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Original Article

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Abstract

Tembusu virus (TMUV) namely KPS54A61 has spread in duck farms in many provinces in Thailand since 2010. The morbidity ranges from 15-30% and the mortality ranges from 10-15%. All infected ducks exhibit nervous signs including ataxia and paralysis. TMUV was successfully isolated from the brain and spinal cord of infected ducks by using C6/36 and Vero cells, but the typical cytopathic effect (CPE) was presented in the Vero cells only. Phylogenetic analysis of the whole genomic sequence of our TMUV-KPS54A61 virus showed that it belonged to the Ntaya virus group of *Flaviviridae* and its genomic sequence was similar to that of Baiyangdian virus (strain BYD-1, isolated from infected ducks in China) with 98% identity. This is the first report of whole genomic sequence of TMUV isolated from domestic ducks in Thailand.

Keywords: domestic duck, flavivirus, genome, Tembusu virus

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Introduction

Flavivirus is an enveloped virus which belongs to the family *Flaviviridae* and contains a single positive strand genomic RNA (Chamber et al., 1990). The genome of flaviviruses is approximately 11 kbs in length, containing methylated cap ($m^7G5'ppp5'A$) at 5′ end, but their 3′ end lacks a polyadenylate tail. This RNA is a messenger RNA encoding three structural proteins (C, prM/M, and E) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) in the single opened reading frame (Lindenbach et al., 2007). Several members of the family *Flaviviridae* are arthropod-borne viruses and cause zoonotic diseases, e.g. yellow fever virus, dengue virus, West-Nile virus, Japanese encephalitis virus, and tick-borne encephalitis virus (Mukhopadhyay et al., 2005). A study reported that flaviviruses could cause poultry diseases although many of them were isolated from wild birds (Bernard et al., 2000). In 1955, Tembusu virus (TMUV) or Sitiawan virus (STWV) was firstly isolated from mosquitoes (*Culex* spp.) in Malaysia. After that this virus was also isolated from broiler chickens in Malaysia in 2000 (Kono et al., 2000).

Recently, Baiyangdian virus (BYDV), which is closely related to TMUV, was reported to cause a severe disease in ducks in southeast provinces in China (Su et al., 2011). TMUV could be transmitted by mosquitoes, especially *Culex* spp., and direct duck-to-duck transmission (Yan et al., 2011). The clinical signs of infected ducks include severe decrease in egg production, anorexia, diarrhea, paralysis and death. At necropsy, gross lesions include severe ovarian hemorrhage and folliculitis and splenomegaly in the infected ducks (Cao et al., 2011; Su et al., 2011). In addition, TMUV infection in other avian species was reported, including ducks, chickens, geese and house sparrows. The disease was also present in chicken and goose farms, resulting in decreased egg production and serious economic loss (Liu et al., 2012; Huang et al., 2013; Tang et al., 2013).

Since 2010, paralytic and egg drop syndrome has mostly occurred in duck farms in several provinces in central, eastern and western Thailand. The clinical signs, gross and histopathological lesions of the infected ducks were similar to those of previously infected ducks in China (Cao et al., 2011). In consideration of biological characters and clinical symptoms, TMUV was suspected of causing decreased egg production with severe central nervous system disorders (Songserm et al., 2014). However, there is few information about nucleotide sequence analysis of TMUV isolated from Thailand.

The purpose of this study was to characterize whole genome of TMUV isolated from domestic ducks in Thailand in 2010-2013 and compare this genomic sequence with those of existing flaviviruses.

Materials and Methods

Outbreaks: From January 2010 to late 2013, an outbreak of paralysis and decreased egg production were found in 31 duck broiler, 27 laying duck and 3 duck breeder farms in the central, eastern and western parts of Thailand. The outbreak areas were mostly the same area of rice and paddy fields. All infected ducks exhibited clinical signs including depression, uncoordinated gait, paralysis, head tilt and retarded growth. The morbidity ranged from 15-30% and the mortality ranged from 10-15%. At least 5 infected ducks from all affected farms were submitted to Center of Duck Health Science, Kasetsart University, Thailand. The clinical and moribund ducks were humanely killed by cervical dislocation under slight level of anesthesia. The procedure of sacrifice was approved by the committee of ethics and animal experimentation, Kasetsart University, Thailand. At necropsy, pieces of brain, spinal cord, ovary, spleen, lung, liver, pancreas, kidney and gastro-intestinal organs were collected and processed for virus isolation and molecular biology.

