Emergence and Transmission of Arbovirus Evolutionary Intermediates with Epidemic Potential

Kenneth A. Stapleford,[1] Lark L. Coffey,[2] Sreyrath Lay,[3] Antonio V. Bordería,[1] Veasna Duong,[3] Ofer Isakov,[4] Kathryn Rozen-Gagnon,[1] Camilo Arias-Goeta,[5] Hervé Blanc,[1] Stéphanie Beaucourt,[1] Türkan Haliloğlu,[6] Christine Schmitt,[7] Isabelle Bonne,[7] Nir Ben-Tal,[8] Noam Shomron,[4] Anna-Bella Failloux,[5] Philippe Buchy,[3] and Marco Vignuzzi[1,*]

[1]Viral Populations and Pathogenesis Unit, Institut Pasteur, Centre National de la Recherche Scientifique UMR 3569, 28 rue du Dr Roux, 75724 Paris Cedex 15, France
[2]Center for Vectorborne Diseases, Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, 5327 VM3A, Davis, CA 95616, USA
[3]Virology Unit, Institut Pasteur in Cambodia 5, Monivong Boulevard, PO Box 983, Phnom Penh, Cambodia
[4]Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
[5]Arboviruses and Insect Vectors Lab, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris Cedex 15, France
[6]Department of Chemical Engineering and Polymer Research Center, Boğaziçi University, Bebek 34342, Istanbul, Turkey
[7]Ultrastructural Microscopy Platform, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France
[8]Department of Biochemistry and Molecular Biology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
*Correspondence: marco.vignuzzi@pasteur.fr
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SUMMARY

The high replication and mutation rates of RNA viruses can result in the emergence of new epidemic variants. Thus, the ability to follow host-specific evolutionary trajectories of viruses is essential to predict and prevent epidemics. By studying the spatial and temporal evolution of chikungunya virus during natural transmission between mosquitoes and mammals, we have identified viral evolutionary intermediates prior to emergence. Analysis of virus populations at anatomical barriers revealed that the mosquito midgut and salivary gland pose population bottlenecks. By focusing on virus subpopulations in the saliva of multiple mosquito strains, we recapitulated the emergence of a recent epidemic strain of chikungunya and identified E1 glycoprotein mutations with potential to emerge in the future. These mutations confer fitness advantages in mosquito and mammalian hosts by altering virion stability and fusogenic activity. Thus, virus evolutionary trajectories can be predicted and studied in the short term before new variants displace currently circulating strains.

INTRODUCTION

A hallmark of RNA viruses is that rapid replication with high mutation rates can result in the emergence of new epidemic variants (Domingo, 2010). Unfortunately, such variants are usually identified retrospective to epidemics (Davis et al., 2005; Schuffenecker et al., 2006) because wide-scale monitoring of circulating strains is time and labor intensive, and predicting long-term evolution is complicated by ecological factors (Holmes, 2013; Weaver and Barrett, 2004). Recent studies in the adaptation of H5N1 influenza virus to droplet transmission in ferrets exemplifies how evolutionary experiments can shed light on the adaptive pathways available to RNA viruses to increase fitness in a new host (Herfst et al., 2012; Imai et al., 2012). These studies underscore the need to better target surveillance of current strains and predict their evolution, at least in the short term, with more confidence (Holmes, 2013). Since surveillance currently relies on consensus sequencing, adaptive mutations are masked by wild-type sequence and only identified once they dominate the virus population, a process that can take months or years. Depending on the virus under study, the likelihood of identifying emerging variants in a circulating strain can be further hindered by the nature of the isolate: (a) amplification and passage of primary isolates in cell culture can alter the composition of mutants within the population; (b) pooling of samples can dilute the abundance of an emerging variant; and (c) the anatomical origin of the sample may not represent the virus population most likely to be transmitted.

Retrospective studies attempting to experimentally recapitulate and mechanistically explain emergence events from previous epidemics have had some success. For example, in 2005/2006 an epidemic of chikungunya virus that normally circulates in *Aedes aegypti* mosquitoes occurred in the Indian Ocean islands when an alanine-to-valine mutation at residue 226 of the E1 glycoprotein (A226V) promoted enhanced infectivity of another mosquito vector, *Aedes albopictus* (Schuffenecker et al., 2006; Tsetsarkin et al., 2007; Vazeille et al., 2007). Subsequent studies uncovering epistatic interactions of A226V with previously acquired E2 glycoprotein mutations revealed a stepwise evolutionary trajectory that partly explains the delay in its emergence (Tsetsarkin et al., 2011). An ecological factor likely impacting this emergence was the abundance of *Ae. albopictus*

