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Original Article

An Ecological Survey of Mosquitoes and the Distribution of Japanese Encephalitis Virus in Ishikawa Prefecture, Japan, between 2010 and 2014

Manabu Murakami[1]*, Kiyoe Hori[2], Yoko Kitagawa[2], Yosaburo Oikawa[1], Kiyoshi Kamimura[3], and Tsutomu Takegami[2]

[1]*Department of Medical Zoology Kanazawa Medical University, Ishikawa;*
[2]*Department of Life Science, Medical Research Institute, Kanazawa Medical University, Ishikawa; and*
[3]*Marusan Pharma Biotech, Toyama, Japan*

SUMMARY: Japanese encephalitis virus (JEV) is a flavivirus, responsible for over 30,000 annual cases of encephalitis worldwide, with a mortality rate of approximately 30%. Therefore, it is important to examine the distribution of mosquitos carrying JEV in the fields, even though recently, the number of Japanese encephalitis cases has been approximately 5 per year in Japan. We report the seasonal dynamics of mosquitoes between 2010 and 2014 in Ishikawa Prefecture, Japan. We collected 39,308 female adult mosquitoes, 98.2% of which were classified as *Culex tritaeniorhynchus* Giles. We identified JEV genomic RNA belonging to genotype 1 from the homogenate of *Cx. tritaeniorhynchus*, collected during our study using reverse transcription-PCR and nucleotide sequencing techniques. Our results indicate that mosquito vectors for JEV are distributed not only in areas in Ishikawa, but also throughout Japan, and the results suggest that we must be careful regarding JEV outbreaks in Japan in the future.

INTRODUCTION

Flaviviridae include many insect-mediated small viruses that cause serious health-related problems worldwide. For example, a flavivirus was responsible for the outbreak of Zika virus infections in Brazil (1–3). Japanese encephalitis virus (JEV) is a flavivirus that is responsible for more than 30,000 annual cases of encephalitis worldwide, with a mortality rate of approximately 30% (2,3). Thus, JEV is a continuing public health threat. JEV has a normal transmission cycle between animals and mosquitoes, particularly a zoonotic transmission cycle, with swine serving as an amplifier host from which infected mosquitoes transmit the virus to humans. JEV is widespread in Southeast Asia, China, Korea, and Japan. It is important to examine the distribution of mosquitos carrying JEV in the fields. However, recently, the number of Japanese encephalitis (JE) cases has been approximately 5 per year, in Japan (4,5). In Ishikawa Prefecture, 3 JE cases were reported in 2002 and 2007 by the National Institute of Infectious Diseases (NIID) (6,7).

The paddy-breeding mosquito, *Culex tritaeniorhynchus* Giles, is thought to be the primary vector for JEV throughout Asia (8). We attempted to isolate new JEV strains from *Cx. tritaeniorhynchus*. For this purpose, we collected many mosquitoes in Ishikawa Prefecture, Japan. Sampling areas were close to swine farms and rice fields. Of several tools used to sample mosquitoes, the Centers for Disease Control and Prevention (CDC) trap has been most commonly used, with varying degrees of success (9,10). To identify the JEV genome, we used reverse transcription PCR (RT-PCR) and sequencing techniques.

In this study, we report the seasonal dynamics of mosquito vectors between 2010 and 2014 in areas of Ishikawa. The report is an important contribution to the surveillance of JE diseases. In addition, we identified the JEV strain Ishikawa12 (genotype 1) from field-collected *Cx. tritaeniorhynchus*. The evaluation of both biological and ecological data is the cornerstone of mosquito control programs from a public health perspective.

