

An updated review of avian-origin Tembusu virus: a newly emerging avian Flavivirus

Wei Zhang,¹† Shun Chen,^{1,2,3,*}† Suresh Mahalingam,⁴ Mingshu Wang^{1,2,3} and Anchun Cheng^{1,2,3,*}

Abstract

Tembusu virus (TMUV, genus *Flavivirus*, family *Flaviviridae*) was first isolated in 1955 from *Culex tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia. In April 2010, duck TMUV was first identified as the causative agent of egg-drop syndrome, characterized by a substantial decrease in egg laying and depression, growth retardation and neurological signs or death in infected egg-laying and breeder ducks, in the People's Republic of China. Since 2010, duck TMUV has spread to most of the duck-producing regions in China, including many of the coastal provinces, neighbouring regions and certain Southeast Asia areas (i.e. Thailand and Malaysia). This review describes the current understanding of the genome characteristics, host range, transmission, epidemiology, phylogenetic and immune evasion of avian-origin TMUV and the innate immune response of the host.

INTRODUCTION

Tembusu virus (TMUV, genus *Flavivirus*, family *Flaviviridae*) is a single-stranded, positive-sense RNA arbovirus first isolated in 1955 from *Culex tritaeniorhynchus* mosquitoes in Kuala Lumpur, Malaysia [1]. However, its relevance to human or animal health has yet to be fully revealed. Many members of the genus *Flavivirus*, including West Nile virus (WNV), dengue virus (DENV), yellow fever virus (YFV), Japanese encephalitis virus (JEV) and Zika virus, are zoonotic and important vector-borne viruses that cause millions of infections each year, ranging from mild febrile symptoms to fatal haemorrhagic/neurologic disease [2–5]. In nature, birds often serve as amplifying and reservoir hosts for many flaviviruses, such as WNV [6–8], Sitiawan virus [9], Usutu virus [10, 11] and Bagaza virus [12]. In April 2010, duck TMUV was first identified as the causative agent of duck egg-drop disease in the People's Republic of China, a disease characterized by a substantial decrease in egg laying, a sudden decline in feed uptake and neurological signs in infected egg-laying and breeder ducks [13]. Ovarian haemorrhage (i.e. hyperaemia, haemorrhage, follicle atresia

and rupture, and lymphocyte infiltration) is the main pathological change consistently observed in almost all diseased birds [13]. Infection and morbidity rates are typically high (up to 90%). Depending on the management conditions of infected birds, total mortality ranges from 5 to 15% while occasionally increasing up to 30% due to secondary bacterial infections. The specific cause of the emergence of this disease in China remains unknown. Avian TMUV has resulted in serious economic loss in the poultry industry, and because of the zoonotic nature of flaviviruses, avian TMUV may be of public health concern. Here, we summarize the current understanding of the viral genome characteristics, host range, transmission, epidemiology, phylogenetics of avian TMUV as well as interactions with the innate immune response of the host.

VIRAL GENOME CHARACTERISTICS

The RNA genome of flaviviruses is single-stranded and approximately 11 kb in length, with one unique ORF that is flanked by a type 1 capped 5'-terminal non-coding region (NCR) and a 3'-terminal NCR [14]. The genomic RNA of

Received 17 May 2017; Accepted 28 July 2017

Author affiliations: ¹Institute of Preventive Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China; ²Research Center of Avian Disease, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan 611130, PR China; ³Key Laboratory of Animal Disease and Human Health of Sichuan Province, Chengdu, Sichuan 611130, PR China; ⁴Emerging Viruses and Inflammation Research Group, Institute for Glycomics, Griffith University, Gold Coast, Australia.

***Correspondence:** Shun Chen, shunchen@sicau.edu.cn; Anchun Cheng, chenganchun@vip.163.com

Keywords: Tembusu virus; virus genome characteristics; host range; transmission; host innate immune response; vaccine.

Abbreviations: aa, amino acids; DEF, duck embryo fibroblast; DENV, dengue virus; IFIT5, IFN-induced protein with tetratricopeptide repeats; ILs, interleukins; ISGs, IFN-stimulated genes; JEV, Japanese encephalitis virus; MAVS, mitochondrial antiviral-signalling protein; MDA5, melanoma differentiation-associated protein 5; NCR, non-coding region; OAS, oligoadenylate synthase; OASL, 2'-5' oligoadenylate synthase-like; PBMCs, peripheral blood mononuclear cells; RIG-I, retinoic acid-inducible gene I; TBEV, tick-borne encephalitis virus; TLR3, toll-like receptor 3; TMUV, Tembusu virus; WNV, West Nile virus; YFV, yellow fever virus.

