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Vaccination of Volunteers with Low-Dose, Live-Attenuated, Dengue Viruses Leads to Serotype-specific Immunologic and Virologic Profiles

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Abstract

There are currently no vaccines or therapeutics to prevent dengue disease which ranges in severity from asymptomatic infections to life-threatening illness. The National Institute of Allergy and Infectious Diseases (NIAID) Division of Intramural Research has developed live, attenuated vaccines to each of the four dengue serotypes (DENV-1 – DENV-4). Two doses (10 PFU and 1000 PFU) of three monovalent vaccines were tested in human clinical trials to compare safety and immunogenicity profiles. DEN4 30 had been tested previously at multiple doses. The three dengue vaccine candidates tested (DEN1 30, DEN2/4 30, and DEN3 30/31) were very infectious, each with a Human Infectious Dose 50% 10 PFU. Further, infectivity rates ranged from 90–100% regardless of dose, excepting DEN2/4 30 which dropped from 100% at the 1000 PFU dose to 60% at the 10 PFU dose. Mean geometric peak antibody titers did not differ significantly between doses for DEN1 30 (92 ± 19 vs. 214 ± 97 , $p = 0.08$); however, significant differences were observed between the 10 PFU and 1000 PFU doses for DEN2/4 30, 19 ± 9 vs. 102 ± 25 ($p = 0.001$), and DEN3 30/31, 119 ± 135 vs. 50 ± 50 ($p = 0.046$). No differences in the incidences of rash, neutropenia, or viremia were observed between doses for any vaccines, though the mean peak titer of viremia for DEN1 30 was higher at the 1000 PFU dose (0.5 ± 0 vs. 1.1 ± 0.1 , $p = 0.007$). These data demonstrate that a target dose of 1000 PFU for inclusion of each dengue serotype into a tetravalent vaccine is likely to be safe and generate a balanced immune response for all serotypes.

Keywords

Dengue virus; dengue vaccine; low dose; viremia; neutralizing antibodies; HID₅₀

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INTRODUCTION

Dengue viruses (DENV) have a large impact on global health and economics. With the resurgence of the DENV mosquito vectors, increasing urbanization, and large-scale global travel, the number of dengue infections has steadily increased for the past 60 years [1]. Currently, the four serotypes of DENV are responsible for an estimated 500 million infections annually in more than 100 countries [2].

Clinical illness from DENV infection ranges broadly from asymptomatic infection to life-threatening disease, including dengue hemorrhagic fever and dengue shock syndrome [3]. While most DENV infections resolve without medical intervention, the number of severe dengue cases reported has risen dramatically in the past quarter century, and have become a leading cause of death in children in several southeast Asian countries [1]. Additionally, the impact of dengue illness on the health sector leads to considerable global economic burden in endemic countries, most of which are developing nations [4, 5]. Thus, there is a critical need for therapeutics and a DENV vaccine that are both inexpensive and efficacious.

DENV are unique amongst flaviviruses in that there are four serotypes, and immunity to one serotype does not confer lasting immunity to the other three serotypes (heterotypic immunity) [6]. However, infection with a single DENV serotype provides long-term, homotypic immunity, along with short-lived heterotypic immunity, to the remaining three DENV serotypes [6–8]. One of the risk factors for developing severe dengue disease is prior DENV exposure: pre-existing immunity to one DENV serotype may be detrimental rather than beneficial in subsequent heterologous DENV infections [9, 10]. Therefore, in addition to being safe and inducing durable protection, the ideal DENV vaccine should elicit a balanced immune response against all four serotypes of DENV [11, 12].

Development efforts toward an efficacious, well-tolerated tetravalent DENV vaccine have made significant progress over the past few decades [13–18]. There are currently five different DENV vaccines in various phases of clinical evaluation [19–21]. Live, attenuated virus (LAV) vaccines for DENV are currently the frontrunners in clinical development and these include tetravalent vaccines developed by Sanofi Pasteur, the National Institute of Allergy and Infectious Diseases (NIAID) Division of Intramural Research, and Inviragen. The Sanofi Pasteur vaccine is currently in late-stage clinical trials in endemic regions.

