



Monitoring Culicine Mosquitoes (Diptera: Culicidae) as a Vector of Flavivirus in Incheon Metropolitan City and Hwaseong-Si, Gyeonggi-Do, Korea, during 2019

Young Yil Bahk¹, Seo Hye Park², Myung-Deok Kim-Jeon³, Sung-Suck Oh³, Haneul Jung², Hojong Jun²,
Kyung-Ae Kim³, Jong Myong Park³, Seong Kyu Ahn², Jinyoung Lee², Eun-Jeong Choi³, Bag-Sou Moon³,
Young Woo Gong³, Mun Ju Kwon³, Tong-Soo Kim^{2,*}

¹Department of Biotechnology, College of Biomedical and Health Science, Konkuk University, Chungju 27478, Korea; ²Department of Parasitology and Tropical Medicine & Global Resource Bank of Parasitic Protozoa Pathogens, Inha University School of Medicine, Incheon 22212, Korea;

³Department of Infectious Diseases Diagnosis, Incheon Metropolitan City Institute of Public Health and Environment, Incheon 22320, Korea

Abstract: The flaviviruses are small single-stranded RNA viruses that are typically transmitted by mosquitoes or tick vectors and are etiological agents of acute zoonotic infections. The viruses are found around the world and account for significant cases of human diseases. We investigated population of culicine mosquitoes in central region of Korean Peninsula, Incheon Metropolitan City and Hwaseong-si. *Aedes vexans nipponii* was the most frequently collected mosquitoes (56.5%), followed by *Ochlerotatus dorsalis* (23.6%), *Anopheles* spp. (10.9%), and *Culex pipiens* complex (5.9%). In rural regions of Hwaseong, *Aedes vexans nipponii* was the highest population (62.9%), followed by *Ochlerotatus dorsalis* (23.9%) and *Anopheles* spp. (12.0%). In another rural region of Incheon (habitat of migratory birds), *Culex pipiens* complex was the highest population (31.4%), followed by *Ochlerotatus dorsalis* (30.5%), and *Aedes vexans vexans* (27.5%). *Culex pipiens* complex was the predominant species in the urban region (84.7%). Culicine mosquitoes were identified at the species level, pooled up to 30 mosquitoes each, and tested for flaviviral RNA using the SYBR Green-based RT-PCR and confirmed by cDNA sequencing. Three of the assayed 2,683 pools (989 pools without *Anopheles* spp.) were positive for *Culex* flaviviruses, an insect-specific virus, from *Culex pipiens pallens* collected at the habitats for migratory birds in Incheon. The maximum likelihood estimation (the estimated number) for *Culex pipiens pallens* positive for *Culex* flavivirus was 25. Although viruses responsible for mosquito-borne diseases were not identified, we encourage intensified monitoring and long-term surveillance of both vector and viruses in the interest of global public health.

Key words: Mosquito, flavivirus, surveillance, insect-specific flavivirus, climate change

INTRODUCTION

Mosquito-borne diseases highlighted and gained increased focus [1-4] as the effects of climate change on public health worldwide have been recognized. Changes in the prevalence of mosquito-borne diseases have attracted attention. In fact, most land regions are experiencing greater warming than the global average [5]. Climate change is expected to accelerate in the future and the outcome for the Korean Peninsula is expected to be relatively large (e.g., temperature rise, rainfall change, etc.) [6]. According to a report by the Korea Meteorological

Administration (KMA), the temperature of the Korean Peninsula has risen geographically with the increase of CO₂ concentrations across the globe and in Korea [7,8]. The Intergovernmental Panel on Climate Change (IPCC) has predicted an average temperature rise globally of 1.5-5.8°C during the 21st century [9].

Global warming is affecting the survival and density of poikilothermal animals, including insects. Korea has the opportunity to implement systematic countermeasures against the flow of mosquito-borne infectious diseases into the country. Almost 350 million estimated cases of mosquito-borne diseases were recognized globally in 2017 [10]. In this sense, vector control has become a pivotal portion of the worldwide strategy to manage mosquito-associated diseases, especially Japanese encephalitis, West Nile encephalitis, malaria, and dengue fever.

