	1					50
PhaV/AB441720	KEVVQQSSHAIQETLKYPRPFKLIMCHLQSSDDSYFSVSAPLTGDASIAT					
PhaV-Bang_Khun_Thian	----------					
PhaV-Bang_Bon	----------					
PhaV-Chom_Thong	----------V--					
	51					100
PhaV/AB441720	KSRILATAILQFKVNYSSHCGVVNSIKTVLNSNHVFEFNSNFEFGFNHYK					
PhaV-Bang_Khun_Thian	----------					
PhaV-Bang_Bon	----------					
PhaV-Chom_Thong	----------					
	101					150
PhaV/AB441720	PDIKAIFSGFLVSEQELLLTRQEELSVLLTSYLENGGTNYVANGLQLGQS					
PhaV-Bang_Khun_Thian	----------					
PhaV-Bang_Bon	----------					
PhaV-Chom_Thong	----------					
	151				185	
PhaV/AB441720	YLHYHLMGLTTTKYFRTYEVLQTILPDPSIGFFVM					
PhaV-Bang_Khun_Thian	----------S----------					
PhaV-Bang_Bon	----------					
PhaV-Chom_Thong	----------					

Figure 1. Alignment of four amino acid sequences of PhaV. AB441720 is amino acid sequences of original PhaV (4), and other three were determined in this study.

and Chom Thong Districts in Bangkok Province, Thailand, during May and June of 2007. Adult individual mosquito was homogenized in 100 µL of modified Eagle's medium supplemented with 2% FBS. Each homogenate was centrifuged at 550 x g for 10 min at 4˚C, and the supernatant was filtered through 0.22 µm Millex-GX filters (Millipore, Billerica, MA, USA). RNA was extracted from 25 µL of homogenates using ISOGEN-LS (Nippon *Gene*, Toyama, Japan) or TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To investigate infection of viruses, RT-PCR was performed using primers for flavivirus (consensus) (Kuno et al., 1998), DENVs (all serotypes) (Lanciotti et al., 1992), and alphavirus (Eshoo et al., 2007). Only DENV was detected in two mosquitoes. The other viruses were not detected. They examined the infectivity of PhaV in 75 adult female mosquitoes (15 groups; five mosquitoes including per group) except for two DENV positive-mosquitoes. Published primers were used for amplification of the PhaV 735 bp partial large segment (Yamao et al., 2009). PhaV was detected in 15 groups by RT-PCR.

The three PCR products of PhaV from the Bang Khun Thian, Bang Bon, and Chom Thong districts were purified from the agarose gel, and nucleic acid sequences were determined. The nucleic acid sequence data are available in the DDBJ/EMBL/Genbank databases under accession numbers AB541986 to AB541988. Three nucleic and amino acid sequences were compared with the original PhaV sequences (DDBJ/EMBL/GenBank accession number AB441720) as determined by the previous study. The three viruses shared nucleotide identities of 93.9 to 96.3%. Amino acid sequence of Bang Bon virus was identical to the original PhaV sequence (Figure 1). In contrast, the Bang Khun Thian and Chom Thong viruses were one amino acid different from the original PhaV.

In this study, we found that over 20% of female mosquitoes were infected by PhaV and these viruses were genetically similar to strains detected in mosquitoes in nearby Bangkok. DENV RNA was detected in only 2.6% of mosquitoes. The results suggested a high prevalence of PhaV in female mosquitoes. Biological transmission of arboviruses can be vertical, which involves the passage of the virus from an infected female vector to its offspring. In addition, horizontal transmission can be venereal, with a vertically infected male directly infecting a female, or can occur orally, from a vector to a vertebrate host via deposition of saliva during blood feeding. Mosquitoes of the *Aedes* and *Culex* genera are important for both human and animals because they transmit viruses causing serious illness. For example, dengue and chikungunya virus, which were transmitted by *Aedes* genus, were occurred epidemic many times at Southeastern Asia region including Thailand. In Asia, chikungunya virus mainly induces high fever and severe arthralgia, and had limited impacts on public health before its emergence in the Indian Ocean in 2005

(Vazeille et al., 2007). These chikungunya outbreaks spread rapidly and caused several million clinical cases in the Indian Ocean Islands and India (Pialoux et al., 2007). PhaV transmission from female mosquitoes to humans and animals will be examined in a future study.

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RESEARCH NOTE

FIELD-COLLECTED PERMETHRIN-RESISTANT *AEDES AEGYPTI* FROM CENTRAL THAILAND CONTAIN POINT MUTATIONS IN THE DOMAIN IIS6 OF THE SODIUM CHANNEL GENE (*KDR*)

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Abstract. One of the mechanisms responsible for pyrethroid resistance in mosquitoes is mutations in domain IIS6 of voltage-gated sodium channel gene (*kdr*). *Aedes aegypti* larvae were collected from the central provinces of Thailand (Bangkok, Prachin Buri and Ratchaburi) and colonized until they became adults. Partial fragment of *kdr* of permethrin-resistant mosquitoes were amplified by RT-PCR and sequenced. Among the four nucleotide mutations detected, two mutations resulted in two amino acid substitutions, S(TCC) 989 P(CCC) and V(GTA)1016 G(GGA). Among 94 permethrin-resistant mosquitoes, the SS genotype (SS/VV) was found to predominate (n = 74), followed by SR (SP/VG) (n = 15) and RR (PP/GG) genotypes (n = 5), with the resistant allele frequency ranging from 0.03 to 0.17. As pyrethroid insecticides are currently being advocated for use in Thailand, investigations of pyrethroid resistance in other regions of the country are needed to prevent potential cross-resistance among different types of insecticides.

Keywords: *Aedes aegypti*, permethrin resistant, voltage-gated sodium channel, *kdr* resistance

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INTRODUCTION

Insecticidal pyrethroids are widely used to control vectors involved in transmission of viral diseases which are of public health concern, such as dengue and chikungunya fever, due to their beneficial

characteristics *viz* low toxicity to humans and mammals and limited persistence in soil. Hence, they are used in indoor residual sprays, for impregnating bed nets, curtains, and screens, as well as in coils, mats and aerosols (Chareonviriyaphap *et al*, 2003).

When a case of dengue fever is reported, the Ministry of Public Health of Thailand initiates spraying of the area surrounding the patient's house with pyrethroid or organophosphate insecticides within 24 hours in order to kill any adult mosquitoes in the vicinity (Limpawitthayakul and Sornpeng, 2006). However, the long-term and frequent usage of these insecticides has led mosquitoes in Thailand to become resistant to these compounds. In Thailand, permethrin and DDT resistance are now widely distributed in *Aedes aegypti* (Prapanthadara *et al*, 2002; Thanispong *et al*, 2008; Paeporn *et al*, 2010), and *Ae. aegypti* is also resistant to deltamethrin in Central Thailand including Bangkok (Yaicharoen *et al*, 2005; Komalamisra *et al*, 2011; Srisawat *et al*, 2011).

The mechanism of pyrethroid and DDT resistance in insects generally involves changes to the sensitivity of the target voltage-gated sodium channels to these insecticides (Brengues *et al*, 2003). The voltage-gated sodium channel contains four homologous repeated domains (I, II, III, and IV), each domain consisting of six putative transmembrane regions arranged to form an ion pore. Mutations to this gene (*kdr*), known as "knockdown resistance", have been linked to resistance of this target in a range of insects (Brengues *et al*, 2003). Single nucleotide substitutions in domain II of the voltage-gated sodium channel gene induce resistance to pyrethroids in *Ae. aegypti*, including Val → Gly or Val →Ile at position 1016, Ile → Met or Ile → Val at 1011, Leu → Trp at 982, Gly → Val at 923 and Ser → Pro at 989 (Brengues *et al*, 2003; Saavedra-Rodriguez *et al*, 2007; Srisawat *et al*, 2010). The S989P mutation was first reported in a laboratory colony selected for deltamethrin resistance.

In this study, we identified a mutation in domain IIS4-6 of *kdr* that is associated with permethrin resistance in field collected *Ae. aegypti*.