The LAV vaccine candidates for DENV developed at the NIAID were created using a directed mutation approach to generate replication-attenuated viruses which are safe and immunogenic [11]. The safety, infectivity, and immunogenicity profiles of each NIAID monovalent DENV vaccine were characterized in healthy, flavivirus-naïve adult volunteers. In order to maximize the probability of generating a balanced neutralizing antibody response to each DENV serotype, only those candidates with favorable safety and infectivity profiles will be selected for inclusion in a tetravalent (TV) formulation [15, 16, 22].

The infectious dose at which at least 50% of individuals are infected (HID_{50}) with a specific serotype of DENV is not known for other live attenuated vaccine candidates. An aim of this study was to define the HID_{50} for each of these candidate vaccines using a series of phase I clinical trials evaluating very low doses of vaccine candidates. Early studies of the DEN4 30 candidate demonstrated the HID_{50} of rDEN4 30 was below 10 PFU [23]. Therefore, a low dose (10 PFU) of three different monovalent vaccines was compared to a higher dose (1000 PFU) to determine the HID_{50} of each vaccine, and to evaluate differences in the adverse event profiles and neutralizing antibody titers elicited by the different doses [23, 24]. These studies allowed the empirical determination of each monovalent component of the DENV TV formulation, a dose of at least 100-fold the HID_{50} .

The vaccines described in this study, rDEN1 30, rDEN2/4 30, and rDEN3 30/31, are highly immunogenic, safe, and well-tolerated at doses of 1000 PFU [25–27]. Prior studies showed similar results for DEN4 30 at doses ranging from 10^1 – 10^5 PFU [23]. All vaccine candidates including DEN4 30 had an HID_{50} 10 PFU, demonstrating that 1000 PFU is a reasonable target dose for a TV formulation. Herein, we describe the differences in clinical outcomes, viral replication, and neutralizing antibody responses in low-dose vaccines which enable us to best identify a formulation for a tetravalent DENV that encompasses many of the facets of the ideal DENV vaccine.

MATERIALS AND METHODS

Study population

Clinical data and serum samples for this study were derived from three separate phase I clinical trials, performed at the University of Vermont (UVM) Vaccine Testing Center and the Center for Immunization Research at the Johns Hopkins School of Public Health (JHSPH). All three trials followed the same design and clinical protocol, and were approved by the Committees for Human Research (UVM) and the Western Institutional Review Board (JHSPH). In accordance with the Code of Federal Regulations Title 21, Part 50, written informed consent was obtained from all subjects following a review of risks and benefits and a comprehension assessment. Clinical trials are described at Clinicaltrials.gov: NCT01084291, NCT01073306, NCT00831012, NCT00473135, NCT00920517, and NCT00831012.

Healthy adult volunteers between the ages of 18–50 were recruited and enrolled using previously described eligibility criteria [23, 25, 26, 28]. Briefly, subjects were excluded for any chronic medical or psychiatric conditions, or previous history of flavivirus exposure as determined by neutralizing antibody titer.

Study Design

Studies were double-blind, placebo controlled phase I trials to evaluate the safety, immunogenicity, and infectivity of low dose (10 PFU) live, attenuated DENV vaccines, rDEN1 30 (DEN1 30), rDEN2/4 30 (DEN2/4 30) and rDEN3 30/31–7164 (DEN3 30/31), previously assessed and reported at higher doses (1000 PFU) [25–27]. Volunteers for DEN1 30 and DEN2/4 30 were enrolled in each of the low dose trials with a vaccine to placebo ratio of 5:1, while the DEN3 30/31 ratio was 5:2. Randomization codes were used to determine whether volunteers received vaccine or a placebo. The vaccines were administered as a single 0.5 mL subcutaneous dose of 10 PFU for each vaccine; placebo recipients received 0.5 mL of vaccine diluent. As described elsewhere, volunteers in each study followed the identical schedule were monitored for safety (physical exams, symptom review, blood draws), serum vaccine viremia (day of onset, duration, and mean peak titer), and serum neutralizing antibody response (days 0, 28, and 42) [23, 25, 26].

Adverse Event Monitoring

Clinical monitoring was performed as described previously [25, 26]. Volunteers were closely monitored for the 28 days following vaccination for adverse events. All solicited or unsolicited, subjective adverse events were graded as mild (easily tolerated), moderate (interfered with daily activity or required treatment), or severe (prevented daily activity). Vaccine infection was defined as a 4-fold increase in serum neutralizing antibody titers or the detection of vaccine virus in serum. Neutropenia was defined as Grade 1 ($\text{ANC } 1,000$ – $1,500/\text{mm}^3$), Grade 2 (750 – $999/\text{mm}^3$), Grade 3 (500 – $749/\text{mm}^3$), or Grade 4 ($<500/\text{mm}^3$).