Virus isolation: All tissues were separately homogenized in Eagle's Minimal Essential Medium (EMEM) in a 20% suspension (w/v). Supernatants were filtered through a 0.2 µm syringe-driven filter (Corning®, Corning Incorporated, Germany) after centrifugation at 8,000 g for 10 min. The filtered suspension was inoculated onto mosquito C6/36 cells (*Aedes albopictus* cells; ATCC® CRL-1660™, VA, USA). The infected cells were incubated at 28°C with 5% CO_2 and daily observed for cytopathic effect (CPE). After four passages in C6/36 cells, the medium of the infected cells was collected for further passage in African green monkey kidney cells (Vero; ATCC® CCL-81™, VA, USA) and incubated at 37°C with 5% CO_2. When the infected host cells showed CPE, including rounding up and cell detachment, the culture media were harvested for viral RNA extraction and detection.

RNA extraction and RT-PCR: The brain and spinal cord tissues or the culture media were prepared for RNA extraction using RNeasy kit (QIAGEN®, Valencia, CA., USA) according to the manufacturer's protocol. The RNA was eluted in a final volume of 60 µl of elution buffer and stored at -80°C until further use.

RT-PCR was employed to detect Tembusu virus in the RNA samples using forward primers (TMUV-NS5F: 5′-CCTCAACCGGTTGGAGAAACTGGC-3′) specific to NS5 gene (position; 9,761-9,784) and TMUV-NS5R: 5′-TGTGGAARTACATYAATAACCACATCT G-3′ (position; 9,952-9,925). These primers were designed from NS5 gene (GenBank accession no. JF895923).

Seven microliters of RNA solution was used for synthesis of viral cDNA by using the SuperScript®III Reverse Transcription system (Invitrogen®, USA) with 3'-NTR specific primers (3'-NTR-R: 5′-AGACTCTGTGTTCTACCACCACC-3′) complementary to virus genome position at 10,968-10,990. The RT step condition followed the manufacturer's recommendations. The PCR cycling protocol was used as initial denaturation step at 95°C for 15 min, followed by 35 cycles of 94°C for 20 sec, 57°C for 20 min and 72°C for 40 sec. The final extension step was performed at 72°C for 5 min. Reactions were performed in the Thermocycler machine (Bio-Rad®, USA).

Genomic sequencing: Specific primers for genomic sequencing were designed according to the conserved region of available 35 complete genomic sequences of TMUV and TMUV-like flaviviruses from GenBank database. The primers used for amplification and sequencing in these studies are listed in Table 1. PCR reactions were obtained to amplify parts of 5′NTR and C-prM-E encoding region by using TMUV-1F and TMUV-1R primers. Moreover, other three primer pairs (TMUV-2F/TMUV-2R, TMUV-3F/TMUV-3R and TMUV-4F/TMUV-4R primer pairs) were used for amplifying NS1-NS2A-NS2B, NS3-NS4A-NS4B, and NS5-3′NTR regions, respectively. The PCR reactions were conducted in a total volume of 50 µl to amplify parts of viral genes. The thermal cycling program consisted of an initial denaturation step at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 sec, annealing at 52-57°C for 20 min, and extension at 72°C for 90 sec. The final extension step was performed at 72°C for 5 min. DNA amplicons were purified using QIAquick gel extraction kit (QIAGEN®, Germany) according to the manufacturer's protocol. Sequences obtained from the amplified samples were used for walking-primer design for next RT-PCR for amplifying other genes and sequencing (Table 1). The PCR amplicons were sequenced at 1st BASE Pte Ltd (Malaysia) by using di-deoxy dye terminator sequencing method.

