Detection of Chikungunya Virus and Arboviruses in Mosquito Vectors[1]

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Abstract Chikungunya (CHIK) is an emerging or reemerging arboviral infectious disease transmitted to humans by the Asian tiger mosquito, *Aedes albopictus* (Skuse), and the yellow fever mosquito, *Ae. aegypti* (L.), particularly in Africa, India, Southeast Asia, and other parts of Europe where numerous outbreaks and epidemics were documented during recent decades. This viral disease is a potential threat to human health worldwide; thus, a quantitative one-step, real-time RT-PCR protocol was developed and assessed as a suitable assay for minimum detection of chikungunya virus (CHIKV) E1 (genus Alphavirus, family Togaviridae) in human serum (7.51 fg/µl) by using the QuantiTect® Probe RT-PCR kit with appropriate synthetic primer (CHIK E1 F and CHIK E1 R) and dual-labeled CHIK E1 probe. Unfortunately, we did not detect any CHIKV from 9 species, namely *Ae. albopictus, Aedes (=Ochlerotatus) cantans* (Meigen), *Ae. cinereus* Meigen, *Ae. geminus* Peus, *Ae. vexans* (Meigen), *Anopheles claviger* Meigen, *An. maculipennis* Meigen *s.l., An. plumbeus* Stephens, and *Culex pipiens* L., but some Kamiti River virus (KRV) were found in *Ae. albopictus* eggs, *Ae. cinereus* and *Ae. vexans* mosquitoes adults. Phylogenetic analyses revealed that these novel insect flaviviruses from *Ae. albopictus* eggs is closely related to Cell fusing agent virus (CFAV), which is different from the other group of arboviruses from extra *Aedes* spp. mosquitoes. These results suggest that *Aedes* spp. in the area of Ticino, Switzerland are likely to be highly infected with two distinct groups of insect flaviviruses, excluding the possibility of CHIKV in mosquito vectors.

Key Words chikungunya, *Aedes albopictus*, E1 gene, real-time quantitative RT-PCR, arbovirus, multiplex PCR, nested PCR

Chikungunya (CHIK), a Swahili word meaning "that which bends up", is an emerging arboviral disease transmitted to humans by the Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae), and the yellow fever mosquito, *Ae. aegypti* (L.) (Diptera: Culicidae). Usually, CHIK causes an acute and painful syndrome with strong fever, asthenia, skin rash, polyarthritis and lethal cases of encephalitis (Sourisseau et al. 2007). It may be confused with dengue fever (DF), but this disease is not associated with hemorrhagic or shock syndromes (Carletti et al. 2007).

Normally, CHIK is geographically distributed in Africa, India and Southeast Asia (Charrel et al. 2007, Enserink 2007) but in recent years has caused several outbreaks

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in Europe, including France and Italy. In 2006, an estimated 110,000 residents of the French island Reunion were infected with the CHIK virus (CHIKV) with 52 mortalities (Enserink 2006). This temperate region arbovirus was confirmed by phylogenetic analysis as the Indian Ocean variant of the African genotype and was the primary example of CHIKV transmission outside the tropics (Rezza et al. 2007), since CHIKV was first isolated from a southern Tanzanian outbreak in 1952 (Ross 1956) and is a single-stranded RNA virus belonging to genus Alphavirus of family Togaviridae, encoding 4 nonstructural (ns1 - 4) and 3 main structural proteins (capsid, E1 and E2) (Tsetsarkin et al. 2007).

The A226V mutation in the glycoprotein envelope 1 (E1) gene of the CHIKV (Charrel et al. 2007) may be linked to the large-scale outbreak of CHIK in the epidemic area and was directly responsible for a significant increase in CHIKV infectivity in *Ae. albopictus* (Kumar et al. 2008, Santhosh et al. 2008). A more alarming finding was that the individual risk of acquiring CHIK fever in a dengue-endemic area was high for local inhabitants and tourists (Massad et al. 2008). In 2008, an emigrant worker returning to China from Ceylon was diagnosed with CHIK by Guangdong Entry-Exit Inspection and Quarantine Bureau (Zheng et al. 2008). Thus, CHIK has evolved from a Third World disease (Enserink 2007) to one that is rapidly undergoing globalization (Charrel et al. 2007).

