Review

New insights in the biology of the emerging Tembusu virus

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Abstract: Reported for the first time in 1955 in Malaysia, Tembusu virus (TMUV) remained for a long time in the shadow of flaviviruses with human health importance such as dengue virus or Japanese encephalitis virus. However, since 2010 and the first large epidemic in duck farms in China, the threat of its emergence on a large scale in Asia or even its spillover into the human population is becoming more and more significant. This review aims to report current knowledge on TMUV from viral particle organization to the development of specific vaccine and therapeutic with a particular focus on host-virus interaction.

Keywords: TMUV; emergent arboviruses; zoonosis; host-pathogen interactions; vector

1. Introduction

Tembusu virus (TMUV) is an emerging arbovirus (arthropod-borne virus), which was initially identified in Malaysia in 1955 from *Culex tritaeniorhynchus* mosquitoes collected in Kuala Lumpur and then occasionally reported in different surveys in Southeast Asia (SEA) during the 70's [1,2]. Like Japanese encephalitis (JEV) and dengue (DENV) viruses, two of the most important endemic arboviruses in SEA, TMUV belongs to the genus Flavivirus in the Flaviviridae family but is a member of the Ntaya group. Until the first major outbreak of TMUV in ducks in 2010, the virus was largely neglected and studies were scarce (Fig. 1). Since 2000, new variants coined Sitiawan virus, Duck Tembusu virus (DTMUV) or Baiyangdian virus (BYD) were identified reported to cause avian outbreaks [3-5]. However, the latter viruses are not isolated species since they are closely phylogenetically related to the original TMUV strain of 1955. Sitiawan virus, DYMUV and BYD are now classified in the TMUV clade/monophyletic group and distributed in different sub-clusters. In this review, we will refer to all these viruses as TMUV, except when specific description have been associated with one sub-cluster.

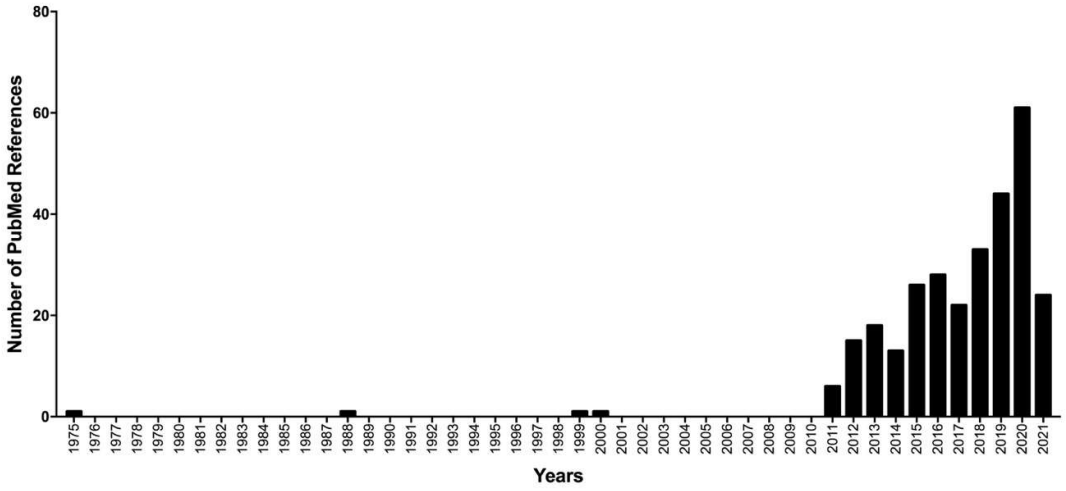

Figure 1. Publication related to TMUV in PubMed database. Articles published between 1975 and 2021. Recording of publication was done using the term "Tembusu virus" and reported annually.

TMUV cause encephalitis and neurological disorders in avian populations, resulting in morbidity rates up to 90%, associated with damage to the female reproductive system leading to severe loss of egg production in farms [4]. Since 2010, TMUV caused several outbreaks and has been detected repeatedly in China and SEA, underscoring the emergence and circulation of this virus outside of its original geographic region. Given the recent regional expansion and associated large economic losses in the poultry industry, TMUV should now be considered an emerging infectious disease.

To date, knowledge on TMUV ecology and biology remains incomplete and the driving cause of sporadic emergence remains unknown. This strongly contrast with other emerging arboviruses causing human diseases such as Yellow fever (YFV), DENV, West Nile (WNV), JEV and Chikungunya (CHIKV) viruses. In the last decade, important progresses in the understanding of TMUV biology were made through both experimental animal models and *in vitro* studies. Here, we review the epidemiology, virus-host interaction and cell biology of avian TMUV infection. We also discuss approaches to increase knowledge for this virus in order to properly evaluate the associated risk of spillover in humans.

Tembusu virus

Genomic organization and replication

As other flaviviruses, viruses from the TMUV group have a ~11 kb positive strand RNA genome, which is composed of a single open reading frame (ORF) flanked by 5'terminal and 3'terminal non-coding regions (UTR) (Fig. 2). The 5' end is capped with m7GppAmp structure (type 1 cap) and 3' end is free from poly A tail. The ORF is predicted to be translated into a single polyprotein that is subsequently cleaved by host and viral proteases in three structural proteins, envelope (E), membrane precursor (prM) and capsid (C), as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Viral proteins are necessary for viral replication and assembly [6]. The virion has an icosahedral capsid enclosed by a lipid envelope with a diameter of 30 to 60 nm [4,5,7].

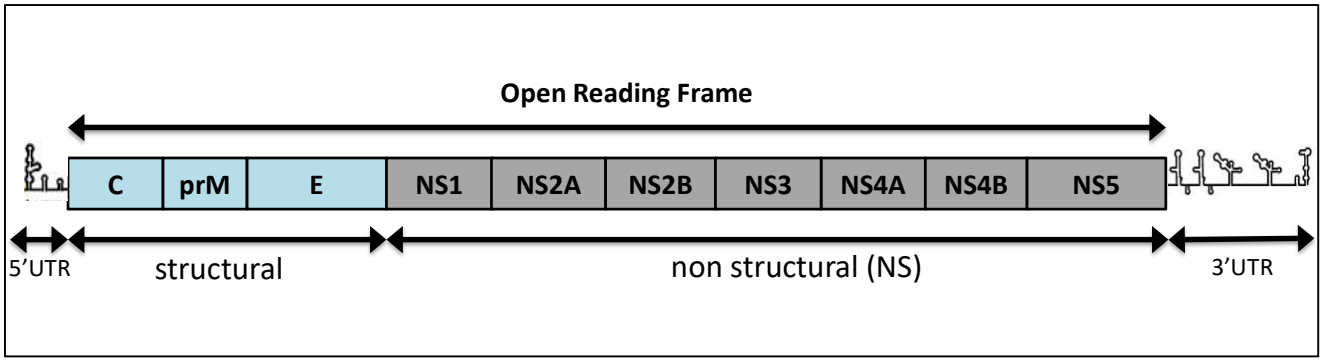

Figure 2. Structure of TMUV genome. The RNA genome is similar to other flaviviruses, with an open reading frame encoding three structural proteins, C, prM and E, and five nonstructural proteins NS1 to NS5. The structural and nonstructural proteins are generated after proteolytic cleavage of the polyprotein. Untranslated regions are shown schematically.

