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14. ABSTRACT - The overall objective of this proposal is to examine the evolutionary consequences of introducing a tetravalent live-attenuated dengue virus vaccine into children in Northern Thailand on naturally occurring endemic wild-type dengue virus. Dengue is considered an emerged global public health problem. It is the most common arbovirus causing human disease in subtropical and tropical regions of the world and estimated that over 50 million DV infections occur each year and over 20,000 deaths. In this grant, an interdisciplinary team of university and military investigators are conducting coordinated studies to determine the effect vaccination with a candidate tetravalent vaccine will have on vaccine related genetic changes on wild-type dengue virus and how these changes will determine risk for severe dengue and serotype-specific dengue virus transmission. Coordinated studies are being performed during the vaccine trial in Kamphaeng Phet Province, Northern Thailand, to isolate wild-type dengue virus and examine genetic diversity in the population, in hospitalized children with severe dengue illness and cluster investigation of their neighborhoods, and by using sophisticated Global Positioning Systems (GPS) technology of isolated viruses and genetic characterization, spatial and temporal analysis are being performed in detail. This study offers a unique opportunity to study the evolutionary consequences of this vaccine on wild-type dengue virus with findings that will have long-term impact on the design of future dengue vaccines and conduct of dengue vaccine efficacy trials.

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Introduction

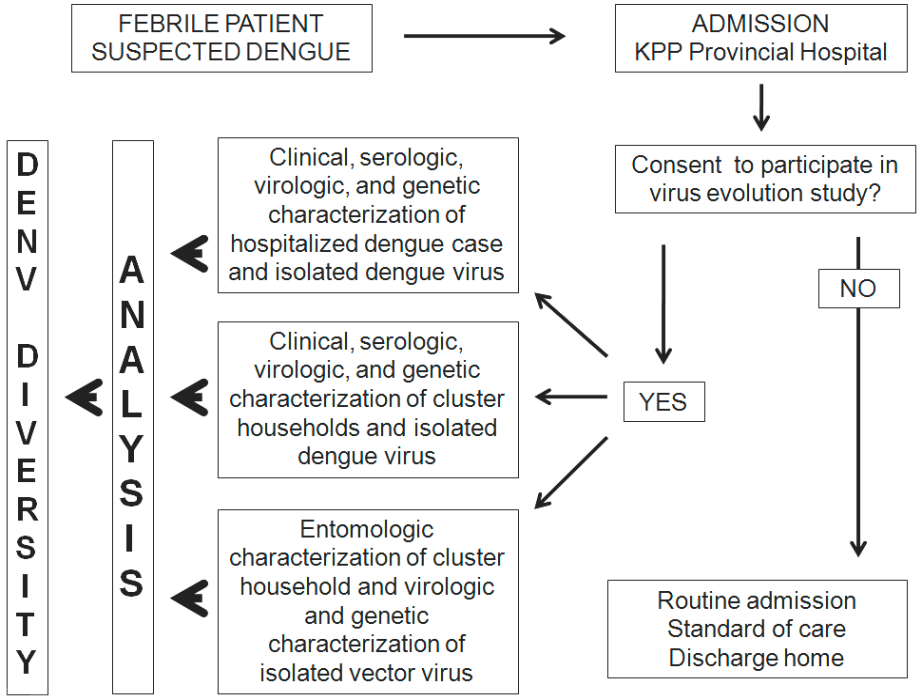
The overall objective of this proposal is to utilize GPS technology to track dengue disease transmission in Northern Thailand establishing a methodological proof of principal for future investigations. This proposal is part of an ongoing NIH supported, Walter Reed Army Institute of Research (WRAIR) approved protocol entitled, “Dengue Virus Circulation, Evolution, Virus-Vector, and Virus-Host Interactions in Kamphaeng Phet Province, Thailand”, WRAIR #1526. Dengue is a worsening global health problem placing deployed soldiers at risk for morbidity and mortality. Dengue is the world’s most important arbovirus and ranks #3 on the DoD infectious diseases threat list. Uncontrolled, dengue-infected vector populations decimated U.S. troops and combat effectiveness during WWII Pacific operations. Nearly one third of hospitalizations for fever of unknown origin during U.S. operations in Viet Nam, Somalia, and Haiti were due to dengue. There is no vaccine or specific drug therapy to prevent or treat dengue. Vector control, except in very rare circumstances, has been unsuccessful at controlling the dengue problem. Personal protective measures require sustained vigilance and may not be possible during a combat or similar operation tempo environment. Increased understanding of vector-virus-host interactions and dengue disease transmission dynamics is required to mount more strategic personal and unit protective measures. Global Positioning System (GPS) technology allows investigators to map, with precision, locations of events of interest; in this case human dengue cases (index), index case contacts that subsequently develop dengue (contact), and mosquito vectors infected with dengue (vector). Advanced molecular techniques allow for complete genetic characterization of viruses (full genome sequencing) isolated from index cases, contacts, and vectors. Tracking events and changes in events clinical/viral/genetic) over time, combined with complex data analysis and geospatial modeling, provide important information about how dengue viruses are transmitted in space in time, how they are evolving, and how viral genotypic evolution impacts disease phenotype (severity). Vector competency assays conducted with viruses and vectors isolated over different geographic regions promote understanding of virus-vector co-evolution and the impact on dengue virus virulence and disease severity. Completing the above before, during, and after execution of a dengue vaccine efficacy trial will provide extremely important information to combatant commanders and vaccine use policy makers about how a dengue vaccine needs to be employed to maintain combat effectiveness during a dengue epidemic or during operations in a dengue endemic environment. The State University of New York-Upstate Medical University (SUNY-UMU) has been awarded a NIH R01 4-year grant to conduct dengue disease surveillance in Northern Thailand, cluster investigations around the index case, and entomologic investigations and vector competency studies. TATRC funding has allowed GPS mapping of all cases and viral isolates in humans and mosquitoes allowing sophisticated temporal-spatial analyses of dengue disease transmission within the community before, during and after conduct of a dengue vaccine efficacy trial. SUNY is the grant awardee and provides project management, dengue expertise, and virologic lab support. AFRIMS Virology manages and supports the field site in Kamphaeng Phet Province (KPP), Northern Thailand, supports all human use study management/execution and the laboratory completing serologic, virologic, molecular, and genetic case characterizations. AFRIMS entomology completes mosquito collections, GPS mapping, and vector competency assays. Wild-type dengue virus is isolated, sequenced and spatial and temporal genetic diversity assessed in individuals hospitalized with dengue, in cluster investigations of their neighborhood contacts, and through integrated intensive vector surveillance and study. A serotype-specific, geospatial specific, genetic diversity index is being determined temporally and spatially. Sanofi Pasteur has selected the AFRIMS Virology field site in KPP to be a participant in a regional phase 3 dengue vaccine efficacy trial. The trial is scheduled to begin in 2Q CY2011. Sanofi will permit the grant to GPS map all vaccine trial volunteers and explore the sharing of data between the RO1 grant and vaccine trial. The collaboration and proposed study represents the first of its kind to geospatially track dengue disease and define dengue virus diversity before, during and after execution of a dengue vaccine trial. Study success will provide proof of principal GPS mapping of infection events in index cases, contacts, and vectors and geospatial modeling can accurately capture and characterize disease transmission dynamics, the impact of interventional measures (vaccine), and guide the development of more strategic protective measures. The study methodology may be applied to other diseases of interest to the DoD and become standard practice for preventive medicine planning and/or disease outbreak investigations among military units. Deliverables: 1) proof of principal for using off the shelf GPS hardware and software technology in rural environments to conduct detailed and state of the

art disease surveillance; 2) detailed information on the dengue serotype-specific virus transmission in the human and vector population in Northern Thailand; 3) geospatial analysis of dengue virus transmission in the human and vector populations; 4) understanding of dengue virus and clinical disease evolution over time and space; and 5) understanding the impact of vaccine introduction on dengue disease and dengue virus evolution over space and time in dengue vaccine recipients and nonrecipients.

Background

Dengue virus has evolved over the last 200 years as four distinct serotypes and an important human pathogen producing severe illness known as dengue hemorrhagic fever (DHF). Dengue is considered an emerged global public health problem. It is the most common arbovirus causing human disease in subtropical and tropical regions of the world and estimated that over 50 million DENV infections occur each year with several hundred thousand cases of DHF, and over 20,000 deaths. DHF is a major cause of hospitalization and disability adjusted life-years (DALYs). A dengue vaccine that offers protection against all four dengue serotypes is a high priority of the Department of Defense and based on cost per DALYs saved, highly cost effective. It is not known what effects vaccination will have on the evolution of naturally occurring dengue viruses. Vaccination may create an environment of relative low-transmission of natural dengue virus, within the human host and its vector, thereby increasing stochastic events that will allow new dengue virus genotypes to emerge. These new genotypes could impact virus-vector and virus-host interactions resulting in increased dengue virus transmission and altered disease phenotype. Dengue viruses will be collected from hospitalized people with dengue and from febrile individuals residing around the index case and identified through cluster investigations. Integrated and intensive vector surveillance will be completed in the area surrounding index cases.

Figure 1 Schematic of Study Activities for Hospital and Cluster Based Investigations.



Dengue vaccine and the effect on DENV evolution

Currently there is no treatment for DHF other than supportive care. A dengue vaccine has been determined to be a high priority and an essential public-health intervention. A number of candidate dengue vaccines are in development. The WRAIR tetravalent live-attenuated DENV vaccine and Sanofi Pasteur chimerivax candidates demonstrated safety and immunogenicity in pre-clinical testing, reduced

transmission and replication in mosquitoes, and safety and immunogenicity in phase 1 and small phase 2 human studies [1-5 6 7,8 9-17]. The nature of severe dengue illness as discussed is a complex interaction between the virus and the host-immune response with severe disease being a result of the hostimmune response. Neutralizing antibody to a DENV serotype, the currently recognized correlate of immunogenicity, may not be predictive of protection as demonstrated in studies conducted by our group in Thailand [18]. Current evidence suggests it will be difficult to predict the short or long-term consequences of a dengue vaccine in a population as it is exposed to wild-type DENV [19-21]. From the data presented, it is clear that the DENVs are evolving within specific localities, generating both abundant genotypic and phenotypic diversity as a result of both virus-human and virus-vector interactions, as well as an intrinsically rapid mutational dynamics. Further, stochastic events that result in new genotypes arising during periods of low-transmission, coupled with the possibility of recombination, suggest that DENVs will continue to evolve and produce severe disease. Dengue vaccination offers the opportunity to protect the population from infection and severe disease. Crucially, however, it is not known what effect vaccination will have on the evolution of naturally occurring DENVs. Indeed, based on available data it is possible that several events could occur during vaccination. For example, vaccination may produce an environment of relative low-transmission of natural DENV, thereby increasing the likelihood of stochastic events, in turn facilitating the emergence of new DENV genotypes. Recombination events with vaccine strains may result in attenuated wild-type virus or new genotypes with greater virulence. Dengue vaccination may produce low-titers of enhancing antibody to specific DENV serotypes resulting in emergence of specific serotypes. The vector population requires the human host to be infected. Following a change in the serotype and potential recombination events, new genotypes may emerge within the vector population posing a risk to humans who are not vaccinated.

Rationale

The AFRIMS Virology field site (KAVRU) is being considered as a site for efficacy studies. It is not known how protective the leading dengue vaccine candidates will be nor the consequences they will have on wild-type circulation and the emergence of genetically unique wild-type DENV. We hypothesize that certain dengue vaccines (live virus or live chimeric) could alter the selection pressure on wild-type DENV by decreasing the transmission pressure on the virus, increasing stochastic events of the virus in the vector population, altering serotype-specific antibody protection or enhancing herd immunity, and potential recombination events with vaccine strains. As a result, DENV genetic diversity will increase in and around the vaccinated population allowing the emergence of genetically unique DENVs. Genetically unique DENVs will interact with the host-immune response that has been primed with previously circulating wildtype viruses producing potentially more severe disease. Our hypothesis suggests that during the dengue vaccine trial there will be more hospitalized severe dengue illness as a result of dengue vaccine failures, emergence of viral neutralizing escape mutants, and genetically unique DENVs that have greater host virulence. This study of hospitalized patients will genetically characterize DENV isolated from patients with severe disease, from febrile patients and mosquitoes in hospitalized patients' homes and neighborhoods, in order to determine the
evolutionary consequences of a dengue vaccine on wild-type DENV.

Military Relevance

Dengue's place in U.S. military history began during World War II (WWII). Soldiers stationed in the Pacific theater introduced dengue viruses throughout Southeast Asia, Japan, and the Pacific Islands. The deployment of non-immune troops to dengue endemic areas with unchecked vector populations resulted in large epidemics of disease. McCoy and Sabin described epidemics among troops in the Northern Territory and Queensland (1942), Espíritu Santo (1943), New Caledonia (1943), New Guinea (1944), and the Philippines (1945). Reports suggest there were over 2 million cases of dengue between 1942 and 1945 in Japan. An extensive dengue outbreak among U.S. forces occurred in 1944 in the Marianas Islands and despite the use of DDT, over 20,000 dengue cases are believed to have occurred on Saipan. Dengue continued to have an adverse impact on the U.S. military during operations in Viet Nam, Somalia, and Haiti. Recent medical literature is documenting significant dengue attack rates among travelers and foreign military personnel deployed to dengue endemic regions. Prevention of dengue through widespread vaccination is an important objective of the U.S.

Department of Defense. The U.S. has made significant contributions to dengue research. Ashburn and Craig provided evidence for the viral etiology of the disease making dengue virus the second human viral pathogen identified after the yellow fever virus. Siler, Hall, and Hitchens researched the role of *A. aegypti* as a vector in the transmission of dengue virus. Research performed by Hotta and Kimura and Sabin and Schlesinger during WWII isolated dengue virus types -1 and -2 (DENV-1, -2) and identified the presence of homotypic immunity following infection. In 1956, the dengue epidemic occurring in Manila resulted in the identification and naming of DENV-3 and -4 by Hammon. In the early 1980s, the U.S. Naval Medical Research Unit (NAMRU) No. 2 described dengue outbreaks among U.S. military personnel at Clark Air Base. NAMRU No. 3 in Peru characterizes dengue epidemiology in Central and South America while AFRIMS has been conducting dengue epidemiology and basic science research in Asia for over 40 years. The U.S. military is a leader in dengue vaccine development. Early efforts date back more than 70 years with attempts to prevent virus transmission using infectious human plasma inactivated with formalin. Sabin and Schlesinger undertook the first attempts to immunize using mouse-passaged live-attenuated DENV-1 and -2 viruses. The U.S. Army continued development of these vaccine candidates for the next decade. In 1962, a field efficacy trial in Puerto Rico using the DENV-1 vaccine candidate demonstrated partial protection during an outbreak of predominantly DENV-3. In 1971, the US Army Medical Research Command (USAMRC) launched a program at the WRAIR to develop a live-attenuated dengue vaccine produced in mammalian cell cultures. Meanwhile, Halstead and colleagues demonstrated that dengue viruses could be attenuated in monkeys by passage in primary dog kidney (PDK) cell cultures. A variety of PDK-attenuated vaccine candidates for all four dengue serotypes were manufactured and tested in humans. A down-selection process based on safety and immunogenicity resulted in four vaccine candidates, one for each DENV type, that were combined into a tetravalent vaccine. In 2000, USAMRMC entered into a Cooperative Research and Development Agreement (CRADA) with GlaxoSmithKline Biologics to co-develop PDK passaged live-virus candidates. Phase 1 human studies demonstrated acceptable safety and immunogenicity in US adults, Thai children and Thai infants. Additional studies evaluated variations in dosing schedule, route and delivery method. Eighteen tetravalent formulations would be tested with two showing promise. Three Phase 2 studies were initiated in the US, Thailand, and Puerto Rico inclusive of over 900 volunteers between the ages of 12 months and 50 years. Adult studies at WRAIR (TDEN-001, N=86) and AFRIMS (TDEN-002, N=120) are complete while the larger study in Puerto Rico (TDEN-003, N=720) continues enrollment (~450 enrolled). In TDEN-001 the frequency of local and general solicited symptoms was similar between the vaccine groups and placebo. Two doses of vaccine were immunogenic, eliciting variable neutralizing antibodies to all DENV types. One dose of vaccine elicited variable CD4+ T-cell responses to all DENV types. An interim analysis of TDEN-002 identified no major safety issues following dose 1 and a significant percentage of volunteers with a baseline naïve or monovalent DENV neutralizing antibody profile being promoted to a tri- or tetravalent antibody profile. An interim analysis of the first 100 pediatric (<21 years) volunteers enrolled in TDEN-003 demonstrated no safety concerns. As seen in previous studies, a single dose of vaccine elicited broad antibody responses in DENV primed volunteers and modest responses in DENV naive volunteers. Final safety and immunogenicity analyses (antibody and CMI) for all studies are pending completion. The WRAIR/GSK dengue vaccine has been safe in over 400 volunteers. Preliminary data supports evaluation of vaccine candidates in a pilot field efficacy trial. After decades of development, the goal of developing a vaccine to protect the Warfighter from dengue appears attainable. As the vaccine development process advances, military investigators and their collaborators are committed to establishing study platforms to understand the near and long term consequences of introducing tetravalent dengue vaccines into endemic populations. Numerous questions and theoretical concerns exist about dengue vaccines and the consequences of their introduction into national immunization programs or, in the case of the military, deployment or entry vaccination schedules.

