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Development of a live attenuated vaccine candidate against duck Tembusu viral disease

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ABSTRACT

Duck Tembusu virus (DTMUV) is a newly emerging pathogenic flavivirus that is causing massive economic loss in the Chinese duck industry. To obtain a live vaccine candidate against the disease, the DTMUV isolate FX2010 was passaged serially in chicken embryo fibroblasts (CEFs). Characterization of FX2010-180P revealed that it was unable to replicate efficiently in chicken embryonated eggs, nor intranasally infect mice or shelducks at high doses of 5.5 $\log_{10}$ tissue culture infectious doses ($TCID_{50}$). FX2010-180P did not induce clinical symptoms, or pathological lesions in ducks at a dose of 5.5 $\log_{10}$ $TCID_{50}$. The attenuation of FX2010-180P was due to 19 amino acid changes and 15 synonymous mutations. Importantly, FX2010-180P elicited good immune responses in ducks inoculated at low doses (3.5 $\log_{10}$ $TCID_{50}$) and provided complete protection against challenge with a virulent strain. These results indicate that FX2010-180P is a promising candidate live vaccine for prevention of duck Tembusu viral disease.

Introduction

Since April 2010, outbreaks of infectious disease in ducks that are characterized by retarded growth and rapid egg drop have spread widely in China. The causative agent has been identified as duck Tembusu virus (DTMUV), a newly emerged duck virus that belongs to the genus *Flavivirus* in Flaviviridea family (P. Yan et al., 2011). The important mosquito-borne pathogens within this virus family include Japanese encephalitis virus (JEV), dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV). The infectious diseases caused by mosquito-borne flaviviruses generally occur in summer and autumn in temperate areas. However, the disease caused by DTMUV is characterized by outbreaks throughout the year. The outbreaks persist even when ambient temperatures are lower than freezing, conditions under which no insects are active in northern China (P. Yan et al., 2011). This is a significant difference compared with the diseases caused by other insect-borne flaviviruses. The duck Tembusu viral disease caused by DTMUV has caused massive economic loss in the Chinese duck industry. However, no vaccine is currently available for prevention of the disease.

Serial passage of parental flaviviruses in cell culture is often used for developing live attenuated vaccine candidates. Yellow fever 17D strain, a classic live-attenuated vaccine, has been obtained by passing the parental virus through modified chicken embryo tissues (Barrett and Teuwen, 2009). Similarly, the SA 14-14-2 strain of the Japanese encephalitis (JE) vaccine was derived from serial passage of the SA14 virus in primary hamster kidney (PHK) cell culture (Yu, 2010). A DTMUV FX2010 strain isolated previously from sick shelducks with rapid egg drop caused systemic infection and lesions in ducks (P. Yan et al., 2011). Here, we report the derivation of FX2010-180P, a strain that emerged from FX2010 after 180 passages and was avirulent in 3- to 5-week-old ducks. However, FX2010-180P induced vigorous immune responses and protected the ducks against the virulent FX2010 strain. The virulence, genetic characteristics, and immunogenicity in ducks of FX2010 derivatives obtained at different passages are also discussed.

Results

Mortality and replication of DTMUVs in chicken embryos

The virulence of a dose of 5.0 $\log_{10}$ $TCID_{50}$ of viruses was evaluated in 8-day-old SPF chicken embryonated eggs. All the embryos in these eggs inoculated with FX2010 died within 36–96 h.p.i, and the survival time was of 56.4 ± 20.4 h (Fig. 1). Viral virulence in the SPF chicken

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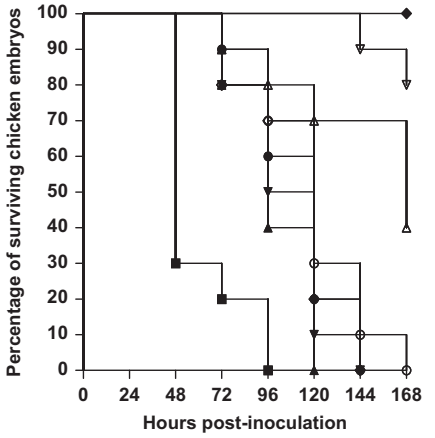

	Surviving time (hours)	Infected embryos	Viral RNA copies in positive embryos (log10 copies/μl)
Control	>168.0	0/10	–
FX2010	56.4±20.4	10/10	5.21±0.26
FX2010-60P	93.6±18.6	10/10	3.95±0.39
FX2010-80P	99.6±22.7	10/10	3.82±0.31
FX2010-100P	106.8±22.9	10/10	3.89±0.25
FX2010-130P	112.8±27.8	10/10	4.03±0.70
FX2010-145P	109.2±23.6	10/10	3.82±0.31
FX2010-168P	>128.0	7/10	3.61±0.24
FX2010-180P	>156.0	2/10	3.70±0.24

Fig. 1. Mortality and replication of DTMUVs in chicken embryos. Ten 8-day-old SPF chicken embryonated eggs were inoculated intra-allantoically with different passage viruses at a dose of 5.0 $\log_{10}$ TCID50. The eggs were incubated at 37 °C and checked daily. The survival times of the inoculated eggs were recorded. Viral infection in chicken embryonated eggs was tested by real-time PCR methods, and embryo samples with a Ct value lower than 35 were considered DTMUV RNA- positive. The DTMUV RNA in the allantoic fluid of the positive eggs was quantified using the same real-time PCR methods. Statistically significant differences between FX2010-infected eggs and the passage virus-infected eggs are marked with asterisks ($P < 0.01$).

embryonated eggs decreased gradually as passage number increased. The survival time of egg embryos inoculated with FX2010-60P, FX2010-80P, FX2010-100P, FX2010-130P, and FX2010-145P increased to 93.6 ± 18.6, 99.6 ± 22.7 h, 106.8 ± 22.9 h, 112.8 ± 27.8 h, and 109.2 ± 23.6 h, respectively (Fig. 1). Both mortality and infection rate associated with eggs inoculated with FX2010, FX2010-60P, FX2010-80P, FX2010-100P, FX2010-130P, and FX2010-145P were 100%. While the mortality rates of eggs inoculated with FX2010-168P and FX2010-180P decreased to 60% and 20% within 168 h.p.i (Fig. 1), the infection rates in these cases were 70% and 20% when the allantoic fluid was tested using real-time PCR, respectively.

The average DTMUV RNA copy number in allantoic fluid of FX2010-infected eggs reached 5.21 $\log_{10}$ copies/μl, while in the eggs infected with FX2010-60P, FX2010-80P, FX2010-100P, FX2010-130P, FX2010-145P, FX2010-168P, and FX2010-180P, the average DTMUV RNA copy number ranged from 3.70 to 4.03 $\log_{10}$ -copies/μl (Fig. 1). The DTMUV RNA copy number in FX2010-infected eggs was significantly higher than that of eggs infected with serially passaged viral strains (Fig. 1; $P < 0.01$, Student's t-test). There were no significant differences (Fig. 1; $P > 0.05$, Student's t-test) in the DTMUV RNA copy number for infected eggs for virus strains with different passage numbers, although the mortality and infectivity associated with both FX2010-168P and FX2010-180P was lower than that for the other strains.