Vaccine Virus

Attenuation of DENV-1 and DENV-4 viruses was achieved by deleting 30 nucleotides from the 3' untranslated region (UTR), creating rDEN1-30 and rDEN4-30 [25, 27]. DENV-3 required two separate deletions (30 and 31 nucleotides) in the 3' UTR for appropriate attenuation for human use (DEN3-30/31) [27, 29]. DEN2/4-30 is a chimeric virus in which the DENV-2 *prM* and *E* genes replace those of vaccine candidate DEN4-30 [26, 30, 31].

Vaccines were produced for human administration using current Good Manufacturing Practices at either Charles River Laboratories (DEN1-30 and DEN2/4-30) or Meridian Life Sciences (DEN3-30/31) [25, 26]. L-15 medium (Cambrex BioScience) was used to dilute the vaccine viruses to yield 10 PFU / 0.5 mL immediately prior to vaccination. Vaccine virus titers were determined using a standard plaque assay and serial dilutions of the vaccine virus immediately following vaccine preparation [23, 25, 26, 28].

Virus Quantitation

Serum virus titers (viremia) were measured using a standard plaque assay as described previously [25, 26, 28]. Viremia was described in three ways: mean day of onset, duration, and mean peak titer in serum.

Serologic Assessments

A 60% plaque reduction neutralization titer assay (PRNT₆₀) was used to quantify the antibody response to each DENV serotype for days 0, 28, and 42 as described elsewhere [25, 26, 28]. A 4-fold increase in PRNT₆₀ on study days 28 or 42 (*i.e.* reciprocal titer ≥ 20) compared to the pre-vaccination titer (day 0) was defined as seroconversion. The dose of vaccine virus required to achieve seroconversion or detectable viremia in ≥ 50% of vaccinees was defined as the human infectious dose 50 (HID₅₀).

Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 and SAS, version 9.2. Comparisons of peak neutralizing antibody titers and viremia data were performed using unpaired, two-tailed t tests. Peak neutralizing antibody titers were log transformed. A mixed effects model was used to analyze differences in viremia or rash between doses for DEN3-30/31, and included a random effect to adjust for clinical trial site or ethnicity. The mixed effects model was used only for DEN3-30/31 data as all other data did not differ in site or did not demonstrate statistical significance between sites. For all other trials, a two-tailed Fisher's exact test was used to determine whether the incidence of viremia, rash, or neutropenia differed significantly between doses for the same DENV serotype. A Chi squared test of independence and multiple logistic regression analyses were performed to determine correlations between ethnicity, rash, and infectivity. Reported values are means ± standard error unless noted. A *p* value of ≤ 0.05 was considered significant.

RESULTS

Demographics

The demographics of the 50 vaccinated volunteers enrolled in the three low dose (10 PFU) trials, compared to 141 vaccinated in the comparable trials at the target dose (1000 PFU) are described in Table 1 [25–27]. There were no significant differences in the mean age or in the male:female ratio between the low and target dose cohorts for the 3 vaccines tested: DEN1-30, DEN2/4-30, and DEN3-30/31. There were significant differences in ethnicity (Black vs. non-Black) between doses for DEN1-30 and DEN3-30/31 (*p* < 0.0001 and *p* = 0.002, respectively).

Reactogenicity

All 3 low dose vaccines were well tolerated by volunteers. Mild and short-lived observed adverse events in vaccine recipients included myalgia, arthralgia, transient anemia, transient neutropenia, and a typical mild vaccine-related asymptomatic rash, from all studies at all doses. No fever or thrombocytopenia were observed. No significant laboratory changes were seen in placebo recipients. The dose of vaccine could affect the incidence of adverse events; therefore, a statistical analysis of the incidence either rash or neutropenia, the two most common vaccine-related adverse events, was performed for each dose cohort. Dose-dependent increases in the incidence of rash were not observed for any of the 3 vaccines (Table 2). Likewise, significant differences in the number of transient neutropenia events for any of the vaccines at either dose were not observed (Table 2).

