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Evolutionarily successful Asian 1 dengue virus 2 lineages contain one substitution in envelope that increases sensitivity to polyclonal antibody neutralization

Chunling Wang[1], Leah Katzelnick[1,2], Magelda Montoya[1], Kien Duong Thi Hue[3], Cameron P. Simmons[3,4], and Eva Harris[1]

[1]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, CA, USA

[2]Center for Pathogen Evolution, Department of Zoology, University of Cambridge, Cambridge, UK

[3]Oxford University Clinical Research Unit, Centre for Tropical Medicine, Ho Chi Minh City, Vietnam; Centre for Tropical Medicine, Nuffield Department of Medicine, University of Oxford, Oxford, UK

[4]Department of Microbiology and Immunology, University of Melbourne, Peter Doherty Institute, Melbourne, VIC, Australia

ABSTRACT

The four dengue virus serotypes (DENV-1-4) cause the most prevalent mosquito-borne viral disease of humans worldwide. DENV-2 Asian 1 (A1) genotype viruses replaced the Asian-American (AA) genotype in Vietnam and Cambodia, after which A1 viruses containing Q or M at envelope (E) residue 160 became more prevalent than those with 160K in both countries (2008-2011). We investigated whether these substitutions conferred a fitness advantage by

measuring neutralizing antibody titer against reporter virus particles (RVPs) representing AA, A1-160K, A1-160Q, and A1-160M using patient sera from Vietnam and a well-characterized Nicaraguan cohort. Surprisingly, we found that A1-160Q and A1-160M RVPs were better neutralized by heterologous antisera than A1-160K. Despite this, Vietnamese patients infected with A1-160Q or A1-160M viruses had higher viremia than those infected with A1-160K. We thus find that independent lineages in Vietnam and Cambodia acquired a substitution in E that significantly increased polyclonal neutralization, but nonetheless were successful in disseminating and infecting human hosts.

Running head

Novel DENV-2 Asian 1 envelope variants

Keywords

Dengue virus; neutralizing antibodies; envelope protein; fitness; evolution; genotype; lineage; Vietnam; Cambodia

Word count of the abstract (154) and of the text (3500)

38 The four dengue virus serotypes (DENV-1-4) cause the most medically important arthropod-
39 borne viral disease of humans worldwide. Up to 96 million dengue cases occur annually,
40 including 500,000 hospitalizations due to severe disease [1]. Within each DENV serotype,
41 multiple genetically distinct lineages have evolved in geographically separated regions [2].
42 Historically in Asia and increasingly in Latin America, all four DENV serotypes and often
43 multiple genotypes and/or clades of each serotype circulate simultaneously for extended periods
44 of time [3]. Antigenic differences between lineages are thought to contribute to disease severity,
45 epidemic cycling, and viral evolution [4-7].

46 Particular DENV genotypes generally have synchronized epidemic cycles and cause the
47 majority of disease for 3-5 years, then become scarce as prevalence of another DENV serotype
48 increases [8]. During periods of serotype dominance, diversification is often observed for all
49 lineages of that serotype, but as serotype incidence wanes, one lineage disappears while another
50 persists [5-7, 9-12]. Some lineage replacements result from competing clades and genotypes that
51 evolve in close proximity for years, while others are due to introduction of a foreign lineage [6,
52 13].

53 One hypothesis is that lineage replacement is stochastic, driven largely by mosquito and
54 virus population bottlenecks, such as annual dry periods with low transmission [9, 12, 14-16].
55 Another hypothesis is natural selection, as lineage replacement events occur over multiple years,
56 suggesting a gradual effect by a selective pressure [6]. Furthermore, some clade replacement
57 events coincide with dynamic changes in the prevalence of co-circulating serotypes, suggestive
58 of interaction/competition between antigenically distinct viruses [5].

59 Two general mechanisms can explain why one virus population may gain a selective
60 advantage over another. First, the dominant clade may have an intrinsic fitness advantage, such

61 as greater viral replication and dissemination in mosquitoes or humans [7, 17]. Alternatively, the
62 dominant clade may have an extrinsic fitness advantage conferred by superior transmission in the
63 presence of host immunity [4, 18]. As for all antigenically variable pathogens, a clade that
64 evades prior host immunity would be able to infect more hosts and replicate better than one
65 constrained by host immunity. However, unique to DENV, a clade that takes advantage of prior
66 host immunity via antibody-dependent enhancement of infection may also have a selective
67 advantage in the face of population immunity [6, 19, 20].

68 DENV is composed of 90 homo-dimers of envelope (E) glycoprotein, the main antigenic
69 target for neutralizing antibodies. E is composed of three domains (EDI, EDII, and EDIII) [21].
70 EDI is the central structural domain, while EDII contains the highly conserved fusion loop that
71 allows for virus-host membrane fusion. Finally, EDIII contains an immunoglobulin-like fold
72 likely involved in receptor binding [22]. Strongly neutralizing human antibodies target EDIII and
73 the hinge region between EDI and EDII [1, 23-28]. The fusion loop region is targeted by both
74 strongly and weakly neutralizing cross-reactive antibodies [29, 30]. Substitution of just a few
75 amino acid positions can substantially alter DENV type-specific neutralization [31], and
76 antigenic differences between genotypes have been described using monoclonal antibodies [32],
77 as well as antisera from experimentally inoculated animals, human vaccine recipients, and
78 naturally infected humans [4, 7, 33, 34]. However, the specific amino acid substitutions/epitopes
79 that determine lineage differences as recognized by polyclonal sera have not been identified.

80 Between 2004 and 2008, the DENV-2 Asian 1 (A1) genotype began replacing the
81 resident Asian-American DENV-2 (AA) genotype in Vietnam and Cambodia. In both countries,
82 the genotype replacement was followed by years of DENV-1 circulation, with DENV-2 again
83 emerging as the dominant serotype a few years later. A study found that A1-infected patients had

84 higher viremia than AA-infected patients, although no significant difference in replication in
85 mosquito cells or mosquito infectivity was noted [13]. We sought to investigate the possible role
86 of pre-existing immunity in driving the genotype replacement and subsequent evolution of the
87 A1 lineage.

88 Here, we describe the unusual observation of a naturally occurring substitution in the E
89 protein of successful DENV-2 A1 lineages that appears to confer an antigenic fitness cost.
90 During the replacement of the AA by the A1 genotype in Vietnam and Cambodia, three separate
91 lineages arose almost simultaneously with substitutions at E position 160 (two with K160M and
92 one with K160Q). We explored the possible role of pre-existing immunity on selection of these
93 viruses by testing polyclonal sera against RVPs representing the previously circulating AA as
94 well as three A1 E-160 variants. Surprisingly, A1-160Q and A1-160M RVPs were better
95 neutralized by polyclonal sera in vitro than A1-160K and AA RVPs. Despite this apparent fitness
96 disadvantage, we found that lineages with 160Q and 160M increased in prevalence in both
97 countries over time and achieved higher viremia in patients than lineages with A1-160K.
98
99 **METHODS**
100 **Cloning of RVPs structural plasmids**
101 RVPs were produced as previously reported [35]. One plasmid encodes the West Nile Virus
102 (WNV) non-structural proteins (NS1-NS5) with a green fluorescent protein (GFP) reporter
103 protein substituting the structural proteins (provided by T.C. Pierson, NIH), and the second
104 plasmid encodes the DENV structural C-prM-E proteins. Structural genes of the DENV-2
105 reference strain 16681 were first cloned into a pcDNA3.1/V5-His-TOPO vector (provided by
106 T.C. Pierson) and served as a cloning intermediate. Substitutions were then made in the 16681 C-

prM-E plasmid using the QuikChange Site-Directed Mutagenesis kit (Stratagene) to construct the AA genotype and A1 genotype variants A1-160K, A1-160Q, and A1-160M (**Table 1**).

Generation of RVPs

To produce RVPs, $4x10^5$ cells were plated per well of a 6-well plate and incubated overnight at 37°C in high-glucose DMEM medium supplemented with 10% Fetal Bovine Serum (FBS; Gibco) and penicillin/streptomycin solution (Gibco). The WNV reporter replicon (1µg), the DENV C-prM-E plasmid (3µg), and MIRUS TransIT-LT1 Transfection Reagent (12µl) were transfected into 293T cells according to the manufacturer's protocol and incubated for 4 hours (h) at 37°C. The medium was then changed to low-glucose DMEM medium supplemented with 10% FBS and penicillin/streptomycin and incubated for 20h at 37°C and 48h at 28°C. The RVP-containing supernatant was harvested and frozen at -80°C.

