



A Single Mutation at Position 156 in the Envelope Protein of Tembusu Virus Is Responsible for Virus Tissue Tropism and Transmissibility in Ducks

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ABSTRACT Duck Tembusu virus (TMUV), like other mosquito-borne flaviviruses, such as Japanese encephalitis virus, West Nile virus, and Bagaza virus, is able to transmit vector-independently. To date, why these flaviviruses can be transmitted without mosquito vectors remains poorly understood. To explore the key molecular basis of flavivirus transmissibility, we compared virus replication and transmissibility of an early and a recent TMUV in ducks. The recent TMUV strain FX2010 replicated systemically and transmitted efficiently in ducks, while the replication of early strain MM1775 was limited and did not transmit among ducks. The TMUV envelope protein and its domain I were responsible for tissue tropism and transmissibility. The mutation S156P in the domain I resulted in disruption of N-linked glycosylation at amino acid 154 of the E protein and changed the conformation of “150 loop” of the E protein, which reduced virus replication in lungs and abrogated transmission in ducks. These data indicate that the 156S in the envelope protein is critical for TMUV tissue tropism and transmissibility in ducks in the absence of mosquitos. Our findings provide novel insights on understanding TMUV transmission among ducks.

IMPORTANCE Tembusu virus, similar to other mosquito-borne flaviviruses such as WNV, JEV, and BAGV, can be transmitted without the presence of mosquito vectors. We demonstrate that the envelope protein of TMUV and its amino acid (S) at position 156 is responsible for tissue tropism and transmission in ducks. The mutation S156P results in disruption of N-linked glycosylation at amino acid 154 of the E protein and changes the conformation of “150 loop” of the E protein, which induces limited virus replication in lungs and abrogates transmission between ducks. Our findings provide new knowledge about TMUV transmission among ducks.

KEYWORDS Tembusu virus, flavivirus, envelope protein, mutation, replication, transmission

Tembusu virus (TMUV) is a member of the Ntaya virus group within the genus *Flavivirus*, family *Flaviviridae* (1). TMUV was first isolated in mosquitos in Malaysia in 1955 (2), and since then several mosquito isolates have been reported in Malaysia and Thailand (3, 4). In 2000, an infectious disease caused by TMUV emerged in a broiler farm in Sitiawan district of Perak state, Malaysia (5). This disease was characterized by encephalitis and retarded growth in broiler chicks (5). Ten years later, TMUV caused outbreaks in ducks characterized by a severe drop in egg production and growth retardation in almost all duck farms in China in 2010 (6). TMUV continues to result in

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annual losses of millions of dollars in China and has spread to duck farms in Southeast Asia (7–10).

As a flavivirus, TMUV was thought to be transmitted by mosquitos at the beginning of duck outbreaks (2, 6). Although arthropod-borne transmission of flaviviruses is still the major route of transmission (11–13), nonvector transmission has occurred between birds and between pigs (14–18). TMUV causes outbreaks in birds in winter, when mosquitos are inactive, suggesting that nonvector transmission routes play a key role in TMUV spread (6). *In vivo* studies indicate that TMUV can be transmitted efficiently among ducks by both direct contact and aerosol transmission (19). However, limited knowledge is available about the molecular basis determining the transmission of flaviviruses without the presence of vectors.

TMUV contains a positive-sense RNA genome with ~10,991 nucleotides that encodes a single polyprotein that is processed by viral and host proteases to generate three structural (capsid, C; pre-membrane, prM; and envelope, E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The structural proteins of flavivirus are involved in virion formation, attachment, and entry into host cells (1, 20), whereas the nonstructural proteins participate in genome replication, virion assembly, and evasion of host antiviral responses (21–23). Comparative genomic and phylogenetic analyses showed multiple nucleotide substitutions in different genes of TMUV that may contribute to the outbreaks in ducks (24, 25). Variation analyses of amino acid loci in the TMUV E protein revealed two mutated amino acid loci in strains isolated from Malaysia, Thailand, and mainland China compared to the prototypical strain of the virus (MM1775) isolated from mosquitos (26). Furthermore, TMUV isolates from the Chinese mainland have six common variations in the E protein that differ from the Southeast Asian strains (26).

Like other flaviviruses, the TMUV E protein contains three different structural domains (β -barrel shaped domain I [DI], finger-like domain II [DII], and Ig-like domain III [DIII]) and a transmembrane helix domain (27–30). The central DI acts as a bridge between DII and DIII, is folded into an eight-stranded β -barrel, and contains about 130 residues in segments: residues 1 to 50, 133 to 197, and 279 to 300. DII is formed by two segments, comprising residues 51 to 132 and 197 to 278. The tip of DII contains the fusion loop, which interacts with the host membrane during membrane fusion (25, 31). In most flaviviruses, DIII contains the putative receptor-binding site and has an important role in virus binding and cell entry (27, 32).

In the present study, we showed that the MM1775, an early TMUV strain isolated from mosquitos, which was recovered from cDNA by using reverse genetics system (33), was able to replicate efficiently in the spleens of ducks inoculated intramuscularly (i.m.) or intranasally (i.n.) but was not transmitted among ducks. In contrast, the recent TMUV duck isolates were able to transmit efficiently among ducks by both direct contact and by aerosol transmission (19). Here, we investigated the underlying molecular mechanisms of TMUV efficient transmission without the presence of mosquito vectors. Our results demonstrate that the envelope protein and its amino acid at position 156 are critical for TMUV transmission in ducks.

RESULTS

Amino acid sequence comparison between TMUVs MM1775 and FX2010. The genome sequences of TMUV strain MM1775 (GenBank accession no. [MH414569](#)), isolated from mosquitos in Malaysia in 1955, and TMUV strain FX2010 (GenBank accession no. [MH414568](#)), isolated from sick ducks in China in 2010, were analyzed using DNASTAR software. A total of 101 amino acid differences among all 10 proteins were detected between 2 viruses depicted in Table 1. There were 25 amino acid differences located at three structural proteins and 76 other differences distributed among 7 nonstructural proteins (Table 1). There were 4 and 5 amino acid differences in the structural PrM and C proteins, respectively, whereas 16 amino acids were different in the E protein. These included 9 amino acids in DI, 3 amino acids in DII, 2 amino acids in DIII, and 2 amino acids in the transmembrane helix domain which acts as a

TABLE 1 Amino acid sequence comparison of TMUVs MM1775 and FX2010

Protein	Positions ^a																						
	Amino acids (MM1775/FX2010)																						
C	42	61	76	109	111																		
	T/A	K/R	S/N	P/S	V/I																		
PrM	22	24	29	150																			
	M/L	I/V	T/A	G/S																			
E	2	38	72	89	148	149	156	157	180	181	185	236	312	332	467	483							
	N/S	R/K	P/S	D/E	A/E	V/A	P/S	V/A	L/M	G/E	A/T	R/K	A/V	S/T	V/A	I/M							
NS1	2	9	21	41	57	69	83	99	105	108	112	113	121	139	147	173	174	205	274	339	351		
	M/T	T/A	I/V	K/R	V/I	A/V	S/A	R/K	S/P	E/D	T/M	F/Y	N/S	R/K	K/R	S/T	G/E	H/K	K/V	V/A	V/M		
NS2A	4	13	15	23	31	35	36	49	63	79	98	147	150	157	165	187							
	D/G	I/M	I/V	V/I	S/P	P/S	S/A	V/I	V/A	I/M	R/K	A/V	L/F	S/L	I/V	N/S							
NS2B	86	95	96	114																			
	K/R	F/L	S/G	L/F																			
NS3	15	107	210	258	324	358	591	610															
	K/R	M/I	R/K	V/I	D/E	R/K	T/I	A/T															
NS4A	5	89	96	110																			
	V/I	A/T	V/I	I/V																			
NS4B	38	46	53	83	203																		
	T/A	N/S	V/M	V/I	T/I																		
NS5	4	76	150	188	197	233	246	258	277	288	385	422	435	506	562	729	828	841					
	A/T	R/K	S/A	T/M	K/R	V/I	R/K	A/V	E/G	K/R	D/N	T/A	N/S	R/K	L/M	V/I	Y/H	I/V					

^aFor each protein, the position numbers are indicated in the first row, and the amino acids are specified in the second row. MM1775 is a TMUV strain isolated from mosquitos in Malaysia in 1955; FX2010 is a TMUV strain isolated from sick ducks in China in 2010.

stem-anchor and is important to the surface architecture of the tick-borne flaviviruses (34). Eighteen amino acids were different in the NS5 protein and 21 amino acid differences were found in the NS1 protein (Table 1). Both NS1 and NS5 are important for flavivirus replication (22, 23, 35).