Table 1 Primers used for amplification and sequencing of TMUV-KPS54A61 genome

Primers[a]	Sequence (5'to3')	Genomic position[b]	Amplicon size (bps)
Amplification primers			
TMUV-1F	AGAAGTCCATCTGTGTGAACTTATTCC	1-27	2,739
TMUV-1R	GGATTTTCTTCAACCACCACAGTC	2,739-2,716	
TMUV-2F	GCATCAAACATGAGTTGAACGCGA	26,67-2,690	1,986
TMUV-2R	CTCCATTGCCTACTTCGGCTC	4,652-4,632	
TMUV-3F	CTGAACGATCCTGGTGTGTCG	4,537-4,577	4,150
TMUV-3R	CTTCACCCTATCAGCGACCATTC	8,506-8,484	
TMUV-4F	CGCAATGGACCCAGGTATGAAG	8,399-8,420	2,591
TMUV-4R	AGACTCTGTGTTCTACCACCACC	10,990-10,968	
Sequencing primers			
TMUV-5F	CGTGACGACAGAATCCAGATGCC	1,156-1,178	
TMUV-5R	GCATATGTATTCAGCATAAGTTGCC	1,231-1,207	
TMUV-6F	CCTATGCAATTGGGAATGCT	3,535-3,554	
TMUV-6R	CGCCTTCTCAAAATCTCCTG	3,589-3,570	
TMUV-7F	CCTAAATGCAAGAGTGGAGAATG	5,774-5,797	
TMUV-7R	GAAGCGGCAGAGGTCAACATTCC	7,506-7,483	
TMUV-8F	CCTCAACCGGTTGGAGAAACTGGC	9,738-9,761	
TMUV-8R	TGTGGAARTACATYAATAACCACATCTG	9,952-9,926	

[a] *F* forward primer, *R* reverse primer
[b] Position referring to the genome of Tembusu virus strain JS804 (GenBank accession number JF895923)

Phylogenetic analysis: Genomic sequences of isolated TMUV were evaluated and adjusted manually using BioEdit version 7.2.3 (available from http://www.mbio.ncsu.edu/bioedit/ page2.html) (Hall, 1999). The sequence homology searches were made by BLASTn analysis of GenBank (available from www.ncbi.nlm.nih.gov/blastn) and multiple alignments were performed with ClustalW (available from; www.ebi.ac.uk). The maximum-likelihood (ML) method from the MEGA version 5.2.1 program was

applied to generate a phylogenetic tree of the aligned sequences. Bootstrap analyses were performed on the ML trees for 1000 pseudo-replicates (Tamura et al., 2011).

Results

Isolation of the virus: The typical CPE of TMUV, as described above, was only presented in the Vero cells at 72-96 hr. after inoculation. In the meantime, it was found that the virus was genomically identified as a duck Flavivirus. The virus was mostly isolated from the brain and spinal cord tissues. This isolated virus strain was named KPS54A61 virus.

Genomic analysis and Phylogenetic analysis: The whole genomic sequence of TMUV-KPS54A61 (GenBank accession no.KF573582) was 10,990 nucleotides in length with single open reading frame (ORF), translating 3,425 amino acids of polyprotein. Similarly to other flaviviruses, this viral polyprotein is composed of three structural proteins (C, PrM and E) and seven non-structural proteins. The amino acid residues at prM-E cleavage site are GRRSRR▼S, clevaged by furin-like proteinases, similar to other flaviviruses.

This TMUV-KPS54A61 virus genomic sequence was compared with other flaviviruses from GenBank via BLAST analysis (www.ncbi.nlm.nih.gov) including Bagaza virus (BAGV; EU684972), Ntaya virus (NTAV; JX236040), Ilheus virus (ILHV; KC481679), St. Louis encephalitis virus (SLEV; JF363695), West Nile virus (WNV; AY632542), Japanese encephalitis virus (JEV; EF363695), Usutu virus (USUV; AY453412), Dengue virus type 1 (DENV1; U88536) and Tick-borne encephalitis virus (TBEV; AB062064). Furthermore, TMUV-KPS54A61 was also compared to other strains of TMUV that were isolated in Thailand (accession no. AB110495, KC810845, KC810846, KC810847, KC810848, KC810849), TMUV strain MM_1775 (accession no. 477685) and Sitiawan virus (STWV; JX477686) in Malaysia, TMUV in China (accession no. JF270480, JN811559 and JX965381), Duck egg-drop syndrome virus in China, strain BYD-1 (DEDSV-BYD1; JQ920420), strain goose (DEDSV-goose; JQ920424), strain pigeon (DEDSV-pigeon; Q920425) and strain JX2 (DEDSV-JX2; JQ920426). The envelope gene of TMUV-KPS54A61 virus was compared in group with other viruses in Japanese encephalitis complex as a mosquito borne encephalitis virus.