†These authors contributed equally to this work.

The GenBank accession numbers of the used sequences are shown in Fig. 3.

flaviviruses is infectious and has a positive polarity with regard to the coding regions of all viral proteins [15]. NCRs of different lengths [a 5' NCR from 67 (DENV) to 132 (tick-borne encephalitis virus, TBEV) bases and a 3' NCR from 114 (TBEV) to 585 (JEV) bases] flank the long ORF in different *Flaviviridae* species [16–21]. Through subsequent cleavage by viral and cellular proteases, the polyprotein is processed into three structural proteins from the 5' end of the genome [i.e. capsid (C), premembrane (PrM) or membrane (M) and envelope (E)]; seven non-structural (NS) proteins are encoded at the 3' end (i.e. NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) [14]. The structural proteins form the viral particle and are involved in viral fusion with host cells (i.e. playing an important role in virus receptor binding, entry and fusion), whereas the NS proteins participate in viral RNA replication, virion assembly and evasion of innate immune responses [14].

Sixty-six full-length duck TMUV genomes are currently available in the NCBI sequence database, and 16 complete sequences of other avian-origin TMUVs have been released, including chicken-, goose- and pigeon-origin TMUV. The genome structure of TMUV is the same as that of mosquito-borne flaviviruses. As previously reported, the length of the TMUV complete sequence ranges from 10 990 to 10 992 nt; a single ORF of the same length is predicted to encode a putative polyprotein of 3425 amino acids (aa) [18, 19, 21, 22]. The putative polyprotein-encoding region of avian TMUV is flanked by a 94- or 95-nt 5' NCR at the 5' end and a 618- to 619-nt 3' NCR at the 3' end. Similar to other flaviviruses, the TMUV genome has a type 1 cap (m7GpppAmp) structure at the 5' end and no poly (A) tail at the 3' end. TMUV RNA encodes 10 proteins, including three structural proteins, i.e. the C protein, PrM protein and E glycoprotein, and seven NS proteins, i.e. NS1, NS2A, NS2B, NS3, NS4A, 2k-NS4B and NS5 (Fig. 1). All available avian-origin TMUVs share the same genome structure and ORF identity. The predicted molecular weights are as follows: C 13 kDa; PrM 18 kDa; E 55 kDa; NS1 39 kDa; NS2A 25 kDa; NS2B15 kDa; NS3 68 kDa; NS4A 14 kDa; 2K 23aa; NS4B 28 kDa; and NS5 100 kDa. The cleavage sites, potential glycosylation sites and unique motifs/modules of avian TMUV have been previously studied. In addition, duck TMUV shares many conserved motifs with other flaviviruses, including the catalytic triad motif, the substrate binding motif and the RNA helicase motif of the NS3 and NS5 proteins [18].

HOST RANGE

TMUV exhibits a wide range of natural host species, including mosquitos, chickens, ducks, geese, pigeons and sparrows [23, 24]. Egg-drop disease affects both meat-type and egg-laying ducks and a variety of duck breeds, including Pekin ducks, Cherry Valley ducks, Shaoxing ducks, Jinyun ducks, Longyan ducks, Jinding ducks, khaki-Campbell ducks, Muscovy ducks and domesticated mallards [25]. *In vitro* laboratory studies have indicated that duck TMUV replicates well in a wide spectrum of mammalian cell lines (i.e. Vero, BHK21, HeLa, HepG2 and SH-SY5Y), cells of avian origin (i.e. DEF, GEF and DF-1) and mosquito cell lines (i.e. C6/36 and *Aedes albopictus*) [26–28]. In addition, an artificial infection study found that BALB/c mice and Kunming mice are susceptible to duck TMUV after intracerebral inoculation, but not subcutaneous (s.c.) or intranasal (i.n.) infection, with resulting clinical lesions that are typical of many other encephalitic flaviviruses and even death. The virus replicates robustly in the brain and spleen, though replication was found to be limited in many visceral organs in infected mice [29–31]. Furthermore, an investigation of duck industry workers reported detection of antibodies against duck TMUV in over 70% of the serum samples tested, with approximately 50% of oral swab samples being positive for TMUV RNA, indicating its potential transmission from ducks to human or from ducks to other non-avian hosts [25]. However, subsequent *in vivo* research showed that duck TMUV failed to cause viraemia or clinical symptoms in subcutaneously inoculated rhesus monkeys but the duck TMUV-specific humoral immune responses could be induced by limited infection [28]. Altogether, these data suggest that duck TMUV replicates well in many types of non-avian cells both *in vivo* and *in vitro* but that the risk of human disease caused by duck TMUV is not as high as that caused by other flaviviruses.