TMUV stain FX2010 causes systemic infection and efficient transmission in ducks compared to an early strain MM1775. To explore why TMUV causes outbreaks in ducks, we compared virus replication and transmission of two related strains, FX2010 and MM1775. At 3 day postinoculation (dpi), all ducks inoculated with 10^{3.5} 50% tissue culture infective dose(s) (TCID₅₀) of FX2010 through either the i.m. or the i.n. route or inoculated with 10^{3.5} TCID₅₀ of MM1775 through the i.m. route showed swollen spleens and severe ovarian follicle distortion, while only two of three ducks inoculated with the same dose of MM1775 through the i.n. route had these clinical symptoms. FX2010 was detected in all tested tissues (including the liver, spleen, lung, kidney, brain, ovary, pancreas, and trachea) of all infected ducks through i.m. inoculation and in most tissues except for the brain through i.n. inoculation (Table 2). In contrast, MM1775 was only detected in the livers and spleens of most of ducks that were inoculated either i.m. or i.n.; in addition, virus was also detected in the ovaries of two of three birds inoculated through the i.m. route (Table 2). Although the range of tropism tissues were different, the viral titer of two viruses reached to a comparable level in spleens in inoculated ducks, suggesting their replication efficiency in this tissue was similar.

The serum samples of the remaining inoculated and contact ducks were tested using ELISAs. All ducks inoculated with FX2010 via either route, as well as all three birds

TABLE 2 Virus titers in different samples of ducks inoculated with TMUV FX2010 or MM1775

Virus	Inoculation route	Test time (dpi) ^a	Virus titer (log ₁₀ TCID ₅₀ /0.1 g) ^b								
			Liver	Spleen	Lung	Kidney	Brain	Ovary	Pancreas	Trachea	Serum
FX2010	i.m.	3	1.17 ± 0.78	3.39 ± 0.34	2.72 ± 0.25	2.33 ± 0.58	1.00 ± 0.33	3.95 ± 0.47	2.32 ± 0.40	2.57 ± 0.35	2.51 ± 0.10
	i.n.	3	-/-/1.00	3.78 ± 0.63	1.72 ± 0.86	0.72 ± 0.54	-/-/-	0.50/2.33/-	0.50 ± 0.3	1.65 ± 0.42	2.10 ± 0.50
MM1775	i.m.	3	1.56 ± 0.10	3.94 ± 0.42	-/-/-	-/-/-	-/-/-	1.67/2.00/-	-/-/-	-/-/-	-/-/-
	i.n.	3	1.67/2.33/-	2.50/3.50/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-

^adpi, days postinoculation.

^bWhere virus was detected in all three ducks sampled, the mean TCID₅₀ ± the standard deviation is shown; otherwise, individual titers are shown for each animal. -, No virus was detected.

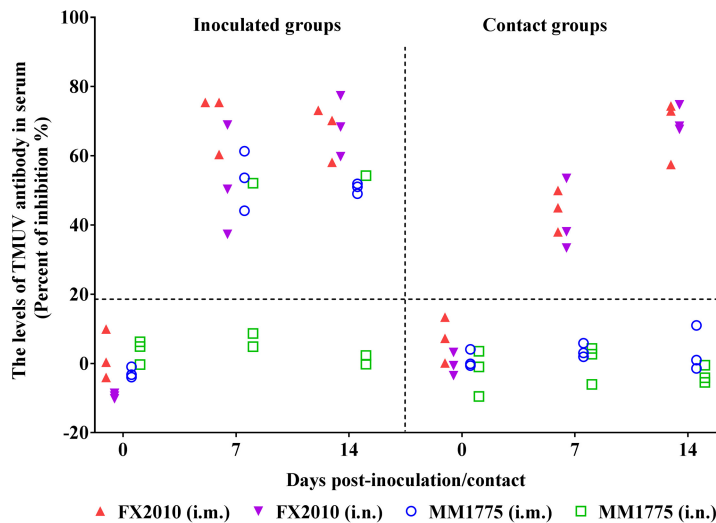


FIG 1 Antibodies against TMUV in FX2010- or MM1775-inoculated and contact ducks. Ducks were inoculated i.m. and i.n. with $10^{3.5}$ TCID₅₀ of FX2010 or MM1775. One day later, naive ducks were introduced into the isolators. Data show the relative levels of TMUV-specific antibodies detected in serum samples by an ELISA. Serum was considered positive conversion when the PI value was $\geq 18.4\%$.

inoculated with MM1775 through the i.m. route were seropositive at both 7 and 14 dpi, while only one of three ducks inoculated with MM1775 through the i.n. route seroconverted (Fig. 1). Seroconversion was detected in all contact ducks in groups of inoculated FX2010 at both 7 and 14 days postcontact (dpc). In contrast, no seroconversion was detected in contact ducks in the MM1775 groups at 14 dpc (Fig. 1). These results indicate that the FX2010 virus, but not the MM1775 virus, transmits efficiently between ducks in the absence of mosquitos.

E protein dictates TMUV tissue tropism and transmissibility in ducks. To identify which protein(s) was/were critical for tissue tropism and transmissibility of TMUVs, the E gene was exchanged between FX2010 and MM1775 viruses. Two chimeric viruses, FX/MME containing the MM1775 E gene in the FX2010 background and MM/FXE containing the FX2010 E gene in the MM1775 background, were rescued by PCR-based reverse genetic method (Fig. 2). Their replication and transmissibility were compared to their respective parental viruses. The growth curves showed that E gene substitution viruses had the similar replication capacities on DF-1 cells infected with each virus at a multiplicity of infection (MOI) of 0.0001 compared to their respective parental viruses (Fig. 3A). The chimeric virus FX/MME was detected in tested spleens, kidneys, and ovaries, but not in the lungs of inoculated ducks at 3 dpi (Table 3). Furthermore, FX/MME was not isolated from tested tissues of contact birds at 6 dpc compared to its parental FX2010 virus (Table 3). On the contrary, the MM/FXE with FX2010 E gene obtained the ability to replicate in the lungs, ovaries, and kidneys of inoculated ducks in contrast to its parental MM1775 virus. Noticeably, MM/FXE was detected in multiple organs, including the spleens, lungs, and ovaries of contact ducks, compared to the parental virus (Table 3).

To further confirm virus replication and transmissibility of chimeric viruses, we performed an enzyme-linked immunosorbent assay (ELISA) to determine seroconversion of originally inoculated and contact ducks. All inoculated ducks, except for two birds inoculated with FX/MME, seroconverted at 4 dpi; however, all inoculated birds, including two seronegative ducks at 4 dpi, were seropositive to TMUV at 7 dpi (Fig. 3B). Seroconversion was only found in contact ducks of either FX2010 or MM/FXE groups at the tested dates; none of the contact birds in either MM1775 or FX/MME groups seroconverted, even at 14 dpc (Fig. 3B). These results indicate that the E gene plays a key role in TMUV replication and transmissibility.

