Evidence of frequent introductions of Japanese encephalitis virus from south-east Asia and continental east Asia to Japan

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The Japanese encephalitis virus (JEV) circulating in Japan consists of viruses with multiple phylogenetic origins. Phylogenetic analysis revealed that some JEV strains have recently migrated from south-east and continental east Asian countries. One phylogenetic subcluster of the JEV strains circulating in Japan was closely related to viruses isolated in Vietnam and China’s inland region while other JEV subclusters were related to viruses isolated in Shanghai, China. One virus subcluster, however, was isolated solely in Japan and was not found in any other Asian country. Therefore, our data suggests that the JEVs that have remained or are circulating in Japan include a mixture of viruses that have previously migrated from south-east and continental east Asian countries.

Japanese encephalitis virus (JEV) is a mosquito-borne member of the genus *Flavivirus* of the family *Flaviviridae*. JEV is now classified into five genotypes by the sequence of its genomic RNA (Solomon et al., 2003; Uchil & Satchidanandam, 2001). Genotype 3 was the major genotype circulating in Japan, Korea and Vietnam until the early 1990s. However, by the mid 1990s, genotype 3 disappeared in Japan and Vietnam and genotype 1 supplanted it. This phenomenon is called ‘genotype shift’ (Ma et al., 2003; Nga et al., 2004). We isolated JEVs from Vietnamese swine blood and mosquitoes in 2001 and 2002, and from Japanese mosquitoes in 2002 (Nga et al., 2004). Phylogenetic data revealed that some Vietnamese strains were phylogenetically similar to Japanese strains. The ‘genotype shift’ and the relationship between Japanese and Vietnamese JEVs support the idea that Japanese JEVs were reintroduced from endemic foci in south-east Asia. In addition, JEVs were isolated from stillborn pigs in Japan even during non-endemic winter seasons (Takashima et al., 1988), suggesting that JEV was overwintering in Japan, although it is still unclear which animal was acting as a JEV reservoir.

Therefore, it is important to understand whether the Japanese population of JEV originates from migrated or overwintering viruses. In this study, we aimed to elucidate the ecology of JEV in Asia. To determine whether the Japanese population of JEVs originated from introduced or overwintering viruses, we sequenced 13 of the most recent JEVs from Japan and Vietnam. Resultant sequences, together with other JEV sequences from 257 strains available in GenBank (shown in Supplementary Table S1, available in JGV Online) were subjected to phylogenetic analysis. To trace the movement of JEVs, we plotted the isolation year and the location of the JEVs belonging to the same phylogenetic subclusters on Asian maps. As a result of our analysis, we were able to conclude that Japanese JEVs contain both migrated JEV and overwintered JEV populations.

Virus isolation and sequencing were performed as previously described (Nga et al., 2004). In short, collected mosquitoes were homogenized and centrifuged. The supernatant was then filtered through a 0.22 μm filter, and inoculated onto a monolayer of C6/36 mosquito culture cells and then incubated at 28 °C for 7 days. Two passages were carried out. RNA was extracted from the fluid of the second cell culture with the use of TRIzol LS reagent (Invitrogen). Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) and random hexamers, following RNA denaturation at 95 °C for 5 min. PCR was conducted using TaKaRa LA Taq DNA polymerase with E region-targeting primers (JE821-840F: gaaagccacacggtatctca, JE2851-2817R: gcaaagagaatgctttttccc-catgctttccagc). We amplified the JEV E protein not only
because it is related to immunogenicity, but also because a number of partial sequences were available for this protein gene. The amplified cDNAs were sequenced in a 3100-Avant Genetic Analyzer (Applied Biosystems). We collected all the JEV-E region-containing sequences from GenBank leaving out sequences from redundant data and artificially modified mutants. Nucleotide sequences were then aligned using the MAFFT program version 6.240 (Katoh et al., 2002). The neighbour-joining phylogenetic tree (Saitou & Nei, 1987) was constructed using the PHYLP package, version 3.68 (Felsenstein, 1989) and drawn using FigTree software, version 1.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Fig. 1 shows the bootstrap consensus phylogenetic tree given by the neighbour-joining method after 1000 replicates. From the phylogenetic clustering pattern, we can postulate that Japanese genotype 1 JEVs can be grouped into eight distinct subclusters by bootstrap values greater than 50%. We compared the geographical distribution of the above-mentioned eight subclusters and found some characteristic patterns of JEV migration (Fig. 2).

Subcluster I contained Japanese, Chinese and Vietnamese strains. In 2001, this subcluster was first isolated in northern Vietnam. In 2004, phylogenetically similar JEVs were isolated in the inland region of China (Henan and Sichuan provinces) and Miyak in Japan. In 2007, subcluster I JEVs were isolated in Nagasaki prefecture in Japan. This observed time-course suggests that Vietnamese JEV migrated to inland China and Japan. A similar pattern was also observed for genotype 3, before the occurrence of the genotype shift event in the 1990s (shown in Supplementary Figure S1, available in JGV Online), in which one genotype 3 subcluster was first isolated in Japan in 1999, as well as in Japan in 2000 and 2002. In addition, subcluster III contained Japanese and Chinese strains. Subcluster III and subcluster V JEVs were first isolated in Yunnan province, southern China in 1979 and 1986, respectively. In 2001 and 2003, these JEV subclusters, together with a new JEV subcluster, subcluster II, were isolated in Shanghai, which is located nearer to Japan. Subcluster V migrated to Japan in 2004, and the JEV strains of subcluster II were isolated in Japan in 2003 and 2004. Subcluster III appeared in Korea in 1999, as well as in Japan in 2000 and 2002. In addition, subclusters II, III and V were isolated in Shanghai, on the east coast of China, but they were not isolated from Sichuan and Henan provinces, which are in the inland region of China. In contrast, strains belonging to subcluster I have yet to be isolated in Shanghai. Further investigation is required to determine whether or not there are two independent migration routes.

