2013

Isolation and genomic characterization of Chaoyang virus strain ROK144 from *Aedes vexansnipponii* from the Republic of Korea

John S. Lee
Military Infectious Diseases Research Program, john.s.lee@amedd.army.mil

Nathan D. Grubaugh
United States Army Medical Research Institute of Infectious Diseases

John P. Kondig
United States Army Medical Research Institute of Infectious Diseases

Michael J. Turell
United States Army Medical Research Institute of Infectious Diseases

Heung-Chul Kim
168th Multifunctional Medical Battalion

See next page for additional authors

Virology 435 (2013) 220–224

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Rapid Communication

Isolation and genomic characterization of Chaoyang virus strain ROK144 from *Aedes vexans nipponii* from the Republic of Korea

John S. Lee [e,*], Nathan D. Grubaugh [a], John P. Kondig [b], Michael J. Turell [a], Heung-Chul Kim [c], Terry A. Klein [d], Monica L. O'Guinn [e]

[a] Virology Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
[b] Diagnostics Systems Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD 21702, USA
[c] 5th Medical Detachment, 168th Multifunctional Medical Battalion, 65th Medical Brigade, Unit # 15247, APO AP 96205-5247, Seoul, Republic of Korea
[d] Force Health Protection and Preventive Medicine, 65th Medical Brigade/ USAMEDDAC-Korea, Unit # 15281, APO AP 96205-5281, Yongsan, Seoul, Republic of Korea
[e] Military Infectious Diseases Research Program, Fort Detrick, MD 21702, USA

ARTICLE INFO

Article history:
Received 25 September 2012
Returned to author for revisions
5 October 2012
Accepted 11 October 2012
Available online 2 November 2012

Keywords:
Arthropod-specific flavivirus
Mosquito flavivirus
Vector surveillance
Arbovirus

ABSTRACT

During June 2003, mosquito surveillance was conducted at a US Army installation and a US Military training site 2 km south of the demilitarized zone, Republic of Korea. Mosquitoes were collected using Mosquito Magnets™, sorted to species, and assayed for the presence of arboviruses. From the 3,149 mosquitoes that were sorted into 126 pools, one *Aedes vexans nipponii* pool (out of 73 pools) tested positive for flavivirus RNA by reverse transcription-PCR . After isolation from C6/36 cell culture supernatant, the viral genome was sequenced and found to be 98.9% related to Chaoyang virus, a potential arthropod-specific flavivirus. This report details the first identification of Chaoyang virus in the Republic of Korea and highlights its relationship to other flaviviruses.

Introduction

The invasion and emergence of arthropod-borne viruses, or arboviruses, have caused an increase in human disease worldwide (Weaver and Reisen, 2010). Many of these important viruses, such as dengue virus (DENV), West Nile virus (WNV), and Japanese encephalitis virus (JEV), belong to the genus *Flavivirus* (family *Flaviviridae*). The genus includes viruses that are transmitted between vertebrate hosts by mosquitoes and ticks and that infect vertebrates with no known vector. The mosquito-borne group can be further divided into viruses transmitted by *Aedes* or *Culex* mosquitoes (Cook and Holmes, 2006). Throughout the last decade, vector surveillance groups from around the world have characterized multiple viruses from mosquitoes that do not appear to infect vertebrate cells. These unclassified viruses formed a tentative new flavivirus group termed arthropod-specific (also termed insect-specific, insect-only, or mosquito-only) because they only replicate in insect cells (Simmonds et al., 2012). The first arthropod-specific flavivirus characterized was the cell fusing agent virus (CFAV) isolated from an *Aedes aegypti* cell line (Stollar and Thomas, 1975) and subsequently isolated from field-collected mosquitoes in Puerto Rico (Cook et al., 2006). Arthropod-specific flaviviruses genetically related to CFAV, such as Kamiti River virus (Crabtree et al., 2003; Sang et al., 2003), *Culex flavivirus* (Hoshino et al., 2007), Quang Binh virus (Crabtree et al., 2009), *Aedes flavivirus* (Hoshino et al., 2009), Nakiwogo virus (Cook et al., 2009), and Calbertado virus (Bolling et al., 2011; Tyler et al., 2011) form a complex that is phylogenetically distinct from other mosquito-borne flaviviruses.