Serum samples

The Nicaraguan Pediatric Dengue Cohort Study [36] was approved by the Institutional Review Boards of the University of California, Berkeley, and the Nicaraguan Ministry of Health; 18 serum samples collected following primary DENV-1 infection (post-primary DENV-1 samples), 10 post-primary DENV-2 samples, 40 post-secondary DENV-2 samples, and 20 post-primary DENV-3 samples were used in neutralization assays. Vietnamese plasma samples used for neutralization tests were from tetanus patients admitted to the Hospital for Tropical Diseases in 1997-1998 (prior to the AA-A1 genotype replacement event; n=25) and 2006-2007 (after the replacement event; n=27). The scientific and ethics committee of the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City approved the use of these anonymized, pre-collected

plasma samples for infectious disease research. De-identified plasma viremia samples analyzed were from Vietnamese patients hospitalized in the HTD with dengue in 2011 [37].

RVP titration and quality control

Raji-DC-SIGN cells (gift from B. Doranz, Integral Molecular), a human B cell lymphoma line with the DENV attachment factor DC-SIGN [29], were used for the RVP neutralization assay as described (**Supplementary Figure 1A**) [38]. For each RVP lot, the optimal working dilution was determined by titration [38]. To ensure that the antibody-RVP interaction only depended on the neutralization capacity of the antibodies in the serum and was not influenced by the amount of RVPs used, consistent with the law of mass action [39], we performed neutralization assays with a polyclonal DENV-positive control (20 pooled sera from Nicaraguan National Blood Center donors) with concentrations of RVPs above and below the optimal dilution determined from the titration of each lot. The neutralization curves, all with similar NT_{50} values for the three dilutions, are shown in **Supplementary Figure 1B** [39].

Neutralization assay

RVPs were diluted in RPMI complete medium (pH 8.0). RVP neutralization assays were performed as previously described using serial 3-fold dilutions of sera/plasma [38]. Infection of cells was quantified after 48h by measuring GFP-positive cells via flow cytometry and analyzed using FlowJo software. Raw data were graphed as percent infection versus the log of the reciprocal serum dilution, and a sigmoidal dose response curve with a variable slope was generated using GraphPad Prism 5.0 to determine the antibody dilution at which a 50% reduction in infection was observed compared to the no-antibody control (NT_{50}) [38, 40]. Stringent QC

153 rules, including ensuring that viral particles were neutralized according to the law of mass action,
154 the absolute sum of squares was <0.2, and the coefficient of determination (R^2) of the non-linear
155 regression was >0.9, were used to ensure reproducibility of results. Monoclonal antibodies
156 (MAbs) were obtained as follows: E76, E87, E60, E28, and E18 (M.S Diamond, Washington
157 University in St. Louis) [41]; 87.1 and 82.11 (Federica Sallusto and Antonio Lanzavecchia,
158 Institute for Research in Biomedicine) [23]; 4G2 (ATCC).
159
160 **Phylogenetic analyses**
161 The sequence set for phylogenetic analyses consisted of all full-length DENV-2 E genes labeled
162 as isolates from Vietnam and Cambodia that were available in GenBank as of June 2015
163 (n=261). For the phylogenetic tree, a set that represented DENV-2 genetic diversity was also
164 included (n=13). Phylogenetic relationships were inferred with the maximum likelihood (ML)
165 method (version 3.0; http://www.atgc-montpellier.fr/phyml/) using the general time reversible
166 (GTR) nucleotide substitution model with four discrete gamma (Γ) categories of among-site rate
167 variation, allowing for invariant sites (GTR+Γ4+I model). The ML tree topology was estimated
168 using Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) branch-
169 swapping. Trees are unrooted but are drawn with American genotype DENV-2 as the out-group.
170
171 **RESULTS**
172 **Greater neutralization of A1-160Q compared to AA RVPs by Vietnamese serum samples**
173 **from two different periods**
174 We hypothesized that the A1 genotype may have succeeded in replacing the AA genotype by
175 acquiring substitutions that allowed it to better escape population immunity. We first generated

RVPs representing AA and A1 to analyze their neutralization profiles with population-level patient sera collected in Vietnam before and after the AA/A1 genotype replacement. Starting with A1 DENV-2 reference strain 16681, we used site-directed mutagenesis to introduce amino acid substitutions to generate the consensus of either the A1 or AA genotype, which differ at thirteen amino acids in E (**Table 1**). We initially constructed an A1 RVP with Q at position 160 (A1-160Q), as it was a major variant in 2006 when AA was almost fully replaced by A1 in Vietnam. We infected human Raji-DC-SIGN cells with A1-160Q and AA RVPs in the presence of two sets of Vietnamese plasma samples: 25 samples collected in 1997-98, prior to the A1/AA lineage replacement, and 27 samples collected in 2006-07, after the lineage replacement. Both sets were from tetanus patients with unknown prior DENV immune history.

DENV neutralization assays can vary from laboratory to laboratory [42]; here, we used a flow cytometry-based system with human cells, implemented with stringent quality control measures to ensure reproducibility [38]. As expected, AA RVPs were better neutralized by Vietnamese sera collected after the major DENV-2 epidemic (2006-07) than before (1997-98), suggesting that years of intense AA transmission increased the magnitude of the neutralizing antibody response against the AA genotype (**Figure 1A**). No significant difference in NT_{50} titers was observed when A1-160Q RVPs were tested with 2006-07 compared with 1997-98 Vietnamese patient sera (**Figure 1B**). However, when we compared the neutralization titers of the A1-160Q relative to the AA RVPs, we found A1-160Q RVPs were better neutralized by both the pre- (**Figure 1C**) and post- (**Figure 1D**) DENV-2 epidemic Vietnamese sera. This observation raised the unusual possibility of a virus with an apparent fitness disadvantage arising naturally in an endemic setting.

Post-primary DENV-2 AA infection sera neutralize A1-160Q and AA equally, but post-secondary DENV-2 AA sera neutralize A1-160Q better than AA

We further probed the difference between A1 and AA DENV-2 genotypes by titrating the RVPs against a panel of sera from the Nicaraguan Pediatric Dengue Cohort Study, which has been ongoing for 12 years and provides continuous monitoring of all DENV infections that occur in the cohort [43, 44]. This allows for identification of the infecting DENV serotype in primary as well as secondary infections [38]. Coincidentally, all DENV-2 viruses circulating in the Nicaraguan cohort were AA genotype, enabling us to test the role of primary AA and secondary AA immunity against the A1 and AA lineages. With primary DENV-2 sera, we did not observe significant differences between neutralization of genotypes A1-160Q and AA (**Figure 2A**). However, post-secondary DENV-2 infection sera better neutralized A1-160Q compared to AA RVPs (**Figure 2B**). This unexpected observation suggested that A1-160Q viruses may be better neutralized by serotype cross-reactive antibodies.

Changes in prevalence of A1-160K, A1-160Q, and A1-160M following the AA/A1 genotype replacement

We estimated the phylogenetic and temporal relationships among all DENV-2 E genes listed as from Vietnam and Cambodia in GenBank and identified two distinct lineages with variation at E-160 that arose in Vietnam in 2006: one with A1-160Q and another with A1-160M (**Figure 3**). The proportion of A1 isolates with 160Q or M increased until 2008, at which point DENV-1 genotype I viruses were the dominant serotype (2007-2010) and no DENV-2 was sequenced from clinical cases, although DENV-2 did circulate at low levels during this period. When DENV-2 re-emerged as the dominant serotype in 2011, the majority of A1 sequenced in Vietnam

contained 160Q (65%), with a smaller number of A1-160M and K viruses still in circulation (**Table 2**).

While the A1-160Q/M substitution arose in Vietnam, a distinct lineage of A1-160M simultaneously emerged in Cambodia. Although fewer sequences were available on GenBank from Cambodia, all isolates of A1 in Cambodia before 2005 contained 160K. However, in 2007, a lineage of A1 emerged containing 160M, and by 2008, when DENV-1 genotype I also dominated in Cambodia, A1-160M was more commonly isolated than A1-160K (**Table 2**). Although few isolates are available on GenBank after 2008, virological data from Cambodia indicates that A1-160M continued to be isolated in 2010 and 2011 (Philippe Buchy, personal communication). Thus, based on analyses of the available sequences in GenBank, it appears that as DENV-1 genotype I became dominant in both Vietnam and Cambodia, A1 DENV-2 viruses evolved two different amino acids substitutions at E position 160 in three independent lineages.