Despite a lack of information on TMUV replication, this process is thought to me more or less identical to that of other members of the flavivirus genus [6]. Prior to cell internalization, flaviviruses bind to a set of the cellular surface factor via cellular receptors. This complex process involves different type of cell surface receptors and interaction with external viral proteins such as E and M proteins [8]. The E protein is involved in surface receptor binding and faciltates viral entry and the subsequent fusion steps between the viral envelope and the intracellular membranes [9]. At the cell surface membrane, Heparan sulfate receptor family, C-type lectin receptors or phosphatidylserine receptors facilitate flavivirus entry, although their exact role remains poorly understood [8]. Given its recent identification, TMUV cellular receptors are less known than for other members of the Flavivirus genus. Experiments with mammalian (BHK-21) and avian (DEF) cells using different drug treatments targeting surface receptors revealed the implication of heparan sulfate molecule in recognition and cell attachment [10]. This precursor study has paved the way to a better understanding of TMUV attachment and entry mechanisms.

A range of endocytic pathways are usually used by flaviviruses to penetrate into the cell [11]. Zhang *et al*, using hamster kidney BHK-21 cell line, reported the involvement of three different endocytosis pathways in the entry process of TMUV. Authors showed an impact on virus infection when they antagonized clathrin-dependent endocytosis by chlorpromazine and dynasore treatment or after depletion of cholesterol, suggesting involvement of clathrin and cholesterol-dependent endocytosis in viral replication. In contrast, treatment of cells using siRNA targeting CAV1 or genistein inhibitor shows that the caveolin pathway is not required for TMUV entry [12]. These results confirmed the first observations reported by Baloch *et al* on the role of the clathrin endocytosis pathway in TMUV entry [13]. In this study, using chemical treatment and knockdown experiments, the authors also showed the involvement of the proteasome and low-pH endosome in the internalization process of TMUV. Indeed, the ubiquitin-proteasome pathway represents an important cellular proteolytic mechanism mediating a range of cellular processes including virus entry. In this respect, TMUV was reported to take advantage of this mechanism for cell entry [13].

To complete its cellular cycle, TMUV has to 1) translate its genomic RNA into viral proteins, 2) replicate its viral RNA genome, 3) encapsidate its genome, 4) assemble the genome into immature virion in the endoplasmic reticulum and 5) mature the virus particles in the Golgi apparatus; and 6) be secreted as infective virus particles in extracellular space by exocytosis. Although these steps are not well known for TMUV, they do not vary among flaviviruses and have been extensively described elsewhere [14,15]. TMUV translation and replication occur in the endoplasmic reticulum (ER). Different cellular mechanisms might likely facilitate replication, evasion and propagation of the virus, and notwithstanding recent progress [16], the exact mechanisms remain to be determined. Moreover, given the importance

of the UTR regions in flavivirus replication, a comprehensive analysis of 5′ and 3′ untranslated regions and their functions in TMUV cycle need to be elucidated as well. Together, the identification and characterization of host factors interacting with viral RNA and proteins is crucial for a better understanding of TMUV replication.

TMUV present a typical flavivirus structured 3′UTR, which plays an important role in the virus fitness [17]. During the intracellular replication, this region leads to the formation of an RNA molecule call subgenomic flavivirus RNA (sfRNA). This highly structured RNA fragment has a critical role in viral replication and pathogenesis [18]. Thus, despite current lack of direct evidence, sfRNA from TMUV is highly likely to have an effect on TMUV cellular cycle and pathogenesis in vertebrate hosts and vectors. It is also possible that sfRNA evolution drives modification in viral pathogenesis pattern and thus host range [19].

Phylogeny

The TMUV members belong to the *Flaviviridae* family, genus *Flavivirus* [9]. Based on the vector type, the Flavivirus genus is divided in four different groups: 1) mosquito-borne, 2) tick-borne, 3) insect-specific and 4) unknown vector flaviviruses [20]. Mosquito-borne flaviviruses (MBF) can be subdivided into *Aedes* and *Culex*-transmitted viruses. *Aedes*-borne viruses, including DENV, YFV, Zika virus (ZIKV), are generally associated with hemorrhagic fevers and non-human primate ancestors and those transmitted by *Culex* genus with meningoencephalitis diseases and bird reservoir as JEV, WNV, TMUV [21]. *Culex* transmitted MBF are classified depending on the serology onto two groups, the Japanese encephalitis serocomplex including JEV and WNV and the Ntaya serocomplex including Ntaya virus and TMUV.

TMUV group is genetically distinct within the Ntaya serocomplex and includes homologous strains that were initially considered as single species, including Sitiawan virus [5], BYD virus [4], Perak virus [3], Duck egg-drop syndrome virus (DEDSV) [4,22] and Duck Tembusu virus (DTMUV) [23]. These viruses are now phylogenetically linked in different sub-clusters within the TMUV group (Fig. 3). The first TMUV was isolated from *Cx. tritaeniorhynchus* in Malaysia in 1955 [24] and represents the TMUV strain the closest to other members of Ntaya serogroup. Virus strains related to the TMUV group were then reported in the 1990s in mosquitoes (refer to dedicated section below) in the north of Kuala Lumpur, in Sitiawan and in Sarawak, on Kalimantan island (Borneo) in Malaysia and in Thailand [1,2,25]. From our phylogenetic analysis with the available sequences, all strains isolated until 2000, except one identified in Thailand in 1992, are grouped in TMUV cluster and are closely related to viruses belonging to the Ntaya serogroup. Interestingly, a recent strain isolated in Taiwan from *Cx. annulus* and *Cx. tritaeniorhynchus* mosquitoes, was grouped in the cluster TMUV, despite the fact that no TMUV has ever been reported in Taiwan [26]. Long-distance travel via migratory birds followed by a low-level maintenance in wild birds in Taiwan may explain the recent identification of TMUV in Taiwan [27,28]. Similar spreading mode was observed for other flaviviruses such as JEV and avian influenza viruses.