Recently, flaviviruses that phylogenetically cluster with the medically important mosquito-borne viruses but without a known vertebrate host have been reported. In 2004, a novel flavivirus termed nounané virus (NOUV) was isolated from *Uranotaenia mashonaensis* mosquitoes collected in Côte d'Ivoire (Junglen et al., 2009). The isolate was able to infect an *Ae. albopictus* C6/36 cell line but was not found to replicate in any of the tested vertebrate cell lines. Phylogenetic analysis of the full-length NOUV genome showed that it forms its own subgroup within the mosquito-borne flaviviruses. Also in 2004, a similar virus named Lammi virus (LAMV) was isolated from *Aedes* mosquitoes in Finland and was found to replicate only in mosquito cells (Huhtamo et al., 2009). Despite the fact that they were only 55% homologous in nucleotide sequences, NOUV and LAMV were phylogenetically positioned closely within the *Aedes*-transmitted group. Numerous isolates of Marisma virus (MMV), another flavivirus potentially specific only to insects, were isolated from *Ae.* (*Ochlerotatus*) *caspius* mosquitoes in

* Correspondence to: Military Infectious Diseases Research Program (MIDRP), US Army Medical Research and Materiel Command, Building 722, 504 Scott Avenue, Frederick, MD 21702, USA.
E-mail addresses: john.s.lee@amedd.army.mil, john.s.lee@us.army.mil (J.S. Lee).

0042-6822/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.virol.2012.10.020

Spain from 2003 to 2010 (Vázquez et al., 2012). A partial MMV nonstructural protein 5 (NS5) sequence was shown to cluster closely with LAMV and Chaoyang virus (CHAOV). The first isolated strain of CHAOV, Deming, was from mosquitoes collected in Liaoning province, China in 2008 (Wang et al., 2009; Liu et al., 2011; GenBank Accession FJ883471). Other strains of CHAOV, BeiBei (GenBank Accession JQ308185) and HLD115 (GenBank Accession NC_017086), were also isolated in China in 2008 and 2010, respectively (unpublished data). CHAOV Deming strain was reported to contain a single open reading frame that encoded three structural proteins and seven nonstructural proteins, with the greatest nucleotide sequence homology between the NS5 gene and that from Sepik virus (yellow fever group). The Deming strain was also reported to replicate in C6/36 cells and to induce a cytopathic effect (CPE) leading to cell deformation, aggregation, and death in C6/36 cells, but it is not known whether it is able to infect and to replicate in vertebrate cells.

A US Military Force Health Protection program has been established in the Republic of Korea (ROK) to survey mosquito populations for pathogens such as DENV and malaria causing parasites. In June of 2003 during routine surveillance, flavivirus RNA was detected from a pool of *Ae. vexans nipponii* mosquitoes which was later found to be related to the CHAOV strains isolated from China. Here we describe the isolation and genomic characterization of CHAOV strain ROK144 and discuss the implications of our findings.

Results

Isolation of CHAOV in the ROK

A below-normal abundance of mosquitoes observed in the ROK during June 2003 resulted in the collection of only 3,149 mosquitoes, representing three genera and nine species (Table 1). Processing and testing of all the mosquitoes by alphavirus and flavivirus RT-PCR identified flavivirus RNA from one pool (pool no. 144) of *Ae. vexans nipponii* collected from Camp Greaves. The clarified supernatant from the mosquito pool was inoculated into cultured C6/36, BHK, primary duck, primary chicken, and Vero cells and observed for cytopathic effects (CPE). No CPE were observed in any of the cell lines, contradictory to what was reported by Liu et al., where they reported that CHAOV Deming strain induced CPE in C6/36 cells (Liu et al., 2011). Flavivirus RNA was detected in the C6/36 cell culture supernatant after the second blind pass, indicating virus replication and release from the infected cells, whereas flavivirus RNA was not detected in any of the vertebrate cell line supernatants after two blind passes.

Table 1
Flavivirus isolate and mosquito species listing for vector surveillance activities in the Republic of Korea, June 2003.