In prior CHIK outbreaks around the world, *Ae. albopictus* has been regarded as one of the principal vectors and tended to replace *Ae. aegypti* in many regions. To date, *Ae. albopictus*, a mosquito native to Asia, has been one of the fastest spreading animal species over the past two decades (Benedict et al. 2007), which is currently the most invasive mosquito in the world and a laboratory-competent vector of 7 alphaviruses (e.g., Eastern equine encephalitis virus and Ross River virus) and 8 bunyaviruses (e.g., LaCrosse virus and Rift Valley fever virus) and 3 flaviviruses (Japanese encephalitis virus, West Nile virus, WNV, and Yellow fever virus, YFV) to humans (Shroyer 1986, Mitchell 1995), due to its aggressive daytime human-biting behavior. In the past 50 years, the anthropophilic *Ae. albopictus* has spread to all continents and adapted to most climates. Although long considered a secondary disease vector, it has been shown to be capable of transmitting arboviruses under both laboratory and field conditions (Charrel et al. 2007).

For this reason, methods must be developed to enhance the detection of CHIKV and other arboviruses under the framework of Sino-Swiss Science and Technology Cooperation (SSSTC). Methods are available to detect the CHIKV E1 gene by real-time RT-PCR allowing quantification of the initial genome concentration, but the viral load to be accurately determined (Carletti et al. 2007, Edwards et al. 2007, Laurent et al. 2007). Here, we developed a specific quantitative real-time one-step RT-PCR and nested PCR procedures for CHIKV, flavivirus, alphavirus and phlebovirus detection from the mosquito vectors.

Materials and Methods

Mosquito source. The total number of samples was 92, with 34 adult samples of 9 mosquito species in 3 genera, namely: *Ae. albopictus*, *Aedes* [=*Ochlerotatus*] *cantans* (Meigen), *Ae. cinereus* Meigen, *Ae. geminus* Peus, *Ae. vexans* (Meigen), *Anopheles claviger* Meigen, *An. maculipennis* Meigen *s.l.*, *An. plumbeus* Stephens, and *Culex pipiens* L.). Another 58 cases of eggs of *Ae. albopictus*, which were collected from ovitraps located in the woods, were identified morphologically under a stereo

microscope (Zeiss Stemi DV4 Stereo microscope, Switzerland). Adult surveillance of *Ae. albopictus* was conducted using BG Sentinel-traps (Biogents, Germany), and the eggs were collected from the woods by ovitraps (London, UK) around parking places in Chiasso and Bellinzona of Ticino areas every 1/2 month from April 2007 to June 2008. Other mosquito adults were provided by A.P. Caminada. All specimens were frozen and stored at -20°C.

RNA extraction. Ten adult mosquitoes of each sample were first frozen in liquid nitrogen and then macerated in 1.5-ml microtubes using plastic pistils (Fischer Scientific, Switzerland). To accurately check the arbovirus, we used the optimum number of eggs of about 15 - 50 per statistical unit. The resulting powder was mixed with 600 μL lysis buffer with β-mercaptoethanol. Total RNA was extracted using the RNeasy® mini kit (Qiagen, Switzerland) according to the manufacturer's protocols. Finally, RNA was eluted in 30 μL of RNase-free water available from the kit and stored at -20°C until used. The viral RNA from positive patient serum for CHIKV infection was supplied by M. Grandadam (Montpellier, France) in the experiment, and purified using QIAamp® viral RNA mini kit (Qiagen, Switzerland) according to the manufacturer's protocols.

