (Saikruang et al., 2013; Vlasova et al., 2017; Yodmeeklin et al., 2016). In this study, all samples were also investigated for the presence of PRVA and found that majority (81.6%) of PAdV positive samples were solely infected with PAdV without co-infection with PRVA. The finding suggests that PAdV could be a potential pathogen of diarrhea. Nevertheless, other bacterial and viral infections which are known to cause diarrhea have not been ruled out in this study. Therefore, further studies are needed to clarify the etiologic role of PAdV in gastroenteritis and other known viral agents of diarrhea such as PEDV and TGEV should also be investigated.

To the best of our knowledge, none of the data reporting on the seasonality of PAdV infection are available in the literature. This study investigated monthly distribution of PAdV in piglets during 2014–2015 and demonstrated that no specific seasonal pattern of PAdV infection was observed. The high rates of PAdV detection vary year by year. The epidemiological survey and genotype distribution of PAdV in pigs are very limited. The only available information on the genotypic characterization of PAdV in domestic pigs was obtained from Spain. The PAdV-3 has commonly been detected in swine fecal samples with the prevalence of 98% (Maluquer De Motes et al., 2004). Other available data were obtained from the study of PAdV in environmental water conducted in New Zealand and Spain (Hundesa et al., 2006; Wolf et al., 2010). The PAdV-5 was a predomiant genotype detected in river water in both New Zealand and Spain while PAdV-3 was detected only in Spain. Our findings are in line with the study conducted in pigs in Spain (Maluquer De Motes et al., 2004) which PAdV-3 was also commonly detected with the prevalence of 97%.

The geographic distribution of PAdV is not fully known, although the virus is suspected to be widespread in domestic swine population. Analysis of the hexon sequences showed that the PAdV-3 strains circulating in Thailand are similar to the PAdV-3 strains identified previously in swine fecal samples collected in Europe (Spain, Germany, Ireland) and Asia (China) with the nucleotide sequence identities ranging from 94.1 to 99.1%. The findings indicate that PAdV is highly endemic in several countries in Europe and less common in Asia and PAdV circulated both Europe and Asia are genetically similar.

In conclusion, the present study provides new information about the molecular epidemiology and prevalence of adenovirus infection in farm animals. To the best of our knowledge, this is the first report of molecular epidemiology of animal adenoviruses in Thailand.

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Statement of animal right

This study was approved by the Research Ethics Committee of the Faculty of Medicine, Chiang Mai University (MIC-2560-04495).

Conflict of interest

The authors declare that they have no conflict of interest.

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