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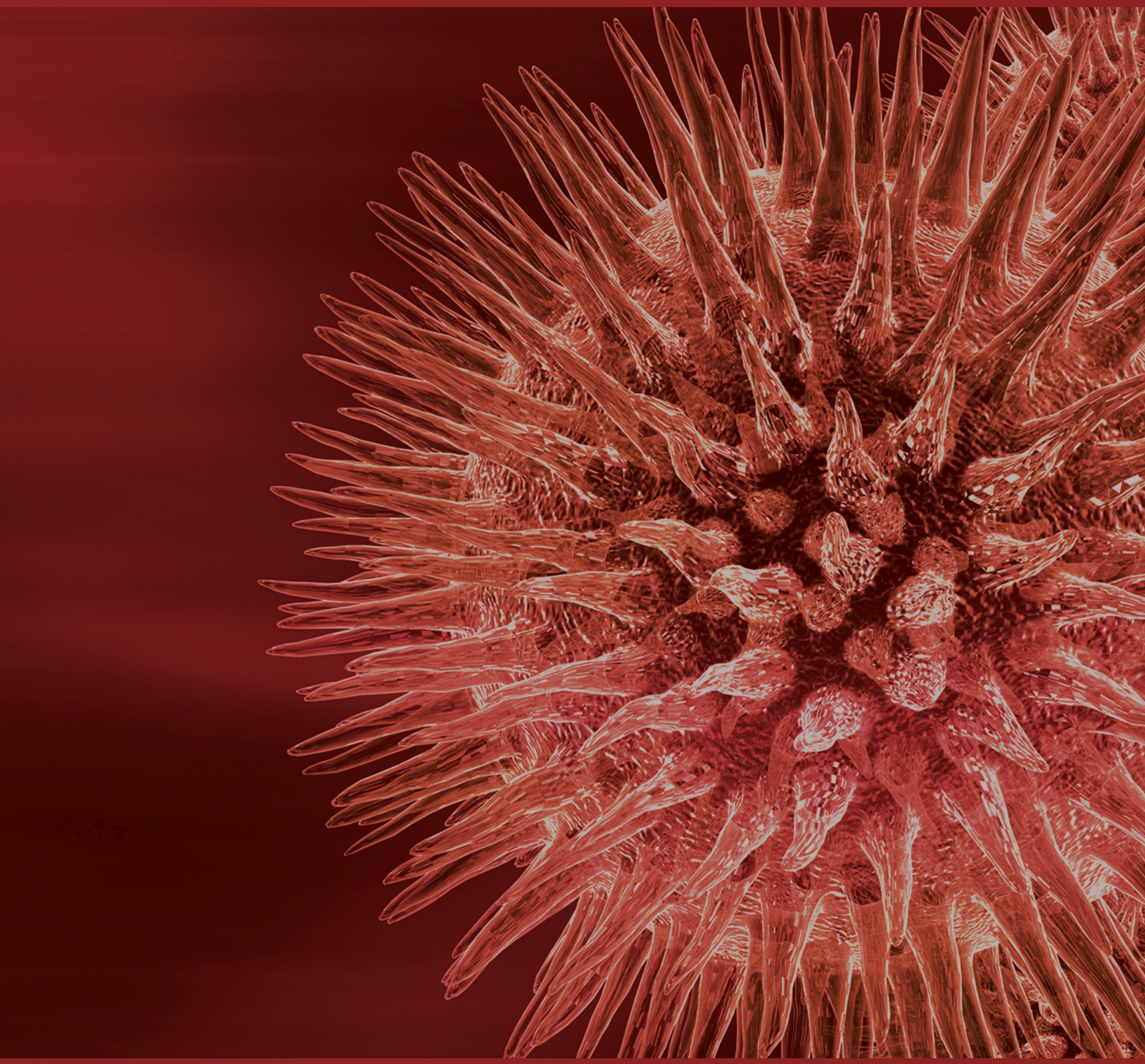
Human Arthropod-Borne Viral Infections

Guest Editors: Aldo Manzin, Byron E. Martina, Ernest A. Gould, Patrizia Bagnarelli, and Vittorio Sambri



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Editorial

Human Arthropod-Borne Viral Infections

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Arthropod-borne viruses, that is, arboviruses, belong to different virus families and genera and are maintained through transmission between vertebrate hosts by virus-infected blood feeding arthropods (mosquitoes, sandflies, biting midges, and ticks). Several factors have contributed to the emergence and reemergence of arthropod-to-human infections in the last decade, also in nonendemic areas: climate changes which are believed to have contributed to increased dispersion of the virus vectors; the international trading system that transports an enormous range of goods, animals, and human beings all over the world, inadvertently increasing the possibility for vectors and viruses to spread; and the global increase of human population density, particularly in areas densely populated by arthropods with transmission capability for several different pathogenic viruses. As a consequence, the incidence of arboviral infections and diseases is increasing worldwide and therefore placing a heavier burden on public health agencies and requiring the need to ensure timely recognition and treatment of virus infections.

This special issue is aimed at the presentation of recent results obtained by prominent colleagues in the field of human infections caused by vector-borne viruses. Faced with a limited number of manuscripts presented, the high quality of these papers will contribute significantly to our knowledge of these important issues.

Pathogens, such as dengue virus, Japanese encephalitis virus, and alphaviruses are addressed by the authors. G. Añez and M. Rios described the epidemiology of what is likely to be the most widespread mosquito transmitted *Flavivirus* infection, that is, dengue fever, in the United States of America where this infection is currently generating huge concerns. In particular, the authors emphasize how, despite the current economic difficulties and restrictions, strict mosquito control policies and activities must be implemented and maintained in localities that have the potential to become the route of entry for viruses and the focus of epidemics.

A second paper by C.-F. Liu and coworkers reported on the complementary role of clinical practice-based laboratory data in facilitating suspicion and diagnosis of dengue in clinical settings, thus ameliorating the overall diagnostic capabilities for this infection. Although the authors state that further studies are needed to confirm the validity of their approach, we believe that the study is important to define which procedures will have the most significant impact on clinical practice in tropical countries, where medical resources are limited. R. Rodriguez-Roche and E. A. Gould contributed

a comprehensive review about the complex epidemiology and pathogenesis of the dengue viruses. They underlined the need for more effort to understand and accelerate the development of suitable vaccines and/or antiviral therapies for the control of this "scourge" of the tropical and subtropical world. Another paper by M. L. Muñoz and coworkers is dedicated to the investigation of the interplay between dengue virus proteins and the receptor polypeptides located on the surface of mosquito cells, since this interaction is the fundamental basis for viral entry into susceptible cells, followed by development and transmission in the vectors. The paper by I.-K. Lee and coworkers provides an insight into the immunopathogenesis of dengue virus in patients with type 2 diabetes mellitus and reports on the ability of mononuclear cells from these patients to be infected by dengue virus. The aim of the paper is to fill a gap in our knowlwdge that concerns epidemiological data and immunological findings of frequent dengue haemorrhagic fever and Th2 cytokines in patients with diabetes mellitus. The authors demonstrated that *in vitro* infection of mononuclear cells from diabetes mellitus patients induces higher levels of both IL-4 and the anti-inflammatory Th2-cytokine IL-10, in addition to increased levels of GM-CSF, and they speculated that these findings might result from a counterbalance to the comparatively highly activated proinflammatory cytokines/chemokines in the diabetic hosts.

Two papers describe different aspects of Japanese encephalitis virus (JEV) related diseases. The paper by G. Kakoti and coworkers reports on the clinical profile and the outcome of Japanese encephalitis virus infection in children up to 12 years old. This is a prospective study based on serological assays and it shows some points of interest from a clinical and epidemiological point of view as it investigates the presence of clinical or technical factors likely to be significantly correlated with increased mortality by JEV in children with acute encephalitis syndrome in North East India. B. Bandyopadhyay and collaborators reported the epidemiology of JEV in West Bengal (India) showing that 22.76% and 5% of the acute encephalitis syndrome cases were positive for JE IgM in 2011 and 2012, respectively, and that JE is mainly prevalent in children and adolescents below 20 years of age with no gender predilection. This is a significant observation from an epidemiological point of view, which shows how active surveillance of JE cases is still warranted. It also emphasizes the need for vigilance to identify the introduction of new genotypes in the endemic areas or to detect evidence of dispersion into newer geographical districts.

This series of papers is completed by a paper by I. Assunção-Miranda that describes the molecular mechanisms involved in the pathogenesis of alphavirus-induced arthritis, the paper by A. Burgueño and coworkers that investigated the seroprevalence of Saint Louis encephalitis related antibodies among horses in Uruguay, where no sharp epidemiological data about this virus were available so far, and the article by G. Sautto and collaborators that speculated about the possible use of candidate monoclonal antibodies to be possibly used in a future passive immunotherapy for arthropod-borne infections. This approach seems of particular importance, given the current absence of specific antiviral drugs as well as effective vaccines for the most diffused arbovirus infections.

Acknowledgment

We believe that any contribution aimed at improving our knowledge of the epidemiology, the clinical aspects, the diagnosis, and the therapeutic and preventive control of these infections is welcome, especially if it comes from countries where economic and budgetary constraints necessitate the reduction of resources that are necessary to address these relevant issues. We warmly thank all the authors that have contributed to this series of informative manuscripts in this special issue and we are confident that these contributions are relevant step forward to the better understanding of emerging diseases that are currently considered to be a major concern in global health.

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Research Article

Clinical Profile and Outcome of Japanese Encephalitis in Children Admitted with Acute Encephalitis Syndrome

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Japanese encephalitis (JE) is an arthropod borne viral disease. Children are most commonly affected in Southeast Asian region showing symptoms of central nervous system with several complications and death. The clinical characteristics and outcomes in pediatric JE patients hospitalized with acute encephalitis syndrome (AES) are still poorly understood. A prospective study was conducted in pediatric ward of Assam Medical College Hospital to evaluate the clinical profile and outcome of JE in children. A total of 223 hospitalized AES cases were enrolled during March to December 2012. Serum and cerebro spinal fluids were tested for presence of JE specific IgM antibody. 67 (30%) were found to be JE positive. The most common presenting symptoms in JE patients were fever (100%), altered sensorium (83.58%), seizure (82.08%), headache (41.79%), and vomiting (29.85%). Signs of meningeal irritation were present in 55.22% of cases. Around 40.29%, JE patients had GCS ≤ 8. Among the JE patients, 14.7% died before discharge. The complete recoveries were observed in 63.9% of cases, while 21.3% had some sort of disability at the time of discharge. JE is still a major cause of AES in children in this part of India. These significant findings thus seek attentions of the global community to combat JE in children.

1. Introduction

Japanese encephalitis (JE) is the most prevalent and significant mosquito borne viral encephalitis of man, occurring with an estimated 30,000 to 50,000 of cases and 15,000 deaths annually [1–3]. About 20% to 30% of JE cases are fatal, and 30–50% result in permanent neuropsychiatric sequelae [3, 4]. Children remain the main victims of the disease [5, 6]. In India, nearly all states have reported JE cases except that of Jammu & Kashmir, Himachal Pradesh, and Uttaranchal [7]. The Northeastern region (NE region) of India, particularly the upper part of the state of Assam, has been experiencing recurrent episodes of JE with different magnitudes from July to October every year [8]. Most JE infections are asymptomatic, and the ratio of symptomatic to asymptomatic infections ranges from 1 in 300 to 1 in 1000 [9, 10]. Japanese encephalitis virus (JEV) targets the central nervous system, clinically manifesting with fever, headache, vomiting, signs of meningeal irritation, and altered consciousness [11]. At present, there is no specific agent available against JE. Treatment of JE is therefore essentially symptomatic and intensive supportive care is important to avoid neurological sequelae [12].

This study was undertaken for a better understanding and to determine the clinical profile and outcome of JE in children hospitalized with AES cases which may help in early diagnosis and initiating prompt supportive care.

2. Materials and Methods

2.1. Case Enrollment and Sample Collection. All the hospitalized AES cases up to 12 years of age in pediatric ward of Assam Medical College hospital were included in this study. This is a tertiary level hospital and provides health care services to mainly seven districts of upper Assam and neighboring state Arunachal Pradesh and Nagaland. Most patients are referred to this apex level institute from periphery for better supportive care and treatment. The study was carried out during March to December 2012.

For investigating AES cases, WHO case definition was adopted. Clinically a case of AES is defined as fever or recent history of fever with change in mental status (including confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures). Other early clinical findings could include an increase in irritability, somnolence or abnormal behavior greater than that seen with usual febrile illness [13, 14]. All enrolled cases were worked up with the help of a predesigned and pretested proforma. After getting written informed consent 2 mL of blood and CSF samples were collected in sterile condition. The samples were then transferred under cold chain to Regional Medical Research Centre Laboratory, ICMR, Dibrugarh and stored at −80°C for further analysis. Reports of CSF samples analyzed for physical, chemical, and cytological examination and other relevant investigations done at the time of admission were recorded from the bed head tickets of the patient.

The study was approved by the Institutional Ethics Committee (Human) of Regional Medical Research Centre (ICMR), Dibrugarh, Assam, India.

2.2. Outcome of JE Cases. The outcome of the patients were recorded at the time of discharge. Few patients were released from the hospital against medical advice and their condition could not be assessed. They were disqualified from the outcome analysis. Outcome was defined as recovered completely, recovered with neurological sequelae, and death.

Neurological sequelae were defined by the presence of one or more of the following at discharge; impaired consciousness, weakness (monoparesis, hemiparesis, and quadriparesis), focal or generalized abnormal limb tone (hypertonia and hypotonia), focal or generalized abnormal limb reflexes (hyperreflexia and hyporeflexia), diagnosis of new onset or recurrent seizures, or new or recurrent extra pyramidal movement disorders [15].

2.3. Serology. JE virus specific IgM antibodies were detected by IgM antibody capture-enzyme-linked immunosorbent assay kits obtained from the National Institute of Virology (NIV), Pune, India. The test was standardized and reported by NIV in 1984 [16]. The performance of the test was evaluated by Christian Medical College (CMC), Vellore in 2002 [17]. The JE IgM kit contains all ready to use reagents and has also been evaluated by Centers for Disease Control (CDC), Fort Collins, CO, USA for its performance. Using the United States' CDC results as the reference standard, the NIV kit had sensitivity in CSF 75%, Serum 71%, and specificity 96% in CSF and 77% in Serum [18]. Serological cross-reactions are common within the flaviviruses that is, Dengue, Japanese encephalitis, and West Nile encephalitis [16, 19] and all are prevalent in this part of the country [20–22]. Therefore, the JEV-IgM positive samples were further tested for the presence of IgM antibody against other flaviviruses namely Dengue and West Nile by using Dengue IgM capture ELISA kit obtained from NIV, Pune, India and PanBio WNV IgM capture ELISA kit (Australia).