Domains I and II of the E protein are crucial for the replication and transmissibility of TMUVs in ducks. To determine which part of TMUV E protein was critical for



FIG 2 Generation of chimeric or mutated TMUVs for duck transmissibility studies. The colored bars indicate the origin of the viral protein (red, FX2010; blue, MM1775). The viruses were rescued in a background of FX2010 (A) or MM1775 (B). Whether virus transmission occurred was determined based on the seroconversion of specific TMUV antibody in contact ducks that were cohoused with originally inoculated ducks at 14 days postcontact. The transmissibility of each virus is specified on the right of the figure.

efficient replication and transmissibility of TMUVs in ducks, the E protein was divided into two parts: domains I and II (DI&II) and domain III and transmembrane helix (DIII&Th) based on its proposed structure (Fig. 2). Chimeric FX/MME-DI&II, FX/MME-DIII&Th, MM/FXE-DI&II, and MM/FXE-DIII&Th were generated as depicted in Fig. 2 in the background of either FX2010 or MM1775, respectively, and characterized in ducks. All chimeric viruses, as well as their parental viruses, were able to replicate in the spleens of inoculated ducks, but only the viruses containing the E-DI&II of FX2010 were detected in the lungs and kidneys of inoculated ducks at 3 dpi (Table 4). In contrast, the viruses containing the E-DI&II of MM1775 were not detected in other tissues of inoculated birds at 3 dpi (Table 4). Substitutions of E-DIII&Th did not significantly impact virus replication and tissue tropisms of TMUVs compared to the parental viruses (Table 4).

ELISA results revealed that seroconversion was found in all inoculated ducks with each parental and chimeric virus at 7 dpi (Fig. 4). Seroconversion was only detected in contact ducks in the viruses that contain the E-DI&II of FX2010 groups. Moreover, no seroconversion was detected in contact ducks in the viruses that contain the E-DI&II of MM1775 groups (Fig. 4). The viruses substituted with E-DIII&Th showed the same transmissibility as their parental viruses, consistent with virus replication patterns (Fig. 4 and Table 4). These data indicate that DI&II, and not DIII&Th, of the E protein are crucial for replication and transmissibility of TMUVs in ducks.

Domain I of E protein is responsible for the replication and transmissibility of TMUVs in ducks. To identify which domain of E protein determined replication and

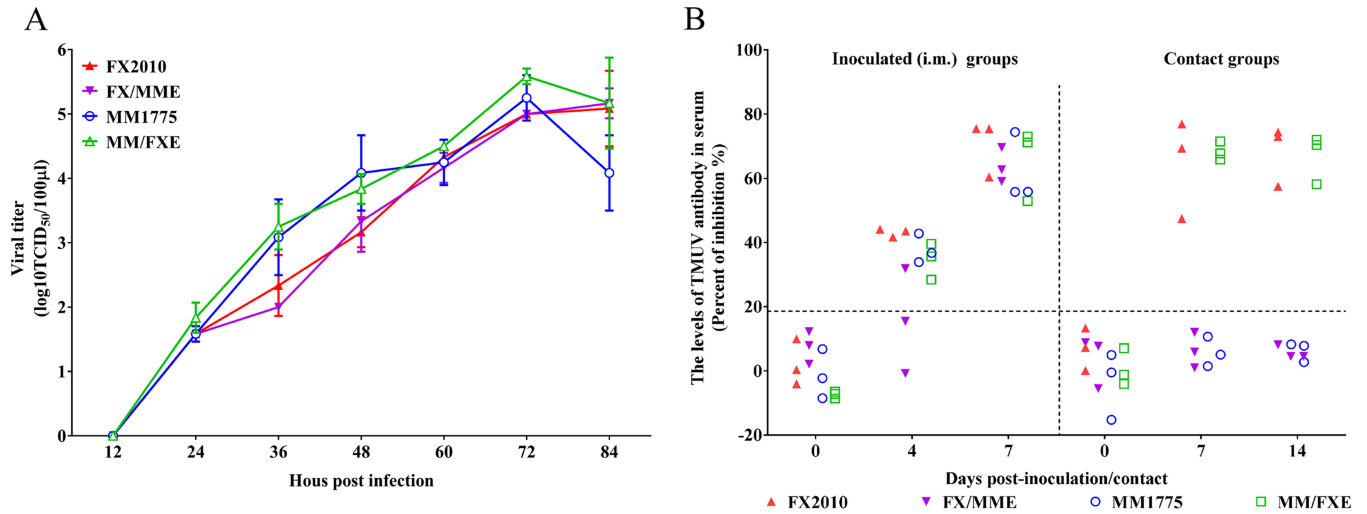


FIG 3 Growth curves of parental and E gene substitution TMUVs on DF-1 cells and antibodies detected in contact and inoculated ducks with these viruses. (A) Replication of parental and E gene substitution TMUVs. DF-1 cells were infected by the parental and E gene substitution TMUVs at an MOI of 0.0001. Virus samples from the supernatant were collected at different time points and titrated on DF-1 cells. The data for virus titers indicate the means of the results of three repeats, and the error bars indicate standard errors of the means (*, $P < 0.05$; **, $P < 0.01$). (B) Relative levels of TMUV-specific antibodies detected in serum samples as a function of time after inoculation with the indicated virus. Serum was considered positive when the PI value was $\geq 18.4\%$.

transmissibility of TMUVs in ducks, the chimeric viruses FX/MME-DI and FX/MME-DII or MM/FXE-DI and MM/FXE-DII, which contain heterogeneous DI or DII in the E protein of FX2010 or MM1775 were rescued as depicted in Fig. 2 and further characterized in ducks. All chimeric viruses substituted with a single domain and parental viruses were able to replicate in the spleens of inoculated ducks (Table 4). Only the chimeric viruses containing the E-DI of FX2010 were detected in the lungs of some inoculated ducks at 3 dpi, while the chimeric viruses containing the E-DI of MM1775 were not detected in the lungs of inoculated ducks at the same date. The FX/MME-DII retained systemic replication in ducks, which is similar to FX2010, while the replication of MM/FXE-DII was still limited in spleens, similar to MM1775 (Table 4).

All ducks inoculated with the chimeric viruses with single E-DI or E-DII showed seropositive at 4 dpi (Fig. 4). One of three contact ducks seroconverted in the MM/FXE-DI infection group, indicating that the parental MM1775 obtained transmissibility in ducks after having the E-DI of FX2010, while the FX2010 lost transmissibility after substitution of the E-DI of MM1775, as none of contact birds seroconverted at 14 dpc (Fig. 4). In addition, substitution of the single E-DII did not impact transmissibility of both FX2010 and MM1775 (Fig. 4). These data indicate that the DI of the E protein is responsible for tissue tropisms and efficient transmissibility of TMUVs in ducks.

TABLE 3 Substitution of the E gene changes the replication capacity of Tembusu viruses in ducks

Virus	Infection route	Test time ^a	Virus titer (\log_{10} TCID ₅₀ /0.1 g) ^b			
			Spleen	Lung	Kidney	Ovary
FX2010	i.m.	3 dpi	2.89 ± 0.78 ^c	1.33 ± 0.87	2.17 ± 1.25	3.27 ± 0.27
FX/MME	i.m.	3 dpi	4.28 ± 0.53	-/-/-	-/0.67/2.00	2.78 ± 0.19
MM1775	i.m.	3 dpi	3.72 ± 0.75	-/-/-	-/-/-	-/0.67
MM/FXE	i.m.	3 dpi	3.17 ± 0.76	2.67/2.33/-	-/2.33/-	2.50 ± 0.33
FX2010	Contact	6 dpc	2.72 ± 0.25	2.33 ± 0.87	2.00 ± 0.44	3.00 ± 0.19
FX/MME	Contact	6 dpc	-/-/-	-/-/-	-/-/-	-/-/-
MM1775	Contact	6 dpc	-/-/-	-/-/-	-/-/-	-/-/-
MM/FXE	Contact	6 dpc	4.83 ± 0.44	2.17 ± 0.58	-/0.50/-	2.57 ± 1.39

^adpi, days postinoculation; dpc, days postcontact.