Species	Number of mosquitoes	Number of pools (+)[a]	MLE[b]
Aedes koreicus	2	1	0
Aedes vexans nopponii	2,129	73 (1)	0.47
Anopheles sinensis (complex)	734	31	0
Anopheles sineroides	1	1	0
Anopheles pullus	167	10	0
Culex orientalis	1	1	0
Culex pipiens	112	7	0
Culex tritaeniorhynchus	1	1	0
Culex vagans	2	1	0
Totals	3,149	126 (1)	0.32

[a] (+), number of flavivirus RNA positive pools by RT-PCR.
[b] MLE, maximum likelihood estimations (estimated number of flavivirus positive mosquitoes per 1,000).

Table 2
Genome organization of Chaoyang virus strain ROK144.

Genome category	Gene or region[a]	Position in ORF (nt)[b]	Protein size (aa)[c]
Structural	VirC	0–312	104
	CTHD	313–366	18
	pr	367–648	94
	prM	649–876	76
	E	877–2370	498
Non-structural	NS1	2371–3573	401
	NS2A	3574–4113	180
	NS2B	4114–4506	131
	NS3	4507–6369	621
	NS4A	6370–6744	125
	2K	6745–6816	24
	NS4B	6817–7668	284
	NS5	7669–10305	879

[a] VirC, mature virion C protein; CTHD, C-terminal hydrophobic domain.
[b] ORF, open reading frame; nt, nucleotides.
[c] aa, amino acids.

Subsequently, the virus has been passed multiple times in C6/36 cells to show that the virus can be passed and that it is stable (data not shown).

Genetic characterization of CHAOV strain ROK144

The complete coding sequence was determined for the flavivirus isolate ROK144 using RNA extracted from the C6/36 cell culture supernatant. The resulting sequence contained 10,308 base pairs (bp) and a single open reading frame (ORF) encoding for 3,435 amino acids (aa). Twelve polyprotein cleavage sites were identified and the protein sizes and positions were determined (Table 2). The ROK144 sequence was found to be 98.9% and 98.0% homologous to the Deming (GenBank ID: FJ883471) and HLD115 (GenBank ID: JQ308158) strains, respectively, of CHAOV isolated in China. This new CHAOV isolate, ROK144 (GenBank ID: JQ068102), was shown to contain 111 nucleotide changes as compared to the Deming strain that resulted in only five amino acid changes: A357V (E gene), V884A (NS1), I1322L (NS2A), R3054M (NS5), and D3099N (NS5). There were 109 nucleotide differences between HLD115 (excluding the 5′ non-coding region) and ROK144 that resulted in six amino acid changes: R272K (prM), V1202L (NS2A), I1322L (NS2A), N2295T (NS4B), V2695F (NS5), and D3099N (NS5). Besides CHAOV, the next highest homology was 74.6% with LAMV (GenBank ID: FJ606789) and 63.2% with Donggang virus (DGV, GenBank ID: JQ086551). The ROK144 strain was found to be only 53.4% homologous to NOUV (GenBank ID: FJ711167). The most distal flaviviruses compared to ROK144 are the arthropod-specific viruses, ranging from 37.4% to 38.3% similarity. Fig. 1A shows the phylogenetic relationship between the complete coding sequence of CHAOV strain ROK144 and other flaviviruses. The CHAOV strains align phylogenetically near LAMV, DGV, and NOUV forming a cluster between the yellow fever virus (YFV) and DENV groups. Sequence analysis of approximately 1000 bp of the NS5 gene was also performed to compare CHAOV to other flaviviruses, including the partial sequence of MMV (GenBank ID: JN603190). Comparing just the partial NS5 sequences, CHAOV strain ROK144 is 98.4% and 97.8% homologous to the CHAOV strains Deming and HLD115, respectively. Strain ROK144 was also 77.3%, 70.2%, and 67.9% similar to the NS5 sequences of LAMV, MMV, and DGV, respectively. Phylogenetic analysis of the NS5 sequences show that CHAOV, LAMV, MMV, and DGV cluster within the *Aedes*-transmitted subgroup (Fig. 1B). The NS5 sequence for NOUV is 61.5% homologous to ROK144. It should be noted that NOUV, as shown in Fig. 1B, often aligns more closely with the DENV and *Culex*-borne groups than with the CHAOV and YFV groups, depending on the sequences used for comparison.