MATERIALS AND METHODS

Description of the investigation area: Mosquitoes were collected weekly in 3 areas, close to swine farms and rice fields in Ishikawa Prefecture (Fig. 1). The 3 areas included 6 points (A-F) and the geographical points were as follows: (i) points A & B in Kahoku city were A: 36°27′31″N, 136°42′35″W and B: 36°43′14″N, 136°43′1″W, respectively; (ii) points C & D in Hakui city were C: 36°49′47″N, 136°46′22″W and D: 36°49′40″N, 136°46′30″W, respectively; (iii) points E & F in Kahoku city were E: 36°42′7″N, 136°41′55″W and F: 36°42′8″N, 136°41′51″W, respectively. These points are surrounded by 12 km^2 of agricultural land, including a rice cultivation area (approximately 50%), forest wilderness (approximately 25%), and residential land (approximately 15%).

Collection, classification, and analysis of mosquitoes: Mosquitoes were collected using CDC miniature light traps (Model 512; John W. Hock Co., Gainesville, FL, USA) baited with CO_2; traps were set from 5:00 pm to 9:00 am from June to October. Mosquitoes collected in traps were transported to the laboratory for further analysis. Mosquito species were determined

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*Corresponding author: Mailing address: Department of Medical Zoology, Kanazawa Medical University, Daigaku 1-1, Uchinada, Kahoku, Ishikawa 920-0293, Japan.
Tel: +81-76-286-2211 (ext. 3935),
E-mail: manabu@kanazawa-med.ac.jp

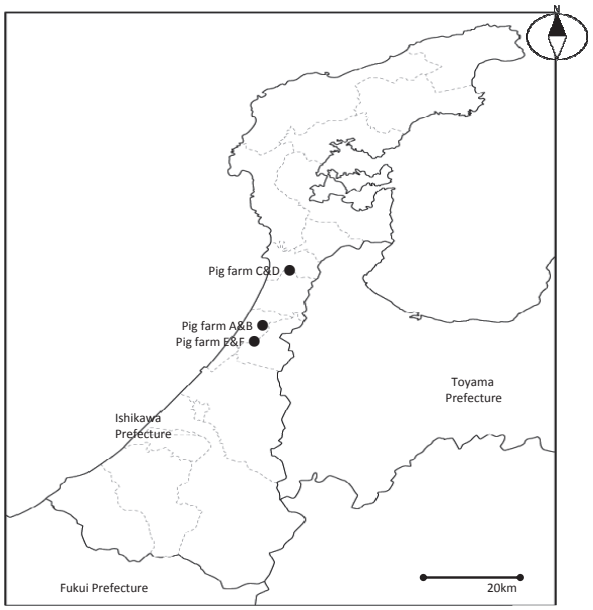

Fig. 1. The position of 3 areas in Ishikawa Prefecture, Japan. We collected mosquitoes in these 3 areas including 6 points from June to October between 2010 and 2014.

based on morphological characteristics. Female *Cx. tritaeniorhynchus* was morphologically identified and labeled based on the date and geographic region of their capture. *Cx. tritaeniorhynchus* pools containing 50 individuals were homogenized in 2 mL of minimum essential medium (MEM) Alpha (Sigma-Aldrich, St. Louis, MO, USA) using a mixer and were then filtered using a combined 0.8/0.2-μm filter and kept at −80°C until viral screening was conducted (10,11).

RNA extraction from mosquitoes: RNA was extracted from the mosquito homogenate using ISOGEN reagents (Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. This was used as the template for cDNA synthesis. The procedure is briefly described as follows: Five hundred microliters of the homogenate were mixed with 200 μL of ISOGEN. RNA was precipitated in 100μL of isopropanol. The RNA pellet was then washed with 70% ethanol and dissolved in 20 μL of ultrapure water.

