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Short communication

Molecular detection and genetic characterization of Salivirus in environmental water in Thailand

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ABSTRACT

Salivirus (SalV), also known as klassevirus, is a newly discovered member of the *Picornaviridae* family, which has been proposed to be a potential causative agent of acute gastroenteritis. This study aimed to provide further insight into the currently limited epidemiological data of SalV in environmental water in Thailand, which could be a potential source of human infection. A total of 95 water samples were collected from six locations in Chiang Mai province, northern Thailand, between November 2016 and February 2018. The molecular screening for SalV was performed by the nested polymerase chain reaction. The SalV genotypes were then determined through nucleotide sequencing and phylogenetic analysis. SalV was detected in 31 out of the 95 (32.6%) water samples and all belonged to the A1 genotype, based on phylogenetic analysis of the 5′UTR and 3D regions. The SalV-A1 strains detected in the environmental water were closely related to the SalV-A1 detected in a patient with diarrhoea in the same geographical area, based on the nucleotide sequence identities of the 5′UTR and 3D regions ranging from 91 to 99% and 96–99%, respectively. This study reports the prevalence of SalV-A1 contamination in environmental water in Chiang Mai, Thailand.

Most recently, several viruses in the *Picornaviridae* family, including enterovirus, Aichi virus, parechovirus, cosavirus, and saffold virus have been reported in association with diarrhoea worldwide (Kumthip et al., 2017; Menage et al., 2017; De Crom et al., 2016; Reuter et al., 2016). The *Picornaviridae* family consists of 12 genera of human and animal non-enveloped viruses with a positive – sense single – stranded RNA genome (Nielsen et al., 2013). Salivirus (SalV), formerly identified as klassevirus, is a newly discovered member of this family, within the genus *Salivirus* (Li et al., 2009). Currently, salivirus A (SalV-A) is the only species within the *Salivirus* genus, and has been identified and subcategorised into two genotypes: salivirus A1 (SalV-A1) and salivirus A2 (SalV-A2). SalV has a linear and monopartite genome of up to 8021 base pairs (bp) long, with a single open reading frame (ORF) that encodes for three structural proteins (VP0, VP1, and VP3), that makes up 60 protomers which encases the genome and forms the icosahedral capsid, and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D) (Reuter et al., 2016).

SalV was first discovered in 2009 in the faecal samples of children in the USA, Australia, and Nepal who were diagnosed with gastroenteritis, whilst in Nigeria and Tunisia patients with SalV in their stool were admitted to hospitals with acute flaccid paralysis (Holtz et al., 2009). SalV is generally responsible for sporadic gastroenteritis in young children with a prevalence of 0.1–8.8% as reported from several countries worldwide, including Germany, Tunisia, South Korea, India, Denmark, Brazil, China, and Hong Kong (Aldabbagh et al., 2015; Ayouni et al., 2016; Han et al., 2010; Itta et al., 2016; Lasure and Gopalkrishna, 2016; Nielsen et al., 2013; Santos et al., 2015; Shan et al., 2010; Yip et al., 2014; Yu et al., 2015). Furthermore, SalV has also been detected in sewage and river water samples in Japan, Spain, the USA, and Thailand (Haramoto et al., 2013; Holtz et al., 2009; Kitajima et al., 2014; Ng et al., 2012).

In Thailand, the first and only report of SalV infection in humans has been reported recently by our group in a child, admitted to hospital with acute gastroenteritis in Chiang Mai city (Kumthip et al., 2017).

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Since SalV has been reported to contaminate sewage and river water in several countries, including Thailand, we hypothesize that other environmental water could also possibly be contaminated and be a source of infection. The aim of the present study was to screen for SalV contamination in environmental water which could be a potential source of human infection in Chiang Mai, Thailand.

A total of 95 water samples were collected, 32 from wastewater canals, 16 from irrigation canals, 16 from a river, and 31 from reservoirs between November 2016 to February 2018 from six different locations (Ang Kaew Reservoir (18°48′19.0″N 98°56′58.5″E), Family Medicine Canal (18°47′25.5″N 98°58′41.4″E), Sompech Moat (18°47′34.8″N 98°59′36.9″E), Ping River (18°47′17.2″N 99°00′17.1″E), Mae Kha Canal (18°47′17.9″N 98°59′57.6″E), Buak Hard Garden (18°46′55.7″N 98°58′46.0″E)) in Chiang Mai province, Thailand.

The virus in water samples was concentrated using the polyethylene glycol (PEG) precipitation method, according to Iwai et al., 2009. Briefly, 100 ml of water sample was mixed with 8 g of PEG and 2.3 g of NaCl and stirred at 4 °C overnight. The mixture was centrifuged at 10,000 ×*g* for 30 min at 4 °C and the pellet was resuspended in 1 ml of RNase-free water. The Geneaid Viral Nucleic Acid Extraction Kit II (Geneaid, Taipei, Taiwan) was used to extract the viral RNA genome from this supernatant. The extracted viral genome was reverse transcribed into cDNA using the Thermo Scientific RevertAid Firststrand cDNA synthesis kit (Thermo Scientific, USA) and detected by nested-PCR using cDNA as a template, SAL-L1/SAL-L2 as forward primers, and SAL-R1/SAL-R2 as reverse primers. The SAL-L1/SAL-R1 and SAL-L2/SAL-R2 primers were used to amplify a 486 bp and 414 bp fragments from the 5′untranslated region (UTR) in a first and second round PCR, respectively (Table 1). The thermocycling conditions for the first round PCR were as follows: 94 °C for 3 min prior to 35 cycles of 94 °C for 40 s, 55 °C for 40 s, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The second round PCR conditions were identical to the first round, with the exception of an annealing temperature of 57 °C.

