

Rapid travel to a Zika vaccine: are we heading towards success or more questions?

Abstract

Introduction: Emergence of the Zika virus (ZIKV) in Latin America in 2015-2016 led to an expeditious search for vaccine candidates, with a DNA-based candidate having progressed to Phase II. However, several features of ZIKV infection and epidemiology are not understood which may be key to maximizing efficacy and ensuring safety of ZIKV vaccines.

Areas covered: Conceivable problems related to vaccine development and policy include: (1) paucity of diagnostics to satisfactorily discriminate between past ZIKV and dengue virus (DENV) exposure; (2) insufficient knowledge of the mechanisms of ZIKV neurovirulence, amongst other unknowns in the biology of this infection, is particularly relevant from a vaccine safety perspective; and (3) the potential for disease enhancement, as observed with DENV infection and vaccine.

Expert opinion: Vaccine candidates that entered phase I/II trials have demonstrated protection in naïve animal models, while ZIKV epidemics occurred in populations that had encountered DENV before. The resulting cross-reactive antibodies pose problems for reliable serologic diagnostic assays, and for the potential of disease enhancement. The alleged neurological complications also warrant further exploration in order to reassure regulators of the safety profile of these vaccines in target populations. These research aspects should be an integral part of the efforts to develop a vaccine.

26 **Keywords:**
27 Antibody Dependent Enhancement
28 Diagnostic
29 Neurological complications
30 Serology
31 Vaccines
32 Zika virus
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1. Introduction

1.1 Zika virus (ZIKV) is the latest arthropod-borne virus (arbovirus) to cause global concern. It is a positive sense RNA virus belonging to the genus *Flavivirus* which comprises several species that affect human populations, mostly through arthropod vectors in areas of endemicity (such as yellow fever virus and dengue virus,^{1,2}). It is transmitted by mosquito bites from several *Aedes* species, spanning tropical areas, Southern US and Southern Europe. The virus can be found in blood, semen, urine, and saliva, and there is evidence of sexual and perinatal transmission, and a theoretical possibility of transmission *via* blood transfusion^{1,3,4}. ZIKV infections are mostly non-symptomatic (in approximately 80% of infections as estimated during the outbreak in the Yap island in 2007⁵), or cause a mild disease with non-specific symptoms over a week. Severe cases involving hospitalization are uncommon, and deaths are rare, even in cases of co-infection with dengue, Chikungunya, and HIV⁶⁻¹⁰. The international attention that ZIKV attracted is due to the serious complications identified during the re-emergence and spread of the disease over the Pacific Islands and the Americas. An 1% risk of microcephaly was estimated in pregnant women following the 2013 outbreak in French Polynesia^{11,12}. In Brazil, a 20-30 fold increase in suspected cases of microcephaly was noted in the areas affected by ZIKV, and a eight fold increase in confirmed cases¹³⁻¹⁷. An increase in the incidence of Guillain-Barré syndrome (GBS) from 1-2/100.000 to 24/100.000 was observed in French Polynesia, and the course of the syndrome was rapid and severe, requiring intensive care and mechanical ventilation¹⁸⁻²⁰. Finally, other neonatal congenital manifestations such as intracerebral calcifications and hearing loss were reported on imaging studies²¹⁻²³.

1 1.2 Major progress has been made in understanding the virus structure²⁴, virulence
2 factors, host response²⁵ and environmental factors facilitating transmission of ZIKV.
3 However, significant advancements are still necessary in the spaces of diagnostic
4 reliability and specificity. as well as efficacious therapeutic and immunization
5 strategies. Announcements from the US Centers for Disease Control and Prevention
6 (CDC) establishing the role of ZIKV in congenital anomalies in newborns stimulates
7 the need to elucidate preventive strategies as well as commitment from US
8 Biomedical Advanced Research and Development Authority (BARDA) for ZIKV
9 vaccine development²⁶, particularly since over half the world's population resides in
10 areas infested with *Aedes aegypti*, the principal vector of ZIKV transmission.

11 Over 20 ZIKV vaccine candidates are currently in development, some based on the
12 vaccine technologies used for Japanese encephalitis virus or DENV, and include
13 inactivated whole virus, chimeric live attenuated vaccines, recombinant proteins, and
14 several vectored vaccine platforms, all listed and described in a recent review²⁷. The
15 DNA plasmid candidate developed by the US NIAID (National Institute of Allergy
16 and Infectious Disease) has progressed to the phase II clinical trial^{27,28}. Major vaccine
17 manufacturers are in the process of developing ZIKV vaccine candidates *via* both
18 novel and traditional platforms. Although one of the big names in vaccine
19 development (Sanofi Pasteur) has prematurely ended its quest²⁹, many other
20 candidates have demonstrated the ability to prevent ZIKV infection in murine and
21 primate animal models^{27,30} as described in reviews by Barzon *et al*²⁷ and Blackman *et*
22 *al*³¹. However, strategies to assess vaccine impact as well as specific and reliable
23 diagnostics still lag behind, raising questions with regard to priorities and prudent
24 approaches to effectively deal with a subsequent outbreak. The target product profiles
25 (TPPs) for ZIKV vaccines as detailed by the WHO suggests a non-replicating

1 platform or platforms which have licensed human vaccines within their class and
2 ideally, should have no contraindication for use during pregnancy or in lactating
3 women in addition to primarily targeting women of reproductive age and boys/men of
4 the same ages³².