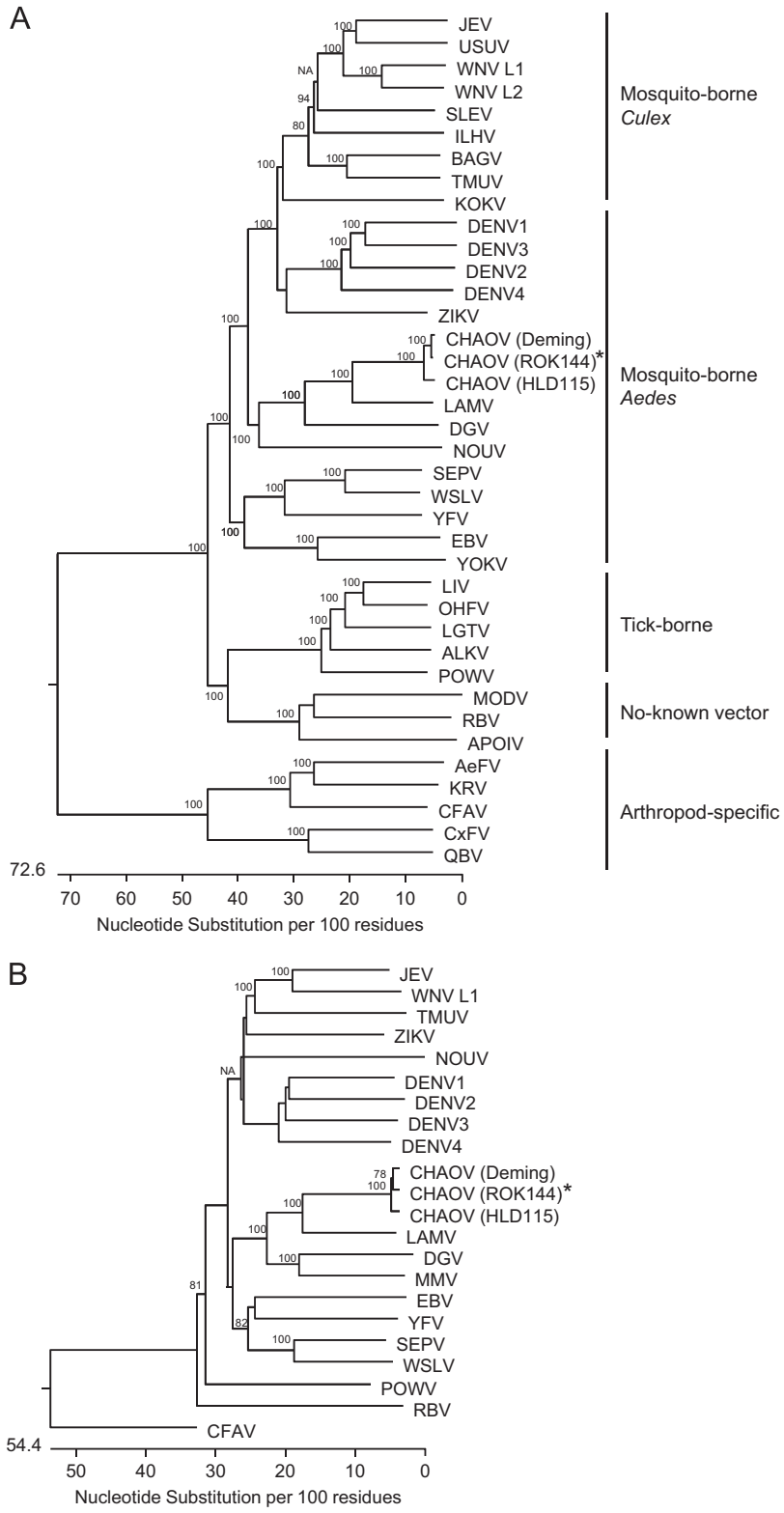

Fig. 1. Phylogenetic trees showing the relationship between the (A) complete coding sequence and (B) partial NS5 sequence of Chaoyang virus (CHAOV) strain ROK144 (GenBank ID: JQ068102) isolated in the Republic of Korea (indicated by *) and other flavivirus sequences. Bootstrap values were calculated with 1,000 trials. Bootstrap values ≥ 75 are shown at the nodes. AeFV, Aedes flavivirus (GenBank ID: AB488408); ALKV, Alkhurma hemorrhagic fever virus (GenBank ID: AF331718); APOIV, Apoi virus (GenBank ID: AF160193); BAGV, Bagaza virus (GenBank ID: AY632545); CFAV, cell fusing agent virus (GenBank ID: M91671); CHAOV strain Deming (GenBank ID: FJ883471); CHAOV strain HLD115 (GenBank ID: JQ308185); CHAOV strain ROK144 (GenBank ID: JQ068102); CxFV, Culex flavivirus (GenBank ID: AB262759); DENV1, dengue virus type 1 (GenBank ID: U88536); DENV2 (GenBank ID: U87411); DENV3 (GenBank ID: AY099336); DENV4 (GenBank ID: AF326825); DGV, Donggang virus (GenBank ID: JQ086551); EBV, Entebbe bat virus (GenBank ID: DQ837641); ILHV, Ilheus virus (GenBank ID: AY632639); JEV, Japanese encephalitis virus (GenBank ID: M18370); KOKV, Kokobera virus (GenBank ID: AY632541); KRV, Kamiti River virus (GenBank ID: AY149905); LAMV, Lammi virus (GenBank ID: FJ606789); LGTV, Langat virus (GenBank ID: AF253419); LIV, louping ill virus (GenBank ID: Y07863); MMV, Marisma virus (GenBank ID: JN603190); MODV, Modoc virus (GenBank ID: AJ242984); NOUV, nounané virus (GenBank ID: FJ711167); OHFV, Omsk hemorrhagic fever virus (GenBank ID: AY193805); POWV, Powassan virus (GenBank ID: L06436); QBV, Quang Binh virus (GenBank ID: FJ644291); RBV, Rio Bravo virus (GenBank ID: AF144692); SEPV, Sepik virus (GenBank ID: DQ837642); SLEV, St. Louis encephalitis virus (GenBank ID: DQ525916); TMUV, Tembusu virus (GenBank ID: JF89523); USUV, Usutu virus (GenBank ID: AY453411); WNV L1, West Nile virus lineage 1 (GenBank ID: DQ311652); WNV L2 (GenBank ID: EU707555); WSLV, Wesselsbron virus (GenBank ID: EU707555); YFV, yellow fever virus (GenBank ID: X03700); YOKV, Yokose virus (GenBank ID: AB114858); ZIKV, Zika virus (GenBank ID: AY632535). GenBank was accessed in April 2012.

Discussion

CHAOV invertebrate hosts and geographic distribution

Here we report the first detection of CHAOV in the Republic of Korea and show that it is genetically related to the CHAOV strains (Deming and HLD115) isolated in China (Wang et al., 2009). The CHAOV strain ROK144 was isolated from a pool of *Ae. vexans nipponii* mosquitoes, an anthropophilic species common to the ROK (Kim et al., 2004). Subsequently, vector surveillance activities conducted from 2008 to 2011 in the ROK have detected and/or isolated an additional 32 strains of CHAOV (unpublished data). The majority of the CHAOV strains were detected in pools of *Aedes* mosquitoes with 26 from *Ae. vexans nipponii* and one each from *Ae. albopictus* and *Ae. bekkui* mosquitoes. Additionally, three pools of *Culex pipiens* and one pool of *Armigeres subalbatus* tested positive for CHAOV. This suggests that *Ae. vexans nipponii* could be the primary invertebrate host for CHAOV but the virus may not be limited to *Aedes* mosquitoes. The mosquito species were not reported for the Deming and HLD115 strains of CHAOV collected in China; however the geographical distribution of *Ae. vexans* extends throughout Asia.