Post-secondary DENV-2 and post-primary DENV-1 and DENV-3 sera neutralize A1-160Q and AI-160M better than A1-160K

The physiochemical properties of the variants at position 160 differ; the initial lysine (K) is positively charged, while glutamine (Q) is polar uncharged and methionine (M) is hydrophobic. Position 160, located in a valley on the surface of EDI (**Supplementary Figure 2A**), is a contact residue of two potently neutralizing type-specific DENV-1 human antibodies, 1F4 [45] and HM14c10 [46], and is adjacent to a site shown to substantially alter DENV-1 type-specific immunity [31]. In DENV-3 viruses, position 160 is adjacent to the site of amino acid deletions (E157 and E158 in DENV-1, -2, and -4 are absent in DENV-3).

To investigate whether the difference in serotype cross-reactive neutralization by human sera was due to amino acid variation at E-160, we generated A1-160K and A1-160M RVPs and compared their neutralization by Nicaraguan sera from post-primary DENV-1 and post-primary DENV-3 infections, as well as post-secondary DENV-2 infections. Interestingly, the high neutralization titers to A1-160Q RVPs were significantly reduced when the A1-160K RVPs were tested with Nicaraguan cohort sera from post-secondary DENV-2 infections (**Figure 4A**, $p<0.0001$), post-primary DENV-1 infections (**Figure 4B**, $p<0.0001$), and post-primary DENV-3 infections (**Figure 4C**, $p<0.0001$). Like A1-160Q, A1-160M RVPs were also significantly better neutralized by post-secondary DENV-2 infection sera than A1-160K RVPs (**Figure 4D**, $p=0.0369$). Thus, A1-160Q, and to some extent A1-160M, changed the neutralization profile of polyclonal sera to A1 RVPs, making them significantly better neutralized by serotype-cross-reactive sera.

Substitution at E-160 does not result in an overall change to virion structure

Amino acid substitutions may affect antibody binding by directly modifying the corresponding epitope, but they can also affect distant sites by causing a global change to virion structure or affecting the number of epitopes exposed by the virion through "breathing" [47]. To test whether the 160Q and 160M substitutions modified cross-reactive antibody binding by inducing a global change to virion structure, we tested the neutralization profiles of A1-160Q and A1-160K RVPs using a panel of MAbs. We tested MAb 87.1 and E76 (which target the EDIII A strand, a cryptic viral epitope only accessible with viral breathing or global changes to virion structure), E87 (which targets the EDIII C-C loop in the lateral ridge), and MAbs E60, 82.11, E28, E18, and 4G2 (which target the EDII fusion loop) against A1-160Q and A1-160K RVPs, but did not find

267 significant differences in their NT_{50} titers (**Supplementary Figure 2B**). The target of E76 in the
268 EDIII A strand is a temperature-dependent epitope, but we observed similar E76 neutralization
269 profiles of A1-160Q and A1-160K at two different temperatures (4°C and 23°C)
270 (**Supplementary Figure 2C**). These data provide preliminary evidence that the substitution at
271 position 160 does not alter the overall virion structure and suggest that heterologous antibodies
272 sensitive to the 160 substitutions may directly target an epitope that includes position 160.
273
274 **Individuals infected with A1-160Q have significantly higher viremia than those infected**
275 **with A1-160K**
276 We hypothesized that Q and M at position 160, rather than K, must have some in vivo fitness
277 advantage to explain their evolutionary success, despite apparently being better neutralized by
278 polyclonal sera. We compared plasma viremia data for 70 Vietnamese adults infected with A1
279 viruses in 2011. Of these, 70% were secondary, 11% primary, and 19% indeterminate DENV
280 infections. Primary and secondary DENV infections were classified as previously described [37].
281 The majority (67%) were drawn on day 3 of illness, with 24% on day 2 and 8% on day 4. Adults
282 infected with A1-160Q/M were statistically more likely to have secondary immune responses
283 than those infected with A1-160K (83% versus 43%, 2-sample test for equality of proportions
284 with continuity correction, $p<0.002$). The day of viremia measurement did not differ
285 significantly between groups (0.17 days, $p=0.23$, two-sided t-test).
286 On average, individuals infected with A1-160Q/M had 4-fold higher viral titers than
287 those infected with A1-160K (A1-160Q/M=8.05 $\log_{10}$(RNA copies/mL), A1-160K=7.42
288 $\log_{10}$(RNA copies/mL), difference in $\log_{10}$(RNA copies/mL)=0.63; $p=0.03$, as measured by
289 linear regression). The difference in viremia between A1-160Q/M and A1-160K remained

significant when controlling for day of illness (difference in $\log_{10}$(RNA copies/mL)=0.59, $p<0.05$) and only modestly decreased when also controlling for immune status (difference in $\log_{10}$(RNA copies/mL)=0.50, p=0.12). **Figure 5** shows the cumulative distributions of viremia titers for those infected with A1-160K compared with A1-160Q/M, along with the raw viremia data. Thus, although A1-160Q and A1-160M were better neutralized by serotype cross-reactive sera in vitro, viruses with these substitutions achieved higher viremia levels in vivo and were more often observed to cause secondary infections, providing a possible explanation for their increasing rate of detection in clinical cases in Vietnam and Cambodia.

DISCUSSION

Here we report a naturally occurring single substitution in E that significantly alters polyclonal neutralization. We observed that A1 DENV-2 lineages circulating in both Cambodia and Vietnam underwent an amino acid substitution at E position 160, making the viruses more susceptible to in vitro polyclonal antibody neutralization, yet more evolutionarily successful. Based on available DENV-2 E gene sequences, three separate lineages, two with 160M and one with 160Q, arose simultaneously in Vietnam and Cambodia in 2006-2007, and in both countries increased in relative rate of isolation over time. Further, individuals infected with A1-160M and A1-160Q viruses had significantly higher early viremia levels than those infected with A1-160K isolates and were more likely to occur in secondary infections. Our findings suggest that A1-160Q and A1-160M substitutions confer a fitness advantage, which allows them to overcome the fitness cost of being better neutralized by serotype cross-reactive sera. However, the specific causal mechanisms underlying the evolutionary success of the A1-160M and A1-160Q lineages remain elusive.

One scenario is that A1-160Q, and possibly A1-160M, are more successful at replicating in the presence of poorly neutralizing heterotypic antibodies by taking advantage of antibody-dependent enhancement. Globally, DENV-2 is more often isolated from secondary infections [48], and based on our findings, it is plausible that A1-160Q and A1-160M replicate better in DENV-immune individuals than A1-160K. An alternate scenario is that the substitution at position 160 results in an intrinsic fitness advantage in humans and possibly mosquitoes, improving viral replication and dissemination independent of pre-existing anti-DENV antibodies [7, 17].

It is possible that another mutation in the genome explains the fitness advantage. We searched for other variants in E, but position 160 was the only highly variable position; the next most variant site, position 201, was 96% conserved. Full genomes are not available for the 70 viremic adults we studied. However, for all full-length sequences of Vietnamese DENV-2 Asian 1 viruses in GenBank (n=127), E position 160 was the only major variant (>20% variation) in the E gene. Two positions in the NS5 gene (one in the methyltransferase and one in the polymerase) were major variants, but distinct amino acids at these positions did not directly correlate with E-160 variants.

In conclusion, the successful emergence and expanded circulation for multiple years in Vietnam and Cambodia of viruses with a substitution in the E protein (K160Q, K160M) that induces increased susceptibility to cross-neutralization calls for an expanded view of the mechanism(s) of selection driving DENV evolution.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author. Preliminary data were presented at the 63rd Annual Meeting of the American Society of Tropical Medicine and Hygiene, November 2-6, 2014, New Orleans, LA, poster 190, and at the Fourth Panamerican Dengue Research Network Meeting, October 20-22, 2014, Belém, Brazil.

Notes

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Author contributions. E.H. and C.P.S contributed the samples and clinical data; C.W., L.C.K., and E.H. conceived and designed the experiments; C.W., M.M.C, and K.D.T.H. performed the

experiments; C.W., L.C.K., M.M.C., and E.H. analyzed the data; and C.W., L.C.K., and E.H. drafted the manuscript; all authors reviewed the manuscript.