TRANSMISSION, CARRIERS, VECTORS AND SPREAD

Most flaviviruses are transmitted by different haematophagous arthropod vectors, specifically mosquitoes and ticks. Therefore, flaviviruses are divided into the following three groups: mosquito borne, tick borne and non-vector borne [29]. The first report, which was published in 2010, indicated that duck TMUV causes a disease that was able to spread across northern China, even in autumn when there

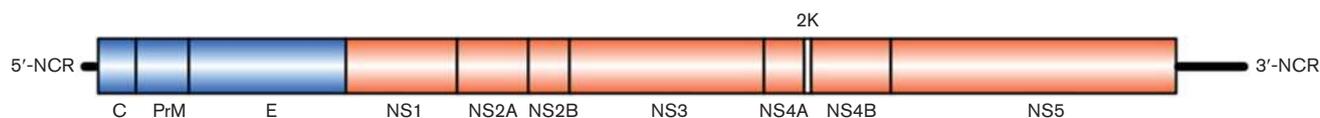


Fig. 1. The genome structure of avian TMUV. The RNA genome of avian TMUV is single-stranded and contains one unique ORF that is flanked by a type 1 capped 5'-terminal NCR and a 3'-terminal NCR. The polyprotein is processed into three structural proteins encoded by the 5' end of the genome [capsid (C), premembrane (PrM) and envelope (E)], and seven nonstructural (NS) proteins are encoded at the 3' end of the genome (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B and NS5).

is low or no mosquito activity [13]. In 2013, isolation of TMUV from *Culex* mosquitoes was first reported in the Shandong Province, China. The field strain of TMUV (TMUV-SDMS) was found to grow well in the mosquito C6/36 cell line, monkey Vero cells and duck embryo fibroblast (DEF) cells. According to phylogenetic analysis of the E and NS5 genes, TMUV-SDMS is closely related to duck-origin TMUV, which causes severe egg-drop disease [27]. Further *in vivo* experimental data have shown that TMUV RNA replicates well in *Cx. tarsalis* mosquitoes, peaking at approximately 4 days and remaining high until the end of the experiment 28 days after inoculation [26]. Both the *Cx. vishnui* and *Cx. vishnui* subgroups, but not the *Cx. Fuscocephala* subgroup, were able to transmit TMUV to naive chickens through feeding on TMUV-infected leghorn chicks, and this finding was confirmed by vector competence studies in the laboratory [32]. Although members of the *Cx. vishnui* subgroup prefer to feed on large animals, there is no evidence to date indicating that mosquitoes serve as a vector for transmitting duck TMUV from birds to large animals. Overall, mosquitoes may play an important role in the life cycle of TMUV, disease outbreak and virus transmission.

Additionally, one publication reported the identification of TMUV in the liver and from cloacal swab samples from healthy house sparrows, with TMUV being isolated from samples positive by reverse transcription (RT)-PCR [33]. The house sparrow (*Passer domesticus*), which has been implicated as an important amplifying host, plays a vital role in the transmission of numerous arboviruses [34]. In addition, bird-to-bird transmission of other flaviviruses has been previously reported [35]. Based on these data, house sparrows carrying and releasing TMUV may play an important role in transmitting the virus among birds via faecal-oral transmission, which might explain the rapid spread of the disease among the observed cases in Malaysia and the occurrence of TMUV infections in duck and goose flocks during winter months in China. However, the transmission route of duck TMUV remains unclear.