Replication of serially passaged viruses in mice

To evaluate the infectivities of the different passage viruses in mice, 5.0 $\log_{10}$ $TCID_{50}$ of viruses were inoculated i.c. or i.n. (30- or 50-μl volumes) into 4-week-old SPF Balb/c mice. At 4 d.p.i., the average RNA copy number of FX2010 in the lung supernatant of the mice inoculated i.n. was 3.68 $\log_{10}$ copies/μl. However, the DTMUV RNAs in mouse lungs decreased significantly as passage number increased, and only very low DTMUV RNA copy numbers were observed in mice inoculated with FX2010-60P, FX2010-80P, and FX2010-100P. There was a statistically significant difference in copy number between the FX2010-infected group and the passaged virus-infected groups (Fig. 2a; $P < 0.01$, Student's t-test). No DTMUV RNAs were detected in the lungs of mice inoculated with FX2010-130P, FX2010-145P, FX2010-168P, or FX2010-180P (Fig. 2a), suggesting that the virus lost its ability to infect lung tissue after 130 passages. In all the brains of mice inoculated i.c. with virus, the viral RNAs could be detected by real-time PCR. The average RNA copy number in the brains of mice inoculated with FX2010 was as high as 5.64 $\log_{10}$ copies/μl, which was significantly higher than that in brains from mice inoculated with passaged viruses (Fig. 2b; $P < 0.01$, Student's t-test). The average RNA copy number in the brains of mice inoculated with FX2010-180P was significantly lower than that observed with the virus obtained from earlier passages (Fig. 2b; $P < 0.01$, Student's t-test).

To evaluate the neurovirulence of viruses in newborn mice, 3-day-old SPF Balb/c mice were inoculated i.c. with 5.0 $\log_{10}$ $TCID_{50}$ of FX2010 or FX2010-180P. At both 4 and 6 d.p.i., the DTMUV RNA copies in the brains of mice inoculated with FX2010-180P were significantly lower than in the brains of mice inoculated with FX2010 (Fig. 2c; $P < 0.01$, Student's t-test).

To test the stability of FX2010-180P in mouse brains, the whole genome of the FX2010-180P was amplified from brains of mice euthanized on day 6 post-infection by reverse transcription polymerase chain reaction (RT-PCR). The PCR product was sequenced, but no mutation was found, suggesting that FX2010-180P was stable in the brain of mice in one passage.

Pathogenicity and infectivity of virus in ducks inoculated intranasally

Clinical symptoms, including lethargy, anorexia, and greenish diarrhea were observed in the ducks inoculated i.n. with FX2010 at 3–4 d.p.i. Mild anorexia and occasional greenish diarrhea were observed in ducks infected with FX2010-60P and FX2010-80P. None of the ducks inoculated with FX2010-100P or FX2010-130P showed any obvious clinical symptoms. Infection with FX2010 caused grossly swollen spleens and mildly swollen kidneys, whereas FX2010-60P, FX2010-80P, and FX2010-100P caused gross to mild spleen swelling. Importantly, infection with FX2010-130P did not cause any visible pathogenic changes in ducks under these conditions.

In order to detect viral replication, the spleen, lung, kidney, and brain of the i.n. inoculated ducks were collected at 4 d.p.i, homogenized, titrated on DF-1 cells, or tested by real-time PCR for DTMUV-specific RNA. A high titer of viruses and DTMUV-specific RNA was detected in the spleen, lung, kidney, and brain of the ducks inoculated with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 (Table 2). Viruses from passages later than FX2010-60P were not able to replicate in the brain under these conditions. However, the replication of 130th passage viruses was not detected in any of the organs tested, and the seroconversion testing using blocking ELISA in the remaining ducks from each group revealed that only one-third

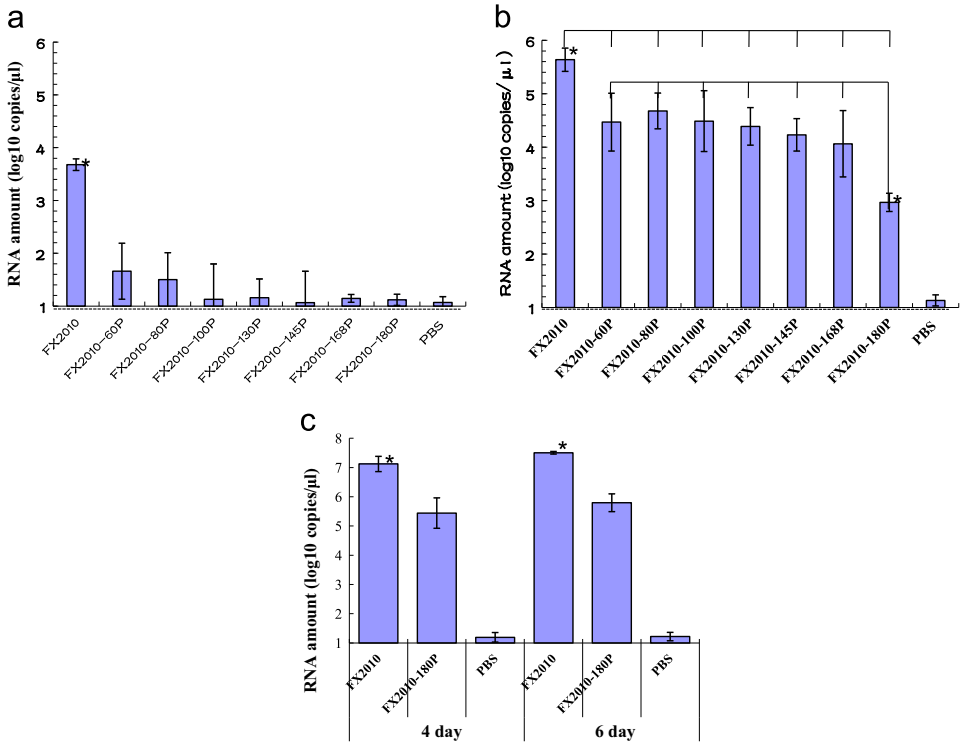

Fig. 2. In vivo replication of viruses isolated from different passages. Groups of three 4-week-old female or six 3-day-old newborn BALB/c mice were lightly anesthetized using carbon dioxide (CO_2). The 4-week-old mice were treated intranasally (i.n.) with 50 μl or inoculated intracerebrally (i.c.) with 30 μl containing 5.0 $\log_{10}$ $TCID_{50}$ of virus, and newborn mice were inoculated i.c. with the same amount of virus. On day 4 post-inoculation, three mice from each group were euthanized and the lungs (a) of those inoculated i.n. and the brains of those 4-week-old (b) and newborn mice (c) inoculated i.c. were collected. On day 6 post-inoculation, the remaining newborn mice (c) were euthanized and the brains were collected. The collected organs were homogenized in PBS to yield 1:1 (ml/organ) tissue homogenates, and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants of tissue homogenates were used for DTMUV RNA quantification. Statistically significant differences between the FX2010-infected group and the passage virus-infected groups inoculated both i.n. and i.c. and the differences between the group inoculated i.c with FX2010-180P and the group inoculated i.c with other passage viruses are marked with asterisks and connecting lines ($P < 0.01$).

of the FX2010-130P inoculated animals underwent conversion at 14 d.p.i.