Real-time one-step RT-PCR. Real-time one-step RT-PCR, based on the E1 gene was performed using QuantiTect® Probe RT-PCR kit (Qiagen, Switzerland) in a total volume of 25 μL containing 12.5 μL 2 × Master Mix, 0.25 μL QuantiTect RT Mix, and 5.0 μL extracted viral RNA. Two pairs of primers, 10 μM CHIK E1 F and 10 μM CHIK E1 R and the dual-labeled CHIK E1 probe (50 μM, see Table 1) were used. The cycler conditions consisted of 30 min reverse transcription at 50°C, 15 min of initial polymerase activation at 95°C, followed by 45 cycles at 94°C for 15 sec and 60°C for 1 min. The final cooling temperature was 40°C for 1 min. Real-time one-step RT-PCR was performed in 7,500 fast real-time PCR system (Applied Biosystems, Switzerland), the real-time data were analyzed using the SDS software (V1.4). A water negative control and a positive control of viral RNA were included in each test.

Sensitivity assay of RT-PCR. Following the results of real-time one-step RT-PCR, this original viral extraction (CHIK1 75.1 ng/μl, ND-1000 spectrophotometer, NanoDrop Technologies Inc., Switzerland) was then serially diluted 10-fold in sterile RNase-free water to assess the sensitivity of this analytical method, to determine the minimum limitation of CHIKV quantity detection. In addition, the optimal diagnostic assay was further assessed in human blood and in mosquitoes. A total of 9 suspected negative sera from human subjects (A-I), including partial samples artificially contaminated with CHIKV strain in advance, was assayed using the real-time RT-PCR. Thirty-four RNA samples originating from field-collected *Aedes*, *Anopheles* and *Culex* species also were tested for CHIKV.

Multiplex PCR reaction. The first-strand cDNA from RNA of *Ae. albopictus* eggs was synthesized at 50°C for 1 h and 70°C for 15 min using SuperScript™ III Reverse Transcriptase Kit (Invitrogen, Switzerland), and was then used as the template in the multiplex-PCR. Degenerated primers (Table 1) were selected for each genus: Flavi 1 + and Flavi 1- (gene NS5, 143 bp), Alpha 1 + and Alpha 1- (region nsP4, 195 bp) and Phlebo 1 + and Phlebo 1- (gene L, 244-bp), the final concentration of each primer was 2 μm, including 25μl of 2 × PCR MultiMaster Mix in a final reaction volume of 50 μL using multiplex PCR kit (Qiagen, Switzerland). The PCR mixtures were initially activated at 95°C for 15 min followed by 40 cycles of 94°C for 30 sec (denaturation), 57°C for 90 sec (annealing) and 72°C for 90 sec (elongation). A final extension step was conducted at 72°C for 10 min in GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Switzerland).

Table 1. Primers used in real-time one-step RT-PCR, multiplex PCR and nested PCR.

Primer and probe	Sequence (5′→3′)	Length of the fragment amplified	Target / gene	Function of gene	Reference
		Real-time One-step RT-PCR			
CHIK E1 F	tcgacgcgccctctttaa	127 bp	E1	encoding envelope glycoprotein	Edwards et al., 2007
CHIK E1 R	atcgaatgcaccgcacact				
CHIK E1 Probe	accagcctgcacccattcctcagac				
		Standard Multiplex PCR (primers 1+/1-) & Nested PCR (2+/2-)			
Alpha1+	gaygcitayytigayatggtigaigg	195 bp	nsP4	encoding RNA polymerase	Sánchez-Seco et al., 2001
Alpha1-	kytcytcigtrtgyttigticcigg				
Alpha2+	giaaytgyaaygtiacicaratg				
Alpha2-	gcraaiarigcigcigcytyiggicc				
Flavi1+	gayytiggitgyggiigiggirgitgg	143 bp	NS5	encoding RNA polymerase	Sánchez-Seco et al., 2005
Flavi1-	tcccaiccigcirtrtcrtcigc				
Flavi2+	ygyrtiyayawcaysatggg				
Flavi2-	ccartgitcykyrttiairaaicc				
NPhlebo1+	atggarggitttgtiwsiciicc	244 bp	TOSV L	encoding L segments of Toscana virus	Sánchez-Seco et al., 2003
NPhlebo1-	aarttrctigwigcyttiarigtigc				
NPhlebo2+	wticciaaicciymsaaratg				
NPhlebo2-	tcytcyttrttyttrarrtarcc				