2.4. Statistical Analysis. Results were presented in the form of percentages, mean ± SD. Statistical association were analyzed with the help of chi square test and Fisher's exact test whichever was applicable.

3. Results

3.1. Demographic Characteristics. A total number of 223 hospitalized pediatric patients with AES were enrolled in the present study. Of which 67 patients (30%) were JE and 156 patients (70%) were non-JE. The JE cases were confirmed following detection of JEV specific IgM antibody either in CSF or serum. All the samples were found to be negative for the presence of IgM antibody against other flaviviruses, namely, Dengue and West Nile prevalent in this region. Among the JE positive patients 18 were diagnosed by only serum testing positive for anti-JEV IgM antibodies and 4 were identified following detection of anti-JEV IgM antibodies in CSF only. In 45 AES patients both serum and CSF were positive for JEV specific IgM antibody. Among the JE positive cases 32 (47.6%) were male and 35 (52.2%) were female. The predominant age group affected was 5 to 12 years (Table 1) and the youngest child affected was 5 months old. Majority of the patients (90%) were from the rural area and belonged to low socioeconomic group (63%). Most of the children (80.5%) were not vaccinated against JE. Vaccination status of 7.5% children was not known. However, only (11.9%) of the care giver could confirm that their children were vaccinated against JE by SA-14-14-2 during 2006–2010 mass vaccination campaign in Assam.

3.2. Clinical Profile. The clinical profile of JE positive patients was presented in Table 2. Patients with JE were presenting vivid signs of AES. The most common presenting symptoms recorded were moderate to high grade fever (100%), altered sensorium (83.58%), seizures (82.08%), headache (41.79%), and vomiting (29.85%). Signs of meningeal irritation were present in 55.22% of cases. Around 40.29% of JE patients had Glasgow comma scale (GCS) within 3 to 8. All the JE patients were presented to the hospital between 1 to 12 days from the onset of illness. Only one patient was admitted on 31st day from the onset.

3.3. Laboratory Parameters. The CSF WBC counts of the 67 patients ranged from 2.0/mm^3 to 520.0/mm^3 (42.63 ± 82.11). Elevated levels of WBC (>5/mm^3) were found in 47 (77%) patients and predominantly lymphocytic in nature. The mean CSF protein and glucose level were 57.0 ± 27.2 mg/dL and 45.6 ± 12.4 mg/dL, respectively. Of these 32 (52.5%) had elevated (>40 mg/dL) level of protein.

3.4. Outcome of JE Patients. Outcome at discharge was recorded for 61/67 patients. The outcome of 6 (8.9%) patients could not be observed as they left the hospital against medical advice. Among the available 61 confirmed JE patients, 39 (63.9%) were recovered completely, while 13 (21.3%) cases had neurological sequelae at the time of discharge. 9 (14.7%) patient died in the hospital (Table 3).

The average duration of illness prior to the admission was 5.4 days. Fatality rate was more (17.95%) in children admitted less than 7 days from the onset of illness. However, it was not found to be statistically significant. The mortality

Table 1: The demographic profile of JE patients.

Parameter	Number of patients ($n = 67$)	Percentage (%)
Age in years		
<1	2	2.98
1 to 5	21	31.34
5 to 12	44	65.67
Sex		
Male	32	47.76
Female	35	52.24
Settings		
Urban	7	10.44
Rural	60	89.56

Table 2: Clinical profile of children with JE ($n = 67$).

Features	Number	Percentage (%)
Fever	67	100
Altered sensorium	56	83.58
Headache	28	41.79
Irritable	3	4.47
Vomiting	20	29.85
Abnormal behavior	2	2.98
Diarrhoea	3	4.47
Seizure	55	82.08
Glasgow coma scale (GCS) ≤ 8	27	40.29
Signs of meningeal irritation	37	55.22

Table 3: Outcome of children with JE at the time of discharge.

Outcome	JE patients* ($n = 61$)	Percentage (%)
Recovered completely	39	63.9
Recovered with neurological sequelae	13	21.3
Death	9	14.7

*JE patient who left the health facility against medical advice was not included in outcome analysis.

was significantly more in patients with GCS between 3 to 8 (26.92% $P < 0.05$). Presences of meningeal signs were not found to be associated with fatal outcome. Similarly, no significant association was observed between high cell counts, elevated level of protein in CSF, and children fatality (Table 4).

4. Discussion

The present study demonstrates that JE is one of the leading forms of viral encephalitis of children in this part of the country. Around 30% of hospitalized children with AES were diagnosed as confirmed JE. Similar study carried out in Cuddalore district, Tamil Nadu also reported 29.3% patients with JE in hospitalized AES children [23]. In our study, children mostly affected were from rural areas (90%) and belong to low socioeconomic group (63%). This correlated well with the earlier studies where the patients were children of farmers or farm laborers of low socioeconomic group residing in rural areas [1, 24]. This may be due to favorable epidemiological factors like presence of water logged paddy field supporting profuse breeding of vector mosquitoes, piggeries in close proximity to residence, nonuse of bed nets and outdoor playing habits of children.

The age group mainly affected was 5 to 12 years and the youngest one was 5 months old. Majority of the affected children were not vaccinated (80.5%). Study of vaccination status of the affected children revealed some striking findings. Currently in Assam, JE vaccination with live vaccine (SA-14-14-2) has been included in routine immunization programme as per National Immunization Schedule (NIS). Previously to clear the backlog in children 1–15 years of age mass vaccination programme was conducted in 11 JE endemic districts of Assam in a phasewise manner since May 2006. However, it was evident from the present study that the vaccination programme could not cover the target children adequately. It has also been noticed from the cases documented in Assam Medical College Hospital registers that the prevalence of JE infection was high amongst the hospitalized AES children in 2006–2010. The highest prevalence was 49.6% in 2006, while lowest was 32.5% in 2008. In other years prevalence was fluctuated as 48.8% in 2007, 37% in 2009, and 41.7% in 2010. In contrary, JE incidences have been declining sharply in pediatric age group in Taiwan after the vaccination programme began in 1967 [25]. This emphasizes the need of quality coverage of JE mass vaccination program and consequently vaccination campaign should be evaluated for appropriate corrective measures [18]. Moreover, continuation of JE vaccination of children in routine immunization in these JE endemic districts of Assam should be a public health priority.

Among the clinical presentation, fever, altered sensorium, seizures, headache, and vomiting was the most common symptoms observed in this study. In children similar manifestation was also noted in earlier studies [25, 26]. Signs of meningeal irritation were frequently observed in more than half of the study patients as recorded in other studies [1, 27].

Elevated cell count (>5 cell/mm^3) in CSF was noted in 77% of patients with lymphocytic predominance and elevated CSF protein level (>40 mg/dL) was recorded in 52.5% of study children. However, in a study by Avabratha et al. observed elevated cell count in 45.06% and protein in 74.67% study patients [26].

In our study, 21.13% JE patients had neurological sequelae at the time of discharge, while 14.7% had died in hospital. Mortality was associated with GCS within 3 to 8. Neurological sequelae in JE are the common observation [26, 28]. In a study of certain prognostic features in 49 patients of JE in Thailand only deep coma was found to correlate with mortality which is in conformity to the present study. Similar association was also noted in other different studies [26, 29–31]. We could not establish any association of mortality with the meningeal signs and elevated level of CSF cell count and CSF protein. In contrary to this, the study conducted by Avabratha et al. in Bellary, Karnataka, revealed association between mortality and meningeal signs [26]. This may be mentioned here that our observation is only from a small number of patients who

Table 4: Association of independent variables and outcome of children with JE.

Symptoms/variables	Total patients ($n = 61$)	Fatal	Percentage (%)	Significance
(1) Age(years)				
<1	2	0	0	NS
1 to 5	17	2	11.76	
5 to 12	42	7	16.66	
(2) Gender				
Male	29	4	13.79	NS
Female	32	5	15.63	
(3) Duration of illness prior to admission				
<7 days	39	7	17.95	NS
≥7 days	13	2	15.38	
(4) GCS				
3 to 8	26	7	26.92	S
(5) Meningeal signs	33	6	18.18	NS
(6) CSF cell count, cell/mm^3 >5 cells/mm^3	47	5	10.64	NS
(7) CSF protein concentration >40 mg/dL	32	4	12.5	NS

NS: Not significant, S: Significant at 95% level.

died in hospital and it is an ongoing study. In future with more number of patients we may be able to shed some more light on mortality and its association with meningeal signs.

In conclusion, JE is still a major cause of AES in children in this part of India. The most common clinical presentations were fever, altered sensorium, seizure, headache, vomiting and signs of meningeal irritation. The case fatality rate was recorded as high as 14.7% due to JE in children admitted with AES. These significant research findings seek the attentions of the global community to combat the menace of this arboviral encephalitis in saving the life of children.

Disclosure

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Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

The Usefulness of Clinical-Practice-Based Laboratory Data in Facilitating the Diagnosis of Dengue Illness

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Alertness to dengue and making a timely diagnosis is extremely important in the treatment of dengue and containment of dengue epidemics. We evaluated the complementary role of clinical-practice-based laboratory data in facilitating suspicion/diagnosis of dengue. One hundred overall dengue (57 dengue fever [DF] and 43 dengue hemorrhagic fever [DHF]) cases and another 100 nondengue cases (78 viral infections other than dengue, 6 bacterial sepsis, and 16 miscellaneous diseases) were analyzed. We separately compared individual laboratory variables (platelet count [PC] , prothrombin time [PT], activated partial thromboplastin time [APTT], alanine aminotransferase [ALT], and aspartate aminotransferase [AST]) and varied combined variables of DF and/or DHF cases with the corresponding ones of nondengue cases. The sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) in the diagnosis of DF and/or DHF were measured based on these laboratory variables. While trade-off between sensitivity and specificity, and/or suboptimal PPV/NPV was found at measurements using these variables, prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L had a favorable sensitivity, specificity, PPV, and NPV in diagnosis of DF and/or DHF. In conclusion, these data suggested that prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L is useful in evaluating the likelihood of DF and/or DHF.

1. Introduction

Dengue is a major medical and public health problem in tropical and subtropical regions. It is estimated that more than 2.5 billion people are living in geographic locales where dengue is endemic, and 50–100 million people have been annually infected by dengue virus (DENV) [1]. The spectrum of clinical manifestations of dengue ranges from a mild-form nonspecific febrile illness, classic dengue fever (DF), to the severe-form dengue hemorrhagic fever (DHF) [2, 3]. DHF is characterized by the presence of hemorrhagia, thrombocytopenia ($<100 \times 10^9$ cells/L), and clinical evidence of plasma leak resulting from increased vascular permeability [2, 3]. Based on the World Health Organization (WHO) criteria, the severity of DHF was categorized into grades I–IV as follows. DHF grade I is manifested by fever accompanied by nonspecific constitutional symptoms, with a positive tourniquet test result; DHF grade II is the appearance of spontaneous bleeding in addition to constitutional symptoms; DHF grade III is circulatory failure with signs of rapid and weak pulse, narrowing of pulse pressure or hypotension, and the presence of cold clammy skin; and DHF grade IV is profound shock with undetectable blood pressure and pulse [4]. Grades III and IV are grouped as dengue shock syndrome (DSS) [4]. The definitive diagnosis of dengue illness is made by positive result(s) of serology testing [5], and these serology tests are unfortunately not always readily available at most clinical laboratories. As a result, the clinically mild-form dengue has been inevitably underreported [6, 7], and it is uncommon that clinicians fail to make a timely detection of the early stage of

a dengue before it evolves into an overt clinical severe-form DHF. Clinicians inexperienced with dengue may not be alert to this infection entity, and this is especially true for clinicians in a nondengue endemic setting. Once DSS developed, the mortality rate in the affected patients might soar to as high as 20% [8]. It is not uncommon that dengue outbreaks are recognized only when hundreds of people are affected [7], making containment of dengue epidemics difficult and challenging. The importance of a timely diagnosis of dengue illness cannot be overemphasized.

Dengue epidemic was once absent in Taiwan after 1942 [9, 10]. It was not until the 1980s that a number of dengue epidemics reemerged, and of them, two remarkably large ones occurred in 1988 and 2002 in the southern part of this island [9, 10]. The rest were sporadic dengue clusters, and there was a small number of silent dengue transmissions between some of these dengue clusters [11, 12]. Owing to the absence of large-scale dengue epidemics like those annually found in southeastern Asian countries [2], most clinicians in Taiwan are not experienced with dengue illness.

In 2002 a dengue epidemic due to dengue virus serotype 2 (DENV-2) developed in southern Taiwan in which more than 5000 symptomatic cases were found and most of the affected patients were adults [10, 13], and thanks to the convenience of medical access in Taiwan, a large number of febrile patients presented to Emergency Services of Kaohsiung Chang Gung Memorial Hospital (KSCGMH) seeking medical help because of their concern for possible dengue illness. KSCGMH is a 2500-bed medical facility serving as a primary care and tertiary referral centre in this area. Complying with the law, clinicians notified Center for Disease Control (CDC, Taiwan) of patients with suspected DF/DHF and sampled patients' blood specimens for the central laboratory of CDC for serologic confirmation of dengue. Of note, blood specimens of a substantial number of suspicious dengue patients were also subject to clinical-practice-based laboratory investigations/tests such as bacterial culture, complete blood count (CBC), coagulation tests, and blood chemistry analysis, as the diagnoses of dengue were doubtful and further testing to exclude other diseases was therefore needed. To elucidate whether the clinical-practice-based laboratory data play a complementary role in facilitating the diagnosis of DF/DHF, the aims of this study were to estimate the sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of the individual data and varied combinations of each of these data retrieved from hemogram (i.e., peripheral white cell count [WBC], platelet count [PC]), coagulation profile (i.e., prothrombin time [PT], activated partial thromboplastin time [APTT]), and blood chemistry (i.e., alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) in the diagnosis of DF/DHF. The above-mentioned data were selected for analyses, because with the exception of PT, they were frequently found to be abnormal in dengue-affected patients [10, 13–17]. The results of this study provide valuable information that potentially helps build up a justified suspicious index for DF/DHF and/or facilitates the diagnosis of DF/DHF before the results of serological tests for dengue are available.