RT-PCR and nucleotide sequencing: RNA extracted from *Cx. tritaeniorhynchus* samples were tested for JEV genome amplification using RT-PCR. The concentration of RNA was determined by measuring the optical density (OD) at 260 nm. RT-PCR was performed according to the manufacturer's instructions using the ReverTra Ace PCR Kit (Toyobo, Osaka, Japan). The primers E6(I) (5′-GATGTCAATGGCACATCCAGT-3′) for the JEV-E protein and NS4BR (5′-ACATGCTCTTGAGGTCTGCTTTGG-3′) for JEV-NS3-NS4a were used. The RT reaction was performed at 42°C for 60 min. Samples were then placed at 95°C for 5 min. During the subsequent PCR, the RT product was mixed with a solution containing 1.25 U of Blend Taq polymerase (Toyobo). Several JEV-specific primers, including the followings, were used: E1(I) (5′-GTCCGTCCGGCTTACCAGTTT-3′), E2(I) (5′-GAGGGAGCGTTTGGAGTTACA-3′), E3(I) (5′-GCGTCTCAAGCAGCAAAGTT-3′), E4(I) (5′-GTCATGTCGTTTAAACTCGC-3′), E5(I) (5′-CCTGTAAAATTCCGATTGTC-3′), E6(I) NS3CS (5′-ATCACCAAGCCCTCAAGTGG-3′), and NS4BR.

PCR was performed under the following conditions for 39 cycles (94°C, 1min; 60°C, 1 min; and 72°C, 2 min) using the thermal cycler PTC-200 DNA Engine (MJ Research, Waltham, MA, USA). Amplified PCR products were detected using agarose gel electrophoresis and ethidium bromide staining. To determine the nucleotide sequence of the JEV-E protein region, JEV-DNA was prepared from the JEV-specific band produced by RT-PCR as follows: the PCR product (JEV-DNA) was excised and extracted from the agarose gel. The nucleotide sequences of JEV-DNA were determined by the dideoxy method using JEV specific primers E1(I) to E6(I). The DNA sequencer (ABI Prism 310; Applied Biosystems, Foster City, CA, USA) was used as previously described (5,12,13).

Virus isolation: To isolate JEV, Vero cells, which were grown in the MEM supplemented with 5 % fetal bovine serum (FBS) at 37°C in 5% CO_2, were used. For virus isolation, PCR-positive mosquito homogenates were selected. The filtered mosquito homogenate in MEM without FBS (1mL) was applied to the monolayer of Vero cells grown in a 6-well plate. After 1 h, sample fluids were replaced with fresh MEM containing 5% FBS, and they were then cultured for several days. Cells were continuously observed to determine whether they showed the cytopathic effect caused by virus multiplication.

RESULTS

Classification of mosquitoes: Between 2010 and 2014, 39,308 female adult mosquitoes belonging to 5 species were captured in the 3 areas of Ishikawa (Table 1). The most abundant mosquito species was *Cx. tritaeniorhynchus* (98.2%) (Table 1 and Fig. 2). The number of other species of mosquitoes (1.8%) was minimal. The other species of identified mosquitoes included *Culex pipiens* complex (1.4%), *Anopheles sinensis* Wiedemann (0.2%), *Armigeres subalbatus* (Coquillett) (0.1%), and *Aedes albopictus* (Skuse) (0.03%) (Table 1). Minor species, accounting for 0.01% of the total mosquitoes, included *Aedes japonicus* Theobald, *Tripteroides bambusa* (Yamada), and *Culex halifaxii* Theobald. *An. sinensis* were collected in the C & D and E & F points but not in the A & B point.

Each year, the number of *Cx. tritaeniorhynchus* collected was lower in the early season and increased to a maximum between August and September as shown in Fig. 2. In 2012 and 2013, the great majority of *Cx. tritaeniorhynchus* were collected (i.e., 9,854 [2012] and 10,401 [2013], respectively) (Table 1 and Fig. 2). The highest number of *Cx. tritaeniorhynchus* was collected from point E & F in 2013 (6,500 per year) (Fig. 2). Our results indicated that the number of *Cx. tritaeniorhynchus* collected gradually increased around June, reaching a maximum level in August, then decreasing around September and October. However, this phenomenon differed in 2012. The mosquito collection level increased in September as shown in Fig. 2.