Phylogenetic alignment with medically relevant flaviviruses

Like other recently discovered novel flaviviruses such as NOUV (Junglen et al., 2009), LAMV (Huhtamo et al., 2009), and MMV (Vázquez et al., 2012), CHAOV seem only, or at least primarily, to infect mosquito cells. These infection/replication restrictive properties seem to be shared with other viruses of the arthropod-specific group and have been found to correlate with the viruses' host range (Kuno, 2007). However, they all phylogenetically aligned more closely with the medically relevant mosquito-borne group than they do with the major group of arthropod-specific flaviviruses. Specifically, CHAOV, LAMV, and DGV, isolated in 2009 from *Aedes* species mosquitoes from the Liaoning province in China (unpublished data), form a cluster separating the YFV group from the rest of the *Aedes*-transmitted viruses. Since primary isolations of viruses in cell culture can be difficult and CHAOV is phylogenetically related to other mosquito-borne viruses, additional experiments are merited to determine if CHAOV could infect a vertebrate host. Furthermore, serological studies involving local vertebrates and clinical patients are needed to determine the pathological potential of CHAOV for humans and animals.

Public health impact of arthropod-specific flaviviruses and vector surveillance

Even if CHAOV truly is arthropod-specific, its potential effect on public health should not be overlooked. The role of arthropod-specific flaviviruses in the fitness of the mosquito and its ability to vector other viruses has yet to be determined. Vector competence studies involving *Culex* mosquitoes co-infected with CxFV and WNV suggest that there may be a competitive interaction between the viruses early in the WNV infection that may have an effect on WNV transmission (Bolling et al., 2012; Kent et al., 2010). Newman et al. discovered a natural positive correlation between WNV and CxFV infection rates, with mosquito pools being more likely to be positive for CxFV if they were also positive for WNV (Newman et al., 2011). While several questions need to be answered first, like whether CHAOV can infect the vectors of DENV, it may be important to conduct similar studies with CHAOV and DENV co-infection .

More viruses similar to CHAOV are bound to be discovered, since there exists a growing trend for increased vector surveillance and improved diagnostic testing. Eventhough the role of CHAOV in animal and human health has yet to be understood, knowing of their existence is important for surveillance programs. Often times a generic flavivirus assay is used to screen numerous pools of mosquitoes, but they may indiscriminately detect both medically relevant and non-relevant viruses. Having a better understanding of the viruses maintained in certain regions along with additional diagnostics to differentiate between them can help alleviate issues of false reporting.

Materials and methods

Vector surveillance and mosquito processing

Adult mosquitoes were collected during June 2003 using Mosquito Magnets® (Woodstream Corp., Lititz, PA) located near barracks at Camp Greaves (US military installation, now closed) and ill-kempt tents at Warrior Base (US military training site) where soldiers were housed. Both were located approximately 2 km from the demilitarized zone separating North and South Korea. Female mosquitoes were pooled according to species, date, and location, and then assayed for the presence of flavivirus and alphavirus RNA using reverse transcription (RT)–PCR as described by O'Guinn et al. (2004), except that MA/ cFD2 flavivirus primers (Kuno, 1998) or VEE 0091/0092 alphavirus primers (O'Guinn et al., 2004) were used. Briefly, Mosquito pools were triturated in a 1.5-mL microcentrifuge tube containing one 4.5-mm spherical copper bead and 0.75 ml of Eagle's minimal essential media supplemented with 10% heat-inactivated fetal bovine serum, 100 units of penicillin and 100 μg of streptomycin per ml, and 0.075% $NaHCO_3$ (EMEM complete) by vortexing the tube for 1–2 min. The mosquito homogenate was clarified by centrifugation in a microcentrifuge at 4 °C and 12,000 rpm for 5 min. RNA was extracted from the clarified supernatant using a QIAamp viral RNA mini-kit (Qiagen, Valencia, CA) according to manufacturer's instructions.

Isolation of CHAOV

Clarified supernatant from the single mosquito pool that tested positive for flavivirus RNA by RT-PCR (no pools tested positive for alphavirus RNA) was subjected to standard plaque assay using Vero cells (African green monkey kidney) and to co-culturing with C6/36, BHK (baby hamster kidney), primary duck, primary chicken, or Vero cells as outlined by Kondig et al. (2007). C6/36 cells were cultured in Hank's minimal essential media supplemented as described above, and BHK, primary duck, primary chicken, and Vero cells were cultured in EMEM complete. Plaque assays were stained with phenol red on days 2 or 5 and evaluated for plaque formation. After two blind passages, the co-cultured cells were observed for cytopathic effects and cell-free cell culture supernatants were assayed for the presence of viral RNA by using RT-PCR with MA/ cFD2 flavivirus primers.