2. Patients and Methods

Potentially eligible included patients who were adults aged ≧18 years with a tentative clinical diagnosis of DF/DHF treated at Emergency Services or during admission at KSCGMH between July and November 2002. After excluding those with missing data, a total of 200 patients were eventually included for analyses. Half of the included patients who were subsequently proven to be serologically dengue-positive were allocated as the overall dengue cases, while another half who were serologically dengue-negative were allocated as the nondengue cases. A definitive diagnosis of dengue was made when at least one of the following serologic test results was found: (i) a positive reverse-transcriptase polymerase detection of DENV, (ii) a positive enzyme-linked immunosorbent assay result for specific immunoglobulin M antibody for DENV in acute-phase serum, and (iii) a four-fold or higher increase in dengue-specific hemagglutination inhibition titers in convalescent serum as compared to that in acute-phase serum [13–16]. The serologic tests for DENV infection were performed by CDC, Taiwan. Patients of the overall dengue cases were separated into those with DF (DF cases) and those with DHF (DHF cases) for further analyses [4].

A retrospective chart review was carried out for collection of demographic, clinical, and clinical-practice-based laboratory information of the included patients. The retrieved WBC, PC, PT, APTT, ALT, and AST were assayed from the specimens sampled from the affected patients when the tentative diagnosis of DF/DHF was made. Each of these data was regarded as an individual variable. Each individual variable and varied combined variables of the overall dengue cases, DF cases, and DHF cases were separately compared to the corresponding ones of nondengue cases. Individual components in combined variables were results of assays of blood specimens sampled on the same day. Because a large number of variables were derived from combination of individual variables and because each of these combined variables were regarded as one individual variable in analysis, to make the complexity more legible, combined variable A and variable B were expressed as a variable A + B and so on. The Chi-squared test or Fisher exact test was used for comparison of dichotomous variables, while the Student's t-test or Mann-Whitney U test was used for comparison of continuous variables. A 2-tailed P was considered statistically significant.

To assess the values of the aforementioned data in facilitating the suspicion/diagnoses of dengue in general and DF/DHF in particular, we calculated the sensitivity, specificity, accuracy, PPV, and NPV based on these individual laboratory variables and varied combinations of each of them [18]. Because of the trade-off between the measurements of sensitivity and specificity, and because of variables with high accuracy suggesting the coexistence of a potentially acceptable sensitivity and specificity, individual laboratory variables and combined variables with a calculated accuracy >0.80 in the diagnosis and DF and/or DHF would be further examined with the receiver-operating-characteristic (ROC) curve analysis plotting sensitivity against 1-specificity

Table 1: Demographic and clinical information of the included patients.

Variable	Overall dengue cases (A) ($N = 100$)	P (A versus D)	Dengue fever cases (B) ($N = 57$)	P (B versus D)	Dengue hemorrhagic cases (C) ($N = 43$)	P (C versus D)	Nondengue cases[1] (D) ($N = 100$)
Demographics							
Age, yr		0.375		0.669		0.279	
Mean (±SD)	46.1 ± 11.5		45.1 ± 12.3		41.2 ± 10.5		44.9 ± 17.6
Median (range)	49 (18–68)		47 (18–68)		49 (18–63)		43 (18–81)
Male gender, no. (%)	44 (44)	0.667	22 (38.6)	>0.99	22 (51.2)	0.270	40 (40)
Underlying condition,[2] no. (%)							
Hypertension	13 (13)	>0.99	6 (10.5)	0.801	7 (16.3)	0.607	13 (13)
Diabetes mellitus	14 (14)	0.834	5 (8.8)	0.603	9 (20.9)	0.199	12 (12)
Old stroke	0	0.121	0	0.297	0	0.316	4 (4)
Chronic kidney disease	0	0.121	0	0.297	0	0.316	4 (4)
Solid tumor	0	0.246	0	0.554	0	0.554	3 (3)
Symptom/sign,[3] no. (%)							
Fever	96 (96)	0.251	57 (100)	0.027	39 (90.7)	>0.99	91 (91)
Bone pain	55 (55)	0.007	33 (57.9)	0.007	22 (51.2)	0.093	35 (35)
Retroorbital pain	8 (8)	>0.99	6 (10.5)	0.782	2 (4.7)	0.505	9 (9)
Arthralgia	10 (10)	0.033	6 (10.5)	0.027	4 (9.3)	0.067	2 (2)
Abdominal pain	40 (40)	0.1	20 (35.1)	0.373	20 (46.5)	0.036	28 (28)
Cough	31 (31)	0.342	16 (28.1)	0.574	15 (34.9)	0.220	24 (24)
Diarrhea	22 (22)	0.197	11 (19.3)	0.497	11 (25.6)	0.148	14 (14)
Nausea/vomiting	36 (36)	0.042	19 (33.3)	0.134	17 (39.5)	0.041	22 (22)
Rash	34 (34)	0.005	24 (42.1)	0.001	10 (23.3)	0.347	16 (16)
Myalgia	15 (15)	<0.001	10 (17.5)	<0.001	5 (11.6)	<0.001	47 (47)
Petechiae	44 (44)	<0.001	19 (33.3)	<0.001	25 (58.1)	<0.001	9 (9)
Gum bleeding	26 (26)	<0.001	13 (22.8)	<0.001	13 (30.2)	<0.001	0
Gastrointestinal bleeding	20 (20)	<0.001	7 (12.3)	0.001	13 (30.2)	<0.001	0

[1] Including viral infections other than dengue illness (78 cases), bacterial sepsis (6 cases), gastrointestinal bleeding with/without liver cirrhosis (4 cases), urinary tract infection (2 cases), and aplastic anemia, colitis, acute hepatitis, acute pancreatitis, hepatocellular carcinoma, biliary tract infection, aseptic meningitis, systemic lupus erythematosus, acute tonsillitis, and pneumonia (each 1 case).
[2] An individual patient might have more than one underlying condition.
[3] An individual patient might have more than one symptom/sign.

[19, 20]. The area under the curve (AUC) with its 95% confidence interval of each separately constructed ROC curve was measured using SPSS 15 software for Windows (SPSS Inc., Chicago, Ill) to obtain the predictive accuracy of these clinical-practice-based laboratory data in the diagnosis of DF and/or DHF. AUC between 0.90 and 1 was considered excellent, between 0.80 and 0.90 good, between 0.70 and 0.80 fair, between 0.60 and 0.70 poor, and between 0.50 and 0.60 fail [20].

3. Results

3.1. Demographic, Clinical, and Laboratory Information of Patients in Dengue and Nondengue Cases. The overall dengue case and nondengue case groups each included 100 patients, and the former was made up of 57 DF cases and 43 DHF cases. Similar demographics but a number of significant differences in clinical manifestations (Table 1) and clinical-practice-based laboratory data (Table 2) were found when the overall dengue cases, DF cases, and DHF cases were separately compared with the nondengue cases.

3.2. Sensitivity, Specificity, PPV, NPV, and Accuracy of Laboratory Data in the Diagnoses of the Overall Dengue, DF, and DHF. The sensitivity, specificity, PPV, NPV and accuracy calculated from individual laboratory variables and varied combinations of them in the diagnosis of DF and/or DHF are summarized in Table 3.

Table 2: Laboratory data of the included patients.

Variable	Overall dengue cases (A) ($N = 100$)	P (A versus D)	Dengue fever cases (B) ($N = 57$)	P (B versus D)	Dengue hemorrhagic cases (C) ($N = 43$)	P (C versus D)	Nondengue cases[1] (D) ($N = 100$)
Leukopenia (WBC $< 3.0 \times 10^9$ cells/L), no. (%)	49 (40)	0.001	31 (54.4)	<0.001	18 (41.9)	0.049	25 (25)
Platelet count < 150.0 ($\times 10^9$ cells/L), no. (%)	100 (100)	<0.001	57 (100)	<0.001	43 (100)	<0.001	71 (71)
Platelet count < 100.0 ($\times 10^9$ cells/L), no. (%)	97 (97)	<0.001	54 (94.7)	<0.001	43 (100)	<0.001	30 (30)
Prolonged APTT,[2] n/N (%)	61/68 (89.7)	0.001	33/36 (91.7)	0.006	28/32 (87.5)	0.030	26/41 (63.4)
Prolonged PT,[3] n/N (%)	1/68 (1.5)	0.023	0/37	0.055	1/31 (3.2)	0.217	5/39 (12.8)
AST > 40 U/L (normal value, < 40 U/L), n/N (%)	68/79 (86.1)	<0.001	34/44 (77.3)	<0.001	34/35 (97.1)	<0.001	27/64 (42.2)
ALT > 40 U/L (normal value, < 40 U/L), n/N (%)	49/65 (75.4)	<0.001	24/37 (64.9)	0.006	25/28 (89.3)	<0.001	19/55 (34.5)

Abbreviations: APTT: activated partial thromboplastin time; PT: prothrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase; n/N: no. of patients/no. of patients with data available.
[1]See footnote in Table 1 for details.
[2]Prolonged APTT was defined as an increased APTT value > 20% of the control value.
[3]Prolonged PT was defined as an increased PT value > 3 seconds than that of control.

In the diagnosis of overall dengue, variables with a high sensitivity, in decreasing order, were PC $< 150 \times 10^9$ cells/L (100%), PC $< 100 \times 10^9$ cells/L (97%), prolonged APTT (89.7%), prolonged APTT + PC $< 150 \times 10^9$ cells/L (89.6%), prolonged APTT + normal PT (89.4%), prolonged APTT + normal PT + PC $< 150 \times 10^9$ cells/L (89.4%), prolonged APTT + PC $< 100 \times 10^9$ cells/L (88.6%), and prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L (87.9%). The specificity of PC $< 150 \times 10^9$ cells/L and PC $< 100 \times 10^9$ cells/L was 48.8% and 70%, respectively. Prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L had an accuracy of 84.6% (sensitivity 87.9%, specificity 78.9%, PPV 87.9%, and NPV 78.9%), while PC $< 100 \times 10^9$ cells/L had an accuracy of 83.5% (sensitivity 97.0%, specificity 70.0%, PPV 76.4%, and NPV 95.9%).

In the diagnosis of DF, variables with a high sensitivity, in decreasing order, were PC $< 150 \times 10^9$ cells/L (100%), PC $< 100 \times 10^9$ cells/L (94.7%), prolonged APTT + normal PT + PC $< 150 \times 10^9$ cells/L (91.7%), prolonged APTT (91.7%), prolonged APTT + normal PT (91.7%), prolonged APTT + PC $< 100 \times 10^9$ cells/L (88.9%), and prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L (88.9%). Among them, prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L had an accuracy of 83.8% (specificity 78.9%, PPV 80%, and NPV 88.2%). The specificity of PC $< 150 \times 10^9$ cells/L and PC $< 100 \times 10^9$ cells/L was 29.0% and 70%, respectively.

In the diagnosis of DHF, variables with high sensitivity, in decreasing order, were PC $< 150 \times 10^9$ cells/L (100%), PC $< 100 \times 10^9$ cells/L (100%), AST > 40 U/L (97.1%), PC $< 150 \times 10^9$ cells/L + AST > 40 U/L (97.1%), PC $< 100 \times 10^9$ cells/L + AST > 40 U/L (97.1%), ALT > 40 U/L + AST > 40 U/L (92.6%), PC $< 150 \times 10^9$ cells/L + ALT > 40 U/L + AST > 40 U/L (92.6%), and PC $< 100 \times 10^9$ cells/L + ALT > 40 U/L + AST > 40 U/L (92.6%). The specificity of PC $< 150 \times 10^9$ cells/L and PC$<100 \times 10^9$ cells/L was 29% and 70%, respectively. Variables with high accuracy (>80.0%) were PC $< 100 \times 10^9$ cells/L + ALT > 40 U/L + AST > 40 U/L (sensitivity 92.6%, specificity 76.6%, PPV 69.4%, and NPV 94.7%), prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L (sensitivity 86.7%, specificity 78.9%, PPV 76.5%, and NPV 88.2%), prolonged APTT + normal PT + AST > 40 U/L (sensitivity 88.5%, specificity 75%, PPV 76.7%, and NPV 87.5%), prolonged APTT + normal PT + ALT > 40 U/L (sensitivity 81%, specificity 81.5%, PPV 77.3%, and NPV 84.6%), and PC $< 100 \times 10^9$ cells/L + ALT > 40 U/L (sensitivity 89.3%, specificity 75.5%, PPV 65.8%, and NPV 93%).

3.3. Sensitivity, Specificity, PPV, NPV, Accuracy, and AUC of Laboratory Variables Included in ROC Curve Analysis. PC $< 100 \times 10^9$ cells/L, prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L, PC $< 100 \times 10^9$ cells/L + ALT > 40 U/L, PC $< 100 \times 10^9$ cells/L + ALT > 40 U/L + AST > 40 U/L, prolonged APTT + normal PT + AST > 40 U/L, and prolonged APTT + normal PT + ALT > 40 U/L were included for ROC analysis. AUC, along with the sensitivity, specificity, PPV, NPV, and accuracy in the diagnosis of DF and/or DHF is summarized in Table 4. PC $< 100 \times 10^9$ cells/L and prolonged APTT + normal PT + PC $< 100 \times 10^9$ cells/L each had a good predictive accuracy

Table 3: Sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of laboratory data in the diagnoses of overall dengue, DF, and DHF[1].