Pathogenicity and infectivity of virus in ducks inoculated i.m.

The clinical symptoms in ducks inoculated i.m. and those inoculated i.n. with FX2010 were similar, and were observed at 3–4 d.p.i. None of the other viral strains induced obvious clinical symptoms. Compared with FX2010, which caused grossly swollen spleens and mild swollen kidneys, FX2010-130P and FX2010-145P caused gross-to-mild spleen swelling at 4 d.p.i. In contrast, neither FX2010-168P nor FX2010-180P induced any visible pathogenic changes under these conditions.

High viral titers and DTMUV-specific RNA were detectable in the spleen, lung, kidney, and brain of the 3 ducks inoculated with FX2010 (Table 2). However, virus and DTMUV-specific RNA was only detectable in the spleens of ducks inoculated with FX2010-180P. Seroconversion was verified with blocking ELISA in all the remaining ducks inoculated with serially passaged viral strains.

Pathogenicity and infectivity of virus in ducklings inoculated i.m.

To evaluate the pathogenicity and infectivity of viruses in ducklings, 5-day-old ducks were inoculated i.m. with 3.5 $\log_{10}$ T-CID50 of FX2010 or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P. The clinical symptoms, including lethargy, anorexia, and greenish diarrhea were also observed in ducklings inoculated with FX2010 at 3–4 d.p.i. None of the obvious clinical symptoms were induced by FX2010-180P. Compared with FX2010, which also caused grossly swollen spleens and mildly swollen kidneys, FX2010-180P did not induce any visible pathogenic changes in ducklings. The viral titers in different organs of ducklings were very similar with which in 8-week-old ducks, inoculated either with FX2010 or FX2010-180P (Table 2).

Viral RNA copy numbers in the sera of ducks inoculated with FX2010 or FX2010-180P

To compare the viremia caused by FX2010 and FX2010-180P, five 3- to 5-week-old ducks each were inoculated either i.n. or i.m. with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P. The highest serum viral RNA copy numbers ranged from 5.16 to 5.81 log10 copies/μl and were most frequently observed in ducks inoculated i.n. with FX2010 on day 2 post-inoculation. Titer values then decreased at 4 d.p.i. and disappeared after 5 d.p.i. (Table 3). In ducks inoculated i.m. with FX2010, the highest serum viral RNA copy numbers ranged from 4.36 to 5.89 $\log_{10}$ copies/μl and appeared 1 day earlier than in intranasally-inoculated ducks. Titers then decreased at 3 d.p.i. and disappeared 4 d.p.i. Strikingly, no FX2010-180P viral RNA was detected in the sera of ducks inoculated by either route over the 14-day period post-inoculation (Table 3).

Antibody responses of ducks inoculated with FX2010 or FX2010-180P

All ducks inoculated i.m. with FX2010 were seroconverted at 3 d.p.i. Three of five ducks inoculated i.n. with FX2010 were seroconverted at 3 d.p.i, with the remaining animals undergoing seroconversion by 4 d.p.i. However, none of the ducks inoculated i.n. with FX2010-180P showed seroconversion at 14 d.p.i. (Fig. 3a). Two of five ducks inoculated i.m. with FX2010-180P were seroconverted at 4 d.p.i and the remaining ducks seroconverted at 6 d.

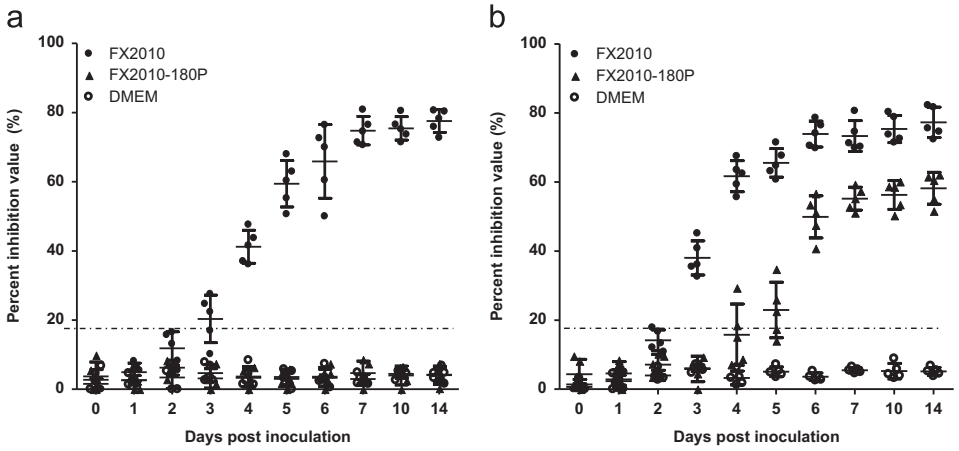

Fig. 3. Antibody responses in ducks inoculated with FX2010 or FX2010-180P. To compare the immune responses caused by FX2010 and FX2010-180P, groups of five 3- to 5-week-old SPF shelducks each were inoculated with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P through i.n. (a) and i.m. (b) routes, respectively. Two groups of 5 control shelducks were inoculated i.m. or i.n. with DMEM containing 2% FBS. Sera of all ducks were collected daily from 1 to 7 d.p.i. and at 10 and 14 d.p.i. for antibody titration using the blocking ELISA method. The serum was considered DTMUV -positive when the PI value was $\geq$ 18.4%.

p.i. Compared with the results for FX2010, the antibody responses induced by FX2010-180P were delayed by approximately 2–3 days, and the PI values of antibodies were 20% lower than those induced by FX2010 (Fig. 3b).

Sequence analysis

Viral virulence gradually decreased as passage number increased. To explore the molecular basis underlying the attenuation of infectivity, the complete viral genomes of all viruses in this study were sequenced. Compared with FX2010, no virus RNA was detectable in the brains of ducks, and only very low amounts of RNA were found in the lungs of partial mice infected with FX2010-60P. The attenuation might be associated with 5 amino acid substitutions and 2 synonymous nucleotide substitutions. Three amino acid substitutions (D120N, V132A, and M349K) were in the E protein, 1 amino acid substitution (V262A) was within the NS1 protein and the other (T322I) was in NS3 (Table 4). Two synonymous nucleotide substitutions were at positions 433 and 3368 in the viral genome. Furthermore, there was a mixture of original and mutated nucleotides at 4 positions (1420, 4865, 6956, and 8105) in the viral genome, indicative of positions that are actively undergoing selection during passaging. The mixed nucleotides at 3 positions (4865, 6956, and 8105) did not change amino acids, and only these at the positions 1420 resulted in mixed amino acids (Y and F) at the position 155 of E protein.

Compared with FX2010-60P, the pathogenicity of FX2010-80P and FX2010-100P was not significantly reduced. Additional mixed nucleotides were found at position 1426, resulting in mixed amino acids (A and V) at the position 157 of E protein, in the genome of FX2010-80P, and at position 1222, resulting in mixed amino acids (E and G) at the position 89 of E protein, in the genome of FX2010-100P.