^bWhere virus was detected in all three ducks sampled, the mean TCID₅₀ ± the standard deviation is shown; otherwise, individual titers are shown for each animal. -, No virus was detected.

TABLE 4 Domain I of the E gene determines the replication capacity of Tembusu virus in the i.m.-inoculated ducks

Virus	Test time (dpi) ^a	Virus titer (log ₁₀ TCID ₅₀ /0.1 g) ^b			
		Spleen	Lung	Kidney	Ovary
FX2010	3	4.83 ± 0.44	2.89 ± 0.19	2.50 ± 0.50	3.23 ± 0.39
FX/MME-DI&II	3	3.67 ± 0.67	-/-/-	-/-/-	-/-/-
FX/MME-DIII&Th	3	3.45 ± 0.39	1.39 ± 0.10	-/1.00/-	3.05 ± 0.48
FX/MME-DI	3	4.39 ± 0.92	-/-/-	-/0.67/-	-/-/-
FX/MME-DII	3	4.22 ± 0.63	-/2.00/2.67	-/-/0.67	2.67 ± 1.53
MM1775	3	2.72 ± 0.86	-/-/-	-/-/-	-/-/-
MM/FXE-DI&II	3	3.56 ± 0.84	2.33/-/-	-/0.67/0.67	-/-/-
MM/FXE-DIII&Th	3	3.89 ± 0.54	-/-/-	-/-/-	-/-/-
MM/FXE-DI	3	4.39 ± 0.35	2.33/-/-	-/-/-	-/-/-
MM/FXE-DII	3	2.72 ± 0.75	-/-/-	-/-/-	-/-/-

^adpi, days postinoculation.

^bWhere virus was detected in all three ducks sampled, the mean TCID₅₀ ± the standard deviation is shown; otherwise, individual titers are shown for each animal. -, No virus was detected.

The E_{S156P} mutation abolishes the transmissibility of TMUV in ducks. To determine which amino acids contribute to efficient replication and transmission of TMUV in ducks, nine mutant viruses (FX-E_{S2N}, FX-E_{K38R}, FX-E_{E148A}, FX-E_{A149V}, FX-E_{S156P}, FX-E_{A157V}, FX-E_{M180L}, FX-E_{E181G}, and FX-E_{T185A}) with a single amino acid change located at DI of the E protein (9 different amino acids in DI of the E protein between FX2010 and MM1775) in the background of FX2010 were generated (Fig. 2) and further characterized in ducks. Viremia was detectable in all ducks inoculated i.m. with each recombinant virus with single mutated amino acid from 1 to 3 dpi. Two mutated viruses (FX-E_{A149V} and FX-E_{S156P}) showed significantly lower titers in serum samples of infected ducks than the parental FX2010 at different tested time points (Fig. 5A). Virus titers in the sera of FX-E_{S156P}-inoculated ducks were significantly lower than FX2010 at both 2 (*P* < 0.05) and 3 dpi (*P* < 0.01) (Fig. 5A). Viremia was also detected in contact ducks of each group, except for the contact birds in the group of FX-E_{S156P} (Fig. 5B). Virus was only detected in the serum samples of contact birds of the FX-E_{E148A} and FX-E_{M180L} groups as early 2 dpc, whereas contact ducks in the FX-E_{S2N} and FX-E_{K38R} groups showed a delayed

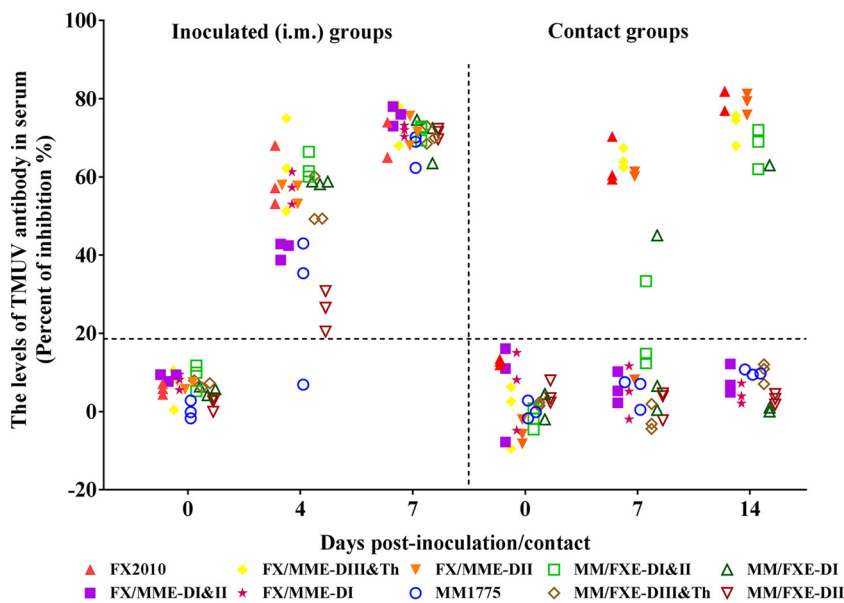


FIG 4 Antibodies detected in contact and inoculated ducks with parental and E domain substitution TMUVs. The data show the relative levels of TMUV-specific antibodies detected in serum samples as a function of time after inoculation with the indicated virus. Serum was considered positive when the PI value was ≥18.4%.

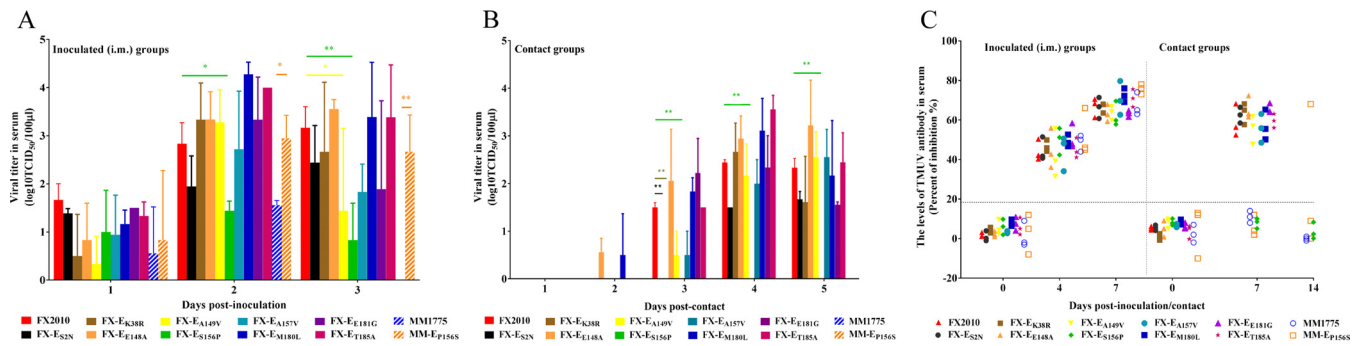


FIG 5 Virus titers and antibody levels in the sera of contact and inoculated ducks with parental and single amino acid mutant TMUVs. (A) Virus titers in sera of infected ducks were determined on DF-1 cells at 1, 2, and 3 dpi. (B) Virus titers in sera of contact ducks were determined on DF-1 cells at 1, 2, 3, 4, and 5 dpc. (C) Serum antibody responses against TMUVs were detected at 0, 4, and 7 dpi for inoculated ducks and at 0, 7, and 14 dpc for contact ducks. Serum was considered positive when the PI value was $\geq 18.4\%$. The data for the virus titers (panels A and B) indicate the means of the results of three ducks, and the error bars indicate standard errors of the means (*, $P < 0.05$; **, $P < 0.01$).

viremia starting at 4 dpc in contrast contact birds in other groups. No virus was detected in the sera of any contact ducks in the FX-E_{5156P} group at all tested time points, indicating that E_{5156P} mutation is critical for virus replication and transmission.