EPIDEMIOLOGY AND PHYLOGENETICS

China

Duck egg-drop syndrome, which is caused by an unknown aetiology, was recognized in Southeast China in 2010, when TMUV was first confirmed in ducks exhibiting illness [36]. During the same year, the disease quickly spread to most of the duck-producing regions in China, including many of the coastal provinces and neighbouring regions, including Beijing Autonomous City, Hebei Province, Shandong Province, Jiangsu Province, Anhui Province, Zhejiang Province, Jiangxi Province and Fujian Province (Fig. 2a). An increasing number of cases were then reported in Southwest and South China, including the Guangdong Province and the Guangxi Zhuang Autonomous Region in 2012, Chongqing Autonomous City in 2013 and Shanghai Autonomous City in 2015 (Fig. 2a, b) [37–39]. The clinical signs of

TMUV-infected ducks included depression, growth retardation, loss of appetite and even paralysis or death. Egg production by affected egg-laying ducks was severely reduced (the decrease in egg laying ranged from 20 to 60 % and was occasionally as high as 90 %), and severe ovarian haemorrhage, ovaritis and regression were consistently observed at necropsy [25, 36]. In addition, molecular phylogenetic analysis grouped together all TMUVs isolated in China (Fig. 3).

Thailand and Malaysia

The first TMUV identified in *Cx. tritaeniorhynchus* was traced back to 1955 in Malaysia [1], and this isolate was closer to all Chinese TMUV strains than to other flaviviruses (Fig. 3). In the 1970s, TMUVs were also isolated from *Cx. vishnui* and *Cx. vishnui* mosquitoes in Malaysia [40]. However, mosquito-origin TMUV had not been documented as an agent of disease in humans and animals. Until 2012, several outbreaks of a neurological disease characterized by ataxia, lameness and progressive paralysis were reported in 4- to 7-week-old broiler duck flocks at several duck farms in Malaysia (Fig. 2a) [41]. After gross pathology and histology observations, virus isolation, reproduction of the disease in ducklings and genome sequencing were performed, the aetiological agent of the disease was confirmed as Malaysian duck Tembusu, and its genomic RNA sequence was found to be similar to that of a related duck TMUV identified in China (Fig. 3).

In early 1982, vector surveillance studies using Centers for Disease Control and Prevention light traps identified TMUV in pools of *Cx. vishnui*, *Cx. tritaeniorhynchus* and *Cx. gelidus* in Northern Thailand [42]. In 1992, TMUV was isolated from *Cx. tritaeniorhynchus* in Chiang Mai, Thailand [43], and in 2002, a study reported the detection of TMUV in *Culex* mosquitoes and sentinel ducks in the same area of Thailand [32]. However, there was no report of any disease caused by TMUV in birds until 2013, a time when a severe contagious disease of egg-drop syndrome newly emerged in layer and broiler duck farms in Thailand (Fig. 2a) [44]. The disease rapidly spread and caused heavy economic losses for both traditional and agro-industrial duck businesses. Duck TMUV was ultimately identified as the causative agent of this emerging disease. Based on phylogenetic analysis, transmission of TMUV to South Asia is believed to have originated from mainland China (Fig. 3). Indeed, mosquito TMUV isolates grouped with duck serum TMUV isolates from China (Fig. 3) and a historical duck TMUV isolate from Thailand was identical to historical duck TMUV isolates from Malaysia and the newly isolated duck TMUV from China.

THE INNATE IMMUNE RESPONSE TO AVIAN TMUV INFECTION

Host innate immune defence mechanisms against avian TMUV infection have been broadly investigated. Duck immune-related genes [i.e. retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), toll-like receptor 3 (TLR3), IFNs, MHCs,

