Patterns of duck Tembusu virus infection in ducks, Thailand: a serological study

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ABSTRACT Duck Tembusu virus (**DTMUV**), a mosquito-borne flavivirus, has been identified as a causative agent of an emerging viral disease in ducks, causing significant economic losses to the duck-producing industry. In Thailand, DTMUV has been detected sporadically in ducks since the first report in 2013. However, information on the patterns of DTMUV infection in ducks in Thailand is limited. In this study, a serological survey of DTMUV on ducks raised in farming and free-grazing systems was conducted during 2015-2016. Blood samples of farm ducks (n = 160) and free-grazing ducks (n = 240) were collected in the summer, rainy, and winter seasons during 2015-2016 and tested for DTMUV infection. Our results showed that DTMUV infection in ducks in Thailand occurred all year-round; however, the patterns of DTMUV infection varied between 2 duck-raising systems. Significant seasonal pattern was found in free-grazing ducks, whereas no seasonality was observed in farm ducks. Notably, DTMUV infection in ducks in Thailand was highest in the winter season. In conclusion, our data indicate distinct patterns of DTMUV infection between farm and free-grazing ducks, and the year-round circulation of DTMUV in ducks in Thailand, with peaks in the winter season. This information will help reduce the risk of DTMUV transmission through prevention and control strategies focusing on the peak period. Routine surveillance of DTMUV in ducks is essential for early detection of DTMUV allowing the implementation of control measures in a timely manner.

Key words: duck Tembusu virus, duck, seasonal pattern, serological survey, Thailand

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INTRODUCTION

Duck Tembusu virus (**DTMUV**) is an emerging mosquito-borne flavivirus that causes a significant decrease in egg production and severe neurological disorders in several species of ducks (Zhang et al., 2017). Duck Tembusu virus was also occasionally detected in various avian species, including chickens, geese, pigeons, and sparrows, but DTMUV-associated disease appears mostly in ducks (Zhang et al., 2017). At present, DTMUV is classified as a new genotype of Tembusu virus (**TMUV**) in the genus *Flavivirus* of the family *Flaviviridae* (Su et al., 2011). Like other mosquito-borne flaviviruses, DTMUV is transmitted by *Culex* mosquitoes, although transmission of DTMUV can also occur through multiple routes, including direct contact (Tang et al., 2013), airborne transmission (Li et al., 2015), and vertical transmission (Zhang et al., 2015). However, the primary mode of transmission for DTMUV remains unknown.

Duck Tembusu virus was first detected in China in 2010 (Su et al., 2011) and was subsequently spread quickly to Malaysia and Thailand (Homonnay et al.,