Variable[2]	Overall dengue cases, n/N (%)	DF cases, n/N (%)	DHF cases, n/N (%)	Nondengue cases, n/N[3] (%)
Prolonged APTT	61/68 (89.7)[4]	33/36 (91.7)[4,7]	28/32 (87.5)[4]	26/41 (63.4)
Leukopenia	49/100 (49.0)	31/57 (54.4)	18/43 (41.9)	25/100 (25.0)
Platelet $< 150 \times 10^9$ cells/L	100/100 (100)[4,7]	57/57 (100)[4,7]	43/43 (100)[4,7]	71/100 (71.0)
ALT > 40 U/L	49/65 (75.4)	24/37 (64.9)	25/28 (89.3)[4,7]	19/55 (34.5)
AST > 40 U/L	68/79 (86.0)[4]	34/44 (77.3)	34/35 (97)[4,7]	27/64 (42.3)
Prolonged APTT + leukopenia	32/68 (47.1)	18/36 (50.0)	14/32 (43.8)	14/41 (34.1)
Prolonged APTT + platelet $< 150 \times 10^9$ cells/L	60/67 (89.6)[4]	32/35 (91.4)[4]	28/32 (87.5)[4,7]	21/41 (51.2)
Prolonged APTT + ALT > 40 U/L	35/50 (70.0)	18/28 (64.3)	17/22 (77.3)[7]	9/30 (30.0)
Prolonged APTT + AST > 40 U/L	50/60 (83.3)[4,6]	26/32 (81.3)[4]	24/28 (85.7)[4,7]	12/31 (38.7)
Prolonged APTT + leukopenia + platelet $< 150 \times 10^9$ cells/L	32/68 (47.0)	18/36 (50.0)	14/32 (43.8)	12/41 (29.3)
Prolonged APTT + leukopenia + ALT > 40 U/L	18/50 (36.0)[5]	8/28 (28.6)[5]	10/22 (45.5)[5]	5/30 (16.7)
Prolonged APTT + leukopenia + AST > 40 U/L	27/60 (45.0)[5,6]	15/32 (46.9)[5]	12/28 (42.9)[5]	5/31 (16.1)
Prolonged APTT + leukopenia + platelet $< 150 \times 10^9$ cells/L + ALT > 40 U/L	18/50 (36.0)[5]	8/28 (28.6)[5]	10/22 (45.6)[5]	5/30 (16.7)
Prolonged APTT + leukopenia + platelet $< 150 \times 10^9$ cells/L + AST > 40 U/L	27/60 (45.0)[5,6]	15/32 (46.9)[5]	12/28 (42.9)[5]	5/31 (16.1)
Prolonged APTT + leukopenia + platelet $< 150 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	15/52 (28.8)[5]	5/31 (16.1)[5]	10/21 (47.6)[5]	4/29 (13.8)
Leukopenia + platelet $< 150 \times 10^9$ cells/L	49/100 (49.0)	31/57 (54.4)	18/43 (41.9)	23/100 (23.0)
Leukopenia + ALT > 40 U/L	22/65 (33.8)[5]	11/37 (29.7)[5]	11/28 (39.3)[5]	7/55 (12.7)
Leukopenia + AST > 40 U/L	32/79 (40.5)[5]	18/44 (40.9)[5]	14/35 (40.0)[5]	9/64 (14.1)
Leukopenia + platelet $< 150 \times 10^9$ cells/L + ALT > 40 U/L	22/65 (33.8)[5]	11/37 (29.7)[5]	11/28 (39.3)[5]	7/55 (12.7)
Leukopenia + platelet $< 150 \times 10^9$ cells/L + AST > 40 U/L	32/78 (41.0)[5]	18/44 (40.9)[5]	14/34 (41.2)[5]	9/64 (14.1)
Leukopenia + platelet $< 150 \times 10^9$ cells/L + ALT > 40 U/L + AST > 40 U/L	21/60 (35.0)[5]	10/33 (30.3)[5]	11/27 (40.7)[5]	6/47 (12.8)
Platelet $< 150 \times 10^9$ cells/L + ALT > 40 U/L	49/65 (75.4)	24/37 (64.9)	25/28 (89.3)[4,7]	17/55 (30.9)
Platelet $< 150 \times 10^9$ cells/L + AST > 40 U/L	67/78 (85.9)[4]	33/43 (76.7)	34/35 (97.1)[4,7]	25/64 (39.1)
Platelet $< 150 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	46/59 (77.9)	21/32 (65.6)	25/27 (92.6)[4,7]	13/47 (27.7)
ALT > 40 U/L + AST > 40 U/L	48/60 (80.0)	23/33 (69.7)	25/27 (92.6)[4,7]	14/47 (29.8)
Prolonged APTT + normal PT	59/66 (89.4)[4]	33/36 (91.7)[4,7]	26/30 (86.7)[4,7]	19/38 (50.0)
Prolonged APTT + normal PT + leukopenia	30/66 (45.6)	18/36 (50)	12/30 (40.0)	12/38 (31.6)
Prolonged APTT + normal PT + platelet $< 150 \times 10^9$ cells/L	59/66 (89.4)[4,6]	33/36 (91.7)[4,7]	26/30 (86.7)[4,7]	14/38 (36.8)
Prolonged APTT + normal PT + ALT > 40 U/L	35/49 (71.4)[5,6]	18/28 (64.3)[5]	17/21 (80.9)[4,5,8]	5/27 (18.5)

Table 3: Continued.

Variable[2]	Overall dengue cases, n/N (%)	DF cases, n/N (%)	DHF cases, n/N (%)	Nondengue cases, n/N[3] (%)
Prolonged APTT + normal PT + ALT > 40 U/L	49/58 (84.5)[4,6,8]	26/32 (81.3)[4]	23/26 (88.5)[4,7,8]	7/28 (25.0)
Prolonged APTT + normal PT + leukopenia + platelet $< 150 \times 10^9$ cells/L	30/66 (45.5)	18/36 (50)	12/30 (40)	10/38 (26.3)
Prolonged APTT + normal PT + leukopenia + ALT > 40 U/L	18/49 (36.7)[5,6]	8/28 (28.6)[5]	10/21 (47.6)[5]	3/27 (11.1)
Prolonged APTT + normal PT + leukopenia + AST > 40 U/L	26/58 (44.8)[5,6]	15/32 (46.9)[5]	11/26 (42.3)[5]	4/28 (14.3)
Prolonged APTT + normal PT + leukopenia + platelet $< 150 \times 10^9$cells/L + ALT > 40 U/L	18/49 (36.7)[5,6]	8/28 (28.6)[5]	10/21 (47.6)[5]	3/27 (11.1)
Prolonged APTT + normal PT + leukopenia + platelet $< 150 \times 10^9$cells/L + AST > 40 U/L	26/57 (45.6)[5,6]	15/32 (46.9)[5]	11/25 (44.0)[5]	4/28 (14.3)
Prolonged APTT + normal PT + leukopenia + platelet $< 150 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	18/46 (39.1)[5,6]	8/26 (30.8)[5]	10/20 (50.0)[5]	3/26 (11.5)
Platelet $< 100 \times 10^9$ cells/L	97/100 (97)[4,7,8]	54/57 (94.7)[4,7]	43/43 (100)[4,7]	30/100 (30.0)
Prolonged APTT + platelet $< 100 \times 10^9$ cells/L	60/68 (88.2)[4,6]	32/36 (88.9)[4,7]	28/32 (87.5)[4,7]	14/41 (34.1)
Prolonged APTT + leukopenia + platelet $< 100 \times 10^9$ cells/L	31/68 (45.6)[5]	17/36 (47.2)[5]	14/32 (43.8)[5]	8/41 (19.5)
Prolonged APTT + leukopenia + platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L	17/50 (34.0)[5]	7/28 (25)[5]	10/22 (45.5)[5]	5/30 (16.7)
Prolonged APTT + leukopenia + platelet $< 100 \times 10^9$cells/L + AST > 40 U/L	26/60 (43.3)[5,6]	14/32 (43.8)[5]	12/28 (42.9)[5]	5/31 (16.1)
Prolonged APTT + leukopenia + platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	17/47 (36.2)[5,6]	7/26 (26.9)[5]	10/21 (47.6) [5]	4/29 (13.8)
Leukopenia + platelet $< 100 \times 10^9$ cells/L	47/100 (47)[5]	29/57 (50.9)[5]	18/43 (41.9) [5]	14/100 (14.0)
Leukopenia + platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L	21/65 (32.3)[5]	10/37 (27)[5]	11/28 (39.3)[5]	7/55 (12.7)
Leukopenia + platelet $< 100 \times 10^9$ cells/L + AST > 40 U/L	31/78 (39.7)[5]	17/44 (38.6)[5]	14/34 (41.2)[5]	9/64 (14.1)
Leukopenia + platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	20/60 (33.4)[5]	9/33 (27.3)[5]	11/27 (40.7)[5]	6/47 (12.8)
Platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L	48/65 (73.8)	23/37 (62.2)	25/28 (89.3)[4,7,8]	13/53 (24.5)
Platelet $< 100 \times 10^9$cells/L + AST > 40 U/L	67/79 (84.8)[4]	33/44 (75)[7]	34/35 (97.1)[4,7]	19/64 (29.7)
Platelet $< 100 \times 10^9$cells/L + ALT > 40 U/L + AST > 40 U/L	46/60 (76.7)[6]	21/33 (63.6)	25/27 (92.6)[4,7,8]	11/47 (23.4)

Table 3: Continued.

Variable[2]	Overall dengue cases, n/N (%)	DF cases, n/N (%)	DHF cases, n/N (%)	Nondengue cases, n/N[3] (%)
Prolonged APTT + normal PT + platelet < 100 × 10^9 cells/L	58/66 (87.9)[4,6,8]	32/36 (88.9)[4,6,7,8]	26/30 (86.7)[4,7,8]	8/38 (21.1)
Prolonged APTT + normal PT + leukopenia + platelet < 100 × 10^9 cells/L	29/66 (43.9)[5,6]	17/36 (47.2)[5]	12/30 (40.0)[5]	6/38 (15.8)
Prolonged APTT + normal PT + leukopenia + platelet < 100 × 10^9 cells/L + ALT > 40 U/L	17/49 (34.7)[5,6]	7/28 (25)[5]	10/21 (47.6)[5]	3/27 (11.1)
Prolonged APTT + normal PT + leukopenia + platelet < 100 × 10^9cells/L + AST > 40 U/L	25/57 (43.9)[5,6]	14/32 (43.6)[5]	11/25 (44.0)[5]	4/28 (14.3)
Prolonged APTT + normal PT + leukopenia + platelet < 100 × 10^9cells/L + ALT > 40 U/L + AST > 40 U/L	17/46 (36.9)[5,6]	7/26 (26.9)[5]	10/20 (50.0)[5]	3/26 (11.5)

Abbreviations: APTT: activated partial thromboplastin time; PT: prothrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase; n/N: no. of patients/no. of patients with data available.
[1]Sensitivity = (number of true-positives) × 100/(number of true-positives + number of false-negatives), specificity = (number of true-negatives) × 100/(number of true-negatives + number of false-positives), accuracy = (number of true-positives + number of true-negatives) × 100/(total instances), PPV = (number of true-positives) × 100/(number of true-positives + number of false-positives), and NPV = (number of true-negatives) × 100/(number of true-negatives + number of false-negatives) [18].
[2]Leukopenia was defined as peripheral white cell count < 3.0 × 10^9 cells/L, prolonged APTT as an increased APTT value > 20% of the control value, and prolonged PT as an increased PT value > 3 seconds than that of control. Variables in bold font are those that had an accuracy > 80% in the diagnosis of the DF and/or DHF and were therefore subjected to the receiver-operating-characteristic (ROC) curve analysis in which the under the curve (AUC) was measured to obtain the predictive accuracy (see Table 4 for details).
[3]See footnote in Table 1 for details.
[4]Variable with a sensitivity > 80%.
[5]Variable with a specificity > 80%.
[6]Variable with a positive predictive value > 80%.
[7]Variable with a negative predictive value > 80%.
[8]Variable with an accuracy > 80%.

Table 4: Sensitivity, specificity, PPV, NPV, accuracy, and AUC of laboratory variables included in ROC curve analysis[1].

Laboratory parameter	Overall dengue						DF						DHF					
	Sn (%)	Sp (%)	PPV (%)	NPV (%)	Ac (%)	AUC (95% CI)	Sn (%)	Sp (%)	PPV (%)	NPV (%)	Ac (%)	AUC (95% CI)	Sn (%)	Sp (%)	PPV (%)	NPV (%)	Ac (%)	AUC (95% CI)
PC < 100×10^9 cells/L	97.0	70.0	76.4	95.9	83.5	**0.835 (0.775–0.895)**	94.7	70.0	64.3	95.9	79.0	**0.824 (0.751–0.890)**	100	70	58.9	100	79.0	**0.850 (0.789–0.911)**
Prolonged APTT + normal PT + PC < 100×10^9 cells/L	87.9	78.9	87.9	78.9	84.6	**0.834 (0.746–0.923)**	88.9	78.9	80.0	88.2	83.8	**0.839 (0.742–0.936)**	86.7	78.9	76.5	88.2	82.4	**0.828 (0.724–0.932)**
PC < 100×10^9 cells/L + ALT > 40 U/L						0.747						0.699	89.3	75.5	65.8	93	80.2	**0.852 (0.761–0.943)**
PC < 100×10^9 cells/L + ALT > 40 U/L + AST > 40 U/L						0.766						0.701	92.6	76.6	69.4	94.7	82.4	**0.846 (0.752–0.939)**
Prolonged APTT + normal PT + AST > 40 U/L						0.791						0.781	88.5	75.0	76.7	87.5	81.5	**0.817 (0.698–0.937)**
Prolonged APTT + normal PT + ALT > 40 U/L						0.765						0.729	81.0	81.5	77.3	84.6	81.3	**0.812 (0.682–0.942)**

Abbreviations: Sn: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value; Ac: accuracy; AUC: area under the receiver-operator characteristic curves; DF: dengue fever; DHF: dengue hemorrhagic fever; CI: confidence interval; PC: platelet count; APTT: activated partial thromboplastin time; PT: prothrombin time; AST: aspartate aminotransferase; ALT: alanine aminotransferase.
[1]Only sensitivity, specificity, positive predictive value, and negative predictive value of variables having an accuracy with an AUC > 0.8 (in bold font) in ROC analysis are shown. Calculations of sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were based on formulae put in the footnote of Table 3.

(AUC > 0.8) in the diagnoses of the overall dengue, DF, and DHF, while the remaining variables had a good predictive accuracy only in the diagnosis of DHF.

4. Discussion

The immunopathogenesis of DF/DHF is characterized by an aberrant immune overactivation and cytokine overproduction that lead to the development of a great array of clinical and laboratory manifestations [17, 21–23]. Some of the cytokines are proinflammatory, while others are anti-inflammatory [17, 21–23]. These cytokines are capable of causing leukocytes to activate synergistically or antagonistically [17, 24], and clinical and laboratory manifestations in the dengue affected patients are the net effect of the interactions between one another among these activated cytokines [17, 21–23].