The ducks inoculated with either the parental FX2010 or its single amino acid mutants showed seroconversion at 4 dpi (Fig. 5C). All single amino acid mutants except for FX-E_{5156P} were transmitted to contact ducks, resulting in seroconversion at 7 dpc. The results further demonstrate that E_{5156P} mutation abolishes the transmissibility of TMUV FX2010 in ducks.

The E_{5156P} mutation restricts tissue tropism of TMUV in ducks. We further compared virus replication of FX-E_{5156P} with the parental FX2010 *in vitro* and in ducks. FX-E_{5156P} had the similar replication capacities as FX2010 on DF-1 cells infected with each virus at an MOI of 0.0001 (Fig. 6A). The ducks were inoculated i.m. with $10^{3.5}$ TCID₅₀ viruses per bird, and at 3 dpi, the virus replication in different tissues was tested. A similar virus titer was detected in spleens of ducks inoculated with either FX-E_{5156P} or FX2010 (Fig. 6B). However, a significantly lower titer was found in both lungs and kidneys of ducks infected with FX-E_{5156P} compared to that detected in FX2010-inoculated ducks. No virus was detected in ovary tissues of any ducks inoculated with

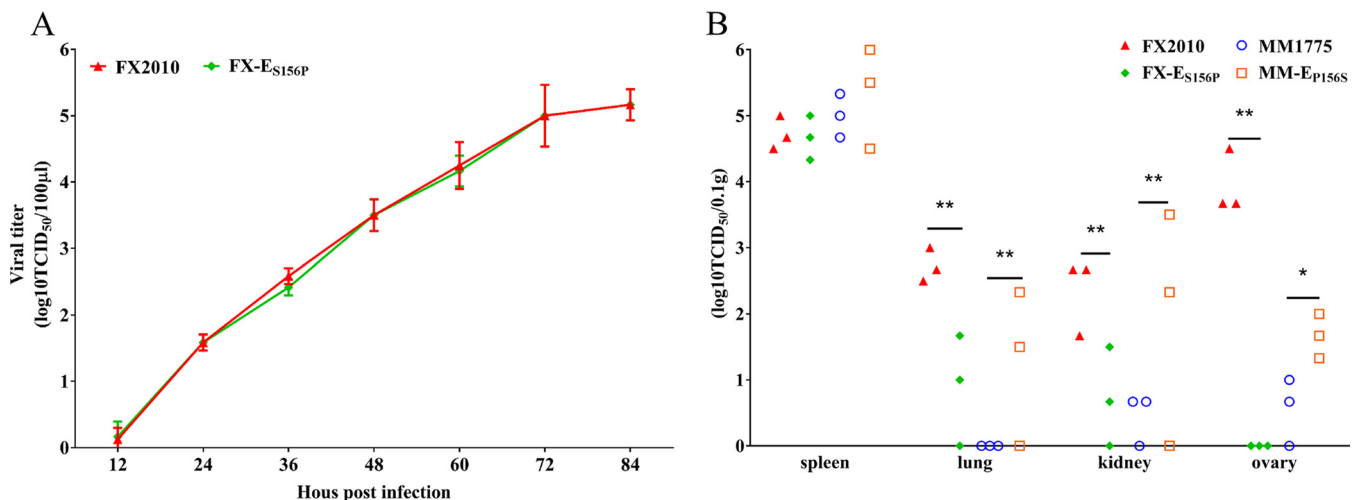


FIG 6 Replication comparison of TMUVs and their E₁₅₆ mutants. (A) Replication of FX2010 or FX-E_{5156P} on DF-1 cells infected at an MOI of 0.0001. Virus samples from the supernatant were collected at different time points and titrated on DF-1 cells. (B) To compare the replication of TMUVs and E₁₅₆ mutants, ducks were inoculated i.m. with $10^{3.5}$ TCID₅₀ of FX2010, FX-E_{5156P}, MM1775, and MM-E_{P156S}, respectively. Three ducks of each group were euthanized at 3 dpi, and the spleens, lungs, kidneys, and ovaries were collected for virus titration. Virus titers were determined on DF-1 cells. The data for virus titers indicate the means of the results of three repeats, and the error bars indicate standard errors of the means (*, $P < 0.05$; **, $P < 0.01$).

FX-E_{S156P}, while virus was found in ovary tissues of all FX2010-inoculated ducks with a high titer (Fig. 6B). These results indicate that the E_{S156P} mutation also restricts the tissue tropism of TMUV in ducks.

The E_{P156S} mutation recovers the transmissibility of TMUV in ducks. To test further the influence of E_{P156S} mutation on tissue tropism and transmissibility of TMUV in ducks, MM-E_{P156S} with a P156S mutation at position 156 of the E protein in the background of MM1775 was rescued and characterized in ducks. Each of six ducks were inoculated i.m. with 10^{3.5} TCID₅₀/bird of MM-E_{P156S} and its parental virus MM1775. The virus replication in different tissues of three inoculated ducks were tested at 3 dpi. A comparable virus titer was detected in the spleens of ducks inoculated with either MM-E_{P156S} or MM1775 (Fig. 6B). However, the virus titers in the kidneys and ovaries of MM-E_{P156S}-inoculated ducks were significantly higher than in those of MM1775-inoculated ducks (Fig. 6B). MM-E_{P156S} was detectable in the lungs of some inoculated ducks, while MM1775 was not detectable in any lungs of inoculated ducks (Fig. 6B). Viremia was detectable in some ducks inoculated both with MM-E_{P156S} and MM1775 at 1 dpi (Fig. 5A). Virus titers in the sera of MM-E_{P156S}-inoculated ducks were higher than those of MM1775-inoculated ducks at 2 dpi ($P < 0.05$) (Fig. 5A). The virus titers in sera of MM-E_{P156S}-inoculated ducks remained high at 3 dpi, while no virus was detected in the sera of MM1775-inoculated ducks at that time point (Fig. 5A). These results indicate that the E_{P156S} mutation enhances TMUV replication in different organs in ducks. Viremia was not detected in contact ducks in either the MM1775 group or the MM-EP156S group from 1 to 5 dpc (Fig. 5B).

All ducks inoculated i.m. with the MM-E_{P156S} or parental MM1775 were seropositive at 4 and 7 dpi (Fig. 5C). One of three contact ducks was seropositive at 14 dpc in the MM-E_{P156S} group, while all contact ducks in MM1775 group were seronegative at 14 dpc (Fig. 5C). These results indicate that E_{P156S} mutation of TMUV enhances transmissibility in ducks.

E_{S156P} mutation demolishes the N-linked glycosylation in “150 loop” of E protein. Sequence analysis showed that the FX2010 and its single amino acid mutants, except for FX-E_{S156P}, encoded only one potential N-linked glycosylation site in the “150 loop” of E protein; this glycosylation site could be amino acid ASN at position 154 (154N/155Y/156S) based on the consensus sequence (N-X-S/T) of N-glycosylation (11, 12). To determine whether the specific site is glycosylated, the molecular weight of E protein of the parental FX2010 and its single amino acid mutants was analyzed by Western blotting using frozen viral stocks. The E protein of FX-E_{S156P} migrated faster than those of the parental FX2010 and its other single-site mutants, indicating that its molecular weight is smaller than others. This result suggests that the S156P mutation ablates the N154 glycosylation site in the E protein (Fig. 7A).