This difference may be due to weather conditions, including temperature and rainfall, in these areas. Information regarding temperature and rainfall in Ishikawa Prefecture during the study period is shown in Fig. 3.

Table 1. The species and numbers of mosquitoes collected in the 3 areas in Ishikawa Prefecture and identification of JEV genomes[1)] among *Cx. tritaeniorhynchus* between 2010 and 2014

Area (Point)		No. of mosquitoes collected						Pool number[2)] *Cx. tritaeniorhynchus* screened (JEV-positive pools)				
		2010	2011	2012	2013	2014	total	2010	2011	2012	2013	2014
A & B	*Cx. tritaeniorhynchus*	958	1,813	3,900	1,223	786	8,680	24 (0)	46 (0)	81 (0)	27 (0)	19 (0)
	Cx. pipiens complex	34	72	48	22	14	190	-	-	-	-	-
	Ae. albopictus	1	2	0	0	0	3	-	-	-	-	-
	An. sinensis	0	0	0	0	0	0	-	-	-	-	-
	Ar. subalbatus	0	1	0	0	0	1	-	-	-	-	-
C & D	*Cx. tritaeniorhynchus*	3,272	2,906	3,657	2,678	1,378	1,3891	74 (8)	68 (0)	78 (3[3)])	58 (0)	27 (0)
	Cx. pipiens complex	4	10	18	27	29	88	-	-	-	-	-
	Ae. albopictus	0	0	0	6	1	7	-	-	-	-	-
	An. sinensis	24	20	13	0	0	57	-	-	-	-	-
	Ar. subalbatus	6	24	4	0	0	34	-	-	-	-	-
E & F	*Cx. tritaeniorhynchus*	3,457	2,649	2,297	6,500	1,142	16,045	74 (4)	64 (1)	50 (0)	115 (0)	31 (0)
	Cx. pipiens complex	73	37	155	15	7	287	-	-	-	-	-
	Ae. albopictus	0	2	0	0	1	3	-	-	-	-	-
	An. sinensis	0	0	0	9	8	17	-	-	-	-	-
	Ar. subalbatus	0	0	0	4	1	5	-	-	-	-	-

[1)]: JEV-genomic RNA was identified using RT-PCR, and RNA was prepared from *Cx. tritaeniorhynchus*.
[2)]: One pool contained 50 mosquitoes.
[3)]: RT-PCR assay indicated JEV-positive products as shown in Fig. 4.

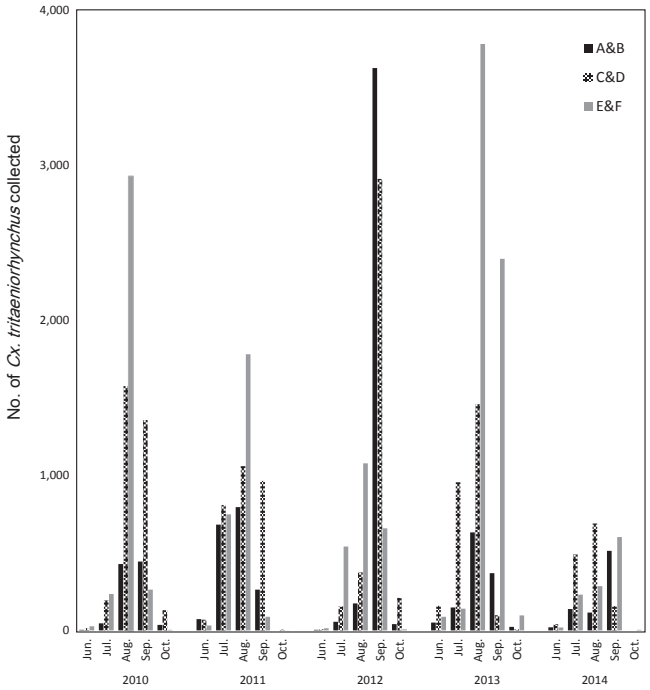

Fig. 2. Seasonal change in *Cx. tritaeniorhynchus* collected in 3 areas between 2010 and 2014.