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Corresponding author: Dr. Eva Harris, Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, 185 Li Ka Shing Center, 1951 Oxford Street, Berkeley, CA 94720-3370; Phone: 1-510-642-4845; FAX: 1-510-642-6350; Email: eharris@berkeley.edu

Alternate corresponding author in the event that the corresponding author is unavailable: Chunling Wang, Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, 185 Li Ka Shing Center, 1951 Oxford Street, Berkeley, CA 94720-3370; Phone: 1-510-643-5404; FAX: 1-510-642-6350; E-mail: ChunlingWang@berkeley.edu

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Figure Legends

Figure 1. Neutralization titers to A1-160Q RVPs and AA RVPs in Vietnamese serum samples from two different periods, 1997-98 and 2006-07. RVPs were incubated with 3-fold serial dilutions of serum from Vietnamese samples. The RVP-serum mixture was then used to infect Raji-DC-SIGN cells, and after 48 hours, GFP expression was recorded by flow cytometry and used to calculate the percentage of infection. The NT_{50} was calculated, and the $\log_2$ of the NT_{50} value were plotted on the Y-axis. Prism Graphpad was used to compare the NT_{50} between **(A)** AA RVPs: Vietnamese serum samples from two different periods, 1997-98 and 2006-07; **(B)** A1-160Q RVPs: Vietnamese serum samples from two different periods 1997-98 and 2006-07; **(C)** A1-160Q RVPs and AA RVPs for Vietnamese serum samples from 1997-98; and **(D)** A1-160Q RVPs and AA RVPs for Vietnamese serum samples from 2006-07.

Figure 2. Neutralization titers to A1-160Q RVPs and AA RVPs in serum samples from the Nicaraguan Pediatric Dengue Cohort Study. Neutralization assays were performed as described in the legend for Figure 1. Neutralization titers were compared between A1-160Q RVPs and AA RVPs using **(A)** Nicaraguan post-primary DENV-2 infection sera; **(B)** Nicaraguan post-secondary DENV-2 infection sera.

Figure 3. Phylogenetic tree of Vietnamese and Cambodian DENV-2 isolates. Maximum likelihood phylogenetic tree of the ancestral relationships among full-length DENV-2 E gene sequences from Vietnam and Cambodia, and 13 DENV-2 reference sequences. Strain names are

colored by amino acid at position 160. The background of the tree is shaded to indicate genotype and country for relevant lineages. A time-series (right) shows the year of virus isolation.

Figure 4. A1-160Q, A1-160M and A1-160K RVP variants were tested against Nicaraguan serum samples from the Pediatric Dengue Cohort Study (2004 to present). Neutralization assay were performed as described in the legend to Figure 1. Neutralization titers were compared between A1-160Q RVPs and A1-160K RVPs using **(A)** Nicaraguan post-secondary DENV-2 infection sera; **(B)** Nicaraguan post-primary DENV-1 infection sera; and **(C)** Nicaraguan post-primary DENV-3 infection sera. **(D)** Neutralization titers were compared between A1-160M RVPs and A1-160K RVPs using Nicaraguan post-secondary DENV-2 infection sera.

Figure 5. Viremia levels of 70 Vietnamese patients infected with A1-160K, Q, and M in 2011. **(A)** Cumulative density plot of $\log_{10}$(RNA copies/mL) for infected with A1-160K or M (n=23 and n=2, respectively), A1-160Q (n=45). Linear regression shows the effect of infecting virus (A1-160Q/M vs. K) on viremia levels: black line corresponds to the difference in viremia levels (a significant difference, p=0.03) and average values for those infected with A1-160Q/M and A1-160K are printed adjacent to the line. **(B)** Jitter plot of $\log_{10}$(RNA copies/mL) in individuals infected with A1-160K or A1-160Q/M.

References

1. Sukupolvi-Petty S, Brien JD, Austin SK, et al. Functional Analysis of Antibodies against Dengue Virus Type 4 Reveals Strain-Dependent Epitope Exposure That Impacts Neutralization and Protection. J Virol **2013**; 87:8826-42.

2. Holmes EC, Twiddy SS. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol **2003**; 3:19-28.

3. Messina JP, Brady OJ, Scott TW, et al. Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol **2014**; 22:138-46.

4. Kochel TJ, Watts DM, Halstead SB, et al. Effect of dengue-1 antibodies on American dengue-2 viral infection and dengue haemorrhagic fever. Lancet **2002**; 360:310-2.

5. Zhang C, Mammen MP, Jr., Chinnawirotpisan P, et al. Clade replacements in dengue virus serotypes 1 and 3 are associated with changing serotype prevalence. J Virol **2005**; 79:15123-30.

6. Adams B, Holmes EC, Zhang C, et al. Cross-protective immunity can account for the alternating epidemic pattern of dengue virus serotypes circulating in Bangkok. Proc Natl Acad Sci U S A **2006**; 103:14234-9.

7. OhAinle M, Balmaseda A, Macalalad AR, et al. Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. Sci Transl Med **2011**; 3:114ra28.

8. Cummings DA, Irizarry RA, Huang NE, et al. Travelling waves in the occurrence of dengue haemorrhagic fever in Thailand. Nature **2004**; 427:344-7.

9. Sittisombut N, Sistayanarain A, Cardosa MJ, et al. Possible occurrence of a genetic bottleneck in dengue serotype 2 viruses between the 1980 and 1987 epidemic seasons in Bangkok, Thailand. Am J Trop Med Hyg **1997**; 57:100-8.

444 10. Wittke V, Robb TE, Thu HM, et al. Extinction and rapid emergence of strains of dengue 3
445 virus during an interepidemic period. Virology **2002**; 301:148-56.
446 11. Klungthong C, Zhang C, Mammen MP, Jr., Ubol S, Holmes EC. The molecular
447 epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology **2004**; 329:168-79.
448 12. Thu HM, Lowry K, Myint TT, et al. Myanmar dengue outbreak associated with displacement
449 of serotypes 2, 3, and 4 by dengue 1. Emerg Infect Dis **2004**; 10:593-7.
450 13. Ty Hang VT, Holmes EC, Duong V, et al. Emergence of the Asian 1 genotype of dengue
451 virus serotype 2 in viet nam: in vivo fitness advantage and lineage replacement in South-East
452 Asia. PLoS Negl Trop Dis **2010**; 4:e757.
453 14. Foster JE, Bennett SN, Carrington CV, Vaughan H, McMillan WO. Phylogeography and
454 molecular evolution of dengue 2 in the Caribbean basin, 1981-2000. Virology **2004**; 324:48-59.
455 15. A-Nuegoonpipat A, Berlioz-Arthaud A, Chow V, et al. Sustained transmission of dengue
456 virus type 1 in the Pacific due to repeated introductions of different Asian strains. Virology **2004**;
457 329:505-12.
458 16. Hay SI, Myers MF, Burke DS, et al. Etiology of interepidemic periods of mosquito-borne
459 disease. Proc Natl Acad Sci U S A **2000**; 97:9335-9.
460 17. Quiner CA, Parameswaran P, Ciota AT, et al. Increased replicative fitness of a dengue virus
461 2 clade in native mosquitoes: potential contribution to a clade replacement event in Nicaragua. J
462 Virol **2014**; 88:13125-34.
463 18. Messer WB, Yount B, Hacker KE, et al. Development and characterization of a reverse
464 genetic system for studying dengue virus serotype 3 strain variation and neutralization. PLoS
465 Negl Trop Dis **2012**; 6:e1486.

19. Ferguson N, Anderson R, Gupta S. The effect of antibody-dependent enhancement on the transmission dynamics and persistence of multiple-strain pathogens. Proc Natl Acad Sci U S A **1999**; 96:790-4.

20. Cummings DA, Schwartz IB, Billings L, Shaw LB, Burke DS. Dynamic effects of antibody-dependent enhancement on the fitness of viruses. Proc Natl Acad Sci U S A **2005**; 102:15259-64.

21. Kuhn RJ, Zhang W, Rossmann MG, et al. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **2002**; 108:717-25.

22. Rey FA, Heinz FX, Mandl C, Kunz C, Harrison SC. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature **1995**; 375:291-8.

23. Beltramello M, Williams KL, Simmons CP, et al. The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe **2010**; 8:271-83.

24. de Alwis R, Smith SA, Olivarez NP, et al. Identification of human neutralizing antibodies that bind to complex epitopes on dengue virions. Proc Natl Acad Sci U S A **2012**; 109:7439-44.

25. Fibriansah G, Tan JL, Smith SA, et al. A highly potent human antibody neutralizes dengue virus serotype 3 by binding across three surface proteins. Nature Communications **2015**; 6.