To further confirm the glycosylation at E_{154N/155Y/156S}, DF-1 cells were infected with either FX2010 or FX-E_{S156P}. Viruses in supernatants and cell lysates were collected and prepared separately, and the E protein was analyzed by Western blotting. The virus was digested with or without either endoglycosidase Hf (Endo Hf, removes only immature carbohydrate structures) or peptide N-glycosidase F (PNGase F, removes all N-linked carbohydrate structures). The E protein of FX2010 migrated more quickly in cell lysates following treatment with either Endo Hf or PNGase F, indicating that the E protein of FX2010 was predominantly in high mannose form in DF-1 cells (Fig. 7B). In contrast to the cell-associated forms, the E protein of FX2010 in the supernatants was Endo Hf resistant but not PNGase F resistant (Fig. 7B). This result is consistent with carbohydrate side chain modification as the envelope proteins were transported through the Golgi apparatus. However, the E protein of FX-E_{S156P} in both cell lysates and supernatants was completely Endo Hf and PNGase F resistant, suggesting that S156P mutation abolished the single glycosylation on the E protein of FX2010.

To locate the amino acid at position 156, the images of E proteins of FX2010 and MM1775 were visualized and analyzed by using PyMOL referring to the JEV E protein. The amino acid at position 156 was presented and exposed on the surface and located

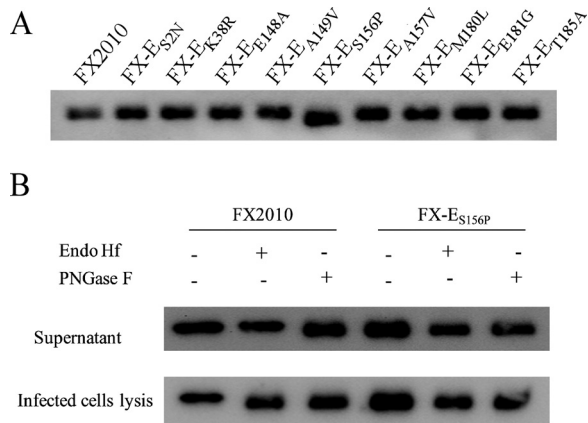


FIG 7 Glycosylation status of FX2010 E protein mutants. (A) The apparent molecular weight of E protein mutants was analyzed by Western blotting against frozen viral stocks. (B) Viruses in supernatants and DF-1 cell lysates from cells infected with FX2010 and FX-E_{S156P} were collected separately at 48 h postinfection and then treated, respectively, with glycosidases Endo Hf, PNGase F, or buffer only (-). Western blots were probed with a monoclonal antibody (1F5) that recognizes TMUV E proteins.

in the “150 loop” of E proteins (Fig. 8A). The amino acid mutations of S156P and P156S changed the loop conformations slightly on the predicted structures (Fig. 8B and C).

DISCUSSION

TMUVs, which belong to the *Flaviviridae* family and have caused significant impacts in the duck industry, have emerged in China since 2010, resulting in millions of dollars worth of losses annually (6, 8, 9, 19). Other flaviviruses such as dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), as well as recently the emerged Zika virus (ZIKV), pose a significant threat to public health (36–40). The diseases caused by JEV and WNV not only occur in seasons depending on mosquito vectors but can also be spread through vector-free transmission (14, 16, 41). Bagaza virus can be transmitted by direct contact in experimental partridges (15). In our

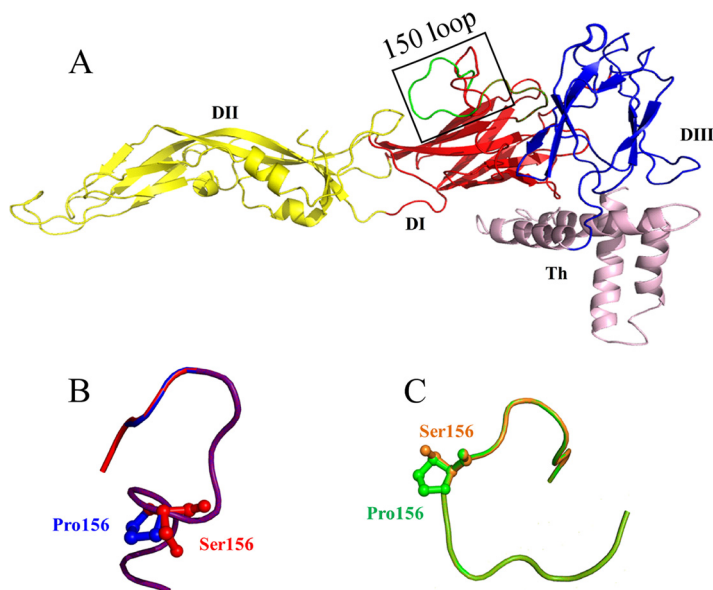


FIG 8 Constructed homology models of E proteins of TMUVs. To localize amino acid at position 156 of the E protein, the images of the E proteins of FX2010 and MM1775 were created with the program Phyre2 by using the JEV E protein structure (PDB accession number 5WSN). Homology models of the E protein of FX2010 (red) and MM1775 (green) (A), the “150 loop” of FX2010 (red) and FX-E_{S156P} (blue) (B), and the “150 loop” of MM1775 (green) and MM-E_{P156S} (orange) (C) are shown.

previous study, TMUV transmitted efficiently among ducks without mosquitos (19). To date, the molecular mechanisms on these pathogens' transmission independent of the mosquito vectors are poorly understood. To determine the genetic determinants that impact the transmissibility of TMUVs in ducks, we used two TMUV viruses: one is a prototypic MM1775 strain, first isolated from *Culex* mosquitos in 1955 (2), and the other is a recently emerged FX2010, isolated from sick ducks in China in 2010, to address this important question. Our results demonstrate that TMUV transmissibility in ducks is largely attributable to the E protein.

Flavivirus pathogenicity is associated with genetic changes in multiple genes and even noncoding regions (30, 34, 42–56). Amino acid changes in E proteins can result in attenuation of several flaviviruses due to the important role of the protein in host-specific adaption, cell tropism, virus attachment, and membrane fusion with target cells (28, 34, 45, 47, 48, 57–59). Like other flaviviruses, the TMUV E protein also has three different structural domains (28). In this study, we show that the DI of E protein is the key domain that crucial for vector-free transmissibility of TMUVs in ducks because the DI directly impacts virus replication in duck lungs, thereby influencing virus shedding and nonvector transmissibility. Finally, we determined that the amino acid Ser at position 156 in the E protein is responsible for virus tropism and transmission in ducks. The amino acid Ser at position 156 located in a “150 loop” region protrudes from the E protein surface. Similar to other flaviviruses, such as WNV and ZIKV (56, 60), the recent TMUV FX2010 has a single glycosylation site (N154) located in the “150 loop,” while the early TMUV MM1775 lacks this glycosylation due to the presence of amino acid proline at position 156. We further demonstrate that the S156P mutation in the E protein of the TMUV FX2010 results in the loss of N-linked glycosylation, leading to the abrogation of vector-free transmission of TMUV in ducks.