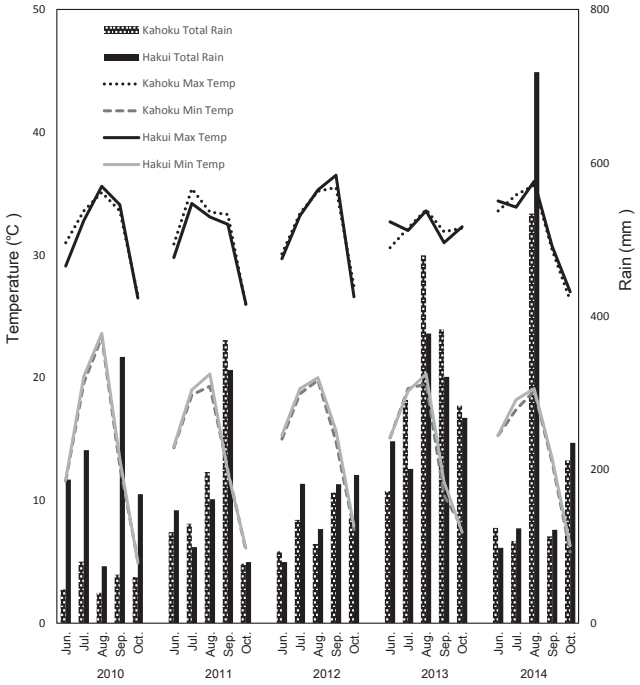

Fig. 3. The situation of temperature and rainfall in Ishikawa Prefecture during the study period.

The maximum and minimum temperature and total rainfall was 25.9–35.7°C, 5.1–23.3°C, and 39.5–534.0 mm in Kahoku (point A & B and E & F), 26.0–36.5°C, 4.9–23.6°C, and 74.0–718.5 mm in Hakui (point C & D). Rainfall was the highest in 2014 as shown in Fig. 3. In 2012, rainfall was relatively lower than that in other years.

Identification of JEV genomic RNA and isolation of JEV: Next, 38,616 *Cx. tritaeniorhynchus* mosquitoes were divided into 836 pools, and they were homogenized to make samples for the surveillance of JEV. The RNA extracted from *Cx. tritaeniorhynchus* was tested using RT-PCR using JEV-specific primers. As shown in Table 1, there were 11 JEV-positive samples among 152 pools collected from point C & D in 2010 and 2012, and 5 among 138 pools collected from point E/F in 2010 and 2011. Three positive PCR products (size, 566 base pairs [bp]) from point C & D in 2012 were clearly identified using gel electrophoresis, although some bands were observed as nonspecific products (Fig. 4). JEV-genomic RNA was detected among 1.9% of the pools of *Cx. tritaeniorhynchus*.

Nucleotide sequence and deduced amino acid sequence of the E protein region: We attempted to iso-

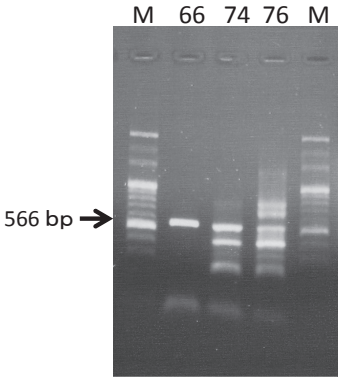

Fig. 4. The products by RT-PCR using RNA prepared from mosquito homogenates. The arrow indicates the JEV-specific PCR product (566 bp) made by PCR using the E3(I) and E4(I) primers. Data show the PCR products from samples in 2012. M and numbers at the top of the panel indicate DNA size markers and the identification numbers of mosquito pools, respectively.

late the virus from PCR-positive samples in 2012 using Vero cells, but we were unsuccessful. Instead, the nucleotide sequence of JEV-specific PCR products was determined (Ishikawa12). The deduced amino acid sequence of E protein of Ishikawa12 is shown and compared with that of JaGAr01 (Fig. 5). There are 6 amino acids that differ between Ishikawa12 and JaGAr01 at positions 123, 129, 222, 327, 366, and 404 of the E protein. It was confirmed that JEV Ishikawa12 belongs to the genotype 1 group.