The genus *Flavivirus* (family Flaviviridae) comprises a diverse

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*Corresponding author (tongsookim@inha.ac.kr)

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group of viruses and contains a variety of important human pathogens causing hemorrhagic fever and encephalitis [11]. A number of flaviviruses are considered major human pathogens. Mosquito-borne viruses can broadly be grouped as those transmitted by *Culex* spp. mosquitoes and associated generally with neurotropic viruses, and those transmitted by *Aedes* spp. mosquitoes and associated more closely with viscerotropic or hemorrhagic disease in humans. In addition, a number of insect-specific flaviviruses have been identified as non-vector-borne flaviviruses and a number of insect-specific flaviviruses have been identified in mosquitoes, such as Chaoyang virus, *Culex* flavivirus, and *Aedes* flavivirus [12], and several viruses have been identified as non-vector-borne flaviviruses. Regardless, the flaviviruses remain elusive about the flaviviruses. To heighten awareness of the risks of flavivirus-caused diseases and the potential for continued spread of these viruses, it is critical to monitor populations of the vectors.

The global environment is changing rapidly as a result of human activities, including population growth, urbanization, economic growth, natural resource use, and energy production, among others. Indeed, climate change has accelerated profound changes in ecosystems that influence human life. Thus, relying on monitoring to enable timely warnings can help protect the health of the public. The aims of this study are: 1) to report on species composition and abundance of vector mosquitoes and the pathogens they are carrying in urban areas and in habitats for migratory birds of Incheon and a rural area in Hwaseong during 2019; 2) to monitor and reduce the potential for the autochthonous transmission of imported mosquito-borne viruses. The approach in this study could provide the basis for epidemiological studies and risk assessment of vector-borne pathogens that enter Korea because of climate change.

MATERIALS AND METHODS

Collection sites

From March to November 2019, observational, descriptive, and prospective monitoring was performed to identify any circulating flaviviruses in different mosquito species in some areas in the middle of Korea Peninsula. Vector mosquitoes were collected in 7 regions: downtown Incheon (Bupyeong dong, Yeonhui dong, and Shinheung dong) as typical urban areas, habitats of migratory birds of Incheon (3 sites in Gyeongseo dong), and the Hwaseong-si area (a cowshed in Hogok-ri, Gyeonggi-do) as a rural area. Supplementary Table S1 presents

the geographic coordinates recorded using a handheld GPS tracker where traps were set for the collection of mosquitoes. Incheon has a humid subtropical/continental climate [13] and the Köppen-Geiger climate classification for Incheon is Dwa (Continental, Dry summer, and Hot summer). Hwaseong-si is a city in Gyeonggi-do and the climate classification of this area based on the Köppen-Geiger method is also Dwa.

Collection of mosquitoes

Four traps were set up at each of the study sites. Adult mosquitoes were continuously collected for 24 hr at 2-week intervals from March to November 2019 using black light traps and BG Sentinel™ traps. Mosquitoes were transferred from the traps into a Styroform cooler on wet ice and transported to the laboratory of Department of Parasitology and Tropical Medicine & Global Resource Bank of Parasitic Protozoa Pathogens, Inha University, School of Medicine. Female mosquitoes were identified and confirmed morphologically using taxonomic keys [14,15] and were compared with standard specimens in the collection of Inha University, School of Medicine and the Korea Centers for Disease Control and Prevention (KCDC). The abbreviations for the mosquito genera and subgenera follow the standardization proposed by Reinert [16]. The collected mosquitoes were pooled (1-30/pool) by species, collection site, and time of survey and quickly transferred to microcentrifuge tubes at 4°C, where they were assayed for flaviviruses including Japanese encephalitis virus, West Nile virus, yellow fever virus, Zika virus and dengue fever virus. In total, 989 mosquito pools were analyzed for flavivirus identification.