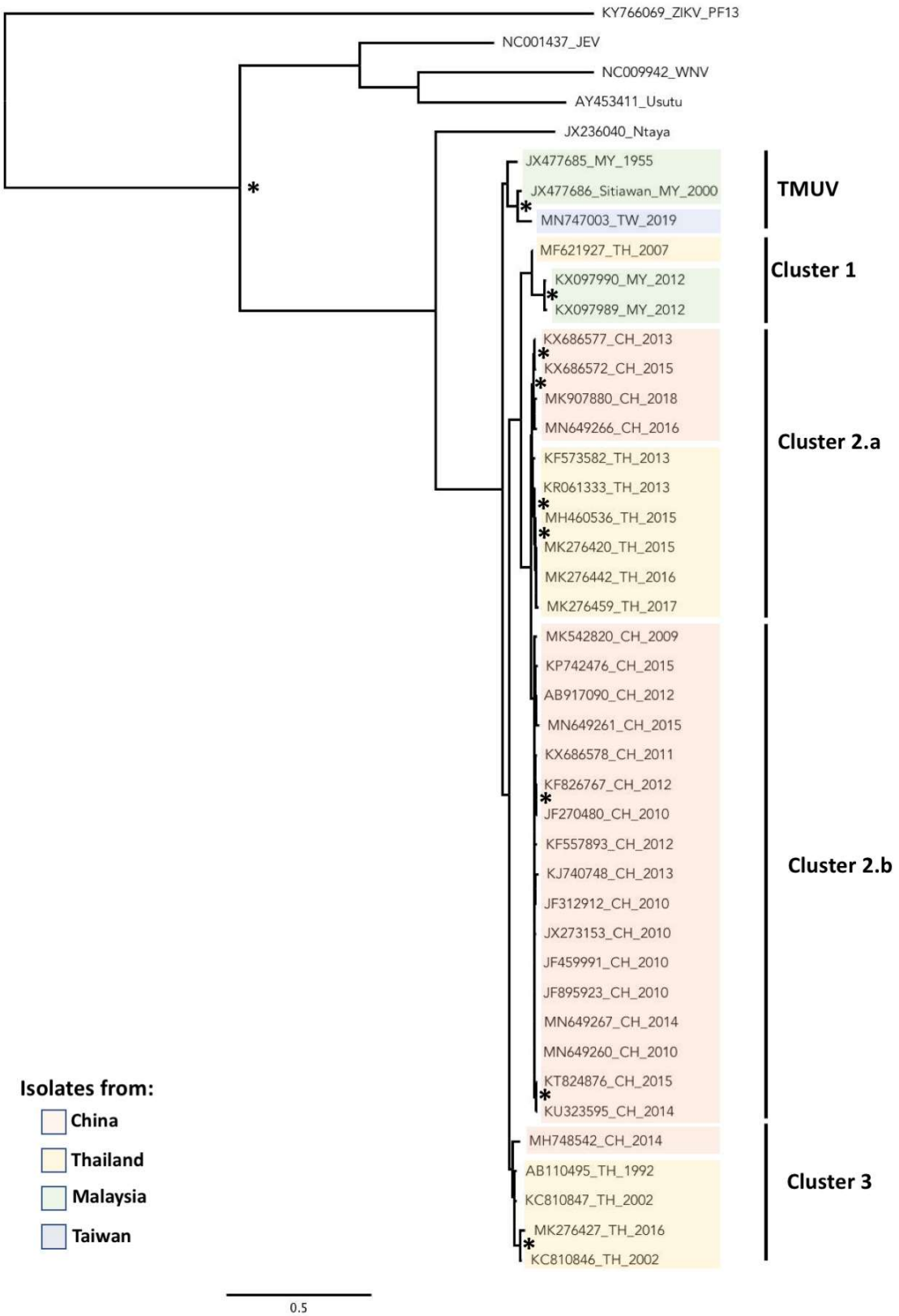

Figure 3 Phylogenetic analysis of TMUV strains. Non-concatenated nucleic acid sequences of the partial E genes from sequences available on GeneBank data base were aligned using MEGA7. Sites that could not be unambiguously aligned were excluded and

divergent regions were excluded from subsequent analyses. Phylogenetic trees were generated using the GTR+G substitution model using PhyML software [29] and edited with FigTree v1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/). Bootstrap method was used to measure the robustness of nodes with 1000 iterations. * represent bootstrap value with a score higher than 80%. Identified TMUV Strains are differentially colored in the tree depending of country origin. Annotations on the right denote TMUV cluster. All sequence accession numbers used in this review are shown in Supplementary table.

Since the beginning of the 21st century, TMUV circulation has been reported in SEA and China [30]. However, these viral isolates were found to be phylogenetically separated from the TMUV cluster and have been grouped in three different lineages comprising strains either isolated in Malaysia, in Thailand or in China. A virus isolated in 2007 in Thailand formed a monophyletic lineage with two strains isolated in Malaysia in 2012 and was named Cluster 1 in our phylogenetic tree (Fig. 3) [3]. In 2010, for the first time, TMUV was reported in China and caused an outbreak in egg-laying ducks [23]. TMUV was then regularly observed in China during the last decade and a pattern of exclusive spreading in China was recently proposed [31]. Most strains isolated in China and Thailand during the last decade form a clade named Cluster 2. However, Cluster 2 is clearly subdivided in two sub-clusters, Cluster 2.a and 2.b. Interestingly, the majority of strains isolated in Thailand since 2010 and a few strains from China belong to the Cluster 2.a, whereas only isolates originated from China are grouped in Cluster 2.b (Fig. 3). Recently, two different research teams identified a novel cluster in the TMUV group named Cluster 3 [31,32]. Based on our phylogenetic analysis (Fig. 3), we also described this Cluster 3 which gathered only viral isolates from Thailand and China. However, while Qiu *et al* described only one strain from China and Ninvilai *et al* reported two strains isolated in 2014 and 2016 in China and Thailand respectively, our analysis identified three more strains associated with this cluster isolated in 2002 and as early as 1992 in Thailand. This observation suggests that this new cluster is specifically located in Thailand with a recent introduction to China. This finding is based on the tree inferred using the available E sequences.

Altogether, phylogenetic analysis suggests a circulation through insular and land territories of SEA up to the 2000s. Since 2010, TMUV has then been circulating actively in China and in SEA with potential different introductions in China from SEA. The isolation in Taiwan of a strain that groups with the original TMUV cluster has led to speculation that the viruses were introduced by migratory birds and this may hold true for earlier introductions in Asia. In Cluster 2 and Cluster 3, the phylogenetic association between strains from China and Thailand and in Cluster 1 with strains from Thailand and Malaysia indicates a spatial segregation of the TMUV genotypes within Asia. However, despite increasing information on the regional phylodynamic of the TMUV, many questions remain about its circulation, particularly about the dynamic of the virus in other Asian countries. Regarding the importance of regional exchange for farming and agriculture trading in Asia and also with regards to migration flows of birds, TMUV can be expected to continue its expansion and this should be investigated.

Epidemiology

Vector

TMVU was first isolated from a *Culex tritaeniorhynchus* mosquito captured in Kuala Lumpur, Malaysia in 1955 and has subsequently been described in different *Culex* species (Table 1). In nature, *Culex* mosquitoes appear to be the preferential vectors of TMUV as for other bird's flaviviruses like WNV, JEV. It is presently unknown whether *Aedes* mosquitoes, which are the major vectors of important flaviviruses such as DENV, are also able to transmit TMUV, although competence study showed that *Aedes albopictus* can be infectd by TMUV [33]. Recently, *Culex tritaeniorhyncus*,

Cx. quinquesfasciatus and *Cx. pallens* were showed to be susceptible with TMUV strains isolated in China. However, despite the presence of virus in salivary gland for *Cx. pallens*, Guo *et al* found that only *Cx tritaeniorhynchus* and *Cx. quinquesfasciatus* were able to transmit the virus to ducks [33]. Despite the latter report and evidence for TMUV infection in *Cx. quinquefasciatus* under natural conditions [34], a recent laboratory study did not confirm the vector competence of this species for TMUV. Unlike *Cx. tritaeniorhynchus* that may be concider has a major vector. In the same line of thought, *Cx tritaeniorhynchus* is mainly found in farms and play a major role in the dissemination of TMUV in bird population in Thailand [35,36]. The widespread detection of TMUV in various *Culex* species in different countries indicates that a more thorough evaluation of the vector capacities of local *Culex* species should be undertaken. This will allow authorities to assess the capacity of TMUV to spread in areas where *Cx. tritaeniorhynchus* is absent, or present in small numbers, and where other *Culex* species are endemic. The intrinsic incubation period during which virus replicates in the mosquito to reach salivary glands should also be evaluated to estimate transmission capacity.

