**Research Vision of the THAIBRA Alliance**

**The Challenge:** The critical barrier to developing effective medical countermeasures (MCMs) to Zika virus (ZIKV) is delineating the risk from immune interactions by dengue virus (DENV). Zika has caused dramatically divergent epidemiological trajectories in different regions: Its introduction in the Americas led to a major pandemic in 2015 whereas the virus has circulated endemically in Southeast Asia for decades, despite endemic transmission of all four DENV serotypes in both locations. These observations highlight that we cannot rely on simple metrics of DENV immunity and phylogenetics to understand ZIKV risk. Instead, we must gain a deeper understanding of the cross-reactive immunity generated by specific circulating lineages, beyond species and serotype, in any one location. Only by comparing the genetic and antigenic profile of specific viruses that circulate at opposite extremes of ZIKV ecology - such as between Brazil and Thailand as we propose here - can we understand this phenomenon.

**The Opportunity:** Our project is built on a unique base of two long-running multigenerational cohorts that will allow us to critically assess the extent to which population immunity shifted following the introduction of ZIKV in Brazil while remaining stable in Thailand. We have also developed a comprehensive antigenic map for DENV*(1)* that can be expanded to include ZIKV and also capture exactly, through construction of antibody landscapes*(2)*, how populations differ between settings and over time. We have also developed the most comprehensive long-term genetic characterisations of DENV and ZIKV from any one setting*(3–5)* and developed state-of-the-art experimental techniques, including to measure cellular mediated immunity (CMI) at scale*(6)*. These tools will allow us to identify incident ZIKV infections, even in the face of DENV cross-reactivity, and to identify individual-level correlates of infection and disease. Our research program consists of three aims:

**Aim 1: Quantify the antigenic relationship between ZIKV and DENV and the link to population immunity profiles.** We will use sera from non-human primates inoculated by DENV or ZIKV and perform neutralisation testing with Thai and Brazilian isolates to build antigenic maps that, for the first time, include both ZIKV and DENV. We will use samples from multigenerational cohorts to characterise the changing antibody and cellular immunity landscape in the two countries.

**Aim 2: Identify how population immunity drives the fitness of Zika and dengue strains** We will sequence DENV and ZIKV from Brazil and Thailand, expanding our existing extensive characterisation of the genetic diversity of the two viruses. We will identify discrete DENV and ZIKV lineages circulating in the two populations and identify how immunity can explain the changing fitness of circulating lineages.

**Aim 3. Define underlying ZIKV infection risk and the relationship between pre-exposure immunity and clinical and immunological outcomes at the individual level.** We will prospectively follow two cohorts to quantify the underlying level of ZIKV and DENV infection, and to identify how antibody landscapes and cellular immunity are linked to infection and disease risk.

## The Approach

**1. <u>Study Team</u>:** We have assembled a multidisciplinary team who have led the field in flavivirus cohort studies (Anderson, Thomas, Buddhari, Ko, Reis), translational immunology (Waickman, Whitehead, Kalimuddin), genomic epidemiology (Salje, Khouri), and mathematical modelling (Salje, Cummings). In addition to establishing two premier cohorts*(7, 8)*, we have made major contributions to the recognition of the 2015 microcephaly outbreak and its link with Zika*(9–15)*, the sustained endemic transmission of ZIKV in Thailand*(5)*, the protective and enhancing role of prior flavivirus immunity*(7, 16–18)* and for pioneering the use of antigenic cartography for DENV*(1, 4)*.

**2. <u>Overview</u>**: To gain a comprehensive understanding of the interplay between ZIKV and DENV immunity (Aim 1), on the fitness of viral populations (Aim 2) and on infection and disease outcomes in individuals (Aim 3), we will leverage longitudinal sampling of cohorts alongside active surveillance (Figure 1A) and harmonised testing and analytic pipelines of data and samples (Figure 1B). Prospective studies, conducted in Kamphaeng Phet, Thailand since 1998 and Salvador, Brazil since 2002, are currently following 3000 and 2243 individuals, respectively. Furthermore, the biorepositories established over decades of longitudinal investigation, prior to and after the 2015 Zika pandemic in the Americas, provide a unique opportunity to interrogate the role of cross-flavivirus protection and enhancement on severe outcomes such as CZS and DHF, in addition to ZIKV and DENV infection.