Serology

The peak serum neutralizing antibody titers were determined for the 10 PFU and 1000 PFU doses of the 3 vaccines (Figure 1 and Table 3). Infectivity, defined as seroconversion or detectable viremia, was achieved in ~60% of vaccinees when any of the three vaccine candidates were administered at 10 PFU, indicating that the HID_{50} is < 10 PFU for each attenuated DENV (Table 3). With the exception of DEN2/4-30, the observed infection rates did not differ greatly from those measured for the higher dose, as previously reported [25–27]. Nearly equivalent infectivity rates at both doses of DEN1-30 paralleled similar peak neutralizing antibody titers (expressed as peak geometric mean titers (GMT) (Table 3). A markedly lower infectivity rate for the 10 PFU dose of DENV2/4-30 was also mirrored by a significantly lower peak antibody GMT than the 1000 PFU dose (Table 3). Notably, 10 PFU of DEN3-30/31 generated higher peak antibody GMTs than the 1000 PFU dose. However the standard error on this result was broad (Table 3).

Viremia

Only a short-lived, low level viremia (< 100 PFU / mL blood) is observed in subjects, because these DENV vaccines are attenuated. Nonetheless, detectable viremia may be important for the development of protective immunity and indicates that the viruses replicate *in vivo*, though to a much lesser extent than wildtype DENV which can reach titers as high as 10^8 – 10^9 infectious units / mL [32, 33]. DEN2/40-30 and DEN3-30/31 demonstrated earlier onset of viremia at the higher dose, with the higher dose of DEN2/4-30 displaying the greatest difference, day 9.4 ± 0.5 versus day 12.4 ± 1.1 ($p = 0.01$) (Table 4). Dose-dependent effects for viremia mean peak titers were only evident for DEN1-30 ($p = 0.007$) (Table 4). Lastly, the lower dose of DEN3-30/31 was associated with a higher incidence of viremia ($p = 0.024$), although the mean peak titer did not differ.

Infectivity

Associations between ethnicity (African-American, non-African-American), rash (presence or absence), and infectivity were sought to determine whether the vaccines performed differently in different populations. No correlations were found except an association of rash and infectivity ($p = 0.045$) for DEN3-30/31. This result may be due to differences in the numbers of volunteers of varying ethnicities in the reported trials as was seen in TV vaccine trials that observed differences in infectivity and incidence of rash [34].

DISCUSSION

As part of an initiative to develop a safe and efficacious live-attenuated tetravalent DENV vaccine, findings from a series of phase I clinical trials were compared to evaluate the performance of 3 low dose (10 PFU) vaccines (serotypes DENV-1, -2, and -3) against

increased doses (1000 PFU) of the same vaccines. As in earlier work with similarly attenuated DENV vaccines, all monovalent vaccines evaluated were uniformly well-tolerated with short-lived and mild adverse events [23, 25, 26, 28, 35, 36]. Peak viremia levels observed for these vaccines remained low, < 100 PFU / mL, and neutralizing antibody responses were robust.

The most intriguing finding from the current work is the observation that the HID_{50} for this set of attenuated vaccine candidates is extremely low, 10 PFU for each of the four serotypes [23]. Greater than 90% seroconversion following vaccination with 10 PFU of DEN1 30 and DEN3 30/31 was achieved; prior work has demonstrated 100% seroconversion with 10 PFU of DEN4 30 [23]. The DENV2/4 30 chimeric virus is likely the most attenuated of the NIAID-developed vaccines; the 10 PFU dose was associated with 60% infectivity and 33% viremia indicating that the HID_{50} is at or just below 10 PFU for this vaccine candidate. As demonstrated in non-human primate studies, this data confirms the strongly-attenuating effects of chimerization strategies [30]. Indeed, another NIAID-developed chimeric vaccine candidate, DEN3/4 30, was found to be over-attenuated with an HID_{50} of > 10^5 PFU, and did not advance further in clinical development (data not shown).

Although a HID_{50} for wild type DENV in humans has never been specifically determined, studies from the World War II era were performed in humans with natural DENV [6]. The determination of a human minimal infectious dose (MID) required a 10^6 serum dilution. Notably, when DENV-infected serum was reinjected back into human volunteers intradermally, the clinical disease caused by 10 MID was equally severe as that caused by 10^6 MID, thus indicating the high infectivity of DENV. The data from the current study demonstrates that despite attenuation, the NIAID live attenuated DENV vaccines remain highly infectious.