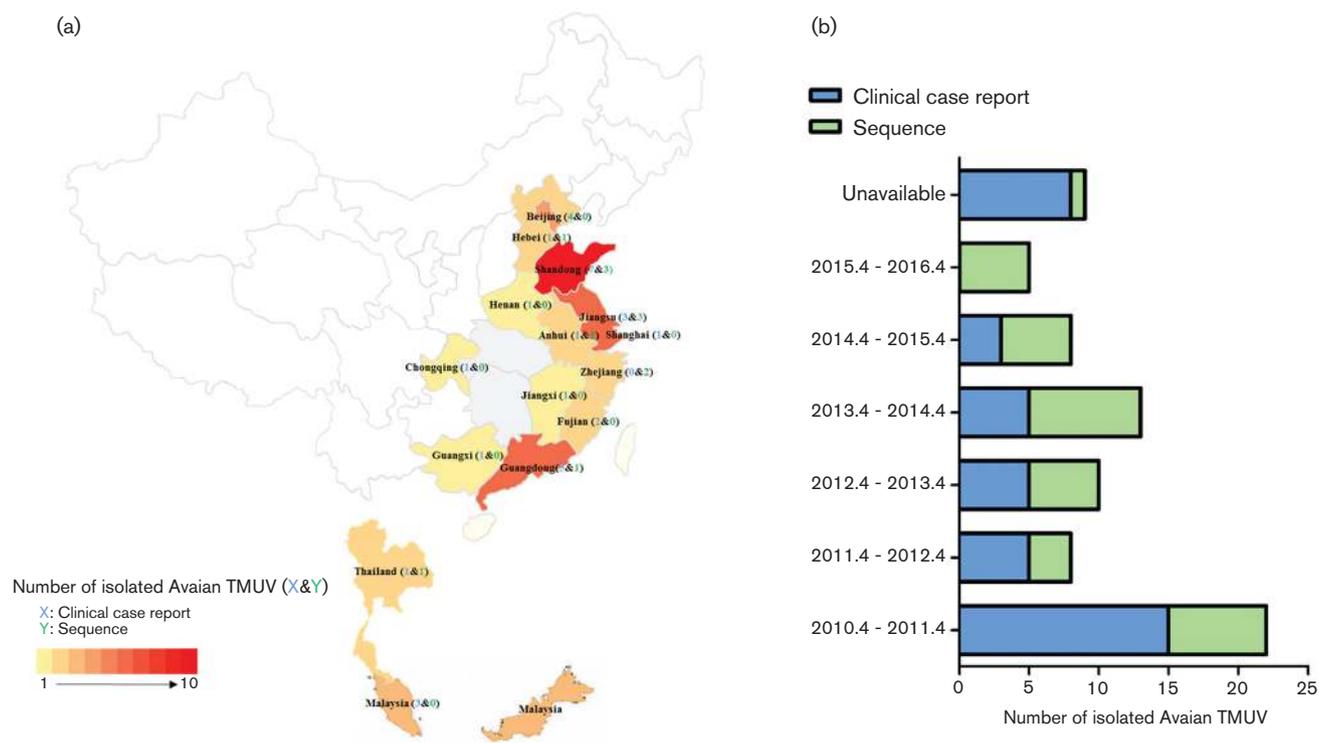


Fig. 2. Distribution of avian TMUV. (a) The area distribution of avian TMUV. The provinces, autonomous regions or municipalities affected are highlighted in gradual colour according to the number of isolated viral cases. The map is drawn based on reports of virus identification with sequence information (X) and records in the NCBI sequence database (Y). (b) The registration of avian TMUV in the GenBank database and the reporting of clinical cases in a time sequence since April 2010.

interleukins (ILs), C-X-C motif ligand 8 and IFN-stimulated genes (ISGs)] and viral distribution in TMUV-infected ducks have been systematically examined by quantitative real-time PCR, with the results indicating that the virus replicates rapidly in many tissues and that host immune responses are activated during the early infection phase [45, 46]. Our previous research indicated that relative transcriptional levels of IFNs and ILs are significantly upregulated in goose peripheral blood mononuclear cells (PBMCs) after stimulation with duck TMUV. More importantly, using TMUV-infected geese as a model, immunohistochemical analysis suggested that the antigen distribution of TMUV is coincident with the location of CD8+T cells, with an association with high production of IFN γ and proinflammatory cytokines (IL1 β and IL6) in virus-preferred tissues [47]. All of the above-mentioned results show that the host actively responds to the invading TMUV pathogen. However, the interaction between upregulated cytokines and TMUV remains unclear. Nonetheless, one study attempted to identify the TMUV-induced signalling pathways downstream of pattern recognition receptors. The mRNA levels of IFNs and ISGs were strongly induced by TMUV both in chicken embryo fibroblasts and inhuman 293T cells, mainly through MDA5 and TLR3-dependent pathways [48]. However, these experiments were performed in human 293T cells and not

in avian cells. Although avian TMUV can proliferate in mammalian cells and is a potential health risk to humans, avian TMUV is only highly pathogenic to domestic poultry, particularly ducks and geese. Therefore, whether avian TMUV infection can trigger IFN-dependent host antiviral immune responses through MDA5 and TLR3-dependent signalling should be further confirmed in avian cells.