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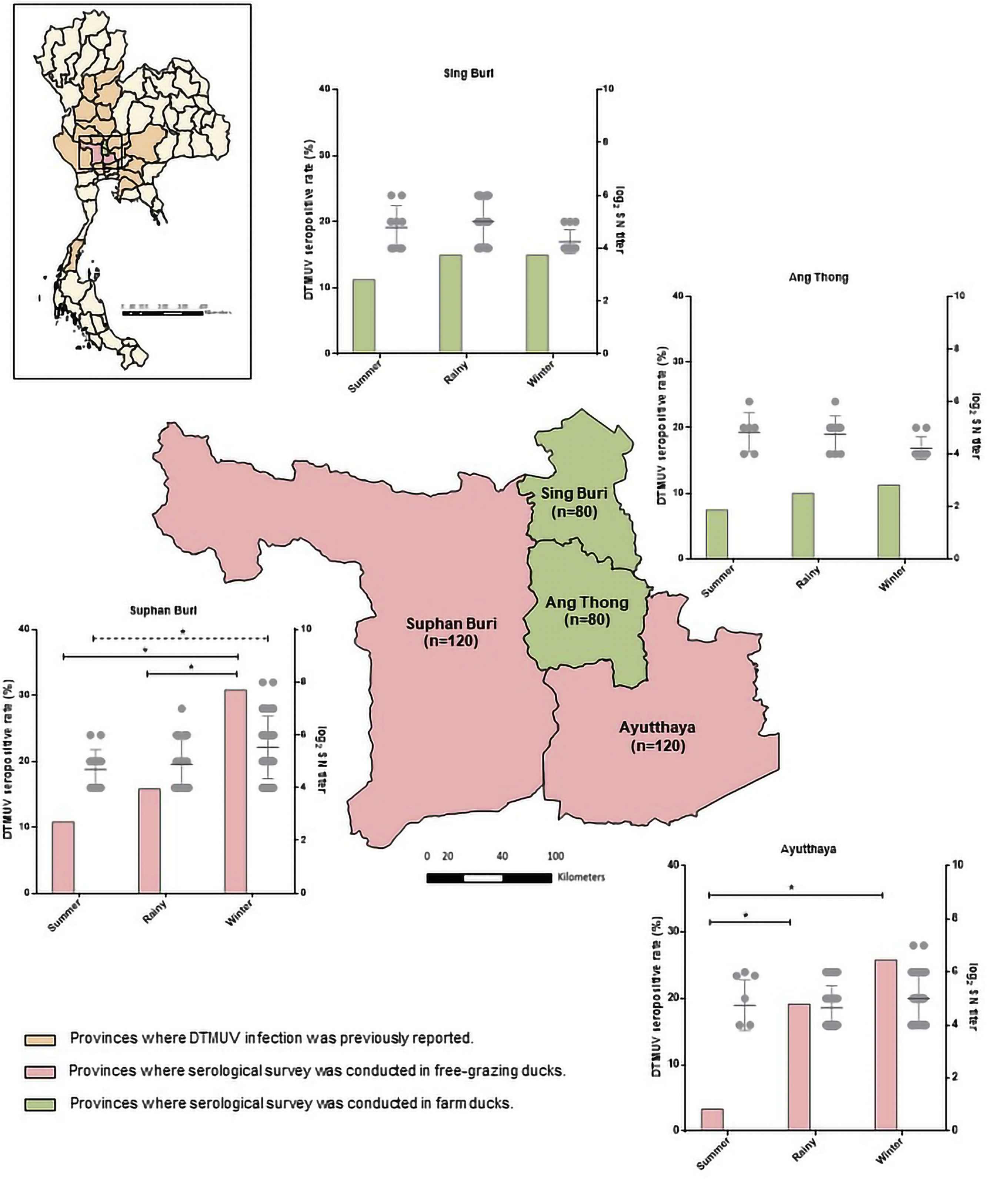

Figure 1. Location of provinces in Thailand where a serological survey on ducks raised in farming and free-grazing systems was conducted in the summer, rainy, and winter seasons during 2015-2016. The number of collected samples in each season is shown in parentheses. Duck Tembusu virus (DTMUV) seropositive rates and mean $\log_2$ SN titers in each season are shown in bar chart. Bars indicate DTMUV seropositive rates and dots indicate mean $\log_2$ SN titers. *, $P < 0.05$ (Pearson's chi-square test for DTMUV seropositive rates and one-way ANOVA for mean $\log_2$ SN titers). Abbreviation: SN, serum neutralization.

2014; Chakritbudsabong et al., 2015; Thontiravong et al., 2015). Currently, DTMUV is widely distributed and becomes endemic in duck populations in Asia, causing significant economic losses to the duck-producing industry. Although DTMUV has been detected sporadically in ducks in Thailand since the first report in 2013 (Thontiravong et al., 2015), little is known about the patterns of DTMUV infection in ducks in Thailand, particularly in free-grazing ducks. In Thailand, ducks were generally raised in 2 major systems, including farming and free-grazing systems (Gilbert et al., 2006). Therefore, to better understand the patterns of DTMUV infection in ducks in Thailand, we conducted a serological survey of DTMUV on ducks raised in farming and free-grazing systems in the summer, rainy, and winter seasons during 2015-2016.