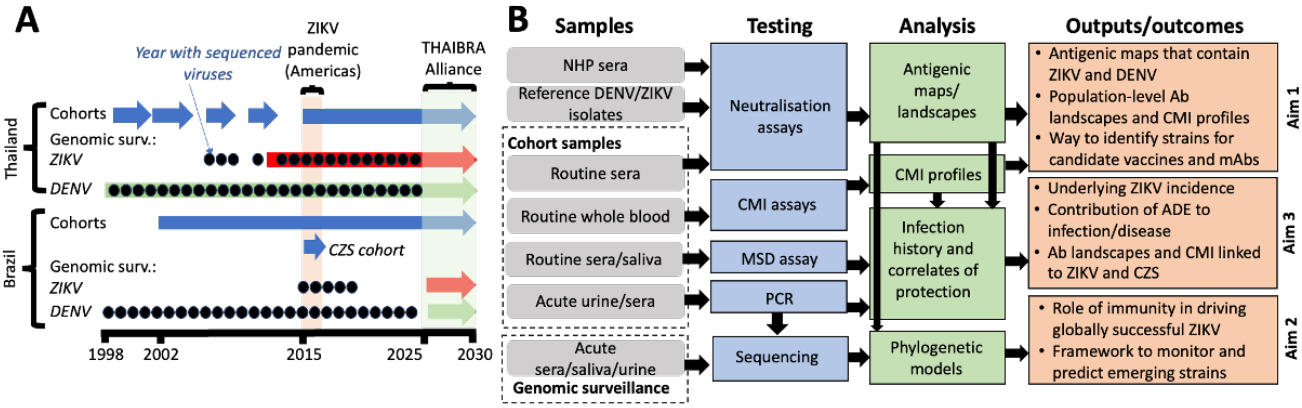


**Figure 1**. **Project overview. (A)** Our existing cohort and genomic surveillance activities (horizontal arrows). (**B)** Overview of source of samples, testing and analysis plan.

**3. <u>THAIBRA foundational principles</u>**: We will use paradigms developed during 30 years of implementing health training programs to promote equitable capacity building and prioritise training of Thai/Brazilian fellows in cutting-edge assays and advanced modelling, such that samples and data generated locally are analysed locally wherever possible. We have established urban health councils in Salvador, alongside work-study programs for community youth to develop into researchers and public health leaders*(19)*. These efforts have resulted in individuals from the community who are now leading research in Brazil*(20)* and are investigators on this proposal. We have similar initiatives in Kamphaeng Phet which are integrated with the universal health system. Our training programs have produced public health leaders, including the CDC Directors (Thailand and Brazil), and Vice-Minister of Health (Brazil), who will facilitate an evidence-to-action plan that comprises of: 1) Real-time data sharing with MoHs; 2) Disseminating findings through a dedicated website, conferences, publications; 3) Annual meetings with MoH leads and SEARO/PAHO staff, ensuring findings

contribute to regional and global policy; and 4) Strategic direction from a steering committee that includes the Thai Inspector General of MOPH, Fiocruz President, Brazilian CDC Secretary, and WHO Arbovirus Initiative Lead.

**4. <u>Outputs that will change the field.</u>** The project will move the field beyond simply tracking genetic diversity to understand pathogen threats. Our quantitative descriptions of changing immune profiles, will instead allow us to finally understand how ZIKV can persist at low levels in one location but cause devastating outbreaks of Zika and CZS in another. It will also allow us to understand the potential impact of existing DENV vaccines, which are based on historic strains that no longer circulate, as well as inform the development of next generation DENV and ZIKV MCMs. We will provide a framework to monitor the emergence of novel 'fitter' DENV and ZIKV lineages with pandemic potential, and an approach to identify populations at heightened risk of Zika and CZS at the global scale due to the specific patterns of historic DENV circulation and address the unmet need for trials sites to evaluate MCMs for Zika. Finally, we will provide a scalable means to quantify CMI immunity overcoming key technical barriers in understanding this often ignored aspect of immunity.