Using flow high-performance liquid chromatography-electro spray tandem mass spectrometry, 131 differentially expressed proteins (53 upregulated and 78 downregulated) were found between TMUV-infected and mock-infected duck ovarian follicles. An abundance of several proteins involved in the immune response and antigen processing and presentation was observed; of these proteins, upregulation of IFN-induced protein with tetratricopeptide repeats (IFIT5) and 2'-5' oligoadenylate synthase-like (OASL) levels by TMUV infection were validated by Western blot analysis [49]. IFIT5, which partially co-localizes to mitochondria and interacts with RIG-I and mitochondrial antiviral-signalling protein (MAVS), is an important enhancer of the innate antiviral immune response [50]. The oligoadenylate synthase (OAS) family belongs to a nucleotidyltransferase family, which is a family of IFN-induced cellular proteins [51]. OAS family proteins exhibit antiviral effects against various viruses (i.e. DENV, WNV, HCV and JEV)

employ different strategies to interfere with the host immune response. The E protein of WNV hinders ubiquitination of RIP-1 to inhibit RIG-I-mediated and TLR3-dependent antiviral pathways [62]. The NS2A, NS4A and NS4B proteins of DENV reduce phosphorylation and nuclear translocation of signal transducer and activator of transcription 1, ultimately suppressing IFN signalling [63, 64]. However, there are only a few reports to date regarding the role of NS1 in interfering with the innate immune response. NS1 is believed to function as a co-factor in viral RNA replication [14], though reports regarding interaction of NS1 with TLR3 are contradictory [65]. Some studies have shown the increased presence of TLR3 in WNV infection *in vivo* [66]. Another study indicated that WNVNS1 inhibits TLR3 signal transduction [67, 68], whereas the NS1 protein of several mosquito-borne flaviviruses cannot inhibit TLR3 signalling [69]. Very recently, by using a reporter assay to screen duck TMUV-encoded structural (i.e. C and E) and non-structural (i.e. NS1, NS2A, NS2B, NS3 and NS5) proteins involved in immune evasion, a study indicated that duck TMUV NS1 markedly suppresses expression of Sev-triggered IFN- β by inhibiting RIG-I-like receptor signalling [70]. Furthermore, based on co-immunoprecipitation and immunoblot analyses of truncated NS1 and MAVS derivatives, authors have reported that all NS1 truncations interact with the C-terminal domain of MAVS; this subsequently impairs the association between RIG-I or MDA 5 and MAVS, thereby downregulating RIG-I-mediated signal transduction and cellular antiviral responses [71]. Altogether, similar to other flaviviruses, duck TMUV may be an effective IFN antagonist in avian cells. Regardless, the strategies and mechanisms employed by duck TMUV proteins to evade the innate and adaptive immune response remain largely unknown.

CONCLUSIONS AND FUTURE PROSPECTS

Avian TMUV is a newly emerging avian pathogenic flavivirus that is causing massive economic losses in the poultry industry in China and Southeast Asia. The underlying reasons for the emergence of duck TMUV in the past 5 years remain unknown. Possible explanations include viral mutations affecting transmission or virulence, leading to an epidemic spread. Although duck TMUV has not been recognized as a disease-causing agent in humans or other mammals, avian TMUV replicates well in mammalian cells, and mice are susceptible to duck TMUV via intracerebral inoculation, exhibiting clinical lesions typical of many other encephalitic flaviviruses. Therefore, the possibility exists that this virus might emerge as a zoonotic pathogen. The tissue distribution profiles of duck TMUV in adult male and female ducks and the effects of age and inoculation route on duck infection have been studied [72–74]. However, the pathogenesis, molecular aetiology and interplay between the TMUV pathogen and bird host remains unclear. Due to these limitations, effective duck TMUV-specific therapeutic protocols and commercial laboratory diagnostics for avian TMUV remain unavailable for its treatment and

discriminating diagnostics. It is important to note that infectious full-length cDNA clones of duck TMUV have been constructed and rescued based on a reverse genetics system [73–75], which will be a useful platform for further studying duck TMUV pathogenesis and facilitating the development of novel vaccine candidates.

Funding information

We are supported by grants from the National Key R&D Program (2016YFD0500800), China Agricultural Research System (CARS-43-8), and National Science and Technology Support Program (2015BAD12N05).

Conflicts of interest

The authors declare that there are no conflicts of interest.

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