Glycosylation is important for the viral protein functions such as entry into host cells, proteolytic processing and protein trafficking (61). The glycosylation of E protein of TMUV FX2010 is Endo Hf resistant, suggesting that the E protein has been folded in the ER and processed through the Golgi network, before being released into the supernatant (24). In most flaviviruses, their E proteins contain one or more potential N-linked glycosylation motifs, but some flaviviruses lack this N-linked glycosylation site (60, 62–67). Among them, a highly conserved glycosylation motif is located at position 153 or 154 of the E protein (11, 12, 60, 68–70). The N153 or N154 glycosylation site is located at the “150 loop” of DI of the E protein, which could be an attachment region and influence virus transmission (25). The isolation of TMUV MM1775 from mosquito, which lacks this N-linked glycosylation site, suggests that N-linked glycosylation of the E protein is not necessary for early TMUV replication in mosquitos. Similar results have been found in DENV2 (57). However, ablation of the N-linked glycosylation site has been reported to reduce infectivity of DENV in mosquito cells (69). In addition, glycosylation of WNV E proteins can affect the efficiency of virus release and infection in a cell type-specific manner (56); further studies indicate that it is required for virus infection, but not spread within mosquito vectors (12). Recent studies have reported that the single N-linked glycosylation at N154 of ZIKV E protein influences E secretion from mammalian cells (60). Similar results have been also found in both WNV and tick-borne encephalitis virus (TBEV) (59, 66). Moreover, this glycosylation is critical for ZIKV infection of mammalian and mosquito hosts (71, 72) and antagonizing to the vector immune defense (73). In the present study, we demonstrate that nonglycosylation on the E protein of TMUVs results in limited virus replication (not systemic infection in ducks) and abrogation of virus transmission.

Analyses based on available E protein sequences show that N-linked glycosylation at N₁₅₃ or N₁₅₄ of E protein is conserved and predominant in most flaviviruses such as ZIKV, JEV, WNV, Murray Valley encephalitis virus, St. Louis encephalitis virus, TBEV, and DENV. This suggests that this glycosylation site is important for the survival of flaviviruses during evolution. Most flaviviruses, such as WNV, DENV, and ZIKV, that pose serious threats to public health contain N₁₅₃ or N₁₅₄ glycosylation (36–40). Viral E protein glycosylation is a molecular determinant of the neuroinvasiveness of WNV (74)

and ZIKV (20). The presence of the single N-linked oligosaccharide side chain on the E protein and its trimming by glucosidases is necessary for secretion of recombinant subviral particles and truncated E dimers (75). Furthermore, the production of WNV (67), DENV (61), and TBEV (75) was inhibited after E glycan structures were disrupted. The TMUV E protein 156S site is located at the “150 loop” which is involved in the dimer contacts of flavivirus E proteins (30). This “150 loop” region varies among flaviviruses, which suggests that differences in this region influence virus transmission and disease (31). We found that the S156P mutation not only disrupted N₁₅₄ glycosylation of the E protein but might also change the E protein loop conformation. Whether the predicted change in the structure of “150 loop” contributes to the abolishment of the transmission of TMUV in ducks needs to be examined. However, TMUV replicates in the spleens of infected ducks independently of the S156P mutation, suggesting that host factors in different tissues most likely impact viral replication.

Taken together, we demonstrate that a single amino acid (S) at position 156 of TMUV E protein is critical for virus replication and transmissibility in ducks, and we further reveal that its mutation (S156P) abolishes N-linked glycosylation at N₁₅₄ and changes the conformation of the “150 loop” of the E protein, thereby influencing virus transmissibility among birds independent of mosquito vectors. Whether this finding also fits other flaviviruses should be explored in future studies.

MATERIALS AND METHODS

Ethics statement. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People’s Republic of China. The protocol (Shvri-po-2015040380) used in the study was approved by the Animal Care Committee of the Shanghai Veterinary Research Institute.

Cells and viruses. DF-1 cells were obtained from the American Type Culture Collection (ATCC) and maintained in Dulbecco modified Eagle medium (DMEM; HyClone, Logan, UT), supplemented with 5% fetal bovine serum (FBS; Biowest, South America), and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂ humidified incubator.

TMUV strain FX2010 was rescued by a PCR-based reverse genetic system using the viral RNAs of FX2010, which was isolated from sick ducks and purified three times in specific-pathogen-free chicken embryonated eggs by a limiting dilution method. TMUV strain MM1775 was rescued by a PCR-based reverse genetic system using the viral RNAs of MM1775 (ATCC VR-1266), which was first isolated from *Culex tritaeniorhynchus* in Malaya in 1955 and propagated in the brain of a suckling mouse. All of the rescued viruses were propagated once on DF-1 cells, aliquoted, and stored at –80°C.

Sequence analysis. The viral RNAs were extracted using RNAiso Plus (TaKaRa Biotechnology, Dalian, China), and the first-strand cDNAs were synthesized with specific reverse transcription primers (see Table S1 in the supplemental material) and SuperScript III transcriptase (Invitrogen, USA) (33). The overlapping segments of PCR products covering the whole genome amplified with primers reported previously (24) were sequenced with ABI 3730 automated sequencers (Applied Biosystems). The sequences were analyzed using DNASTAR software.

Plasmid construction. To prepare the templates for PCR-based virus rescue, the first-strand cDNAs of FX2010 and MM1775 were used to amplify the overlapping segments covering the whole genome by a PCR method using High Fidelity DNA polymerase *Pfx* (Invitrogen). Four plasmids, pFXT7-1-956, pFX942-2459, pFX2433-3831, and pFX3656-10991, containing nucleotides 1 to 956 with the addition of a T7 promoter at the 5’ terminus, nucleotides 942 to 2459, nucleotides 2433 to 3831, and nucleotides 3656 to 11001 of FX2010 were generated to provide the template for FX2010 genome amplification. For MM1775, the primers were modified to improve the amplification efficiency and, accordingly, the four plasmids pMMT7-1-955, pMM941-2458, pMM2433-3827, and pMM3549-11001, containing nucleotides 1 to 955 with the addition of a T7 promoter at the 5’ terminus, nucleotides 941 to 2458, nucleotides 2433 to 3827, and nucleotides 3549 to 11001 of MM1775, respectively, were generated. Plasmids containing a domain substitution or single-site mutation on E protein were generated through two-step PCR methods or using a site-directed mutagenesis kit (TransGene, Inc., Strasbourg, France) based on the background of the plasmids pFX942-2459 and pMM941-2458, which contained the E genes of each virus.

PCR-based Tembusu virus rescue. The full-length cDNA with T7 promoter was generated by two rounds of PCR using High Fidelity DNA polymerase *pfx* (Invitrogen) as reported previously (33). First-round PCR was performed to produce four overlapped fragments, using each of four plasmids (pFXT7-1-956, pFX942-2459, pFX2433-3831, and pFX3656-10991; or pMMT7-1-955, pMM941-2458, pMM2433-3827, and pMM3549-11001) as the templates. For the rescue of the viruses with domain substitution or a single-site mutation, one of four plasmids pFX942-2459 or pMM941-2458 was substituted with the accordingly modified plasmid. The full-length cDNA was then produced by fusion PCR, using the PCR fragments as the templates.

The transcription of infectious viral RNA from full-length cDNA was performed *in vitro* using an mMESSAGE mMACHINE T7 kit (Ambion, USA). The transcripts were purified by lithium chloride precipitation and used to transfect DF-1 cells on 6-well plate at an amount of 5 µg using Lipofectamine LTX

and Plus Reagent (Invitrogen). The cell culture medium was changed to the DMEM containing 2% FBS at 6 h posttransfection. When cytopathic effects appeared in the transfected cells, the supernatants were collected, and rescued viruses were amplified on DF-1 cells, aliquoted, and stored at -80°C . The rescued viruses were confirmed by sequencing.

Growth of TMUVs in DF-1 cells. To test the viral replication *in vitro*, DF-1 cells grown in T-25 flask were infected with TMUVs at an MOI of 0.0001. The cells were incubated with DMEM containing 2% FBS at 37°C . Virus samples from the supernatant were harvested every 12 h postinfection and subjected to virus titration on DF-1 cells to determine the virus titer.

Virus titration. To determine the virus titer, organs were weighed and homogenized in PBS to yield 1:1 (ml/g) tissue homogenates. Tissue homogenates or cell cultures were clarified by centrifugation, and undiluted and 10-fold serially diluted supernatants were titrated for virus infectivity on DF-1 cells in 96-well plates. The lower limit of virus detection was $0.5 \log_{10}$ TCID₅₀ per 0.1 g of tissue. The virus titer was calculated by the method of Reed and Muench.