Myelosuppression in DF/DHF leads to leukopenia [24], and some of the dengue-affected patients experience prior transient neutrophilia and monocytosis before development of leukopenia [24]. DEV-2 was reported to be able to bind to human platelets in the presence of virus-specific antibodies [25]. As a result of molecular mimicry, autoantibodies produced in DF/DHF patients are capable of coating human platelets [17], and IFN-γ activates macrophages to phagocytosize the auto antibody-coated platelets, rendering thrombocytopenia [24]. During acute DENV infection, both coagulation and fibrinolysis are activated, leading to alterations in coagulation parameters (e.g., platelet count and APTT) and fibrinolytic parameters (e.g., tissue-type plasminogen [tPA] and plasminogen activator inhibitor [tAPI]). APTT prolongs as tPA increases. The activations of coagulation and fibrinolysis are much more drastic in DHF/DSS than in DF [24, 26].

An APTT prolongation and normal PT often found in DHF suggest a defect in the intrinsic pathway of coagulation, which is caused by either downregulation of the synthesis or overconsumption of specific factor(s) that are presumably produced by hepatocytes [24]. Hepatitis is usually found in the acute phase of DF/DHF [24, 27]. Data derived from the analysis of the linear correlation and regression between the levels of AST/ALT and APTT show a strong association between them, suggesting that hepatic dysfunction might be responsible for the decreased synthesis of specific factors in the coagulation intrinsic pathway [24, 28]. Increased factor consumption as indicated by the high levels of tPA is also associated with APTT prolongation [26]. Elevated liver enzymes are especially found in patients with DHF [27], and this may explain the findings that variables made up of PC < 100×10^9 cells/L and/or prolonged APTT + normal PT with an elevated AST and/or an elevated ALT had a good accuracy in the diagnosis of DHF but not in the diagnosis of the overall dengue or DF.

Given significant differences in clinical manifestations between the overall dengue/DF/DHF cases and the non-dengue cases in this series and in others [29], clinicians experienced with these infectious disease entities may not often have difficulty making the diagnosis of DF/DHF on clinical basis, especially in areas where DF/DHF is always endemic [29]. On the other hand, for inexperienced clinicians the clinical diagnosis of DF/DHF is often a big challenge. The scenarios in which clinical-based suspicion/diagnosis of DF/DHF is challenging include inexperienced clinicians' facing febrile patients in a small dengue cluster or encountering febrile travelers from dengue-endemic locales to non-dengue-endemic area. The significant differences in the daily-practice-based laboratory data between patients with DF/DHF and the nondengue cases (Table 2) suggested these individual data alone and/or in combination with other(s) potentially facilitate the suspicion/diagnosis of DF/DHF. One study from Singapore where dengue was found all year round reported that a model combining clinical feature (skin rash) and laboratory parameters (white cell count, hemoglobin, PT, creatinine, and bilirubin levels) was able to distinguish dengue illness (mainly DF) from other infections with a sensitivity of 84% and specificity of 85% [29].

While ROC plots provide a global comprehensive view of the test, sensitivity and specificity describe the test's ability to correctly distinguish between DF/DHF and nondengue patients [19]. As PC < 100×10^9 cells/L had a high sensitivity but low specificity in the diagnosis of DF and/or DHF, it may be useful in screening dengue illness when this viral infection is rarely encountered. Prolonged APTT + normal PT + PC < 100×10^9 cells/L with high sensitivity (87.9% for the overall dengue, 88.9% for DF, and 86.7% for DHF) and a comparatively high specificity of 78.9% for DF and/or DHF in this series suggest that the combined variable is especially useful in screening dengue illness during a dengue epidemic or in countries where dengue is always endemic, as under these circumstances, it is likely that clinicians tend to make a tentative diagnosis of dengue in most febrile patients lacking obvious localizing signs to suggest an alternative diagnosis [29, 30].

In the diagnosis of DHF, APTT + normal PT + PC < 100×10^9 cells/L, PC < 100×10^9 cells/L + ALT > 40 U/L, PC < 100×10^9 cells/L + ALT > 40 U/L + AST > 40 U/L, prolonged APTT + normal PT + AST > 40 U/L, and prolonged APTT + normal PT + ALT > 40 U/L were found to have a comparable sensitivity and specificity in the diagnosis of DHF. However, when facing a patient with an underlying liver dysfunction due to viral hepatitis and/or fatty liver, APTT + normal PT + PC < 100×10^9 cells/L is the variable of choice for screening DHF.

Predictive value, a calculation of the percentage of correct negative or correct positive result, is applicable once the prevalence of a disease is taken into consideration [19]. The potential roles played by individual variable/varied combined variables in the diagnoses of the overall dengue/DF/DHF are summarized in Table 4. While trade-off between sensitivity and specificity, and/or suboptimal PPV/NPV was found at measurements using other variable/varied combined variables, prolonged APTT + normal PT + PC < 100×10^9 cells/L had a favorable sensitivity, specificity, PPV, and NPV in diagnosis of DF and/or DHF. To make the applicability of these clinical-practice-based laboratory data simplified and user-friendly, we propose prolonged APTT + normal PT + PC < 100×10^9 cells/L be used for screening and evaluating the likelihood of DF and/or DHF.

The present study implies that daily-practice-based laboratory data play a complementary role in prompting the suspicion and/or facilitating the diagnosis of DF and/or DHF, which is especially important for clinicians who are inexperienced with these infectious entities. In addition to providing an appropriate therapeutic guidance, a timely suspicion and diagnosis of dengue infection may help the public health authorities launch necessary containment measures earlier, thus diminishing the amplitude of a dengue epidemic that would otherwise be a much larger one.

As DF/DHF features dynamically changing clinical and laboratory manifestations within a few days [14, 16, 31, 32], clinicians may repeatedly sample serum specimens for daily-practice-based laboratory tests if the initial ones do not disclose clear enough information for evaluation of the likelihood of DF/DHF.

Our data were obtained from adult patients during a dengue epidemic due to DENV-2 in Taiwan. Of our serologically dengue-negative patients, 78% suffered viral infections other than dengue and 6% suffered bacterial sepsis, while the rest 22% experienced miscellaneous diseases (see footnote of Table 1 for details). The entities of febrile illness in the nondengue patients might affect the measurements of sensitivity, specificity, PPV, and NPV for dengue illness using the clinical-practice-based laboratory data in our study, and this is one of the limitations that deserves attention. As clinical and laboratory manifestations in DF/DHF result from sophisticated immunologic reactions [17, 24, 33], which may vary from patients in one series to another depending on the genetics of the hosts and the culprit viruses [34, 35], additional limitations of our study must be addressed. It is uncertain whether these daily-practice-based data are applicable in facilitating diagnosis of DF/DHF in adults of other race and/or DF/DHF caused by DENV of other serotypes. Likewise, it is uncertain whether these daily-practice data are applicable in facilitating diagnosis of DF/DHF in pediatric patients. Further study is merited to clarify these important questions, as the answers potentially greatly impact medicine practice in dengue epidemics which are distributed worldwide, mainly in tropical areas where medical resources are deficient.

Ethical Approval

These data were analyzed anonymously, and the study was conducted with a waiver of patient consent approved by the Institutional Review Board of Chang Gung Memorial Hospital (Document number 99-3533B).

Conflict of Interests

The authors have declared that they have no conflict of interests.

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Research Article

Incidence of Japanese Encephalitis among Acute Encephalitis Syndrome Cases in West Bengal, India

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Background and Objectives. Japanese encephalitis (JE) is the most important cause of acute and epidemic viral encephalitis. Every year sporadic JE cases are reported from the various districts of West Bengal, indicating its endemicity in this state. JE vaccination programme has been undertaken by the State Health Department of West Bengal. This study was aimed at seeing the present scenario of JE among acute encephalitis syndrome (AES) cases in West Bengal. *Materials and Methods*. Blood and/or CSF samples were referred from suspected AES cases to the referral virology laboratory of the Calcutta School of Tropical Medicine from different hospitals of Kolkata. IgM antibody capture ELISA was performed on the CSF and serum samples by JE virus MAC ELISA kit supplied by the National Institute of Virology, Pune. *Results*. The present study reveals that 22.76% and 5% of the AES cases were positive for JE IgM in 2011 and 2012, respectively. JE is mainly prevalent in children and adolescents below 20 years of age with no gender predilection. Although the percentages of JE positive cases were high in 2011, it sharply decreased thereafter possibly due to better awareness programs, due to mass vaccination, or simply due to natural epidemiological niche periodicity due to herd immunity.

1. Introduction

The mosquito-borne Japanese encephalitis virus (JEV) is an enveloped, positive-sense single-stranded RNA virus and member of the genus *Flavivirus* under the family Flaviviridae [1]. JEV is the sole etiologic agent of Japanese Encephalitis (JE), a neurotropic killer disease being one of the major causes of viral encephalitis in human. Since the isolation of this virus in Japan in 1935 [2], it has spread worldwide becoming a major public health problem. JE is a disease of major public health importance due to its high epidemic potential, high case fatality rate (CFR), and sequelae among survivors [3].

Approximately 2 billion people live in countries where JE presents a significant risk to humans and animals, particularly in China and India, with at least 700 million potentially susceptible children [4]. In Southeast Asia around 50,000 cases and 10,000 deaths occur per year affecting essentially children below 10 years of age [5]. Further threats to humanity are there because the JE virus has shown a tendency to extend to other geographic areas. The combined effects of climate change, altered bird migratory patterns, increasing movement of humans, animals, and goods, increasing deforestation, and development of irrigation projects will also help this geographic dispersal of the virus, producing an enhanced threat. The disease is also highly prevalent in animals. In Nepal, seroprevalence of JE in pigs, ducks, and horses was 48.11%, 26.79%, and 50.0%, respectively [6]. Phylogenetic analysis showed that JE isolates in India belonged to genogroup III [7].

Although most human infections are mild or asymptomatic, about 50% of patients who develop encephalitis suffer permanent neurologic defects and 30% of them die due to the disease [8]. In 1973, JE outbreak was first recorded in the districts of Burdwan and Bankura in West Bengal where 700 cases and 300 deaths were reported [9–13].

Since 1973, epidemics of JE have occurred in West Bengal, Bihar, Uttar Pradesh, Assam, Andhra Pradesh, Tamil Nadu, and Karnataka [14]. Every year sporadic JE cases are

Table 1: Distribution of JE positive cases in 2011 and 2012.

Sex	Samples tested in 2011	Samples reactive in 2011	Samples tested in 2012	Samples reactive in 2012
Male	151	36 (23.84%)	206	10 (4.8%)
Female	95	20 (21.05%)	154	8 (5.2%)
Total	246	56 (22.76%)	360	18 (5%)

reported indicating their endemicity in this state [15]. JE vaccination programme has been undertaken by the State Health Department, Government of West Bengal.

This study was aimed to see the present scenario of JE among acute encephalitis syndrome cases in West Bengal.

2. Materials and Methods

2.1. Human Blood and or CSF Samples. Blood and/or CSF samples were referred and submitted to the referral Virology laboratory at the Calcutta School of Tropical Medicine, from 606 clinically diagnosed cases of acute encephalitis syndrome during the period from January 2011 to December 2012. Specimen collection and transportation of samples were strictly monitored. 1 mL CSF and 2–5 mL of clotted blood sample were collected as per standard procedures. The samples were transported to the virology laboratory maintaining cold chain. The CSF and serum samples were stored at 4°C in the refrigerator if tested within 3 days or minus 80 degree freezer for long-term storage.

2.2. Serological Study for JE. IgM antibody capture (MAC) ELISA was performed on the CSF and serum samples by JE virus MAC ELISA kit supplied by the National Institute of Virology, Pune, as an integral part of the National Vector Borne Disease Control Program. The samples were tested strictly following the manufacturer's protocol.

3. Results

The present study was carried out in the Virology unit of the Microbiology Department of the Calcutta School of Tropical Medicine, Kolkata, and comprised 606 clinically diagnosed cases of acute encephalitic syndrome. Among them 357 (59.92%) were males and 249 (41.08%) were females. 74 (12.21%) cases were found to be positive for JE. Table 1 shows that 23.84% and 21.05% of the JE positive cases were males and females, respectively, in 2011, whereas 4.8% and 5.2% of the JE positive cases were males and females, respectively, in 2012. In general, the differences between male and female distributions of JE positive cases were not statistically significant at a 95% level.

However, there was a remarkable change in the percentages of JE positive cases in the years 2011 and 2012 (Table 1). In males, the percentage of JE positive cases dropped from 23.84% to only 4.8% (P value is highly significant below 0.01 level); similarly in females it dropped from 21.05% to 5.2% (P value is highly significant below 0.01 level).

Figure 1 shows the distribution of the percentage of JE positive cases in the different age groups in the years 2011 and 2012. It was found that 48.21% and 61.11% of JE positive cases were below 20 years of age in 2011 and 2012, respectively.

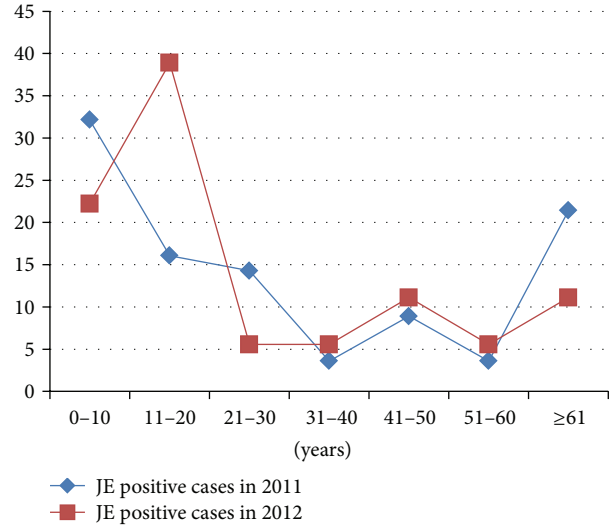

Figure 1: Percentage of JE positive cases in the various age groups, 2011-2012.

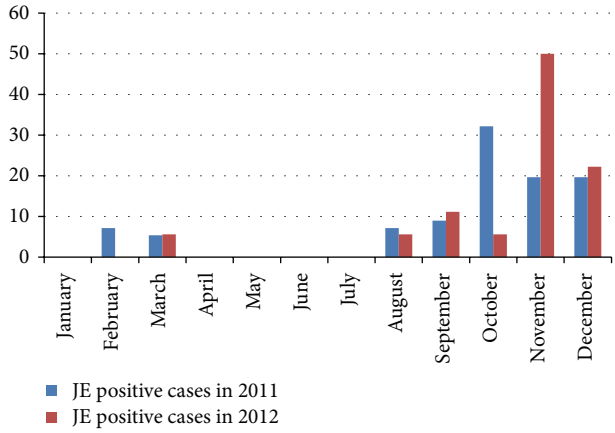

Figure 2: Monthly distribution of JE positive cases (in percentage), 2011-2012.