**4. <u>Aim 1</u>: Quantify the antigenic relationship between ZIKV and DENV and the link to population immunity profiles.**

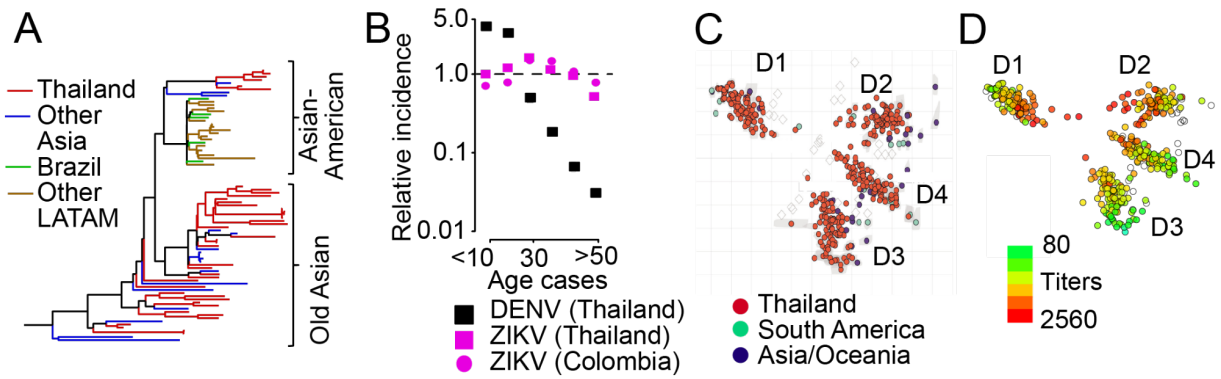


**Figure 2. Existing work. (A)** Phylogenetic ZIKV tree. **(B)** Age distribution of ZIKV/DENV cases(5). **(C)** Antigenic DENV map(1). **(D)** Antigenic landscape for a single multitypic individual..

***Rationale**:* We have used over 4,000 DENV and 40 ZIKV sequences to demonstrate that both viruses have circulated endemically in Thailand for decades (Figure 2A)*(5)*. However, surprisingly, Zika cases are evenly distributed across all ages, similar to that observed in South America, inconsistent with high population immunity (Figure 2B). A ZIKV seroprevalence study in our cohort also found low immunity (~10%). We hypothesise that the specific antigenic characteristics of the DENV viruses that circulate in the two countries in relation to ZIKV explains the different risk of ZIKV infection. Specifically, we propose that the DENV that circulates in Thailand leads to wide cross-reactivity (i.e., heterotypic immunity) with ZIKV, whereas the DENV that circulates in Brazil does not (Figure 3). Supporting this hypothesis is our antigenic map for DENV, developed using sera from inoculated NHPs*(1, 21)*, which showed that Thai viruses are consistently more cross-reactive (i.e., closer to the map centre) than global strains (Figure 2C).