MATERIALS AND METHODS

Field collected permethrin-resistant *Ae. aegypti*

Ae. aegypti larvae were collected from areas of Central Thailand in which permethrin resistance had been reported (Bang Khae District, Bangkok; Mueang District, Prachin Buri Province; and Pong Sawai District, Ratchaburi Province) (Paeporn *et al*, 2004; Srisawat *et al*, 2011). The global positioning system (GPS) coordinates of the study sites were recorded. Larvae were colonized in the laboratory of the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand until adults emerged. *Ae. aegypti* that survived 1 hour exposure to 0.75% permethrin (WHO diagnostic dose) were considered to be permethrin-resistant. The susceptible strain originating 10 years ago from Buri Ram Province was colonized at the Department of Medical Entomology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Insecticide susceptibility test

Twenty-five 2-5-day-old, non-blood fed female mosquitoes were tested for insecticide susceptibility according to standard WHO procedure (WHO, 1998). In brief, mosquitoes were kept in a holding tube for 1 hour and then transferred

to an exposure tube lined with 0.75% permethrin paper for 1 hour before being transferred back to the holding tube and provided with 10% sugar solution. Mortality was examined after 24 hours. Susceptibility tests were carried out using 100 female mosquitoes from each strain. The control mosquitoes were exposed to paper containing silicone oil. When the mortality of control mosquitoes was > 20%, the experiment was terminated. When the mortality of the control group ranged from 5% to 20%, the mortality rate was adjusted using Abbott's formula:

$$\frac{(\%\ \text{test mortality} - \%\ \text{control mortality}) \times 100}{(100 - \%\ \text{control mortality})}$$

Amplification and sequencing of *kdr* from permethrin-resistant *Ae. aegypti*

Total RNA was extracted from individual mosquitoes of permethrin-resistant strain using TRIzol® reagent (Invitrogen, Carlsbad, CA). Subsequently, RT-PCR was performed using primers NaF (forward primer 5' CGT GGC GCT GTC GTT GCT C 3') and NaR (reverse primer 5' CTT GTT CGT TTC GTT GTC GGC 3'), which cover *kdr* regions S4 and S6 of domain II (Srisawat *et al*, 2010). The PCR mixture 12.5 µl total volume contained 10 picomole of each primer, 1X SuperScript™ One Step RT-PCR PLATINUM Taq reaction mixture (Invitrogen, Carlsbad, CA), and 200 ng of RNA template from each mosquito. Thermal cycling consisted of 50°C for 30 minutes, 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 65°C for 30 seconds and 68°C for 1 minute, and final heating step at 68°C for 2 minutes. PCR amplicons were analyzed by electrophoresis at 100 V in 1.5% agarose gel for 30 minutes, stained with ethidium bromide, and visualized under UV light. Amplicons then were purified and sequenced by Macrogen (Seoul, Korea), using ABI BigDye Terminator Cycle Sequencing kit and a 3730xl Automated DNA sequencer (PE Applied Biosystems, Foster City, CA).

Effect of the two mutations (989, 1016) of *kdr* in relation to permethrin exposure time

Permethrin-resistant Pongsawai strain of *Ae. aegypti*, collected in Ratchaburi Province, Thailand in 2003 (Paeporn *et al*, 2004), was subjected to an examination of the relationship between the frequency of the two resistance alleles and permethrin exposure time according to standard WHO protocol (WHO, 1998). Mosquitoes were exposed to 0.75 % permethrin for 60, 270, or 360 minutes, and only the surviving *Ae. aegypti* were examined for their resistant allele frequency.

RESULTS

All *Ae. aegypti* were highly resistant to 0.75% permethrin [mortality rate of <80%, the criterion used by World Health Organization (1998), to designate resistant mosquitoes]. Mortality rate of *Ae. aegypti* in Bangkok ranged from 0% (BK-2) to highest of 63% (BK-5) (Table1). *Ae aegypti* from Ratchaburi and Prachin Buri Provinces also showed high levels of permethrin resistance.

Four nucleotide substitutions were detected in domain IIS4-S6 of the sodium channel gene in the surviving permethrin-resistant *Ae. aegypti* mosquitoes (data not shown). One nucleotide substitution at 982 position and one at 1011 were synonymous. However the other two resulted in S(TCC) 989 P(CCC) and V(GTA) 1016 G(GGA), both occurring together in the same permethrin-resistant mosquito. The mutant *kdr* allele is designated R and wild type allele S.

An examination of *kdr* genotypes in

Table 1
Percentage mortality of *Ae. aegypti* exposed to 0.75% permethrin for 1 hour.

Collection site	Code	GPS coordinates	Mortality rate (%)
Bang Khae District, Bangkok	BK-1	13° 70′ 91.5″ N, 100° 42′ 59.5″ E	9
	BK-2	13° 74′ 88.9″ N, 100° 35′ 42.7″ E	0
	BK-3	13° 41′ 47.8″ N, 100° 25′ 44.6″ E	52
	BK-4	13° 41′ 98.6″ N, 100° 23′ 32.3″ E	35
	BK-5	13° 42′ 13.4″ N, 100° 24′ 51.2″ E	63
Pong Sawai District, Ratchaburi Province	RB-1	ND	35
Mueang District, Prachin Buri Province	PR-1	14° 06′ 49.1″ N, 101° 19′ 52.1″ E	51
Laboratory susceptible strain (Buri Ram Province)	SBU	ND	100

ND, no data

Table 2
Genotypic frequency of mutations in domain IIS6 of *kdr* in field collected permethrin-resistant and laboratory susceptible strain of *Ae. aegypti*.

			Genotype frequency at 989/1016			Frequency of R allele
			SS	SR	RR	
Insecticide status	Locality	No.	SS/VV	SP/VG	PP/GG	
Permethrin-resistant strain	Bangkok	73	0.75 (55)[a]	0.09 (13)	0.07 (5)	0.17
	Prachin Buri	15	0.93 (14)	0.03 (1)	0 (0)	0.03
	Ratchaburi	6	0.83 (5)	0.08 (1)	0 (0)	0.08
Permethrin-susceptible strain	Buri Ram	6	1 (6)	0 (0)	0 (0)	0

[a] Number of mosquitoes

94 samples, SS genotype was found to predominate (74 mosquitoes), followed by SR (15) and RR (5) (Table 2). The resistant allele frequency was highest in *Ae. aegypti* from Bangkok (0.17), followed by those from Ratchaburi (0.08) and Prachin Buri (0.03), while the susceptible strain possessed homozygous susceptibility allele. The permethrin-resistant allele increased in frequency in *Ae. aegypti* from Ratchaburi Province surviving longer exposure time and SS genotype was no longer found in these mosquitoes (Table 3).

DISCUSSION

At present, most *Ae. aegypti* in Thailand are resistant to permethrin including those in Bangkok, Ratchaburi and Prachin Buri (Jirakanjanakit *et al*, 2007;

Table 3
Genotypic frequency of two mutations sites in *Ae. aegypti* collected from Ratchaburi Province, Thailand after exposure to 0.75% permethrin for various time.

Exposure time (min)	N	Frequency of mutation site (989/1016)			Frequency of R allele
		SS	SR	RR	
		SS/VV	SP/VG	PP/GG	
60	6	0.83 (5)[a]	0.08 (1)	0 (0)	0.08
270	5	0 (0)	0.3 (3)	0.4 (2)	0.7
360	6	0 (0)	0.33 (4)	0.33 (2)	0.7

[a] Number of mosquitoes

Thanispong *et al*, 2008; Paeporn *et al*, 2010; Komalamisra *et al*, 2011; Srisawat *et al*, 2011; Yanola *et al*, 2011). The permethrin- resistant allele (R) frequency in *Ae. aegypti* in Bangkok (an urban area) was higher than those in Prachin Buri and Ratchaburi (rural areas). Houses in urban areas are small and enclosed, and once an insecticide (*viz*, permethrin) has been sprayed inside a house, mosquitoes are likely to become exposed to it because of the limited space within the house. On the other hand, in rural areas, houses are large, open and distant from each other, thereby mosquitoes have less chance to come into contact with insecticides. In addition, mosquito coils, which repel mosquitoes, are the preferred anti-mosquito measure in rural areas.