RNA extraction and quantitative real-time (qRT)-PCR analysis of mosquito pools for flavivirus detection

To improve extraction and achieve high efficiency, and to reduce variability in the total RNA yields, bead beaters with QIAamp™ viral RNA mini kit were used in accordance with the manufacturer's protocols (Qiagen 52904, Qiagen GmbH, Hilden, Germany). After RNA extraction from the homogenized mosquito samples, the extracted total RNA was resuspended in RNase-free water containing RNAsin™ Plus RNase inhibitor (Promega Corporation, Madison, Wisconsin, USA) and then stored at -70°C until use. qRT-PCR assays were conducted using a Verso SYBR Green 1-step qRT-PCR kit (Thermo AB4104, Thermo-Fisher Scientific, Waltham, Massachusetts, USA) in accordance with the manufacturer's protocols for the detection of pathogens in mosquitoes [17]. The specific prim-

ers for nonstructural protein 5 (NS 5) used were as follows: PFlav-fAAR (sense) 5'-TACAACATGATG-GGAAAGAGAGAGA-ARAA-3', PFav-rKR (anti-sense) 5'-GTGTCCCAKCCRGCTGT-GTCATC-3'. qRT-PCR was performed using 1.0 µl PrimeScrip™ 1-step enzyme mix, 12.5 µl 2×1 step SYBR RT-PCR buffer, each with 1 µM primer, and 1 pg to 100 ng total RNA in a 25 µl total reaction volume. qRT-PCR conditions for each reaction were 30 min at 50°C, followed by 15 min at 95°C, then 15 sec at 95°C, 20 sec at 58°C and 30 sec at 72°C for 45 cycles, and finally annealed for 1 min at 60°C. Samples that generated a detectable fluorescence signal when assayed were determined to be flavivirus positive by monitoring the increase in fluorescence of a dye labeled oligonucleotide probe [17,18]. Using a Verso SYBR Green 1-step qRT-PCR kit with the positive samples, the cDNAs were re-assayed using the same primer set at the PCR condition of 2 min at 94°C, then 30 sec at 94°C, 20 sec at 58°C and 30 sec at 72°C for 30 cycles, and finally annealed for 5 min at 72°C. The PCR products (202 bp in size) were then sent to Macrogen company (Macrogen, Seoul, Korea), where the obtained DNA sequences were edited and assembled for flavivirus classification.

RESULTS

Mosquitoes collected

In total, 79,209 (2,683 pools) female mosquitoes comprising 15 species (*Aedes albopictus*, *Ae. vexans nipponii*, *Ae. vexans vexans*, *Armigeres subalbatus*, *Coquillettidia ochracea*, *Culex inatomi*, *Cx. bitaeniorhynchus*, *Cx. orientalis*, *Cx. pipiens* complex, *Cx. tritaeniorhynchus*, *Cx. vegans*, *Mansonia uniformis*, *Ochlerotatus dorsalis*, *Och. koreicus*, and *Anopheles* spp.) were collected from March to November 2019 (Table 1) at the 7 locations in the downtown and habitats of migratory bird in Incheon and one location in a cowshed in Hwaseong-si areas. *Ae. vexans nipponii* was the most frequently collected mosquitoes (56.5%), followed by *Och. dorsalis* (23.6%), *Anopheles* spp. (10.9%), and *Cx. pipiens* complex (5.9%). The remaining 11 species comprised <1.00% of the total number of mosquitoes collected. The mosquitoes had periods of high prevalence (Fig. 1; Table 2). *Ae. vexans nipponii* populations increased from late May until late August peaking in late June; *Och. dorsalis* populations increased from late June to late September peaking in late June. *Ae. vexans nipponii* and *Och. dorsalis* showed clear 3 peaks in abundance through the year, with highest numbers collected during the late June (42.9% and 40.6%, respectively). *Ae. vex-*