MATERIALS AND METHODS

Serum Samples and Sampling Sites

To assess the patterns of DTMUV infection in ducks in Thailand, a serological survey of DTMUV on ducks raised in farming and free-grazing systems was conducted in the summer, rainy, and winter seasons during 2015-2016. For the estimation of DTMUV seroprevalence for each season in ducks raised in farming and free-grazing systems, the sample size was calculated by using Cochran's (1977) sample size formula based on an estimated seroprevalence of 50%, a precision of 10% and a confidence level of 90%, giving a required sample size of 68 per province in each season in both duck-raising systems (Cochran, 1977). In this study, blood samples were randomly collected from ducks raised in open house farming system from Ang Thong (n = 80) and Sing Buri (n = 80) provinces and from ducks raised in free-grazing systems from Ayutthaya (n = 120) and Suphan Buri (n = 120) provinces, all of which are located in the central region of Thailand (Figure 1). These provinces were selected as study sites based on the high-density duck-raising areas of Thailand, DTMUV infection history, and the farmers' cooperation (Niamsang, 2015; Thontiravong et al., 2015; Ninvilai et al., 2018; Tunterak et al., 2018). In farming system, ducks were kept in moderate biosecurity open houses, which contained 3,000-5,000 ducks each. These farms were located close to the pond and rice field but far from the village. In contrast to farm ducks, free-grazing ducks were raised freely on the postharvest rice paddy fields, where they fed on leftover rice, insects and snails, frequently sharing fields with domesticated and wild birds. The flock size ranged from 1,000 to 3,000 ducks. In both duck-raising systems, the egg production rate varied between 80 and 90% depending on the duck age and health status. In Thailand, the summer, rainy, and winter seasons generally occur in February–May, June–October, and November–January, respectively (Thai Meteorological Department, 2014). Therefore, a serological survey of DTMUV was conducted in the summer (March–May), rainy (July–September), and winter (November–January) seasons during 2015–2016. For each season, blood samples of 80 farm ducks and 120 free-grazing ducks from each province were randomly collected from the same flock/farm (Table 1). It is noted that tested ducks in each season were not identical to the ones that had already tested in other seasons to ensure that the DTMUV seropositive rates of 3 seasons were independent. All blood samples were taken from clinically healthy laying ducks older than 3 mo of age. Ducks included in this study were Khaki Campbell, native laying ducks, and crossbreed of Khaki Campbell and native laying duck, which are common breeds of domestic laying ducks in Thailand (Songserm et al., 2006). All these ducks were routinely vaccinated against fowl cholera and duck plague virus; however, all of them were not vaccinated with DTMUV vaccine. All samples were maintained at 4°C after collection and submitted to the laboratory within 2 to 6 h of collection. Sera were separated and stored at -80°C until serum neutralization (**SN**) testing.

Serum Neutralization Test

To detect DTMUV-specific antibodies in serum samples, SN test was performed with the 2013 Thai DTMUV (DK/TH/CU-1) as previously described (Tunterak et al., 2018). Briefly, duplicate of serial two-fold diluted heat-inactivated sera were incubated with 100 $TCID_{50}$ of DK/TH/CU-1 for 1 h at 37°C. The virus-serum mixture was then transferred onto monolayers of baby hamster kidney (BHK-21) cells grown in 96-well plates. The plates were incubated at 37°C and checked daily for the presence of cytopathic effects for 5 d. Reference DTMUV antibody positive and negative sera obtained from our previous study (Ninvilai et al., 2020), uninfected BHK-21 cells, and back titration of used virus were included in each test. Serum neutralization antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited cytopathic effects. Serum with SN antibody titer $\geq$16 was defined DTMUV antibody positive (Tunterak et al., 2018). Because the cross-neutralizing reactivity between DTMUV and other flaviviruses, including Japanese encephalitis virus (**JEV**) and West Nile virus, were possibly found in ducks (Kalaiyarasu et al., 2016), all of the DTMUV antibody positive sera were further tested for the presence of JEV-specific antibodies by SN test using BHK-21 cells and JEV Beijing strain as described previously (Saito et al., 2009; Singh et al., 2015). Only JEV was used for comparison in this study because West Nile virus has never been identified in Thailand (Changbunjong et al., 2012). A serum sample was considered positive for DTMUV-specific antibodies if DTMUV antibody titer was at least four-fold higher than the corresponding JEV antibody titer (Kalaiyarasu et al., 2016). In this study, none of the DTMUV seropositive ducks were positive for JEV neutralizing antibodies (SN titer < 2).

Table 1. Duck Tembusu virus (DTMUV) seropositive rates in ducks raised in farming (A) and free-grazing (B) systems in Thailand during 2015-2016.