Figure 2 shows the monthly distribution of the JE positive cases (in percentage). It is evident that a larger number of JE cases occurred in the rainy season and after the rainy season.

Figure 3 shows that sporadic JE positive cases were reported from almost all rural districts of West Bengal. Maximum number of JE IgM positive cases occurred in Hooghly district followed by Birbhum in 2011. However, comparatively a larger number of cases were reported from

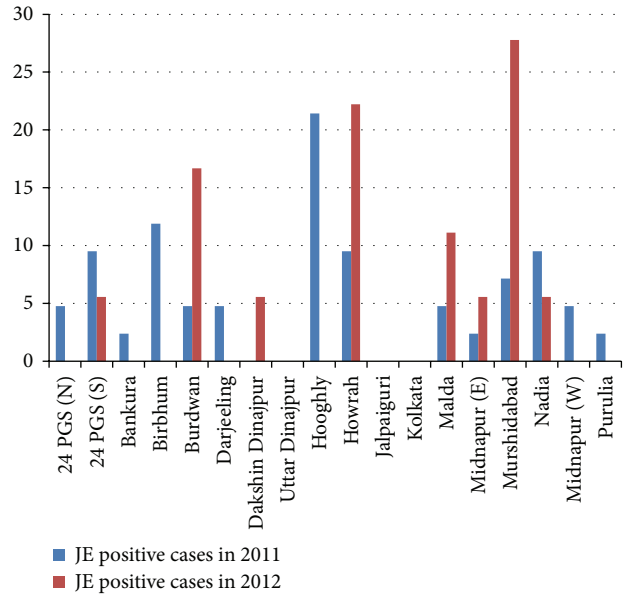

Figure 3: District wise distribution of JE positive cases (in percentage), 2011-2012.

Murshidabad, Bardhaman, and Howrah districts of West Bengal in 2012.

Out of 56 JE cases in 2011 line listing could be done in 42 cases. No address was available for 14 patients in 2011 as these cases were referred from other Medical colleges of West Bengal.

4. Discussion

Patients with high grade fever (≥39°C) for 5–15 days including any 2 of the following symptoms, namely, headache, vomiting, stupor, delirium, abnormal movements, presence of kernig's sign, convulsions, neck rigidity, altered sensorium, and unconsciousness were considered as acute encephalitis syndrome (AES) cases [16]. Although these manifestations can occur in manifold infectious diseases, in West Bengal, JE is an important disease particularly in rural areas and should be considered first in such cases. In general population the incidence of acute encephalitis syndrome ranges between 3.5 and 7.4 cases per 100,000 patient-years [17].

The incidence of JE varied in each month in our study. However, the most of the cases were reported during the monsoon and after the monsoon period. No patients were admitted during April to July. Anuradha et al. [3], Sarkar et al. [18], Benakappa et al. [19], and Reuben and Gajanana [20] have also reported higher incidence of JE during similar months due to increased prevalence of the vector mosquitoes. *Culex* mosquitoes breed abundantly in the paddy fields covered with stagnant water during the rainy season. Most of the JE cases occurred in children and adolescents below 20 years of age. Children and adolescents are probably directly exposed to the mosquito vector (*Culex* sp.) bite, as they often visit the fields with their parents or may take active part in the cultivation where vectors are abundant. Also lack of immunity against JE virus in the younger age group could be responsible for the increased incidence of disease in this age group [21–23].

Our study also indicates that most of the JE cases occurred in the rural districts of West Bengal, where the main occupation is farming. This finding is also similar to the findings of Anuradha et al. [3], Benakappa et al. [19], and Reuben and Gajanana [20].

There are currently believed to be four distinct genotypes of JEV, genotypes I to IV [24], although some studies support the existence of a fifth JEV genotype [25, 26], all of which are thought to have arisen from a common ancestor virus present in the Indonesian-Malaysian region [24]. While some genotypes are present in multiple countries (such as genotype III), others are present in only one country, such as genotype IV which is found only in Indonesia [24]. Conversely, countries may experience the circulation of several genotypes, such as Indonesia where genotypes II, III, and IV circulate [24]. However, genotypic shift, with the replacement of one genotype by another, has occurred recently in several countries [27–29]. Currently, JEV is considered hyperendemic in northern India and southern Nepal as well as in parts of central and southern India. More than 3 billion people are living in the current JE-endemic region [30, 31].

However, during the past decade, JEV GI has been introduced into Republic of Korea, Thailand, and China and has replaced the GIII strains that had been circulating in Japan and Vietnam during the mid 1990s [32]. Until 2007, all known JEV strains isolated in India belonged to GIII [31, 33–35].

Sarkar et al. reported the prevalence of genotypes III and I among the JE cases of West Bengal [18]. Studies from Gorakhpur also indicate the presence of genotypes I and III isolates among the AES cases [36].

The present study reveals that 22.76% and 5% of the AES cases were positive for JE IgM in 2011 and 2012, respectively. There was no sex predilection among the JE cases in the population of West Bengal, India. Results from a previous study in 2010 done by Sarkar et al. [18] on JE seropositivity in West Bengal and a similar study done in Uttar Pradesh of India in 2011 by Bhatt et al. [37] were comparable to our findings of 2011. Thus, it appears that although the percentages of JE positive cases were more or less stable in 2010 and in 2011, after 2011 it decreased sharply. This may be due to better awareness programs, mass vaccination, and/or simply due to natural epidemiological niche periodicity due to herd immunity. A changing epidemiological trend of flavivirus mediated diseases from JE to dengue has also been noted in recent years possibly due to increased urbanisation of the remote villages [38–40]. Cross-protection by other flaviviral diseases, namely, dengue, could be a reason for decline of the JE cases to some extent. The State Health Department of Government of West Bengal undertook mass vaccination programme against JE in several endemic districts using live attenuated JE vaccine SA-14-14-2. The significant decline of JE cases in our study in 2012 could be attributed to this as a major factor for the controlling of JE cases in the previously endemic district. However, active surveillance of JE cases is still warranted in order to be vigilant about any new genotype

introduction in the endemic districts or to find out any spread into newer geographical areas.

Conflict of Interests

All the authors declared that there is no financial conflict of interests.

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Research Article

Proteomic Identification of Dengue Virus Binding Proteins in *Aedes aegypti* Mosquitoes and *Aedes albopictus* Cells

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The main vector of dengue in America is the mosquito *Aedes aegypti*, which is infected by dengue virus (DENV) through receptors of midgut epithelial cells. The envelope protein (E) of dengue virus binds to receptors present on the host cells through its domain III that has been primarily recognized to bind cell receptors. In order to identify potential receptors, proteins from mosquito midgut tissue and C6/36 cells were purified by affinity using columns with the recombinant E protein domain III (rE-DIII) or DENV particles bound covalently to Sepharose 4B to compare and evaluate their performance to bind proteins including putative receptors from female mosquitoes of *Ae. aegypti*. To determine their identity mass spectrometric analysis of purified proteins separated by polyacrylamide gel electrophoresis was performed. Our results indicate that both viral particles and rE-DIII bound proteins with the same apparent molecular weights of 57 and 67 kDa. In addition, viral particles bound high molecular weight proteins. Purified proteins identified were enolase, beta-adrenergic receptor kinase (beta-ARK), translation elongation factor EF-1 alpha/Tu, and cadherin.

1. Introduction

Dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) are the most important arthropod-borne diseases nowadays, affecting people living mainly in tropical and subtropical regions, where environmental conditions favor the proliferation of the mosquito vector *Ae. aegypti*, as this has been spread to other regions in the world likely due to gradual climatic changes [1, 2]. Though, this may contribute to the spread of this disease, this has not been demonstrated [3].

The etiological agent of dengue is a positive-stranded RNA virus containing 3 structural proteins (C, prM, E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). It belongs to the family *Flaviviridae*, genus *flavivirus*, known as dengue virus (DENV), and includes serotypes from 1 to 4. Each serotype is also classified into a series of genotypes or subtypes [3–6]. Dengue virus genotypes differ in virulence, including their human pathogenicity and epidemic potential.

Dengue virus is transmitted to humans in America mainly by the mosquito vectors *Aedes aegypti* [7] infecting primary human cells such as peripheral blood leukocytes, blood monocytes/macrophages, dendritic cells, and B lymphocytes [7]. Dengue virus attaches to the host epithelial cell receptors protein E-mediated [8, 9] and enters the cell mainly via this receptor by clathrin-dependent endocytosis [10–13].

In mammalian cells, several DENV receptors have been described [14–19] as well as in mosquito cells; however the molecular identity of the receptors in mosquito cells has not

been completely elucidated. The apparent molecular weights described for these proteins are between 20 to 40 kDa and 57 to 130 kDa in size and bind dengue virus particles *in vitro* [8, 20–24]. In addition, Mercado-Curiel et al. [23] reported that specific antibodies against the membrane proteins R67 and R80 inhibited infection of C6/36 cells. Further, a protein with molecular mass of 57 kDa was also purified by affinity chromatography using a DEN2-Sepharose 4B column [23].

Viral envelope (E) protein of DENV as other *Flavivirus* has a homology of about 40% among different members of the family [25], and the crystal structures of this protein revealed three domains (I, II, and III) containing significant structural conservation [26–28]. DENV E protein is a class II fusion protein responsible for host cell attachment, entry, and virus-mediated cell membrane fusion.

It has also been shown that domain III of the envelope glycoprotein is an immunoglobulin-like structure and that the main viral region interacts with receptors on the host cells [29–36]. It has been also demonstrated that EIII domain of DENV-2 inhibits infection of DV on C6/36 cells and mammalian cells, suggesting that EIII domain binds molecules on the cell membrane that may participate in receptor-mediated DV entry [37]. However, the molecular mechanism of DENV-receptors has not been characterized in mosquitoes until now. We would expect that such molecular interactions in mosquito vector would influence virus passage through the different mosquito barriers: the first one is that after the virus established a midgut (MG) infection (MI) by overcoming the MG infection barrier (MIB): next replication in the MG epithelium, and then that virus must pass through a MG escape barrier (MEB) and replicate in other tissues to establish a disseminated infection (DI). Finally, virus must infect salivary glands and be shed in the saliva to be transmitted to a vertebrate host [38, 39].

It is accepted that the mechanisms by which DENV infects its target host cell should be the major determinant of the virus cellular tropism and critical for viral pathogenesis. Erb et al. [37] demonstrated that the FG loop located in DIII, where DENV2 has an extended loop motif between the F and G beta strands, was critical for the infection of *Aedes aegypti* mosquito MGs and mammalian cells by mutational studies. In addition, Butrapet et al. [40] identified critical amino acids within the hinge region of DENV-2, that are vital for virus fusion and replication. While domain III has already been used to study immunological and pathological mechanisms [40–46], its suitability for isolating specific receptors has not yet been investigated; then, we are showing in this communication the purification of DENV binding proteins from *Ae. aegypti* MG tissue and C6/36 cells by affinity chromatography using particles of DENV-2, -1, and -4 or rE2-DIII covalently bound to Sepharose 4B. In addition the identity of these proteins was determined by proteomic analysis.

2. Materials and Methods

2.1. Virus. DENV-2 Jamaica was expanded in Vero cells [47], purified from the culture supernatants as previously described [48], and kept frozen at −70°C until use. Briefly, Vero cells were cultured at 37°C, 2% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM; HyClone, Logan, Utah, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Vero cells (2×10^6/100 mm plate) were infected with 0.2 mL of DENV-2 inoculums with an input MOI of 600 PFU/plate and incubated for 10 days. We also included in our studies DENV-1 Hawaii, DENV-2 S1656OAX05 (Asian/American genotype), DENV-3 H-87, and DENV-4 H-341 strains.

2.2. Virus Purification. Viruses were purified on sucrose gradients essentially as described by Srivastava et al. [49] with minor modifications previously described [23]. The virus was recovered, suspended in PBS, and kept frozen at −70°C until use. The titer of the viral stock was adjusted to 6×10^6 PFU/mL. Virus purity was examined for total protein and by RT-PCR and transmission electron microscopy.

2.3. Virus Biotinylation. NHS-coupled biotinyl compounds have been used to label cell surface proteins previously [8]. The procedure to biotinylate DENV particles was as follow. Briefly, dissolve (+)-Biotin N-hydroxysuccinimide ester (Sigma, Catalog Number H1759) in DMSO immediately prior to use protecting solution from the light at a concentration of 11 mg/mL. Purified viruses were biotinylated by suspension of the viral pellet (2 mg of protein) in 0.8 mL of ice cold Phosphate buffer, pH 7.5 (PB) by adding 0.2 mL of NH-D-Biotin solution with gentle stirring and incubated overnight at 4°C or 3 h at ambient temperature. Biotinylated virus recovered after centrifugation at 100,000 ×g for 2 h was suspended in PBS (500 μL). The degree of biotinylation was determined by dot blot, and the viral stock was kept at −70°C until use [8].

2.4. Mosquito Culture. Aedes aegypti mosquitoes from the strains DS3 (susceptible to DENV), IBO-11 (refractory to infection), DMEB (midgut escape barrier), and Mori (collected in Monterrey, México) were laboratory-reared and maintained at 32°C and 80% RH with a 12 h photoperiod using standard mosquito-rearing procedures [50]. The entire MG was dissected from more than 1500 mosquitoes at day 5 after egg hatching. The procedure was carried out in 10 μL phosphate buffered saline (PBS). After dissection, each MG was rinsed twice in the same solution, quickly removed, and snap-frozen at −70°C until use.

2.5. Protein Extract Preparation. To optimize MG protein extraction, frozen MGs were homogenized in buffer E (0.05 M Tris-HCl, pH 7.2, 1 mM EDTA), containing 1 μL/mL of protease inhibitor cocktail (Sigma P9599) and 0.01, 0.05, 0.1, 0.5, or 1.00% v/v of Triton X-100. Protein extracts were centrifuged for 10 min at 29000 ×g at 4°C. Total protein concentration was determined as described previously by Bradford [51].

2.6. Virus Overlay Protein Binding Assay (VOPBA). Mosquito MG proteins were separated by 10% SDS-PAGE according to the method described by Laemmli [52] and blotted onto PVDF membranes (BioRad) by Towbin's technique [53].