5 This current manuscript focuses on ZIKV vaccine development in light of the recent
6 outbreak in South America and the vaccine trials planned in these areas.

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2. Paucity of ZIKV serological diagnostics and impact on strategies to assess

ZIKV vaccine efficacy and impact

2.1 Difficulty to diagnose ZIKV infections by serological assay

The routine diagnostic tools used since the 1970s to identify arbovirus infections have been based either on virus isolation or on capture ELISA. The ELISA formerly used a crude antigen mix produced in suckling mouse brain infected with the flavivirus of interest³³. Flaviviruses are phylogenetically and serologically related, and this mix contains several antigens that have a degree of conservation across the different flavivirus species. The sequence identity between ZIKV and DENV polyprotein is 55.1%-56.3%³⁴. Within the envelope protein, the domains I, II and III (DI, DII, and DIII) of ZIKV and DENV share 35, 51, and 29% amino acid identity, respectively²⁵. The non-structural 1 (NS1) proteins also shares 51 to 53% of amino acid identity between ZIKV and DENV²⁵. The cryo-electron microscopy structure of ZIKV was resolved recently, and high similarity was observed with the structure of other known flavivirus structures²⁴. This extensive cross-reactivity between flaviviruses hampers the accurate identification of the causative agent in areas where several flaviviruses are endemic and co-circulating³³. Currently, the diagnostics available for laboratory and research purposes include virus isolation, detection of viral RNA and neutralisation assay³⁵. The plaque reduction neutralising assay is more specific than the ELISA mentioned above, however cross-reactivity is observed after secondary flavivirus infection¹⁹, and there is variability between laboratories. In order to support vaccine development, particularly in phase II and phase III trials, serological assays offer the most promise in assessing vaccine impact as opposed to detection of viral RNA, which is only transient.

1 Moreover, the cross-reactivity of the current serologic assays poses difficulties in
2 quantifying the immunogenicity of the vaccines. Pre-clinical and phase I studies
3 involved only hosts that were immunologically naïve to all flaviviruses, and therefore
4 made estimating immunological responses to ZIKV vaccine candidates relatively easy
5 by simply estimating neutralising antibody titres in these study participants. The
6 challenge of estimating immunological responses in populations exposed to various
7 flaviviruses still remains, as currently available serological tests do not have a
8 satisfactory discriminatory capability in distinguishing between ZIKV and other co-
9 circulating flaviviruses. This is imperative in order for gains to be made in subsequent
10 phases of ZIKV vaccine development. The VRC-ZKADNA090-00-VP candidate, a
11 DNA-based vaccine developed by the US NIAID is well into its phase II trial²⁸. This
12 multi-centre trial has study sites in Florida, Texas, Peru, Mexico, Puerto Rico and
13 Panama, and the population studied will encompass adolescents and adults. A
14 confirmed past infection of ZIKV is one amongst various exclusion criteria²⁸, which
15 is essential at this stage of vaccine testing but is likely to miss the majority of ZIKV
16 exposed participants who have had sub-clinical or mild infections and did not seek
17 medical care¹. Since the virus has been circulating in the aforementioned regions for
18 at least two years^{36,37}, it would seem likely that a significant proportion of the
19 population would already have been exposed to ZIKV. Difficulties in identifying if
20 this group has also been exposed to DENV stem from this fact that current serological
21 tests fail to discriminate between DENV and ZIKV past infections.

22 The poor specificity of most currently available serological tests have been
23 repetitively demonstrated during serologic surveys conducted in areas endemic for
24 multiple, co-circulating flaviviruses, including ZIKV where cross-neutralising
25 antibodies against DENV was a common occurrence^{35,38}. This has hampered the

1 evaluation of population wide estimates of ZIKV exposure in South and Central
2 America. Evaluation of acute-phase sera from patients during the ZIKV outbreak in
3 the Yap Islands demonstrated extensive cross-reactivity with other flaviviruses in
4 both IgM capture ELISA and plaque reduction neutralization tests (PRNT)³⁵. Reports
5 of natural ZIKV infection producing false positive DENV results have come to light
6 in specimens collected from travellers returning from Indonesia³⁹, Thailand^{3,40} French
7 Polynesia⁴⁰ and Maldives⁴¹. DENV IgM assays demonstrating false-positive results in
8 ZIKV infection include Focus Diagnostics DENV IgM Capture^{3,35,41} and SD Bioline
9 Dengue Duo NS1 Ag + Ab Combo³⁵. These results prompted the CDC to caution that
10 serologic data alone are insufficient to confirm acute ZIKV infection in patients with
11 secondary flavivirus infections³⁹.