Table 1. Mosquito species found infected by TMUV

Country/location of origin	Species	Year	References
Kuala Lumpur, Malaysia	*Cx tritaeniorhynchus*	1955	[37]
Malaysia	*Cx. vishnui*	1970	[2]
Kamphaengphet Province, Thailand	*Cx. gelidus* *Cx. tritaeniorhynchus* *Cx. vishnui*	1982	[25]
Chiang Mai Province, Thailand	*Cx. tritaeniorhynchus*	1992	[1]
Kamphaeng Phet Province, Thailand	*Cx. vishnui*	2005	[38]
Shandong Province, China	*Cx. pipiens*	2010-2012	[39]
Yunnan Province, China	*Cx. tritaeniorhynchus*	2012	[40]
Shandong Province, China	*Cx. spp*	2012	[41]
Sing Buri Province, Thailand	*Cx. tritaeniorhynchus*	2015	[36]
Kanchanaburi Province, Thailand	*Cx. quinquefasciatus*	2015	[34]
Taipei, Taiwan	*Cx. annulus*	2019	[26]
Taichung, Taiwan	*Cx. tritaeniorhynchus*	2019	[26]

Vertebrate Host

The first presence of TMUV in a vertebrate host was reported in 2000 in chicks in Malaysia [5]. TMUV host range was then reported to be similar to avian hosts of the Avian Influenza virus, which causes disease in poultry in over 50 countries worldwide since 1997 [42]. TMUV was mostly isolated from ducks in industrial farms. However, the presence

of the virus in free-grazing ducks has been reported in Thailand and may play an important role in the spreading of the virus through avian fauna [43]. Besides ducks, a wide range of birds, including geese, chickens, sparrows and pigeons are naturally infected by TMUV (Table 2). These wild birds are likely to play a crucial role in the spread of the virus to farm birds, not only via arthropod vectors but also as a result of close contact or expectoration between animals.

Several members of the flavivirus family, such as DENV and ZIKV, induce pathology in humans [44,45]. However, the presence of TMUV in humans or other vertebrates is not well-documented. TMUV infections in humans and non-human primates were mainly detected by post-infection serological surveys and pathogenesis in mammals remains unclear [46]. Finally, it is not yet known whether vertebrates other than birds can serve as reservoirs or amplifying hosts or whether they are potentiel dead-end hosts with viral loads insufficient for active transmission to mosquito vectors. Moreover, as for other flaviviruses, a high percentage of TMUV-infected individuals may be asymptomatic or present only with weak symptoms, thereby hiding the spread of the virus in the human population and the risk for potentially large threat outbreak.

Table 2. Vertebrate hosts found naturally infected by TMUV

Country/location of origin	Species	Year	References
Perak state, Malaysia	Broiler Chick	2000	[5]
Thailand	Duck	2007	[30]
Shandong province, China	Human	2010-2012	[47]
Shanghai, China	Duck	2010	[48]
Shandong province, China	Meat duck Layer duck	2010	[49]
Henan province, China	Layer duck	2010	[49]
China	Pekin duck, Cherry Valley Pekin duck Shaoxing duck Breeder duck	2010	[4,23,50,51]
China	Goose	2010	[50]
China	Chicken	2010	[52]
Jiangsu Province, China	Goose	2010	[53,54]
Shandong Province, China	House sparrow (*Passer domesticus*)	2010-2011	[55]
Guangdong province, China	Layer duck	2011	[56]
Guangxi province, China	Layer duck	2011	[49]
Shandong Province, China	House sparrow	2012	[41]
Beijing Autonomous City, China	Pigeon	2012	[22]

Shandong province, China	Goose	2012	[41,57]
Hebei province, China	Duck	2012	[22]
Jiangsu province, China	Egg-Laying duck	2012	[49]
Shandong province, China	Duck	2012	[49,57]
Fujian province, China	Duck	2012	[58]
Malaysia	Pekin duck	2012	[3,59]
China	Duck	2013	[60]
Guangxi Province, China	Cherry Valley duck	2013	[61,62]
Shanghai Province, China	Pekin duck	2013	[63]
Shandong province, China	Layer duck	2013	[41]
Anhui province, China	Layer duck	2013	[41]
Thailand	Duck	2013	[64]
Thailand	Broiler chicken	2013	[32,65]
China	Chicken	2013	[41]
China	Goose	2014-2015	[66]
China	Chicken	2014	[67]
China	Layer Duck	2014	[68]
China	Mallard *(Anas platyrhynchos)*	2014	[69]
China	Broiler Duck	2015	[70]
China	Layer Duck	2015	[71]
Guangdong province, China	Muscovy duck	2015	[72]
Thailand	duck	2015	[73]
Shandong province, China	Meat duck	2016	[41]
Inner Mongolia Autonomous Region, China	Meat duck	2017	[41]
Thailand	Free-grazing duck	2018	[43]

Geographic distribution

Following the first isolation of TMUV in mosquitoes in Malaysia in the mid 50s [37], TMUV has been sporadically reported in SEA, mainly in wild and domestic birds and in trapped mosquitoes [2,5,25] (Fig. 4). Subsequent entomological, serological and virus isolation studies pointed out a geographical distribution restricted to a few countries. While TMUV was discovered more than 70 years ago, the presence of the virus in human was not reported before 2000 with the finding of TMUV in natives and migrants in Borneo, Indonesia, and in farm workers in China [46,47]. The first marked TMUV outbreak was reported in 2010 in China in egg-laying ducks [23]. The dramatic decrease

in egg production (eggs drop syndrome), associated with neurological manifestations in ducks, reaching up to 90% of animals locally, highlighted the potential nuisance of its threat to intensive duck farming. Since then, several epizooties were reported in China, Thailand, Malaysia and in Taiwan [3,26,64]. Following the identification of new TMUV variants in Thailand in 2015, a retrospective study confirmed the presence of the virus as early as 2007 [30].

Figure 4 Geographic distribution of TMUV in Asia. Map representing countries where TMUV were reported. Created with map-chart.net.

Since 1955, the presence of TMUV has been reported in few countries in SEA as well, although it has to be noted that these countries are geographically very distant with no common land border for some. Despite the lack of information about the virus distribution and its mode of transmission, human population movements and regional trade may provide a partial explanation for the distribution. The impact of bird migration may also explain the emergence of the virus in countries distant from its site of origin. Finally, as TMUV mainly affects birds, especially wildlife species, its presence may have been unreported or underestimated, until farms with large numbers of fowls in close proximity were affected [47,74].

Transmission

TMUV transmission occurs during mosquito biting on a vertebrate host (Fig. 5). However, although the bite of an infected mosquito is probably the main mode of transmission, the existence of other modes may exist. Vertical transmission within mosquitoes was examined for *Cx tritaeniorhynchus* and *Cx. Quinquefasciatus*, but the progeny was not infected [35]. Nevertheless, further studies are needed to clearly assess the vertical maintenance of infection in mosquitoes.