Consistent with its reduced virulence compared to viruses isolated at earlier passages, more nucleotide substitutions were evident in the genome of FX2010-130P. A comparison with FX2010-60P, FX2010-80P, and FX2010-100P revealed that amino acid E-155 in FX2010-130P was fixed, as it had changed from Y to F, and additional mixed amino acids were found at 5 positions in M (106), NS1 (205), NS4A (110), and NS4B (50 and 112) proteins. With regard to synonymous nucleotide changes, 2 substitutions were fixed, as they had changed from A to T at position 4865 and form C to T at position 6956, and additional mixed bases were found at 4 positions (1992, 2060, 8105 and 8264) in the genome of FX2010-130P. Two amino acid substitutions, E89G and A157V, were fixed in the E protein of FX2010-145P, when compared with FX2010-130P. No mixed nucleotides at single position were found in the sequence of FX2010-168P. The mixed amino acid at 5 positions (M-106, NS1-205, NS4A-110, NS4B-50, and NS4B-112) in early passages were fixed in FX2010-168P, and 2 additional amino changes E-R166K and NS5-V793A were found in FX2010-168P.

The virulence of FX2010-180P was attenuated dramatically in mice and in chicken embryonated eggs, but was not significantly different to FX2010-168P with respect to pathogenicity in ducks. Four amino acids (E-D37N, NS1-R192G, NS4AF54L, and NS5-R273G) and 2 synonymous nucleotides in the stop codon and 3′ noncoding region (NCR) were different between FX2010-168P and FX2010-180P. Compared with the results for FX2010, a total of 34 nucleotide changes were found in the entire genome of FX2010-180P. Of these, 19 missense mutations resulted in 19 amino acid changes. These included the following: one change in prM/M; eight in E; three in NS1; one in NS3; two changes each in NS4A, NS4B, and NS5 (Table 4); and 15 synonymous changes (Table 5). In addition, 2 synonymous changes were found in the 3′-NCR.

Immunoprotection in ducks vaccinated with FX2010-180P

To evaluate the immunogenicity of FX2010-180P, ducks were vaccinated i.m. with a 3.5 $\log_{10}$ $TCID_{50}$ dose of the virus. The antibody titers (PI value) of immunized ducks ranged from 48.2% to 64.5% at 2 w.p.i., 54.9% to 64.3% at 4 w.p.i., 47.0% to 62.0% at 8 w.p.i., and 45.0% to 61.8% at 12 w.p.i., while that of control ducks were lower than 12.9% (Fig. 4a). After challenge with FX2010 at 2 or 12 w.p.i., the control ducks had typical clinic symptoms as described previously. However, none of the ducks vaccinated with FX2010-180P showed any obvious clinical symptoms. Three ducks in each group were euthanized and dissected at day 4 post challenge. The ducks in control groups showed grossly swollen spleens, ruptured ovarian follicles, and vitelline peritonitis (Fig. 4c). In contrast, the ducks vaccinated with FX2010-180P had normal spleens and ovaries (Fig. 4d). Whereas virus was isolated from the spleens, lungs, kidneys, ovaries, and brains of all control ducks (Fig. 4b), it was absent from vaccinated animals, suggesting that FX2010-180P affords complete protection against FX2010 challenge at 2 and 12 w.p.i.

Discussion

The newly emerged duck Tembusu viral disease caused by DTMUV had resulted in a huge economic loss to the Chinese duck industry since 2010. In addition to ducks and geese, DTMUV can infect other avian species, including chicken and house sparrows

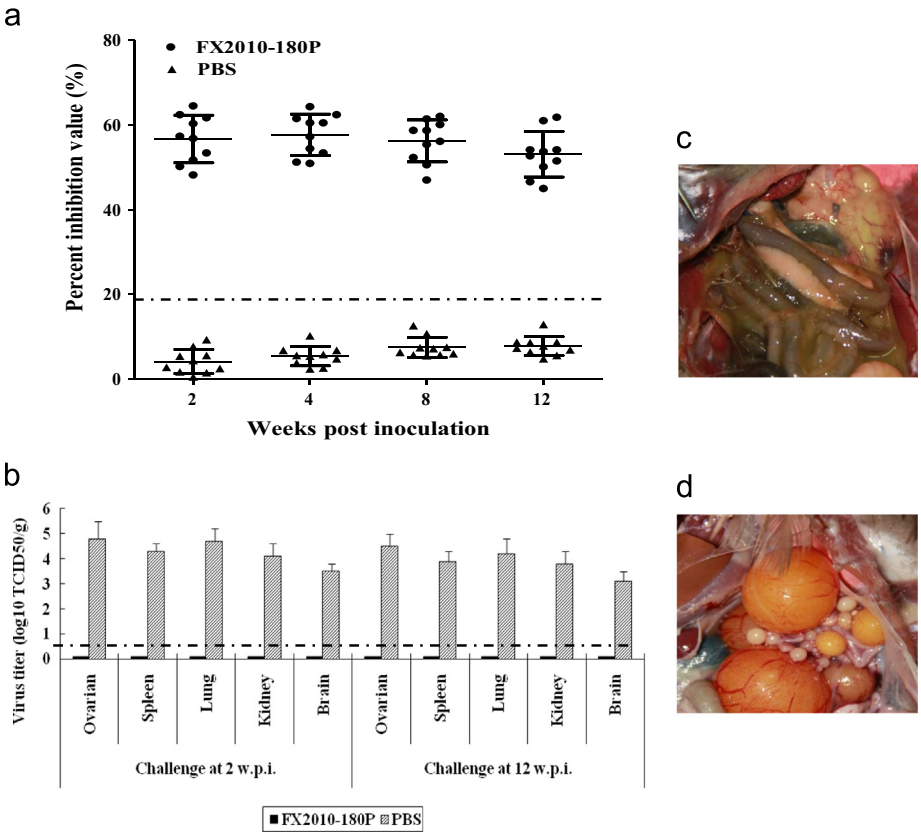

Fig. 4. Vaccination with FX2010-180P completely protects ducks against FX2010 challenge. To evaluate the immunogenicity of FX2010-180P, twenty 18-week-old shelducks were vaccinated (i.m.) at a dose of 3.5 $\log_{10}$ $TCID_{50}$. Twenty control shelducks were inoculated (i.m.) with PBS. The serum samples were collected for antibody titer testing using blocking ELISA at 2, 4, 8 and 12 w.p.i (a). At 2 and 12 w.p.i, ducks were challenged i.m. with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010. Samples from the spleen, lung, kidney, brain, and ovary were collected for virus titration, and high virus titers were detected from all organs of control ducks (b). The ducks in control groups showed grossly ruptured ovarian follicles and vitelline peritonitis (c). In contrast, the ducks vaccinated with FX2010-180P had ovarian tissue with a normal appearance (d).

(Huang et al., 2012; Liu et al., 2012; Tang et al., 2012; P. Yan et al., 2011). As a member of the Flaviviridae, DTMUV also poses a potential health threat to mammals, including humans (S. Li et al., 2013; Liu et al., 2013; Tang et al., 2013). To date, DTMUV is widespread in China, but no effective measures are taken to prevent the disease.