Body

Hypothesis

We hypothesize that the introduction of a tetravalent dengue vaccine will have important

consequences for the evolution of naturally occurring DV (DV) by altering the ecologic pressure on the virus through changes in the virus-host and virus-vector interactions. **The goals of this project are to determine the effect that vaccination with a candidate live-attenuated tetravalent vaccine will have on (1) vaccine related genetic changes that increase risk for DHF in hospitalized children with acute dengue; (2) serotype-specific DV transmission and genetic diversity in the placebo population and breakthrough viremia in the vaccinated population; and (3) the role of mosquito vectors in the emergence and spread of novel DV genotypes.** We propose that dengue vaccination with a live-attenuated tetravalent vaccine will not result in sterile immunity, but produce low-level viremia with subclinical infection from wild-type DV. This will result in selective pressure on specific serotypes to emerge with greater mutational events occurring stochastically as a result of reduced transmission of DV. Mutational events will be reflected in isolated DV in the non-vaccinated population and mosquito vector. We also hypothesize that the effects of selective pressure and cross-reactive antibody protection or enhancement in the vaccinated population will allow the emergence of genetic changes not previously observed in DVs. This will include defective viruses with little pathogenicity as well as the potential for new genetic diversity and recombination events that may result in viruses with greater virulence. An understanding of the genetic events that occur in DV during the field testing of a dengue vaccine will improve our understanding on the longterm consequences of using a dengue vaccine and other candidate DV vaccines on a population, on naturally occurring DVs and its potential effect on unprotected hosts.

Technical Objectives

1. Establish baseline data of wild-type DENV genetic diversity and microevolution in a geographically defined area over time
2. Determine the role of mosquito vectors in the emergence and spread of novel DENV genotypes that may arise from the introduction of a dengue vaccine in a population.

Specific Aims:

1. To conduct a prospective hospitalized-based study of dengue infections and cluster investigations, in order to:
    A. GIS map hospitalized dengue patients and perform cluster investigations of neighborhoods of hospitalized cases to establish data on DENV genetic diversity within a population to determine if diversity varies spatially as a result of local variation in the level of herd immunity and circulating dengue serotypes.
    B. Identify and virologically define virus genetic changes that result in severe hospitalized symptomatic dengue disease.
    C. Characterize virus-host interactions and how DENV genetic diversity impacts these interactions.
2. Evaluate, in a cluster study design, the role of mosquito vectors in the emergence and spread of novel DENV genotypes in order to:
    A. Define the impact of mosquito-virus interactions on the genetic diversity and number of novel DENV genotypes.
    B. Determine whether the emergence and spread of novel DENV genotypes depends on viral adaptation to the local mosquito vector populations.

Methods:

Ethics Statement

The study protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR), and the State University of New York (SUNY), Upstate Medical Center. The IRB's of the University of Massachusetts Medical School (UMMS), University of California, Davis (UCD), and University at Buffalo deferred review to the WRAIR IRB via an inter-agency agreement. All study volunteers and subjects engaged in the informed consent or assent process, as applicable, and documented the same prior to participating in any study activities. In the event the volunteer or subject was unable to participate in the informed consent/assent process, a recognized health care proxy represented them in the process and documented consent. From this point forward, when the authors discuss consent, assent is also implied as applicable.

Prospective Hospital-Based Study of Suspected Dengue Virus Infections and Cluster Investigation- This proposal involves children and adults who present to the hospitals in Kamphaeng Phet with suspected dengue. We will examine the consequences of DENV evolution on dengue disease severity by: (1). GIS mapping of hospitalized dengue patients and cluster investigation in their neighborhoods to determine the level of standing DENV genetic diversity in KPP. To see if diversity varies spatially as a result of local variation in the level of herd immunity and circulating dengue serotypes; (2) identify, quantify and virologically define virus genetic changes that result in severe hospitalized symptomatic dengue disease and determine if these changes are reflected in the larger population; and (3) examine the virus-host and virus-vector interactions by examining the host immune response to variations in the virus.

Monitor Serotype-Specific DENV Transmission and Genetic Diversity in the Vector Population - Our entomological research will be an extension of cluster investigations currently being conducted in Kamphaeng Phet. In this study, we will evaluate the role of mosquito vectors in the emergence and spread of novel DENV genotypes. This is a unique and timely opportunity to study the evolutionary consequences of herd immunity on DENV-vector interactions. Using our existing GIS for the proposed study area, hospitalized symptomatic dengue cases will initiate cluster investigations and collection of infected mosquitoes. Based on prospective longitudinal cluster investigations, we will (1) characterize and compare DENV isolates obtained from mosquitoes collected in areas of index cases and cluster investigations and; (2) monitor the evolution of serotype-specific DENV transmission by conducting lab-based vector competence assays using field-derived mosquito populations and viral isolates from humans with clinical outcomes ranging from severe disease to sub-clinical infection. Mosquito genotyping and virus sequence data will define genetic variation among the mosquitoes and viruses we study. Results of this study will determine whether the evolutionary success of novel DENV genotypes is constrained by genetically diverse mosquito populations.

Study Design

Subject Enrollment: Kamphaeng Phet Province is served primarily by the public health hospital, Kamphaeng Phet Provincial Hospital (KPPPH). Children and adults who are admitted with a diagnosis of suspected dengue illness will be enrolled in this study. There can be considerable seasonal variation in the annual number of hospitalized dengue cases. Based on a conservative annual clinical attack rate between 1.5-2.0%, PCR positive rates >70% in hospitalized cases and known logistic limitations, investigators anticipate enrolling between 200-800 cases from the hospital each year (seasonal variation) and conducting between 80-150 clusters investigations annually. If enrollment exceeds the upper limit of these projections by
>10% investigators will immediately inform the IRB and request an increase in enrollment.

Hospitalized Subjects

Hospital admission evaluation, clinical course and discharge: Patients with an admission diagnosis of acute dengue infection will be contacted by an AFRIMS study nurse and the informed consent and assent, if applicable, process implemented. AFRIMS performs research assays for all suspected dengue cases; all potential volunteers will be known to AFRIMS and captured using this mechanism. Therefore, there will be no advertisements used for this study.

The following information will be recorded from enrolled subjects: demographic information including home address, chief complaint/reason for hospitalization, date of onset of symptoms, symptoms during illness and oral or axillary temperature. Clinical information will be collected from direct interviews and the hospital record. Information will be recorded in the study chart. A blood specimen will be obtained at the time of admission; this specimen is collected by the hospital as part of routine medical care for suspected DENV infections. The clinical course will be charted and based on clinical criteria a classification as DF, DHF and DHF grade, if applicable, assigned. A 2 week (± 5 days) follow-up visit will be completed to obtain a
convalescent blood sample. The convalescent visit will occur at the subject's home/school or, in very rare circumstances, the KAVRU field site. Once a parent provides consent for enrollment of their child, the parent does not have to be present for blood collections related to the study. In the hospital and home setting, parents will almost always be present. In the school setting parents will likely not be present. The previous 10 years of dengue studies in KPP were schoolbased studies and blood collection occurred without parents and without incident. In the event a collection needs to occur at KAVRU there will almost always be a parent.

Blood specimens: see submitted proposal for details.

Non-hospitalized Subjects (Cluster Investigations)

Study Population: Cluster investigations of DENV RT-PCR confirmed cases (index case), will be performed throughout the study year. The number of cluster investigations performed per week will be based on the number of index cases and logistical constraints (i.e. index cases residing > 2 hours from KAVRU can not be investigated). Based on past experience, investigators anticipate performing 4-8 cluster investigations per week (total 80-150 clusters per year). Homes of index cases will be GIS mapped and up to approximately 25 susceptible contacts within the household and neighborhood homes will be surveyed for fever or history of fever. Based on the past 5 yrs of cluster studies in KPP, sampling up to 25 contacts was determined to be necessary to obtain a sufficient number of dengue virus susceptible volunteers to consistently isolate viruses, generate useful data for analysis, yet not exceed logistical constraints. Clinical information and acute and convalescent blood samples from consenting subjects will be collected. Mosquito collections will be performed in the household and surrounding neighborhood.

Investigation: Index cases (hospitalized PCR+ subjects) 'triggering' a cluster investigation will be identified between Monday and Thursday of each week throughout the year. Cases will occur during the winter months and these viruses will be a source of diversity during the interepidemic period. Most specimens from index cases will arrive at the field station laboratory by 3pm each day. The DENV RT-PCR result will usually be available by 11AM the following morning. A study nurse will visit the index case and begin the consent process and assent, if applicable, for the performance of a cluster investigation. After the informed consent process is complete and consent has been provided the study nurse will notify an entomological team supervisor to visit the village and begin identifying houses to potentially participate in a cluster investigation (usually within 48 hours of the +PCR result). The exact location of all houses in each participating village will have their location GIS mapped using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100-200 meter radius (the exact radius to be predetermined based on the prevalent average density of homes across all villages) of the index case. The standard cluster radius will be 100m based on the known restricted flight range and the anthropophilic resting and breeding behavior of peri-domestic *Ae. aegypti* populations and the density of housing in most villages. However, in areas where human dwellings are less concentrated, and there are fewer houses, it may be necessary to extend the sampling unit (cluster) to include more houses between 100-200m from the index case. This approach has been devised based on 5 years of previous cluster investigation experience in the same province and is designed to maximize the scientific output of every investigation. We expect that most clusters will be within a radius of 100 m, but none will exceed a radius of 200 m.Study nurses will visit houses within the 100-200 meter radius starting closest to the index case house and moving outward in a concentric fashion. Each home will be queried for active fever (oral equivalent of >38°C) or a history of fever within the past 7 days. If there is a current fever or history of fever within the household, all household occupants will be offered an opportunity to participate in the cluster investigation. The absence of fever or history of fever will result in no enrollments from that household. A clinical study nurse will complete the informed consent process (Volunteer aged > 18 years old) and assent (Volunteer aged 7-17 years old), if applicable, with potential subjects or the parents or guardians of potential subjects. Those parents (and children) who are unavailable to be consented will be visited that same evening or the following morning; this will be the extent of enrollment attempts. Once up to approximately 25 contacts have been consented, the field teams will be dispatched to the village to begin the investigation. If 25 contacts have been enrolled and there are still residents within the household, investigators will complete enrolling the household (i.e. there may be slightly more than 25 enrollees in a cluster investigation). Clinical information and a blood sample will be obtained from each consenting and assenting, if applicable, subject. DENV RT-PCR will be performed on all acute specimens. Investigators will return to the village in approximately 2 weeks (15 days ±5 days) to acquire a convalescent blood sample and additional clinical information. DENV IgM/IgG ELISA will be performed on all acute and convalescent samples. In the event a subject who is not ill at the time of the acute sample collection becomes ill with fever, investigators will collect a blood sample at the time of the illness and restart the "clock" counting to 15 days ±5 days to acquire a convalescent blood sample. The convalescent visit will occur at the subject's home or, in very rare circumstances, the KAVRU field site. This strategy is designed to maximize acquiring DENV isolates. If

a cluster subject becomes ill with a DENV infection and requires hospitalization he or she will NOT become a new index case but followed to completion as a cluster subject. Blood samples will be labeled with a study identifier; the study subject's unique study number, an indicator the sample was collected as part of the hospital or cluster portion of the study, and an indicator that the sample is an acute or convalescent sample (see Annex C). Village leaders will be briefed on the study objectives and design once IRB approval is acquired and before study implementation. Many of the leaders will be familiar with cluster investigations due to recently completed, and similar, activities in the same region. A reasonable effort will always be made to contact the village leader prior to initiating an investigation. If the village leader is available, he or she will be requested to facilitate contact and communication with identified households. AFRIMS has established a precedence for more than 10 years of incorporating the village leadership and residents into the execution of KPP-based dengue studies.

Entomological Investigation: Kamphaeng Phet Province is the site of an AFRIMS' vector field station. Our proposed entomological research will complement the other two aims of this project by addressing the aim to test the following 2 assumptions of our hypothesis (see below) in the context of prospective longitudinal cluster investigations:

a. Genetic diversity of DENVs in mosquito vectors changes as prevailing trends in dengue herd immunity among the local human population changes. Viruses isolated from field-collected mosquitoes will be sequenced and compared phylogenetically across different locations and time-points. The extent and structure of DENV diversity in the mosquito population will be compared with that of the corresponding human population across different geographic locations and across the time course of the study. Based on 5 years of previous work in Kamphaeng Phet we know we will maximize DENV isolations by capturing adult mosquitoes in and around the homes of index cases (see Preliminary Data). Hospitalized symptomatic dengue cases will initiate cluster investigations and collection of infected mosquitoes.

b. The efficiency of DENV transmission by mosquitoes changes as prevailing trends in dengue herd immunity among the local human population changes. We will monitor the evolution of serotype-specific DENV transmission by conducting lab-based vector competence assays with viral isolates from mosquitoes (obtained in *a* above) and humans with clinical outcomes ranging from severe disease to sub-clinical infection. Mosquito genotyping and virus sequence data will define genetic variation among the mosquitoes and viruses we study. Several vector competence indices (rate of midgut infection, rate of virus dissemination from the midgut to the salivary glands, and virus transmission potential) will be compared experimentally across a matrix of different vector and DENV populations from the same and different locations and the same and different times. The experimental design will allow us to determine whether over time viruses adapt to local mosquitoes populations so that they infect mosquitoes and are transmitted more efficiently.

Research Assays see submitted proposal for details.

Project Milestones: This project is ongoing with the first patients enrolled January 2010 and cluster investigations ongoing. DENV have been isolated from patients, clusters and mosquitoes and currently being sequenced. The dengue peak season will start in May and the protocol is fully engaged to capture all DENV infections per protocol

Results:

Results based on a manuscript in preparation for submission for publication, Thomas SJ et al, "Improved Dengue Virus Capture Rate in Humans and Vectors Using Improved Index Case and Contact Surveillance Methods During the 2010 Dengue Season in Kamphaeng Phet Province, Thailand".

Characteristics of the Index Cases

For this analysis, 149 PCR + cases dengue cases were identified at the KPPPH. All met criteria for inclusion as index cases; 93/149 (62.4%) were enrolled. Multiple PCR+ samples in a single day and samples collected outside of the 24-hour window (e.g., Friday afternoon) accounted for the difference between the 149 PCR+ cases meeting inclusion criteria and the 93 ultimately enrolled. All index cases were chosen randomly from those meeting inclusion criteria and all approached for enrollment consented to do so. The minimum age was 2.6 years and the maximum 56 years, with a mean age of 18.7 years (SD 9.4 years). Male to female ratio was 1.1: 1.

Occurrence and Clinical Spectrum of DENV Infections in Index Cases

By definition, each of the 93 index cases was PCR+. Infections with serotypes DENV-1, -2, -3, and -4 were detected in 15 (16%), 56 (60%), 22 (24%), and 0 cases, respectively. Serologic diagnosis indicated acute primary infection in 2 (2%), acute secondary infection in 79 (85%), recent secondary infection in 2 (2%), no serologic diagnosis due to single specimen in 7 (8%); and serology data were unavailable in 3 (3%). The final clinical diagnosis indicated 45 (48%) dengue fever (DF), 24 (26%) dengue hemorrhagic fever grade I (DHFI), 17 (18%) DHFII, 7 (8%) DHFIII, and 0 DHFIV.

Contacts Available for Enrollment

There were a total of 1063 households with contacts eligible for study inclusion; of these 208/1063 (19.6%) contained a person eventually enrolled as a contact. The potential number of contact houses within 200 meters of any index case ranged from 1 to 232 with a mean of 47.7 (SD 42 houses). The range of houses actually enrolled was from 1 to 9 with a mean of 2.2 (SD 1.5 houses). For each index/contact investigation there was a range of potential contacts from 0 to 18 people with a mean of 4.7 (SD 4.0 people) enrolled. See figure 1.

Characteristics of the Contacts

A total of 793 potential contacts were identified, 438/793 (55.2%) were enrolled and serologic data was available for 409/438 (93.4%). The minimum age was 7 months and maximum age 94.2 years; the mean age was 31.4 years (SD 22.3 years). Males made up a slightly lower percentage of contacts than females (46% vs 54%; 1:1.2). People who declined to enroll did so due to fear of donating a blood sample, not being available at the time of enrollment, not willing to participate for unspecified reasons, and having been involved in an index case/contact investigation within the previous 6 months (exclusion criteria).

Occurrence and Clinical Spectrum of DENV Infections in Contact Cases

Of the 438 enrolled contacts 409/438 (93.4%) had a serologic diagnosis available. Evaluable subjects ranged in age from 7 months to 94.2 years with a mean age of 31.4 years (SD 22.3 years). Acute primary DENV infection was diagnosed in 14 (3%), acute secondary infection in 69 (17%), and recent secondary infection in 3 (1%); 322 (79%) had no serologic evidence of infection. There was 1 case (0.2%) with serology consistent with a Japanese encephalitis infection. Among the 86 (21.0%) contacts with a positive dengue serology result, the minimum age was 10 months and maximum 82 years, with a mean of 23.1 (19.6 years). Females represented 55% of cases. Nested PCR results among the 86 revealed DENV-1, -2, -3, and -4 infections in 1 (1%), 14 (16%), 21 (24%), and 0 cases, respectively. PCR was negative in 50 (58%) cases. There were 156/409 (38.1%) subjects with a serologic test result and fever ranging in age from 7 months to 77 years with a mean age of 20.1 (SD 19.5 years). Females accounted for 57%of cases. Acute primary DENV infections were diagnosed in 11 (6%), acute secondary DENV infections in 49 (25%), recent secondary DENV infection in 1 (1%), and no serologic evidence of DENV infection was found in 95 (67%). Therefore, 11/14 (78.6%) acute primary DENV infections, 49/69 (71%) acute secondary DENV infections, and 1/3 (33.3%) recent secondary DENV infections were associated with fever. Fever was associated with 95/322 (29.5%) of contact cases without serologic evidence of DENV infection.

Presentation of symptom data by primary and secondary DENV infection can be found in Figure 2. A significant difference in symptoms was found only in the occurrence of nausea (0% of primary DENV infections versus 35% of secondary DENV infections).

There was a statistically significant difference in the probability of a contact becoming infected based on the DENV type of the corresponding index case. DENV-2 and -3 index case infections carried a much higher probability of infecting a contact (Table 1). Further evaluation of the probability of infection among contacts by age group revealed significant differences within the contacts of index cases with DENV-2 infections.