Further genetic characterization of the SalV strains detected in this study was carried out by amplification of the 3D region. In the first round of amplification, a 439 bp amplicon was generated using the primers SalV-3DF as a forward primer and SalV-3DR as a reverse primer (Yip et al., 2014). In the second round of amplification, a 363 bp amplicon of the SalV 3D region was generated by semi-nested PCR using primers SAL-3DIF as a forward primer and SalV-3DR as a reverse primer. All primer sequences used in this study are listed in Table 1. The thermocycling conditions were as follows: 94 °C for 3 min prior to 35 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR products of both 5′UTR and 3D regions of SalV detected in this study were purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Tapei, Taiwan). Purified amplicons were subsequently sequenced by a fluorescence-based cycle sequencing method using a BigDry® Terminator V3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA, USA) and compared with those of SalV reference strains available in the NCBI GenBank database using the BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic trees were constructed using the MEGA7.0 software (Kumar et al., 2016) based on the neighbour-joining method. GraphPad Prism 7.04 (GraphPad, La Jolla, CA) was used to perform the analysis of the quantitative data in this study, where *p*. values < 0.05 were considered significant.

The nucleotide sequences of 5′UTR and 3D regions of SalV strains detected in this study have been deposited in the GenBank database under the accession numbers MH287282 - MH287312 for the 5′UTR and MH287313 - MH287334 for the 3D region.

Among 95 water samples tested, 31 (32.6%) were positive for SalV by the nested PCR screening method. SalV was detected in every month during the period of the study (November 2016–February 2018), with the highest detection rates in December 2016, October and December of 2017, and January and February of 2018 (Fig. 1). The highest detection rate was observed at Mae Kha canal with SalV being detected at this location in every month during the study period, whilst the lowest detection rate was seen at Ang Kaew reservoir where no SalV was detected during the 16-month sampling period. The positive detection frequency of SalV at the six locations were as follows: 0 at Ang Kaew Reservoir; 8 at Family Medicine Canal; 2 at Sompech Moat; 3 at Ping River; 16 at Mae Kha Canal; and 2 at Buak Hard Garden.

The phylogenetic tree demonstrated that all 31 5′UTR sequences were clustered with SalV-A1 reference strains from China detected in 2009, 2011, and 2013 (GU376738 - GU376746, JN379039, KT182636), Germany in 2013 (KP247439), Guatemala in 2009 (KT310068), Hungary in 2013 (KT240115), Nigeria in 2007 (GQ179640), Tunisia in 2011 and 2012 (KU362791, KU362792), USA in 2002 and 2012 (GQ184145, AB893308), with the nucleotide sequence similarities ranging from 88 to 100%. Comparison among the nucleotide alignments of the 31 SalV strains showed a 90–100% sequence similarity. It was interesting to observe that all 31 5′UTR sequences of SalV strains isolated from environmental water in Chiang Mai area showed high nucleotide sequence identities ranging from 91 to 99% with the SalV-A1 isolated previously from a patient with diarrhoea in Chiang Mai in 2016 (KY798519). Phylogenetic analysis also confirmed that the 5′UTR sequences of these SalV strains clustered together with this Thai - SalV - A1 strain (Fig. 2). The CMW 45/17 strain was the SalV-A1 strain that was most closely related to the Thai reference strain on the phylogenetic tree, with a nucleotide sequence identity of 99%.

Among the 31 confirmed SalV strains detected, the 3D region of 22 strains were successfully amplified. Based on a phylogenetic analysis of the 3D region, all 22 sequences of SalV were identified as SalV-A1. Comparison among the nucleotide sequences of these 22 strains demonstrated a 97–100% sequence similarity. Twenty- one SalV-A1 clustered together within the same branch and were closely related to SalV-A1 isolated previously in China in 2013 (KT182636), India in 2014 (KU872014, KU872016, KU872017), and were also similar to the Thai SalV-A1 isolated from a patient with diarrhoea in 2016 (KY798521). This group shared nucleotide sequence identities with the latter ranging from 96 to 99%. Again, the CMW 9/16 was the SalV-A1 strain that was most closely related with the Thai reference strain on the phylogenetic tree, with a nucleotide sequence identity of 99%. Only one SalV-A1 strain (CMW 91/18) was distantly related to those of other strains detected in the water in Chiang Mai. This strain was most closely related to a SalV strain detected previously in China in 2009 (GU245894) with a 98% nucleotide sequence identity (Fig. 3).

Table 1
List of specific primers used for the detection and characterization of saliviruses.

Primer Name	Primer nucleotide sequence (5′-3′)	Orientation	PCR application	Amplicon length (bp)	Target region	Reference
SAL-L1	CCCTGCAACCATTACGCTTA	Forward	First PCR	486	5′UTR	Shan et al., 2010
SAL-R1	CACACCAACCTTACCCCACC	Reverse	First PCR		5′UTR	
SAL-L2	ATTGAGTGGTGCAT(C)GTGTTG	Forward	Nested PCR	414	5′UTR	Shan et al., 2010
SAL-R2	ACAAGCCGGAAGACGACTAC	Reverse	Nested PCR		5′UTR	
SalV-3DF	GAGGGCACCGACCTGGATGC	Forward	First PCR	439	3D	Yip et al., 2014
SalV-3DR	TGGTTGATGAGAGAACCAAG	Reverse	First/Nested PCR		3D	
SAL-3DIF	AGAGGCGGCYGATTGGTAYTT	Forward	Nested PCR	363	3D	This study