Nested PCR reaction. In the case of a negative result from multiplex PCR, 10 μL of the multiplex product was used for the nested PCR reaction which was conducted separately for each arboviral group using HotstarTaq DNA polymerase kit (Qiagen, Switzerland). Final reaction volumes of 50 μL containing 5 μL of 10 × PCR buffer, 2.5 mM of $MgCl_2$, 200 μM of each dNTP, 0.8 μM of each Flavi 2 + and Flavi 2-, 0.25 μl of HotstarTaq DNA polymerase were run on a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Switzerland), under the following conditions: 95°C for 15 min (initial activation) and 35 cycles of 94°C for 30 sec, 49°C for 30 sec and 72°C for 1 min, followed by final extension at 72°C for 10 min.

The PCR product was analyzed by electrophoresis through a 1.5% agarose gel stained with ethidium bromide at 100 V for 25 min, using RunOne electrophoresis cell (Embi tec., Switzerland). The gel will be visualized under UV light and documented using BioDOC-ItTM system (UVP Inc., Switzerland).

DNA sequencing. The positive PCR products were first purified using the Microcon®-PCR centrifugal filter devices (Millipore, Switzerland) according to the manufacturer's protocols, the mixture reaction was conducted 25 cycles at 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min using an ABI prism BigDye terminator cycle sequencing kit (Applied Biosystems, Switzerland). The sequence reaction products were further purified by using Sephadex G-50 solution at 2700 rpm for 3 min. DNA sequencing was performed in both directions on an ABI Prism 310 Genetic Analyser (Applied Biosystem, Switzerland) using specific primers for nested PCR, according to the manufacturer's instructions.

DNA sequence analysis. The obtained sequences were first blasted through online nucleotide sequence database of the National Center for Biotechnology Information (NCBI) and the identity of virus was primarily determined according to pairwise sequence alignments. Then, these available sequences were assembled and analyzed using DNASTAR 6.0 software. The phylogenetic tree was built using MEGA 4.0.2 software (Tamura et al. 2007) based on the database of relative NS5 gene sequence of mosquito-borne disease downloaded from NCBI.

Results

Our results confirmed that the quantitative real-time one-step RT-PCR detected specific CHIKV in the human blood samples (see Fig. 1) using the QuantiTect Probe RT-PCR kit (Qiagen, Switzerland). Furthermore, the assay detected a minimum quantity of CHIKV at a dilution of 10^{-7} (7.5 fg/μl based on the estimate of molecular weight) when the positive CHIKV strain was used in 10-fold serious gradient dilution. Moreover, the threshold cycle (Ct) was about 16 when the fluorescence passed the threshold, which suggested that the CHIK E1 P probe in the experiment was prone to excite the acceptor fluorochrome to produce higher detectable CHIKV antibody signals during the reaction.

Using this modified CHIKV assay and the original viral RNA from the CHIKV strain (CHIKV1) diluted 100 times with RNAase-free water as the positive template, we easily detected the virus in 4 artificially contaminated blood samples (Fig. 2). This confirmed that this improved assay of real-time RT-PCR maybe suitable for the study of CHIKV in a latent mosquito-borne epidemic.

Unfortunately, we found no positive CHIKV in the 34 field-collected samples of 9 species of mosquitoes, although some adults of *Aedes* spp. have been infected by alphavirus and flavivirus (Fig. 3), as detected by multiplex PCR and nested PCR.