Spatial Distribution of Contact Households

The spatial distribution of all households and enrolled households demonstrated a statistically significant relationship between the proximity of the household to the index case and the likelihood of reported fever in the household. As distances increased from the index case house, the likelihood of enrollment became lower (see Table 2 and 3).

Also of note and consistent with previous studies, households further from the index case had a lower rate of DENV infection among contacts living within the household. There was a significant decline in the proportion of infected contacts living >120 meters from the index household.

DENV Infection in *Ae. aegypti* Mosquitoes Residing Among Human Dengue Cases

For entomological studies, households were divided into 5 categories (1) all households with potential for enrollment (N=4388), (2) index case households (N=93), (3) non-index households with a PCR+ contact enrolled (N=41), (4) non-index households without a PCR+ contact (N=75), and (5) non-index households without enrolled contacts (N=4229). There were significant differences in the likelihood of collecting mosquitoes and the likelihood that one or more collected mosquitoes was PCR+ for DENV among the sub-groups of households.

A total of 3,565 mosquitoes were collected from 4,438 households (i.e. all households) (Table 4). From all households, 1,288 (29%) had female *Ae. aegypti*; between 0 and 34 female *Ae. aegypti* were collected per household (mean 0.80, SD 2.08). A total of 63 of the 3,565 (1.8%) mosquitoes collected were PCR+ females. A total of 36 of all 4,438 households (0.81%) had a PCR+ female *Ae. aegypti* mosquito. Among index case households (N=93), a mean of 2.49 (SD 4.98) females were collected per house, 51 of 93 households (54.8%) had females, and 23 of all 63 PCR+ females (35.6%) were collected in index case households. Nine of all index case households (9.7%) had a PCR+ female *Ae. aegypti* mosquito. In contact households with PCR+ contacts there was a mean of 2.07 (SD3.66) *Ae. aegypti* females were collected per household, 24 of the 41 (58.5%) had *Ae. aegypti* females, and 1 was PCR+ (1.6% of all PCR+ mosquitoes). In contact homes without a PCR+ contact (N=41), a mean of 2.07 *Ae. aegypti* females (SD 3.66) were collected per home yielding 1 PCR+ mosquito from 1 household (1.6% of all PCR+ mosquitoes). The remainder of non-index case households, those without contacts (N=4,229), had a mean of 0.75 *Ae. aegypti* females collected per household (SD 1.94) and 1,177 (27.8%) households had female *Ae. aegypti*. A total of 36 PCR+ mosquitoes, 57.1% of all PCR+ mosquitoes, were collected from 23 households (0.54% of all households in this category).

There was a significant association between a contact household's distance from the index case household and the finding of PCR+ mosquitoes collected in the contact household. The greatest percentage of positive mosquitoes was in households between 0-40 meters from the index case household; 33 PCR+ of the 561 collected (5.9%). Between 40-80 (7 PCR+ of 545 collected, 1.3%) and 80-120 (13 PCR+ of 733 collected, 1.8%) meters from the index case household rates of PCR+ mosquitoes were roughly equivalent. Contact households 120-160 (7 PCR+ of 749 collected, 0.9%) and 160-200 (3 PCR+ of 981 collected, 0.3%) meters from the index household had very few PCR+ mosquitoes.

Tables and Figures:

Figure 1.

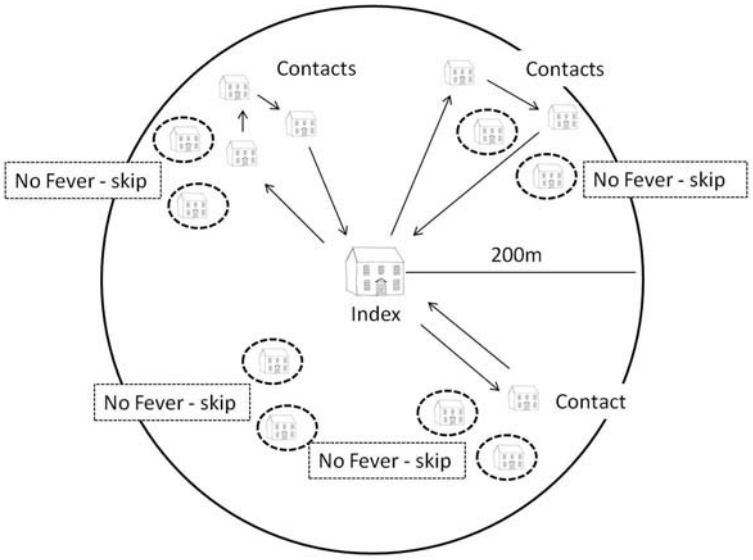


Figure X – Basic design of conducting contact home identification within a 200 meter radius of the index home and identifying those homes with fever or history of fever for possible enrollment.

Figure 2

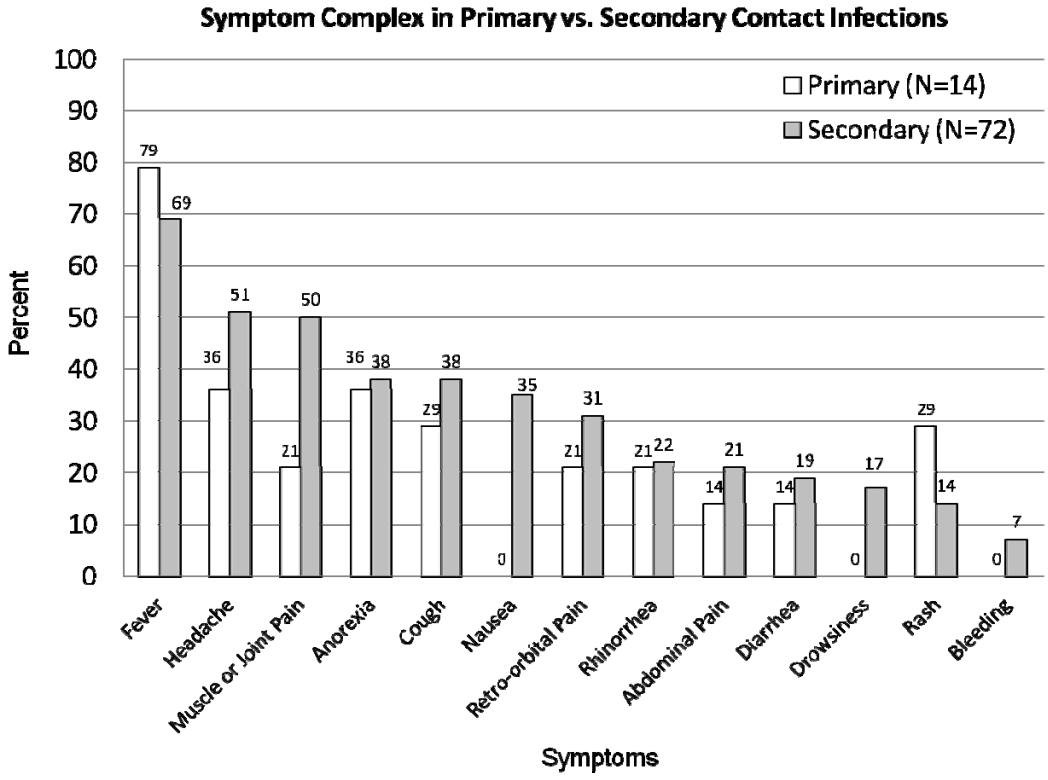


Table 1.
Probability of Dengue Virus Infection in Contacts According to Index Case Infecting DENV type. Numbers in parentheses are the proportions EIA positive. The infecting DENV type is taken from the index case. The differences are significant (Fisher's Exact Test, p-value = 0.003).

Group	DENV-1	DENV-2	DENV-3
EIA Positive	4 (0.09)	44 (0.19)	38 (0.30)
EIA Negative	43	191	88

Table 2.
Spatial Distribution of Households and Enrolled Households – Numbers in parentheses indicate proportions of houses enrolled for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

		Distance to Index Household			
	0-40	>40-80	>80-120	>120-160	>160-200
Total Households	539	814	971	1051	1063
Enrolled Households	103 (0.191)	53 (0.065)	32 (0.033)	13 (0.012)	7 (0.007)

Table 3.

Spatial Distribution of Contacts and EIA Positive Contacts – Numbers in parentheses indicate proportions of contacts that are EIA positive for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

		Distance to Index Household			
	0-40	>40-80	>80-120	>120-160	>160-200
Total Contacts with Serology	228	67	51	38	25
EIA Positive Contacts	56 (0.246)	12 (0.179)	16 (0.313)	0 (0.000)	2 (0.080)

Table 4. Spatial Distribution of Mosquitoes within Households and PCR Positive Mosquitoes within Households – Numbers in parentheses indicate proportions of mosquitoes that are PCR positive for each distance category. Differences are significant - Fisher's Exact Test – p-value < 0.001.

		Distance to Index Household			
	0-40	>40-80	>80-120	>120-160	>160-200
Total Mosquitoes	561	545	733	745	981
PCR Positive Mosquitoes	33 (0.059)	7 (0.013)	13 (0.018)	7 (0.009)	3 (0.003)

Key Research Accomplishments

1. Validated the study design in capturing hospitalized ill index cases and cluster associated dengue infections in a spatially defined area.
2. Demonstrated the association between probability of infection and living close to an index case with dengue infection and virus isolates in mosquitoes.
3. Sequencing of viruses are currently ongoing and will demonstrate unique findings in the evolution of the dengue viruses.
4. Demonstrated the value of GPS technology in understanding the spatial and temporal transmission of dengue virus (example in Figure 3 appendix). Two key studies were reported recently from these findings discussed below (papers included in appendix).

Reportable Outcomes

1. In a publication by Yoon et al, "Fine Scale Spatiotemporal Clustering of Dengue Virus Transmission in Children and Aedes aegypti in Rural Thai Villages" geographic cluster investigations of 100-meter radius were conducted around DENV positive and DENV-negative febrile ''index'' cases (positive and negative clusters, respectively) from a longitudinal cohort study in rural Thailand [22]. Child contacts and *Ae. aegypti* from cluster houses were assessed for DENV infection. Spatiotemporal, demographic, and entomological parameters were evaluated. In positive clusters, the DENV infection rate among child contacts was 35.3% in index houses, 29.9% in houses within 20 meters, and decreased with distance from the index house to 6.2% in houses 80–100 meters away (p,0.001). Significantly more *Ae. aegypti* were DENV-infectious (i.e., DENV-positive in head/thorax) in positive clusters (23/1755; 1.3%) than negative clusters (1/1548; 0.1%). In positive clusters, 8.2% of mosquitoes were DENV-infectious in index houses, 4.2% in other houses with DENV-infected children, and 0.4% in houses without infected children (p,0.001). The DENV infection rate in contacts was 47.4% in houses with infectious mosquitoes, 28.7% in other houses in the same cluster, and 10.8% in positive clusters without infectious mosquitoes (p,0.001). *Ae. aegypti* pupae and adult females were more numerous only in houses containing infectious mosquitoes. The conclusion was that human and mosquito infections are positively associated at the level of individual houses and neighboring residences. Certain houses with high transmission risk contribute disproportionately to DENV spread to neighboring houses. Small groups of houses with elevated transmission risk are consistent with over-dispersion of transmission (i.e., at a given point in time, people/mosquitoes from a small portion of houses are responsible for the majority of transmission).

2. Aldstadt et al recently published the analysis of GPS data from this grant entitled, “Space-time analysis of hospitalized dengue patients in rural Thailand reveals important temporal intervals in the pattern of dengue virus transmission” [23]. Spatial coordinates of each patient’s home were captured using the Global Positioning System. A novel method based on the Knox test was used to determine the temporal intervals between cases at which spatial clustering occurred. These intervals are indicative of the length of time between successive illnesses in the chain of dengue virus transmission. The strongest spatial clustering occurred at the 15–17-day interval. There was also significant spatial clustering over short intervals (2–5 days). The highest excess risk was observed within 200 m of a previous hospitalized case and significantly elevated risk persisted within this distance for 32–34 days. The conclusion was that fifteen to seventeen days are the most likely serial interval between successive dengue illnesses.

Conclusions

Dengue is an important global and military health problem with no currently licensed vaccine for protection, nor antiviral for treatment. In this novel prospective study of index hospitalized cases of dengue infection and spatial investigation of their surrounding neighborhood using GPS technology, important information is being developed to under the spatial restrictions of dengue transmission. The findings from this study will translate into better interventions to disrupt transmission and guidelines to protect soldiers deployed to dengue endemic areas.

References

1. Innis BL, Eckels KH. Progress in development of a live-attenuated, tetravalent dengue virus vaccine by the United States Army Medical Research and Materiel Command. Am J Trop Med Hyg 2003;69:1-4.
2. Eckels KH, Dubois DR, Putnak R, et al. Modification of dengue virus strains by passage in primary dog kidney cells: preparation of candidate vaccines and immunization of monkeys. Am J Trop Med Hyg 2003;69:12-6.
3. Edelman R, Tacket CO, Wasserman SS, et al. A live attenuated dengue-1 vaccine candidate (45AZ5) passaged in primary dog kidney cell culture is attenuated and immunogenic for humans. J Infect Dis 1994;170:1448-55.
4. Bancroft WH, Scott RM, Eckels KH, et al. Dengue virus type 2 vaccine: reactogenicity and immunogenicity in soldiers. J Infect Dis 1984;149:1005-10.
5. Vaughn D, Hoke C, Yoksan Sea. Testing of dengue 2 live-attenuated vaccine (strain 16881 PDK 53) in ten American volunteers. Vaccine 1996;14:329-36.
6. Sun W, Nisalak A, Gettayacamin M, et al. Protection of Rhesus monkeys against dengue virus challenge after tetravalent live attenuated dengue virus vaccination. J Infect Dis 2006;193:1658-65.
7. Sun W, Edelman R, Kanesa-Thasan N, et al. Vaccination of human volunteers with monovalent and tetravalent live-attenuated dengue vaccine candidates. Am J Trop Med Hyg 2003;69:24-31.
8. Edelman R, Wasserman SS, Bodison SA, et al. Phase I trial of 16 formulations of a tetravalent live-attenuated dengue vaccine. Am J Trop Med Hyg 2003;69:48-60.
9. Guirakhoo F, Kitchener S, Morrison D, et al. Live attenuated chimeric yellow fever dengue type 2 (ChimeriVax-DEN2) vaccine: Phase I clinical trial for safety and immunogenicity: effect of yellow fever pre-immunity in induction of cross neutralizing antibody responses to all 4 dengue serotypes. Hum Vaccin 2006;2:60-7.
10. Guirakhoo F, Pugachev K, Arroyo J, et al. Viremia and immunogenicity in nonhuman primates of a tetravalent yellow fever-dengue chimeric vaccine: genetic reconstructions, dose adjustment, and antibody responses against wild-type dengue virus isolates. Virology 2002;298:146-59.
11. Guirakhoo F, Pugachev K, Zhang Z, et al. Safety and efficacy of chimeric yellow Fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J Virol 2004;78:4761-75.
12. Guirakhoo F, Weltzin R, Chambers TJ, et al. Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. J Virol 2000;74:5477-85.
13. Higgs S, Vanlandingham DL, Klingler KA, et al. Growth characteristics of ChimeriVax-Den vaccine viruses in Aedes aegypti and Aedes albopictus from Thailand. Am J Trop Med Hyg 2006;75:986-93.

14. Johnson BW, Chambers TV, Crabtree MB, Guirakhoo F, Monath TP, Miller BR. Analysis of the replication kinetics of the ChimeriVax-DEN 1, 2, 3, 4 tetravalent virus mixture in Aedes aegypti by real-time reverse transcriptase-polymerase chain reaction. Am J Trop Med Hyg 2004;70:89-97.
15. McGee CE, Lewis MG, Claire MS, et al. Recombinant chimeric virus with wild-type dengue 4 virus premembrane and envelope and virulent yellow fever virus Asibi backbone sequences is dramatically attenuated in nonhuman primates. J Infect Dis 2008;197:693-7.
16. McGee CE, Tsetsarkin K, Vanlandingham DL, et al. Substitution of wild-type yellow fever Asibi sequences for 17D vaccine sequences in ChimeriVax-dengue 4 does not enhance infection of Aedes aegypti mosquitoes. J Infect Dis 2008;197:686-92.
17. Monath TP, McCarthy K, Bedford P, et al. Clinical proof of principle for ChimeriVax: recombinant live, attenuated vaccines against flavivirus infections. Vaccine 2002;20:1004-18.
18. Endy TP, Nisalak A, Chunsuttitwat S, et al. Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. J Infect Dis 2004;189:990-1000.
19. Stephenson JR. Understanding dengue pathogenesis: implications for vaccine design. Bull World Health Organ 2005;83:308-14.
20. Seligman SJ, Gould EA. Live flavivirus vaccines: reasons for caution. Lancet 2004;363:2073-5.
21. Thomas S, Redfern JB, Lidbury BA, Mahalingam S. Antibody-dependent enhancement and vaccine development. Expert Rev Vaccines 2006;5:409-12.
22. Yoon IK, Getis A, Aldstadt J, et al. Fine scale spatiotemporal clustering of dengue virus transmission in children and Aedes aegypti in rural Thai villages. PLoS neglected tropical diseases 2012;6:e1730.
23. Aldstadt J, Yoon IK, Tannitisupawong D, et al. Space-time analysis of hospitalised dengue patients in rural Thailand reveals important temporal intervals in the pattern of dengue virus transmission. Trop Med Int Health 2012;17:1076-85.

Appendices

Figure 3. One cluster investigation with GIS mapping using Google Maps.

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PLoS NEGLECTED TROPICAL DISEASES

Fine Scale Spatiotemporal Clustering of Dengue Virus Transmission in Children and *Aedes aegypti* in Rural Thai Villages

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Abstract

Background: Based on spatiotemporal clustering of human dengue virus (DENV) infections, transmission is thought to occur at fine spatiotemporal scales by horizontal transfer of virus between humans and mosquito vectors. To define the dimensions of local transmission and quantify the factors that support it, we examined relationships between infected humans and *Aedes aegypti* in Thai villages.