A. Farm ducks

	Ang Thong			Sing Buri		
Season	No. of positive sera/ No. Of sera tested	DTMUV seropositive rate, % (95%CI)[1]	GMT SN titer[2] (mean $\log_2$ SN titer)	No. of positive sera/ No. of sera tested	DTMUV seropositive rate, % (95%CI)	GMT SN titer (mean $\log_2$ SN titer)
Summer (Mar–May)	6/80	7.5 (1.73-13.27)	28.44 (4.83)	9/80	11.25 (4.33-18.17)	27.47 (4.78)
Rainy (Jul–Sep)	8/80	10 (3.43-16.57)	26.91 (4.75)	12/80	15 (7.18-22.82)	32 (5)
Winter (Nov–Jan)	9/80	11.25 (4.33-18.17)	18.64 (4.22)	12/80	15 (7.18-22.82)	19.03 (4.25)
Total	23/240	9.58 (5.86-13.31)	23.75 (4.57)	33/240	13.75 (9.39-18.11)	25.46 (4.67)

B. Free-grazing ducks

	Ayutthaya			Suphan Buri		
Season	No. of positive sera/No. of sera tested	DTMUV seropositive rate, % (95%CI)[1]	GMT SN titer[2] (mean $\log_2$ SN titer)	No. of positive sera/No. of sera tested	DTMUV seropositive rate, % (95%CI)	GMT SN titer (mean $\log_2$ SN titer)
Summer (Mar–May)	4/120	3.33 (0.12-6.55)	26.91 (4.75)	13/120	10.83 (5.27-16.39)	25.81 (4.69)
Rainy (Jul–Sep)	23/120	19.17 (12.12-26.21)[3]	25.11 (4.65)	19/120	15.83 (9.30-22.36)[4]	29.65 (4.89)
Winter (Nov–Jan)	31/120	25.83 (18.00-33.67)[3]	32 (5)	37/120	30.83 (22.57-39.10)[3]	46.53 (5.54)[5]
Total	58/360	16.11 (12.31-19.91)	28.64 (4.84)	69/360	19.17 (15.10-23.23)	36.76 (5.20)

[1]95% confidence interval of the percentage.
[2]Geometric mean (GMT) serum neutralization (SN) titers (= 2^n; n = mean $\log_2$ SN titer) were calculated from DTMUV seropositive samples in each season.
[3]*P*-value < 0.01 when compared with DTMUV seropositive rate in the summer season.
[4]*P*-value < 0.01 when compared with DTMUV seropositive rate in the winter season.
[5]*P*-value < 0.05 when compared with mean $\log_2$ SN titer in the summer season.

Statistical Analyses

Data obtained from a serological survey were descriptively presented in DTMUV seropositive rate with 95% confidence intervals. To compare the significance of differences in DTMUV seropositive rates between seasons, Pearson's chi-square test was used. In addition, differences in mean $\log_2$ SN titers of 3 seasons were evaluated by analysis of variance (**ANOVA**). Probability (*P*) values < 0.05 were considered statistically significant. All statistical analyses were conducted using the SPSS Statistics software version 22 (IBM Corp., NY).

Ethical Considerations

This study was conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee of Chulalongkorn University (approval number 1531081). Informed consents were obtained from duck flock/farm owners.

RESULTS AND DISCUSSION

To investigate the pattern of DTMUV infection in ducks raised in farming system, a total of 160 blood samples of farm ducks were collected from 2 provinces in each season during 2015-2016. These serum samples were then tested for DTMUV neutralizing antibodies. Our results showed that DTMUV seropositive samples were detected in both provinces in all seasons, including the summer (7.5 and 11.25%), rainy (10 and 15%), and winter (11.25 and 15%) seasons (Table 1A; Figure 1). The trend in DTMUV seropositive rates was similar in both provinces, in which the winter season had the highest DTMUV seropositive rate, followed by the rainy and summer seasons (Table 1A; Figure 1). However, no significant difference in DTMUV seropositive rates was found between seasons in both provinces (Table 1A; Figure 1). Collectively, these findings indicate that DTMUV infection in ducks raised in farming system occurred all year round without significant seasonal variation.

To determine the pattern of DTMUV infection in ducks raised in free-grazing system, a total of 240 blood samples of free-grazing ducks were collected from 2 provinces in each season during 2015-2016. In both provinces, DTMUV seropositive samples were detected in all seasons; however, the rate of DTMUV seropositivity varied between seasons. In Ayutthaya province, the winter (25.83%) and rainy (19.17%) seasons had significantly higher DTMUV seropositive rates than the summer season (3.33%) ($P < 0.01$) (Table 1B; Figure 1). Correspondingly, DTMUV seropositive rates in Suphan Buri province also significantly higher in the winter season (30.83%) than the rainy (15.83%) and summer (10.83%) seasons ($P < 0.01$) (Table 1B; Figure 1). Interestingly, the highest geometric mean SN titer against DTMUV in both provinces was found in the winter season (Table 1B; Figure 1). Overall, in contrast to farm ducks, DTMUV infection in free-grazing ducks showed significant seasonality with the highest occurrence in the winter season.