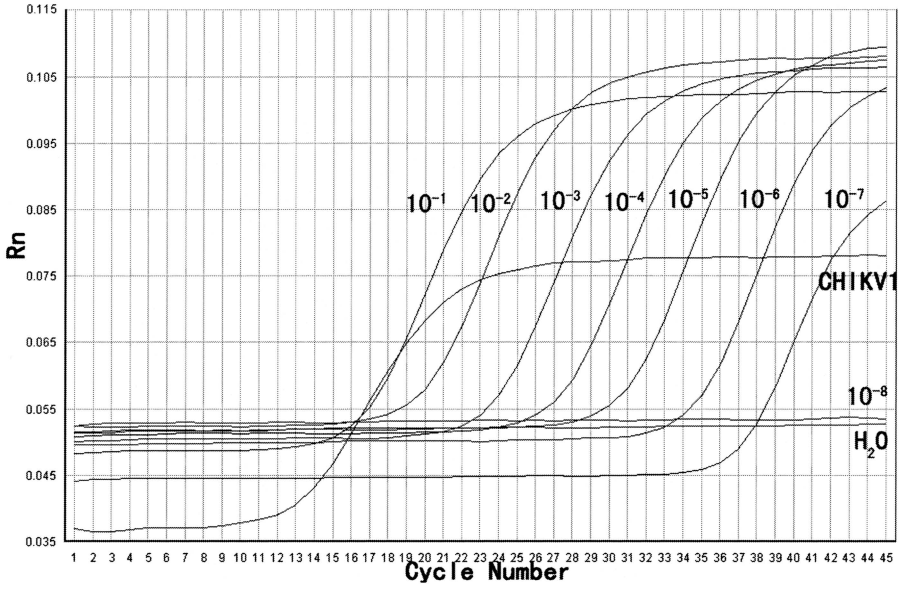

Fig. 1. The chart of serial dilution for detection of CHIKV by using real-time PCR. The horizontal ordinate and longitudinal coordinate stand for the cycle number (Ct) and fluorescence signal intensity (Rn).

Some insect flaviviruses were previously discovered from adults of 3 species namely: *Ae. geminus* ($n = 1$), *Ae. cinereus* ($n = 2$) and *Ae. vexans* ($n = 6$). An unknown alphavirus was also only found in *Ae. cinereus* ($n = 1$) collected from Bolle meridionali, centrale and settentrinali. Therefore, we used another 3 alphavirus, flavivirus and phlebovirus primers to check whether there were some potential arboviruses infecting the offspring of *Ae. albopictus*. Our results showed that the offspring of *Ae albopictus* were not infected with alphavirus, but 3 positive flaviviruses were detected from the egg mass. However, these fragments from *Ae. albopictus* egg, such as 6#, 22# and 36#, have identical sequences (146 bp) with the same group related to Kamiti River virus (KRV, see Fig. 3). The sequences are also closely related to Cell fusing agent virus (CFAV) and belong to the same family Flaviviridae containing dengue virus. At the same time, phylogenetic analyses revealed that these available novel KRV from *Ae. albopictus* eggs are different from the other group of insect flaviviruses from adults of *Ae. geminus*, *Ae. cinereus* and *Ae. vexans*. Our study was a great breakthrough in the mosquito-borne disease research because it confirmed that assays based on multiplex and nested PCR were useful in detecting alphavirus, flavivirus and phlebovirus from *Aedes* spp. adults and eggs.