26. Fibriansah G, Ibarra KD, Ng TS, et al. DENGUE VIRUS. Cryo-EM structure of an antibody that neutralizes dengue virus type 2 by locking E protein dimers. Science **2015**; 349:88-91.

27. Rouvinski A, Guardado-Calvo P, Barba-Spaeth G, et al. Recognition determinants of broadly neutralizing human antibodies against dengue viruses. Nature **2015**; 520:109-13.

28. Brien JD, Austin SK, Sukupolvi-Petty S, et al. Genotype-specific neutralization and protection by antibodies against dengue virus type 3. J Virol **2010**; 84:10630-43.

29. Smith SA, de Alwis AR, Kose N, et al. The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein. MBio **2013**; 4:e00873-13.

30. Lai CY, Williams KL, Wu YC, et al. Analysis of cross-reactive antibodies recognizing the fusion loop of envelope protein and correlation with neutralizing antibody titers in Nicaraguan dengue cases. PLoS Negl Trop Dis **2013**; 7:e2451.

31. VanBlargan LA, Mukherjee S, Dowd KA, Durbin AP, Whitehead SS, Pierson TC. The type-specific neutralizing antibody response elicited by a dengue vaccine candidate is focused on two amino acids of the envelope protein. PLoS Pathog **2013**; 9:e1003761.

32. Shrestha B, Brien JD, Sukupolvi-Petty S, et al. The development of therapeutic antibodies that neutralize homologous and heterologous genotypes of dengue virus type 1. PLoS Pathog **2010**; 6:e1000823.

33. Kochel TJ, Watts DM, Gozalo AS, Ewing DF, Porter KR, Russell KL. Cross-serotype neutralization of dengue virus in Aotus nancymae monkeys. J Infect Dis **2005**; 191:1000-4.

34. Katzelnick LC, Fonville JM, Gromowski GD, et al. Dengue viruses cluster antigenically but not as discrete serotypes. Science **2015**; 349:1338-43.

35. Ansarah-Sobrinho C, Nelson S, Jost CA, Whitehead SS, Pierson TC. Temperature-dependent production of pseudoinfectious dengue reporter virus particles by complementation. Virology **2008**; 381:67-74.

36. Kuan G, Gordon A, Aviles W, et al. The Nicaraguan pediatric dengue cohort study: study design, methods, use of information technology, and extension to other infectious diseases. Am J Epidemiol **2009**; 170:120-9.

510 37. Nguyet MN, Duong TH, Trung VT, et al. Host and viral features of human dengue cases
511 shape the population of infected and infectious Aedes aegypti mosquitoes. Proc Natl Acad Sci U
512 S A **2013**; 110:9072-7.
513 38. Montoya M, Gresh L, Mercado JC, et al. Symptomatic versus inapparent outcome in repeat
514 dengue virus infections is influenced by the time interval between infections and study year.
515 PLoS Negl Trop Dis **2013**; 7:e2357.
516 39. Pierson TC, Sanchez MD, Puffer BA, et al. A rapid and quantitative assay for measuring
517 antibody-mediated neutralization of West Nile virus infection. Virology **2006**; 346:53-65.
518 40. Mattia K, Puffer BA, Williams KL, et al. Dengue reporter virus particles for measuring
519 neutralizing antibodies against each of the four dengue serotypes. PLoS One **2011**; 6:e27252.
520 41. Sukupolvi-Petty S, Austin SK, Engle M, et al. Structure and function analysis of therapeutic
521 monoclonal antibodies against dengue virus type 2. J Virol **2010**; 84:9227-39.
522 42. Thomas SJ, Nisalak A, Anderson KB, et al. Dengue plaque reduction neutralization test
523 (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions
524 impact performance. Am J Trop Med Hyg **2009**; 81:825-33.
525 43. Gordon A, Kuan G, Mercado JC, et al. The Nicaraguan pediatric dengue cohort study:
526 incidence of inapparent and symptomatic dengue virus infections, 2004-2010. PLoS Negl Trop
527 Dis **2013**; 7:e2462.
528 44. Balmaseda A, Standish K, Mercado JC, et al. Trends in patterns of dengue transmission over
529 4 years in a pediatric cohort study in Nicaragua. J Infect Dis **2010**; 201:5-14.
530 45. Fibriansah G, Ng TS, Kostyuchenko VA, et al. Structural changes in dengue virus when
531 exposed to a temperature of 37 degrees C. J Virol **2013**; 87:7585-92.

46. Teoh EP, Kukkaro P, Teo EW, et al. The structural basis for serotype-specific neutralization of dengue virus by a human antibody. Sci Transl Med **2012**; 4:139ra83.

47. Dowd KA, Mukherjee S, Kuhn RJ, Pierson TC. Combined effects of the structural heterogeneity and dynamics of flaviviruses on antibody recognition. J Virol **2014**; 88:11726-37.

48. OhAinle M, Harris, E. Dengue pathogenesis: viral factors. In: Gubler DJ, Ooi, E. E., Vasudevan, S., Farrar, J., ed. Dengue and dengue hemorrhagic fever. 2nd ed. Wallingford, UK: CABI, **2014**:231-50.

Table 1. Amino acid positions of DENV-2 E protein at which DENV-2 prototype strain (16681) and representative Vietnamese Asian 1 and Asian/American genotypes differ.

Position	**6**	**83**	**120**	**129**	**141**	**160**	**164**	**203**	**226**	**228**	**308**	**346**	**461**	**478**	**484**	**485**	**491**
Prototype 16681	M	N	R	V	I	K	I	N	T	G	V	H	V	T	I	V	V
VN Asian1	I	K	T	I	V	Q/K/M	V	N	K	E	V	Y	V	S	I	V	V
VN Asian/American	I	N	T	I	I	K	I	D	T	G	I	H	A	S	V	I	A

Table 2. Increasing percentage of A1_160Q virus over time in Vietnam and A1-160M in Cambodia.*

Year	1988	2001	2002	2003	2004	2005	2006	2007	2008	2011
Vietnam										
160K	1	-	-	11	3	2	44	34	1	23
160Q	0	-	-	0	0	0	12	22	5	47
160M	0	-	-	0	0	0	5	3	1	2
% Q (Vietnam)	0	-	-	0	0	0	20%	37%	71%	65%
Cambodia										
160K	-	2	5	9	3	5	0	5	6	2
160M	-	0	0	0	0	0	0	2	18	2
% M (Cambodia)	-	0	0	0	0	0	0	28%	75%	50%

* Analysis was based on sequence analysis of E protein aa 160 using all isolate with Vietnam and Cambodia in name from GenBank.

- = not available

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Supplementary Figure 1. RVP flow cytometry-based neutralization assay. (A) Experimental design for the flow cytometry-based neutralization assay. RVPs are incubated with serial dilutions of the serum/antibody mixture, and then the RVP-serum mixture is used to infect Raji-DC-SIGN cells. After 48 hours, GFP expression is recorded by flow-cytometry and used to obtain the percentage of infected cells. **(B)** Neutralization curves of two representative preparations of Vietnamese AA (left panel) and A1-160Q (right panel) RVPs. Three concentrations of RVPs were used to infect Raji-DC-SIGN cells in the presence or absence of 3-fold serial dilutions of pooled Nicaraguan polyclonal sera (NPS). The percentage of cells expressing GFP was calculated relative to that without NPS to calculate the relative percentage of infection.

Supplementary Figure 2. Substitution at E protein position 160 does not result in an overall change in virion structure. (A) PyMOL structural analysis reveals that aa160 is located in a valley on the surface of E (Domain I; EDI) based on the crystal structure of DENV-2 E protein (1OAN). EDI is shown in red, EDII in yellow, EDIII in blue, and the second monomer in the dimer is shown in cyan. Arrows indicate the residue of interest highlighted in green. The top panel is the cartoon representation, while the bottom panel shows the surface of the E dimer. **(B)** Similar neutralization profile of A1-160Q and A1-160K as tested by a panel of monoclonal antibodies, including MAbs 87.1 and E76 (which target the EDIII A strand), MAb E87 (which targets the EDIII C-C loop in the lateral ridge), and MAbs E60, 82.11, E28, E18, and 4G2 (which target the EDII fusion loop). **(C)** Similar neutralization profile of MAb E76, which targets the temperature-dependent A strand epitope on EDIII, against A1-160Q and A1-160K RVPs at two different temperatures (23°C and 4°C).