Permethrin-resistant *Ae. aegypti* possesses two point mutations within domain IIS6 of *kdr*, with a number of mutations being reported *viz* G923V, L982W, S989P, I1011M/V, and V1016G (Brengues *et al*, 2003; Rajatileka *et al*, 2008; Srisawat *et al*, 2010). Rajatileka *et al* (2008) reported I1011V mutation in a mosquito sample collected from the southern part of Thailand, but a synonymous change was found in our samples, which were collected from the central part of the country. The two non-synonymous nucleotide substitutions found in this study, resulting in S989P and V1016G mutations, were the same as those found in deltamethrin-resistant *Ae. aegypti* (Srisawat *et al*, 2010). In contrast, Yaicharoen *et al* (2005) could not find either S989P or V1016G in field *Ae. aegypti* from Bangkok.

The frequency of resistant allele in permethrin-resistant *Ae. aegypti* in this study is low compared with those reported from Vietnam, Brazil, and Mexico (Saavedra-Rodriguez *et al*, 2007; Kawada *et al*, 2009; Martins *et al*, 2009). It might be due to the fact that only 20% of permethrin-resistant mosquitoes were tested. The mosquitoes in those countries have demonstrated an increasing *kdr* resistant genotype frequency. However, there are signs of increasing *kdr* resistant allele frequency in Thai populations, especially in Bangkok population when compared to the result of Yaicharoen *et al* (2005).

A possible reason for the discrepancy between low *kdr* resistant allele frequency and high level of permethrin resistance detected in this study is the presence of other resistance mechanisms, increases in the expression of detoxification enzymes

might also be involved in insecticide resistance mechanism (Hemingway and Ranson, 2000; Brooke, 2008). Mixed function oxidase and esterase activities are elevated in pyrethroid resistant *Ae. aegypti* (Paeporn *et al*, 2004; Yaicharoen *et al*, 2005). In addition, mutations in domain I and domain III associated with permethrin resistance should be considered (Yanola *et al*, 2010). Recently, Yanola *et al* (2011) reported that *Ae. aegypti* with F/F1534 domain III susceptibility genotype was able to survive after being exposed to permethrin because of the presence of homozygous mutations in domain II, S989P and V1016G. Thus enzyme detoxification mechanism and/or mutations in other domains may contribute permethrin resistance, which needs further studies.

This study clearly demonstrated that loss of SS genotype and appearance of R allele are associated with high resistance to permethrin in field collected *Ae. aegypti*, as was reported in a deltamethrin-resistant *Ae. aegypti* laboratory strain (Srisawat *et al*, 2010). However, the distribution of resistant alleles remains unknown in many parts of Thailand. It is therefore necessary to investigate insecticide resistance status and to prevent potential cross-resistance among different type of insecticides.

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Short Communication

Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon

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A method for rapid production of single-round infectious particles (SRIPs) of flavivirus would be useful for viral mutagenesis studies. Here, we established a DNA-based production system for SRIPs of flavivirus. We constructed a Japanese encephalitis virus (JEV) subgenomic replicon plasmid, which lacked the C-prM-E (capsid–pre-membrane–envelope) coding region, under the control of the cytomegalovirus promoter. When the JEV replicon plasmid was transiently co-transfected with a JEV C-prM-E expression plasmid into 293T cells, SRIPs were produced, indicating successful *trans*-complementation with JEV structural proteins. Equivalent production levels were observed when C and prM–E proteins were provided separately. Furthermore, dengue types 1–4, West Nile, yellow fever or tick-borne encephalitis virus prM-E proteins could be utilized for production of chimaeric flavivirus SRIPs, although the production was less efficient for dengue and yellow fever viruses. These results indicated that our plasmid-based system is suitable for investigating the life cycles of flaviviruses, diagnostic applications and development of safer vaccine candidates.

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Japanese encephalitis virus (JEV) is the leading cause of viral encephalitis with severe mortality in eastern and south-eastern Asia, and is estimated to be responsible for 67 900 cases annually, mostly in children (Campbell *et al.*, 2011). The virus is transmitted by *Culex* mosquito vectors between pigs and/or wild birds, and humans and horses are thought to be dead-end hosts. JEV is a member of the genus *Flavivirus* within the family *Flaviviridae*, which includes dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV) and tick-borne encephalitis virus (TBEV). JEV is an enveloped single-stranded positive-sense RNA virus with an 11 kb genome that is translated as a single large polyprotein. The polyprotein is co-translationally cleaved by host and viral proteases into three structural proteins – capsid (C), pre-membrane (prM) and envelope (E) – and seven non-structural (NS) proteins (Sumiyoshi *et al.*, 1987).

For several flaviviruses, subgenomic replicons, which lack structural protein genes but can replicate in cells, have been constructed (Khromykh & Westaway, 1997; Pang *et al.*, 2001; Shi *et al.*, 2002). In addition, the expression of viral structural proteins in cells harbouring replicon RNA has been shown to produce single-round infectious particles (SRIPs), which are infectious, but progeny viruses cannot be spread from the infected cells, as the packaged genome lacks structural protein genes (Gehrke *et al.*, 2003; Jones *et al.*, 2005; Khromykh *et al.*, 1998; Ng *et al.*, 2007; Scholle *et al.*, 2004; Yun *et al.*, 2009). Furthermore, *trans*-packaging of replicons by the prM-E proteins from heterologous flaviviruses have been reported (Ansarah-Sobrinho *et al.*, 2008; Yoshii *et al.*, 2008).

A method for rapidly producing SRIPs of flaviviruses would be useful for viral mutagenesis studies, diagnostic applications and the production of vaccines with reduced

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One supplementary figure is available with the online version of this paper.

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risk of infection. In this study, to establish a DNA-based production system for SRIPs, we constructed a JEV subgenomic replicon plasmid, which lacked the C-prM-E coding region, under the control of the cytomegalovirus (CMV) promoter. As DNA-based replicons can be transfected directly into eukaryotic cells without *in vitro* transcription, SRIPs can be rapidly produced by co-transfection with structural protein-expression plasmids.

In order to generate a subgenomic replicon from the JEV Nakayama strain (McAda *et al.*, 1987), viral RNA was extracted from infected Vero cells, reverse transcribed into cDNA and amplified in individual dsDNA fragments containing T7 RNA polymerase promoter and hepatitis delta virus ribozyme (HDV-RZ) as shown in Fig. 1(a). For deletion of the C-prM-E region, a synthetic antisense oligonucleotide was used to add a *Bsp*TI site at codons 17–18 of the C-coding region following the cyclization sequence, and a sense oligonucleotide was designed that added the *Bsp*TI site before the C-terminal transmembrane domain, which consists of 30 aa of the E protein coding sequence, in order to permit ligation of C to NS1. The five individual fragments required to produce a replicon-length cDNA were readily assembled into the low-copy-number plasmid pACYC177, designated pJEVrep#97. Replication of *in vitro*-transcribed RNAs derived from the plasmid was confirmed in RNA-transfected cells (data not shown). Next, to construct a DNA-based replicon plasmid, the T7 RNA polymerase promoter was replaced with the CMV promoter, and the simian virus 40 polyadenylation signal was inserted downstream of the HDV-RZ; the resulting plasmid was designated pCMV-JErep (Fig. 1a). pCMV-JErep-fs, which contains a frameshift mutation through a 4 nt insertion upstream of the GDD motif of RNA-dependent RNA polymerase in NS5, was also constructed as a negative control with no replication activity. To characterize the replication activity of the plasmid-derived replicon, 293T cells were transfected with plasmids as described previously (Suzuki *et al.*, 2013). Indirect immunofluorescence with an anti-dsRNA antibody showed positive staining in the cytoplasm of cells transfected with pCMV-JErep plasmid or infected with JEV Nakayama strain, whereas no signal was detected in the cells transfected with pCMV-JErep-fs, indicating the ability of viral RNAs transcribed intracellularly from the plasmid pCMV-JErep to replicate in cells (Fig. 1b). It should be noted that NS1 protein was detected in the cytoplasm of cells transfected with both pCMV-JErep and pCMV-JErep-fs.

