

# The spread of Tembusu virus in China from 2010 to 2019

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## Abstract

Tembusu virus (TMUV) is a positive-sense RNA virus that is associated with severe reduction in egg production and even death in ducks. TMUV infection shows high incidence and is a threat to the global duck industry. However, the possible origin, genotype, and codon usage bias of TMUV are not very clear. Here, we addressed these questions by analyzing the available genomic sequences from China. The results showed that the ancestor of avian TMUV was most likely a mosquito TMUV. Moreover, three TMUV clades were identified by three different phylogenetic analysis methods. The TMUV genome exhibits a stronger mutation pressure than natural selection pressure. TMUV has the potential to cause an infection outbreak among humans, thereby posing a major threat to public health. Our findings provide important insights that reveal the ongoing TMUV spread in China and can aid in future prevention and control.

## Introduction

China has a wide range of duck farming industries and has been a global leader in commercial duck production. However, with the rapid development of large-scale industries and increase in stocking density, infectious disease of ducks, especially those caused by the avian influenza virus (AIV), Newcastle disease virus (NDV), Tembusu Virus (TMUV), and duck circovirus (DuCV) have become increasingly frequent (Fan et al., 2014; L. Xie et al., 2012; Z. Xie et al., 2013; Yun et al., 2012). These infectious diseases not only cause severe losses in the duck industry, but also affect the safety of human life, such as in case of AIV and TMUV infections (Cao et al., 2011; S. Su et al., 2015).

TMUV belongs to the genus *Flavivirus* and family *Flaviviridae*. Its genome encodes three structural proteins and seven nonstructural proteins (Bai et al., 2013; Mackenzie & Williams, 2009; Zhao et al., 2015). TMUV was first isolated in 1955 in Kuala, Malaysia, and was reported in China in 2010 (Platt et al., 1975; J. Su et al., 2011; Yun et al., 2012). The clinical symptoms caused by TMUV include depression, thin body condition, severely reduced egg production, severe neurological signs, and even death (Ti et al., 2015; Zhu et al., 2012). Since 2010, TMUV has become one of the most economically important infectious diseases of ducks in China (Wang et al., 2011; Yu et al., 2013).

As a member of the genus *Flavivirus*, the relevance of TMUV to human and animals is unclear (Zhang, Chen, Mahalingam, Wang, & Cheng, 2017). However, other members of *Flavivirus* such as West Nile virus (WNV), dengue virus (DENV), yellow fever virus (YFN), Japanese encephalitis virus (JEV), and Zika virus cause serious diseases in humans, including mild febrile symptoms and fatal hemorrhagic or neurologic disease every year worldwide (Q. Chen et al., 2020; Gubler, 2002; Hayes et al., 2005; Lanciotti et al., 1999). In nature, birds usually serve as amplifying hosts, including for WNV, Sitiawan virus, Usutu virus, and Bagaza virus. Notably, there has been a survey reporting evidence that TMUV has the potential to be transmitted to humans by infected birds, emphasizing the need for further study of the virus (Aguero et al., 2011; Barnard, Buys, Du Preez, Greyling, & Venter, 1980; Becker et al., 2012; Gould & Solomon, 2008; Kono et al., 2000; Niu et al., 2018; Weissenböck et al., 2002; Zhang et al., 2017).

In this study, we obtained the TMUV gene sequences reported across China from NCBI. Then, the epidemiologic, genetic diversity, and evolutionary dynamics of TMUV were analyzed through bioinformatics research methods. The findings of this study are expected to aid in the prevention and control of TMUV in the future.

## Materials and methods

### Sequence datasets

All sequences used in this study were obtained from the GenBank database of NCBI (<https://www.ncbi.nlm.nih.gov/>). A total of 40 strains of avian TMUV, including duck TMUV, goose TMUV, and chicken TMUV were obtained. Furthermore, a total of 4 related viral strains were also obtained from GenBank, including 4 strains of mosquito TMUV, 2 strains of Zika virus, 1 strain of WNV, and 1 strain of DENV. The details of these sequences are presented in Table 1.

### Tracking the origin of TMUV

Since the first report of TMUV in China, it has spread quickly. However, until now, the exact origin has not been elucidated. To investigate the origin of TMUV, a maximum likelihood (ML) tree was constructed using open reading frame (ORF) gene sequences. Furthermore, to verify the result of the ML tree, a maximum clade credibility (MCC) tree was constructed with the complete ORFs.

### Epidemiology of TMUV

To investigate the phylogeny trend of TMUV, 40 strains were obtained from GenBank, and were used as representative strains of the complete genome of TMUV obtained from mainland China from 2010 to 2019. All 40 sequences were aligned using ClustalW, which was applied in MEGA 6. The best substitution model was selected by jModelTest according to the Bayesian information criterion (BIC) score. Furthermore, to study the epidemiological trends of TMUV, a Bayesian skyline plot (BSP) was established. Additionally, to ensure the feasibility of the results, two independent runs of the chain were performed and compared to ensure adequate sampling; a chain length comprised 10,000,000 generations with sampling every 1,000 generations with 10% burn-in removed.