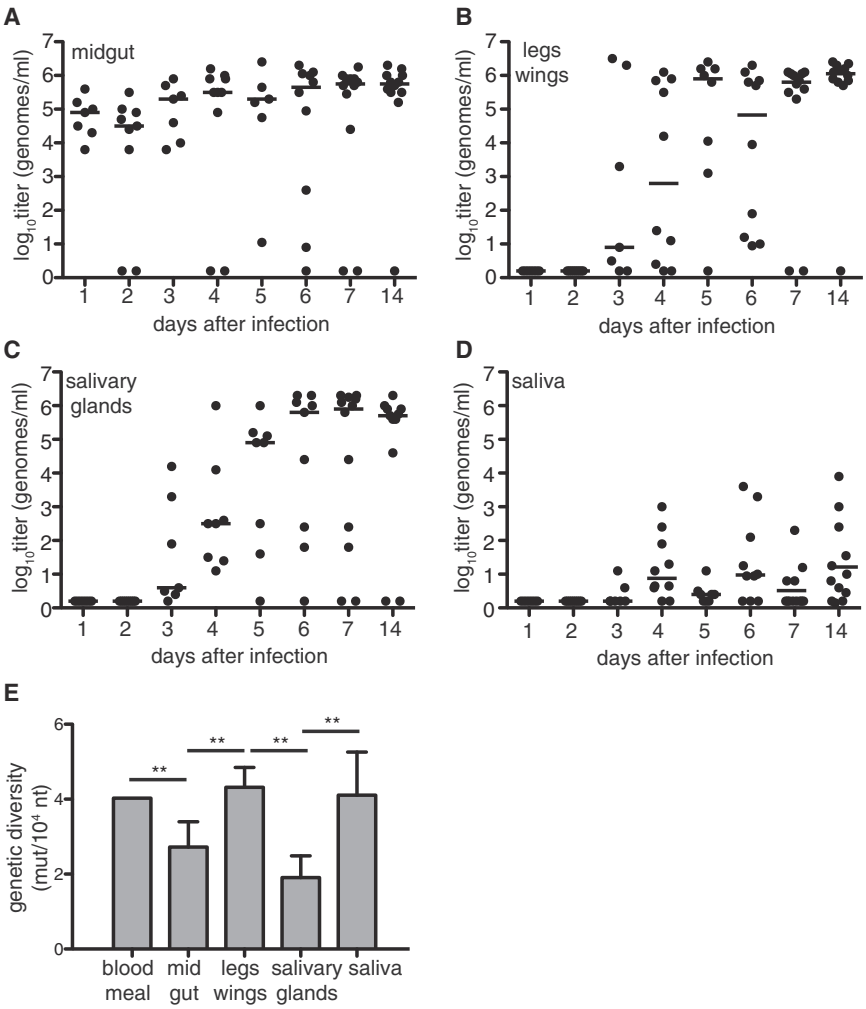

Figure 1. Infection Kinetics and Population Dynamics of Chikungunya Virus in Mosquitoes

(A–D) At indicated days after feeding on blood meals containing 10^6 pfu/ml of chikungunya virus, virus titers were assayed by qRT-PCR in (A) midguts, (B) legs/wings, (C) salivary glands, and (D) saliva from *Aedes aegypti* mosquitoes. Titers for individual mosquitoes (scatter plot) and median (bar) are shown.

(E) The mean genetic diversity present in blood meals, midguts, legs/wings, salivary glands, and saliva from individual mosquitoes 7 days after infection, represented as the average number of mutations per 10^4 nt sequenced. A general trend of reduction in genetic diversity at known anatomical barriers confirms the presence of population bottlenecks (midgut, salivary glands). Between 70 and 96 clones and an average of 82,000 nt were sequenced per sample; n = 6 mosquitoes; **p < 0.01 by X^2 test.

and near lack of *Ae. aegypti* in the Indian Ocean islands compared to endemic regions (Tsetsarkin et al., 2007). Taken together, these conditions created an optimal environment for the emergence of A226V, a problematic event given the world-wide distribution of *Ae. albopictus*. However, although the adaptive mutation required a single nucleotide substitution, the emergence of this variant in nature required years to occur, and as a result the aforementioned studies were performed after the fact. Here, we show that emergence events that require years to identify in nature can be more readily identified and predicted by monitoring transmission site-specific virus subpopulations during experimental infection and transmission in vivo.

RESULTS

Chikungunya Virus Infection Dynamics and Population Bottlenecks

Given that higher fitness variants remain undetected until they displace wild-type after repeated infection and spread across hosts, we hypothesized that studying the spatial and temporal evolution of these viruses in vivo could more quickly identify evolutionary intermediates prior to emergence. Since mosquitoes are persistent carriers of arboviruses, we first optimized experimental conditions by infecting *Ae. aegypti* mosquitoes with chikungunya virus and monitoring replication in midguts (first site of replication), legs and wings (disseminating population), salivary glands (final site of replication), and saliva (transmitted population) (Figures 1A–1D). The initial infection of midguts was followed by increasing titers of circulating and transmitted virus that peaked 7 days after infection. To study whether virus populations differed upstream and downstream of known anatomical barriers, we determined the relative viral genetic diversity in compartment-specific subpopulations at peak titers. Fluctuations in the global genetic diversity in each compartment are in accordance with two population bottlenecks expected to coincide with anatomical barriers in midguts and salivary glands (Figure 1E) that were previously confirmed for West Nile and Venezuelan equine encephalitis viruses (Ciota et al., 2012; Forrester et al., 2012).

Emergence of the Chikungunya Virus E1 A226V Indian Ocean Epidemic Strain in the Saliva of Mosquitoes Infected with the Pre-epidemic Strain

To study the predictive power to identify evolutionary intermediates with this approach, we focused on the viral subpopulations transmitted in the saliva of individual mosquitoes after the peak of infection. We asked whether we could recapitulate the emergence of the E1 A226V amino acid change responsible for the Indian Ocean epidemic (Schuffenecker et al., 2006; Tsetsarkin et al., 2007; Vazeille et al., 2007). We infected both *Ae. aegypti* and *Ae. albopictus* mosquitoes with the pre-epidemic strain (alanine at residue 226) and deep sequenced individual mosquito saliva samples 10 days after infection (Table 1). All ten *Ae. aegypti* mosquitoes presented only the original strain with no detectable changes at residue 226 (limit of detection < 0.01%). In contrast,