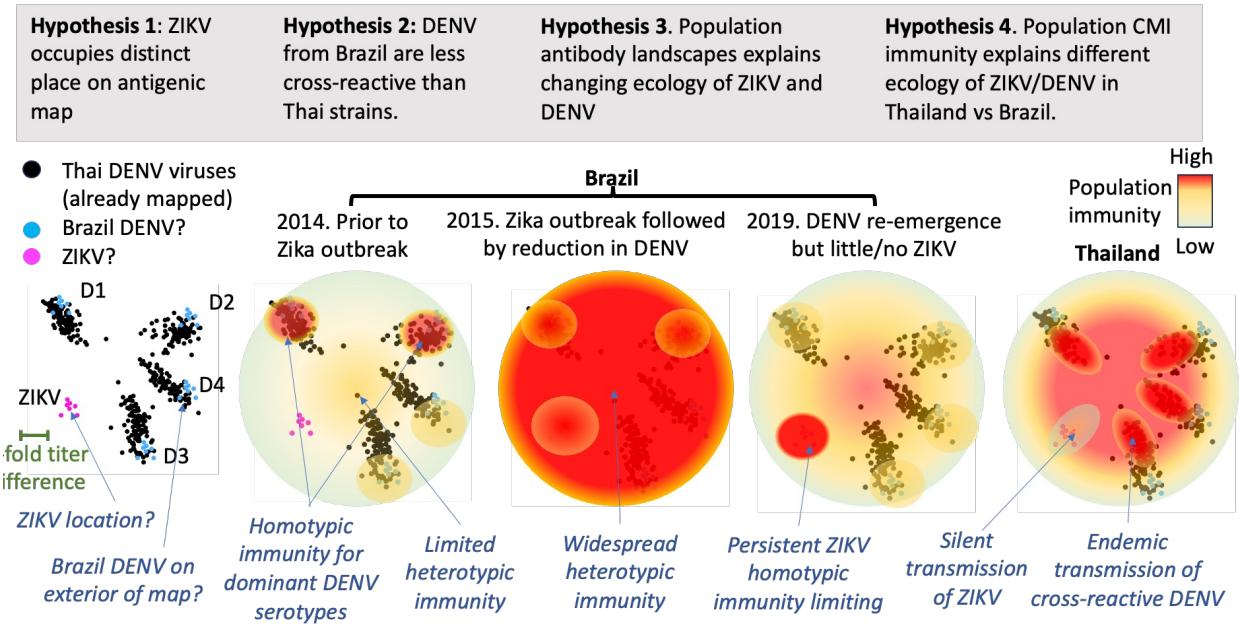


**Figure 3. Motivation for Aim 1.** We will expand our existing antigenic maps to include Brazilian DENV and ZIKV. We will then build population landscapes to understand how specific immune profiles explain the different ZIKV/DENV ecologies in the two countries.

***Constructing antigenic maps to interrogate ZIKV and DENV cross-reactivity (Hypotheses 1 and 2)***: We will add ZIKV and Brazilian DENV onto our antigenic map using existing sera from NHPs infected with DENV (N=20) and ZIKV (N=3) (source: Steve Whitehead, collaborator). This will represent the first antigenic map that contains both viruses, a major advancement for the field. We will conduct neutralisation testing on the sera using ZIKV (N=10, including from Brazil, Thailand, African genotypes) and DENV (N=16 from Brazil, N=16 from Thailand -8 from each serotype) using a high-throughput protocol we have used extensively (>20,000 assays)*(1, 22)*. We will use multidimensional scaling to build a joint ZIKV-DENV antigenic map*(23)* and compare the distances between ZIKV and Thai DENV viruses on the map and between ZIKV and Brazilian DENV viruses.

***Generating antigenic landscapes to interrogate population-level cross-immunity in Brazil pre/post the Zika pandemic and during endemic transmission in Thailand (Hypothesis 3):*** By measuring the neutralisation titre of an individual's serum to a panel of viruses from our antigenic map, we can capture the magnitude and breadth of antigenic portfolios (Figure 2D)*(2, 24)*. In this way, we will generate the first population-representative antigenic landscape for DENV and ZIKV. We will choose a random subset of samples from each cohort (N=100 from each). In Brazil we will cover periods pre/post ZIKV emergence. We will conduct neutralisation testing using a reference set of 16 DENV and 4 ZIKV from our antigenic map and build population-average immune landscapes for each population, where we weight the contribution of each individual based on their age/sex and population demography. We will use generalised additive models to fit the titres. We will then compare the difference in the fitted landscapes over time and between Thailand and Brazil to identify where the landscapes differ.

***Understanding whether CMI contributes to ZIKV and DENV cross immunity at the population level (Hypothesis 4)***: Cellular-mediated immunity (CMI) has been linked to

infection and disease risk*(25)*. However, our population-level understanding of CMI has been limited by the requirement for PBMCs and the high cost of assays. However, a novel CMI-based assay for SARS-CoV-2 has been developed using whole blood*(6)*. The assay works by stimulating whole blood with peptide pools spanning the entire complement of immunogenic proteins and measuring the resulting abundance of T-cell derived cytokines (IFN-g, IL-2, TNF-a, GZMB) quantified using a high-sensitivity/multiplexed-cytokine assay (MSD). This protocol mirrors results obtained with conventional CMI assays (ELISPOT, flow cytometry). In collaboration with Dr. Kalimuddin (NUS, collaborator), we will adapt this assay to generate the first population-level CMI descriptions for DENV and ZIKV. We will test 100 individuals from each cohort to reconstruct average CMI profiles for the two populations. Taking each virus and each of the CMI measures in turn, we will calculate an average response for each population.

**5. <u>Aim 2</u>: Identify how population immunity drives the fitness of Zika and dengue strains**

***Rationale**:* As we have shown for other pathogens such as SARS-CoV-2, there exist multiple co-circulating lineages within an endemic population, which are continuously evolving in response to population immunity*(26–28)*. However, the extent to which this occurs with DENV and ZIKV has not been explored. We will apply our novel analytical methods to identify DENV and ZIKV lineages and assess whether the emergence of the globally successful Asian-American ZIKV clade that caused the 2015-16 pandemic was linked to specific immune landscapes in Asia, where its parent clade was regularly identified.