We also constructed expression plasmids for JEV C-E, mature C consisting of 105 aa, and prM-E, which we designated pCAG-JECE, pCAG-JEC and pCAG-JEprME, respectively (Fig. 2a). To reduce sequence homology and intergenomic recombination potential with the truncated C and E genes in the subgenomic replicon, 21 nt mutations were incorporated into the 5′ region of the C gene and 3′ region of the E gene. These changes also include two nucleotides in the conserved 5′ cyclization sequence (CS) (Hahn *et al.*, 1987; Khromykh *et al.*, 2001), producing a sequence that was non-complementary to the 3′ CS of the replicon genome, thereby preventing replication of a recombinant genome. To produce SRIPs of JEV, 293T cells were transfected with a mixture of two (pCMV-JErep and pCAG-JECE) or three (pCMV-JErep, pCAG-JEC and pCAG-JEprME) plasmids. The infectivity of SRIPs was determined by inoculating the culture supernatant of transfected cells into Vero cells, followed by immunostaining with anti-NS1 antibody. 293T cells produced a titre of 6.9×10^5 IU ml^{-1} (Fig. 2b) or 7.9×10^5 IU ml^{-1} (Fig. 2c) 3 days after transfection with two or three plasmids, respectively. In contrast, no infectious particles were detected in the supernatant when one of the two or three plasmids was omitted or the replicon containing a frameshift mutation was introduced. The production levels of SRIPs from cells transfected with two or three plasmids were similar, as shown in Fig. 2(d).

In order to confirm that the SRIPs have only single-round infectivity potential, Vero cells were inoculated with medium harvested from 293T cells transfected with replicon and structural protein plasmids, and were examined for antigen-positive cells. SRIPs were demonstrated to be infectious in the first round (Fig. 3a). However, no antigen-positive cells were observed in a second round, in which the supernatants of the cells infected with SRIPs were transferred to naive Vero cells (Fig. 3a). As a control, supernatant from JEV-infected cells produced antigen-positive cells in second-round infection.

We then evaluated whether the SRIPs could be used in neutralization tests instead of infectious live virus by using anti-JEV sera raised in rabbits as a representative antibody. Serial fourfold dilutions of serum were mixed with aliquots of SRIPs or virus of equivalent infectivity. The virus–antibody mixture was incubated for 1 h at room temperature, followed by titration for infectivity on Vero cell monolayers in a 96-well plate. The neutralizing activity of each antibody dilution was expressed as a percentage of the infectivity obtained with the control, which was tested in the absence of any serum. Infection with SRIPs and JEV Nakayama strain were similarly neutralized by anti-JEV antibody in a dose-dependent manner, although normal serum did not affect infection with SRIPs and JEV (Fig. 3b).

Next, to examine whether SRIPs derived from other flaviviruses could be generated using our plasmid-based method, we used prM-E expression plasmids for the following viruses: DENV1, Mochizuki strain; DENV2, New Guinea C strain; DENV3, H87 strain; DENV4, H241 strain (Konishi *et al.*, 2006); WNV, NY99-6922 strain (Ishikawa *et al.*, 2007); YFV, 17D strain; and TBEV, Oshima 5-10 strain (Yoshii *et al.*, 2003). Detection of each E protein in cells transfected with prM-E expression plasmids by immunofluorescence revealed indistinguishable efficiency of transfection as shown in Fig. S1 (available in JGV Online). Efficient production of chimaeric flavivirus SRIPs by co-transfection with JEV C and JEV replicons was achieved for

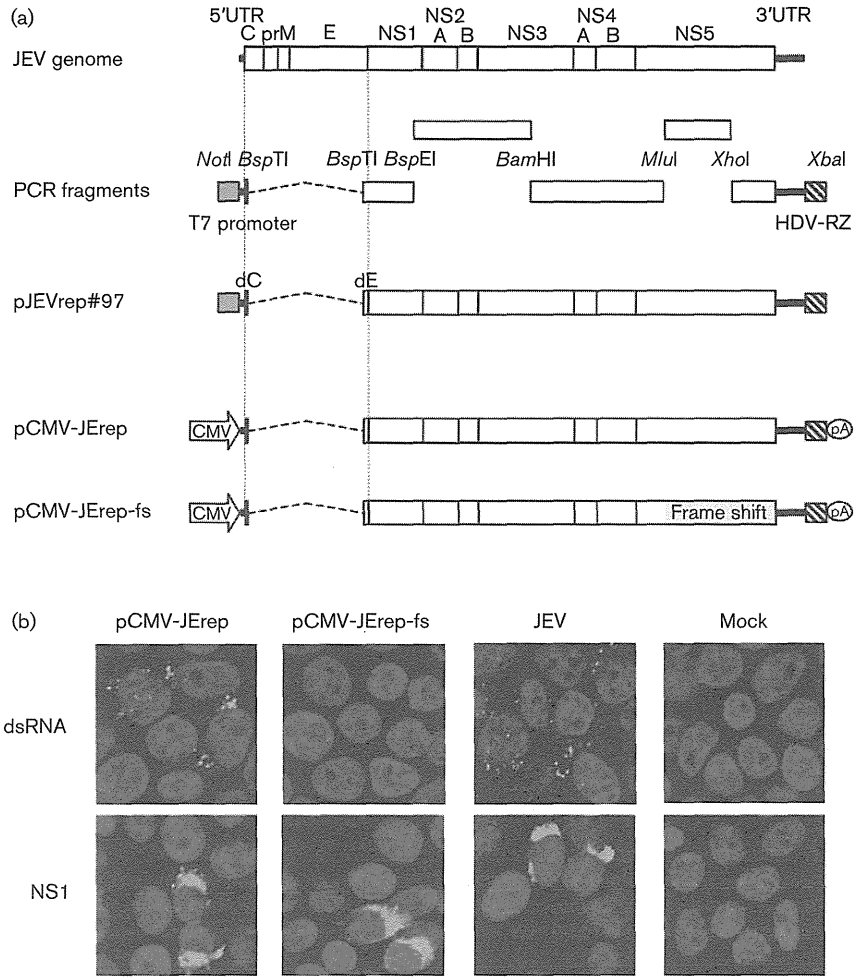

Fig. 1. (a) Schematic representation of the position of the JEV genome showing restriction enzymes sites (*Not*I, *Bsp*TI, *Bsp*EI, *Bam*HI, *Mlu*I, *Xho*I and *Xba*I), fragments used to assemble for replicon construction, position of the T7 promoter, CMV promoter (CMV), HDV-RZ and polyadenylation signal (pA). (b) 293T cells were transfected with the indicated plasmids or were infected or mock-infected with JEV. Two days post-transfection or post-infection, cells were fixed and permeabilized as described previously (Suzuki *et al.*, 2013). Samples were then incubated with anti-dsRNA antibody (J2; English & Scientific Consulting) or anti-NS1 antibody (2D5; Konishi *et al.*, 2004). Green signals were obtained with Alexa-Fluor-488-labelled goat anti-mouse IgG secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI.

WNV and TBEV, although production of SRIPs was less efficient for DENV1-4 and YFV (Fig. 3c).

It is curious that TBEV prM-E protein can be utilized efficiently for assembly of SRIPs in combination with the JEV C protein and replicon RNA producing equivalent titres to JEV and WNV, as TBEV is a tick-borne virus and is classified as a distinct serogroup from JEV. In contrast, production of DENV- and YFV-SRIPs was less efficient. The low infectious titre of SRIPs containing at least dengue prM-E may be explained by the low specific infectivity of particles encapsidated in DENV envelope protein (van der Schaar *et al.*, 2007; Winkelmann *et al.*, 2011), although we were unable to exclude the possibility that the viral assembly and/or secretion with dengue prM-E is not efficient (Chang *et al.*, 2003; Hsieh *et al.*, 2008). Adaptive mutations in structural and NS proteins could possibly enhance the production of infectious particles by improving the specific infectivity of the resulting particles (Winkelmann *et al.*, 2011). In addition, it has been reported that a chimaeric WNV genome with DENV2 prM-E genes but lacking the C gene replicates much better in DENV2-C-expressing cells than in WNV-C-expressing cells (Suzuki *et al.*, 2009), thus suggesting that the combination of homologous C protein and prM-E proteins improves the production of viral particles. Therefore, it is possible to obtain a better yield of dengue SRIPs by using DENV C protein instead of JEV C protein. Such DENV-SRIPs can be useful for studying infection-enhancing and neutralizing antibody activities.