ans nipponii showed other peaks at late May and early August, while *Och. dorsalis* showed them in early August and late September. Prevalence patterns for urban and rural regions were as expected, with more mosquitoes collected in the rural region of Hwaseong (89.8%) and in the habitat regions of Incheon (6.9%), a finding similar to many studies [19,20] (Table 1). In rural regions of Hwaseong, *Ae. vexans nipponii* maintained the highest population (62.9%), followed by *Och. dorsalis* (23.9%) and *Anopheles* spp. (12.0%). In another rural region of Incheon (habitat of migratory birds), *Cx. pipiens* complex maintained the highest population (31.4%), followed by *Och. dorsalis* (30.5%), and *Ae. vexans vexans* (27.5%). However, *Cx. pipiens* complex was the predominant species in the urban region (84.7%) and the other species made up <5% of the total mosquitoes collected during the collection period (Table 1). The other mosquito species made up <1% of the total mosquitoes collected during the monitoring period and the sizes of the populations of these species were too small to be considered pivotal vectors in this monitoring study.

Flaviviruses in mosquito vectors

After qRT-PCR using a Verso SYBR Green 1-step qRT-PCR kit, the amplicons of PCR using the first amplified cDNA from the positive pooled mosquito samples were sequenced. The sequences were edited and assembled to obtain small DNA fragments of 202 bp in size. From the analysis of the derived nucleotide sequences for matching genotypes using NCBI-BLAST service, 3 pools of *Cx. pipiens pallens* (from 989 total pools) tested positive for *Culex* flavivirus (Table 3) [21]. The maximum likelihood estimations (the estimated number of virus-positive mosquitoes/1,000 mosquitoes) for *Cx. pipiens pallens* was 25 (Table 3). These mosquitoes were collected from the habitat for migratory birds (Gyeongseo-dong (#2 site)) in Incheon during the first and second weeks of April and the first week of May 2019. *Cx. pipiens pallens* is a nuisance mosquito that exists widely throughout Korea and was the most frequently captured mosquito in the habitat of migratory birds (Table 1). Other types of flaviviruses were not identified using Verso SYBR Green 1-step qRT-PCR assays.

DISCUSSION

The climate in Korea has changed gradually from temperate to subtropical, making the Korean Peninsula environmentally preferable for the proliferation of vector mosquitoes in the

Table 1. Female mosquitoes and mosquito pools collected in Incheon Metropolitan City and Hwaseong-si areas, 2019