Methodology/Principal Findings: Geographic cluster investigations of 100-meter radius were conducted around DENV-positive and DENV-negative febrile ''index'' cases (positive and negative clusters, respectively) from a longitudinal cohort study in rural Thailand. Child contacts and *Ae. aegypti* from cluster houses were assessed for DENV infection. Spatiotemporal, demographic, and entomological parameters were evaluated. In positive clusters, the DENV infection rate among child contacts was 35.3% in index houses, 29.9% in houses within 20 meters, and decreased with distance from the index house to 6.2% in houses 80–100 meters away ($p<0.001$). Significantly more *Ae. aegypti* were DENV-infectious (i.e., DENV-positive in head/thorax) in positive clusters (23/1755; 1.3%) than negative clusters (1/1548; 0.1%). In positive clusters, 8.2% of mosquitoes were DENV-infectious in index houses, 4.2% in other houses with DENV-infected children, and 0.4% in houses without infected children ($p<0.001$). The DENV infection rate in contacts was 47.4% in houses with infectious mosquitoes, 28.7% in other houses in the same cluster, and 10.8% in positive clusters without infectious mosquitoes ($p<0.001$). *Ae. aegypti* pupae and adult females were more numerous only in houses containing infectious mosquitoes.

Conclusions/Significance: Human and mosquito infections are positively associated at the level of individual houses and neighboring residences. Certain houses with high transmission risk contribute disproportionately to DENV spread to neighboring houses. Small groups of houses with elevated transmission risk are consistent with over-dispersion of transmission (i.e., at a given point in time, people/mosquitoes from a small portion of houses are responsible for the majority of transmission).

Citation: Yoon I-K, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, et al. (2012) Fine Scale Spatiotemporal Clustering of Dengue Virus Transmission in Children and *Aedes aegypti* in Rural Thai Villages. PLoS Negl Trop Dis 6(7): e1730. doi:10.1371/journal.pntd.0001730

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Introduction

Dengue is the most widespread mosquito-borne viral disease with 3.6 billion people at risk of infection world-wide each year [1]. *Aedes aegypti* is the principal mosquito vector of dengue virus (DENV). Indirect transmission occurs by horizontal transfer of virus between humans and female *Ae. aegypti* [2]. A key component of understanding DENV transmission dynamics is to understand the spatial and

Author Summary

Dengue is the leading cause of mosquito-borne viral infections globally. An improved understanding of the spatial and temporal distribution of dengue virus (DENV) transmission between humans and the principal vector, *Aedes aegypti*, can enhance prevention programs. Human DENV infection is known to occur at very fine spatiotemporal scales. We sought to link and quantify human DENV infections with infectious mosquitoes at these fine scales by conducting geographic cluster investigations around febrile children with and without DENV infection. We found that DENV infection in children was positively associated with houses in which infectious mosquitoes were captured. These houses also had more *Ae.* aegypti pupae and adult female mosquitoes than neighboring houses. However, the neighboring houses still had elevated rates of human DENV infection. Our results indicate that certain houses with high risk of DENV transmission contribute disproportionately to DENV amplification and spread to surrounding houses. At a given point in time, people and mosquitoes from a small portion of houses are responsible for the majority of DENV transmission.

temporal scale at which human-mosquito encounters and virus transmission occur. DENV infection in humans has been shown to have substantial spatial and temporal variation at relatively small scales. Cohort studies in rural Thailand, where dengue is hyperendemic, indicate that dengue epidemiology and clinical presentation can differ dramatically between children in close geographic and temporal proximity at the level of a school and village [3–5]. Clusters of human DENV infections have also been detected in and around individual households [6–8]. Much of this fine scale spatiotemporal heterogeneity has been thought to be due, at least in part, to the behavior of the mosquito vector. Flight patterns and feeding behavior of the female *Ae. aegypti*, which have been studied extensively, indicate that this is a relatively sedentary species that feeds frequently and almost exclusively on human blood [9–15]. Much less is known about the interactions between humans and *Ae. aegypti* in natural settings that result in DENV transmission.

Results from our combined longitudinal cohort and geographic cluster study in Kamphaeng Phet, Thailand are consistent with focal DENV transmission occurring at a fine scale [5,6]. Within 100 meters of a house with a DENV-infected child (as detected by school absence-based surveillance), the likelihood of another house with a DENV-infected child decreased with increasing distance from the original infected child's house. In the current report, we present additional data from the geographic cluster component of our larger cohort/cluster study that more specifically defines the dimensions of local transmission and quantifies the factors that support it. We detected a positive association between DENV infection in children and female *Ae. aegypti* at fine geographic and temporal scales. Our results add new details to the understanding of focal DENV transmission that can be used to further inform dengue surveillance and prevention strategies, and provide currently missing data for the construction, parameterization and validation of mathematical and simulation models of DENV transmission and control.

Methods

Ethics Statement

The study protocol was approved by the Institutional Review Boards of the Thai Ministry of Public Health (MOPH), Walter Reed Army Institute of Research (WRAIR), University of Massachusetts Medical School (UMMS), University of California at Davis (UCD) and San Diego State University (SDSU). Written informed consent was obtained from the parents of study participants and assent was obtained from study participants older than seven years.

Study Location and Population

Our study methodology was previously described [5,6]. Briefly, the geographic cluster study presented here was part of a larger combined longitudinal cohort and geographic cluster study conducted from 2004 to 2007 among children living in Muang district, Kamphaeng Phet province in north-central Thailand. Children came from 11 schools and 32 villages consisting of >8,445 houses. Demographics of house residents and house spatial coordinates were geo-coded into a Geographic Information System (GIS) database (MapInfo [2000] version 6·0; MapInfo Corporation).

Geographic Cluster Investigations

Geographic cluster investigations were initiated by "index" cases selected from a longitudinal cohort of approximately 2000 primary school children. Active school absence-based surveillance was used to detect symptomatic DENV infection in the cohort from June to November of each study year [5]. Cohort children who were DENV-positive by semi-nested reverse transcriptase polymerase chain reaction (RT-PCR) [16] from an acute blood sample drawn within three days of illness onset served as an "index" case to initiate a positive cluster investigation around the index case house. Cohort children who were dengue PCR-negative from an acute illness blood sample served as an "index" case for a negative (i.e., control) cluster investigation. In each geographic cluster, ten to 25 child contacts aged six months to 15 years living within 100 meters of the index case were enrolled regardless of clinical status. The child contacts were evaluated at days 0 (i.e., the same day as cluster initiation), 5, 10, and 15 by temperature measurement and symptom questionnaire. Blood samples were collected on days 0 and 15. Paired day 0 and 15 blood samples from child contacts were tested by both dengue PCR and an in-house dengue/Japanese encephalitis IgM/IgG capture EIA [17]. Dengue EIA-positive results were categorized as "recent dengue" (RD) if IgM was negative but IgG was positive with a declining titer between days 0 and 15 [18], "enrollment seroconversion" (ES) if IgM was positive on both days 0 and 15, and "post-enrollment seroconversion" (PES) if IgM was negative on day 0 but positive on day 15. Based on estimated antibody kinetics and human incubation period [19,20], the approximate interval between infection and day 0 blood collection for RD infections was thought to be about 3 weeks or more, ES infections up to about 2 weeks, and PES infections to be several days. Day 15 PCR-positive infections were thought to have occurred at or soon after cluster initiation.

Entomological Procedures

On day 1 of each cluster investigation, adult *Ae. aegypti* were collected using backpack aspirators from inside and within the immediate vicinity of each house within a cluster. *Ae. aegypti* larvae and pupae were collected from water-holding containers [21]. After mosquito collections were completed, a pyrethrin mixture insecticide spray (BP-300: Pyronyl oil concentrate OR-3610A, Prentiss Inc.) was applied by ultralow volume aerosol inside and around each house to kill adult mosquitoes with the intention of terminating local DENV transmission [22]. Temephos was applied to artificial water holding containers to kill immature mosquitoes. On day 7, the Thai Ministry of Public Health (MOPH) sprayed deltamethrin or permethrin 10% in and around each house in a cluster according to their standard procedures.

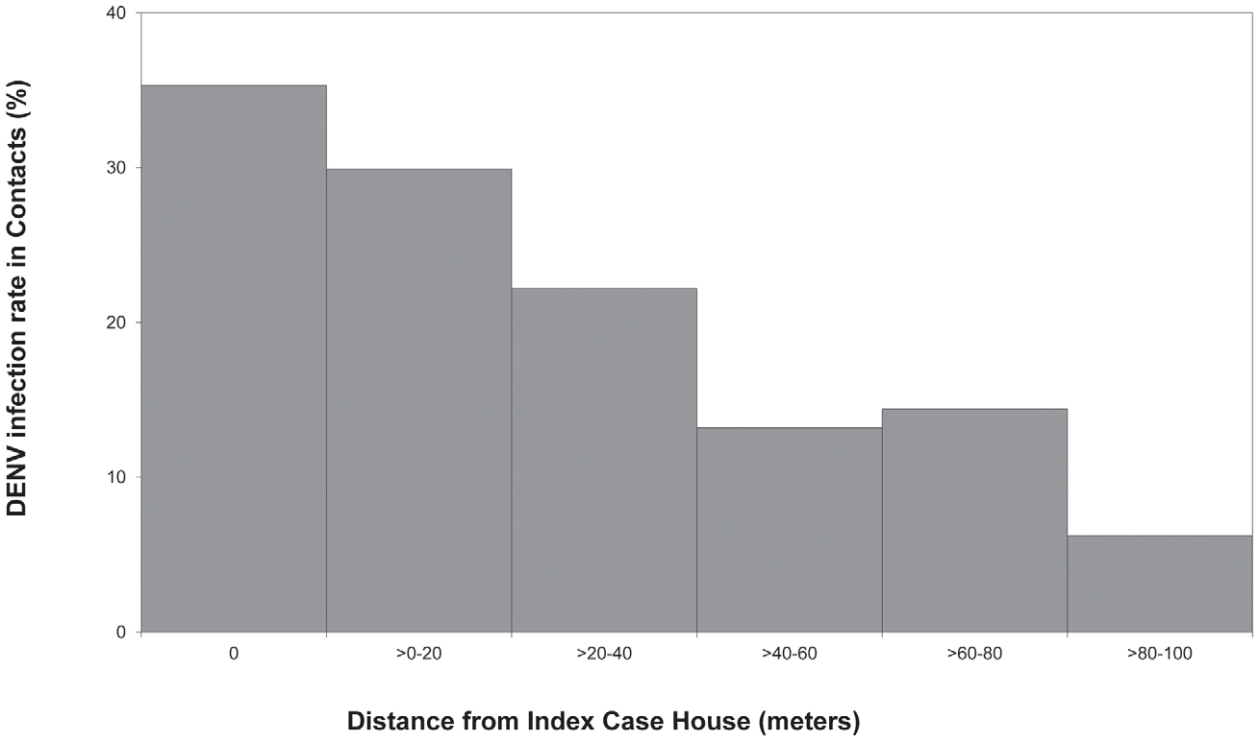

Figure 1. Focal aggregation of DENV-infected child contacts in positive clusters. Relationship between DENV infection rate and distance from index case house was significant (Chi square, $p<0.001$). Distance from index case house 0 m: N = 18/51; >0–20 m: N = 20/67; >20–40 m: N = 28/126; >40–60 m: 23/174; >60–80 m: 28/194; >80–100 m: 12/193 (N = Infected Contacts/Enrolled Contacts).
doi:10.1371/journal.pntd.0001730.g001

Female *Ae. aegypti* were processed so that individual, serotype-specific rates for DENV-infectious mosquitoes could be detected. Mosquito abdomens were removed so that only those females that had virus particles in the head or thorax (i.e., disseminated infections with presumably infective salivary glands) were identified. Individual heads and thoraces were stored at $-70^{\circ}C$ in the field laboratory and transported weekly on dry ice to the Armed Forces Research Institute of Medical Sciences (AFRIMS) laboratory in Bangkok. At the AFRIMS laboratory, heads and thoraces of individual mosquitoes were ground and suspended in 100 µL of RPMI with 1% L-glutamine and 10% heat-inactivated FBS. Ten mosquito suspensions were pooled by combining 14 µL from each individual suspension. Pools were then tested by dengue PCR and each individual sample from a PCR-positive pool was tested by using 14 µL of the individual suspension diluted times ten [23,24].

Statistical Analysis

Data were analyzed using SPSS (SPSS for Windows version 19). Demographic, environmental and entomological parameters were analyzed at the cluster and house levels. Student's *t*-test or analysis of variance (ANOVA) was used to determine differences in continuous variables including distances between houses. Chi-square or Fisher's exact test was used for proportions. A mixed-effects logistic regression model was used to analyze the probability of infection of cluster contacts, while accounting for the nesting of observations within cluster investigations.

Results

DENV Infections in Child Contacts in Geographic Clusters

Of 805 child contacts enrolled in 50 positive cluster investigations, 129 (16.0%) had evidence of DENV infection; 119 (14.8%) were dengue EIA-positive on day 0 and/or 15 of which 40 were PCR-positive on day 0, and an additional 10 (1.2%) were DENV-positive only by PCR on day 15. In comparison, nine (1.1%) of 794 enrolled child contacts in 53 negative clusters had evidence of DENV infection; seven (0.9%) were dengue EIA-positive of which three were PCR-positive on day 0, and an additional two (0.3%) were DENV-positive by day 15 PCR alone [5].

Within positive clusters, the percentage of enrolled contacts that were dengue EIA-positive varied significantly according to distance from the index case house. The DENV infection rate among contacts from the same house as a positive index case was 35.3%. If the child contact lived in a different house but within 20 meters of the index case house, the infection rate was 29.9%. The infection rate decreased with increasing distance from the index case house, down to 6.2% when the contact lived 80–100 meters away. The inverse relationship between DENV infection rate among contacts and distance from the index case house was significant (Chi square, $p<0.001$) (Figure 1). A mixed-effects logistic regression model confirmed that this association remained significant after controlling for age and gender (Table 1).

Of the 119 dengue EIA-positive child contacts in the positive clusters, 15 (12.6%) were categorized as having RD infection, 41 (34.5%) as ES infection, and 63 (52.9%) as PES infection. RD, ES and PES infections in the positive clusters all tended to decrease as the distance from the index case house increased (Figure 2). DENV infections based solely on a day 15 PCR-positive result did not appear to decrease with increasing distance from the index case house; however, the number of these cases was low (Figure 2).

Table 1. Mixed-effects logistic regression analysis of the probability of DENV infections in child contacts in positive clusters.

Independent Variable	Coefficient	Standard Error	p-value
Distance from Index Case House (m)	−0.022	0.004	<0.001
Age (yrs)	0.006	0.028	0.823
Female	−0.165	0.224	0.460
Intercept	−0.982	0.390	0.012

doi:10.1371/journal.pntd.0001730.t001

DENV Infections in Female *Ae. aegypti* in Geographic Clusters

Twenty-three (1.3%) of 1755 female *Ae. aegypti* were dengue PCR-positive in positive clusters, while one (0.1%) of 1548 was PCR-positive in negative clusters ($p<0.001$). Considering only those houses with index cases or enrolled child contacts, all 19 DENV-infectious female *Ae. aegypti* collected from these houses were in positive clusters. These 19 mosquitoes came from 16 different houses in 14 different positive clusters (Table 2). All four DENV serotypes were represented.

Houses with DENV-infectious mosquitoes had significantly more *Ae. aegypti* pupae and total female *Ae. aegypti* mosquitoes than houses without infectious mosquitoes (Table 3). Two of the houses with infectious mosquitoes (houses 1 and 3) contained the largest and second largest number (164 and 129) of *Ae. aegypti* pupae collected from any house in the entire study (Table 2).

Relationship between DENV Infection in Children and Female *Ae. aegypti*

There were 17 DENV infections in children from the 16 houses with DENV-infectious mosquitoes; 9 DENV infections were in child contacts and 8 were in index cases. Within the houses with infectious mosquitoes, the serotype, when available, of DENV in infected children was identical to that in the infectious mosquito from the same house (Table 2).

DENV infection in children was positively associated with the presence of DENV-infectious mosquitoes in the house. The DENV infection rate among child contacts in houses with infectious mosquitoes was 47.4% compared to 28.7% in houses from the same cluster but without infectious mosquitoes, and 10.8% in houses from other positive clusters (Fisher's exact, $p<0.001$; Table 3). Conversely, the DENV infection rate among female *Ae. aegypti* from houses with a positive child index case was 8.2% (Table 4). Excluding index case houses, the rate of infectious mosquitoes from positive cluster houses with a DENV-infected child was 4.2%. This rate was only 0.4% when no child was infected in a positive cluster house (Fisher's exact, $p<0.001$).

Within the 100-meter radius of positive clusters, almost all DENV-infectious *Ae. aegypti* were collected from index case houses or from houses within 40 meters of the index case house (Figure 3). This negative correlation of infectious mosquitoes with distance from the index case house was most likely due to the positive association between houses containing infected children and infectious mosquitoes.