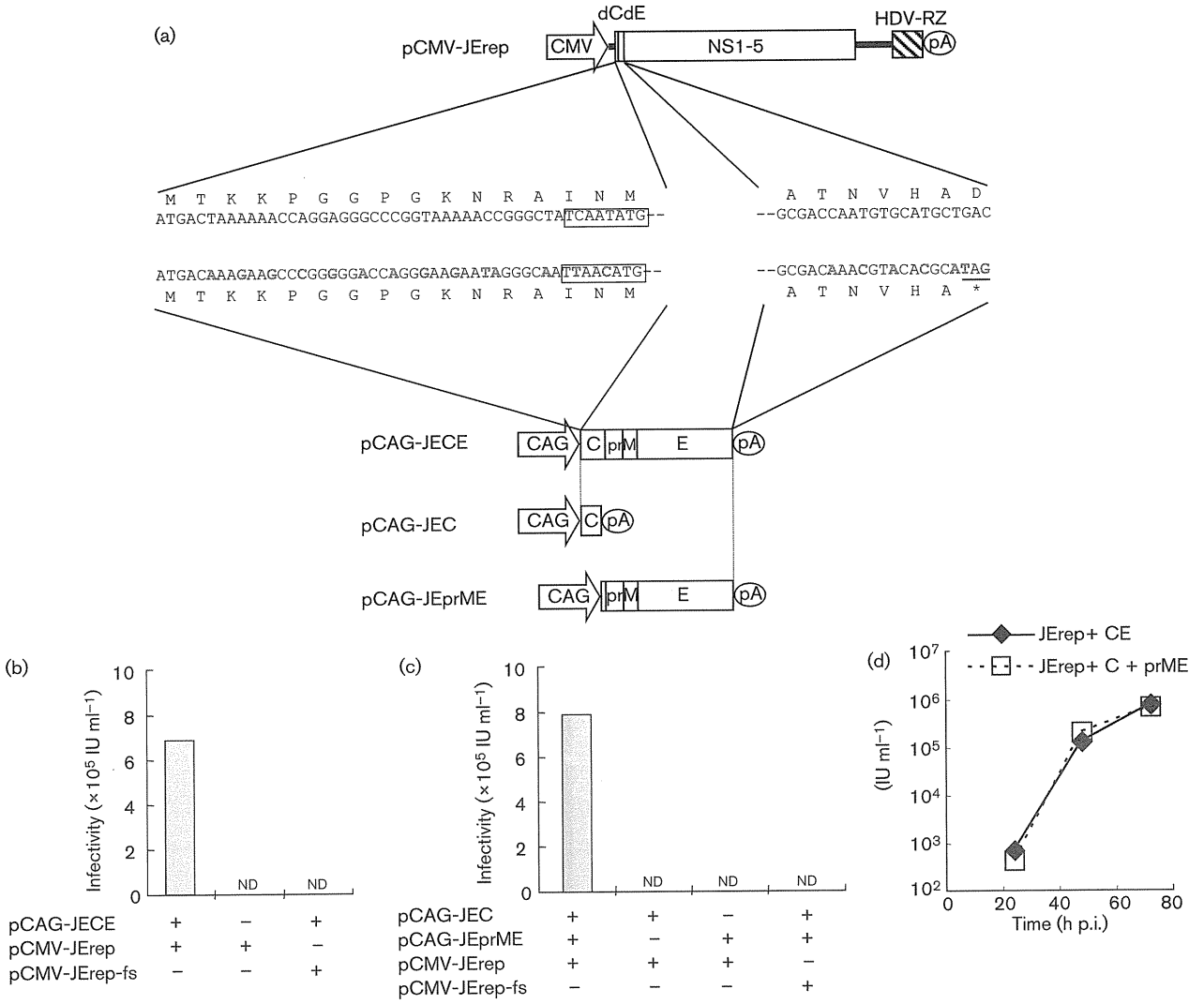

Fig. 2. Schematic representation of JEV replicon and structural protein-expression plasmids. (a) Top: JEV subgenomic replicon with deletion of structural proteins. This replicon contains a partial C and E gene. Bottom: JEV structural protein-expression plasmids showing the region of overlap with JEV replicon. Boxes indicate the 8 nt in the 5′ CS that are 100 % conserved among all mosquito-borne flaviviruses. The JEV C-E, C and prM-E coding sequences harbour silent mutations designed to prevent homologous recombinations that included two changes to the CS sequence, which must be 100 % complementary to the 3′ CS of JEV in order to permit genome replication. The termination codon is underlined. Nucleotide substitutions are shown in red. (b, c) Titres of JEV-SRIPs produced by transfection of 293T cells with replicon plasmid and structural protein-expression plasmids. Dilutions of supernatant collected at 3 days post-transfection were used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-NS1 antibody. Stained cells were then counted to determine the titres (IU ml^{-1}) produced by transfections. ND, Not detected. (d) Time course of JEV SRIP production from transfected cells. At each time point, medium was removed and frozen for subsequent titration and fresh medium was added. JEV SRIP titres were determined by assaying infectivity in Vero cells.

The plasmid-based production system offers an advantage for vaccine production in terms of stability and safety, as this method is able to reduce the chance of mutations in the structural protein region, as well as the risk of infection when compared with live virus production. In addition, our replicon plasmids have the potential for application to DNA-based vaccines, as described previously (Cao *et al.*, 2011; Chang *et al.*, 2008; Huang *et al.*, 2012).

In conclusion, we established a DNA-based production system for SRIPs of flaviviruses. This system has potential value as a basic research and diagnostic tool, and could be used to enhance the safety of neutralization assay, as well as vaccine production.