DISCUSSION

This study reported the distribution and isolation of JEV from *Cx. tritaeniorhynchus* mosquitoes collected in areas close to the swine farms and rice fields in Ishikawa Prefecture, Japan. In addition, natural and man-made water reservoirs covered with water plants are located in these areas. *Cx. tritaeniorhynchus* might play an important role as a vector for JE in Japan, including in Ishikawa Prefecture, even though only 3 cases of JE infection, 1 in 2002 and 2 in 2007 in Ishikawa, have been reported by Japan NIID (6,7).

Here mosquitoes including *Cx. tritaeniorhynchus* were collected using the CDC trap method in areas in Ishikawa (Fig. 1). It is known that the major flying activity of *Cx. tritaeniorhynchus* is observed at night from June to October in most areas in Ishikawa. Some mosquitoes, including *Ae. albopictus* and *Ar. subalbatus*, were collected during the night, even though these mosquitoes were thought to be mainly active during the day (14,15). As shown in the results, the great majority of *Cx. tritaeniorhynchus* were collected in 2012 (9,854) and 2013 (10,401). Temperature and rainfall (Fig. 3) might have affected the number of *Cx. tritaeniorhynchus*, which varied each year. Our results indicated that the number of *Cx. tritaeniorhynchus* collected gradually increased around June, reached a maximum level in August, and then decreased around September. We should note that this phenomenon was different in 2012. The number of mosquitoes collected was greater in September in 2012 as shown in Fig. 2. The rainfall in September in 2012 was relatively lower than other years (Fig. 3). However, the weather conditions in 2012 were not very different from other years. We think that great numbers of mosquitoes appeared due to not only the temperature and rainfall, but also to some kinds of other factors including feeding. To describe the relationship between weather conditions and the number of mosquitoes, we must know the exact amount of rainfall and temperature specific to the local area. The Japan NIID reports on the rate of JE antibodies in swine sera each year. The rate of swine with JE antibodies has gradually increased and

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Ishikawa12  FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEASQLAEVRSYC  60
JaGAr01     FNCLGMGNRDFIEGASGATWVDLVLEGDSCLTIMANDKPTLDVRMINIEASQLAEVRSYC

            YHASVTDISTVARCPTTGEAHNEKRADSSYVCKQGFTDRGWGNGCGLFGKGSIDTCAKFS 120
            YHASVTDISTVARCPTTGEAHNEKRADSSYVCKQGFTDRGWGNGCGLFGKGSIDTCAKFS

            CTNKAIGRMIQPENIKYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVTPNAPSITLKL 180
            CTRKAIGRTIQPENIKYEVGIFVHGTTTSENHGNYSAQVGASQAAKFTVTPNAPSITLKL

            GDYGEVTLDCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLSLPWTSPSSTAWRNRELLM 240
            GDYGEVTLDCEPRSGLNTEAFYVMTVGSKSFLVHREWFHDLALPWTSPSSTAWRNRELLM

            EFEEAHATKQSVVALGSQEGGLHQALAGAIVVEYSSSVKLTSGHLKCRLKMDKLALKGTT 300
            EFEEAHATKQSVVALGSQEGGLHQALAGAIVVEYSSSVKLTSGHLKCRLKMDKLALKGTT

            YGMCTEKFSFAKNPADTGHGTVVIELTYSGSDGPCKIPIVSVASLNDMTPVGRLVTVNPF 360
            YGMCTEKFSFAKNPADTGHGTVVIELSYSGSDGPCKIPIVSVASLNDMTPVGRLVTVNPF

            VATSSSNSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAGSTLFKAFSTTLKGAQRLAAL 420
            VATSSANSKVLVEMEPPFGDSYIVVGRGDKQINHHWHKAGSTLGKAFSTTLKGAQRLAAL

            GDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMSWITQGLMGALLLWMGVNARDRSI 480
            GDTAWDFGSIGGVFNSIGKAVHQVFGGAFRTLFGGMSWITQGLMGALLLWMGVNARDRSI

            ALAFLATGGVLVFLATNVHA 500
            ALAFLATGGVLVFLATNVHA
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Fig. 5. Alignment of amino acid sequences of E protein from Ishikawa12 and JaGAr01. Nucleotides from JEV-DNA were directly sequenced by the method described in the text and deduced amino acid sequences are shown. Mutated amino acids are boxed.

spread from southwestern areas to northeastern areas in Japan during the early summer to fall season. Interestingly, the number of positive cases of swine carrying JEV antibodies in their sera was higher in 2012 in Japan.

As shown in Figs. 4 and 5, we detected JE genomic RNA in the homogenate of *Cx. tritaeniorhynchus* using RT-PCR and identified the nucleotide sequence of the JEV E protein. However, virus isolation was unsuccessful. One of the reasons may be that most of the mosquitoes were dead after being stored for several months before conducting RT-PCR. According to our long-term experience and a previous study, virus recovery is not expected under such conditions (16). In our case, most collected mosquitoes were already dead and left overnight before being placed in storage at −80°C. In addition, we isolated JEV in 1998, 2005, and 2010 from *Cx. tritaeniorhynchus* that we collected via a different method using nets and dry ice in the same areas in Ishikawa (17). Using this method, we quickly put the collected mosquitoes in the deep freezer at −80°C, and then we performed the virus isolation process as soon as possible. During that study period, we attempted to isolate JEV using a similar procedure, with the exception being the preparation of mosquito homogenates as soon as possible after collection. In the comparison of amino acid sequences, Ishikawa12 showed 6 amino acid changes in the E protein region (Fig. 5). Interestingly, the amino acid arginine (R) at position 123 of the E protein was changed to asparagine (N). Previously reported JEV strains have either R or serine (S) at position 123 (18–20). Some research groups have reported that the amino acid at position 123 is related to virulence (21). Therefore, the virulence of Ishikawa strains including Ishikawa12 might change.

In this study, 1.9% of the total mosquito pools contained JEV-positive cases based on RT-PCR: these results were not very high. However, it is important to confirm that JEV-carrying mosquitoes are still flying in areas in Ishikawa. In Japan, JE infections have been reported every year. The NIID report has shown that the incidence of human JE patients in Japan was less than 10 between 2010 and 2014. There are several possible explanations. One is virus mutation inducing attenuation or different interaction between virus and host (5,20–23). It is also possible that JE can be controlled in Japan by a vaccination program and a change in human lifestyle (3).

Japan produces and consumes a lot of pork, as this meat is the foundation of the food industry along with rice and vegetables. However, even though the biosecurity of farms has increased, it does not eliminate swine with arthropod-borne diseases including those infected with JEV. Rice fields near peripheral farms are popular larval breeding places for *Cx. tritaeniorhynchus*, the major competent vector for JEV. It is well known that JEV-infected swine are asymptomatic, although pregnant swine infected with JEV give birth to stillborn offspring. *Flaviviridae* including JEV and Zika virus, could easily infect brain tissues and cause severe effects in the human body (1,2,4,5). JEV can cause severe and acute meningitis and encephalomyelitis, resulting in mortality rates of approximately 30% worldwide. Thus, JEV is a continuing public health threat. We must be cautious regarding the outbreak of JEV infections in future because many mosquitoes still carry the JEV in fields, even in Japan or Europe (24–28). Therefore, it is essential to continue to survey mosquitoes carrying JEV.

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Conflict of interest None to declare.

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