The procedure was followed as previously described [8]. Previous results in our laboratory have showed that biotinylated virus recognized the same proteins as compared to virus without any treatment [8].

2.7. Affinity Chromatography. To perform the affinity chromatography assays, DENV-2, -1, -4 (5.7×10^8 PFU/mL), or domain III of E protein (500 μg, rE2-DIII) (ProSpec-Tany TechnoGene LTD) were covalently bound to 1 mL of CNBr-activated Sepharose 4B as recommended by the manufacturer (Amersham Biosciences) as described elsewhere [23]. Both affinity columns were stored in 0.002% sodium azide at 4°C until use.

Midgut protein extract obtained as described above (300 μg) was applied to the DENV-Sepharose 4B column, or rE2-DIII-Sepharose 4B column equilibrated in Buffer E containing 0.5 M NaCl, and washed with the same buffer thoroughly. The DENV-2 binding proteins were eluted with 0.1 M glycine-HCl pH 2.7 or buffer E containing 1 M NaCl. Fractions of 0.500 mL were collected, and the protein concentration was monitored by the Bradford method [51]. Eluted proteins in each fraction were concentrated by acetone-precipitation [54], separated by 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) [52] and Coomassie Brilliant Blue or silver stained [55]. Total protein extracts from *Ae. aegypti* mosquito DS3 (susceptible to DENV), IBO-11 (refractory to infection), and DMEB (the membrane escape barrier infected exclusively in the midgut epithelial cells) strains were also separated by SDS-PAGE, and then the proteins that migrated as the purified proteins (57 and 67 kDa) were also excised from the gels and the proteomic analysis was carried out (Table 1). Protein assignment was done by at least two peptide matches.

2.8. Protein Sequencing. The protein bands of interest were excised from a Coomassie Brilliant Blue R-250-stained gel, digested with trypsin, and identified by mass spectrometry (3200 TRAP hybrid tandem mass spectrometer, Applied Biosystem/MDS Sciex, Concord, ON, Canda). LC/MS/MS analysis of tryptic peptides was carried out using a NanoAcquity ultraperformance liquid chromatograph (UPLC) (Waters Corporation), coupled to a Q-ToF Synapt High Definition Mass Spectrometer (Waters Corporation), and equipped with a NanoLockSpray ion source. Protein identification was performed from the MS/MS spectra data sets using the MASCOT search algorithm (Version 1.6b9, Matrix Science, London, UK) available at http://www.matrixscience.com/ [56]. Peptide mass tolerance was set to ±1.2 Da and fragment mass tolerance to ±0.6 Da and the taxonomy parameter set to all species. Each MS/MS spectrum was also searched for *Ae. aegypti* against the data sets at VectorBase [57].

3. Results

To optimize solubilization of membrane proteins from mosquito MGs tissue was homogenized with buffer E containing Triton X-100, 0.01, 0.05, 0.1, and 0.5 or 1.00% v/v. Each protein extract was separated by SDS-PAGE and stained with Coomasie Blue. Figure 1(a) shows protein integrity and the same protein pattern at all Triton X-100 concentrations. To detect DENV-2 binding proteins, MG protein extracts were separated by SDS-PAGE, blotted onto a PVDF membrane, and incubated with biotinylated DENV-2 as mentioned in the Materials and Methods section. Figure 1(b) displays the proteins recognized by DENV-2 labeled with biotin. The optimal concentration of Triton X-100 to extract maximal DENV binding protein amount was 0.05% v/v (Figure 1(b), lane 2), since protein bands revealed by DENV-2 labeled with biotin are of greater intensity. Four major proteins with molecular masses of 57, 67, 80, and 115 kDa were observed in all lines (Figure 1(b)). Extraction of proteins with apparent molecular weight of 67 and 115 (Figure 1(b), lane 2) with the buffer containing 0.05% v/v Triton X-100 displayed higher densities, suggesting higher concentrations. This suggests that both proteins may be located at the membrane. Consequently, protein extraction was subsequently performed at a concentration of 0.05% Triton X-100. Negative control without virus showed no bands (data not shown).

In order to recover all proteins bound to the affinity column, after passing protein extracts from C6/36 cells through DENV-2-Sepharose 4B column, the proteins were eluted from independent columns with buffer E containing 1 M NaCl (Figure 2, lines 1 and 2), or 0.1 M Glycine pH 2.7 (Figure 2, lines 3 and 4).

Once the protein extraction procedure was optimized, dengue virus binding proteins were purified by affinity chromatography by passing protein extracts from C6-36 cells through a rE2-DIII-Sepharose 4B column and eluted with 0.1 M Glycine pH 2.7 containing 0.5 M NaCl (Figure 2, lines 6–9). Proteins with apparent molecular weights of 57 and 67 were mainly eluted with this column (Figure 2).

Then, dengue virus binding proteins were purified by affinity chromatography by passing protein extracts from *Ae. aegypti* MG through a DENV-2 or rE2-DIII-Sepharose 4B columns (Figure 3). Representative patterns of MG proteins retained and eluted from the column (from at least four experiments) are shown in Figure 3. Proteins with apparent molecular weights of 57, 67 kDa were eluted with buffer E containing 1 M NaCl (Figure 3, lines 1-2) or 0.1 M Glycine pH 2.7 (Figure 3 lines 3-4) from DENV-2 Sepharose 4B column. Proteins showing the same apparent molecular weights were eluted from rE2-DIII-Sepharose 4B column with buffer E containing 1 M NaCl (Figure 3, lines 5) or 0.1 M Glycine pH 2.7 (Figure 3, lines 6-7). The eluted proteins (EP) were stored at −70°C for a further analysis.

3.1. Identification of Mosquito Proteins That Interact with Dengue Virus. Proteins identified from the MS/MS spectra data sets using the MASCOT search algorithm [56] with trypsin enzyme specificity are shown in Table 1. Peptide sequence of each protein is displayed in Table 2. Proteins are ordered from the top to the bottom for the number of peptides identified as well as for the number of the experiments. Proteomic analysis was performed in protein extract purified

Table 1: Mosquito proteins bound to DENV.

DENV-Sepharose 4B*	Cell/tissue expression/gel slice**	Name****	Accession number	Accession swissprot	Predicted mass (Da)	Size (aa)	Mascot score	Theoretical isoelectric point*	Number of peptides	Protein coverage (%)
DENV-2	C6/36 57 kDa	Enolase[1]	gi|157121051|ref|XP_001653750|	Q17KK5	46621	433	76.1	5.6	4	15.9
DENV-2	C6/36 67 kDa	Enolase[2]	gi|157121051|ref|XP_001653750|	Q17KK5	46621	433	60.5	5.6	1	7.4
DENV-2	DS3 (extract)	Enolase[3]	gi|157121051|ref|XP_001653750|	Q17KK5	46621	433	20.2	5.6	1	1.4
None	DMEB*** 67 kDa	Enolase[4]	gi|157121051|ref|XP_001653750|	Q17KK5	46621	433	75	5.6	2	13
DENV-1	DMEB and DS3 67 kDa	Beta-adrenergic receptor kinase[1]	gi|157114479|ref|XP_001652291|	Q174J9	66217	580	40/26.9	6.74	1	2.6
DENV-4	C6/36 67 kDa	Beta-adrenergic receptor kinase[2]	gi|157114479|ref|XP_001652291|	Q174J9	66217	580	40/26.9	6.74	1	2.6
DENV-2	C6/36 57 kDa	Translation elongation factor EF-1 alpha/Tu[1]	gi|94468780|gb|ABF18239.1|	Q1HR88	50,473	463	82.9	9.61	1	5.4
DENV-2	MORI (extract)	Translation elongation factor EF-1 alpha/Tu[2]	gi|94468780|gb|ABF18239.1|	Q1HR88	50,473	463	21.8	9.61	1	1.7
None	IBO-11 57 kDa	Translation elongation factor EF-1 alpha/Tu[3]	gi|94468780|gb|ABF18239.1|	Q1HR88	50,473	463	63	9.61	2	10.8
DENV-4	C6/36 80 kDa	Cadherin	gi|157115805|ref|XP_001658290|	Q17LY6	186427	1653	52.0	4.74	1	1%

*Affinity chromatography was performed with DENV-2, -1, or -4.
**Bands of interest were excised at the molecular weight of interest (57 or 67 kDa).
***Total extract of MGs from *Ae. aegypti* mosquitoes strain DMEB was separated by SDS-PAGE and the band with apparent molecular weight of 67 kDa was excised for a further analysis.
****Superscript number in the name of the protein indicates the number of the experiment.

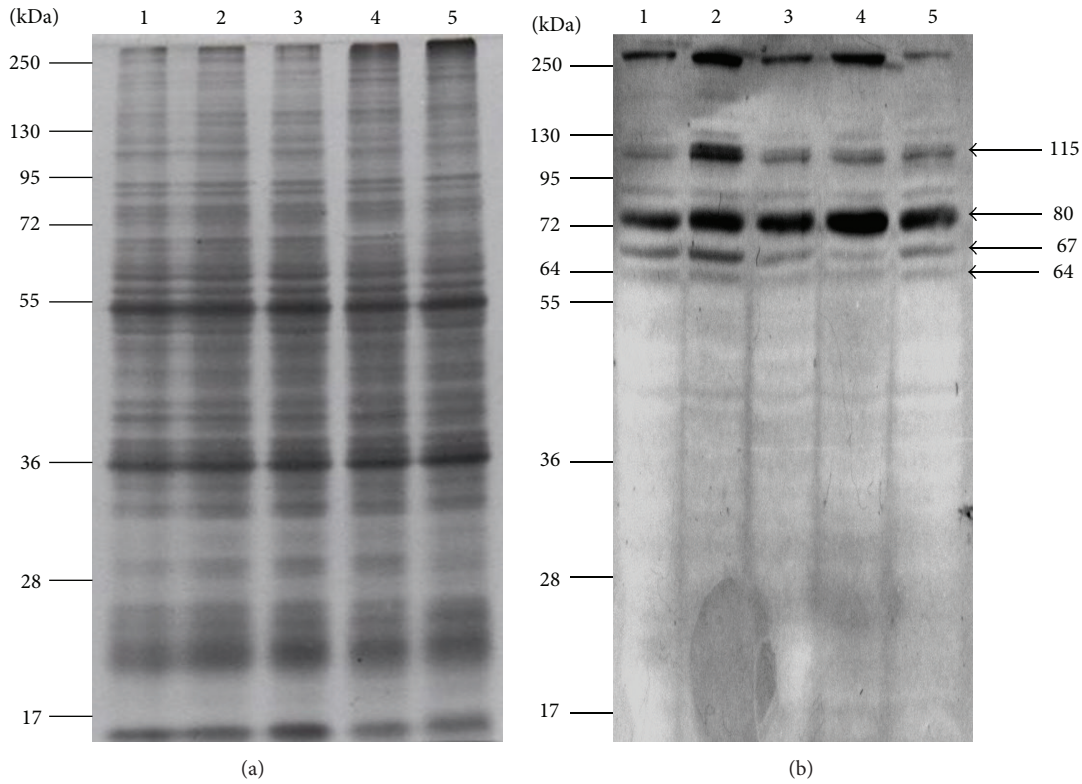

Figure 1: Midgut total protein extraction with Triton X-100 and VOPBA. (a) Proteins were extracted from mosquito MG tissue at different Triton X-100 concentrations, separated by SDS-PAGE, and stained with Coomasie Blue. Triton X-100 concentrations were 0.01, 0.05, 0.1, 0.5, and 1% corresponding to lane 1 to 5, respectively. (b) Proteins, separated by SDS-PAGE, were blotted onto PVDF and incubated with biotinylated DENV-2 and then with AP-Streptavidin. Proteins recognized by DENV-2 were developed with BCIP/NBT according to the procedure previously described [8]. The apparent molecular weights of these proteins are shown on the right side of (b). Molecular weight markers are shown on the left side in (a) and (b).

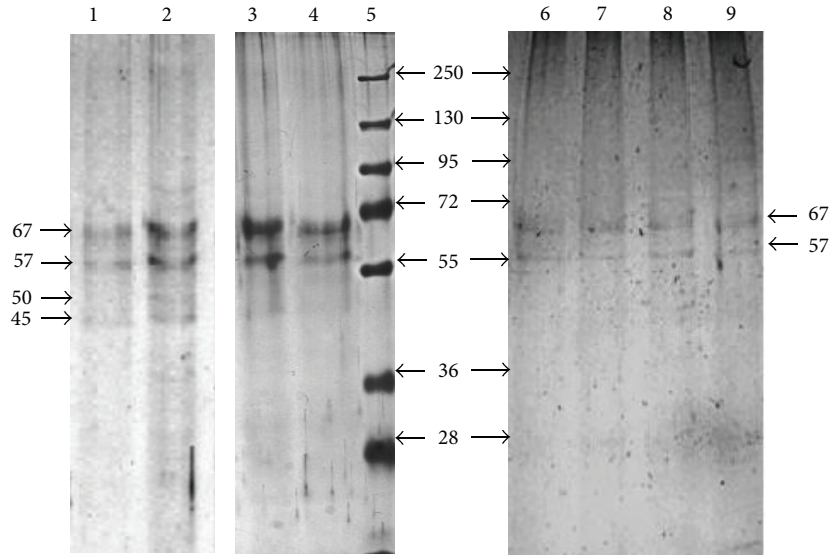

Figure 2: Affinity chromatography of C6/36 cell extracts. Proteins were purified from C6/36 cells by affinity chromatography using DEN-2, -1, -4, or rE2-DIII-Sepharose 4B column as described in the methods section. Aliquots of 500 μL were collected from each column and proteins were acetone-precipitated. Proteins eluted from DENV-2-Sepharose 4B columns with buffer E containing 1 M NaCl are displayed in lines 1 and 2, or 0.1 M Glycine pH 2.7 in lines 3 and 4. Proteins eluted from rE2-DIII-Sepharose 4B column with 0.1 M Glycine pH 2.7 are displayed in lines 6–9. Proteins were separated by 10% SDS-PAGE and Coomassie Brilliant Blue or silver stained. The apparent molecular weights of these proteins are shown on the right side. Molecular weight markers (line 5) are shown on the left side.