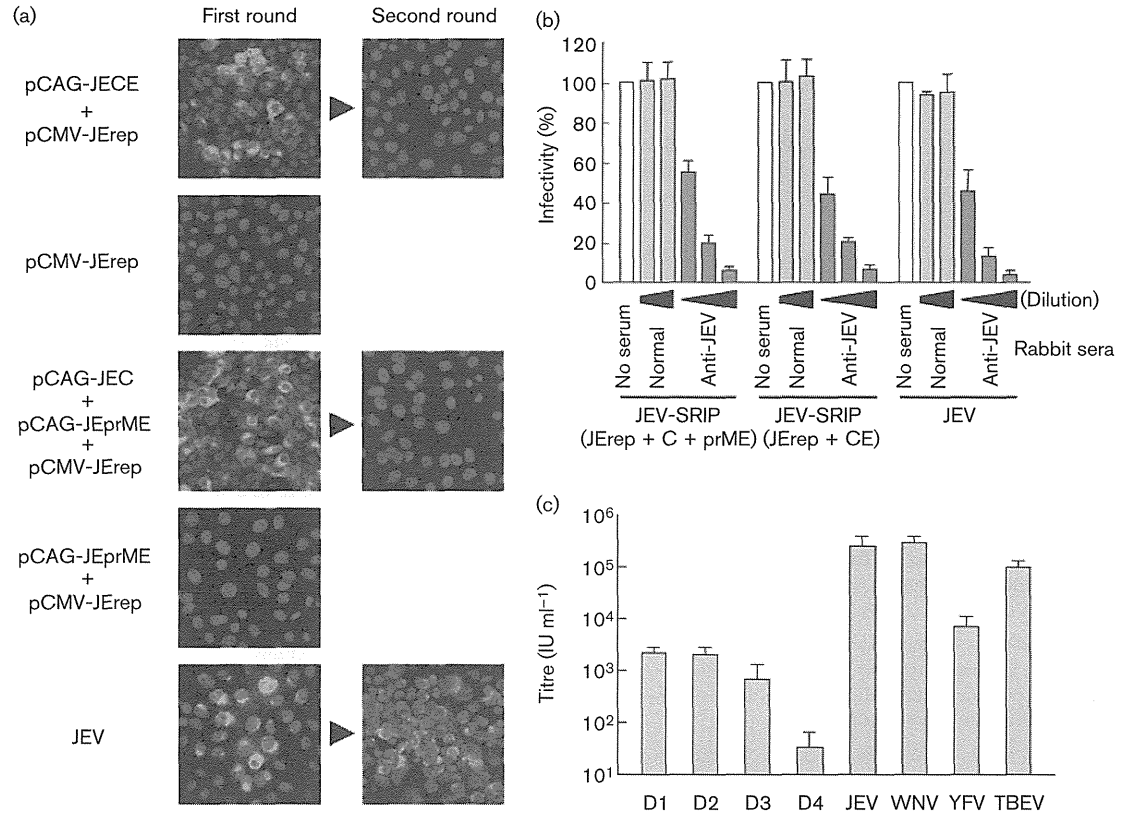

Fig. 3. Vero cells were inoculated with supernatant of 293T cells transfected with the indicated plasmids or infected with JEV. Two days post-inoculation, culture supernatants were collected, and cells were fixed and stained with NS1 antibodies (first round). Naive Vero cells were reinfected with culture supernatants from the first round. Two days post-inoculation, cells were fixed and stained with NS1 antibodies (second round). Cell nuclei were counterstained with DAPI. (b) JEV SRIP inoculum was incubated with serially diluted (1 : 2000, 1 : 8000 and 1 : 32 000) rabbit normal serum or anti-JEV serum for 1 h at room temperature, followed by inoculation onto Vero cells. Cells were immunostained with anti-NS1 antibody at 2 days post-infection, and antigen-positive cells were counted and used to calculate a titre based on f.f.u. ml^{-1} for spreading infections or IU ml^{-1} for non-spreading infections. Data for each condition are means of values obtained from three independent experiments with error bars showing SD. The value for controls without serum (no serum) was set at 100 %. (c) Infectious titres of flavivirus SRIPs, including dengue types 1–4 (D1–4), produced by transfection of 293T cells with pCMV-JErep, pCAG-JEC and flavivirus prM-E expression plasmids. Dilution of supernatant collected at 3 days post-transfection was used to inoculate monolayers of Vero cells. Cells were fixed at 2 days post-infection and stained with anti-JEV NS1 antibody, and stained cells were counted in order to determine titres.

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Two cases of Zika fever imported from French Polynesia to Japan, December 2013 to January 2014

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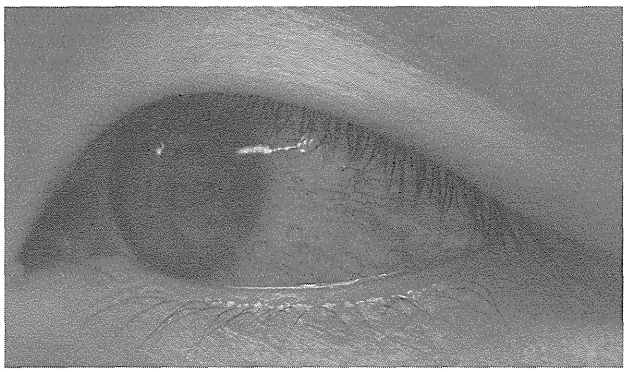
We present two cases of imported Zika fever to Japan, in travellers returning from French Polynesia, where an outbreak due to Zika virus (ZIKV) is ongoing since week 41 of 2013. This report serves to raise awareness among healthcare professionals, that the differential diagnosis of febrile and subfebrile patients with rash should include ZIKV infection, especially in patients returning from areas affected by this virus.

We report two cases of Zika fever in Japan, which were imported from French Polynesia, where on 6 November 2013 public health authorities reported an outbreak of subfebrile illness with rash due to Zika virus (ZIKV). The epidemic started spreading across the archipelago beginning in week 41 of 2013 [1]. During weeks 42 to 52, the syndromic surveillance network reported 6,630 suspected ZIKV infection cases to the Bureau de Veille Sanitaire. About 500 of these cases were tested at the Institute Louis Malarde laboratory in Papeete for confirmation; 333 were confirmed by real-time reverse transcription-polymerase chain reaction (RT-PCR) as ZIKV infections [2]. The outbreak is currently ongoing and as of 13 January 2014, 361 laboratory-confirmed cases have been reported [3]. Symptoms of most ZIKV infection cases are mild and self-limited (mean duration of symptoms is 3–6 days). No hospitalisations for acute infection have been reported.

FIGURE 1

Conjunctivitis in a case of imported Zika virus infection from French Polynesia, Japan, January 2014



Although the patient was afebrile upon examination, both bulbar conjunctivas appeared congested.

Case 1

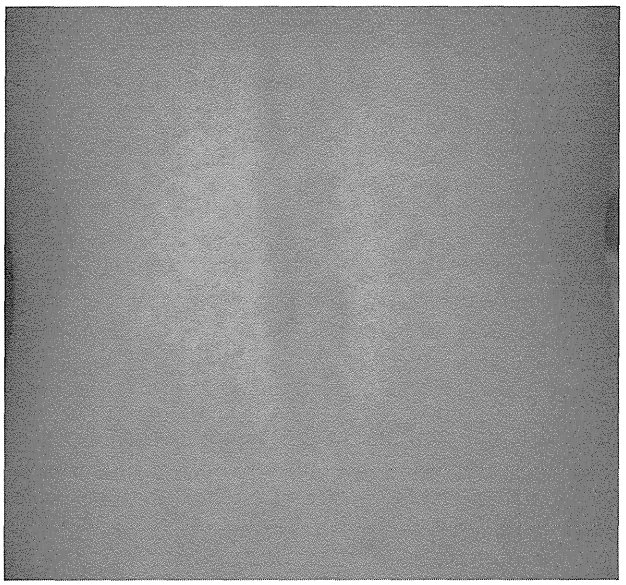
A previously healthy Japanese man in his mid-20s presented to our hospital in mid-December 2013 after four days of fever (self-reported), headache, and arthralgia and one day of rash. He had visited Bora Bora in French Polynesia, in the first week of December 2013 for six days for sightseeing with his partner. He did not use insect repellent during the trip. Upon examination, his body temperature was 37.2°C (99°F) and he had maculopapular rash on his face, trunk, and extremities. Other clinical examination results were normal. Laboratory tests revealed leucopenia (3,300 $\times 10^6$/L; norm: 3,500–8,500$\times 10^6$/L) and thrombocytopenia (14,900$\times 10^6$ /L; norm: 15,000–35,000$\times 10^6$ /L). ZIKV RNA was detected in serum using real-time RT-PCR performed at the National Institute of Infectious Diseases in Japan with primer-probe sets previously described [4]; thus, we diagnosed the patient with Zika fever. His fever and other symptoms subsided a day after first presentation and his rash disappeared over the next few days.

Case 2

A previously healthy Japanese woman in her early 30s presented to our hospital in the beginning of January 2014 for retro-orbital pain, slight fever (self-reported), rash, and itches. Her retro-orbital pain and mild fever had appeared five days prior to her visit at our hospital, while the rash and itches appeared on the day before the visit. She had travelled to Bora Bora where she stayed for 10 days starting mid-December 2013 for sightseeing with a companion. The first symptoms occurred six days after this journey. She had used insect repellent during her travels, but reported mosquito bites. She was afebrile and in good general condition at the first presentation to the hospital. On examination, both bulbar conjunctivas appeared

FIGURE 2

Maculopapular rash on the back in a case of imported Zika virus infection from French Polynesia, Japan, January 2014



congested (Figure 1). She had maculopapular rash on her face, trunk, and extremities (Figure 2).