Species	Cx. inatomi	Cx. bitaeniorhynchus	Cx. orientalis	Cx. pipiens complex*	Cx. tritaeniorhynchus	Cx. vegans	Man. uniformis	Anopheles spp.	Ae. albopictus	Ae. lineatopennis	Ae. vexans nipponii	Ae. vexans vexans	Och. koreicus	Och. dorsalis	Ar. subabatus	Total
Downtown																
Bupyeong																
No.	0	0	0	129	0	0	0	1	0	0	0	0	1	2	0	133
(%)	0	0	0	(96.99)	0	0	0	(0.75)	0	0	0	0	(0.75)	(1.50)	0	(100)
pool	0	0	0	5	0	0	0	1	0	0	0	0	1	1	0	8
Yeonhui																
No.	0	0	2	234	0	1	0	6	7	0	0	33	4	36	1	324
(%)	0	0	(0.617)	(72.22)	0	(0.31)	0	(1.85)	(2.16)	0	0	(10.19)	(1.24)	(11.11)	(0.31)	(100)
pool	0	0	1	8	0	1	0	1	1	0	0	2	1	2	1	18
Shinheung																
No.	0	0	0	1,859	0	0	0	3	263	0	0	24	12	3	2	2,166
(%)	0	0	0	(85.83)	0	0	0	(0.14)	(12.14)	0	0	(1.11)	(0.55)	(0.14)	(0.09)	(100)
Pool	0	0	0	62	0	0	0	1	9	0	0	1	1	1	1	76
Habitats of migratory birds																
Gyeongseo (#1)																
No.	0	1	3	618	0	2	0	26	0	114	3	285	2	528	2	1,584
(%)	0	(0.06)	(0.19)	(39.02)	0	(0.13)	0	(1.64)	0	(7.2)	(0.19)	(17.99)	(0.13)	(33.33)	(0.13)	(100)
pool	0	1	1	22	0	1	0	1	0	4	1	10	1	18	1	60
Gyeongseo (#2)																
No.	0	5	59	655	8	2	3	32	0	248	2	942	1	1,067	0	3,024
(%)	0	(0.17)	(1.95)	(21.66)	(0.27)	(0.07)	(0.10)	(1.06)	0	(8.2)	(0.07)	(31.15)	(0.03)	(35.28)	0	(100)
pool	0	1	2	22	1	1	1	2	0	9	1	32	1	36	0	109
Gyeongseo (#3)																
No.	0	0	2	450	6	0	1	21	1	33	0	282	2	76	1	875
(%)	0	0	(0.23)	(51.43)	(0.69)	0	(0.11)	(2.40)	(0.11)	(3.77)	0	(32.23)	(0.23)	(8.69)	(0.11)	(100)
pool	0	0	1	15	1	0	1	1	1	2	0	10	1	3	1	37
Hwaseong (cowshed)																
No.	1	0	0	699	0	15	0	8,562	0	0	44,771	0	41	17,014	0	71,103
(%)	0	0	0	(0.98)	0	(0.02)	0	(12.04)	0	0	(62.97)	0	(0.06)	(23.93)	0	(100)
pool	1	0	0	24	0	1	0	286	0	0	1,493	0	2	568	0	2,375
Total																
No.	1	6	66	4,644	14	20	4	8,651	271	395	44,776	1,566	63	18,726	6	79,209
(%)	0	(0.01)	(0.08)	(5.86)	(0.02)	(0.03)	(0.01)	(10.92)	(0.34)	(0.50)	(56.53)	(1.98)	(0.08)	(23.64)	(0.01)	(100)
pool	1	2	5	157	2	4	2	293	11	15	1,495	55	8	629	4	2,683

*Cx. pipiens complex comprised to 2 Cx. pipiens molestus and 4,642 Cx. pipiens pallens.

Table 2. Female mosquitoes collected every other week in 2019

Species	March		April		May		June		July		August		September		October		November		Total(%)
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	
<i>Cx. inatomii</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 (>0.01)
<i>Cx. bitaeniorhynchus</i>	0	0	0	0	0	0	0	0	0	0	4	0	0	2	0	0	0	0	6 (>0.01)
<i>Cx. orientalis</i>	0	0	0	0	0	0	0	0	0	0	61	0	0	3	1	1	0	0	66 (0.08)
<i>Cx. pipiens complex</i>	1	11	1	15	87	81	244	687	404	895	280	125	194	458	357	363	441	0	4,644 (5.86)
<i>Cx. tritaeniorhynchus</i>	0	0	0	0	0	0	0	0	0	0	0	1	1	10	2	0	0	0	14 (0.02)
<i>Cx. vegans</i>	0	0	0	1	2	14	0	0	0	0	0	0	0	2	0	0	0	0	20 (0.03)
<i>Coq. ochracea</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0 (0)
<i>Man. uniformis</i>	0	0	0	0	0	0	0	1	0	1	0	2	0	0	0	0	0	0	4 (>0.01)
<i>Anopheles spp.</i>	0	0	0	8	0	12	25	839	4,690	888	1,020	416	389	311	45	7	1	0	8,651 (10.92)
<i>Ae. albopictus</i>	0	0	0	0	0	0	0	2	7	4	77	6	33	81	30	27	4	0	271 (0.34)
<i>Ae. lineatopennis</i>	0	0	0	0	0	0	0	0	0	0	184	48	3	47	70	43	0	0	395 (0.5)
<i>Ae. vexans nipponii</i>	0	0	0	6	4	4,008	835	19,227	9,550	697	7,989	1,436	170	507	328	19	0	0	44,776 (56.53)
<i>Ae. vexans vexans</i>	0	0	0	0	0	0	0	0	0	10	677	325	21	471	55	7	0	0	1,566 (1.98)
<i>Och. koreicus</i>	0	0	0	0	0	0	0	40	0	3	8	0	3	0	4	4	1	0	63 (0.08)
<i>Och. dorsalis</i>	0	0	0	3	529	1,363	147	7,597	1,241	96	2,808	1,127	145	3,327	306	37	0	0	18,726 (23.64)
<i>Ar. subalbatus</i>	0	0	0	0	0	0	0	0	1	0	1	1	0	3	0	0	0	0	6 (>0.01)
Total (%)	1	11	1	33	622	5,478	1,252	28,393	15,894	2,594	13,109	3,487	959	5,222	1,198	508	447	0	79,209 (100)
	(>0.01)	(0.01)	(>0.01)	(0.05)	(0.79)	(6.92)	(1.58)	(35.85)	(20.07)	(3.27)	(16.55)	(4.4)	(2.21)	(6.59)	(1.51)	(0.64)	(0.56)		