The ability of live attenuated DENV vaccines to replicate in vaccinees and induce an immune response in the absence of undesirable clinical signs is exemplified by the findings of these clinical trials. Despite a 100-fold decrease in dose, the 10 PFU dose of each vaccine was generally associated with similar or slightly increased incidences of either rash or viremia; however, 1000 PFU doses were associated with earlier onset of viremia for DEN2/4 30 and DEN3 30/31, and slightly higher viremia for DEN1 30. The reported adverse events remained mild at either dose. However, the antibody response to DEN2/4 30 was less robust at the lower dose, presumably due to its decreased infectivity at the 10 PFU dose. Alternatively, virus interference may have occurred, resulting in decreased apparent infectivity of DEN2/4 30 [37].

These findings also highlight DENV serotype differences. Each DENV serotype demonstrated unique patterns of viremia and neutralizing antibody responses. For example, minimal dose differences were noted in the 10 PFU vs. target dose evaluation of DEN1 30. The low dose of DEN2/4 30 was associated with the weakest neutralizing antibody response, and clearly a higher dose than 10 PFU will be necessary to elicit a satisfactory immune response[28]. Of interest, following vaccination with DEN3 30/31, volunteers receiving the 10 PFU dose had a higher incidence of viremia and higher neutralizing antibody titers than the 1000 PFU dose. Given the large standard error on this result, we suspect it may not be generalizable to DENV-3 vaccines unless confirmed with larger numbers of subjects. Nevertheless, an inverse correlation with dose and neutralizing antibody responses has been seen previously with other flaviviruses and flavivirus vaccines, including Yellow Fever and Japanese Encephalitis vaccines[38–40]. As noted, this association may be due to small subject numbers; alternatively, lower doses of some serotypes may better activate innate immune responses. More broadly, these findings may

highlight serotype and strain differences that will be important to confirm as tetravalent formulations are constructed and tested in research and field settings.

Although these clinical trials were closely monitored for the time periods described, safety and immunology data collection was limited to six weeks following vaccination for the low dose studies. Immune responses that occurred or peaked beyond this time would have been missed. Particularly in the low dose studies, if viral replication was delayed, the development of neutralizing antibodies might be later than anticipated, leading to an underestimation of the neutralizing antibody response. This is most important for the low dose of DEN2/4 30, in which the onset of viremia was markedly later than for the 1000 PFU dose, and, accordingly, the available mean peak antibody titers were significantly lower. However, the onset of viremia was delayed for the 10 PFU dose of DEN3 30/31 and higher antibody titers were detected, so clearly viremia is not the only factor in the development of a robust neutralizing antibody response. Extended follow-up periods in future studies will be helpful to determine the kinetics of the neutralizing antibody response to vaccination, especially with DEN2/4 30.

With increases in urbanization, travel, and the geographic distribution of mosquito vectors, there is an urgent need for an effective, inexpensive DENV vaccine that can be delivered rapidly in a minimum number of doses. These studies are a part of a large NIAID-led effort towards such a vaccine. Individual evaluation of monovalent vaccine candidates prior to their incorporation into tetravalent formulations has provided extensive information critical to the selection of strains and doses for a tetravalent formulation. The initial dose of each monovalent component included in the TV formulation will be 100-fold higher than the HID_{50} in order to ensure a vaccine “take” and a balanced immune response in vaccinees. It is anticipated that these efforts will result in the induction of a neutralizing antibody response to all four DENV serotypes by the TV vaccine with a minimum of administered doses. Furthermore, studies with these monovalent vaccines provided useful insights about each DENV serotype and may be useful in understanding asymptomatic, natural DENV infections. The data obtained from these studies have, to-date, been used to formulate tetravalent dengue vaccines which yielded at least trivalent, balanced neutralizing antibody responses in a majority of vaccinees in early phase studies [34]. Doses will require confirmation in endemic settings, but we hope the strategy employed in these clinical trials will yield a safe, efficacious tetravalent DENV vaccine that is accessible to regions with the greatest need.

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Highlights

- Two doses of three monovalent DEN vaccines were compared in human clinical trials.
- The human infectious dose 50% was identified for three DEN viruses as 10 PFU.
- No significant differences in safety profiles were observed between doses.
- Dose did affect neutralizing antibody titers for two of the vaccine candidates.
- The ideal target PFU (1000) for each DENV in a tetravalent vaccine was identified.