Laboratory tests on the day of first presentation at the hospital revealed leucopenia ($3{,}500\times10^6$/L; norm: $3{,}500–8{,}500\times10^6$/L) and thrombocytopenia ($14{,}400\times10^6$/L; norm: $15{,}000–35{,}000\times10^6$/L). Real-time RT-PCR assays, performed at the National Institute of Infectious Diseases, gave negative results for ZIKV RNA in serum but presence of the virus was detected in urine. The patient was diagnosed with Zika fever. Her leucocyte and platelet levels returned to the normal range 12 days after first presentation at the hospital. The positive versus negative ratios (P/N ratio) of Zika-specific IgM antibodies were positive in two serum samples collected on the first day at the hospital and five days later (P/N ratios = 2.4 and 9.8, respectively; ratios were considered positive when greater than or equal to 2.0). The neutralising antibody titres of the serum in these two consecutive samples were $PRNT_{50}$=1:20 and $PRNT_{50}$=1:1,280, respectively.

Background

Zika fever is a febrile or subfebrile illness caused by ZIKV, which mainly spreads through the bite of infected mosquitoes. ZIKV is a member of the family Flaviviridae, which includes dengue viruses, West Nile, and yellow fever viruses [5]. The most common symptoms reported in confirmed ZIKV infections are fever, headache, malaise, maculopapular rash, fatigue or myalgia, and arthritis and arthralgia [6].

ZIKV was first isolated from the blood of a sentinel rhesus monkey from the Zika Forest in Uganda [7]. Serological studies and isolation of ZIKV strains have subsequently demonstrated that the virus has a wide geographical distribution, including eastern and western Africa, south and south-east Asia, and Micronesia [8], where in 2007, an outbreak of Zika fever was reported on Yap Island [9].

Phylogenetic analysis of the Zika virus sequence retrieved from case 2

Phylogenetic analysis of the partial ZIKV E-protein genome sequence (470 bp, GenBank accession number: AB908162*) obtained from the urine sample of case 2, shows that this sequence has 99.1% identity with the sequence of a ZIKV strain isolated from Cambodia in 2010 (GenBank accession number: JN860885), and 97.9% identity with the sequence of a ZIKV strain isolated in Yap islands in 2007 (GenBank accession number: EU545988) (Figure 3). The sequence from case 2 sample was also similar to previously identified ZIKV sequences of strains in Asia and Micronesia [8]. In the phylogenetic tree, these sequences formed a distinct cluster from that of sequences from Zika viruses of African origin. Further studies using full-length genome of the ZIKV will address the similarity between virus strains of the African and Asian clusters.

Discussion and conclusion

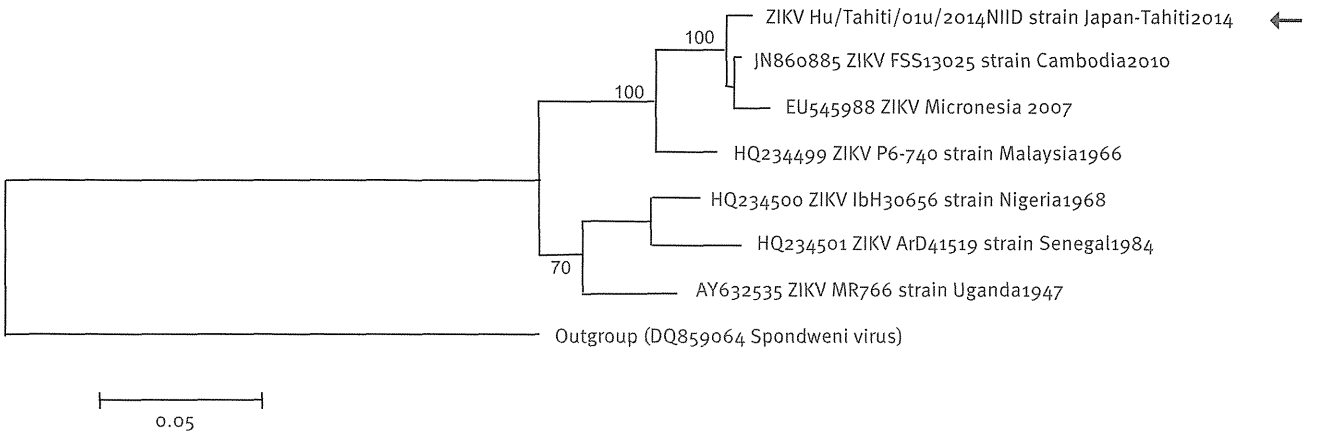
Our two cases are among the first imported cases found linked to the recent outbreak in French Polynesia starting in 2013. They occur shortly after 26 imported cases into New Caledonia from the same outbreak, as well as the report of one indigenous case [10]. Aside from cases related to French Polynesia, imported Zika fever cases have been previously identified in travellers returning from Africa and south-east Asia. These include a case of sexually transmitted Zika fever following two imported cases from Senegal into the United States, and an imported case of Zika fever from Indonesia to Australia [11,12]. Two imported cases from Thailand, one to Canada [13] and one to Germany [14] have also recently been reported.

Although the numbers of imported cases described so far are limited, the possibilities of ZIKV infections to be underdiagnosed and underreported are high due to generally mild symptoms and self-limited disease. Additionally, due to the similarity of ZIKV disease symptoms to those of dengue and chikungunya, differential diagnosis is required to define the extent of ZIKV epidemic. Importantly, as dengue virus (DENV) outbreaks also occur in French Polynesia [2], differential diagnosis between ZIKV infection and dengue is required in cases related to this area. Because of the ongoing dengue epidemic in Bora Bora, DENV infection was excluded in both cases in this study, by confirming that the serum samples were negative for both dengue virus nonstructural glycoprotein-1 (NS1) antigen and IgM/IgG antibodies, using rapid diagnostic kits (SD Bioline Dengue Duo Combo, Alere Medical, Inc.).

In this study, the two cases of ZIKV infection had not only leucopenia but also mild thrombocytopenia.

FIGURE 3

Phylogenetic analysis of a Zika virus sequence derived from a case of imported Zika virus infection from French Polynesia, Japan, January 2014



The phylogenetic tree was based on partial E-protein nucleotide sequences and compiled using the neighbour joining method (Genetyx, Japan). The sequence of the Spondweni virus (GenBank accession number DQ859064) was used as an outgroup. Bootstrap percentages based on 1,000 replicates are shown on the tree nodes. The sequence of the case of imported Zika virus infection from French Polynesia to Japan in January 2014 is indicated with an arrow. Scale bar (0.05) indicates nucleotide substitutions per site.

Previous investigators reported leucopenia, but not thrombocytopenia in patients with ZIKV infection [12]. Our two cases suggest that ZIKV infection can be associated with clinical features including thrombocytopenia and leucopenia, and shares similar clinical features to those of dengue fever and yellow fever.

In the second case identified in this study, viral RNA was negative in the serum sample but was positive in the urine sample. To our knowledge, this is the first case diagnosed by detection of Zika viral particles in urine. Detection of DENV genome in urine after disappearance of the viral genome in serum samples by real-time RT-PCR has been a useful laboratory diagnostic method [15]. Our case suggests that detection of Zika virus genome in urine by real-time RT-PCR is useful to confirm ZIKV infection, particularly after disappearance of viraemia in serum.

Phylogenetic analysis revealed that the ZIKV genome sequences of case 2, had a high sequence homology with recent strains from Asia and Micronesia, including those detected in Cambodia in 2010, but sequence homology was low with a strain isolated in 1947, the Ugandan prototype MR766 strain [4].

The ongoing ZIKV outbreaks in French Polynesia and the confirmation of ZIKV viraemic travellers in our study suggests that in addition to enhanced and continued surveillance efforts, awareness among healthcare professionals should be raised that ZIKV infection ought to be considered as differential diagnosis in febrile patients with rash returning from areas affected by this virus. Further prevention measures, such as offering advice on the use of insect repellents during travel to regions with outbreaks, would be important for ZIKV disease control.

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Conflict of interest

None declared.