12 A recently published report however suggests that using a recombinant DI and DIII
13 which only possess epitopes recognised by ZIKV specific IgG antibodies, it is
14 possible to satisfactorily discriminate between DENV and ZIKV convalescent cases
15 but not acute cases⁴². However the sensitivity and specificity of this assay is still
16 unknown. The NS1 blockade-of-binding (BOB) assay employs a recombinant ZIKV
17 NS1 protein as the solid-phase antigen and a labelled monoclonal antibody (ZKA35)
18 as the probe⁴³. The labelled monoclonal antibody (ZKA35) was selected for its
19 specificity to a site S2 on ZIKV NS1 not targeted by cross-reactive antibodies and
20 binds equally potently to both African and Asian lineages. This assay was developed
21 to measure the presence of serum antibodies capable of preventing the binding of
22 ZKA35 to the ZIKV NS1 *in-vitro*. This assay has been shown to be easily transferable
23 across multiple laboratories in South-America and Europe. The sensitivity of this
24 assay increases from 92% to 95% as the day of sampling increases suggesting that
25 sensitivity is greater for convalescence when compared with acute disease. The

1 specificity ranged from 80% to 95.9%⁴³. The reliability of this assay, though
2 seemingly better than currently available serological assays, still does not meet the
3 target product profiles (TPPs) of ZIKV diagnostics laid out by the WHO i.e. >95%
4 (ideally 98%) sensitivity and specificity. In addition the TPPs also recommend the
5 ability for diagnostic tests to simultaneously detect other locally endemic arboviral
6 diseases⁴⁴.

7

8 **2.2 Impact on vaccine development and evaluation of vaccine efficacy**

9 These observations are a cause for concern particularly with regard to gauging
10 vaccine immunogenicity in field trials with multiple co-circulating flaviviruses and
11 the subsequent planning of vaccine policy and strategy. ZIKV vaccine efficacy (VE)
12 would be directly related to the protection of vaccinated individuals under optimal
13 conditions⁴⁵. Measuring VE would require describing the risk (R) of acquiring the
14 infection in the vaccinated and unvaccinated groups, respectively [$VE = (R_{unvaccinated} -$
15 $R_{vaccinated})/R_{vaccinated}$]. This is difficult in the current Latin American setting, as
16 clinically apparent ZIKV infection is declining, and a large proportion of the
17 population has already been exposed to the disease³¹. Unlike VE, vaccine impact (VI)
18 is a measure of the downstream effects and the longer-term effects of vaccination.
19 Vaccine impact requires incidence rate of the primary end points (clinically
20 apparent/laboratory confirmed ZIKV infection) and secondary end points⁴⁵ (herd
21 immunity/complications of ZIKV). VI is measured using incidence rate ($VI =$
22 $IR_{prevaccine} - IR_{postvaccine} / IR_{prevaccine}$). Therefore, VI will also be difficult to assess in the
23 current scenario of ZIKV infection in the Americas owing to the lack of specificity of
24 ZIKV clinical manifestations and to the scarcity of ZIKV specific diagnostic
25 modalities. It is thus foreseeable that the absence of reliable serological assays and/or

1 protective correlates for ZIKV vaccines, coupled with the difficulties of assessing VE
2 and VI will make it particularly hard for vaccine experts, policy makers and
3 authorities to make decisions regarding vaccine deployment and subsequent cost-
4 benefit analyses. The fate of ZIKV vaccine success thus hinges on development of
5 reliable serological diagnostic tools and on the ability to estimate VE and VI in an
6 appropriate at-risk population. Currently available confirmatory diagnostics such as
7 nucleic acid detection *via* PCR will prove to be a challenging modality in assessing
8 vaccine impact if ZIKV infection is the end point in phase II trials: the study
9 population will need to be sampled frequently in order to identify all cases of ZIKV
10 infection, as a significant proportion (up to an estimated 80%) will be sub-clinical¹.
11 This assay will be useful only during acute viremia. It will also be difficult to show
12 protective efficacy given the apparent declining incidence of ZIKV infection in North,
13 South and Central America and the Caribbean³⁷ as the infection rates may be low.
14 Therefore, new assays based for example on less conserved antigens or regions such
15 as NS1 may be of particular interest²⁵.

16 Serological approaches also serve to inform another aspect of vaccine development
17 namely correlates of protection. A correlate of protection is important though not
18 mandatory for initial WHO pre-qualification and recommendation by the strategic
19 advisory group of experts (SAGE) committee for immunisation. A correlate of
20 protection will also help with licensure of future vaccine candidates based on non-
21 inferior immunogenicity. The correlates of protection may be in the form of
22 neutralising antibodies as are seen with yellow fever and Japanese encephalitis
23 vaccines. Total binding antibody responses and neutralising antibodies are correlates
24 for the tick-borne encephalitis vaccine³⁰. It is thus important to delineate a correlate of
25 protection for ZIKV infection in order to ensure a strong future for ZIKV vaccine

development and deployment. Neutralising antibodies generated in humans as well as non-human primates and subsequently administered to ZIKV infected mice models demonstrated protection, and while the requirement for protection in non-human primate is estimated to be log 2.0-2.1⁴⁶⁻⁴⁸, but it remains to be determined what the protective titres are in humans exposed to ZIKV and whether there is waning of neutralising antibody titres over time.