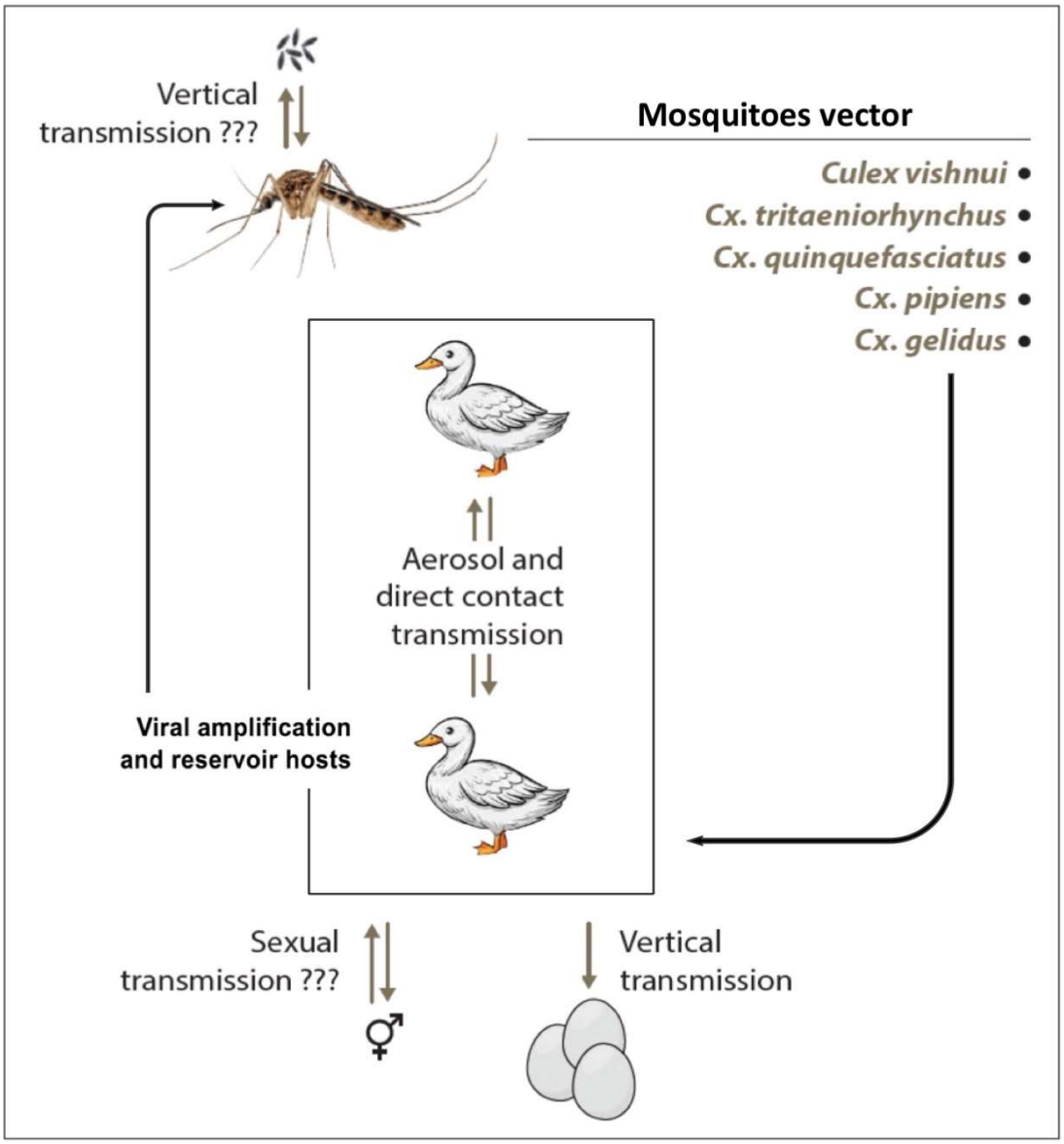

Figure 5. Summary of the transmission cycle for TMUV.

Birds are the main hosts of the virus and vectors of *Culex* genus associated with birds play a major role in transmission. Observation of TMUV-affected animals shows that the virus has been detected in the ovaries of birds and in eggs. Intrauterine transmission from mother to chick is therefore possible and leads to abortion of the gestating eggs. This clinical presentation is reminiscent of the ability of ZIKV to be transmitted in humans from mother to child during

pregnancy, resulting in abortion or congenital malformations [75]. Although sexual transmission has not yet been demonstrated, non-vector transmissions were reported in birds by airborne contamination or by close contact between animals particularly during winter season and the periods of arthropod inactivity [73,76,77]. Therefore, the regular presence of wild birds on farms, especially outdoors, could allow transmission between domestic birds and maintenance of the virus on the farm without the intervention of an arthropod vector. Finally, the transmission of TMUV to non-human primates and humans has been demonstrated by results of serological studies, as well as by the presence of viral RNA [46,47]. The role of vertebrate hosts other than birds in viral transmission is not known at present but should be evaluated.

Clinical features and pathogenicity

Symptomatology/diseases presentations

TMUV is a mosquito-borne epornitic flavivirus, like WNV and Usutu virus (USUV), with its natural maintenance cycle involving birds and mosquitoes [6]. TMUV is an avian epizootic agent which was mainly reported in ducks during several outbreaks in China and in Thailand. The etiology of the infection in birds is characterized by sudden acute signs including rhinorrhea, diarrhea, anorexia and perturbation of social behavior. Infected animals also show severe neurological disorders including encephalitis, difficulty to move or imbalanced movement, ataxia and paralysis [30]. The infection rate can reach 90% in a farm, depending on husbandry conditions, whereas the mortality rate is variable ranging from 5% to 30% of the infected flocks and is increased by secondary infections [23]. Spleen, liver, kidney, brain usually present gross lesions. Histologic analysis shows multiple microscopic changes in infected organs with hemorrhage, inflammation, hyperplasia and macrophage and lymphocyte infiltration [3,64]. The common clinical presentation of the disease observed in infected female ducks is a generalized damage to the ovaries with large inflammation, hemorrhage, hyperemia associated with organ degeneration [4,30,78]. TMUV infection is associated with a degeneration of embryos and a dramatic decrease in eggs production coined “egg-drop syndrome”, leading to important economic losses both in traditional or industrial poultry production in Asia [55]. A recent study reported that ducks of all ages are potentially susceptible to infection with TMUV – cluster 2. Although a greater severity was reported in younger animals in some duck populations [79], a higher susceptibility to the infection was reported in old breeding ducks [78]. Moreover, older infected ducks present a longer shedding period with a high viral loads without clinical signs suggesting the potential role of these animals in the spread of the virus during outbreaks [76].

It is now clearly established that TMUV is a neurotropic virus for birds although the mechanisms of diffusion at the neuronal level are still poorly understood. However, a recent study on a duckling model had shed light on the modalities of passage from the blood-brain barrier (BBB) to the central nervous system [80]. Thus, in the early stages there are few clinical symptoms and, although there is a propagation of virus in the brain with a corollary induction of inflammatory cytokines, the BBB seems to remain relatively impermeable to the virus. It is therefore necessary to wait for a more advanced stage of the infection to see the first neurological symptoms associated with a disruption of the BBB and a diffusion in the microvascular endothelial cells causing an inflammatory storm and fatal encephalitis in the infected animal.

While symptoms due to TMUV infection are remarkably well described in birds, disease presentations in other vertebrate hosts are sparse. In 2020, Yurayart *et al* reported neuropathogenesis and global dissemination in mice intracerebrally infected with TMUV. The animals exhibited a wide range of clinical signs with additional severe internal organ lesions that led to death [81]. Because TMUV impacts poultry farms, Tang *et al* investigated the presence of TMUV infection in avian farm workers [47]. Oral swab and serum samples were collected for molecular and serological

screening for the presence of TMUV. Semi-nested RT-PCR was used as a molecular method whereas IgG ELISA and a virus neutralization test were performed for serological assays. More than 70% of serum samples contained detectable levels of anti-TMUV antibodies, whereas TMUV could be isolated from 48% of oral swabs. Asymptomatic presentation was observed in infected workers as well. Moreover, the authors reported for the first time a potential spillover of TMUV to humans and suggested to consider TMUV as an emergent zoonotic pathogen, although, like for other avian flavivirus members, human spillover events may represent self-limited, dead-end cases with no further human to human transmission. A recent study reported TMUV seropositivity in Thailand in humans without previous specific symptoms [74]. The latter study highlighted the importance of prospective surveillance and survey in the human community, particularly the population at high risk for exposure to avian fauna. Further studies are also needed to better evaluate the potential outcomes in mammals, particularly neurological presentations and their role in transmission.