Sequencing and phylogenetic analysis

Twelve pairs of overlapping primers for the RT-PCR amplification and genome sequencing of the ROK144 isolate were designed by using the genomic sequence of CHAOV strain Deming (GenBank ID: FJ883471). The primer sequences are available upon request. Sequencing was accomplished as described by O'Guinn et al., except that an ABI 3100 genetic analyzer and Big Dye™ 3.1 were used (PE Biosystems, Inc., Foster City, CA) (O'Guinn et al., 2004). The nucleotide BLAST program was used to compare the ROK144 sequence with other GenBank sequences (http://www.

ncbi.nlm.nih.gov). Phylogenetic analysis was conducted according to Kondig et al. (Kondig et al., 2007) using the Clustal W program (version 9.1.0) to calculate bootstrap values with a default setting of 1,000 trials (iterations) and a seed value of 111. The polyprotein signalase cleavage sites were predicted by using SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and by comparing the amino acid sequences to the cleavage sites for LAMV (GenBank ID: FJ606789).

Acknowledgments

This work was supported through the joint partnership between the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD; 65th Medical Brigade, Yongsan, Seoul, ROK; and the Armed Forces Health Surveillance Center-Global Emerging Infections Surveillance and Response System, Silver Spring, MD, and was funded in part by the Military Infectious Disease Research Program project # U0176_09_RD.

The mention of trade names or commercial products does not constitute endorsement or recommendation for use by the Department of the Army or the Department of Defense. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

References

Bolling, B.G., Eisen, L., Moore, C.G., Blair, C.D., 2011. Insect-specific flaviviruses from *Culex* mosquitoes in Colorado, with evidence of vertical transmission. Am. J. Trop. Med. Hyg. 67 (1), 85–92.

Cook, S., Holmes, E.C., 2006. A multigene analysis of the phylogenetic relationships among the flaviviruses (Family: *Flaviviridae*) and the evolution of vector transmission. Arch. Virol. 151 (2), 309–325.

Cook, S., Bennet, S.N., Holmes, E.C., De Chesse, R., Moureau, G., de Lamballerie, X., 2006. Isolation of a new strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J. Gen. Virol. 87 (Pt 4), 735–748.

Cook, S., Moureau, G., Harbach, R.E., Mukwaya, L., Goodger, K., Ssenfuka, F., Gould, E., Holmes, E.C., de Lamballerie, X., 2009. Isolation of a novel species of flavivirus and a new strain of *Culex flavivirus* (*Flaviviridae*) from a natural mosquito population in Uganda. J. Gen. Virol. 90 (Pt 11), 2669–2678.

Crabtree, M.B., Sang, R.C., Stollar, V., Dunster, L.M., Miller, B.R., 2003. Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch. Virol. 148 (6), 1095–1118.

Crabtree, M.B., Nga, P.T., Miller, B.R., 2009. Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch. Virol. 154 (5), 857–860.

Hoshino, K., Isawa, H., Tsuda, Y., Yano, K., Sasaki, T., Yuda, M., Takasaki, T., Kobayashi, M., Sawabe, K., 2007. Genetic characterization of a new insect flavivirus isolated from Culex pipiens mosquito in Japan. Virology 359 (2), 405–414.

Huhtamo, E., Putkuri, N., Kurkela, S., Manni, T., Vaheri, A., Vapalahti, O., Uzcategui, N.Y., 2009. Characterization of a novel flavivirus from mosquitoes in northern europe that is related to mosquito-borne flaviviruses of the tropics. J. Virol. 83 (18), 9532–9540.

Junglen, S., Kopp, A., Kurth, A., Pauli, G., Ellerbrok, H., Leendertz, F.H., 2009. A new flavivirus and a new vector: characterization of a novel flavivirus isolated from *Uranotaenia* mosquitoes from a tropical rain forest. J. Virol. 83 (9), 4462–4468.