3. An incomplete understanding of the neurological complications associated with ZIKV infection.

The unequivocal evidence supporting the neurotropism of ZIKV has been generated in cell lines and animal models⁴⁹⁻⁵¹. However, the manifestations of Guillain-Barré syndrome (GBS) and microcephaly need to be further understood particularly in light of ZIKV vaccine safety.

3.1 Link between ZIKV infection and Guillain-Barré syndrome.

GBS is one of the probable complications of ZIKV infection which was brought to light following the increased number of GBS cases in ZIKV endemic areas such as French Polynesia, Colombia and Martinique^{19,52,53}. Other arboviral infections are reported to trigger GBS: this includes DENV, chikungunya, Japanese encephalitis and West Nile viruses¹⁹. However a proportion of GBS cases attributable to ZIKV infection have also showed co-circulating antibodies to DENV. This points either towards an increased susceptibility to GBS in hosts infected with ZIKV with prior exposure to DENV, or to a problem related to cross-reactive antibodies which could then potentiate the onset of GBS^{19,54,55,55,55}. Among the post-ZIKV GBS patients in French Polynesia, 74% had IgM antibodies to ZIKV and none to DENV using an

1 immunofluorescence assay based on virus-infected cell lines, whereas 19% with IgM
2 antibodies to DENV also had IgM against ZIKV. The situation was more ambiguous
3 when IgG antibodies of DENV and ZIKV were estimated: all GBS affected patients
4 who had neutralizing IgG antibodies to ZIKV also had neutralizing IgG antibodies to
5 at least one of the four DENV serotypes¹⁹. Another study in Colombia reported that
6 86% of patients with ZIKV associated GBS had IgG antibodies against circulating
7 flaviviruses⁵². The authors suggest that the IgG antibodies were an “anamnestic
8 response to DENV”, but the possibility of increased susceptibility to GBS due to prior
9 DENV exposure could not be ruled out^{19,52}. Both the aforementioned papers included
10 small numbers of ZIKV infected GBS patients (41 patients in French Polynesia and
11 37 patients in Colombia). These preliminary epidemiological data need to be
12 validated in larger studies in order to gain robust mechanistic insight establishing or
13 affirmatively ruling out the relationship of simultaneous and/or successive infections
14 with these viruses in the occurrence on GBS. Although past DENV infection was not
15 significantly different between cases and controls in ZIKV induced GBS in French
16 Polynesia¹⁹, these findings put forward a hypothesis of cross-reactive DENV
17 antibodies being produced on exposure to ZIKV. Finally, and as alluded to in the
18 previous section, it is also likely that in both studies, the antigen/assay, based on an
19 Indirect immunofluorescent assay on Vero cells (African green monkey kidney cells)
20 infected with either ZIKV (PF13-251013-18) or DENV (D1-Hawaii 1944), did not
21 have an acceptable level of discriminatory power.

22 In the cases where GBS followed infections with DENV, chikungunya, Japanese
23 encephalitis and West Nile viruses¹⁹, it was reported that the GBS cases were
24 secondary to the production of anti-ganglioside (GM₁)⁵⁶ antibodies. There is
25 considerable interest in neural gangliosides as a link between ZIKV, microcephaly

1 and GBS⁵⁴, potentially through molecular mimicry between these gangliosides and
2 surface moieties of ZIKV. It is not currently known which ZIKV antigens may be
3 specifically implicated in molecular mimicry, and identifying this is essential in order
4 to exclude these antigens from ZIKV vaccine candidates. It is also unknown whether
5 DENV exposure prior to immune stimulation with ZIKV either through natural
6 infection or immunization, may increase the risk of GBS.

8 **3.2. The risk of microcephaly**

9 Microcephaly came to prominence as a potential congenital sequel of ZIKV during
10 the recent South American outbreak. Two theories hypothesizing the mechanism of
11 microcephaly currently exist. The first suggests a direct transplacental transfer of the
12 virus and subsequent foetal brain infection. The second proposes an immunological
13 response in the placenta to the virus which alters inflammatory markers in foetal
14 tissues⁵⁷. The second theory, if established, rules out pregnant women as a target
15 population for vaccination.

16 ZIKV is neurotropic, possibly targeting the neural gangliosides and/or cortical neural
17 progenitors particularly during foetal life^{54,57}. Further evidence of its neurotropism
18 stems from recent evidence of the inhibitory action of ZIKV on glioblastoma stem
19 cells⁴⁹. It is however unclear whether the neurotropism is due to molecular mimicry or
20 direct toxicity which again has important implications in choosing appropriate
21 antigens for vaccine candidates.