Detection and diagnostics

Since the first isolation of TMUV, several techniques have been developed to study the virus or detect it for diagnostic purposes. The development of diagnostic tools was particularly motivated to prevent large outbreaks in farms and preserve poultry industry from economic losses. Indeed, TMUV is harmful to birds and prevention of outbreaks in countries producing and exporting a large amount of poultry is a major issue, especially for China as it is the first producer of duck in the world.

Kono *et al* identified viral particles by electronic microscopy in the transformed chicken B lymphocyte cell line BK3 (LSCC-BK3) infected with TMUV [5]. Although virus isolation and electronic microscopy remain the gold standard, they are not suitable for diagnostic purposes. Isolation of TMUV is time-consuming and implementation requires a dedicated laboratory with costly equipment and high-skilled trained personnel, making isolation difficult outside research laboratories. Moreover, fresh specimens must be used to obtained viable viruses. Finally, TMUV isolation from cells or embryos is mainly carried out in laboratories for basic research.

Nowadays, common methods for diagnosis of TMUV include serological detection by enzyme-linked immunosorbent assay (ELISA), based on NS1 protein detection, and plaque reduction neutralization test (PRNT) [82,83]. The spatial organization of the E protein has been well described in DENV, WNV, and JEV showing a high degree of similarity between flaviviruses [84-86]. Since this glycoprotein is exposed on the surface of the virion, it is responsible for its immunogenicity [88,89]. Indeed, the E protein has been widely used to produce mono- or poly-clonal antibodies. Recently, a new neutralizing antibody (1G2) cross-reacting with JEV, WNV and ZIKV and targeting a minimal epitope located in the domain II of the E protein, was reported to provide a large protection against TMUV in mice suggesting a potential usage in detection and a valuable candidate for diagnostic and therapeutic purposes [93]. Reverse transcriptase coupled with Polymerase chain reaction (RT-PCR) is commonly used to evaluate the presence of viruses in biological fluids. Several approaches using multiplex PCR targeting avian viruses were developed to detect TMUV [94-96]. Finally, a new and promising detection method using Reverses Transcriptase-PCR coupled with mass spectrometry detection, has been developed to screen the presence of specific duck's viruses [97]. With this technique, various viral infections of interest to duck farming can be simultaneously identified with low detection threshold.

Cell biology tools

Transmission and dissemination of arboviruses requires virus replication in infected host cells. Like other Flavivirus members, to ensure its transmission, TMUV must replicate in mosquito vectors prior to a vertebrate host (Fig. 5). In parallel with virus isolation in embryonated chicken eggs, C6/36 mosquito cell lines were rapidly used for virus isolation

[5,25]. However, the lack of information on the virus in the vertebrate host has led to use primary cells and cell lines to investigate the biology of the virus (Table 3).

Different types of avian cells have been used to evaluate TMUV biology in birds and include BK3-cells, a transformed chicken B lymphocyte cell line, the chicken macrophage-derived cell line HD-11, the DF1 cell line, isolated from chicken embryo fibroblasts. Besides specific usage for research, chicken embryo fibroblast, duck and chicken eggs are routinely used for virus isolation and maintenance [3-5,26,30,98,99].

TMUV is also able to replicate in a wide range of non-avian cells which are used to assess the impact of TMUV in mammalian hosts. The primary cells and cell lines used in TMUV investigation are summarized in Table 3. Human cell lines were used to evaluate tropism of the virus and determine molecular mechanisms involved in TMUV infection. The HEK293T cell line and mouse primary cell line (MEF) were used to investigate innate immune response and how TMUV evades the immune response [100] (see specific paragraph). A recent investigation on TMUV cell tropism was carried out using various human cell lines. The authors pointed out the susceptibility of liver, kidney and nerve cell lines at various degrees, while lung, muscle, B cells, T cells and monocytes were largely resistant to the infection [101]. All these reports indicate that TMUV is able to replicate in a wide range of vertebrate cells. Results suggest that targeted organs are similar in mammals and birds. However, knowledge on pathogenesis in human cells are parceled and further investigation is needed. Furthermore, while TMUV is transmitted via mosquitoes' bite, no information is available on the biology of the virus in insects both *in vivo* and *in vitro*, except for effective replication in *Aedes albopictus* cell line (C6/36 cell line).

Table 3. Cell models used for TMUV investigations.

	Name	Organism, Tissue	Type	reference
Avian	DF-1	Chicken, embryo fibroblast	Cell line	[3,26,98]
	HD11	Chicken, bone marrow macrophage	Cell line	[102]
	DEF	Duck embryonic fibroblast	Primary cells from 9 days old duck embryos	[10,103]
	goose PBMCs	Goose blood	Primary cell	[104]
	LSCC-BK3	Chicken, B lymphocyte	Cell line	[5]
Mammalian	MARC145	Monkey Kidney	Cell line	[5]
	VERO	African green monkey kidney	Cell line	[3,5,26,100, 105]
	BHK-21	Hamster, kidney,	Cell line	[10,22,26]
	CPK	Porcine, kidney	Cell line	[5]
	MEF	Mouse embryonic fibroblast	Primary cells	[103]
	A549	Human, epithelial lung	Cell line	[100,101]
	HeLa	Human, epithelial cervix	Cell line	[100]
	SH-SY5Y	Human, epithelial bone marrow	Cell line	[100,101]
	HEK293T	Human, epithelial kidney	Cell line	[101,103]
	HUH7	Human, liver	Cell line	[101,103]
	RD	human rhabdomyosarcoma cell	Cell line	[101]

	SUP-T1	human T-cell lymphoblastic lymphoma cell	Cell line	[101]
	Z-138	Human B-cell non-Hodgkin's lymphoma cell	Cell line	[101]
	U937	human histiocytic lymphoma cell	Cell line	[101]
	imHC	Human hepatocyte-like cell	derived cell from hiPSC	[101]
Insect	C6/36	*Aedes albopictus*, larva	Cell line	[22,26,105]

Innate immune response and viral evasion

Flaviviruses trigger a strong innate immune response, which induces the production of a broad range of complementary antiviral molecules. Innate immunity is initiated by recognition of specific viral components, named pathogen associated molecular patterns (PAMPs), via cellular pattern-recognition receptors (PRRs) and was largely investigated in mammals and in birds [106-109]. Three classes of PRRs sense the presence of PAMPs in infected cells: Toll-like receptors (TLRs), retinoic acid-inducible gene I – like receptor (RLRs) and NOD-like receptor (NLRs). The first two ones are crucial for Interferon (IFN) response and proinflammatory cytokines production. These molecules are both essential for eliminating viruses and for recruitment of innate and adaptative immune cells [108,110].