Furthermore, the envelope gene revealed 98% identical nucleotide to DEDSV-BYD1, the first strain from ducks in 2010 and from geese (99% amino acid identity) isolated from China. It also showed 91% identical nucleotide to Thailand's TMUV (strain AFRIMS-D119-014/150) previously isolated from duck sera in 2002 (Fig 1). In addition, the NS5 gene revealed a nucleotide identity of 99% and 92% with DEDSV-BYD1 and Thailand's TMUV AFRIMS-D119-014/150 (data not shown), respectively.

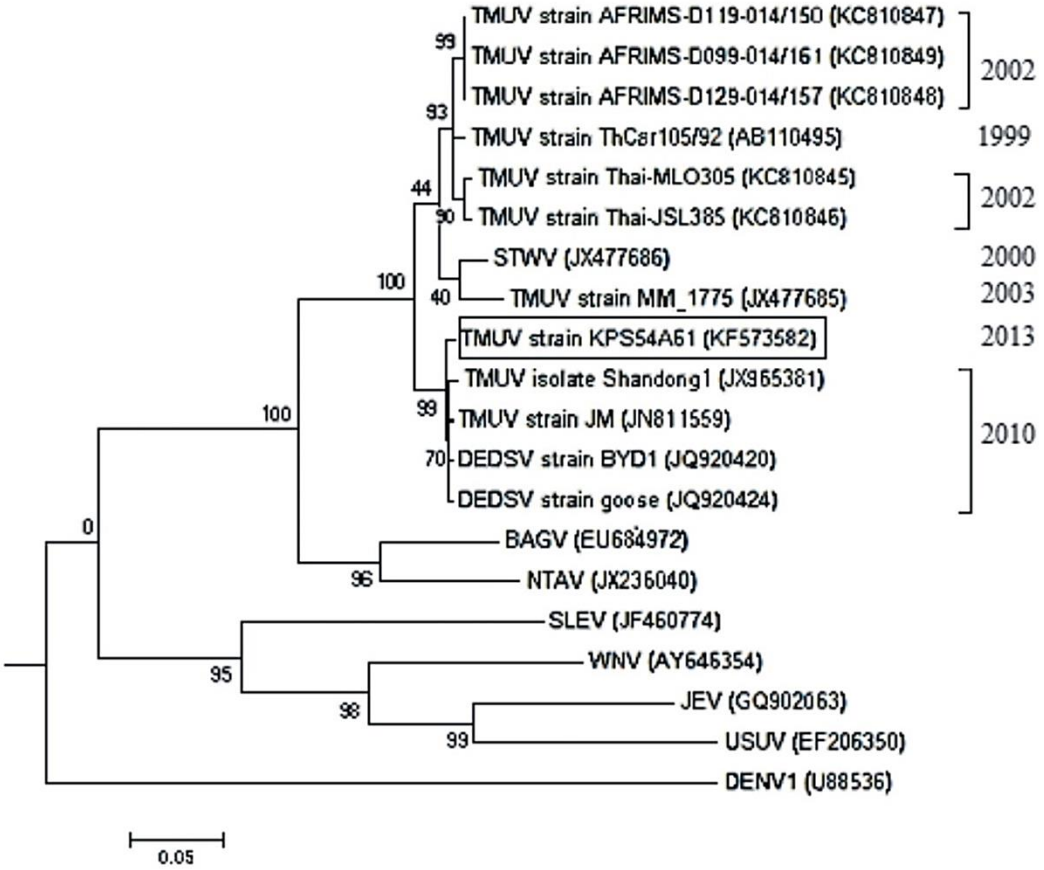

Figure 1 The envelope encoding gene of TMUV-KPS54A61 was compared to other TMUV isolated in Thailand and same selective Flaviviruses.