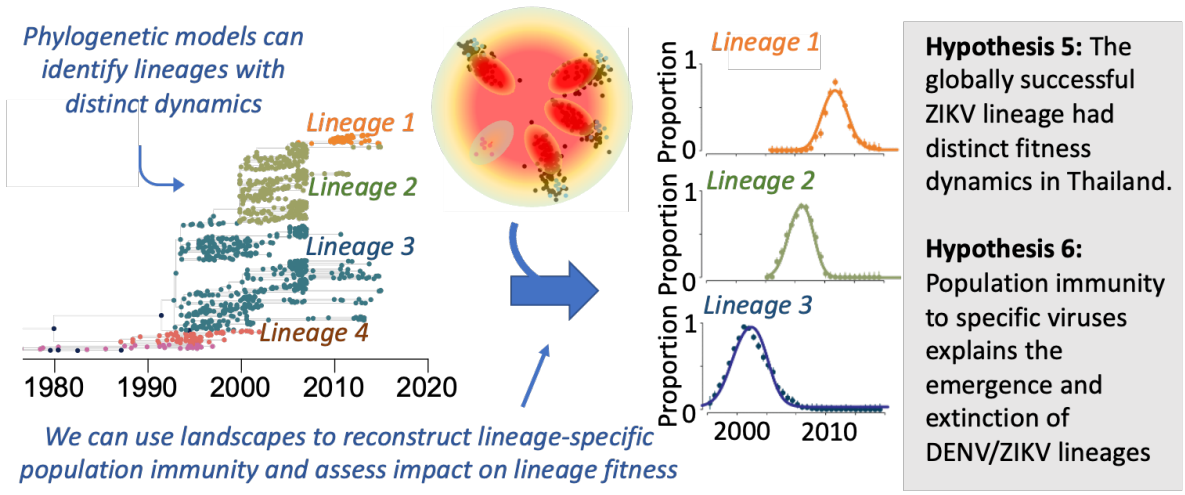


**Figure 4. Motivation for Aim 2.** We have applied *phylowave* to DENV1-4 (DENV-1 shown here) to identify lineages with distinct fitness. We can track the fitness of lineages and use reconstructions of population immunity to understand if fitness is linked to population immunity.

***Genomic surveillance in Thailand and Brazil:*** We and others have obtained ZIKV sequences from Thailand annually since 2013 (106 total ZIKV sequences since 2006) (Figure 1A). This represents the most comprehensive long-term characterisation of ZIKV diversity from anywhere. We will enhance these existing activities to sequence additional PCR positive samples from around Thailand held by the MOPH and from our cohort. We anticipate obtaining 50 prospective and 30 retrospective sequences from these activities. Furthermore, we have already sequenced 4,812 DENV sequences from 1973-2020 from the results of our service sample testing and through collaborations with the MOPH(3, 15). We will continue these

activities (funded externally) to provide sequences through to 2030, providing nearly 60 years of continual dengue diversity from a single setting. In Brazil, we will test samples from suspected Zika/dengue cases at urgent care centres in Salvador and sequence positive samples. We will conduct full genome sequencing of DENV and ZIKV using established protocols on Illumina NGS instruments at sites in Thailand and Brazil.

***Understanding the relative fitness of different ZIKV strains (Hypothesis 5)***: To identify and quantify lineage fitness, we have developed an analytical platform (*phylowave*) that uses the relative success of individual viruses in a phylogenetic tree to identify lineages circulating in a population*(26)*. This framework also identifies characteristic SNPs that distinguish different lineages that can be used to provide hypotheses as to the mechanism for fitness differences. We have previously applied these approaches to a range of pathogens*(26–28)*.

***Understanding whether population immunity can explain the fitness of ZIKV and DENV lineages (Hypothesis 6)***: We will reconstruct ZIKV- and DENV-lineage-specific immunity by age and year using catalytic models applied to observed ZIKV and DENV serotype-specific case data from the country, our observed distribution of lineages by year and our antigenic maps, expanding our existing models for DENV*(4)*. We will then compare the fitness of each lineage with the specific population immunity against that lineage at the time. We will fit models in a Bayesian framework (RStan) and use standard model comparison metrics (WAIC, LOO) to compare the performance of models where we do, and do not include a role for landscape specific (rather than e.g., serotype-specific) population immunity.