22 Current data from ZIKV vaccine animal models⁵⁸ have demonstrated protection in the
23 offspring of mice vaccinated against ZIKV⁵⁰, however, sterilizing immunity has not
24 been consistently achieved⁵⁰. There is no consensus whether this is a requirement for
25 protection during pregnancy³⁰. Richner *et al* vaccinated mice at 4 weeks and 8 weeks

1 before insemination with an mRNA vaccine and a live-attenuated vaccine, and found
2 that 11 of 19 (58%) foetal heads from the mRNA vaccinated group and 19 of the 23
3 (83%) in the live-attenuated vaccinated group had viral RNA levels at the limit of
4 detection of the assay, which suggested substantially decreased placental transmission
5 in these vaccinated mice (13,000 fold mean-reduction). Moreover, the remainder
6 offspring of the vaccinated mice had significantly lower viral RNA levels than those
7 detected in samples from the placebo group⁵⁰. However an increasing duration of time
8 between vaccination and subsequent pregnancy and infection needs to be tested to
9 ascertain temporal waning of protective antibody titres and the protective threshold of
10 these antibodies. Such data and knowledge would support adequate timings for
11 vaccinating in pregnancy.

12
13 Studying both these complications in human studies are difficult as the incidences are
14 rare and the number of ZIKV positive cases are declining^{31,37}. Human challenge
15 studies have been considered, if restricted to healthy celibate volunteers, to help
16 alleviate concerns including potential risks to pregnant women and their unborn
17 children, uncertainties about reservoirs of viral persistence and the possibility of
18 sexual transmission³¹. This approach has been employed before by Plotkin and
19 colleagues who studied CMV transmission and vaccine protection in a closed group
20 of celibate priests who volunteered to be infected with CMV⁵⁹. However, recent
21 recommendations regarding the ethical considerations for ZIKV human challenge
22 trials stated that human challenge studies were currently not ethically justified⁶⁰.

23 24 **4. The potential for disease enhancement**

1 Antibody dependent enhancement (ADE) is an immunological event which
2 potentiates DENV infection in individuals with a sub-protective DENV-specific
3 antibody threshold⁶¹. It was first observed in 6-12 month-old infants in whom an
4 unexpected number of severe dengue syndrome cases occurred, when maternal
5 derived-antibodies waned below neutralizing levels^{62,63}. Evidence of ADE induced
6 *via* vaccination came from a phase III clinical trial: young DENV-infected vaccine
7 recipients had an increased risk of DENV-related hospitalization more than one year
8 after vaccination as compared with placebo controls. It was postulated that
9 vaccination of DENV-naïve individuals induced poorly neutralizing anti-DENV
10 antibodies, which translated to an increased risk of severe dengue disease via the
11 mechanism of ADE⁶⁴. A recent publication studying well characterised cohort of
12 children from a DENV endemic region in Nicaragua revealed that the correlate of risk
13 for severe disease (DENV-Ab titre 1:21-1:80) was independent from the correlate of
14 protection (DENV-Ab titres at and above 1:80-1:320) against symptomatic DENV
15 infection⁶². Notably, the Japanese encephalitis virus, tick-borne encephalitis virus and
16 yellow fever virus vaccine platforms have not raised this concern.

17 There is some evidence to suggest that this mechanism might be at play in ZIKV
18 pathogenesis too^{31,65}, involving antibodies to a domain of the structural envelope
19 protein (E)^{25,66}. ZIKV is most similar to DENV as far as the molecular structure is
20 concerned, although there is only one serocomplex. The E protein, present on all
21 flaviviruses, is essential for viral attachment and entry into the host cell. It elicits an
22 immune response to three of its domains namely DI, DII and DIII. Stettler *et al.*
23 compared monoclonal antibodies (mAbs) from ZIKV-infected donors with mAbs
24 from DENV-infected donors, and showed that anti DI/DII monoclonal antibodies
25 enhanced ZIKV and DENV infection *in vitro*, and enhanced DENV disease *in vivo* in

1 mice²⁵. In contrast, neutralizing antibodies targeting the DIII of the envelope protein,
2 or quaternary epitopes on the infectious virus, were able to protect mice from ZIKV
3 infection²⁵. A longitudinal study showed that on natural ZIKV exposure, the
4 antibodies to the DI/II domains are the first to peak, that they are weakly neutralizing
5 in function and show cross-reactivity to DENV⁶⁶. The anti-DIII antibodies peaked
6 later, were neutralizing, weakly cross-reactive and protective in mice⁶⁶. Work done by
7 Dejnirattisai *et al* also suggested that human antibodies to DENV envelope isolated
8 from patients were cross-reactive to ZIKV, poorly neutralized ZIKV but, most
9 worryingly, induced ADE of ZIKV infection *in vitro*⁶⁷. If this translated to an *in vivo*
10 phenomenon similar to DENV, it would have serious implications from a vaccine
11 deployment perspective: administering the vaccine would have the potential risk to
12 increase susceptibility to ZIKV, DENV or their complications *via* the mechanism of
13 ADE⁶⁸. A cautious approach is required particularly in light of the announcements
14 regarding Dengvaxia where Sanofi Pasteur issued a statement that its product poses
15 higher risks to people without prior DENV infection^{69,70}. In the Philippines, following
16 the alleged deaths of 62 children due to severe dengue above the age of 9 years
17 following the administration of Dengvaxia is a warranted cause for concern and is
18 thought to be mediated through ADE⁶³.