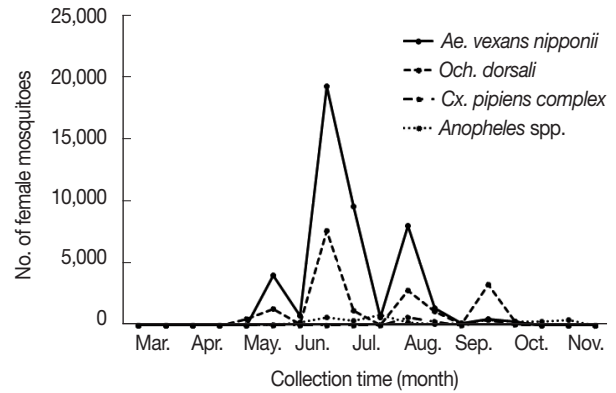


Fig. 1. Populations of each mosquito species including *Aedes vexans nipponii*, *Ochlerotatus dorsalis*, *Anopheles* spp., and *Culex pipiens complex* collected from March to November 2019.

Table 3. Incidence of flavivirus in culicine mosquitoes detected using qRT-PCR and determined using maximum likelihood estimation (MLE) in 2019

Mosquitoes	No. of mosquitoes collected	No. of pools	No. of flavivirus positive pools (%)	Virus identification (MLE) ^a
<i>Ae. albopictus</i>	271	20	0	
<i>Ae. lineatopennis</i>	395	21	0	
<i>Ae. vexans nipponii</i>	44,697	320	0	
<i>Ae. vexans vexans</i>	1,566	70	0	
<i>Ar. subalbatus</i>	6	5	0	
<i>Cx. bitaeniorhynchus</i>	6	3	0	
<i>Cx. inatomii</i>	1	1	0	
<i>Cx. orientalis</i>	66	6	0	
<i>Cx. pipiens molestus</i>	2	1	0	
<i>Cx. pipiens pallens</i>	4,642	211	3 (1.42)	<i>Culex flaviviruses</i> (25)
<i>Cx. tritaeniorhynchus</i>	16	6	0	
<i>Cx. vagans</i>	17	4	0	
<i>Man. uniformis</i>	5	4	0	
<i>Och. dorsalis</i>	18,705	304	0	
<i>Och. koreicus</i>	63	13	0	
Total	70,558	989	3 (0.30)	

^aMLE, estimated number of flaviviral RNA positive mosquitoes per 1,000.

near future [22]. Climate variables such as precipitation and temperature, which have changed significantly as a result of global warming, are major driving forces of vector-borne diseases and altered the exposure pathways. The determinants could influence the fate and transport of pathogens, as well as their stability, reproduction rates, and variability in the environment, especially mosquitoes for serious vector-borne diseases, such as dengue fever, Japanese encephalitis, Zika fever, Chikungunya fever, yellow fever, and West Nile fever—the majority of