Kent, R.J., Crabtree, M.B., Miller, B.R., 2010. Transmission of West Nile virus by *Culex quinquefasciatus* Say infected with *Culex* flavivirus Izabal. PLoS Negl. Trop. Dis. 4 (5), e671.

Kim, H.C., Chong, S.T., Pike, J.G., O'Guinn, M.L., Pacha, L.A., Lee, H.C., Klein, T.A., 2004. Seasonal prevalence of mosquitoes collected from light traps in the Republic of Korea, 2002. Entomol. Res. 34 (3), 177–186.

Kondig, J.P., Turell, M.J., Lee, J.S., O'Guinn, M.L., Wasieloski Jr., L.P., 2007. Genetic analysis of South American eastern equine encephalomyelitis viruses isolated from mosquitoes collected in the Amazon Basin region of Peru. Am. J. Trop. Med. Hyg. 76 (3), 408–416.

Kuno, G., 1998. Universal diagnostic RT-PCR protocol for arboviruses. J. Virol. Methods 72, 27–41.

Kuno, G., 2007. Host range specificity of flaviviruses: correlation with in vitro replication. J. Med. Entomol. 44, 93–101.

Liu, H., Gao, X., Liang, G., 2011. Newly recognized mosquito-associated viruses in mainland China, in the last two decades. Virol. J. 8 (1), 68–80.

Newman, C.M., Cerutti, F., Anderson, T.K., Hamer, G.L., Walker, E.D., Kitron, U.D., Ruiz, M.O., Brawn, J.D., Goldberg, T.L., 2011. *Culex* flavivirus and West Nile virus mosquito coinfection and positive ecological association in Chicago, United States. Vector Borne Zoonotic Dis. 11, 1–10.

O'Guinn, M.L., Lee, J.S., Kondig, J.P., Fernandez, R., Carbajal, F., 2004. Field detection of eastern equine encephalitis virus in the Amazon Basin region of Peru using reverse transcription-polymerase chain reaction adapted for field identification of arthropod-borne pathogens. Am. J. Trop. Med. Hyg. 70 (2), 164–171.

Sang, R.C., Gichogo, A., Gachoya, J., Dunster, M.D., Ofula, V., Hunt, A.R., Crabtree, M.B., Miller, B.R., Dunster, L.M., 2003. Isolation of a new flavivirus related to cell fusing agent virus (CFAV) for field-collected flood-water *Aedes* mosquitoes sampled from a dambo in central Kenya. Arch. Virol. 148 (6), 1085–1093.

Simmonds, P., Becher, P., Collet, M.S., Gould, E.D., Heinz, F.X., Meyers, G., Monath, T., Pletnev, A., Rice, C.M., Stiasny, K., Thiel, H.-J., Weiner, A., Bukh, J., 2012. Family Flaviviridae. Virus Taxonomy, 9th Report of the International Committee on Taxonomy of Viruses. Academic Press, San Diego, pp. 1003–1020.

Stollar, V., Thomas, V.L., 1975. An agent in the *Aedes aegypti* cell line (Peleg) which causes fusion of *Aedes albopictus* cells. Virology 64 (2), 367–377.

Tyler, S., Bolling, B.G., Blair, C.D., Brault, A.C., Pabbaraju, K., Armijos, M.V., Clark, D.C., Calisher, C.H., Drebot, M.A., 2011. Distribution and phylogenetic comparisons of a novel mosquito flavivirus sequence present in *Culex tarsalis* Mosquitoes from western Canada with viruses isolated in California and Colorado. Am. J. Trop. Med. Hyg. 85 (1), 162–168.

Vázquez, A., Sánchez-Seco, M., Palacios, G., Molero, F., Reyes, N., Ruiz, S., Aranda, C., Marqués, E., Escosa, R., Moreno, J., Figuerola, J., Tenorio, A., 2012. Novel flaviviruses detected in different species of mosquitoes in Spain. Vector Borne Zoonotic Dis. 12 (3), 223–229.

Wang, Z., An, S., Wang, Y., Han, Y., Guo, J., 2009. A new virus of flavivirus: Chaoyang virus isolated in Liaoning province. Chin. J. Pub. Heal. 25, 769–772.

Weaver, S.C., Reisen, W.K., 2010. Present and future arboviral threats. Antiviral Res. 85 (2), 328.