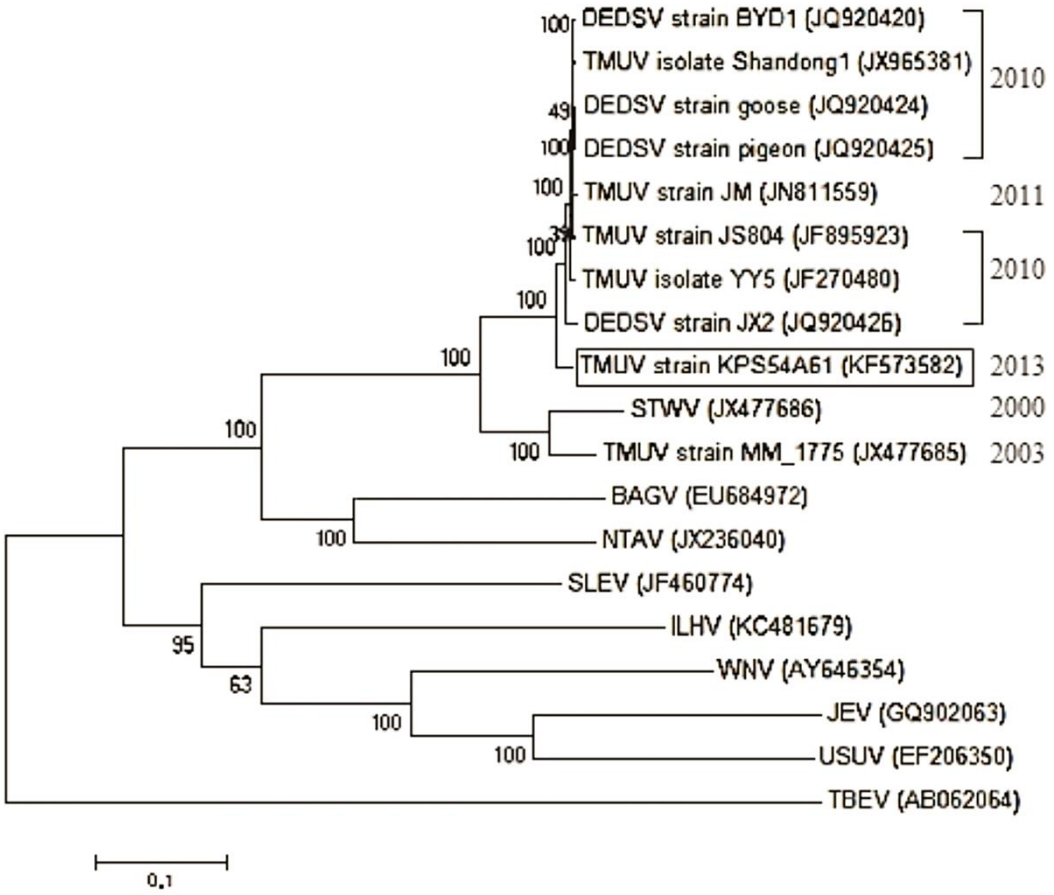

Figure 2 Phylogenetic analysis of TMUV-KPS54A61 virus compared with other Flaviviruses. The polyprotein encoding nucleotide sequence of TMUV-KPS54A61 was compared to TMUV in China, Duck egg-drop syndrome viruses (DEDSV) in China, TMUV and Sitiawan virus (STWV) in Malaysia, Bagaza virus (BAGV), Ntaya virus (NTAV), Ilheus virus (ILHV), St. Louis encephalitis virus (SLEV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Usutu virus (USUV), Dengue virus type 1 (DENV1) and Tick-borne encephalitis virus (TBEV).

The result of whole genomic sequence analysis of TMUV-KPS54A61 virus showed high similarity to that of DEDSV-BYD1 (98% nucleotide identity and 99% amino acid identity). In addition, the whole coding sequences of strain KPS54A61 were 88% and 87% identical to TMUV strain MM1775 and STWV, respectively (Fig 2). The analysis of whole virus genome indicated that TMUV-KPS54A61virus genome was closely related to DEDSV-BYD1 in China.

Discussion

TMUV outbreaks have predominantly occurred in the rainy season in Thailand during the past recent years. Detected by RT-PCR, Thailand's TMUV-KPS54A61 was preferentially replicated in C6/36 cells without CPE, different from previous studies which found a significantly present CPE in C6/36 cells 3-4 days after inoculation (Yun et al., 2012; Tang et al., 2013).