Long distance transport of arboviruses occurs through either vertebrate host or vector movement. In the case of West Nile virus, onset cases were reportedly observed along a wild bird migration route (Rappole et al., 2000; Rappole & Hubalek, 2003). JEV can also infect wild birds and therefore it is possible that it moves with migrating birds (Nga et al., 2004). Mosquitoes could also be involved in long distance transport of the virus. When JEV spread into northern Australia in 1995, mosquitoes were suspected to be JEV carriers (Ritchie & Rochester, 2001; Chapman et al., 2003) because population structure analysis of mosquitoes revealed that windblown Culex annulirostris and Culex palpalis were moving between Papua New Guinea and Cape York, northern Australia, over the Torres Strait (Chapman et al., 2003; Hanna et al., 1996; Hemmerer et al., 2007).

In Asia, some insects are known to migrate over the sea. Windblown leafhoppers, major pests of rice plants, are carried by low atmospheric pressure systems moving from eastern China to Japan with westerly winds (Kisimoto, 1983; Iwanaga et al., 1985; Crummay & Atkinson, 1997; Syobu & Mikuriya, 2000). A major vector of JEV, Culex tritaeniorhynchus, is known to be carried for long distances by winds (Ming et al., 1993), and it was collected by observatory ships on the East China Sea and on the north-west Pacific, 500 km south of Honshu island, Japan (Asahina, 1970). These data suggest that Culex tritaeniorhynchus is brought from China to Japan by westerly winds like the leafhopper. In addition, the leafhopper is brought to Japan from south-east Asia in early summer by means of a seasonal low-level jet stream during the rainy season (Watanabe & Seino, 1991). The small brown leafhopper, Laodelphax striatellus, is known to be a migratory pest, but it cannot overwinter in Japan. Therefore, new leafhopper populations migrate to Japan every year from south-east Asia (Watanabe & Seino, 1991; Kisimoto, 1983). If the atmospheric circulation in Asia can carry mosquitoes as well as leafhoppers and JEV migration is always from west to east, mosquitoes carried by the jet stream and westerly winds could be a vehicle for JEV from continental east and south-east Asia to Japan. In this context, we speculate that the JEV distribution revealed by our analysis can be explained by the migration of windblown mosquitoes, as was the case in Australia.

Subcluster VIII, which contained only one JEV strain, Ishikawa, was the first genotype 1 strain isolated in Japan in 1994. Subcluster IV contained one strain isolated in Oita.

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**Fig. 1.** Bootstrap consensus phylogenetic tree of JEVs. This phylogenetic tree was constructed after 1000 replicates of bootstrap analysis using the neighbour-joining method. Bootstrap values (%) greater than 50% are shown above branches. Numbers in parentheses indicate the phylogenetic distance of the truncated branches (substitutions/site). Genotype 1 JEVs (103 strains) are shown in this figure. Other genotypes, genotype 2 (6 strains), genotype 3 (158 strains) and genotype 4 (3 strains) are used as outgroups. Labels of strains conform to the following format: (GenBank accession no.)(strain name)_(country-region)_(year of isolation). NA, Not available.
Fig. 2. Locations and isolation years for JEVs.
prefecture and subcluster VII contained two strains isolated on Okinawa island. Unfortunately, there are limited data available about these subclusters, and therefore additional information is required to explain their phylogenetic positions.

Subcluster VI was isolated only in Japan. From 1994 to 2002, subcluster VI appeared annually in Japan, which implies that this JEV subcluster was overwintering there. The overwintering mechanism of JEV in Japan is still unclear. *Culex tritaeniorhynchus* can overwinter as adults in Japan. In Korea, JEV was isolated from overwintering *Culex pipiens* (Rosen, 1986). Laboratory experiments demonstrated that the adults of *Culex* mosquitoes can harbour JEV during winter (Hurlbut, 1950; Mifune, 1965) and that JEV can be transmitted vertically to the progeny of infected mosquitoes via eggs (Rosen et al., 1978, 1980, 1989). However, JEV was not isolated from overwintering mosquitoes in Japan, although exhaustive mosquito surveys were conducted (Buei et al., 1986; Ito et al., 1986; Nakamura et al., 1968; Rosen, 1986). Vertebrate hosts of JEVs were also investigated in relation to the overwintering of JEVs. Wild birds can be a potential reservoir of JEV; however, JEV was not isolated from birds in winter (Miura, 1973). One report showed that JEV was isolated from wild bats, *Miniopterus schreibersi* (Kimura, 1970). JEV has not been subsequently isolated from those bats (Doi et al., 1983). Reptiles were investigated as the potential wild reservoir of JEV. It was experimentally confirmed that JEV was maintained during winter in hibernating lizards, *Takydromus tachydromoides*. Although HI antibody was detected from field caught lizards in Japan, JEV was not successfully isolated from hibernating wild lizards (Doi et al., 1983). Hence, the overwintering mechanism of JEV in Japan remains to be elucidated by future studies.

In conclusion, phylogenetic analysis has revealed that Japanese genotype 1 JEV regularly comes to Japan from south-east and continental east Asia and, after its arrival, can overwinter for some years in Japan.

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