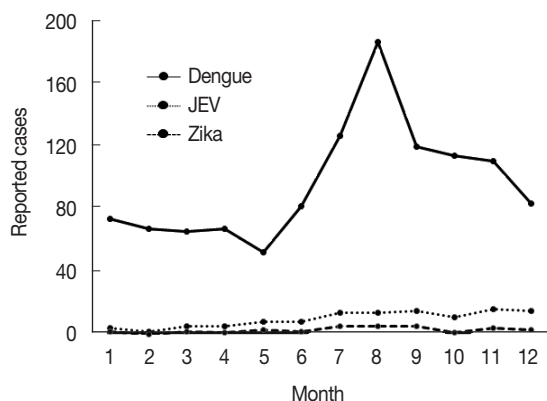


Fig. 2. Monthly accumulated cases of indigenous mosquito-borne diseases including dengue fever, Japanese encephalitis, and Zika fever [23] in Korea during 2015-2019.

which are zoonoses that infect a range of vertebrate hosts via mosquitoes and can cause serious public health issues. In Korea, these infectious diseases are classified as group III infectious diseases according to the Korean Act on the Prevention and Control of Infectious Diseases. Currently, the major indigenous mosquito-borne infectious diseases in Korea are vivax malaria and dengue virus infection and the minor ones are Japanese encephalitis and Zika fever (Fig. 2). During a 5-year period (2015-2019), 1,171 clinical cases of dengue fever were reported in Korea [23]. Although approximately 1,600 cases of Japanese encephalitis were reported annually until 1970s, total cases decreased dramatically to 129 from 2015 to 2019 because of the introduction of an effective vaccine and a mandatory vaccination program [24]. In case of Zika virus infection in Korea, 33 total clinical cases were reported during 2016-2019 [23]. Although no cases of other flaviviral diseases, such as Chikungunya fever, yellow fever, and West Nile fever, have been reported as indigenous to Korea to date, it is highly likely, based on the existence of the vector mosquitoes in Korea that the context might change if the mosquito-borne diseases are introduced from the outside of country.

Kim et al. [25] studied the population densities of culicine mosquitoes and reported *Ae. vexans nipponii* maintained the highest population (50.7%), followed by *Cx. tritaeniorhynchus* (36.6%), *Cx. pipiens* complex (7.5%), *Och. dorsalis* (2.1%), and *Cx. bitaeniorhynchus* (1.3%). The present study found that *Ae. vexans nipponii* was the most frequently collected species, followed by *Och. dorsalis*, *Anopheles* spp., and *Cx. pipiens* complex. Obvious differences exist between Incheon (an urban area) and Hwaseong (a rural area) in the species collected. In rural regions of Hwaseong, *Ae. vexans nipponii* maintained the high-

est population, followed by *Och. dorsalis* and *Anopheles* spp. In another rural region of Incheon (habitat of migratory birds), *Cx. pipiens* complex maintained the highest population, followed by *Och. dorsalis*, and *Ae. vexans vexans*. In urban area, *Cx. pipiens* complex was the predominant species. From the surveillance of the vector mosquitoes carrying flaviviruses during April to November 2015 in Incheon and Hwaseong [26], the *Cx. pipiens* complex was the most collected species (81.9%) in downtown Incheon followed by *Ae. vexans nipponii* (11.8%) and *Och. koreicus* (2.9%), whereas *Ae. vexans nipponii* was the most collected species in Hwaseong (58.9%) followed by *Cx. pipiens* complex (15.3%), *Och. koreicus* (13.9%), and *Och. dorsalis* (11.1%). The other species made up less than 2% of the total mosquitoes collected during the collection period [26]. From the surveillance data collected during 2016-2018 in Incheon and Hwaseong [19], *Ae. vexans nipponii* comprised 49.1% of the population and *Cx. pipiens*, *Och. dorsalis*, *Och. koreicus*, and *Cx. tritaeniorhynchus* comprised 31.8%, 13.6%, 1.7%, and 1.5% of the population, respectively. Although *Cx. tritaeniorhynchus* is the major mosquito vector throughout Asia, including Korea, and *Cx. bitaeniorhynchus* has been implicated recently as a vector of Japanese encephalitis in Korea, the populations of these 2 species made up less than 0.02% of the total number of mosquitoes collected and were not considered important vectors in our study sites.