Within an individual positive cluster, houses with DENV-infectious mosquitoes tended to be closer to houses containing

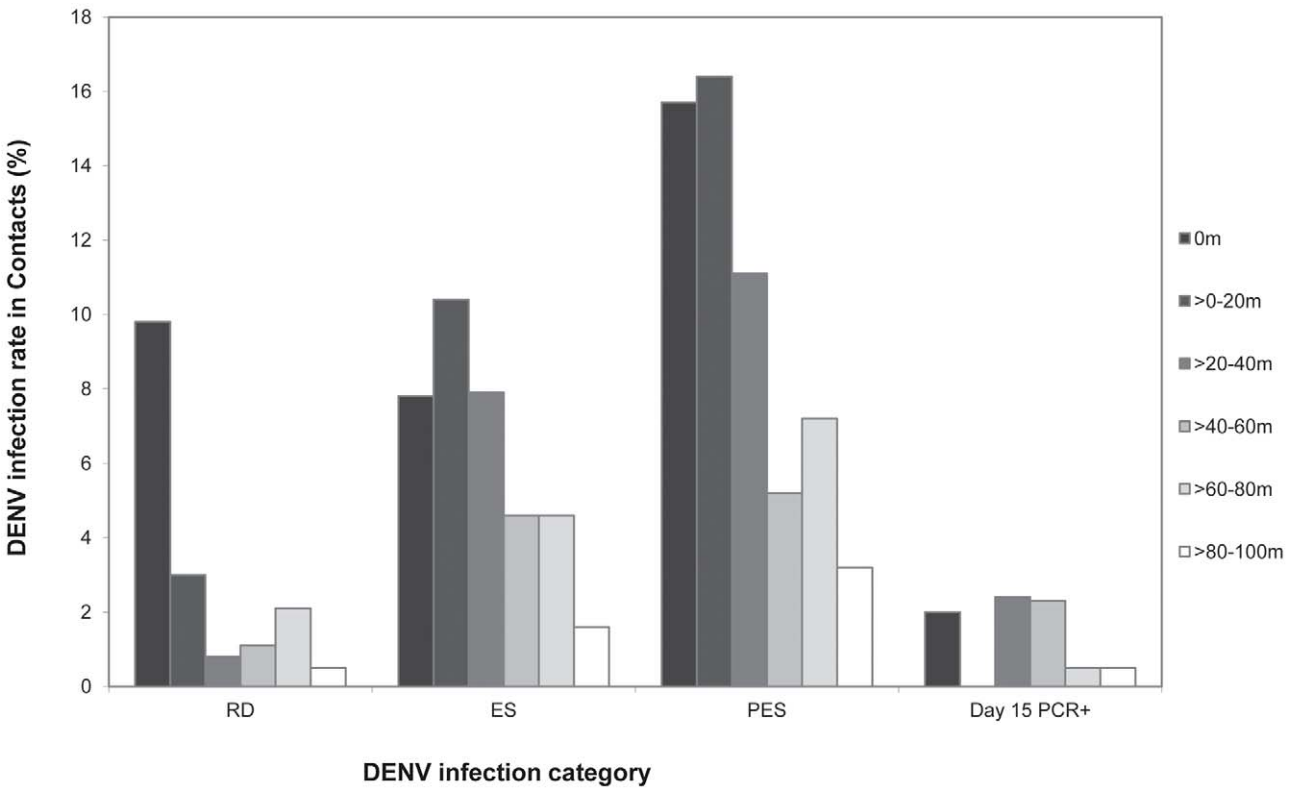

Figure 2. Focal aggregation of DENV-infected child contacts in positive clusters categorized by estimated time of infection. RD = recent dengue (N = 15); ES = enrollment seroconversion (N = 41); PES = post-enrollment seroconversion (N = 63); day 15 PCR-positive (N = 10).
doi:10.1371/journal.pntd.0001730.g002

Table 2. Description of houses with DENV-infectious female *Ae.* aegypti.

House	Cluster month/year	Index case house (Y/N)	Female *Ae. aegypti* collected	Infectious female *Ae. aegypti*	*Ae. aegypti* pupae	Human adults	Human children	Enrolled child contacts	DENV infection in enrolled contacts	DENV serotype of infectious mosquito	DENV serotype of index case in same cluster	DENV serotype of infected child contact
1	Jun 04	N	7	1	164	3	2	2	1	DENV-4	DENV-4	ND
2	Jun 04	Y	3	1	5	2	3	2	1	DENV-4	DENV-4	ND
3	Sep 04	N	8	2	129	4	1	1	1	DENV-4, DENV-4	DENV-4	ND
4	Nov 05	N	17	1	57	1	1	0	0	DENV-3	DENV-3	-
5	Nov 05	Y	2	1	6	2	1	1	0	DENV-3	DENV-3	-
6	Jun 06	Y	9	1	1	6	2	1	0	DENV-1	DENV-1	-
7	Jun 06	Y	7	1	2	2	1	0	0	DENV-4	DENV-4	-
8	Jun 06	N	1	1	3	3	2	2	1	DENV-4	DENV-4	DENV-4
9	Aug 06	N	5	1	3	3	3	2	2	DENV-1	DENV-1	ND, ND
10	Aug 06	Y	2	1	5	4	2	1	1	DENV-4	DENV-4	ND
11	Oct 06	Y	2	1	0	3	3	2	1	DENV-1	DENV-1	DENV-1
12	Nov 06	Y	3	1	2	4	1	0	0	DENV-2	DENV-2	-
13	Jun 07	N	4	1	0	2	3	3	0	DENV-1	DENV-1	-
14	Jun 07	N	3	1	1	3	1	1	0	DENV-1	DENV-1	-
15	Jul 07	Y	15	3	0	3	1	0	0	DENV-1, DENV-1, DENV-1	DENV-1	-
16	Jul 07	N	4	1	0	2	1	1	1	DENV-2	DENV-2	ND
Mean per-house (s.d.)		-	**5.75 (4.65)**	**1.19 (0.54)**	**23.6 (50.3)**	**2.94 (1.18)**	**1.75 (0.86)**	**1.19 (0.91)**	**0.56 (0.63)**	-	-	-

Footnote: Includes only houses with index cases or enrolled child contacts. ND = not detected by PCR but positive by EIA.

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Table 3. Selected entomological indices and DENV infection rates in houses with and without infectious mosquitoes.

	Positive Clusters				Houses in Negative Clusters	Difference p-value between positive and negative cluster houses
	Houses with Infectious Mosquitoes (19 Infectious Mosquitoes)	Houses in same cluster as Infectious Mosquitoes but with no Infectious Mosquitoes	Houses in clusters with no Infectious Mosquitoes	Difference p-value		
Houses, N	16	158	324	-	480	-
Female *Ae. aegypti*, N per person (s.d.)	1.57 (2.05)	0.49 (1.00)	0.45 (0.81)	<0.001	0.31 (0.49)	<0.001
***Ae. aegypti* pupae, N per person (s.d.)**	5.84 (11.59)	1.86 (3.76)	1.22 (2.56)	<0.001	1.39 (2.99)	0.383
Human adults, N per house (s.d.)	2.94 (1.18)	2.92 (1.31)	2.70 (1.44)	0.239	2.74 (1.25)	0.607
Human children, N per house (s.d.)	1.75 (0.86)	1.68 (0.88)	1.77 (1.04)	0.598	1.82 (1.10)	0.272
DENV-infected child contacts/Enrolled child contacts (%)	9/19 (47.4)	56/195 (28.7)	64/591 (10.8)	<0.001	9/794 (1.1)	<0.001

Footnote: Only houses with index cases or enrolled child contacts were included. Person refers to adults and children. P-values are for one-way ANOVA comparisons for means and Fisher's exact test for proportion of infected contacts.
doi:10.1371/journal.pntd.0001730.t003

DENV-infected children than to all houses. Figure 4 shows the mean distance between each of the 16 houses with infectious mosquitoes and other houses in their respective clusters. In three houses (#10, 11 and 12), the only infected children in the cluster were in the houses with the infectious mosquito(es). Of the 13 possible comparisons, 12 houses with infectious mosquitoes were closer to houses with infected children than to all houses within the cluster. On average, houses with infected children were closer to houses with infectious mosquitoes than to houses with no infectious mosquitoes within their respective positive clusters ($p = 0.028$).

Discussion

This study demonstrates a positive association between DENV-infectious *Ae. aegypti* and DENV-infected children living in the same and neighboring houses. Spatiotemporal clustering of DENV infection in children and mosquitoes was detected at a fine scale, consistent with focal aggregation well within a 100-meter radius area. Houses with infectious mosquitoes had an especially high risk (47.4%) of human DENV infection along with elevated measurements of mosquito density; neighboring houses also had elevated risk of human infection. Our results are consistent with the notion that houses with high DENV transmission risk contribute disproportionately to virus amplification and spread. Infections followed a pattern of over-dispersion, which has been reported for other infectious diseases to include indirectly transmitted, mosquito-borne infections [25–28]. At a given point in time, people and mosquitoes in a relatively small portion of houses were responsible for the majority of DENV transmission.

Table 4. Rates of DENV-infectious *Ae. aegypti* in houses with and without DENV-infected children in positive clusters.

	Houses with index case (N = 50)	Houses with DENV-infected contacts, not including houses with index case (N = 86)	Houses with no DENV-infected contacts (N = 395)	Difference p-value
DENV-infectious *Ae. aegypti*/Collected female *Ae. aegypti* (%)	10/122 (8.2)	6/142 (4.2)	3/735 (0.4)	<0.001
Human adults, N per house (s.d.)	2.70 (1.18)	2.83 (1.30)	2.78 (1.44)	0.879
Human children, N per house (s.d.)	2.14 (1.51)	1.90 (1.01)	1.65 (0.88)	0.001
Female *Ae. aegypti* per person (s.d.)	0.65 (1.35)	0.46 (1.19)	0.49 (0.83)	0.492
DENV-infectious *Ae. aegypti* per person (s.d.)	0.049 (0.140)	0.016 (0.067)	0.002 (0.024)	<0.001

Footnote: Person refers to adults and children. Only houses with index cases or enrolled child contacts were included. P-values are for one-way ANOVA comparisons for means and Fisher's exact test for proportions of infectious mosquitoes.
doi:10.1371/journal.pntd.0001730.t004

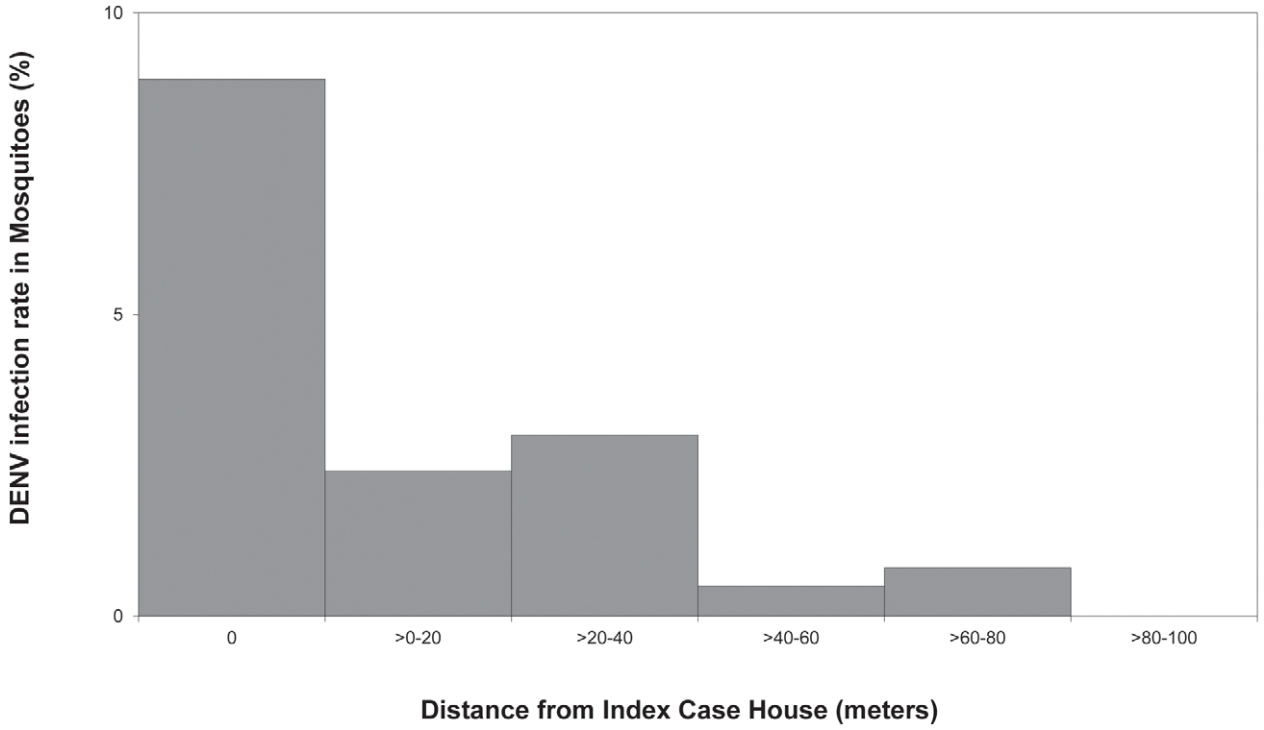

Figure 3. Focal aggregation of DENV-infectious mosquitoes in positive clusters. Relationship between proportion of female *Ae. aegypti* that were DENV-infectious and distance from index case house was significant (Fisher's exact, $p<0.001$). Distance from index case house 0 m: N = 10/112; >0–20 m: N = 2/85; >20–40 m: N = 4/138; >40–60 m: 1/204; >60–80 m: 2/243; >80–100 m: 0/206 (N = Infectious mosquitoes/Collected mosquitoes).
doi:10.1371/journal.pntd.0001730.g003

Our results are the first to demonstrate a direct relationship between DENV infection in humans and mosquitoes at very fine spatiotemporal scales in the natural setting. Other researchers have reported heterogeneity of human DENV infection across space and time [4,6,8,29]. Many entomological studies have shown the limited flight range and preferential and frequent human feeding behavior of *Ae. aegypti* that would be expected to enhance DENV transmission [11,13–15,22,30]. Prior studies of DENV infections in mosquitoes tended to focus on mosquitoes collected in or around houses of people with dengue-like illness [31,32]; and when these studies were done across communities, infected mosquitoes were not explicitly linked to human infection [33]. Perhaps because of the difficulty in collecting adult *Ae. aegypti*, there has been relatively little research done on mosquito DENV infections in relation to human infection dynamics. Our study expands on this picture by showing that human and mosquito infections are positively associated with each other at small geographic and temporal scales. The strongest association was at the level of the individual house.

We did not directly evaluate the role of human adults in DENV transmission. It is possible that spatiotemporal dynamics of DENV transmission is different in adults and children, perhaps due to age-specific differences in existing immunity, the rate at which they are bitten [34], or in their movement patterns and exposure to daytime-biting *Ae. aegypti* [35]. We would not expect our overall conclusions to change, however, because both adults and children would have contributed to our findings whether or not adults were separately evaluated.

Fine scale spatial aggregation of DENV transmission may persist for three weeks or longer. Given the estimated time of infection of RD, ES and PES infections and because all of these categories of infection appeared to show focal aggregation within the ≤100-meter radius of the clusters, the spatial pattern we detected could have been present for greater than three weeks. This pattern, which is similar to what was observed for DENV-infected *Ae. aegypti* in households in Mexico [12], may have persisted for a longer period if not truncated by the vector control interventions instituted on day 1 (by the study team) and day 7 (by the MOPH) of the cluster investigations. The lack of focal aggregation among day 15 PCR-positive child contacts supports this notion, although the small number of those infections may have been insufficient for a meaningful analysis.

Our testing method favored identification of PCR-positive mosquitoes that were infectious. The DENV incubation period in mosquitoes from the time that they imbibe an infectious blood meal to the time they become infectious (i.e., extrinsic incubation period) typically lasts for 10–14 days under environmental conditions like those in Kamphaeng Phet, Thailand [2,36]. This implies that DENV-infectious mosquitoes in our study fed on an infected human considerably earlier than the time of cluster initiation and, thus, the transmission chain in houses with infectious mosquitoes had been taking place for some time before the "index" case was detected and the cluster investigation initiated. Consequently, as with infected children in the clusters, focal aggregation of infectious mosquitoes within the clusters may have been going on for two weeks or longer prior to initiation of each cluster investigation. So although "index" cases were used to initiate cluster investigations, they were not necessarily the first infection to occur within the cluster. Again, because vector control measures were instituted on day 1 and 7 and no further entomological collections were performed afterwards, we were not able to determine how long the focal pattern of DENV

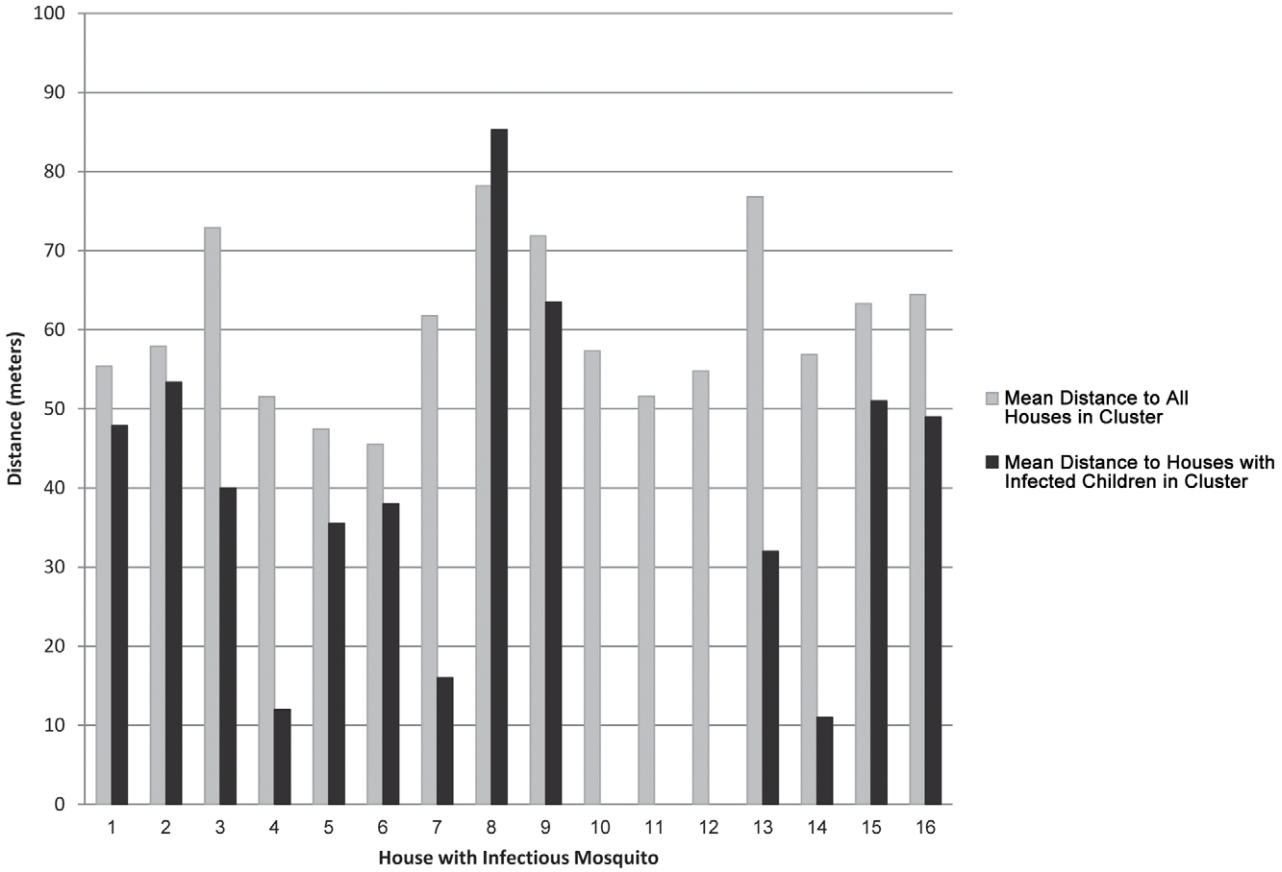

Figure 4. Mean distance of houses with DENV-infectious mosquitoes to other houses in the same cluster.
doi:10.1371/journal.pntd.0001730.g004

infection in mosquitoes would have persisted. We speculate that the duration of these focal areas of higher risk is limited more by the availability of susceptible humans than by susceptible mosquitoes. Future studies could investigate the required duration of interventions, which may need to be continued for one month or more.