19 A modified mRNA vaccine candidate with a modified DII that abolishes fusion-loop
20 specific antibodies⁵⁸, and virus-like particle (VLP) with only a DIII domain^{71,72} may
21 therefore avoid this problem, but only under the assumption that the anti-DI/DII
22 antibodies represent the only pathway causing ADE. This may not be the case in light
23 of previous hypothesis on DENV ADE: Halstead⁶¹, Boonnak⁷³ and Ubol⁷⁴ all
24 independently suggested that ADE is likely to encompass a variety of factors such as
25 the host genetic susceptibility, duration between “sensitizing” and “enhancing”

1 exposure, pathogen virulence (*eg* DENV1 vs DENV2), the role of the innate immune
2 system⁶¹ and a possible role of T cell function⁷⁵. There is currently no compelling
3 evidence to suggest that these factors play a role but there is no evidence disproving it
4 either. A more comprehensive understanding of ADE with a keen focus on the host-
5 pathogen interaction at the genomic and transcriptomic level is thus warranted,
6 coupled with various temporal models of highly controlled sensitizing and enhancing
7 exposure including DENV => ZIKV, ZIKV => DENV and ZIKV => ZIKV
8 combinations of exposure. It is difficult to envision how such a study involving
9 human participants would take place outside of a human challenge model where strict
10 monitoring of vitals, packed cell volume (PCV) and haematological cell counts are
11 possible. Blackman *et al* suggest that in order to better understand the
12 association/causality between ZIKV and other flaviviruses, one method is to conduct
13 studies with robustly controlled conditions randomly allocating subjects to exposure
14 of ZIKV and DENV, and then crossing-over subsequent exposure, with blood
15 sampling at multiple intervals to establish temporal signals between baseline immune
16 signatures and outcomes of infection.³¹ This may be an ideal approach but will be a
17 challenge to execute given the large sample sizes required and difficulty replicating
18 natural exposure.

19 This phenomenon of ADE activity between ZIKV and DENV has been successfully
20 shown *in vitro* but its significance *in vivo* remains controversial³¹, thus adding another
21 layer of complexity to understanding this mechanism. Primates infected with ZIKV a
22 year after DENV infection failed to show enhanced infection with ZIKV, despite
23 blood samples from these primates demonstrating enhancing ZIKV activity *in vitro*⁶⁵.
24 Additionally, the possibility of ADE contributing to the inclination of the virus to
25 permeate anatomical barriers could not be ruled out⁶⁵. There are also suggestions that

1 ADE mediates enhanced infection of FcR-bearing neural cells, which is perhaps
2 relevant for ZIKV-associated neurological complications^{31,65}. Notably, there is *in*
3 *vitro* evidence of ADE for West Nile virus and yellow fever virus^{70,76}, but lack of
4 evidence that this phenomenon occurs in humans. Experiments in cell culture and in
5 mice showed that low concentration of DENV or West Nile virus antibodies^{67,77} will
6 enhance infection, while this is not observed in non-human primates^{78,79}. There is
7 suggestion that prior infection of ZIKV could enhance subsequent DENV-2 infection
8 ⁸⁰. Finally, sub-neutralizing level of ZIKV antibodies did not enhance the replication
9 of following ZIKV infection in non-human primates even when it showed
10 enhancement effects in cell culture⁴⁶. Further well-designed *in vivo* experiments are
11 needed to better characterize infection and pathology in specific anatomical sites
12 unique to ZIKV infection.

13 The current ZIKV vaccine candidates which confer protection *via* neutralising
14 antibodies²⁷ thus introduce the theoretical possibility of producing antibodies that
15 mediate ADE, and therefore put the population of interest at risk of enhanced
16 infections with ZIKV and DENV. As evidenced by the last outbreaks of ZIKV (in
17 Micronesia, French Polynesia, Singapore, Thailand and The Americas), it is probable
18 that the next major outbreak may also be in a region already endemic for DENV, and
19 thus understanding the role of ADE in the context of DENV pre-exposure is
20 imperative. A possible and safe approach to avoid this issue might be to investigate
21 the potential of T-cell-based vaccines, an approach considered by some research
22 groups for DENV. While some of the ZIKV vaccine candidates detailed in the recent
23 review by Barzon *et al* elicit a T cell response to ZIKV antigens, this is in the context
24 of induction of neutralising antibodies as well²⁷. However, there is currently no
25 evidence that antigen-specific T-cells may be sufficient to prevent ZIKV infection.