Figure 1. Geometric mean peak neutralizing antibody titers differ significantly between doses for DEN2/4 30 and DEN3 30/31

The geometric mean peak neutralizing antibody titers are shown for each low dose (10 PFU) and corresponding target dose (1000 PFU) of vaccine. An asterisk (*) denotes $p = 0.046$, (**) denotes $p = 0.001$, and *ns* = not significant.

Table 1

Study demographics for each DEN vaccine study at two doses.

Vaccine candidate	Dose (PfU)	# Vaccinated/Total ^d	% Female (n)	Mean Age ± SE	African-American/Non-African-American ^e
DEN1 30	10	15/18	27(4)	27.6 ±2.2	0/15
	1000 ^a	71/90	49 (35)	31.0 ± 1.1	44/27
DEN2/4 30	10	15/18	53(8)	35.4 ±2.4	11/4
	1000 ^b	20/25	50(10)	30.4 ±1.6	14/6
DEN3 30/31	10	20/28	60(12)	33.0 ±2.3	18/2
	1000 ^c	50/70	31 (62)	30.4 ±1.3	25/25

^aData described in [25]. One subject was lost to follow-up prior to Day 28.

^bData described in [26].

^cData described in [27]. Two subjects were lost to follow-up prior to Day 28.

^dThese numbers show the number of subjects vaccinated over the total number of subjects enrolled in a trial.

^eEthnicity data is shown only for vaccinees in each trial. Placebos are not shown.

Table 2

Vaccine dose does not affect the incidence of rash or neutropenia.

	DEN1 30 (PFU)			DEN2/4 30 (PFU)			DEN3 30/31 (PFU)		
	10	1000	p value	10	1000	p value	10	1000	p value
Rash									
n (%)	2 (13%)	22 (31%)	0.22	3 (20%)	5 (25%)	1.0	3 (15%)	22 (44%)	0.47 ^a
Neutropenia									
n (%)	3 (20%)	17 (24%)	1.0	2 (13%)	4 (20%)	0.68	2 (10%)	2 (4%)	0.57

^a A mixed effects model was used to account for ethnicity effects on incidence of rash [41]. Values shown are means ± SE.

Table 3

DEN vaccine candidates are highly infectious even at 10 PFU dose..

Vaccine candidate	Dose (PFU)	Infectivity ^a (%)	Seroconversion ^b (%)	Peak neutralizing antibody titer (GMT ± SE)	Peak GMT antibody titer p values
DEN1 30	10	93	93	92 ± 19	0.08
	1000	96	94	214 ± 97	
DEN2/4 30	10	60	53	19 ± 9	0.001
	1000	100	100	102 ± 25	
DEN3 30/31	10	90	90	119 ± 135	0.046
	1000	90	82	50 ± 50	

^aInfectivity is defined as the presence of detectable viremia or a neutralizing antibody titer 20 (seroconversion).
^bSeroconversion defined as a 4-fold increase in PRNT₆₀ on study days 28 or 42 (titer 20) compared to the pre-vaccination titer (day 0).

Table 4

DEN vaccine candidate serotype and dosage affect incidence of viremia and viral load.

Vaccine candidate	Dose (PFU)	Volunteers with Viremia, n (%)	Peak titer ^a (log ₁₀ PFU / mL)	Day of Onset ^a	Duration (Days) ^a
DEN1 30	10	8 (53%)	0.5 ± 0 ^b	11.8 ± 0.7	3.5 ± 0.8
	1000	43 (61%)	1.1 ± 0.1 ^b	10.1 ± 0.4	3.5 ± 0.3
DEN2/4 30	10	5 (33%)	0.7 ± 0.2	12.4 ± 1.1 ^c	1.4 ± 0.4
	1000	13 (65%)	0.5 ± 0	9.4 ± 0.5 ^c	3.4 ± 0.9
DEN3 30/31	10	13 (65%) ^b	0.8 ± 0.1	9.5 ± 0.9 ^c	4.5 ± 1.0
	1000	17 (34%) ^b	0.6 ± 0.1	7.6 ± 0.4 ^c	3.2 ± 0.5

^aMean ± SE

^bA mixed effects model was used to correct for site and ethnicity effects on incidence of viremia [41]. Data from the 10 PFU cohort was significantly different from the 1000 PFU cohort ($p = 0.024$).

^cData from the 10 PFU cohort was significantly different from the 1000 PFU cohort ($p = 0.05$).