**6. <u>Aim 3:</u> Quantify underlying ZIKV infection risk and the relationship between pre-exposure immunity and clinical and immunological outcomes at the individual level.**

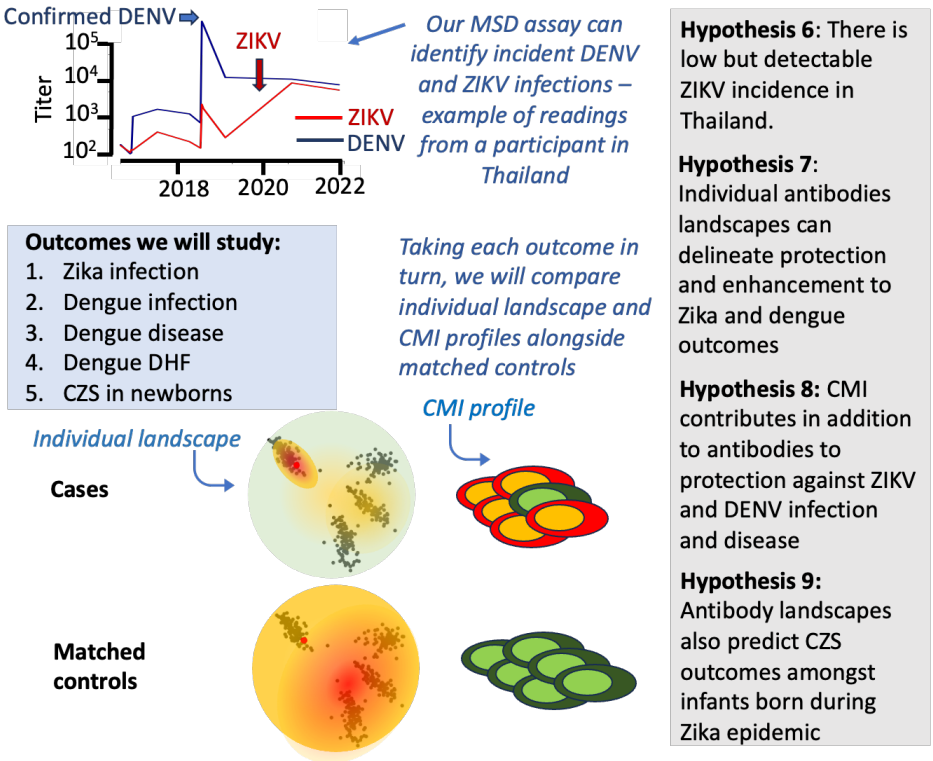


**Figure 5. Motivation for Aim 3.**

***Rationale**:* ZIKV infection is the critical outcome on the natural history pathway and the target for MCMs, since inapparent infections can cause maternal-to-foetal transmission*(29)*. We will delineate how immunity landscapes influence ZIKV and DENV outcomes at the individual level. Well-designed prospective investigations are needed to evaluate the individual level risk for cross-flavivirus enhancement and protection as preexisting flavivirus responses may increase or decrease the risk of ZIKV infection and disease, and vice versa*(7, 18, 30–32)*. Our overall hypothesis is that the quantity and quality of immune responses influence the interplay between protection and enhancement and that delineating such signatures requires constructing individual level immune landscapes, since strains, in addition to serotype and species, drive the complex nature of flavivirus cross-reactivity.

***Quantify ZIKV infection risk (Hypothesis 6)***: Across our cohorts, we will conduct active case finding to identify virologically-confirmed infections through RT-PCR testing on blood/urine from all individuals with symptoms consistent with Zika (rash regardless of fever) and dengue (acute fever). We will separately identify subclinical ZIKV infections through evaluation of anti-DENV/ZIKV antibodies (MSD) in prospective biannual blood/saliva collections. In preliminary studies, NS1 responses alone demonstrated 98% sensitivity and 92% specificity in differentiating ZIKV and DENV infections in a Brazilian population with high (86%) prior DENV exposure*(7)*. Moreover, the platform detected ZIKV exposures (0.7% per year) in a sample of the Thai cohort. In addition to incorporating anti-DENV EIII and saliva antibody responses, we will further improve performance by applying analytical tools, including machine learning, mixture models and Bayesian data augmentation that specifically account for cross-reactivity*(18, 33–35)*. The latter approach will also fully reconstruct individual responses over time, providing a quantification of the duration of homotypic and heterotypic immunity after exposure to ZIKV and DENV.