Significantly more *Ae. aegypti* pupae and adult females were collected from houses containing infectious mosquitoes than from those without. In addition, the risk of DENV infection in children was high in houses with infectious mosquitoes and, notably, remained elevated in neighboring houses. The higher entomological indices, however, were detected only in houses that actually contained infectious mosquitoes. These findings indicate that certain individual houses with high DENV transmission risk may disproportionately contribute to virus transmission within neighboring houses, likely due to local human and mosquito movement. Our study did not specifically evaluate when these elevated entomological measurements began or how long they persisted. They could have been present for some time prior to detection. Therefore, even in clusters with high DENV transmission, there may be individual houses that are responsible for the bulk of the transmission risk. Dengue management interventions that fail to include these individual, high-risk houses may have less impact than expected on reducing overall DENV spread. Similarly, surveillance programs that average measurements or indices of risk over a large area may fail to detect individual high-risk houses that disproportionately contribute to persistence and expansion of local transmission [25].

Fine scale spatiotemporal clustering of human-mosquito DENV transmission supports the hypothesis that DENV spread to more distant locations is driven by human movement [35]. Whether DENV is successfully transmitted at those distant locations is likely related to a suite of factors including susceptibility of the local human population, mosquito vector density and infection status, vector competence, degree of human-vector contact, and intrinsic virus factors. Locations with high levels of human movement and potential for high interaction between people and mosquitoes merit additional investigation. These components of transmission may need to be factored into dengue surveillance and control efforts more than is currently being done [37].

Results from our study have implications for strategies to prevent DENV transmission. Transmission models that address DENV spread and the impact of vaccines alone or in combination with vector control need to account for the spatiotemporal scale and dynamics of DENV transmission. Depending on the questions being asked, these models and the interpretation of surveillance data that feed into them will need to account for the presence of high-risk hotspots of human-vector virus exchange that have a high impact on DENV spread to surrounding areas [27,28,38]. These efforts should be integrated into an overall multifaceted strategy that takes into account DENV spread by movement of viremic humans among focal areas of concentrated, high levels of transmission.

Supporting Information

Checklist S1 STROBE checklist.

(DOC)

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Author Contributions

Conceived and designed the experiments: IY AG JA ALR ACM AN MPM ST AS SG DHL TE TWS. Performed the experiments: IY DT CJMK TF JWJ RGJ AN MPM AS RVG CP. Analyzed the data: IY AG JA ALR DT AS RVG TWS. Wrote the paper: IY AG JA ALR DT CJMK TF ACM RGJ MPM SG RVG TWS.

References

1. Pediatric Dengue Vaccine Initiative, International Vaccine Institute. Global burden of dengue. Available: http://www.pdvi.org/about_dengue/GBD.asp. Accessed 2011 May 22.
2. Scott TW, Morrison AC (2010) Vector dynamics and transmission of dengue virus: implications for dengue surveillance and prevention strategies: vector dynamics and dengue prevention. Curr Top Microbiol Immunol 338: 115–128.
3. Endy TP, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, et al. (2002) Spatial and temporal circulation of dengue virus serotypes: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol 156: 52–59.
4. Endy TP, Anderson KB, Nisalak A, Yoon IK, Green S, et al. (2011) Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS Negl Trop Dis 5: e975.
5. Yoon IK, Rothman AL, Tannitisupawong D, Srikiatkhachorn A, Jarman RG, et al. (2012) Under-Recognized Mildly Symptomatic Viremic Dengue Virus Infections in Rural Thai Schools and Villages. Journal of Infectious Diseases DOI: 10.1093/infdis/jis357.
6. Mammen MP, Pimgate C, Koenraadt CJ, Rothman AL, Aldstadt J, et al. (2008) Spatial and temporal clustering of dengue virus transmission in Thai villages. PLoS Med 5: e205.
7. Morrison AC, Getis A, Santiago M, Rigau-Perez JG, Reiter P (1998) Exploratory space-time analysis of reported dengue cases during an outbreak in Florida, Puerto Rico, 1991–1992. Am J Trop Med Hyg 58: 287–298.
8. Thai KT, Nagelkerke N, Phuong HL, Nga TT, Giao PT, et al. (2010) Geographical heterogeneity of dengue transmission in two villages in southern Vietnam. Epidemiol Infect 138: 585–591.
9. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal of the dengue vector Aedes aegypti within and between rural communities. Am J Trop Med Hyg 72: 209–220.
10. Morrison AC, Gray K, Getis A, Astete H, Sihuincha M, et al. (2004) Temporal and geographic patterns of Aedes aegypti (Diptera: Culicidae) production in Iquitos, Peru. J Med Entomol 41: 1123–1142.
11. Getis A, Morrison AC, Gray K, Scott TW (2003) Characteristics of the spatial pattern of the dengue vector, Aedes aegypti, in Iquitos, Peru. Am J Trop Med Hyg 69: 494–505.
12. Garcia-Rejon J, Lorono-Pino MA, Farfan-Ale JA, Flores-Flores L, Del Pilar Rosado-Paredes E, et al. (2008) Dengue virus-infected Aedes aegypti in the home environment. Am J Trop Med Hyg 79: 940–950.
13. Scott TW, Chow E, Strickman D, Kittayapong P, Wirtz RA, et al. (1993) Blood-feeding patterns of Aedes aegypti (Diptera: Culicidae) collected in a rural Thai village. J Med Entomol 30: 922–927.
14. Scott TW, Amerasinghe PH, Morrison AC, Lorenz LH, Clark GG, et al. (2000) Longitudinal studies of aedes aegypti (Diptera: Culicidae) in Thailand and Puerto Rico: Blood feeding frequency. J Med Entomol: 89–101.
15. Scott TW, Takken W (2012) Feeding strategies of anthropophilic mosquitoes result in increased risk of pathogen transmission. Trends Parasit In press.
16. Klungthong C, Gibbons RV, Thaisomboonsuk B, Nisalak A, Kalayanarooj S, et al. (2007) Dengue virus detection using whole blood for reverse transcriptase PCR and virus isolation. J Clin Microbiol 45: 2480–2485.
17. Innis BL, Nisalak A, Nimmannitya S, Kusalerdchariya S, Chongswasdi V, et al. (1989) An enzyme-linked immunosorbent assay to characterize dengue infections where dengue and Japanese encephalitis co-circulate. Am J Trop Med Hyg 40: 418–427.
18. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (1997) Dengue in the early febrile phase: viremia and antibody responses. J Infect Dis 176: 322–330.
19. Vazquez S, Cabezas S, Perez AB, Pupo M, Ruiz D, et al. (2007) Kinetics of antibodies in sera, saliva, and urine samples from adult patients with primary or secondary dengue 3 virus infections. Int J Infect Dis 11: 256–262.
20. Koraka P, Suharti C, Setiati TE, Mairuhu AT, Van Gorp E, et al. (2001) Kinetics of dengue virus-specific serum immunoglobulin classes and subclasses correlate with clinical outcome of infection. J Clin Microbiol 39: 4332–4338.
21. Koenraadt CJ, Jones JW, Sithiprasasna R, Scott TW (2007) Standardizing container classification for immature Aedes aegypti surveillance in Kamphaeng Phet, Thailand. J Med Entomol 44: 938–944.
22. Koenraadt CJ, Aldstadt J, Kijchalao U, Kengluecha A, Jones JW, et al. (2007) Spatial and temporal patterns in the recovery of Aedes aegypti (Diptera: Culicidae) populations after insecticide treatment. J Med Entomol 44: 65–71.
23. Klungthong C, Gibbons RV, Thaisomboonsuk B, Nisalak A, Kalayanarooj S, et al. (2007) Dengue Viral Detection using Whole Blood for RT-PCR and Viral Isolation. J Clin Microbiol.
24. Johnson BW, Chambers TV, Crabtree MB, Guirakhoo F, Monath TP, et al. (2004) Analysis of the replication kinetics of the ChimeriVax-DEN 1, 2, 3, 4 tetravalent virus mixture in Aedes aegypti by real-time reverse transcriptase-polymerase chain reaction. Am J Trop Med Hyg 70: 89–97.
25. Bousema T, Griffin JT, Sauerwein RW, Smith DL, Churcher TS, et al. (2012) Hitting hotspots: spatial targeting of malaria for control and elimination. PLoS Med 9: e1001165.
26. Mwangi TW, Fegan G, Williams TN, Kinyanjui SM, Snow RW, et al. (2008) Evidence for over-dispersion in the distribution of clinical malaria episodes in children. PLoS One 3: e2196.
27. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM (2005) Superspreading and the effect of individual variation on disease emergence. Nature 438: 355–359.
28. Woolhouse ME, Dye C, Etard JF, Smith T, Charlwood JD, et al. (1997) Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proc Natl Acad Sci U S A 94: 338–342.
29. Balmaseda A, Standish K, Mercado JC, Matute JC, Tellez Y, et al. (2010) Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua. J Infect Dis 201: 5–14.
30. Harrington LC, Scott TW, Lerdthusnee K, Coleman RC, Costero A, et al. (2005) Dispersal Of The Dengue Vector Aedes Aegypti Within And Between Rural Communities. Am J Trop Med Hyg 72: 209–220.
31. Morales A, Groot H, Russell PK, McCown JM (1973) Recovery of dengue-2 virus from Aedes aegypti in Colombia. Am J Trop Med Hyg 22: 785–787.
32. Russell PK, Quy DV, Nisalak A, Simasathien P, Yuill TM, et al. (1969) Mosquito vectors of dengue viruses in South Vietnam. Am J Trop Med Hyg 18: 455–459.
33. Chan YC, Ho BC, Chan KL (1971) Aedes aegypti (L.) and Aedes albopictus (Skuse) in Singapore City. 5. Observations in relation to dengue haemorrhagic fever. Bull World Health Organ 44: 651–657.
34. De Benedictis J, Chow-Shaffer E, Costero A, Clark GG, Edman JD, et al. (2003) Identification of the people from whom engorged Aedes aegypti took blood meals in Florida, Puerto Rico, using polymerase chain reaction-based DNA profiling. Am J Trop Med Hyg 68: 437–446.
35. Stoddard ST, Morrison AC, Vazquez-Prokopec GM, Paz Soldan V, Kochel TJ, et al. (2009) The role of human movement in the transmission of vector-borne pathogens. PLoS Negl Trop Dis 3: e481.
36. Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A (1987) Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. Am J Trop Med Hyg 36: 143–152.
37. Honorio NA, Nogueira RM, Codeco CT, Carvalho MS, Cruz OG, et al. (2009) Spatial evaluation and modeling of Dengue seroprevalence and vector density in Rio de Janeiro, Brazil. PLoS Negl Trop Dis 3: e545.
38. Galvani AP, May RM (2005) Epidemiology: dimensions of superspreading. Nature 438: 293–295.

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Space-time analysis of hospitalised dengue patients in rural Thailand reveals important temporal intervals in the pattern of dengue virus transmission

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Abstract

OBJECTIVE To determine the temporal intervals at which spatial clustering of dengue hospitalisations occurs.

METHODS Space-time analysis of 262 people hospitalised and serologically confirmed with dengue virus infections in Kamphaeng Phet, Thailand was performed. The cases were observed between 1 January 2009 and 6 May 2011. Spatial coordinates of each patient's home were captured using the Global Positioning System. A novel method based on the Knox test was used to determine the temporal intervals between cases at which spatial clustering occurred. These intervals are indicative of the length of time between successive illnesses in the chain of dengue virus transmission.

RESULTS The strongest spatial clustering occurred at the 15–17-day interval. There was also significant spatial clustering over short intervals (2–5 days). The highest excess risk was observed within 200 m of a previous hospitalised case and significantly elevated risk persisted within this distance for 32–34 days.

CONCLUSIONS Fifteen to seventeen days are the most likely serial interval between successive dengue illnesses. This novel method relies only on passively detected, hospitalised case data with household locations and provides a useful tool for understanding region-specific and outbreak-specific dengue virus transmission dynamics.

keywords dengue, transmission, serial interval, space-time, clustering, Thailand

Introduction

Dengue fever and dengue haemorrhagic fever are important and expanding public health problems in tropical and subtropical regions (Endy *et al.* 2010; Gubler 2002). Dengue has spread from being endemic in just nine countries in 1970 to more than 100 countries as of 2009, with an estimated 2.5 billion people at risk of dengue virus (DENV) infection annually (WHO 2009). There are no licensed vaccines or drugs for dengue, and current prevention and control efforts focus on vector population reduction. More accurate characterisation of the spatial and temporal pattern of DENV transmission can inform improved surveillance and efficient control programmes. It can also help to provide data for models that are key tools for evaluating the cost and effectiveness of proposed control efforts, whether the approach is based on vaccination alone or a combined vaccine and vector control effort. Characterising the variation in the length of time between successive infections in the chain of transmission would help to better understand region-specific transmission dynamics. This study examined the spatial and temporal pattern of hospitalised dengue patients in Kamphaeng Phet, Thailand. The results indicate that the most likely interval between successive dengue illnesses can be estimated using only the severe cases that reach healthcare facilities.

Dengue transmission cycle

A cycle in the chain of DENV transmission (Figure 1) is initiated when an infective mosquito takes a blood meal from a susceptible person, injecting virus in the process. The latent intrinsic incubation period is thought to range from 2 to 12 days and is most commonly between 4 and 6 days (Sabin 1952; Siler *et al.* 1926; Nishiura & Halstead 2007). The infectious period in the host varies from 1 to 7 or more days, and viraemia is most often detected for a duration of 3–5 days (Gubler *et al.* 1981; Vaughn *et al.* 2000, 1997). Siler *et al.* (1926) reported that infectiousness preceded the onset of symptoms by 6–18 h in a group of volunteers challenged with near wild-type DENV. During the infectious period, a mosquito that feeds on the host may become infected. The extrinsic incubation period varies with ambient temperatures and can be as short as 8 or as long 20 days (Lambrechts *et al.* 2011; Siler *et al.* 1926; Watts *et al.* 1987). After the extrinsic incubation period, the infectious mosquito may transmit the virus with each subsequent blood meal (Putnam & Scott 1995), and female *Aedes aegypti* bite humans frequently, almost daily (0.76–0.63 human blood meals per day) (Scott *et al.* 2000). The serial interval between successive dengue illnesses along the chain of transmission is at least as long as the sum of the intrinsic and extrinsic incubation periods (Fine 2003). In the explanation of the DENV transmission cycle, it should also be noted that the duration of the extrinsic incubation period is long relative to the estimated life expectancy of *A. aegypti* (Bartley *et al.* 2002). There is considerable uncertainty associated with the estimates of daily survival, but a majority of adult *A. aegypti* are expected to die before they are able to transmit DENV (Harrington *et al.* 2001; Sheppard *et al.* 1969).

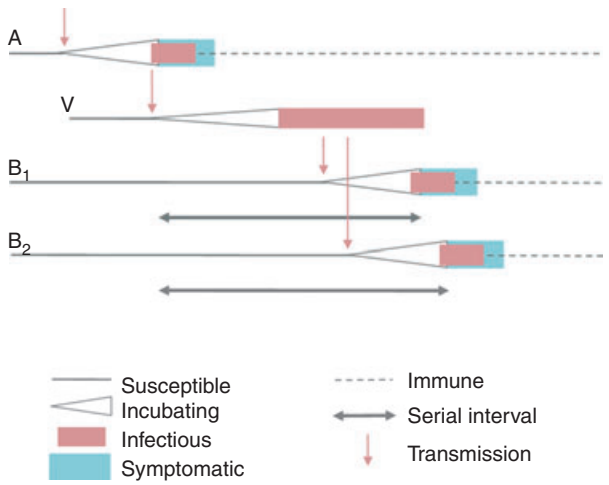

Figure 1 The DENV transmission cycle. Human hosts are represented by timelines A, B_1 and B_2. The vector is represented by timeline V. The cycle begins when susceptible host A acquires an infection from a vector (not shown) at the upper left. The vector, V, takes a blood meal from host A during the infectious period. After the extrinsic incubation period, the cycle is completed when DENV is transmitted to susceptible hosts B_1 and B_2. The serial interval between successive illnesses is depicted with the thick grey arrows.