The phylogenetic analysis of TMUV-KPS54A61 isolate presented that this virus was classified as a mosquito-borne flavivirus of the Ntaya virus group and the whole nucleotide of this virus was closely related to other TMUV virus strains, especially DEDSV-BYD1 from China (Su et al., 2011), TMUV strain MM1775 and STWV from Malaysia (Kono et al., 2000). Previously, Takagi et al. (1997) reported that the abundance of *Culex* mosquitoes, main vectors of TMUV, was directly related to the rice crop production in northern Thailand. In 2002, TMUV was detected in two pools of *Culex* mosquitoes in Kanchanaburi province as well as from sera of sentinel ducks in the same area. The result of virus detection in the mosquitoes provides evidence for the involvement of *Culex* mosquitoes in the transmission of TMUV in the environment and in some parts of envelope and NS5 encoding genes. This indicates either the existence of locally persistent viral reservoirs or the reintroduction of viruses from other regions. Although the introduction of a new viral strain (TMUV-AFRIMS-D119-014/150) in 2002 into Kanchanaburi province (western Thailand) was reported, the viral sequences from this province remained the same as previous TMUV linage. This indicates that yearly re-emerging viruses in central Thailand belong to a similar strain and suggests that they originate from local *Culex* mosquito vector (O'Guinn et al., 2013). The genomic similarity between DEDSV-BYD1 and TMUV-KPS54A61 is intriguing, as the locations of the two outbreaks were more than 1500 kilometers apart. This might suggest that long-range carrier such as birds and regional transportation might be involved in the transmission. Understanding how the virus spreads over long distances is crucial to the successful control of the epidemic and deserves further investigation.

Additionally, a previous study supported that human antibody to TMUV could be detected

without severe disease for humans (Tang et al., 2013). Whether these geographical characteristics contribute to the TMUV outbreaks requires further investigation. The limited geographical area of the sporadic outbreaks in 2010-2013 in Thailand suggests that the control of the virus vector and outbreaks should be focused on.

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บทคัดย่อ

การศึกษาลักษณะยีโนมของฟลาวิไวรัสเทมบูซูสายพันธุ์ใหม่ที่แยกได้จากเป็ดในประเทศไทย

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เชื้อไวรัส Tembusu (TMUV) ได้สร้างความเสียหายให้กับฟาร์มเป็ดในหลายจังหวัดของประเทศไทยตั้งแต่ปี พ.ศ. 2553 เป็ดที่ติดเชื้อ TMUV จะมีอัตราป่วยร้อยละ 15-30 และมีอัตราตายร้อยละ 10-15 เป็ดที่ติดเชื้อจะแสดงอาการทางระบบประสาท เช่น อาการเดินเซ และอัมพาต เป็นต้น รายงานการศึกษานี้แสดงการแยกเชื้อ TMUV จากสมองและไขสันหลังของเป็ดที่ติดเชื้อ โดยใช้เซลล์เพาะเลี้ยงชนิด C6/36 และ Vero ซึ่งไวรัสที่แยกได้ใหม่นี้ถูกตั้งชื่อว่า TMUV สายพันธุ์ KPS54A61 เมื่อดูลักษณะของเซลล์พบ cytopathic effect ของเชื้อไวรัสเฉพาะในเซลล์เพาะเลี้ยงชนิด Vero เท่านั้น การวิเคราะห์โดยแผนภาพต้นไม้วิวัฒนาการ (Phylogenetic analysis) พบว่าลักษณะยีโนมของเชื้อ TMUV สายพันธุ์ KPS54A61 อยู่ในกลุ่มเดียวกับเชื้อไวรัส Ntaya ซึ่งจัดอยู่ในวงศ์ *Flaviviridae* นอกจากนี้ยังพบว่าลำดับสารพันธุกรรมในยีโนมของเชื้อไวรัส TMUV มีความคล้ายคลึงกับเชื้อไวรัส Baiyangdian สายพันธุ์ BYD1 ที่แยกได้จากเป็ดในประเทศจีน โดยมีความเหมือนกันถึงร้อยละ 98 รายงานนี้เป็นรายงานแรกที่แสดงลำดับสารพันธุกรรมทั้งสายในยีโนมของเชื้อไวรัส TMUV ที่แยกได้จากเป็ดในประเทศไทย

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