Concerning the distribution of infected *Aedes*, all positive eggs samples were collected from Chiasso, nearby Italy, with 2 of them acquired in August, and the third in October 2007. The average number of *Ae. albopictus* eggs collected from the woods per month was more than 30, with the highest peak in September 2007. Fortunately, we did not find any positive samples with flavivirus and other arbovirus in 2008, whereas the positive infection rate was about 21.1% in 2007. We believe that there are

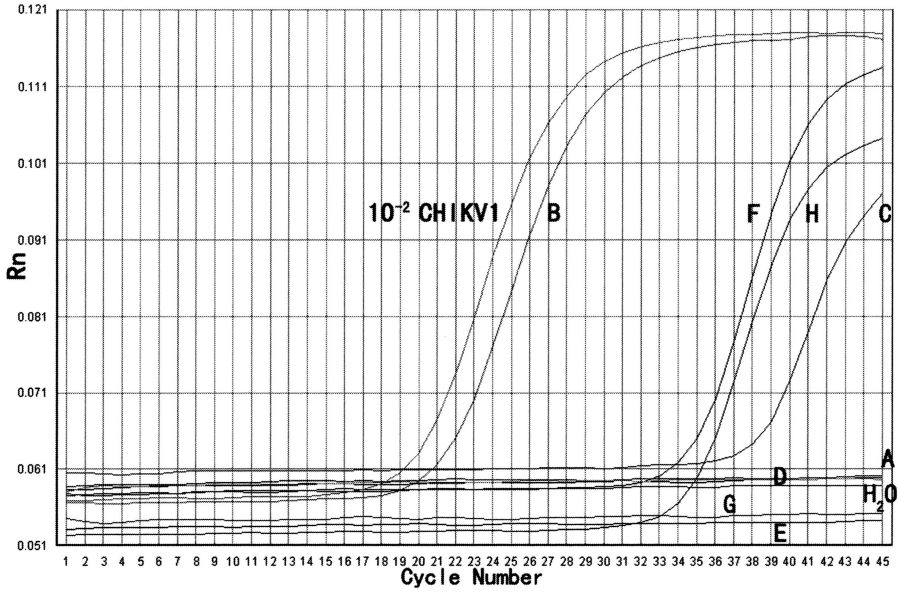

Fig. 2. The curve of positive results from contaminant samples based on real-time RT-PCR.

enormous latent risks of mosquito-borne diseases in Chiasso, considering the high level of egg dispersal of *Ae. albopictus* populations, particularly those infected with YFV, DF or WNV. This also indicates that locally-occurring wild mosquitoes from 3 genera (*Aedes, Culex*, and *Anopheles*) have not yet been infected by CHIKV. Although some insect arboviruses are present in the Ticino area of Switzerland, the risk of CHIK outbreak was low until now.

Discussion

Various factors including global warming and globalization have triggered the spread of CHIK (Enserink 2006, Rezza et al. 2007). The Asian tiger mosquito, a secondary vector of CHIKV, has spread throughout tropical and subtropical regions of the world (Delatte et al. 2008) and posed a threat for the transmission of mosquito-borne diseases (Gubler 1998, Monath 1994, Vainio and Cutts 1998). The A226V mutation in E1 gene increases the risk for infection and epidemics in human populations in various regions of the world (Kumar et al. 2008, Santhosh et al. 2008), especially in areas where *Ae. albopictus* is endemic (Wymann et al. 2008).

Over the past few years, some CHIK viruses were isolated from mosquitoes in Yunnan and Hainan provinces of China, and Yunnan remains the focus of natural infection of CHIK in China (Fang et al. 2005). In 2008, there was a report of a CHIKV-infected patient in one hospital in China; however, this was not confirmed by our China Center for Disease Control and Prevention (CDC). Yet, in 2004, one traveler to Southeast Asia contracted dengue fever, returned to China, and initiated an outbreak of the disease in Cixi in southeastern Zhejiang province (Yang et al. 2009), with *Ae. albopictus*