Space-time analysis of DENV transmission

The spatial and temporal dynamics of DENV transmission are governed by the dispersal of infected vectors and hosts. Release-recapture studies have shown that *A. aegypti* do not generally disperse over long distances (Harrington *et al.* 2005; Sheppard *et al.* 1969; Trpis & Hausermann 1986). Harrington *et al.* (2005) observed that most recaptured *A. aegypti* were located within the house where they were released or in a neighbouring house. Garcia-Rejon *et al.* (2008) reported that DENV-infected *A. aegypti* were collected from dengue patients' homes up to 27 days after the onset of symptoms, indicating that infected mosquitoes do not disperse far from the likely site of transmission. It is thought, therefore, that human movement is responsible for most DENV transmission over long distances and between human settlements. The extent and role of human movement in dengue transmission is not yet well characterised, but modelling results indicate that movement is important in understanding individual risk of infection (Stoddard *et al.* 2009).

Clustering of dengue cases in space and time has been observed in a variety of settings. Early epidemiological studies described the clustering of cases within households and an apparent diffusion through communities from first observed cases (Halstead 1966; Neff *et al.* 1967). More recent examinations have employed spatial statistics to identify significant clusters and describe the evolution of patterns through time (Kan *et al.* 2008; Vazquez-Prokopec *et al.* 2010). With the focal nature of DENV transmission as a guide, several cluster sampling strategies have been employed to detect dengue infections across the clinical spectrum (Beckett *et al.* 2005; Mammen *et al.* 2008; Reyes *et al.* 2010). In the same region under study here, Mammen *et al.* (2008) found that DENV infection risk surrounding an index case identified through a school-based surveillance programme was clustered within 100 m radius sampling areas. Morrison *et al.* (1998) used the Knox test to examine clinically apparent case data from an outbreak in Puerto Rico. They determined that there was significant clustering of cases within 5 m and 3 days of each other and also within 35 m and 3 days of each other. Tran *et al.* (2004) employed the Knox test in an exploratory mode and examined the space-time clustering of

cases over a large range of distances and times during an outbreak in French Guiana. Their study also identified focal dengue risk, with the highest risk within 15 m and 6 days of a previously identified case. Additionally, the Knox test results showed a temporal periodicity in risk at 3-, 6- and 9-day intervals. The authors suggest that this pattern reflects the feeding interval of infectious *A. aegypti*.

Observations and analysis of the space-time pattern of dengue cases have provided guidance for vector control and disease surveillance efforts (Scott & Morrison 2010). For the most part, however, existing examinations of case data have provided little information on the timing and spatial extent of successive illnesses along the chain of transmission. This is in part a limitation of existing space-time clustering methods (Jacquez *et al.* 2007). The techniques employed in the studies outlined previously are designed to test hypotheses about clusters within a cumulative space-time window (i.e. cases within 5 m and 3 days of each other). The incremental Knox test (IKT) was introduced to overcome this limitation and examine the clustering of cases at a series of time intervals (Aldstadt 2007). The IKT can be used to examine the clustering of cases that occur at a given time interval apart. For example, the proximity of cases occurring 7 days apart could be examined. The interval could also be defined as a range of values to account for uncertainty in the reporting of onset of illness and effectively smooth the data. Given the typically limited flight range of *A. Aegypti,* we would expect that successive cases in the chain of transmission would be clustered in space. Those time intervals between cases when significant spatial clustering occurs likely indicate the most frequent lengths of intervals in the dengue transmission cycle. The case study included in Aldstadt (2007) found the most frequent serial interval between cases during an outbreak in Puerto Rico to be approximately 18–19 days.

Useful estimates of the serial interval between consecutive infections are important for understanding DENV transmission dynamics. It has been hypothesised that variations in the extrinsic incubation period are responsible for the timing of seasonal patterns and its importance is borne out by modelling efforts (Bartley *et al.* 2002; Focks *et al.* 1993; Lambrechts *et al.* 2011). Estimates of the serial interval and the distribution of spatial distances between cases occurring at that interval can be used to inform the proper extent and duration of vector control in response to an outbreak. Similarly, an accurate characterisation of the interval between successive illnesses can help to improve surveillance and sampling techniques designed to capture DENV infections across the clinical spectrum of disease and to estimate the capacity for virus population expansion; that is, R_o or basic reproductive rate. The ability to detect differences in the distribution of serial intervals between outbreaks could provide answers to other open questions in dengue research. For example, variability in extrinsic incubation period could be a component of viral fitness that drives replacement of one virus genotype by another within a serotype; that is, clade replacement (Anderson & Rico-Hesse 2006; Hanley *et al.* 2008; Lambrechts *et al.* 2012). In this research, the IKT methodology was used to examine the space–time interactions of people with hospitalised, serologically confirmed dengue illnesses.

Materials and methods

Study site and sample

The study site was the Muang District of Kamphaeng Phet Province in north-central Thailand (Figure 2). There were 206 456 registered residents of the district in 2009 (Department of Provincial Administration 2010). The district is served primarily by Kamphaeng Phet Provincial Hospital in the Nai Muang Subdistrict (capital subdistrict). Dengue illness occurs year round; however, there is a regular seasonal pattern with higher rates of illness in the wet months from June through September. The local Thai

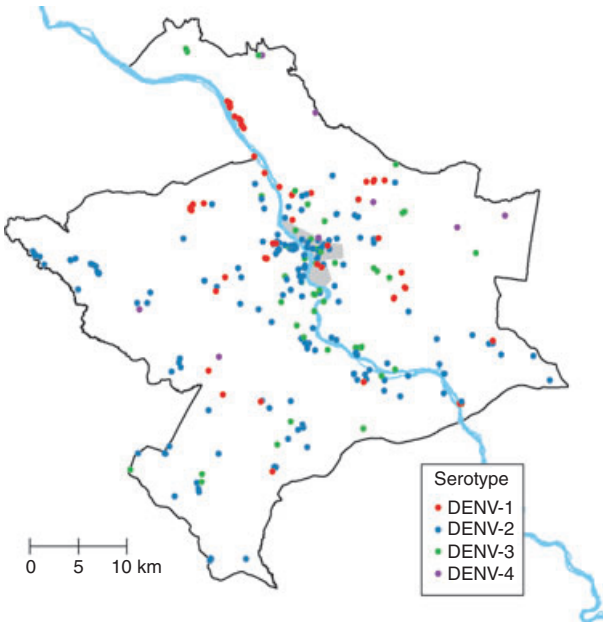

Figure 2 The study site and household locations of DENV-infected, hospitalised individuals. The black line is the boundary of the Mueang District of Kamphaeng Phet, Thailand. The grey shaded area is the Nai Mueang Subdistrict, and the Ping River is depicted in light blue.

Ministry of Public Health performs insecticide spraying on notification of a dengue illness and again after 7 days (Mammen *et al.* 2008). The policy requires spraying houses within 100 m of the infected household.

The space-time analysis was performed using data on subjects admitted to the hospital with serologically confirmed DENV infections and virus serotype identifications by nested PCR (Lanciotti *et al.* 1992; Klungthong *et al.* 2007). Over the period from 1 January 2009 through 6 May 2011, 262 cases were identified for analysis. There were 42 DENV1 infections, 163 DENV2 infections, 53 DENV3 infections and 4 DENV4 infections (Figure 3). The household of each study participant was located using the Global Positioning System (GPS) and entered into a geographic information system (GIS). The reported date of onset of symptoms was used as the time for each case.

Statistical analysis

An incremental Knox test was carried out to examine the level of spatial clustering at sequential overlapping time intervals. This test is a modification of the widely used Knox (1964) test for space–time interaction. The Knox test statistic, X, is the number of pairs of cases that are close in both space and time. The statistic is calculated as

$$X(s,t) = \sum_{i=1}^{N} \sum_{j=1}^{i-1} a_{ij}^{s} a_{ij}^{t} \qquad (1)$$

where N is the number cases, a_{ij}^{s} is equal to 1 if cases i and j are close in space, and 0 otherwise, a_{ij}^{t} is equal to 1 if cases i and j are close in time and 0 otherwise, and s and t represent pre-specified spatial and temporal distances. The Knox test statistic amounts to the number of cases occurring within a given distance and time interval apart. For example, when s is equal to 100 m and t is = 7 days, the resulting value would indicate the number of pairs of cases occurring within 100 m and 7 days of each other. The resulting statistic is useful for testing the null hypothesis of no space–time interaction *vs.* the alternative hypothesis of a contagious or focal process. The test does not, however, indicate which time intervals within t that the spatial clustering occurs. The significance of the observed value is most often determined using the randomisation procedure suggested by Mantel (1967). In this Monte Carlo significance test, the N occurrence times are randomly permuted among the cases to estimate the distribution of the statistic under the null hypothesis of no space–time interaction. The interval Knox statistic is formulated as

$$IK(s,t) = \sum_{i=1}^{N} \sum_{j=1}^{i-1} a_{ij}^{s} b_{ij}^{t} \qquad (2)$$

where b_{ij}^{t} is equal to 1 if cases i and j occur t units apart, and 0 otherwise. This modification considers only pairs of cases that occur at a given time interval, for example, 7–9 days. When calculated for a series of temporal intervals, the IKT results indicate the intervals when spatial clustering of cases occurs.

For this analysis, we employed a series of overlapping time intervals, t, to account for uncertainty in date of illness onset. The first test examined cases occurring on the same day or 1 day apart. The second case interval is

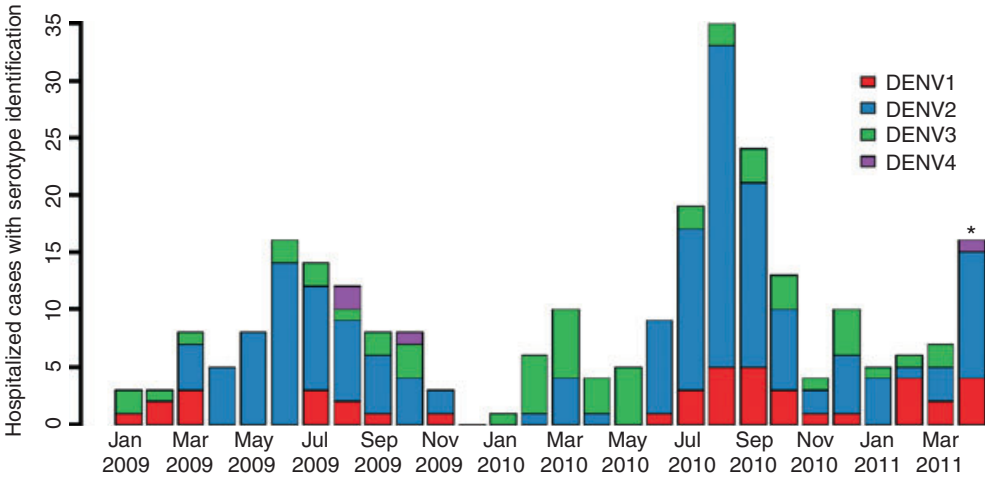

Figure 3 The times series plot for hospitalised dengue patients included in this research. The asterisk denotes that the count for April 2011 includes five cases with onset of illness between 1 and 3 May 2011.

0–2 days apart, and the series continued with 3-day overlapping intervals to cases 39–41 days apart. Based on previous observations of the focality of DENV transmission in the study area (Mammen *et al.* 2008) and to ensure that the results would be robust to choice of distance cut-off, the analysis was performed for distances of 100, 200, 300, 400 and 500 m. Only pairs of hospitalisations resulting from infection with the same serotype were considered. Simulation studies have shown that space–time interactions tests are liberal in rejecting the null hypothesis (Kulldorff & Hjalmars 1999), and therefore, the conservative Bonferroni adjustment was employed to correct for multiple testing (Shaffer 1995). The test results are also reported as the epidemiological meaningful notion of excess risk; in this case, owing to the space–time interaction present in the observed data set (Diggle *et al.* 1995). Excess risk is calculated as the ratio of the observed statistic divided by the permutation mean $\mu_x(s,t)$.

$$ER(s,t) = \frac{IK(s,t)}{\mu_x(s,t)} \quad (3)$$

Excess risk was computed for the same time intervals and distances as above. A total of 100 000 permutations under the null hypothesis were completed to estimate $\mu_x(s,t)$ and critical values of the distribution for each test.

Ethical approval

The study protocol and consent forms were approved by Upstate Medical University's Institutional Review Board, the Thailand Ministry of Public Health Ethics Committee, and the Walter Reed Army Institute of Research's Institutional Review Board.

Results

The results of the IKT analysis are displayed in Figure 4. The temporal intervals with the strongest spatial clustering are 2–5 days and 15–17 days. These results are consistent for all five distances. For the shortest distances, 100 and 200 m, there are no pairs of hospitalisations observed at the 6–8-day interval, which has the lowest excess risk at all distances examined. There is also clustering observed, although to a lesser extent, at intervals of 9–13, 19–22, 23–27 and 30–34 days. The excess risk of hospitalised dengue because of the space–time interaction of the transmission process is shown in Figure 5. This graph shows that the temporal pattern of clustering observed at 200 m is similar to the pattern observed at 100 m. The largest estimated excess risk attributable to space–time interaction of hospitalisations was 15.2 and occurred at the 200 m distance and 15–17-day interval. This means that the probability of being hospitalised for dengue at this temporal interval owing to spatial proximity is 15.2 times that which would occur at a randomly selected case location in the study area.

Discussion

This research demonstrates that spatiotemporal methods can be used to detect temporal patterns of excess risk within a focus of DENV transmission. These patterns are detectable using only hospitalised illnesses, which represent a small proportion of total infections and are indicative of important temporal intervals in the diffusion of DENV. The spatial clustering of hospitalisations over short-time intervals (2–5 days) confirms the results of previous studies. There are several mechanisms that are potentially responsible for this pattern. The first is that multiple mosquitoes became infected at approximately the same time by feeding on one or more infectious hosts. They have survived the extrinsic incubation period and infected subsequent individuals that have onset of symptoms a few days apart. Second, *A. aegypti* often take multiple blood meals from multiple hosts with each egg laying cycle (Scott *et al.* 1993a,b; Gould *et al.* 1970; Macdonald 1956). This multiple feeding behaviour increases the likelihood that multiple people are infected by a single infectious vector over a short-time period. Third, the gonotrophic cycle of *A. aegypti* can be as short as 3 days (Christophers 1960), although it is assumed to last longer (Watts *et al.* 1987). This means that cases occurring within a 3–5-day period could be the result of the same infected mosquito(s) feeding on nearby hosts during consecutive egg laying cycles. The combination of these factors is likely responsible for the focal and sometimes explosive nature of DENV transmission, but the relative contribution of these three aspects of DENV transmission cannot be untangled as part of the current analysis.

The strongest spatial clustering occurred at the 15–17-day interval, and this likely indicates the most frequent serial interval between successive human DENV infections. This estimate fits with prior knowledge regarding the length of the intrinsic and extrinsic incubation periods (Lambrechts *et al.* 2011; Nishiura & Halstead 2007). We would also expect that the strongest clustering signal is associated with the most direct link in the chain of transmission. The spatial extent of the clustering is consistent with the normally limited migration of female *A. aegypti*, although we cannot determine from our analysis the relative contribution of virus movement in flying infected mosquitoes *vs.* movements by humans. Of the 14 pairs of hospitalisations within 500 m of each other at the 15–17-day interval, 13 were within 200 m and 8 were

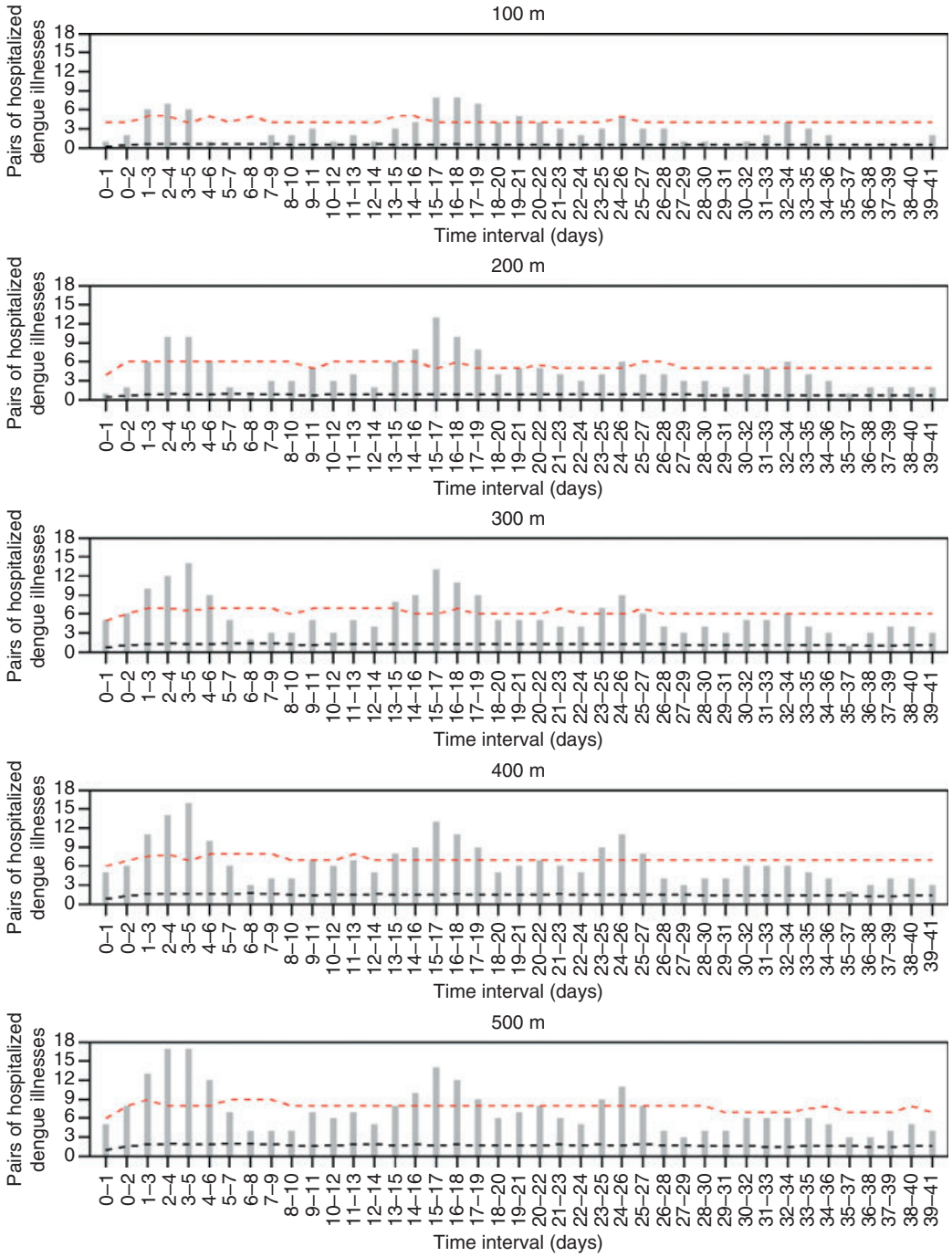

Figure 4 Space-time clustering results. The bars indicate the number of hospitalised dengue patients with the same infecting serotype that resided within the stated distance of each other. The results are shown for distances of 100, 200, 300, 400 and 500 m. The black dashed line indicates the expected number of cases under the null hypothesis of no space–time interaction. The dashed red line indicates the 99.98th percentile of the permutation distribution. Bars exceeding this line have a nominal significance level <0.0002, and a Bonferroni adjusted significance level <0.05.