Formalin-inactivated whole-virion vaccines produced in mouse brain tissue or cell culture have been successfully developed for other flaviviruses, including those causing Japanese encephalitis (McArthur and Holbrook, 2013), tick-borne encephalitis, louping ill, and Kyasanur Forest disease (Monath, 2001). For preparation of an inactivated vaccine, the virus must replicate to high titer, in order to provide sufficient antigenic mass. However, DTMUV does not reach very high titers in cell culture during replication, which has thwarted the development of inactivated vaccine against this virus. To work around this issue, two doses of inactivated vaccine have instead been employed in order to stimulate a significant immune response (Hoke et al., 1988). However, the higher prices of inactivated flavivirus vaccines (Hennessy et al., 1996) limit the utility of this approach in animals, and in particular in poultry.

Live attenuated virus vaccines against flaviviruses are widely used. The most successful live attenuated virus vaccine is that against yellow fever (YF). The 17D strain was developed in 1936 by serial passaging and has been used in over 400 million persons, with an excellent record of safety and efficacy (Monath, 1999). A single dose induces neutralizing antibodies in nearly 100% of vaccinated individuals. Immunity is probably lifelong, although revaccination is recommended every 10 years (Monath et al., 2002). In 1988, an inexpensive live-attenuated vaccine against JE (SA14-14-2) was first licensed in China. SA14-14-2 demonstrated an excellent safety profile, effectiveness (88–96%), and efficacy in large-scale trials (involving $>200{,}000$ children) (Halstead and Thomas, 2010; Hennessy et al., 1996; Kumar et al., 2009; Liu et al., 1997). More than 300 million doses have been produced and administered to >120 million children. Recently, the vaccine has been licensed for use, and millions of doses have been administered in Nepal, India, Sri Lanka, and South Korea (Halstead and Thomas, 2010; Solomon, 2006). Preventive vaccine strategies based on the construction of chimeric viruses of these licensed live attenuated vaccines are being developed against WNV, DENV, and others (Monath, 2001). However, all these licensed vaccines are indicated for use in mammalians (including humans), and there is no evidence to demonstrate the efficacy of the backbone of these vaccines in avian species. In this study, we developed an attenuated vaccine candidate (FX2010-180P) against DTMUV by serial passage of FX2010 in CEF cells. We believe this is the first report of an attenuated vaccine candidate against avian flaviviruses. FX2010-180P could not i.n. infect ducks, nor replicate in the lungs of i.n. infected mice, indicating that its horizontal transmission in natural avian and artificial mammalian hosts was eliminated by serial passaging. Moreover, the limited replication of FX2010-180P in embryonated eggs and its low lethality to egg embryos indicate its low virulence in vivo. Although FX2010-180P stimulated a robust protective immune response in ducks, the viral replication was limited strictly to the spleen. Similar to SA14-14-2 (Hase et al., 1993), the titers of FX2010-180P in the brains of mice inoculated i.c. were still low at 4 d.p.i., suggesting that the vaccine poses a low safety risk to mammals.

Many reports have shown that virus adaptation to cell lines during passaging resulted in reduced virulence in vivo(Barrett and Teuwen, 2009; Yu, 2010). Additionally, the host-specific adaption

and pathogenicity of flavivirus may be associated with genetic modifications in the 5′-NCR, preM/M, E, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5, and 3′-NCR of the virus (Butrapet et al., 2000; Chiou and Chen, 2001; Ciota et al., 2007; Engel et al., 2010; Holzmann et al., 1990, 1997; Mandl et al., 2000; Mitzel et al., 2008; Puig-Basagoiti et al., 2007; Ruzek et al., 2008; Wicker et al., 2012, 2006; Yamshchikov et al., 2004; Yoshii et al., 2010; Yu et al., 2008; Zhang et al., 2006; Zhao et al., 2005). In this study, we indeed found a number of mutations associated with passaging of the virus in cell culture. Of these, those that affect the E protein may play a more important role in the host-specific adaption and attenuation of virus, since this protein mediates the essential functions of attachment to, and fusion with, host cell membranes (Rey et al., 1995). Mutations E-89 (Glu→Gly) and E-120 (Asp→Asn) are located in domain II, which contains the fusion peptide and has the ability to bind target membranes. Mutations near this region change the fusion properties of the E protein in cell culture and have been associated with alterations in neurovirulence. An amino acid change in domain II resulted in a reduced neuroinvasiveness of tick-borne encephalitis virus (TBEV) and WNV (Goto et al., 2003; Zhang et al., 2006). In particular, mutation E-120 (Asp→Asn) lies near to the fusion loop and hence may contribute to reduced neuroinvasiveness (Zhang et al., 2006). In addition, this mutation introduces a potential glycosylation site (DCT→NCT) that might affect virulence, since the envelope protein glycosylation status is reported to influence the mouse neuroinvasion capacity of WNV (Beasley et al., 2005). The mutations E-312 (Val→Ala) and E-349 (Met→Lys) are located within the receptor-binding domain III, a region in which mutations that attenuate neurovirulence and neuroinvasiveness of flaviviruses have been identified (Holzmann et al., 1990, 1997; Jiang et al., 1993; Mandl et al., 2000). Mutations E-37 (Tyr→Phe), E-155 (Tyr→Phe), E-157 (Ala→Val) and E-166 (Arg→Lys) are located in domain I, another region that is a hotspot for attenuating mutations (Jiang et al., 1993).

In addition, the M proteins may also be determinants of host-specific replication of flaviviruses (Mitzel et al., 2008). A mutation in the M protein of ChimeriVax-JE led to a significant change in the susceptibility of the virus to changes in pH and to accelerated growth kinetics (Maier et al., 2007). Structural studies of the M and E proteins suggested that the first ~20 residues of M interact with the ij loop in domain II of the E protein (Zhang et al., 2003). The prM-106 (Ala→Val) mutation is located in a region of M required for modulation of E protein function during early events of infection such as membrane fusion (Maier et al., 2007). This suggests that the prM-106 mutation might modulate membrane fusion in a host-specific manner. On the other hand, the attenuation of flaviviruses during serials passaging in cells is associated with frequent non-conservative changes in non-structural proteins, such as NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Butrapet et al., 2000; Chiou and Chen, 2001; Ciota et al., 2007; Engel et al., 2010; Puig-Basagoiti et al., 2007; Ruzek et al., 2008; Wicker et al., 2012, 2006; Yamshchikov et al., 2004; Yoshii et al., 2010; Yu et al., 2008). In this study, 10 mutations were found in non-structural proteins including NS1, NS3, NS4A, NS4B, and NS5 in the FX2010-180P strain. Most of these mutations arose in high passage variants, and their appearance coincided with the decline in replicative capacity in vivo (Tables 2 and 4). These mutations might contribute to attenuation at the level of RNA replication or other stages of the virus infectious cycle (Yamshchikov et al., 2004). The mutations E-120, E-312, E-349, NS1-262, and NS3-322 first arose in the FX2010-60P and might play important roles in viral neurovirulence or neuroinvasiveness, as viruses with these mutations were not detected in the brains of inoculated ducks. On the other hand, the two nucleotide mutations in the 3′-NCR probably do not play important roles in viral attenuation since they were not localized in the functionally important 3′ stem-loop structure (3′-SL) common to many flaviviruses (Markoff et al., 2002; Yu and Markoff, 2005).