Several studies investigated innate immune response against TMUV both *in vivo* and *in vitro* in avian and mammalian species. After experimental infection of female ducks or ducklings, Li *et al* and Zhang *et al* showed a rapid multiplication of the virus in different organs (brain, spleen, kidney, heart, pancreas, thymus or Bursa of Fabricius) associated with severe lesions. The infection triggers activation of the RLR and TLR pathways and overexpression of PRRs such as RIG-I or MDA-5. This activation leads to the upregulation of Interferon Stimulated Genes (ISGs) from the Mx and OAS families in brain and spleen [111,112]. Up-regulation of the RLR genes RIG-I and MDA-5 induced increase in type I IFN expression in the early phase of the infection *in vivo* [113]. A set of experimental infections in CEFs and in 293T cells have also shown the implication of RLR and TLR pathways in the innate immune response to TMUV infection. Through activation of the molecular adaptors MDA5 and TLR-3, involved in the RLR and TLR signaling pathways respectively, TMUV infection strongly increased expression of a set of type I IFN genes and some critical ISGs (Mx1, OAS1, IFITM3 and OASL), diminishin virus replication [114]. Hua *et al* characterized the implication of TBK1, a molecule involved in several type I IFN signaling pathways, in an experimental duck model. Using overexpression and knockdown experiments, they suggested a key role of TBK1 in the antiviral innate immune response in DEFs via IFN-ß production [115]. TBK1 signaling in mammals is intitiated by DDX3 interaction with TBK1/IKKε, to activate ISGs synthesis through IRF3/7 activation [116]. In TMUV-infected ducks, the duck-DDX3 (duDDX3) modulates innate immune response and duDDX3 over-expression inhibits TMUV. Nevertheless, albeit duDDX3 may influence TMUV replication, TMUV is able to inhibit duDDX3 expression, suggesting an underlying mechanism to evade to immune response [117].

In geese, TMUV infection triggers immune response with an increase of pro-inflammatory cytokines and interferons in several organs as well as in PMBCs. Interestingly, immunohistochemical analysis presented co-localization of CD8+T cells and TMUV, associated with high cytokine expression, suggesting an activation cascade due to viral infection and leading to the establishment of an antiviral status [104]. Molecular mechanisms in specific tissues involved in the etiology of the TMUV disease affecting the brain and female reproductive system were partially addressed. A global proteomic analysis of duck ovaries from infected animals revealed differential protein expression including proteins involved in cellular structure, RNA processing, innate immune response, protein biosynthesis and modification, vesicle transportation, signal transduction. Interestingly, some modulations of expression may be related

to immune evasion strategies [118]. In TMUV-infected duck brains, a recent transcriptomic analysis provides information on molecular mechanisms engaged in neurovirulence and host responses. Several pathways and genes specifically related with nervous system and innate immune responses were modulated, providing evidence on the neuro-immune interactions in TMUV infection [119]. Despite efforts to shed light on molecular mechanisms triggered by TMUV infection, particularly about activation of innate immune pathways, intimate modalities of host responses to the infection remain incompletely known and further studies will be necessary to uncover host-pathogen interaction.

Flaviviruses have developed different immune evasion strategies [120]. Viral proteins, particularly non-structural (NS) proteins antagonize crucial signaling pathways, such as virus recognition pathways and IFN pathway, needed for an effective response [121]. Several studies also reported immune evasion mechanisms developed by TMUV. Interferon treatment of TMUV-infected avian cells have shown the absence of impact on virus replication, contrary to the IFN treatment on mammalian TMUV-infected cells [100]. This observation suggests specific cellular mechanisms allowing TMUV to overcome IFN mediated effects in avian cells specifically. Different strategies might be implemented by TMUV to prevent anti-viral responses. Involvement of TMUV NS1 in the inhibition of RLR receptor signaling by impairment of the interaction of RIG-I and MDA5 with IPS1 was reported using reporter assays experiments. NS1 protein interacted with CARD domain of RLR adaptors impeding recognition and association with virus molecule adaptors. Disruption of this interaction lead to a suppression of RLR-mediated IFN-β production to finally facilitate immune evasion [103]. In 2019, Wu *et al* also infected HEK293T to study the impact of the viral protein NS2B and reported interaction with actors of the RIG-I pathway, resulting in suppression of type I interferon response. TMUV-NS2B3 viral protease may also inhibit IFN-β production. Contrary to TMUV-NS1, NS2B3 protease acts directly on mitochondrial duck STING (duSTING) protein, which is a key intermediate in RLR pathway, to inhibit signal transduction to decrease the production of IFN-β. In this context, synthesis rates of a subset of ISGs related proteins (Mx1 and OASL) are also down regulated. Interestingly, ZIKV and DENV NS2B3 present the same enzyme cleavage site as TMUV NS2B3 on duSTING [16]. In fact, ZIKV and DENV NS2B3 are able to hydrolyze duSTING, while TMUV NS2B3 is reciprocally able to cleave human STING, suggesting a potential relevance in case of spillover of the virus to humans. A study by the same research group on the role of the NS2A protein found similar results with alteration of STING protein activity in RLR signal transduction. In the same way as NS2B, the binding of NS2A on STING blocks the interaction with TBK1, decreasing its phosphorylation and leading to the inhibition of IFN-β production [122]. A recent study has reported that NS4B protein can inhibit IFN-β production as previously observed with DENV. Thus, the TMUV NS4B protein has been identified as a major inhibitor of RLR pathway decreasing expression of RIG-I, MDA5, MAVS, STING and also TBK1 unlike NS2-A and -B. Moreover, specific mutations in NS4B protein modifying interactions with TBK1 lead to phenotypic changes decreasing the pathogenicity of TMUV. Thus, NS4B appears to strongly interact with TBK1 and inhibit its recruitment by STING, resulting in a blockage of the signaling pathway [123]. These studies confirm the importance of TBK1 in the implementation of an efficient antiviral response as reported by Hua *et al*. [115] and strategies developed by TMUV to overcome immune response. Finally, flavivirus nonstructural protein 5 (NS5) is a RNA dependent RNA polymerase (RdRp) that catalyzes the replication of the viral RNA genome in the replication complex [124]. Little is known about the molecular mechanisms of replication with TMUV, however NS5 also appears to play an important role in infection. Indeed, specific amino acid substitution in the NS5 protein decreases infectivity both *in vitro* in BHK21 and DEF cells and *in vivo* in ducklings. This decrease is associated with a weaker innate immune response and a reduction of expressions of IFN–α –β –γ, interleukin IL-1β and IL-6. Development of attenuated viral models stimulates new approaches to study molecular mechanisms involved in the pathogenicity and replication of TMUV.

Autophagy is a cellular mechanism of recycling and degradation of cytoplasmic components to prevent cell death and promote homeostasis of cells exposed to an extrinsic or intrinsic stress, including viral infection [125]. Despite host immunity defense against several pathogen infections, autophagy can be hijacked by a range of viruses, including flaviviruses such as ZIKV [126], DENV [127] or JEV [128], to promote their own replication. Induction of autophagy by TMUV was confirmed in avian cells, acting as a viral strategy to evade host immune response [99]. In these studies, stimulation of autophagy promoted TMUV replication and, inversely, treatment with a chemical inhibitors of autophagy led to a decrease in TMUV virions. Moreover, Hu *et al* have shown that TMUV infection promotes degradation of p62-autophagy-adaptor, leading to a downregulation of innate immune response mediated through TBK1 protein and evasion of antiviral response. Indeed, p62-adaptor is not only a cargo adaptor but also plays an important role in the immune response via activation of the IFN and NF-kB pathways. p62 degradation may be a strategy developed by TMUV to evade innate immune responses [99,129].