1 Another approach is to improve the design of the E antigen, as suggested by Barba-
2 Spaeth *et al.*: an epitope that can induce cross-neutralising antibodies, which
3 neutralised ZIKV, was identified, using antibodies from DENV infected patients. The
4 conserved epitope was conformational, on the site of interaction of two envelope
5 proteins (forming the envelope dimer) and the precursor membrane (prM) antigen⁸¹.
6 In contrast, longitudinal evidence suggests that neutralising antibody titres may
7 distinguish between ZIKV and DENV infections, and cross-neutralising antibodies
8 persist only for a short period of time in early ZIKV convalescence (14-28 days) but
9 this observation needs further investigation with a larger sample size and the
10 interaction with other flaviviruses^{82,83}. There is also an association with ZIKV
11 infection and a reduction in DENV infection in Salvador, a city in Brazil endemic for
12 DENV, ZIKV and Chikungunya virus suggesting a protective effect of ZIKV against
13 DENV infection. However, this observation is not causal and randomised prospective
14 studies are needed to understand this relationship between these two viruses⁸⁴. While
15 ZIKV is most similar to DENV as far as the molecular structure is concerned, the
16 concerns over ADE in ZIKV vaccine development may also be balanced by the more
17 positive outcomes of the Japanese encephalitis virus, tick-borne encephalitis and
18 yellow fever vaccine platforms.

19

20 **Conclusions**

21 The arboviruses belonging to the flaviviridae family have peculiar characteristics with
22 respect to transmission, host immune response and clinical features. ZIKV is the latest
23 to cause global concern and its epidemiological features need to be understood
24 predominantly with regard to transmission dynamics and clinical effects of co-
25 circulating flaviviruses. However, despite the plethora of data produced in the last two

1 years, a number of uncertainties still need further insight in order to drive policy
2 related decisions particularly with regard to vaccine development. ZIKV is also the
3 latest addition to the list of congenital infections, stimulating the need to elucidate
4 preventive strategies, which may include, but not necessarily be limited to
5 vaccination.

6 The development of effective vaccines against flaviviruses is possible: vaccines
7 against yellow fever virus, Japanese encephalitis, tick-borne encephalitis and dengue
8 viruses are licensed. However vaccine development for ZIKV poses unique problems.

9 The small number of symptomatic infections and the incidence of the severe
10 endpoints cause difficulties in designing efficacy trials, as these will require a rather
11 large sample size in both, the experimental and control arms to demonstrate protective
12 efficacy. Acquiring these prohibitively large numbers may prove to be challenging
13 given the recent trend of decreasing symptomatic cases, particularly in the current
14 context of declining incidence of ZIKV infection. The declining rates of ZIKV
15 infection in South and Central America is also a rate limiting step for developing new
16 diagnostic tools as reported in the proceedings of a recently held meeting discussing
17 ZIKV diagnostics⁸⁵. The absence of a correlate of protection, as well as difficulties in
18 choosing meaningful endpoints in gauging vaccine impact, both contribute to the
19 difficulties in ZIKV vaccine development. Moreover, the animal challenge models
20 used to establish preclinical efficacy (interferon-gamma deficient mice, non-human
21 primates where ZIKV infection is asymptomatic) might not be relevant, specifically
22 with regard to the potential safety issues surrounding ADE and neurovirulence.

23 The interaction between virulence factors of ZIKV, including antigens and epitopes as
24 well as the immune response are also important in understanding certain sequelae
25 such as GBS and microcephaly, which will subsequently have implications in the

1 design of a successful vaccine. Finally, The complex interplay between viruses of the
2 flaviviridae family in endemic settings also needs to be carefully evaluated and
3 understood. For instance, the ZIKV outbreaks in the recent past were preceded by
4 DENV epidemics and it is yet to be determined whether simultaneous/successive
5 flavivirus infections or the immune response to them accentuate the transmission of
6 other viruses in the family, or if their persistence in a geographic region is solely
7 related to the vector. Analogous to the difficulties in studying ADE in DENV, insight
8 into mechanisms of ADE in the light of ZIKV infection is also limited. This peculiar
9 immunological occurrence needs to be investigated thoroughly in relation with
10 sensitising and enhancing exposures of both DENV and ZIKV.
11 ZIKV vaccine success relies on the estimation of VE and VI in an appropriate at-risk
12 population, which will be a difficult mission in a setting of declining incidence and
13 unreliable point-of-care diagnostics.

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1 **Expert Opinion**

2 The international focus on the spread and consequences of ZIKV infections, as well
3 as the rapid increase of new knowledge and data regarding ZIKV epidemic in the
4 Americas in the last 2 years has pushed research groups and industries into a hasty
5 search for vaccine candidates. However, several aspects of ZIKV infection and
6 epidemiology need to be clearly understood in order to make an educated plan for
7 potential vaccine development. The fact that a large majority of ZIKV infections are
8 sub-clinical combined with the lack of reliable diagnostics for past exposure presents
9 numerous difficulties ranging from accurate evaluation of vaccine impact to
10 differentiating other flavivirus infections. If immunological endpoints and safety data
11 are used for licensure, vaccine efficacy will only be able to be estimated post-
12 licensure. The cross-reactive antibodies between DENV and ZIKV infection
13 complicates approaches to vaccine development. The DNA vaccine candidate
14 currently in phase II trials confers protection *via* neutralising antibodies⁸⁶. However, if
15 lessons from DENV vaccine trials are to be learnt, and if the ADE phenomenon is
16 confirmed for ZIKV infection as well as for vaccine-induced antibodies, an approach
17 involving the harnessing of T cells to provide protection⁶³, or antibody-based vaccines
18 designed to avoid epitopes involved in ADE may need to be employed.