In short, we have successfully developed a live attenuated vaccine candidate against duck Tembusu viral disease, in which 19 amino acid mutations in both structural and non-structural proteins were found. Further work is required to map the virulence determinants within these regions by reverse genetic technology.

Materials and methods

Serial passage of the virus

DTMUV FX2010 isolated from sick shelducks in Shanghai was used in this study (P. Yan et al., 2011). Primary CEFs prepared from 10-day-old Specific-pathogen-free (SPF) chicken eggs (Marival Vital Laboratory Animal Technology Co., Ltd., Beijing, China) were trypsinized and seeded in 75-cm^2 flasks with Dulbecco′s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Biowest, South America), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) and were maintained in a 5% CO_2 humidified incubator at 37 °C. When the cells reached 80–90% confluence, they were washed with phosphate-buffered saline (PBS) and infected with FX2010 at a multiplicity of infection (MOI) between 0.1 and 0.5. After 1.5 h incubation at 37 °C, the inoculum was removed. The monolayer was washed 3 times with PBS to remove the unattached virus, and 10 ml of DMEM containing 2% (v/v) FBS was added to the culture and incubated at 37 °C. Culture medium was harvested after 72 h incubation or until 70% cytopathic effect (CPE) was evident. Cell debris was removed by centrifugation, and the virus was stored at −70 °C. The same procedure was used to perform 172 additional passages of the virus in CEF. FX2010-180P was developed by two additional passages of FX2010-178P that was purified from the 173rd passage virus of FX2010 through 5 limited dilution titration passages in CEF.

Animals

Three- to 4-week-old weanling SPF Balb/c mice and 3-day-old newborn SPF Balb/c mice (Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, China) were used in mouse experiments. Shelducks were hatched from SPF Shelduck eggs (Harbin Veterinary Research Institute), housed in isolators for 3 days to 8 weeks, and used for duck experiments. All animal experiments were approved by the Animal Care and Use Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

Infection of chicken embryos

To evaluate the mortality of the different passages in chicken embryos, groups of ten 8-day-old SPF chicken embryonated eggs were subjected to intra-allantoic injection with 100 μl of inoculum containing 5.0 $\log_{10}$ $TCID_{50}$ of FX2010, 60th passage virus (FX2010-60P), 80th passage virus (FX2010-80P), 100th passage virus (FX2010-100P), 130th passage virus (FX2010-130P), 145th passage virus (FX2010-145P), 168th passage virus (FX2010-168P), and 180th passage virus (FX2010-180P). The eggs were incubated continually at 37 °C and checked daily. The survival time of the inoculated eggs was recorded. To verify viral infection in chicken embryonated eggs, DTMUV-specific RNA in the supernatants of the homogenated dead or alive embryos was evaluated by real-time PCR (L. Yan et al., 2011). When the viral RNA copy number in total allantoic fluid of these eggs was 10-fold higher than that in

the inoculated column, the embryos of these eggs were assumed to be positive for DTMUV infection. Subsequently, DTMUV RNA in the allantoic fluid of the positive eggs was determined using real-time PCR.

Mouse experiments

To evaluate the replication abilities of the different passages in mammalian models, groups of three 4-week-old female BALB/c mice were lightly anesthetized using carbon dioxide (CO_2) and inoculated intracerebrally (i.c.) with 30 μl or intranasally (i.n.) with 50 μl of 5.0 $\log_{10}$ $TCID_{50}$ of each viral strain. Four days post-inoculation (d.p.i.), the mice were euthanized and the brain and lung tissues of mice inoculated via i.c. and i.n. routes were collected, respectively. The tissues collected were homogenized in PBS to yield 1:1 (ml/organ) tissue homogenates, and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants of tissue homogenates were used for DTMUV RNA quantification.

To evaluate the neurovirulence of FX2010 and FX2010-180P, each of six 3-day-old newborn mice was inoculated i.c. with 30 μl of 5.0 $\log_{10}$ $TCID_{50}$ of each viral strain. At 4 and 6 d.p.i., the brains of mice were collected, homogenized and used for DTMUV RNA quantification as outlined above. Whole genome of the FX2010-180P was amplified from brains of mice euthanized on day 6 post-infection by reverse transcription polymerase chain reaction (RT-PCR). The PCR product was sequenced to test the stability of FX2010-180P in the brains of mice.

Duck experiments

To evaluate intranasal infectivities of different passages, groups of six 8-week-old SPF shelducks were inoculated i.n. with 100 μl of 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-60P, FX2010-80P, FX2010-100P, and FX2010-130P, respectively. Six control shelducks were inoculated with DMEM containing 2% FBS. Ducks were observed twice a day for signs of disease. Three ducks in each group were euthanized at 4 d.p.i, and samples from the spleen, lung, kidney, and brain were collected for viral titration and DTMUV RNA detection. The seroconversion of the remaining 3 ducks was tested at 14 d.p.i.

Studies of intramuscular activities were performed on 8-week-old or 5-day-old SPF shelducks as outlined above, except that the virus was injected intramuscularly. Other than 100 μl of 3.5 $\log_{10}$ TCID50 of FX2010, same volumes of 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-130P, FX2010-145P, FX2010-168P, or FX2010-180P were used to inoculate 8-week-old ducks. In addition, 100 μl of 3.5 $\log_{10}$ TCID50 of FX2010 or identical volumes of 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P were used to inoculate 5-day-old ducklings. Each of the six 8-week-old or 5-day-old control shelducks were inoculated intramuscularly with DMEM containing 2% FBS.

To track the growth and decline of viral RNA and antibody titers in the sera, groups of five 3- to 5-week-old SPF shelducks each were inoculated with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010, or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P through either intranasal or intramuscular routes. In each case, a control group of 5 shelducks inoculated with DMEM containing 2% FBS was included. Sera of all the ducks were collected daily from 1–7, and at 10 and 14 d.p.i. for viral RNA quantification by real-time PCR and antibody titration using the blocking ELISA method (X. Li et al., 2013).

Viral titration

Organs were weighed and were homogenized in PBS containing 100 U/ml penicillin and 100 μg/ml streptomycin to yield 1:1 (ml/g) tissue homogenates. Tissue homogenates were clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants were titrated in 96-well tissue culture plates containing the spontaneously immortalized chicken embryo fibroblast (DF-1) cells (ATCC CRL-12203™). The virus titer for each organ was determined by the method described by (Reed and Muench (1938)) and expressed as $\log_{10}$ $TCID_{50}$/g of tissue.