Figure 1

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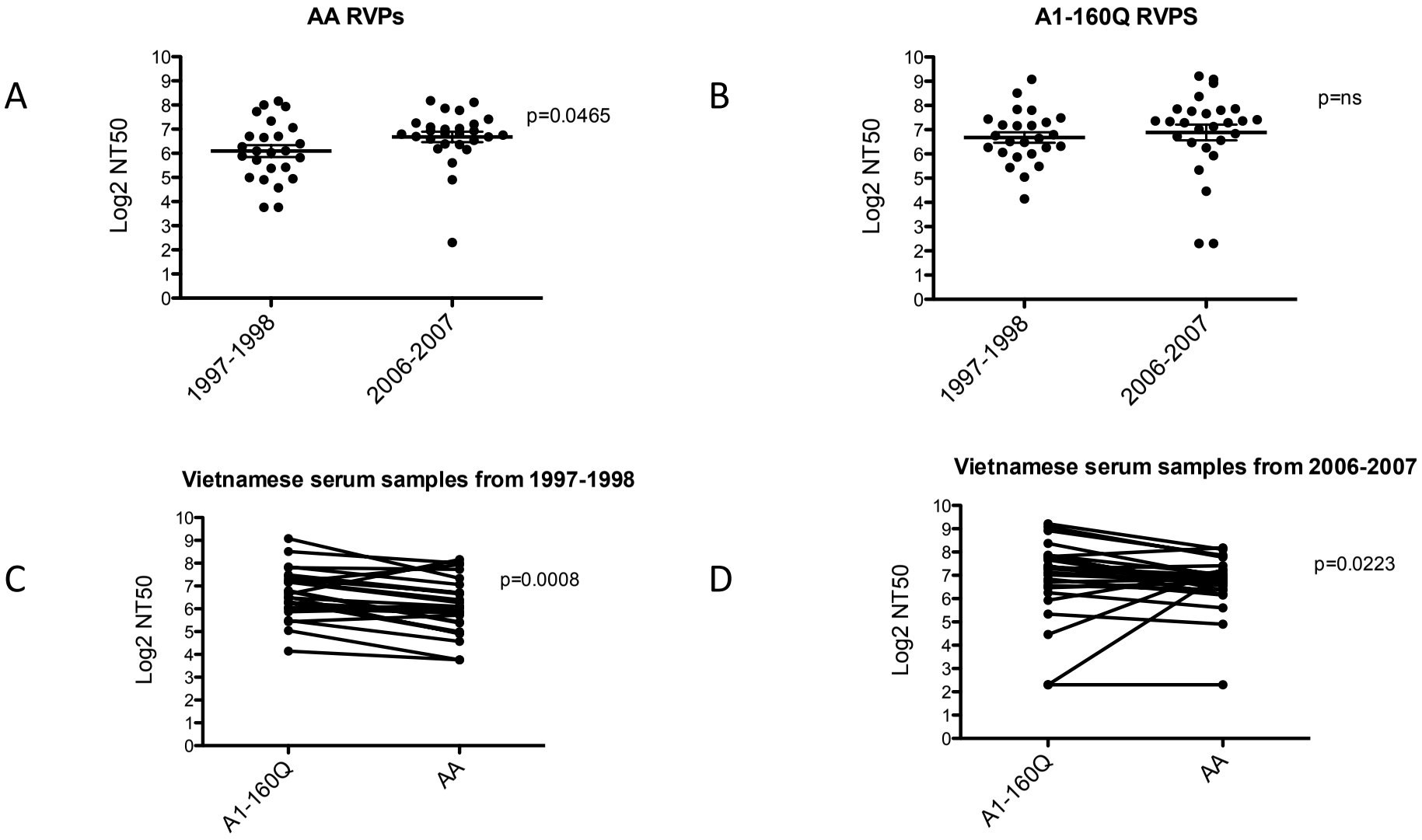

Figure 2

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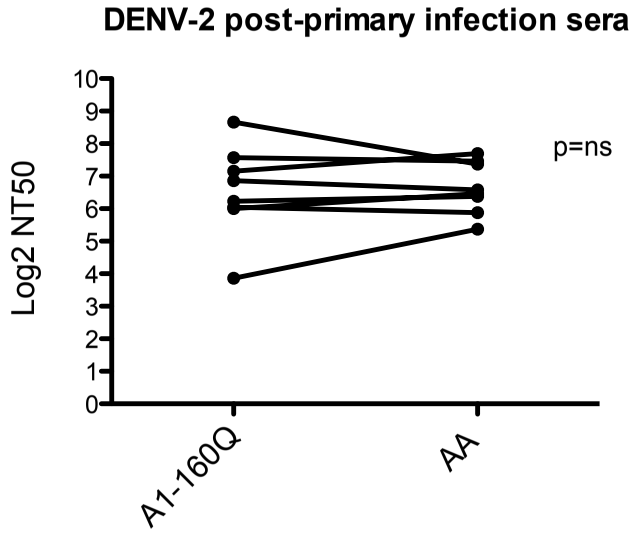

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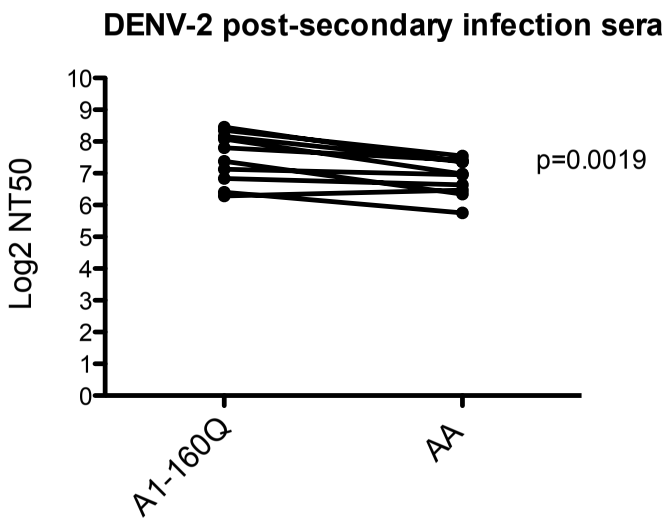

Figure 3

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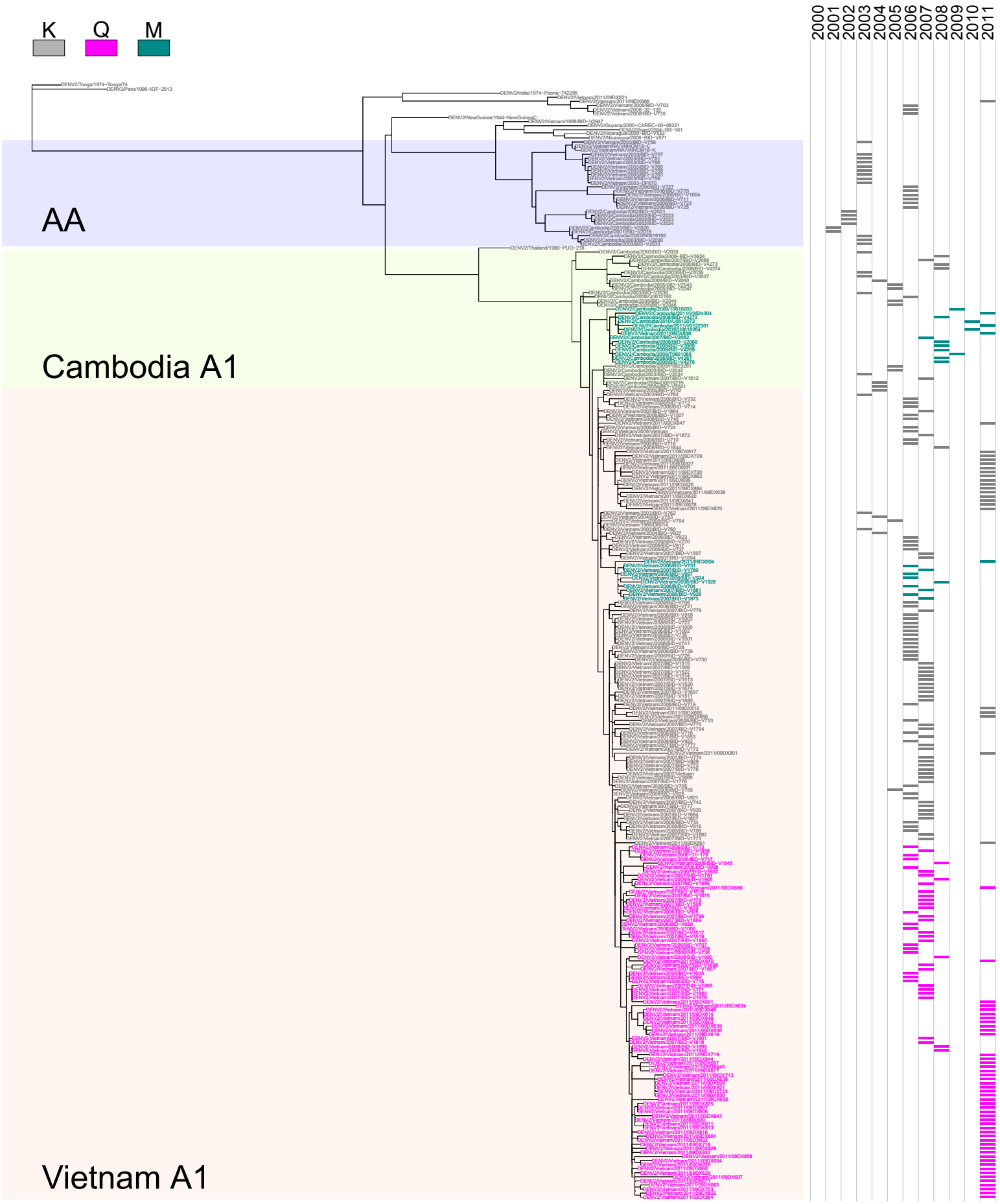