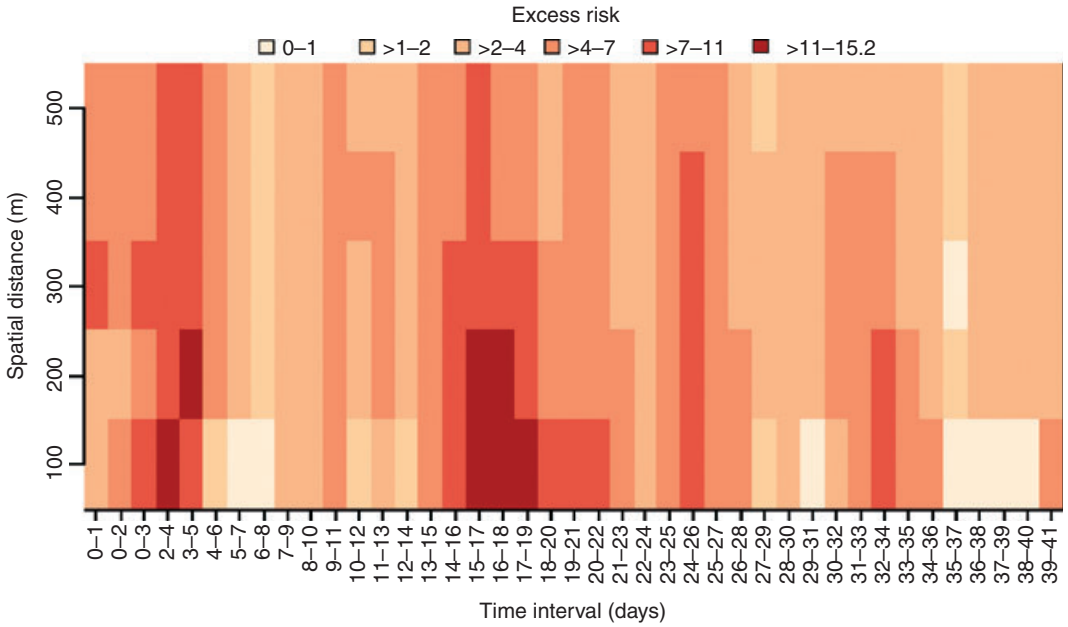

Figure 5 The excess risk of dengue patient hospitalisation because of the space–time interaction between cases. Spatial and temporal distances are from a previous hospitalised case.

within 100 m of each other. The significant result was not because of temporally or spatially isolated events. The first case for each of the pairs observed at this interval occurred from March through October, and the pairs of infections resulted from the three predominant serotypes (4 DENV1, 7 DENV2, and 2 DENV3). The general trend of declining risk held for nearly all time intervals; however, the number of pairs observed at the 3–5-day interval continued to increase at larger distances (from 10 pairs at 200 m to 17 pairs at 500 m). The significant excess risk at longer intervals shows that transmission may persist within a small area. The high excess risk at the 32–34-day interval seems to indicate that two complete transmission cycles can be completed within a small area.

There are several limitations to this study. The first is that we used residential distance as a proxy for relatedness of hospitalisations. Previous work in the same region has demonstrated the focal nature of transmission, but it is possible that geographically proximate infections, even owing to the same serotype, were not closely linked in the chain of transmission. DENV infections may also be acquired at locations far from the place of residence (Mondini *et al.* 2009). Data on patient movement patterns were not collected, and so there is no way to link cases associated with transmission at schools, workplaces or other activity spaces. With the advent of inexpensive full genome sequencing and deep sequencing, it may soon be possible to use genetic similarity of viruses on its own or in concert with geographic information as a more reliable measure of the relatedness of infections. Another concern is that the current analysis employed only hospitalised illnesses. Studies have indicated that virulence varies by serotype and strain of infecting virus (Hesse 2007; Nisalak *et al.* 2003). Host-virus interactions have also been implicated in disease severity (Endy *et al.* 2004). If the lengths of incubation periods are correlated with clinical severity, then the results presented here may not hold for milder DENV infections. Hospitalised cases comprise a small proportion of total DENV infections (Endy *et al.* 2011), and very few nearby pairs were observed in the cooler portion of the year. A more sensitive surveillance system might be required to perform a similar analysis in periods of low transmission or low virulence.

The strength of space-time permutation tests is that they require few assumptions other than that nearby cases are more likely to be related than cases that occur far away. Special populations and resource intensive techniques are not required, nor is it assumed that cases are directly linked in the chain of transmission. One drawback of this type of test is that the results may be subject to population shift bias (Kulldorff & Hjalmars 1999). The effect of population shift was limited here by examining only temporal intervals that are short relative to the overall study period. The results of simulation studies indicate that the inclusion of data on the susceptibility of individuals within a cohort improves the power to detect significant interaction

(Aldstadt 2007). This type of information is, however, rarely available. Despite reduced power, the same simulation studies found that the permutation approach employed here provided unbiased estimates of the serial interval between illnesses even when only a small proportion of total infections were observed.

This novel approach to space-time analysis revealed temporal intervals at which the homes of people hospitalised because of DENV infection were spatially clustered. These patterns in the diffusion of the infection are linked to the temporal intervals in the DENV transmission cycle. The most likely serial interval was observed as the 15–17-day period, and significant excess risk of dengue illness persisted as long as 32–34 days. The IKT methodology is a tool that can be used to better understand region-specific and outbreak-specific transmission dynamics. Systematic characterisation of differences between settings can lead to a more complete understanding of the variation of the force of transmission, guide locally appropriate dengue control efforts and constitutes valuable new information for construction and parameterisation of DENV transmission models.

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References

Aldstadt J (2007) An incremental Knox test for the determination of the interval between successive cases of an infectious disease. *Stochastic Environmental Research and Risk Assessment* **21**, 487–500.

Anderson JR & Rico-Hesse R (2006) *Aedes aegypti* vectorial capacity is determined by the infecting genotype of dengue virus. *The American Journal of Tropical Medicine and Hygiene* **75**, 886.

Bartley L, Donnelly C & Garnett G (2002) The seasonal pattern of dengue in endemic areas: mathematical models of mechanisms. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **96**, 387–397.

Beckett CG, Kosasih H, Faisal I *et al.* (2005) Early detection of dengue infections using cluster sampling around index cases. *The American Journal of Tropical Medicine and Hygiene* **72**, 777–782.

Christophers SR (1960) *Aëdes aegypti* (L.) the Yellow Fever Mosquito: its Life History, Bionomics and Structure. Cambridge University Press, London.

Department of Provincial Administration (2010) *Population Statistics*. Department of Provincial Administration, Available at: http://203.113.86.149/xstat/xstat.html (accessed August 31 2011).

Diggle P, Chetwynd A, Haggkvist R & Morris S (1995) Second-order analysis of space-time clustering. *Statistical Methods in Medical Research* **4**, 124–136.

Endy TP, Nisalak A, Chunsuttitwat S *et al.* (2004) Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. *Journal of Infectious Diseases* **189**, 990–1000.

Endy TP, Weaver SC & Hanley KA (2010) Dengue virus: past, present and future. In: *Frontiers in Dengue Virus Research* (KA Hanley & SC Weaver) Caister Academic Press, Norfolk, pp. 265–297.

Endy TP, Anderson KB, Nisalak A *et al.* (2011) Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in kamphaeng phet, Thailand. *PLoS Neglected Tropical Diseases* **5**, e975.

Fine PEM (2003) The interval betwen successive cases of an infectious disease. *American Journal of Epidemiology* **158**, 1039–1047.

Focks D, Haile D, Daniels E & Mount G (1993) Dynamic life table model for *Aedes aegypti* (Diptera: Culicidae): analysis of the literature and model development. *Journal of Medical Entomology* **30**, 1003–1017.

Garcia-Rejon J, Loroño-Pino MA, Farfan-Ale JA *et al.* (2008) Dengue virus–infected *Aedes Aegypti* in the home environment. *The American Journal of Tropical Medicine and Hygiene* **79**, 940–950.

Gould DJ, Mount GA, Scanlon JE, Ford H & Sullivan MF (1970) Ecology and control of dengue vectors on an island in the Gulf of Thailand. *Journal of Medical Entomology* **7**, 499–508.

Gubler DJ (2002) Epidemic dengue/dengue hemorrhagic fever as a public health, social, and economic problem in the 21st century. *Trends in Microbology* **10**, 100–103.

Gubler D, Suharyono W, Tan R, Abidin M & Sie A (1981) Viraemia in patients with naturally acquired dengue infection. *Bulletin of the World Health Organization* **59**, 623–630.

Halstead SB (1966) Epidemiological studies of Thai haemorrhagic fever 1962–1964. *Bulletin of the World Health Organization* **35**, 80–81.

Hanley K, Nelson J, Schirtzinger E, Whitehead S & Hanson C (2008) Superior infectivity for mosquito vectors contributes to competitive displacement among strains of dengue virus. *BMC Ecology* **8**, 1.

Harrington LC, Buonaccorsi JP, Edman JD *et al.* (2001) Analysis of survival of young and old *Aedes aegypti* (Diptera: Culicidae) from Puerto Rico and Thailand. *Journal of Medical Entomology* **38**, 537–547.

Harrington LC, Scott TW, Lerdthusnee K *et al.* (2005) Dispersal of the dengue vector *Aedes aegypti* within and between rural communities. *The American Journal of Tropical Medicine and Hygiene* **72**(20), 9.

Hesse RR (2007) Dengue virus evolution and virulence models. *Clinical Infectious Diseases* **44**, 1462–1466.

Jacquez GM, Meliker J & Kaufmann A (2007) In search of induction and latency periods: space-time interaction accounting for residential mobility, risk factors and covariates. *International Journal of Health Geographics* **6**, 35.

Kan C-C, Lee P-F, Wen T-H *et al.* (2008) Two clustering diffusion patterns identified from the 2001–2003 dengue epidemic, Kaohsiung, Taiwan. *American Journal of Tropical Medicine and Hygiene* **79**, 344–352.

Klungthong C, Gibbons RV, Thaisomboonsuk B *et al.* (2007) Dengue virus detection using whole blood for reverse transcriptase PCR and virus isolation. *Journal of Clinical Microbiology*, **45**, 2480.

Knox EG (1964) Detection of space-time interactions. *Applied Statistics* **13**, 25–29.

Kulldorff M & Hjalmars U (1999) The Knox method and other tests for space-time interaction. *Biometrics* **55**, 544–552.

Lambrechts L, Paaijmans KP, Fansiri T *et al.* (2011) Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. *Proceedings of the National Academy of Sciences* **108**, 7460.

Lambrechts L, Fansiri T, Pongsiri A *et al.* (2012) Dengue-1 virus clade replacement in Thailand associated with enhanced mosquito transmission. *Journal of Virology* **86**, 1853–1861.

Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ & Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology* **30**, 545.

Macdonald W (1956) *Aedes aegypti* in Malaya. II. Larval and adult biology. *Annals of Tropical Medicine and Parasitology* **50**, 399.

Mammen MP Jr, Pimgate C, Koenraadt CJM *et al.* (2008) Spatial and temporal clustering of dengue virus transmission in Thai villages. *PLoS Medecine* **5**, e205.

Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research* **27**(20), 9–220.

Mondini A, Bronzoni RVDM, Nunes SHP *et al.* (2009) Spatiotemporal tracking and phylodynamics of an urban dengue 3 outbreak in São Paulo, Brazil. *PLoS Neglected Tropical Diseases* **3**, e448.

Morrison AC, Getis A, Santiago M, Rigau-Perez JG & Reiter P (1998) Exploratory disease analysis of reported dengue cases during an outbreak in Florida, Puerto Rico 1991–1992. *American Journal of Tropical Hygiene and Medicine* **58**, 287–298.

Neff JM, Morris L, Gonzalez-Alcover R, Coleman PH, Lyss SB & Negron H (1967) Dengue fever in a Puerto Rican community. *American Journal of Epidemiology* **86**, 162–184.

Nisalak A, Endy TP, Nimmannitya S *et al.* (2003) Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *The American Journal of Tropical Medicine and Hygeine* **68**(19), 1–202.

Nishiura H & Halstead SB (2007) Natural History of Dengue Virus (DENV)â€"1 and DENVâ€"4 Infections: Reanalysis of Classic Studies. *Journal of Infectious Diseases* **195**, 1007.

Putnam JL & Scott TW (1995) The effect of multiple host contacts on the infectivity of dengue-2 virus-infected Aedes aegypti. *The Journal of Parasitology* **17**, ???–174.

Reyes M, Mercado JC, Standish K *et al.* (2010) Index cluster study of dengue virus infection in Nicaragua. *The American Journal of Tropical Medicine and Hygiene* **83**, 683–689.

Sabin AB (1952) Research on dengue during world war II. *The American Journal of Tropical Medicine and Hygiene* **1**, 30.

Scott TW & Morrison AC (2010) Vector dynamics and transmission of dengue virus: implications for dengue surveillance and prevention strategies. In: *Dengue Virus* (ed. AL Rothman) Springer, Berlin Heidelberg.

Scott TW, Chow E, Strickman D *et al.* (1993a) Blood-feeding patterns of *Aedes aegypti* (Diptera: Culicidae) collected in a rural Thai village. *Jounal of Medical Entomology* **30**, 922–927.

Scott TW, Clark GG, Lorenz LH, Amerasinghe PH, Reiter P & Edman JD (1993b) Detection of multiple blood feeding in *Aedes aegypti* (Diptera: Culicidae) during a single gonotrophic cycle using a histologic technique. *Journal of Medical Entomology* **30**, 94–99.

Scott TW, Amerasinghe PH, Morrison AC *et al.* (2000) Longitudinal studies of *Aedes aegypti* (Diptera: Culicidae) in Thailand and Puerto Rico: blood feeding frequency. *Journal of Medical Entomology* **37**, 89–101.

Shaffer JP (1995) Multiple hypothesis testing. *Annual Review of Psychology* **46**, 561–584.

Sheppard P, Macdonald W, Tonn R & Grab B (1969) The dynamics of an adult *Aedes aegypti* in relation to dengue hemorrhagic fever in Bangkok. *The Journal of Animal Ecology* **38**, 661–702.

Siler JF, Hall MW & Kitchens A (1926) Dengue: its history, epidemiology, mechanism of transmission, etiology, clinical manifestations, immunity and prevention. *Philippine Journal of Science* **29**, 1–304.

Stoddard ST, Morrison AC, Vazquez-Prokopec GM *et al.* (2009) The role of human movement in the transmission of vector-borne pathogens. *PLoS Neglected Tropical Diseases* **3**, e481.

Tran A, Deparis X, Dussart P *et al.* (2004) Dengue spatial and temporal patterns, French Guiana 2001. *Emerging Infectious Diseases* **10**, 615–621.

Trpis M & Hausermann W (1986) Dispersal and other population parameters of *Aedes aegypti* in an African village and their

possible significance in epidemiology of Vector-Borne diseases. *American Journal of Tropical Medicine and Hygiene* **35**, 1263–1279.

Vaughn DW, Green S, Kalayanarooj S *et al.* (1997) Dengue in the early febrile phase: viremia and antibody responses. *Journal of Infectious Diseases* **176**, 322.

Vaughn DW, Green S, Kalayanarooj S *et al.* (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. *Journal of Infectious Diseases* **181**, 2.

Vazquez-Prokopec GM, Kitron U, Montgomery B, Horne P & Ritchie SA (2010) Quantifying the Spatial Dimension of Dengue Virus Epidemic Spread within a Tropical Urban Environment. *PLoS Neglected Tropical Diseases* **4**, e920.

Watts D, Burke DS, Harrison BA, Whitmire RE & Nisalak A (1987) Effect of temperature on the vector efficiency of *Aedes aegypti* for dengue 2 virus. *American Journal of Tropical Hygiene and Medicine* **36**, 143–152.

WHO (2009) Fact sheet no.117, Dengue and dengue haemorrhagic fever. http://www.who.int/mediacentre/factsheets/fs117/en/ (accessed September 1 2011).

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