Blocking ELISA

To test for specific antibodies against DTMUV, a Blocking ELISA method was used as described previously (X. Li et al., 2013). Briefly, ELISA plates were coated with approximately 3 μg/well purified FX2010 in 0.1 M carbonate–bicarbonate buffer (pH 9.6) and incubated 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 μl of blocking buffer (PBS containing 5% skim milk) for 1 h at 37 °C. Serum samples were initially diluted 10-fold with PBS, and then further diluted through a series of 2-fold dilutions. Diluted serum (100 μl) was added to each well and incubated for 1 h at 37 °C. The wells were then washed 3 times with PBST and incubated with mAb 1F5 (20 ×) for 1 h at 37 °C. After the wells were rinsed 3 times with PBST, goat anti-mouse IgG (Sigma, USA) conjugated to HRP was added, and the mixture was incubated at room temperature (RT) for 1 h. After the wells were rinsed with PBST 3 times, 100 μl of 3,3′,5,5′-tetramethyl benzidine was added, and the mixture was incubated at RT 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) value was determined using the formula: PI (%)=[1−(OD450 nm of test serum/OD450 nm of negative control serum)] × 100%. The serum was considered positive for DTMUV reactivity when the PI value was ≥ 18.4%. When the PI value was ≤ 12.6%, the serum was considered negative. Intermediate PI values were considered as "borderline positive". Repeated analyses were performed on sera with borderline PI values and were considered negative when the re-tested values were less than 18.4%. The highest dilution of the serum considered positive to DTMUV was determined as the blocking ELISA titer for the anti-DTMUV antibody.

Real-time PCR

To quantify DTMUV RNA copy number in the samples, a previously described real-time RT-PCR assay with minor modifications was employed (Yan et al., 2012; L. Yan et al., 2011). The tissues were homogenized in PBS to yield 1:1 (ml/g of ducks tissue or ml/organ of mouse tissue) tissue homogenates, and clarified by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatants of tissue homogenates were used for DTMUV RNA quantification. Total RNA from 300 μl of serum, the supernatant of tissue homogenate, or allantoic fluid was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions and was eluted in 20 μl of DEPC-treated water. Reverse transcription was performed using AMV reverse transcriptase (Takara Biotechnology, Dalian, China) with DTMUV-specific primers (5′-CGTATGGGTTGACTGTTATCA-3′) in a final volume of 20 μl containing 5 μl of RNA solution, and then 1 μl of cDNA was used in real-time RT-PCR. Briefly, real-time PCR was performed on a Mastercycler ep realplex system (Eppendorf, Hamburg, Germany) using cycling conditions described previously (Yan et al., 2012; L. Yan et al., 2011): 95 °C for 2 min, followed by 40 cycles each at 95 °C for 20 s and 54 °C for 1 min. The total reaction system contained 2.5 μl of 10 × buffer, 2.5 μl of dNTP, 1 μl of each primer (10 μM of EF and ER), 0.6 μl of the probe (10 μM of EP), 1 μl of cDNA, 0.2 μl of Ex Taq Hot Start, and 16.2 μl of sterile water. The RNA copies in the sample were calculated by comparing 1 μl of cDNA with a standard sample.

Table 1
Primers used for full-genome sequencing of DTMUV.

Primer name	Sequence (5′–3′)	Map position
DTMUV-1F	5′-AGAAGTTCATCTGTGTGAACTTATTCC-3′	1–27
DTMUV-1R	5′-CGGTACCATAATCCTCCATCTCAGC-3′	1488–1512
DTMUV-2F	5′-GGAAGCGAGCACCTACCACAAT-3′	1397–1418
DTMUV-2R	5′-CTGGGCACTCTTTAGTTTTTGGTCC-3′	2868–2892
DTMUV-3F	5′-GGAGAGCTCATGTACGGATGGAAGA-3′	2784–2808
DTMUV-3R	5′-TCTATCCCCACTATTCTGAGCCCTG-3′	3994–4018
DTMUV-4F	5′-CTTGGCGTTGCGTTAGCACTCAT-3′	3834–3856
DTMUV-4R	5′-CCTTTCAGTGCTTCCGCTATTTCAG-3′	5278–5302
DTMUV-5F	5′-AAAAGGCAACTAACAGTGCTGGACC-3′	5145–5169
DTMUV-5R	5′-GGCTGGGACTTCTGCTATCCATAAC-3′	6725–6749
DTMUV-6F	5′-TGACTACAGCTGAGAAAGGGAGCAG-3′	6535–6559
DTMUV-6R	5′-AGCAGTGTGTCAGATGGTTCAGTCG-3′	8062–8086
DTMUV-7F	5′-GTGAGAGGTTACACAAAAGGAGGGC-3′	7953–7977
DTMUV-7R	5′-CTTGCAGGTGCAGTTCTCTCTCTCT-3′	8970–8994
DTMUV-8F	5′-GAGAAGGTGAATAGTAACGCAGCCC-3′	8862–8886
DTMUV-8R	5′-TCCTTCCTGTGGGGACCCATGAGAC-3′	10023–10047
DTMUV-9F	5′-GTGGTTCCATGTCGAGACCAGGATG-3′	9843–9867
DTMUV-9R	5′-AGACTCTGTGTTCTACCACCACCAG-3′	10967–10991

Genome sequencing

RNAs from different passages were extracted using the RNeasy Plus Mini Kit. The 5′ and 3′ ends of the genomes were amplified using 5′ and 3′ rapid amplifications of cDNA ends (RACE) methods. Specific primers (Table 1) for DTMUV designed according to the sequence of FX2010 were used for RT-PCR to generate nine overlapping PCR products. The PCR products were purified using the TIANquick Midi Purification Kit (Tiangen, Beijing, China) and then used for sequencing with ABI 3730 automated sequencers (Applied Biosystems). Genome sequences were analyzed using DNASTAR software.

Vaccination and challenge experiments

To evaluate the immunogenicity of FX2010-180P, twenty 18-week-old shelducks were vaccinated i.m. with a 3.5 $\log_{10}$ $TCID_{50}$ dose of virus. Twenty control shelducks were inoculated i.m. with PBS. At 2 weeks post-inoculation (w.p.i.), 10 vaccinated and 10

Table 2
Infectivity of different passages of FX2010 in ducks inoculated intranasally or intramuscularly.

Virus	Age of ducks	Inoculation[a]	DTMUV RNA positive (virus titers [$\log_{10}$ $TCID_{50}$/g])			
			Spleen	Lung	Kidney	Brain
FX2010	8-week-old	i.n.	3/3 (3.8 ± 0.8[b])	3/3 (4.1 ± 1.2)	3/3 (3.3 ± 1.1)	3/3 (2.2 ± 0.5)
FX2010-60P	8-week-old	i.n.	3/3 (4.8 ± 1.1)	3/3 (−/4.5/2.3/[c])	3/3 (−/2.8/2.3)	0/3 (−[d])
FX2010-80P	8-week-old	i.n.	3/3 (4.3 ± 1.4)	2/3 (−/2.5/2.0)	2/3 (−/−/2.5)	0/3 (−)
FX2010-100P	8-week-old	i.n.	3/3 (−/3.3/4.5)	1/3 (−/−/2.0)	1/3 (−)	0/3 (−)
FX2010-130P	8-week-old	i.n.	0/3 (−)	0/3 (−)	0/3 (−)	0/3 (−)
DMEM control	8-week-old	i.n.	0/3 (−)	0/3 (−)	0/3 (−)	0/3 (−)
FX2010	8-week-old	i.m.	3/3 (3.9 ± 0.9)	3/3 (4.2 ± 0.9)	3/3 (3.1 ± 1.3)	3/3 (2.4 ± 0.4)
FX2010-130P	8-week-old	i.m.	3/3 (4.4 ± 1.7)	0/3 (−)	0/3 (−)	0/3 (−)
FX2010-145P	8-week-old	i.m.	3/3 (4.2 ± 1.0)	0/3 (−)	0/3 (−)	0/3 (−)
FX2010-168P	8-week-old	i.m.	3/3 (−/3.0/3.3)	0/3 (−)	0/3 (−)	0/3 (−)
FX2010-180P	8-week-old	i.m.	3/3 (−/2.3/3.0)	0/3 (−)	0/3 (−)	0/3 (−)
DMEM control	8-week-old	i.m.	0/3 (−)	0/3 (−)	0/3 (−)	0/3 (−)
FX2010	5-day-old	i.m.	3/3 (3.7 ± 0.6)	3/3 (4.0 ± 0.7)	3/3 (3.1 ± 0.8)	3/3 (2.3 ± 0.5)
FX2010-180P	5-day-old	i.m.	3/3 (2.1 ± 0.5)	0/3 (−)	0/3 (−)	0/3 (−)
DMEM control	5-day-old	i.m.	0/3 (−)	0/3 (−)	0/3 (−)	0/3 (−)