Blocking ELISA. To test for specific antibodies against TMUV, a blocking ELISA method was used as described previously (76). Briefly, each well of ELISA plates was coated with $\sim 0.1 \mu\text{g}$ of purified FX2010 in 0.1 M carbonate-bicarbonate buffer (pH 9.6), followed by incubation overnight at 4°C . Antigen-coated plates were washed with PBS (pH 7.4) containing 0.05% Tween 20 (PBST), and the nonspecific binding sites were blocked with $100 \mu\text{l}$ of blocking buffer (PBS containing 5% skim milk) for 1 h at 37°C . After a 10-fold dilution in PBS, serum samples were added to the wells, followed by incubation for 1 h at 37°C . The wells were then washed three times with PBST and incubated with monoclonal antibody (MAb) 1F5 (1:20) for 1 h at 37°C . After the wells were washed three times with PBST, goat anti-mouse IgG (1:2,000; Sigma, USA) conjugated to horseradish peroxidase (HRP) was added, and the mixture was incubated at room temperature for 1 h. After the wells were rinsed with PBST three times, $100 \mu\text{l}$ of 3,3',5,5'-tetramethyl benzidine was added, and the mixture was incubated at room temperature for 5 min. The reaction was then stopped by adding 0.1 N sulfuric acid. The optical density (OD) was measured at 450 nm, and the percent inhibition (PI) was determined using the following formula: $\text{PI} (\%) = [1 - (\text{OD}_{450} \text{ of test serum} / \text{OD}_{450} \text{ of negative-control serum})] \times 100$. The serum was considered positive for TMUV reactivity when the PI value was $\geq 18.4\%$.

Duck experiments. To test the pathogenicity and transmissibility of TMUV strain FX2010, TMUV strain MM1775, or the modified viruses in ducks, 12- to 15-week-old Shelducks (a local outbred strain) were inoculated i.n. or i.m. with $10^{3.5}$ TCID₅₀ of each virus at a volume of 0.2 ml, respectively. One day later, naive ducks were introduced into the isolators where the inoculated ducks were housed. The ducks were monitored daily for clinical signs. The specific method is described as follows.

To compare the replication and transmission of TMUV FX2010 and MM1775, groups of six ducks were inoculated i.m. and i.n., respectively, and three naive ducks were introduced 1 day later. On day 3 postinoculation, three inoculated birds in each group were euthanized by CO₂ inhalation, and tissues from the spleen, lung, kidney, brain, ovary, pancreas, and trachea, as well as serum samples, were collected to measure viral replication. The serum samples were collected from the remaining inoculated ducks at 0, 4, and 7 dpi and from the contact ducks at 0, 7, and 14 dpc for antibody detection.

To identify the proteins related to the replication and transmissibility of TMUVs, the chimeric viruses with E gene substitution and their parental viruses were inoculated i.m. into six ducks, respectively, and six naive ducks were introduced 1 day later. In each group, three inoculated ducks were euthanized at 3 dpi, and three contact ducks were euthanized at 6 dpc by CO₂ inhalation, and tissue samples from the spleens, lungs, kidneys, and ovaries were collected for viral titration. The serum samples were collected from the remaining inoculated ducks at 0, 4, and 7 dpi and from the remaining contact ducks at 0, 7, and 14 dpc for antibody detection.

To determine further which domain of TMUV E protein influenced the replication and transmissibility of TMUVs in ducks, the E domain substitution recombinant viruses were inoculated i.m. into six ducks, and three naive ducks were introduced 1 day later. Three inoculated ducks were euthanized by CO₂ inhalation at 3 dpi, and tissue samples from the spleens, lungs, kidneys, and ovaries were collected for viral titration. The serum samples were collected from the remaining inoculated ducks at 0, 4, and 7 dpi and from the contact ducks at 0, 7, and 14 dpc for antibody detection.

To determine the key amino acids attributed to the replication and transmission of TMUV FX2010 in ducks, viruses with single amino acid mutations located at E-DI were inoculated i.m. into three ducks, following by the introduction of three naive ducks 1 day later. Serum samples were collected from the inoculated ducks at 0, 1, 2, 3, 4, and 7 dpi or from the contact ducks at 0, 1, 2, 3, 4, 5, 7, and 14 dpc for virus titration or antibody detection.

To compare the virus replication of FX-E_{5156P} with the parental FX2010 in ducks, each virus was inoculated i.m. into three ducks. The inoculated ducks were euthanized by CO₂ inhalation at 3 dpi, and tissue samples from the spleens, lungs, kidneys, and ovaries were collected for viral titration.

To test the influence of E_{P156S} mutation in the replication and transmission, each of six ducks was inoculated i.m. with MM-E_{P156S} and MM1775, respectively, and three naive ducks were introduced into each of isolator 1 day later. Three inoculated ducks were euthanized by CO₂ inhalation at 3 dpi, and tissue samples from the spleens, lungs, kidneys, and ovaries were collected for viral titration. The serum samples were collected from remaining inoculated ducks and the contact ducks at different dpi or dpc points for virus titration or antibody detection.

Western blotting. To analyze the influence of single amino acid mutation on the posttranslational modification of E proteins, Western blot assays were performed. Briefly, samples were denatured in sample preparation buffer (2% sodium dodecyl sulfate[SDS], 50 mM Tris [pH 6.8], 10% glycerol) at 95°C for 10 min. The samples were then analyzed under denaturing conditions by SDS-PAGE (10%) and then

transferred to nitrocellulose membrane (PALL, USA) by wet transfer process. The membrane was blocked with 5% (wt/vol) skim milk powder in PBS at room temperature for at least 1 h before probing the protein bands with E MAb 1F5 (1:500) (76) overnight at 4°C.

After three washes with PBST, the membranes were incubated with HRP-linked anti-mouse IgG goat antibody (1:2,000; Sigma) for 2 h at room temperature. Blots were imaged using SuperSignal West Femto maximum sensitivity substrate (Thermo) on a Tanon 5200 automatic chemiluminescence analyzer (Tanon, Inc., Shanghai, China).

Glycosylation assays. DF-1 cells were plated in a six-well plate at 10^6 cells/well and infected with each indicated virus at an MOI of 0.0001. At 48 h postinfection, both supernatants and cells were collected. The cells were solubilized in lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100 [pH 7.5] with protease inhibitors [Roche Molecular Biochemicals, Indianapolis, IN]). Lysates were centrifuged at $10,000 \times g$ for 10 min to clear cellular debris. Both supernatants and lysates were denatured in sample preparation buffer (2% SDS, 50 mM Tris [pH 6.8], 10% glycerol) at 55°C for 20 min. Aliquots from each sample were then treated with 50 U of PNGase F or Endo Hf according to the manufacturer's instructions (New England BioLabs, Inc., Beverly, MA) or with PNGase F buffer without enzyme as a control as described previously (56). Samples were then analyzed under denaturing conditions by SDS-PAGE (7.5% or a 10 to 20% gradient) and Western blotting.

Homology modeling. To analyze the E protein structure, images of the E proteins of FX2010 and MM1775 were created with the program Phyre2 (77). The E protein structure was referred to JEV E protein (Protein Data Bank [PDB] accession number 5WSN) and visualized and analyzed with PyMOL (Schrodinger, 2015).

Statistical analysis. Virus titers between groups were evaluated using analysis of variance with GraphPad Prism software (GraphPad Software, Inc.). A *P* value of ≤ 0.05 was considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JVI.00427-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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Z.L. designed the study. D.Y., Y.S., H.W., G.L., X.L., B.W., X.S., J.W., Q.T., J.Y., H.C., and Q.L. performed the experiments. D.Y., W.M., and Z.L. analyzed the data and wrote the paper.

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