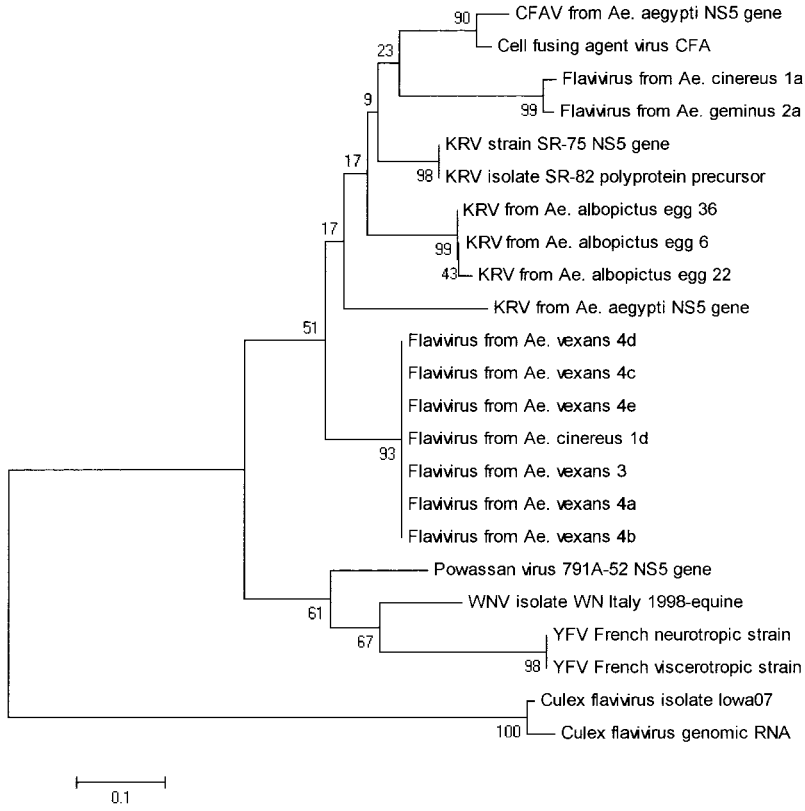

Fig. 3. Phylogenetic relationships of KRV from *Ae. albopictus* eggs and other *Aedes* spp. mosquitoes adults based on the nucleotide sequence of their NS5 genes generated by Neighbor-Joining (NJ) analysis using Mega 4.0.2 software.

serving as a major vector. The other traveler also caused another DF outbreak in 2009 in Yiwu city, which is known as the first international commodities market in China, located in the central area of Zhejiang province. Schilling et al. (2009) reported a case of coinfection with DENV and CHIKV, because both viruses share the same vector. Moreover, we have found a novel insect flavivirus, KRV, from the eggs of *Ae. albopictus* again. This KRV has been formerly reported in *Ae. macintoshi* (Crabtree et al. 2003) and *Cx. pipiens* (Hoshino et al. 2007). Lutomiah et al. (2007) noted that *Ae. aegypti* adults were highly susceptible to KRV and were able to vertically transmit this virus to their progeny. Roiz et al. (2009) suggested that no alphavirus was present in wild *Ae. albopictus* in Northern Italy. We agreed with this point because no CHIKV have been detected until July in Southeast Switzerland, nearby Italy, during our surveillance of the arbovirus.

According to the mosquito surveillance in Zhejiang province, during the last 5 years *Ae. albopictus* was one of the dominant vectors in Shaoxin, Shangyu, Jinhua, Huzhou

and Jiaxin, with population densities ranging from 20% to over 53% (Yang and Fu 2006). We should strengthen the surveillance of *Ae. albopictus* and the diagnosis of arbovirus from suspected hosts cases of mosquito-borne diseases through Sino-Swiss cooperative projects. Fortunately, we have made some progress in the inspection of CHIKV in China. Real-time quantitative RT-PCR assay for CHIKV E1 gene detection from the samples uses only one ChikProbe without extra fluorescent-labeled probes (Edwards et al. 2007, Laurent et al. 2007), which has proven more sensitive than conventional serological identification of the new strain of CHIKV. However, we do not know whether there were any missed or erroneous diagnoses particularly in Zhejiang province; thus it is imperative that we reinforce the detection of CHIKV and other arbovirus from mosquitoes in the event that an infected individual will return from endemic areas including Southeast Asia and Africa.

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