### Selection pressure and codon usage bias analysis

To analyze the selection pressure on the ORF of TMUV, 4 different methods were used: Single Likelihood Ancestor Counting (SLAC), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian AppRoximation for inferring selection (FUBAR), and mixed effects model of evolution (MEME) on DATAMONKEY (<http://www.datamonkey.org/>) (Kato & Standley, 2013; Murrell et al., 2013; Murrell et al., 2012; Smith et al., 2015). The basic nucleotide composition (A%, T%, C%, and G%), nucleotide at the third position of synonymous codons (A3s%, T3s%, C3s%, and G3s%), GC content on the third synonymous codon position (GC3s), and the effective number of codons (ENC) were calculated for the *VP2* gene sequence using CodonW (1.4.2).

The ENC value ranged from 20 to 61. The higher the ENC value, the smaller is the codon usage bias. Therefore, when the evaluation value is 20, the deviation is stronger. When the ENC value is less than 35, codon usage bias is considered very strong (Comeron & Aguade, 1998; He et al., 2019). Subsequently, an ENC-plot (ENC plotted against GC3s) was drawn to reflect the factors influencing codon usage bias (Fuglsang, 2004; Wright, 1990). The expected ENC was calculated as follows ('s' is the frequency of G + C at the third position of synonymous codons):

$$ENC_{expect} = 2 + s + \frac{29}{s^2 + (1 - s)^2}$$

At the same time, the neutrality plot analysis was also performed, which can reflect the degree of the influence of mutation pressure and natural selection on codon use patterns. We can judge whether natural selection

or mutation is dominant based on the slope of the curve. All data were plotted using Origin 2018 software.

## Results

### Origin of TMUV in China

To trace the origin of TMUV, we constructed an ML tree based on the ORF gene sequence (Fig. 1a). We found that all the avian TMUV strains were closely related to the mosquito TMUV with high confidence. The West Nile virus lineage 2 (NC001563) was selected as outgroup of the mosquito TMUV and avian TMUV strains, which might indicate a potential mosquito TMUV origin. To further verify this result, an MCC tree was constructed (Fig. 1b). The MCC tree showed a topology similar to that of the ML tree. Although this provides evidence that a close relationship exists between avian TMUV and mosquito TMUV, it is worth noting that only 3 strains of mosquito TMUV could be obtained from GenBank; we will update the analysis once more mosquito TMUV is available.

### TMUV genetic diversity and epidemiological data in China

To investigate the genetic diversity of TMUV, we used three different methods (NJ, ML, and MCC) to construct the phylogenies using the ORF sequence of the TMUVs. All of the strains represent four different avian species that were isolated in China from 2010 to 2019, and the strain XZW10694 (Accession number: KY937189) as outgroup. Three different clades were observed using the three different methods (Fig. 2a, 2b, and 2c). Compared with a previous study (Dai, Li, & Tao, 2015), a new clade was observed in the three different phylogenetic trees. This new clade (clade 3) was isolated in Shandong in 2014 and the host was *Anas platyrhynchos* (Accession number: MH748542). Clade 3 showed a longer evolutionary distance compared to clades 1 and 2, which might suggest that clade 3 is a potential new genotype. Based on the BSP, the TMUV might have diversified from 2010 to 2012, after which it entered the platform period (Fig. 3).

### Selection pressure and codon usage bias analysis

An analysis of selection pressure can be used to explore whether the existence of variability is related to selection pressure. The results showed three positive selection sites of TMUV detected by three methods, and six positive selection sites detected by two methods; details are shown in Table 2.

To investigate the evolutionary characteristics of TMUV, the ORF gene sequence was analyzed for codon usage bias. The mean value of G% was the highest, followed by A%, C%, and T%, which indicates a strong compositional bias in favor of G or A. Furthermore, in terms of synonymous codons, we found that at the third position (A3%, T3%, C3%, G3%), the highest value was observed for nucleotide G for all genotypes.

At the same time, to investigate the effects of selective pressure and mutation pressure on the evolution of TMUV, an ENC-plot and neutrality plot analyses were conducted. The mean ENC value was  $53.2575 \pm 0.458$ , which was higher than 35, indicating a low codon bias in TMUV. The ENC-plot and neutrality plot analyses (Fig. 4a and 4b) indicated mutation pressure rather than natural selection pressure.