[a] Three of shelducks were each inoculated intranasally or intramuscularly with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 or 5.5 $\log_{10}$ $TCID_{50}$ of the strains derived by serial passaging. Ducks were euthanized on day 4 post-inoculation and tissue samples were collected for viral titration and DTMUV RNA detection.
[b] Virus titers are shown as mean ($\log_{10}$ $TCID_{50}$/g) ± SD.
[c] Virus titers of individual organs are shown when organs from three ducks were not all virus-positive on DF-1 cells.
[d] None of the organs from three ducks were virus-positive on DF-1 cells.

Table 3
Viral RNA copies in the sera of ducks inoculated with FX2010 or FX2010-180P.

Virus	Inoculation[a]	Ducks	0 day	1 day	DTMUV RNA copies (log10 copies/μl)							
					2 day	3 day	4 day	5 day	6 day	7 day	10 day	14 day
FX2010	i.n.	7026	–[b]	–	5.16	4.90	1.70	–	–	–	–	–
		7027	–	–	5.81	3.21	–	–	–	–	–	–
		7028	–	–	5.33	5.69	3.01	–	–	–	–	–
		7029	–	–	5.21	3.53	–	–	–	–	–	–
		7030	–	–	5.32	5.08	1.37	–	–	–	–	–
	i.m.	7021	–	4.36	4.35	–	–	–	–	–	–	–
		7022	–	5.89	4.99	1.27	–	–	–	–	–	–
		7023	–	5.62	4.78	1.92	–	–	–	–	–	–
		7024	–	5.82	5.07	1.45	–	–	–	–	–	–
		7025	–	4.98	4.18	–	–	–	–	–	–	–
FX2010-180P	i.m.	7031–7035	–	–	–	–	–	–	–	–	–	–
	i.n.	7036–7040	–	–	–	–	–	–	–	–	–	–
DMEM	i.m.	7041–7045	–	–	–	–	–	–	–	–	–	–
	i.n.	7046–7050	–	–	–	–	–	–	–	–	–	–

[a] Five 3- to 5-week-old ducks each were inoculated intranasally or intramuscularly with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010, or 5.5 $\log_{10}$ $TCID_{50}$ of FX2010-180P. Sera were collected 1–7, 10 and 14 days post-inoculation and used for DTMUV RNA detection by real-time PCR.
[b] –, Ct > 35 was considered negative.

Table 4
Amino acid mutations in viruses with different serial passages of FX2010.

Viruses	Amino acid mutation																		
	M	E								NS1			NS3	NS4A		NS4B		NS5	
	106	37	89	120	155	157	166	312	349	192	205	262	322	54	110	50	112	273	793
FX2010	A	D	E	D	Y	A	R	V	M	R	K	V	T	F	V	F	S	R	V
FX2010-60P	–[a]	–	–	N	Y/F	–	–	A	K	–	–	A	I	–	–	–	–	–	–
FX2010-80P	–	–	–	N	Y/F	A/V	–	A	K	–	–	A	I	–	–	–	–	–	–
FX2010-100P	–	–	E/G[b]	N	Y/F	A/V	–	A	K	–	–	A	I	–	–	–	–	–	–
FX2010-130P	A/V	–	E/G	N	F	A/V	–	A	K	–	K/R	A	I	–	V/A	F/Y	S/L	–	–
FX2010-145P	A/V	–	G	N	F	V	–	A	K	–	K/R	A	I	–	V/A	F/Y	S/L	–	–
FX2010-168P	V	–	G	N	F	V	K	A	K	–	R	A	I	–	A	Y	L	–	A
FX2010-180P	V	N	G	N	F	V	K	A	K	G	R	A	I	L	A	Y	L	G	A

[a] Constant amino acid.
[b] Co-existent amino acids (constant/mutant).

Table 5
Synonymous mutations in viruses with different serial passages of FX2010.

Viruses	Nucleotide mutation sites in whole genome (location in proteins or NCR)														
	443 (C 116)	1454 (E 166)	1992 (E 346)	2060 (E 368)	3368 (NS1 303)	3866 (NS2A 117)	4076 (NS2B 40)	4865 (NS3 92)	6293 (NS3 568)	6956 (NS4B 21)	8105 (NS5 150)	8264 (NS5 203)	10373 (stop codon)	10560 (3′-NCR)	10619 (3′-NCR)
FX2010	G	A	C	T	G	A	C	A	C	C	T	A	A	C	T
FX2010-60P	A	–	–[a]	–	T	–	–	A/T[b]	–	C/T	T/C	–	–	–	–
FX2010-80P	A	–	–	–	T	–	–	A/T	–	C/T	T/C	–	–	–	–
FX2010-100P	A	–	–	–	T	–	–	A/T	–	C/T	T/C	–	–	–	–
FX2010-130P	A	–	C/T	T/C	T	–	–	T	–	T	T/C	A/G	–	–	–
FX2010-145P	A	–	C/T	C	T	–	C/T	T	–	T	C	A/G	–	–	–
FX2010-168P	A	G	T	C	T	T	T	T	A	T	C	G	–	–	C
FX2010-180P	A	G	T	C	T	T	T	T	A	T	C	G	G	T	C

[a] Constant nucleotide.
[b] Co-existent nucleotides (constant/mutant).

control ducks were challenged i.m. with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 after their blood samples were collected from the wing (alar) veins. Afterwards, the serum samples were used in antibody titer testing by blocking ELISA. Ducks were observed twice a day for signs of disease, and 3 ducks in each group were euthanized on day 4 post-challenge. Samples from the spleen, lung, kidney, brain, and ovary were collected for virus titration. The remaining ducks were bled for sera which were then used in antibody titer testing at 4, 8 and 12 w.p.i; the ducks were then challenged i.m. with 3.5 $\log_{10}$ $TCID_{50}$ of FX2010 at 12 w.p.i. Studies of vaccine protection against virulent DTMUV were performed as outlined above.

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