Authors' contributions

Satoshi Kutsuna collected the data and drafted the manuscript; Yasuyuki Kato participated in the coordination and concept of the manuscript and edited the manuscript and helped with the draft of the manuscript; Tomohiko Takasaki, Meng Ling Moi, Akira Kotaki performed real-time RT-PCR and performed the phylogenetic analysis; Haruka Uemura, Takashi Matono, Yoshihiro Fujiya, Momoko Mawatari, Nozomi Takeshita, Kayoko Hayakawa collected the data and participated in the concept of the manuscript; Shuzo Kanagawa, Norio Ohmagari revised the article for intellectual content. All authors read and critically revised the first as well as the subsequent and final drafts of this manuscript.

* Addendum:

The GenBank accession number of the partial Zika virus nucleotide sequence derived from a sample obtained from case 2 was added on 07 February 2014.

* Erratum:

The title of this manuscript was initially wrong at the time of publication: 'Two cases of Zika fever imported from French Polynesia to Japan, December to January 2013'. The mistake was corrected on 31 January 2014.

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海外渡航にともなう健康問題に関する意識調査

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要　旨

我々は，ロングステイ財団の調査モニターを対象に，海外渡航中の健康問題に関する意識調査を行い，1648人より回答を得た．旅先での健康問題に関心があると答えた者は80%近くに達した．関心の高い健康問題としては，20歳代～30歳代で「感染症」や「環境変化による病気」（高山病など），60歳代以上は「旅先でかかる医療費」や「旅先の医療機関」が多かった．海外医療情報の入手方法に関しては，ホームページや旅行会社から入手する者が多く，国内の医療機関から入手する者は少なかった．これから先に希望する医療支援としては，ワクチン接種と医療情報提供が多くあげられた．今後はトラベルクリニックなどの専門医療機関で，こうした医療支援を海外渡航者に提供していく必要があるものと考える．

序

海外に渡航する日本人は年々増加している．その渡航目的は，観光，仕事，留学など多様であるが旅先で健康問題が発生し，目的を達せられずに帰国するケースも少なくない．こうした旅先での健康問題に対処するため，わが国では渡航医学が急速に発展してきた．その結果，トラベルクリニックなど渡航者の健康問題に対応する医療体制が築き上げられているが，まだ日本では対応が遅れているのが現状である[1]．そこで今回我々は一般の旅行者が旅先での健康問題に対してどのような医療支援を求めているかを明らかにするため，ロングステイ財団が毎年行っているモニター調査の機会を利用し，旅先での健康問題に関する意識調査を実施した．

方　法

ロングステイ財団は1992年２月に通商産業省（現経済産業省）の認可を受けて設立された財団法人で，ロングステイ（海外滞在型余暇）の普及と啓発活動に努めている．この財団では海外渡航に興味のある一般の成人を対象にロングステイに関する実態調査を毎年行っている．今回の我々の調査は2012年４月に行われたこの実態調査に医療・健康に関するテーマを加えることで実施した．

具体的にはインターネット調査会社であるマクロミル社に今回の調査を委託した．同社の調査モニター（20歳以上）1,008,327人を対象に「各年代同数」「男女比率同数」「全国の都道府県対象」を条件に調査対象6,827人をランダム抽出した．この調査対象にインターネット上のアンケートへの記載を依頼したところ1,648人から回答が得られた．

アンケートの内容はロングステイ財団との共通項目として回答者の特性（年齢居住地職業）と海外渡航歴を聴取する質問（渡航回数渡航目的）が３項目ある．また同財団の質問とは別に海外渡航中の医療・健康に関する質問が10項目あり，いずれも多肢選択方式とした．各質問の最後には回答者が自由に意見を記入できる項目を設けた．

なお，本調査は調査会社に委託して実施されており，ロングステイ財団ならびに我々は個人情報を特定できない集計データのみを扱った．また調査委託先であるマクロミル社はプライバシーマークを取得の上，個人情報規約にのっとり個人情報の管理を行っている．さらに同社の調査モニターは登録時点でアンケートへの参加規約に同意している．

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結　果

１．回答者の特性

今回の調査の回答者総数は1648人であった．地域別には北海道85人，東北地方72人，関東地方673人，中部地方268人，近畿地方313人，中国地方77人，四国地方41人，九州地方119人で，関東地方が最多だった．年代別では20代126人，30代286人，40代412人，50代412人，60代347人，70代以上65人で，40代～50代が最も多かった．職業は男性では会社員（技術系）が多く女性は専業主婦が多かった．

回答者のうち海外渡航経験者は1222人で74.2%を占めた．経験者の渡航回数は２～５回（480人）が最も多かった．海外渡航の目的としては「観光」（1126人）「出張」（262人）が多く，「ロングステイ」（51人）は少なかった（**表１**）．今後の海外渡航の予定としては「観光」（1378人）「ロングステイ」（209人）「家族や友人を訪問」（120人）の順であった．なお本調査でロングステイとは「生活の主たる源泉を日本に置きながら海外の一箇所に２週間以上滞在しその国の文化や生活に触れ，現地社会での貢献を通じて国際親善に寄与する海外滞在型余暇」（ロングステイ財団刊行「ロングステイ調査統計2012」総論より）と定義した．

表１　回答者の海外渡航の目的

渡航目的	過去の海外渡航 (N=1222)		今後予定する海外渡航 (N=1648)	
	人数	%	人数	%
観光	1126	92.1	1387	84.2
ロングステイ	51	4.2	206	12.5
出張	262	21.4	97	5.9
駐在(帯同家族も含む)	44	3.6	23	1.4
留学	78	6.4	34	2.1
家族・友人訪問	162	13.3	120	7.3
その他	44	3.6	178	10.8

表２　海外渡航中に発生した健康問題

（N=1222）複数回答可

	人数	%
下痢	287	23.5
便秘	189	15.5
感冒	216	17.7
持病の悪化	20	1.6
けが	39	3.2
歯の痛み	41	3.6
時差ぼけ	375	30.7
皮膚病	28	2.3
高山病	18	1.5
感染症	11	0.9
不眠	130	10.6
乗り物酔い	167	13.7
メンタル面の病気	25	2.0
その他	239	19.6

２．海外渡航中に発生した健康問題

海外渡航経験者1222人を対象に「今までの海外渡航中に発生した健康問題」について質問した．健康問題の種類を**表２**に示すが「時差ぼけ」（375人）「下痢」（278人）「感冒」（216人）が頻度の高いものだった．その一方で「感染症」（11人）や「高山病」（18人）は少なかった．また海外渡航経験者のうち旅先で医療機関を受診した者は100人（8.1%）にのぼり入院した者は10人（0.8%）だった．

３．関心のある健康問題

以下の質問は全回答者1648人を対象に行った．「旅先での健康問題にどれだけ関心があるか」を質問したところ「大変関心がある」と答えた者が515人（31.3%）で「やや関心がある」（798人）を含めると79.7%に達した．また「大変関心がある」と答えた者の割合は年齢が高くなると多くなり60歳代で43.5%，70歳代で47.7%になった．次に「関心のある健康問題の種類」を質問したところ「旅先でかかる医療費」（919人）「旅先の医療機関」（824人）「感染症」（816人）「環境変化による病気（高山病など）」（668人）の順に多かった（**表３**）．年齢別では20歳代～30歳代で「感染症」や「環境変化による病気」への関心が高く60歳代以上は「医療費」「医療機関」への関心が高い傾向であった．「持病の悪化」（238人）と回答した者は全体的に少なかったが60歳代以上でやや多かった．

４．海外医療情報の入手方法

「海外医療情報の入手方法」を質問したところ「ホームページから入手」が政府機関と民間団体を合わせて875人（53.1%）と最も多かった（**表４**）．また「旅行会社から入手」と回答した者も822人（49.9%）と多かったが「国内の医療機関から入手」は209人（12.7%）と少なかった．「旅行会社から入手」は年齢が高くなると増える傾向だった．

５．希望する医療支援

「海外渡航者が希望する医療支援サービス」を質問したところ「ワクチン接種」（1132人）と「旅先の医療情報提供」（1114人）が多くあげられた（**表５**）．年齢別に見ると20歳代で「旅先での健康指導」70歳以上で「旅先で服用する薬剤の処方」が他の世代に比較して多かった．