***Delineate the relationship between pre-existing immunity and ZIKV and DENV outcomes (Hypotheses 7 and 8)***: We will evaluate the association between pre-existing neutralisation and CMI measures with four outcomes (Figure 5): (**1**) ZIKV infection, (**2**) DENV infection, and (**3**) DENV disease from the prospective cohorts, and (**4**) DHF in 20 individuals from previous Thai cohorts (1998-2005, Figure 1)*(18)*. We will employ a nested case-control design, where controls will be matched by age, sex, location and time. We will analyse pre-infection samples and build individual-level antibody landscapes and CMI profiles (Aim 1). Taking each outcome in turn, we will compare reconstructed antigenic landscapes and CMI measurements between cases and controls using generalised additive models within a conditional logistic framework. We will thereby provide a refined understanding of how ZIKV and DENV antibody cross-reactivity at the strain-level influences infection and disease outcomes, which goes beyond current serotype and species level inferences on protection and ADE. If our analyses indicate a role for CMI, we can explore the contribution of ZIKV- and DENV-derived peptides using stored PBMCs.

***Contribution of antibody landscapes in mothers to CZS outcomes in their newborns (Hypothesis 9)**:* We will conduct a similar analysis using samples from our Brazilian cohort of 1,093 mother-newborn pairs during the 2015-2016 outbreak (N=102 with CZS-associated microcephaly)*(36)*. Although samples were collected at birth rather than when infection occurred, antibody profiles at delivery reflect foetal exposure to potentially protective and enhancing antibodies. Our preliminary studies found that low titres to DENV2 were associated

with higher CZS risk whereas titres to other serotypes appeared protective. We will expand these analyses by constructing antibody landscapes from 20 cases and controls and identifying specific signatures of the resulting landscape which are associated with protection or enhancement. These analyses, in synergism with Aim 1, will provide a comprehensive insight into how population and individual level immunity influences CZS, the critical public health outcome of Zika.

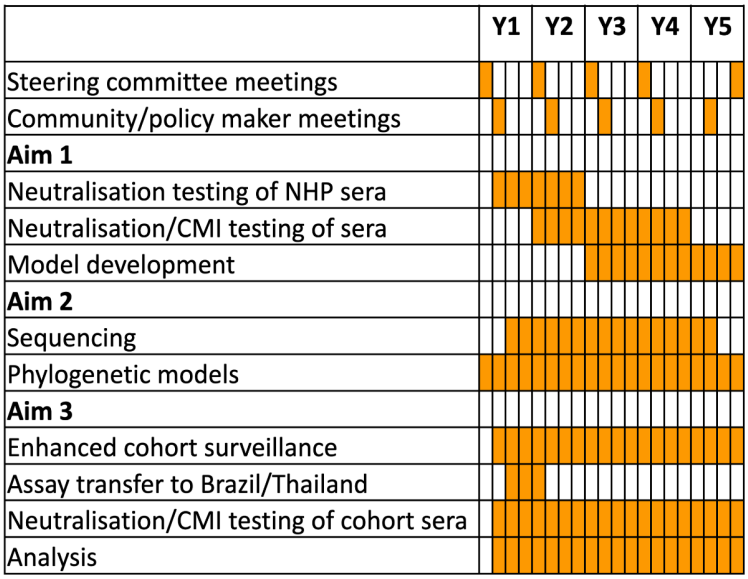


**Figure 6**. **Timeline**.

**7. Key milestones and pitfalls**

*Milestone 1. Develop a reference antigenic map.* We have established testing protocols and mapped 348 DENV, existing NHP sera and have identified ZIKV and Brazilian DENV isolates for the map.

*Milestone 2. Develop population level antigenic landscapes.* We have existing sera from our cohorts to build the landscapes.

*Milestone 3. Develop population level CMI profiles.* We have developed protocols and Dr. Kalimuddin who developed the original assay is a collaborator.

*Milestone 4. Identification of ZIKV/DENV lineages.* We already have sufficient DENV/ZIKV sequences from Thailand to identify lineages. While we may not identify additional ZIKV sequences from Brazil, this will not impact our ability to explore the impact of population landscapes on DENV and ZIKV fitness.

*Milestone 5. Identifying incident ZIKV infections.* Preliminary analysis show ZIKV incidence in our Thai cohort, and confirmed cases are detected by the MOPH annually, suggesting widespread incidence.