Endoplasmic reticulum (ER) is an important intracellular organelle in the protein synthesis pathways. In ER, proteins are folded and matured before intracellular or extracellular release. The replication cycle of TMUV, as other flaviviruses, mainly takes place in close interaction with the ER of the infected cell [130]. ER acts as the site where the viral genome is translated in protein and replicated in de novo genome RNA in vesicle packets, which are formed by invagination of ER membrane. ER homeostasis is firmly regulated by a quality control process preventing accumulation of misfolded proteins in the ER lumen. However, similar to other virus infections, flaviviruses trigger ER stress. To alleviate ER stress and maintain homeostasis, infected cells activate a pro-survival mechanism pathway known as the unfolded protein response (UPR). Nonetheless, flaviviruses have evolved to exploit the UPR pathway to facilitate their own replication by preventing apoptosis, promoting autophagy and evading innate immune responses [131,132]. Similarly, TMUV has been reported to activate UPR via its three arms (IRE1, ATF6 and PERK signaling branches). UPR activation was shown in avian and mammalian cell lines [133]. However, the exact role of the UPR pathway in TMUV replication remains unclear and needs to be further investigated.

Treatment and Vaccine development

As for some other Flavivirus infections, there is currently no specific treatment for TMUV. The anti-viral efficacy of different molecules is currently being evaluated. For example, minocycline, a tetracycline analogue, has shown some efficiency *in vitro* against neuronal cell death infected by neurotropic viruses. This neuroprotective effect was also found with duck neuronal cells infected with TMUV [134]. Epigallocatechin-3-gallate (EGCG), a polyphenol-like active molecule, has already shown broad-spectrum activity against different viruses and appears as promising treatment against TMUV infection. EGCG use in BHK-21 cells showed a significant decrease of TMUV replication, probably due to an increase in the type I IFN production. This effect was then confirmed by an increased survival rate of TMUV-infected ducklings treated with EGCG [135]. New therapeutic strategies are being evaluated to prevent or directly treat TMUV. The use of Capsid-targeted viral inactivation (CTVI) strategy, an antiviral strategy targeting capsid protein originally developed to target retroviruses [136] and which has shown success against DENV and JEV [137,138], has also been recently evaluated for the treatment of TMUV [139].

Currently, there is no commercial vaccine against TMUV. However, regarding the large spread of the virus in Asia since the first outbreak in China in 2010 and the association with high economic impact on poultry industry, the development of an efficient vaccine is a primordial objective. Since the first report of the virus, different research teams have taken on the challenge. Rapidly after the first TMUV report in China, attenuated viruses by passaging in embryonated chicken eggs were developped as a live vaccine strategy [140,141]. The attenuation of virulence might be due to amino acid substitution in structural and non-structural proteins but the attenuated virus kept their

immunogenicity, thereby providing effective protection for animals against TMUV infection. Different kinds of oil-emulsion containing inactivated TMUV vaccines were developed to protect duck against TMUV [142]. In 2017, Zhang *et al* proposed to use an inactivated TMUV from the TMUV-JXSP strain which was successfully propagated in the BHK-21 cell line then inactivated using β-propiolactone and associated with medical-grade white oil to obtain an oil-emulsion vaccine amenable for injection. Vaccinated ducks presented an increase in antibody titers after the first injection and experienced a diminution of viral load without apparition of the egg-drop effect [143]. In the same way, after several passages of TMUV isolated from sparrows in China, attenuated viruses possessed a strong immunogenicity and provided an effective protection for ducks exposed to TMUV [144]. Recently, the team of Yang *et al*. worked with a variant of TMUV which developed attenuated virulence after several passages. This work sheds new light on the development of an effective vaccine against TMUV infection, determining that the residue 304 of the E protein was essential for virulence and cell attachment [145]. However, despite the interest of these candidate vaccines, further investigations are needed to optimize certain aspects of vaccination such as the route of inoculation, the optimal number of inoculations to achieve correct immunity or possible interactions with other animal vaccination programs.

Two recombinant duck enteritis viruses were also designed to express a part of TMUV E protein and pre-membrane protein. Immunization of ducks led to the production of protective neutralizing antibodies and further TMUV challenged animals exhibited resistance to the infection [146]. A reverse genetics strategy was used to produce a chimeric recombinant TMUV based on JEV backbone, which was successfully used in mice [147]. In 2016, Ma *et al*. used liposomes containing recombinant TMUV E protein produced in BL21 cells to immunize ducks. Two injections induced an effective immune response leading to a full protection to ducks challenged with TMUV compared to animals challenged with Freund's adjuvant as a control. This study suggested that a liposome-based vaccine is an interesting candidate. The use of a combination of liposomes (for delivery) and protein E (for immunization) presents several advantages such as better delivery, higher stability, longer immunization and lower toxicity [148]. Vaccine based on adenovirus platform was also experimented. The E protein of DTMUV expressed in a recombinant adenovirus triggered immune response and antibody production in immunized duck. Vaccine-challenged ducks presented up to 80% survival rate, indicating efficient protection [149].

Recently, vaccine development efforts were dedicated to provide a recombinant vaccine against TMUV. Several studies reported the development of antibodies targeting immunogenic domain regions of the virus particles. In particular against the loop of Domain II of the E protein [93]. Among them, a DNA-based vaccine was recently constructed to target viral prM-E proteins. The DNA-based vaccine provided effective immunization and opened up an interesting avenue for vaccination methods. By using attenuated Salmonella SL7207 bacteria as a vehicle to deliver the prM and E proteins, a trial showed the development of effective protection in ducks [150]. Other teams used a DNA vaccine also based on the integration of prM and E proteins but coupled with CpG oligodeoxynucleotide as an adjuvant to boost protection efficiency and showed effective production of neutralizing antibodies providing protection to ducks challenged with TMUV[151]. Chimeric Virus-like particles (VLPs) containing the E protein of TMUV showed their potential immunization effect in birds, leading to the decrease of the viral load in immunized birds [152].

Strategies using attenuated pathogens are widely used for vaccine development. However, an interesting way to develop new vaccine would be to use the strategy based on messenger RNA (RNA vaccine) as currently developed with success against SARS-CoV2. This type of strategy, coupled with liposome delivery, is already used to try to develop a vaccine against other Flaviviruses like DENV and ZIKV [153]. Despite extensive research on vaccine and treatment, a lack of effective means against TMUV calls for further research.

Conclusion

Like other epornitic arboviruses, TMUV has taken advantage of increasingly favorable conditions over the last few decades to spread across Asia and invades new territories. The first outbreaks in China in 2010, followed by the evidence of transmission to humans, has led to a reconsideration of the danger of this virus. Its transmission is largely facilitated by the conditions of intensive poultry rearing in farms, containing several thousands of animals. In such conditions, TMUV even seems to spread between animals without the intervention of mosquito vectors. Finally, the impact of migratory birds has not been clearly demonstrated but could play a significant role in the expansion of the virus across Asia. Thus, intensive animal husbandry, increasing trade, and environmental changes have facilitated the wide spread of TMUV.

This situation is all the more worrying since the passage of this virus to humans has been demonstrated and thus could lead to the emergence of a new zoonotic disease. The clinical presentation of TMUV infections in birds is similar to that of JEV, WNV or ZIKV, and it is easy to imagine the impact on the human population if TMUV were to develop therein. Due to the lack of widespread surveillance, the virus has so far been detected in only three Asian countries and actively studied only in China and Thailand. However, the remoteness of these countries and the phylogenetic pattern of the virus suggest that the virus is more widely established in Asia and SEA.

In recent years, research on host-pathogen interactions has been mainly focused on avian models, but a better understanding of the biology of the virus in mammals and in particular in humans is important. Finally, the presence of potential vectors in non-Asian regions may facilitate spreading outside Asia as it has been the case recently for other arboviruses. Thus, in order to respond to the challenges posed by this emerging virus, an integrated One Health approach could allow a broader understanding of the different parameters involved in the ecology of TMUV.

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Supplementary Materials: Table S1: Refermdpienced virus strains used in this study

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