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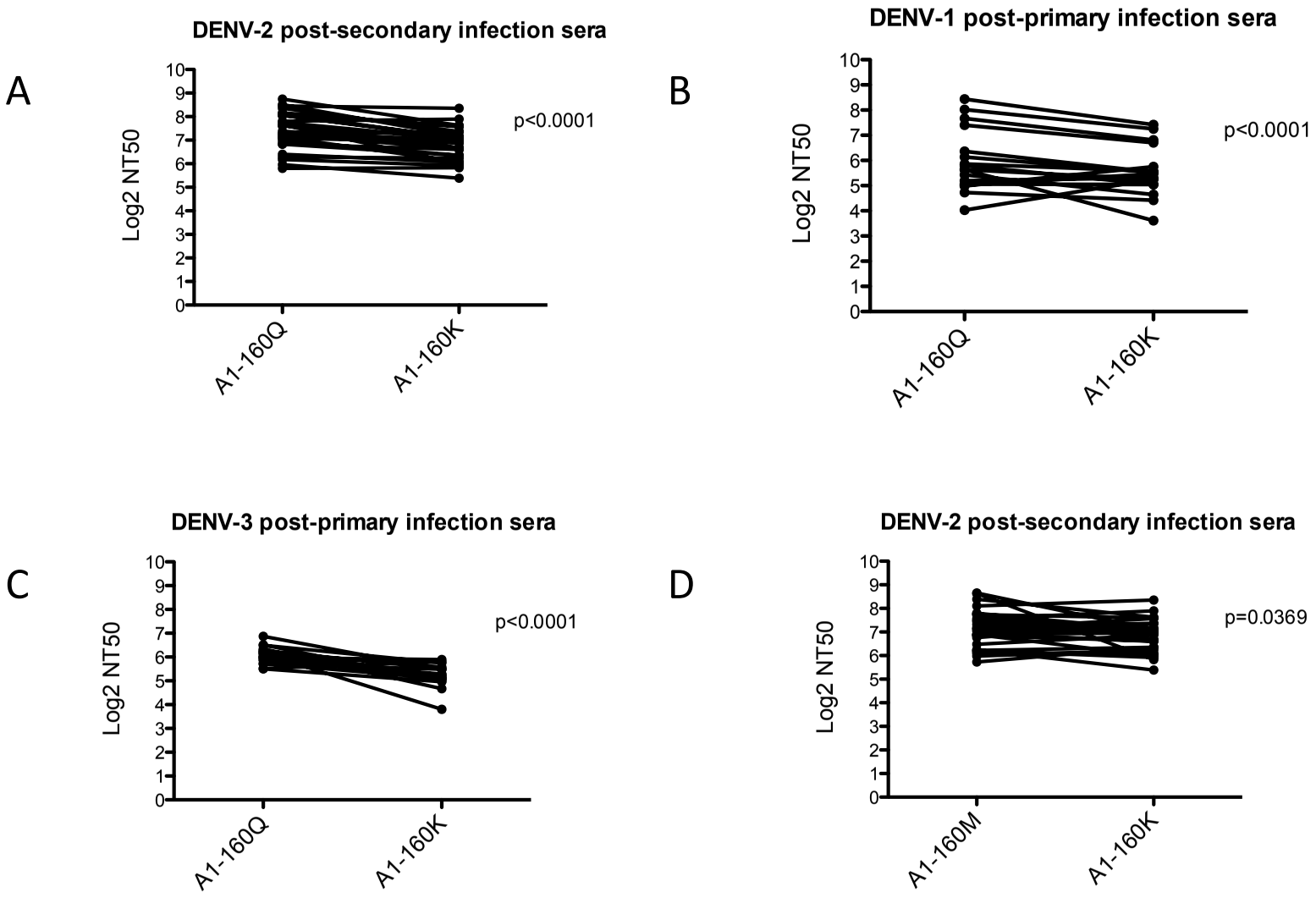

Figure 5

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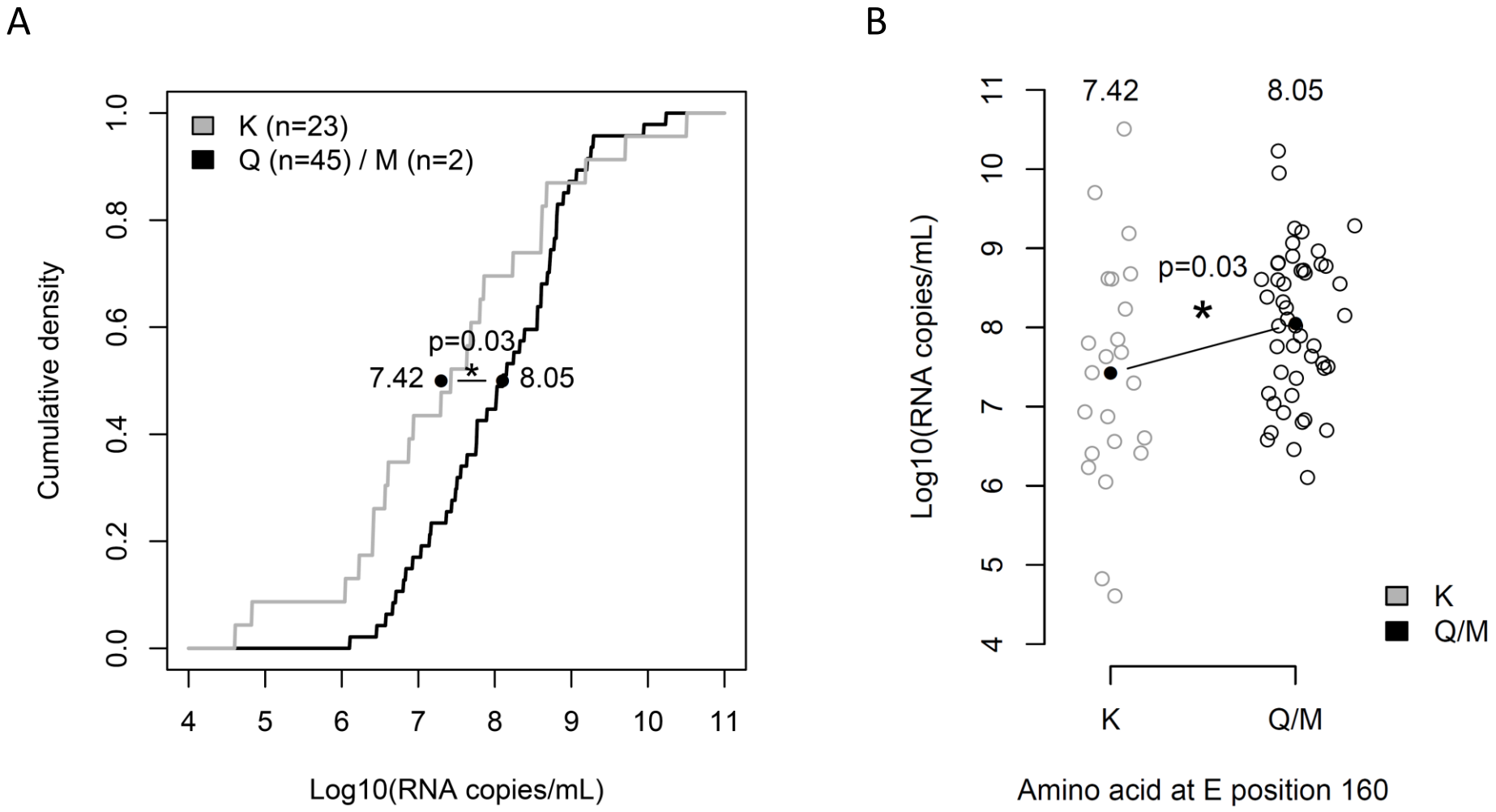