## Discussion

Since TMUV was first reported in 2010, it had widely spread in China (Liu et al., 2013; Z. Xie et al., 2014; Yan et al., 2017; Yun et al., 2012; X. Zhou et al., 2016; Zhu et al., 2012). Many studies have focused on the mechanism of virus action and the establishment of detection methods (Bai et al., 2015; L. Chen et al., 2019; S. Chen et al., 2018; Cui et al., 2018; Fu, Ji, Liu, Dafallah, & Zhu, 2015; Hu et al., 2020; Jiang et al., 2012; Wu et al., 2016). In this study, we provide new insights into the origin, genetic diversity, and codon usage of TMUV. Compared with the previous studies, we provide better evidence for the origin, genotype diversity, and codon usage bias of TMUV (Dai et al., 2015; H. Zhou et al., 2015). We found that mosquito TMUV, which was isolated from 2015 to 2019, shared the most recent common ancestor with avian TMUV. Furthermore, we found a new potential clade that was isolated in the Shandong province; this new clade also showed high homology with the mosquito TMUV. Overall, the mosquito TMUV is the most likely origin of avian TMUV based on the currently available sequences. Mosquitoes are considered the main

transmission hosts of *Flaviviridae*. TMUV host jumps pose a major threat to the duck industry and public safety. According to previous research, TMUV virus has also been detected in humans (Cao et al., 2011).

Previously, Tao et al. used ML trees to type TMUV (Dai et al., 2015). In this study, we explored the phylogenetic history of TMUV isolated in China from 2010 to 2019. To identify the genotype of TMUV, three different methods (NJ, ML, and MCC) were used. All three methods showed that Chinese TMUV can be divided into three different clades (TMUV 1, TMUV 2, and TMUV 3). Additionally, within the TMUV 1 clade, 3 stable subclades were identified (TMUV 1a, TMUV 1b, and TMUV 1c), and in the TMUV 2 clade, 2 stable subclades were identified (TMUV 2a and TMUV 2b). Compared with the previous study (Dai et al., 2015; Ninvilai, Tunterak, Oraveerakul, Amonsin, & Thontiravong, 2019), the TMUV in the current study showed more genetic diversity. Nonetheless, in recent years, the rate of emergence of new genotypes has slowed, which is also supported by the Bayesian skyline plot analysis.

The codon usage bias can be used to understand the changes in codon usage and its influencing factors during the evolution of TMUV. Gene mutation pressure, secondary protein structure, natural selection pressure, and external environment were found to be the main factors causing codon bias (Bera et al., 2017; Hershberg & Petrov, 2008). In this study, ENC-plot analysis and neutrality plot analysis showed that mutation pressure played a decisive role in codon usage bias, which is similar to the results of previous research (H. Zhou et al., 2015). Notably, the preferred codons consisted of the optimal codons for human cells, which indicates that TMUV has the potential to infect humans after cross-species transmission; this is also supported by previous studies that have detected TMUV in humans (Tang et al., 2013; H. Zhou et al., 2015).

Cross-species transmission of viruses is a serious threat to public safety. There are already many such cases that have adversely affected public health, including SARS, MERS, SARS-CoV-2, and Zika virus infections (Dye & Gay, 2003; Lazear, Stringer, & de Silva, 2016; Verdoni et al., 2020; Zumla, Alagaili, Cotten, & Azhar, 2016). TMUV has the potential to spread across species and cause great damage. With the rapidly increasing population and increasing constant contact of humans with nature, there is an increasing possibility of an interaction between different hosts (avian to mosquito to human). Therefore, it is likely not a matter of if but when TMUV will cause a major infection outbreak in the human population.

In summary, we collected the sequences of TMUV that were reported in China over the past 10 years. We assigned the possible mosquito origin of TMUV and divided the TMUV into three clades. Furthermore, we raised the possibility that in future TMUV may infect people and cause serious consequences. Future research should focus on the following aspects: recombinant TMUV in animals, cross-species transmission of TMUV within the animal, and finally, establishing a detection network to prevent the sudden emergence of TMUV with high virulence that can infect humans. Overall, our study characterized TMUV spread in China and provided insights that may aid in future prevention and control of the virus spread.

## Data Availability Statement

The data used to support the findings of this study are included within the article.

## Declaration of conflicting interests

The authors declare that they have no conflict of interest.

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## Compliance with Ethical Standards

All experiments were compliant with the ethical standards of Anhui Agricultural University.

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## Figure captions

Fig. 1 The origin of Tembusu virus (TMUV) was deduced using the complete ORF gene. a) Maximum likelihood tree reconstructed with MEGA 6.0 using different species of TMUV. b) Maximum clade credibility tree reconstructed using BEAST 2.0.

Fig. 2 Phylogenetic analysis of Tembusu virus (TMUV). a) Maximum likelihood tree reconstructed using MEGA 6.0. b) Neighbor-Joining phylogenetic tree reconstructed using MEGA 6.0. c) Maximum clade credibility tree reconstructed using GTR+I+G substitution model and with an uncorrected relaxed clock (lognormal) of TMUV complete ORF gene sequence. The different genotypes are shown using different colors.

Fig. 3 Bayesian skyline plot of Tembusu virus (TMUV). The genetic diversity of TMUV is shown on the y-axis with 95% highest posterior density (HPD) shown in blue.

Fig. 4 Effective number of codons (ENC)-plot analysis and neutrality plot of TMUV complete genome. a) ENC-plot analysis show that the observed ENC values were below the expected curve regardless of genotype. b) Neutrality plot analysis indicated that the correlations between GC12 and GC3 were not significant in the Tembusu virus (TMUV) genotypes.