In this study, we found that DTMUV infection in ducks in Thailand occurred all year round; however, the patterns of DTMUV infection differed between duck-raising systems. Seasonality of DTMUV infection was found in free-grazing ducks with significant highest occurrence in the winter season, whereas no significant seasonality was observed in farm ducks. This difference can be explained partly by difference in management practices between 2 duck-raising systems. In free-grazing system, ducks are raised freely on flooded rice fields that provide suitable habitats for mosquitoes and wild birds. This possibly results in a higher level of exposure to infected mosquitoes and wild birds than ducks kept in a confined housing. Notably, DTMUV seropositivity in free-grazing ducks was highest in the winter season, when the number of *Culex* mosquitoes in Thailand is high (Sanisuriwong et al., 2020). Because *Culex* mosquito has proven to be a vector for DTMUV transmission (O'Guinn et al., 2013; Tang et al., 2015), high DTMUV seropositivity in the winter season is probably related to increased vector activity. In concordance with this observation, our recent study demonstrated that DTMUV was detected in *Culex* mosquitoes only during the winter season in Thailand (Sanisuriwong et al., 2020). In contrast to free-grazing system, ducks raised in farming system are kept in confined housing at a high density, resulting in a high contact rate between susceptible and infected ducks but low level of exposure to mosquitoes and wild birds in the environment. As a result, the virus may be transmitted between farm ducks mainly through nonvector routes, potentially leading to the year-round occurrence of DTMUV infection in farm ducks without significant seasonality. Correspondingly, previous studies showed that DTMUV can also be transmitted via nonvector routes, including direct contact (Tang et al., 2013; Ninvilai et al., 2020) and airborne transmission (Li et al., 2015). However, the exact underlying factors associated with the pattern of DTMUV infection in both systems require further investigation.

In both duck-raising systems, DTMUV seropositive ducks could be observed in all seasons, indicating the endemicity of DTMUV infection in domestic ducks in Thailand. This finding is in line with the recent study reporting the widespread distribution and endemicity of DTMUV infection in free-grazing ducks in Thailand (Tunterak et al., 2018). However, the highest DTMUV seropositive rates in both duck-raising systems were generally detected in the winter season, which is consistent to previous studies reporting increased DTMUV outbreaks in domestic ducks in Thailand during the rainy and winter seasons (Ninvilai et al., 2019). A similar observation has been documented in China, in which a high seropositive rate of DTMUV was detected during the winter season (Li et al., 2015). However, although DTMUV seropositive rate in the summer season was lowest, DTMUV infection still occurred in both farm and free-grazing ducks, indicating that DTMUV could circulate in the summer season, when the climate is usually hot and dry, and *Culex* mosquitoes nearly disappeared (Sanisuriwong et al., 2020). Our observations suggested that, besides vector transmission, nonvector route may also involve in the spread of DTMUV in ducks in Thailand. However, the mechanisms that allow the maintenance of DTMUV in the summer season remain unknown and require further investigation. Interestingly, our results also showed that ducks raised in farming system were generally less likely to have DTMUV infection than ducks raised in free-grazing system. This finding may be explained by biosecurity measures, which are generally better implemented in farming system compared with free-grazing system (Beaudoin et al., 2014).

In conclusion, this study demonstrated distinct patterns of DTMUV infection in ducks in Thailand, in which significant seasonality was found in free-grazing ducks. Our data also indicate the year-round circulation of DTMUV in both farm and free-grazing ducks in Thailand, with the highest occurrence in the winter season. To the best of our knowledge, this is the first study presenting the patterns of DTMUV infection in ducks in Thailand. This information will help reduce the risk of DTMUV transmission through prevention and control strategies focusing on the peak period. Routine combined surveillance of DTMUV in ducks and mosquitoes is essential for early detection of DTMUV allowing the implementation of control measures in a timely manner.

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DISCLOSURES

The authors declare that they have no conflict of interest.

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