Previous surveillances during April to November 2015 and during 2016 to 2018 found 3 Japanese encephalitis genotype V viruses from *Cx. pipiens* complex pools and of one Chaoyang virus from *Ae. vexans nipponii* pools, respectively [19,26]. In the present study, we found 3 *Culex* flavivirus positive cases from the *Cx. pipiens pallens* pooling samples at the habitat for migratory birds in Incheon. However, other genotype- and insect-specific flaviviruses and pathogenic flaviviruses were not found in this study. An insect-specific flavivirus isolated in Korea has been identified recently as 98.9% homologous to the first-reported Chaoyang virus from Liaoning Province, China, grouped genetically within the mosquito-borne flavivirus cluster [27,28]. The genus *Flavivirus* includes many pivotal human pathogens causing hemorrhagic fever and encephalitis, such as dengue fever, Japanese encephalitis, Zika fever, Chikungunya fever, yellow fever, and West Nile fever. The Chaoyang virus, which is limited to insects alone, is referred to as an 'insect-specific' virus that naturally infects mosquitoes and replicates in mosquito cells in vitro, but does not appear to replicate in vertebrate cells or to infect humans or other vertebrate [29,30]. *Culex* flavivirus

has been isolated and characterized from *Culex* mosquitoes in Japan [21], Guatemala, Mexico, and the USA [31]. The insect-specific flaviviruses are speculated to represent the genetic primordial form of the genus [21]. *Culex* flaviviruses are distributed widely in the natural environment and can infect various mosquito species [31]. *Culex* flaviviruses have been detected in *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. tritaeniorhynchus*, and *Anopheles sinensis* in Japan, Indonesia, the USA, Uganda, China, Mexico, and Brazil [32]. In the present monitoring, the 3 *Culex* flavivirus positive cases from the *Cx. pipiens pallens* samples were detected during the first and second weeks of April and the first week of May 2019. We continued to sample *Culex* mosquitoes to November 2019 but did not detect any more flavivirus-positive mosquito pools. In fact, *Culex* mosquitoes are seasonal in this study and the highest peak of appearance of these mosquitoes was in late July, followed by late June (Table 2). However, the total number of individuals collected from the *Cx. pipiens* complex is relatively small compared to other species. We recognize that an important limitation in this study is lack of data for more than one year with which to test the consistency of seasonal trends.

Biological control measures involve the use of natural predators or pathogens to reduce mosquito abundance or vector competence. From recent studies with *Wolbachia*, a bacterial that infects arthropod species, maternally inherited bacterial endosymbionts found in a variety of arthropod species have been used to reduce insect populations by way of cytoplasmic incompatibility [33]. Certain *Wolbachia* strains have the potential to reduce the vector competence of mosquitoes by rendering them refractory to certain human pathogens [34]. If a bacterial symbiont can alter the vector competence of a mosquito for arboviruses, then it seems likely that certain viral symbionts may have similar effect [35]. Although this phenomenon is not yet fully characterized, it may be feasible to create a population of mosquitoes in which the individuals are unable to transmit certain human pathogenic arboviruses [30]. This study might be leveraged as part of future mosquito surveillance for a better comprehension of insect-specific flaviviruses circulating in mosquitoes in Korea. Furthermore, research to elucidate any effect of the interaction between insect-specific flavivirus and human pathogenic flaviviruses is of critical importance.

In conclusion, we examined the status of flaviviral diseases transmitted by mosquitoes and investigated the abundance of mosquito populations in downtown and in habitats for migra-

tory birds of Incheon and a cowshed of Hwaseong, Korea from March to November 2019. Climate change is a driving forces in altering the distribution, reproduction, and activity of mosquito populations and influencing the prevalence of mosquito-borne pathogens causing public health issues. Intensified monitoring and long-term surveillance of both the vectors and viruses they transmit are significant to global public health.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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