Supplementary Figure 1

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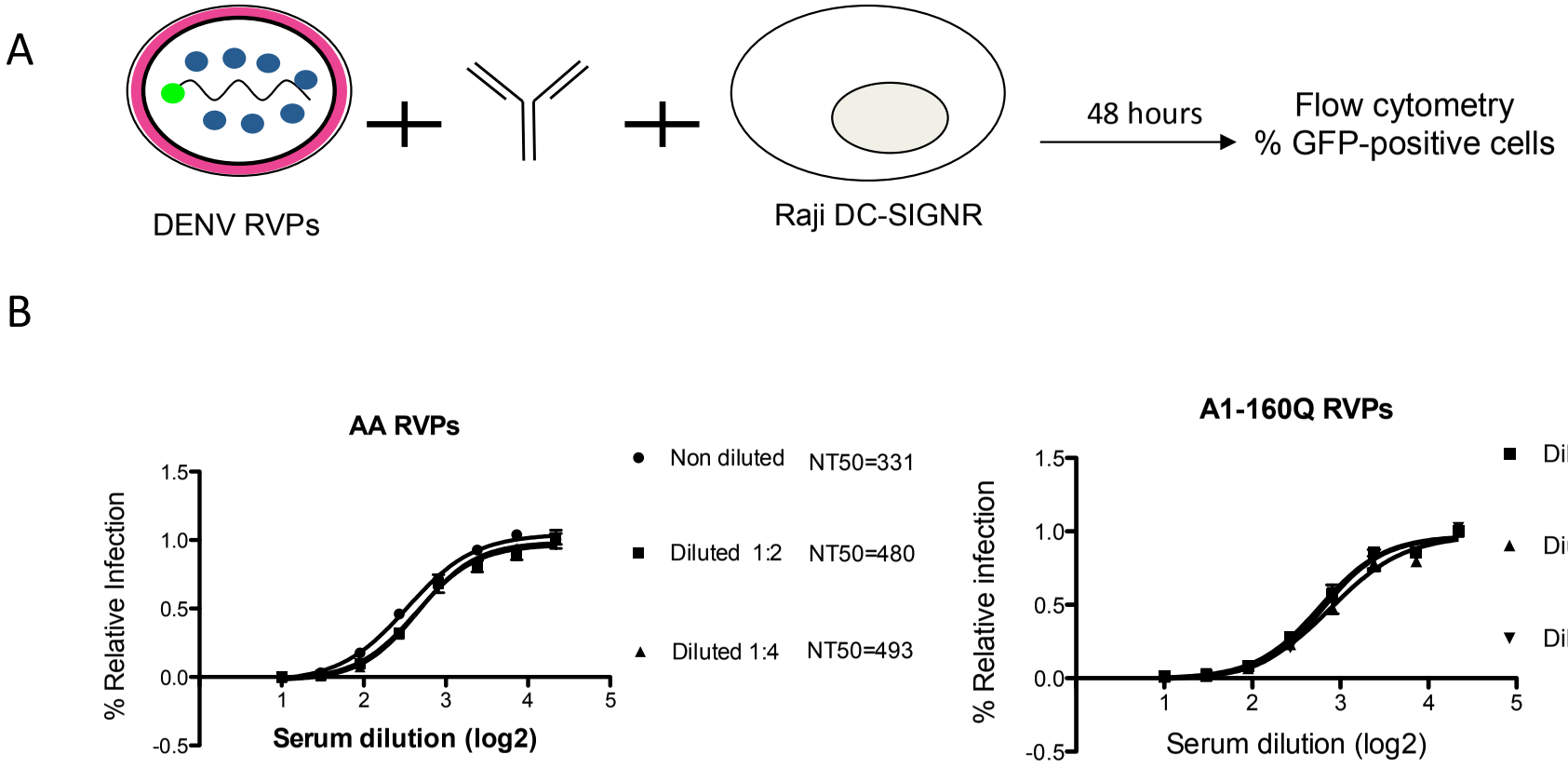
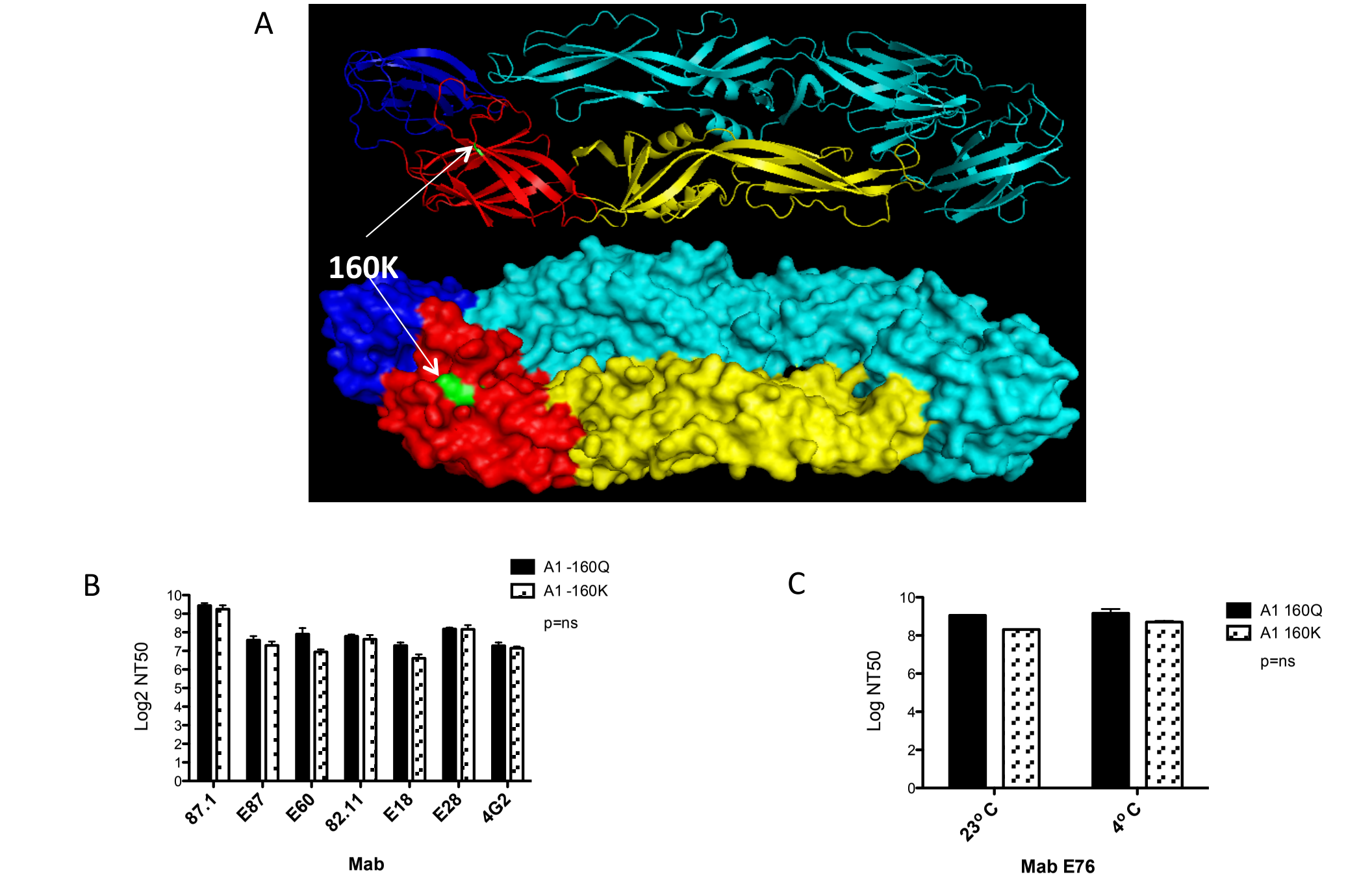


Supplementary Figure 2

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