19 Drawing from experiences of other flaviviral vaccines, a correlate/surrogate of
20 protection is essential for policy makers and vaccine regulators to incorporate them
21 into vaccine schedules. Neutralising antibodies are the correlates for the yellow fever
22 and Japanese encephalitis vaccines, while total binding antibody responses (formerly
23 used) and neutralising antibodies are correlates for the tick-borne encephalitis
24 vaccine³⁰. The assays for these diseases correlated well with protection even in the
25 setting of multiple circulating flaviviruses. Experience from DENV vaccine trials

1 however suggests that even when *in-vitro* correlates of protection are promising in the
2 form of neutralising antibody titres, these serve as a poor surrogate of DENV vaccine
3 induced protection in field studies⁶³, and it is plausible that ZIKV candidates may
4 encounter a similar obstacle. It will be essential for ZIKV vaccines to also have a
5 correlate of protection in order to convince regulators of its ability to reduce the
6 burden of ZIKV infection. ZIKV vaccination may theoretically carry the risk of
7 enhancing a subsequent ZIKV or DENV infection *via* ADE. Notably, the Japanese
8 encephalitis virus, tick-borne encephalitis virus and yellow fever virus vaccine
9 platforms have not raised concerns of ADE, however, it is hard to see how the ZIKV
10 vaccine candidates will achieve licensure unless ADE is clearly ruled out as a
11 consequence of vaccination, and the lack of an increased risk of either ZIKV or
12 DENV in vaccines demonstrated.

13 Finally, the scientific community must reach a consensus on the neurological
14 complications associated with ZIKV infection, and prove beyond reasonable doubt
15 that the ZIKV vaccine candidates do not increase the risk of these complications. As it
16 is unknown if GBS is caused by direct infection or by an immune response to a ZIKV
17 antigen, a ZIKV vaccine may increase the risk for GBS, particularly in the case of a
18 live attenuated vaccine. However, there are too few numbers of these complications,
19 and it is thus likely that these questions may only be resolved post licensure.

20 With so many uncertainties, identifying the target populations for efficacy trials and
21 for use of ZIKV vaccines is complex. Objectives and readouts may include: (1)
22 Prevention of virologically confirmed clinical illness (representing 20% of infections,
23 thus requiring high numbers of participants in efficacy trials); (2) Prevention of
24 infection, with the caution that sterilizing immunity is not achieved with other
25 flavivirus vaccines; (3) prevention of congenital disease. Though congenital Zika

1 syndrome is a worrying possible complication one would theorise that it would be too
2 rare to be a relevant readout in efficacy trials and can only be estimated post-
3 licensure. If immunological endpoints and safety data are used for licensure, vaccine
4 efficacy will be only measured post-licensure.

5 A recent systematic review on congenital ZIKV-related syndrome, suggests that the
6 highest risk of harm to the foetus occurs during the first two trimesters of pregnancy,
7 particularly during the stages of embryogenesis and neural development⁸⁷. If the
8 objective of a vaccine is to prevent the cases of microcephaly, then immunising
9 women prior pregnancy may be the most efficient approach (women of reproductive
10 age). However, it will be essential to establish that (1) the vaccine does not induce an
11 immune response responsible of ADE or able to increase the potential for
12 reproductive toxicity, (2) the vaccine induces a reasonable persistence in protection
13 and (3) the antibody decay with time does not predispose to ADE. Finally, the general
14 population may be considered, however distinction may still need to be made between
15 primed (previously exposed to ZIKV or to DENV) versus naïve population, as seen
16 with the dengue virus vaccine.

17 At best, a vaccine candidate may reach licensure in 2022, by which time the epidemic
18 may have subsided, or be sporadic (there is possibility of ZIKV outbreaks in areas of
19 flavivirus endemic areas including Asia and Africa) and is thus unlikely to make the
20 widespread use of a vaccine cost-effective. It is therefore likely that the international
21 community, as well as non-commercial stake-holders will be required to sustain
22 funding the development of a vaccine against ZIKV along with the necessary research
23 into diagnostic and potential complications.

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Article highlights box

- The development of vaccine candidates against ZIKV infections is hampered by the lack of specific serological diagnostic assay, the unknowns relating to the neurological complications and the possibility of antibody-dependent enhancement.
- Accurate evaluation of vaccine efficacy may only be possible after licensure, if only immunological endpoints and safety data are used for clinical development.
- The cross-reactive antibodies between DENV and ZIKV infection complicates approaches to vaccine development and implementation, if an antibody-dependent enhancement phenomenon is confirmed for ZIKV infection and vaccine-inducing antibodies.
- The identification of a correlate/surrogate of protection is required.
- The scientific community must reach a consensus on the neurological complications associated with ZIKV infection, and prove beyond reasonable doubt that the ZIKV vaccine candidates do not increase the risk of these complications.

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