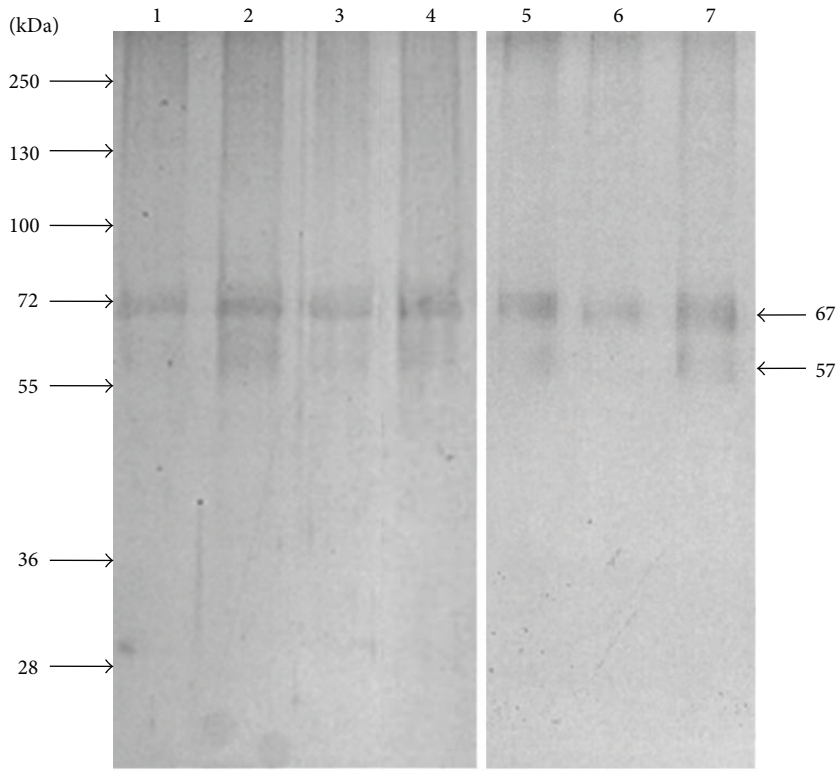

Figure 3: Affinity chromatography of MG protein extracts. MG proteins were purified from extracts of different *Ae. aegypti* strains (DMEB, DS3, IBO-11, or Mori) by affinity chromatography using DENV-2, -1, -4 or rE2-DIII-Sepharose 4B column as described in Section 2. Midgut proteins were eluted from DENV-2-Sepharose 4B columns with buffer E containing 1 M NaCl (lines 1-2), or 0.1 M Glycine pH 2.7 (lines 3-4) and from rE2-DIII-Sepharose 4B column with 1 M NaCl (line 5) or 0.1 M Glycine pH 2.7 containing 0.5 M NaCl (line 6-7). Aliquots of 500 μL were collected from each column and proteins were acetone-precipitated and separated by 10% SDS-PAGE and Coomassie Brilliant Blue or silver stained. The apparent molecular weights of these proteins are shown on the right side. Molecular weight markers are shown on the left side.

by the affinity columns or separated by SDS-PAGE and then excised from the gel.

Proteomic analysis of proteins obtained from total protein extracts of *Ae. aegypti* mosquito DS3, IBO-11, and DMEB strains separated by SDS-PAGE that migrated as the purified proteins (57 and 67 kDa) with at least two peptide matches is shown in Table 1. The proteins identified were enolase, beta-ARK, translation elongation factor EF-1 alpha/Tu, and cadherin. Translation elongation factor EF-1 alpha/Tu and cadherin had been identified previously, thus ensuring that the procedure described in this work is suitable to the identified proteins bound to DENV and E protein domain III. Peptide sequence AKPGAEAHPPFRQHK has partial alignment with beta-ARK (ref|XP_001652291|) and with ATP-dependent RNA helicase (ref|XP_001648042.1|); however, the identification of beta-ARK was confirmed by the match of ESQELLGSMAKK peptide with beta-ARK identified in two mosquito strains (DS3 and DMEB). Although, cadherin is showing only one peptide, the peptide match to this protein has a very high score of 52 (16/17 amino acids). Proteins identified in C6/36 cells or mosquito MGs from DMEB, DS3, IBO-11, or Mori strains are also included in Table 1. Manual analysis was used to confirm peptide identity (Figure 4). Peptide sequence coverage was 35% for enolase, 2.6% for beta-ARK, and 20% for translation elongation factor EF-1 alpha/Tu. Because translation elongation factor EF-1 alpha/Tu matched two proteins, we manually verified mass spectra for presence of unique peptides for each homologous assignment. In Figure 5 we demonstrate the alignment for these two homologous proteins EJY57625 and ABF18239 and peptides identified in each of those two proteins. Peptides NNPPKQAA and K.GASDFTAQVIVLNHPGQIANGYTPVLDCHTAVIACKFAEIQQK.V were specific for protein EJY57625 (Figure 5).

4. Discussion

Flavivirus vector competence studies in *Ae. aegypti* have indicated that the MIB is a major determinant of transmission [58, 59] and have shown wide variation among *Ae. aegypti* populations and *flaviviruses* including DENV [9, 38, 60]. Studies on mosquito receptors have displayed protein receptors on MG epithelial cells that may be the base to develop a strategy to control mosquito vector through blocking virus infection. In order to elucidate the nature of these receptors, mass spectrometry-based proteomic analysis of the purified proteins was performed. In our study, we are showing the isolation of proteins by affinity columns bound to the virus or domain III of the E protein of dengue 2 virus. Considering

Table 2: Distinct host peptides identified by mass spectrometry bound to DENV.

Cell/tissue expression	Protein name	Experment number	Peptide identified	Score
C6/36 (57 kDa)	Enolase	1	K.EALNLIQDAIAK.A	45.6
			R.GNPTVEVDLVTDLGLFR.A	62.1
			K.VNQIGTVTESINAHLLAK.K	76.1
			R.SGETEDTFIADLVVGLSTGQIK.T	76.1
C6/36 (67 kDa)		2	FGLDATAVGDEGGFAPNILNNKEALDLINEAISK	60.5
DS3		3	GVLKAVTQ	20.2
DMEB (67 kDa)		4	R.AAVPSGASTGVHEALELR.D	53.2
			K.NLILPVPAFNVINGGSHAGNKQAMQEFMILPTGACSFTEAMK.M	21.7
DMEB (67 kDa)	Beta-adrenergic receptor kinase	1	ESQELLGSMAKK	40.1
DS3 (67 kDa)		2	ESQELLGSMAKK	40.1
C6/36 (67 kDa)		3	AKPGAEAHPPFRQHK	26.9
C6/36 (57 kDa)	Translation elongation factor EF-1 alpha/Tu	1	SGDAAIVNLVPSWPLCVESFQEFPPLGR	82.9
Mori (extract)		2	NNPPKQAA	21.8
IBO		3	K.GASDFTAQVIVLNHPGQIANGYTPVLDCHTAVIACKFAEIQQK.V R.LPLQDVYK.I	63
C6/36 (80 kDa)	Cadherin	1	FLIDYGSGTLELRIATK	52

* Proteomic analysis was performed in protein from C6/36 cells, mosquito MGS purified by affinity chromatography (extract), or in the bands of interest excised after separation by SDS-PAGE.

that *Ae. aegypti* MG is the best candidate to disrupt the virus life cycle within the mosquito because it is the earliest interface between insect and virus and that DENV attachment to MG epithelial cell receptors is also critical for understanding the initial virus-vector interactions, this will help to explain MIB to DENV infection and variations in vector competence.

Accordingly, identification of viral receptors in the MG would represent a critical step in understanding vector competence and designing possible targets for preventing viral entry to cells and therefore inhibiting the infection. Published data have shown that domain III of the viral E protein is involved in target cell recognition [29] and binding of host cell surface receptors [32, 34–37]. Consequently, identification of dengue virus binding proteins by affinity chromatography using rE2-DIII will help to understand virus cell entry and to design strategies to block virus infection in the mosquito cells. Thus, in order to purify DENV binding proteins, rE2-DIII or viral particles were covalently bound to Sepharose 4B matrix.

Our results suggest that purified proteins by rE2-DIII-Sepharose 4B affinity column correspond to the same proteins purified by dengue particles with apparent molecular weights of 57 and 67 that were also consistently and previously reported in C6/36 cell membranes [23]. Specific antibodies against the 67 kDa protein inhibited virus infection [8, 23]. Although, DENV-Sepharose 4B bound additional proteins, we focused our studies to the proteins with apparent molecular weight of 57 and 67 bound to DENV particles and E protein domain III (Figures 2 and 3, Table 1). We also showed that DENV-1, -2, and -4 bound the same proteins with apparent molecular weights of 57 and 67 kDa.

These results are very important since the identity of specific MG mosquito proteins bound to viral particles and domain III of E protein has not been previously reported. The proteins identified by the proteomic analysis were enolase, elongation factor 1, beta-ARK, and cadherin. Enolase is a glycolytic enzyme and has been found in small vesicles outside the cell [61, 62]; it binds to plasminogen and helps pathogens to invade [63]. Enolase is also found in viral particles [64–66] and is required for the transcription of Sendai virus [67]. Furthermore, enolase has been identified in the MG brush border of *Ae. aegypti* mosquitos [68]. We identified enolase in protein extracts of C6/36 cell, and in the MG of *Ae. aegypti* mosquitoes from DS3 and DMEB strains and also showed that this protein is bound to DENV-2. In our previous reports we established that the 67 kDa protein is a membrane DENV binding protein [8, 9]. Therefore, our results agree with previous reports as enolase is in the brush border of mosquito MGs [68]; This reinforces the idea that enolase may be a DENV receptor of *Ae. aegypti* MGs. In addition, enolase has been also reported to bind to West Nile and DENV virus envelope and capsid proteins, respectively [69].

The second protein identified by the proteomic analysis was the beta-ARK with apparent molecular weight of 67,000. This protein specifically phosphorylates the agonist-occupied form of the beta-adrenergic and closely related receptors, probably inducing a desensitisation of them in higher eukaryotic organisms. This kinase is a member of the G protein-coupled receptor kinase (GRKs) family and catalyzes the phosphorylation of the activated forms of the beta-adrenergic receptor (beta-AR). As member of GRKs, this protein is also very important, because it has been implicated in

ORIGIN

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  1 MPFKSIKARQ IFDSRGNPTV EVDLVTDLGL FRAAVPSGAS TGVHEALELR DNVKADWHGK
 61 GVLKAVENIN KTIAPAVLNS GLCVTQQKEL DELMLKLDGT ENKSKLGANA ILGVSLAVCK
121 AGAAKKGIPL YKHIAELSGN GNIILPVPAF NVINGGSHAG NKLAMQEFMI LPTGASSFTE
181 AMKIGSEVYH HLKNVIKAKF GLDATAVGDE GGFAPNILEN KEALNLIQDA IAKAGYTGKV
241 EIGMDVAASE FHKDGKYDLD FKNPNSDKSA WLTPDALEGM YQGFIKDFPI VSIEDPFDQD
301 HWDAWAKMTA NTSIQIVGDD LTVTNPKRIA TAVEKKACNC LLLKVNQIGT VTESINAHLL
361 AKKNGWGTMV SHRSGETEDT FIADLVVGLS TGQIKTGAPC RSERLAKYNQ ILRIEEELGS
421 DAKFAGKNFR HPQ
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(a)

ORIGIN

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  1 MKNEVPVNLF EPYIEEIFHH LRGEPFRKFL ESDKYTRFCQ WKNLELNIQL TMNDFSVHRI
 61 IGRGGFGEVY GCRKADTGKM YAMKCLDKKR IKMKQGETLA LNERTMLSLV STGVDCPFIV
121 CMTYAFHTPD KLCFILDLMN GGDLHYHLSQ HGVFNESDMK FYAAEVILGL EHMHKRFIVY
181 RDLKPANILL DENGHVRISD LGLACDFSKK KPHASVGTHG YMAPEVLSKG TPYDSSADWF
241 SFGCMLYKLL KGHSPFRQHK TKDKHEIDRM TLTMNVELPE SFSKELRDLL EGLLQRDIDK
301 RLGCKGGGAD EVKAHPFFTG IDWNQVYYQK YTPPLIPPRG EVNAADAFDI GSFDEEDTKG
361 IKLTEQDQEL YKYFPLTISE RWQQEVAETV FETVNLEADR VEQKRKAKQK QRFDADEKDS
421 DCILHGYLKK YSGSFASVWQ TRYAKLYPNR LELHTESSST KPDLVFMDQI EEIAPDYIQF
481 KNEQCIQIKF RDGIRDGRLI LTMADEIGLK EWSLSLRGAH KESQELLGSM AKKAGKIYGT
541 ERDASKANVL ISSSTTSYSN ASATTNAASG GQRNANGSSN
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(b)

ORIGIN

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  1 MGKEKTHINI VVIGHVDSGK STTTGHLIYK CGGIDKRTIE KFEKEAQEMG KGSFKYAWVL
 61 DKLKAERERG ITIDIALWKF ETSKYYVTII DAPGHRDFIK NMITGTSQAD CAVLIVAAGT
121 GEFEAGISKN GQTREHALLA FTLGVKQLIV GVNKMDSTEP PYSESRFEEI KKEVSSYIKK
181 IGYNPAAVAF VPISGWHGDN MLEVSTKMPW FKGWNVERKE GKADGKCLIE ALDAILPPTR
241 PTDKALRLPL QDVYKIGGIG TVPVGRVETG VLKPGTVVVF APVNLTTEVK SVEMHHEALQ
301 EAVPGDNVGF NVKNVSVKEL RRGYVAGDTK NNPPKGAADF TAQVIVLNHP GQISNGYTPV
361 LDCHTAHIAC KFAEIKEKVD RRSGKSTEEN PKSIKSGDAA IVNLVPSKPL CVESFQEFPP
421 LGRFAVRDMR QTVAVGVIKS VNFKDASGGK VTKAAEKAQK GKK
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(c)

Figure 4: Identification of enolase, beta-adrenergic receptor kinase, and translation elongation factor EF-1 alpha/Tu as DENV-binding proteins by LC MS/MS analysis of the excised protein bands corresponding to the apparent molecular weights of 57 and 67 kDa. The colored sequences represent the amino acid peptides identified as enolase (a), beta-ARK (b), and translation elongation factor EF-1 alpha/Tu (c) using MS/MS spectrometry after in-gel digestion of the protein-staining band (Tables 1 and 2). The protein sequence refers to gi|157121051|ref|XP_001653750|, gi|157114479|ref|XP_001652291|, and gi|94468780|gb|ABF18239.1|, respectively.

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EJY57625   1 MGKEKIHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKF  80
ABF18239   1 MGKEKTHINIVVIGHVDSGKSTTTGHLIYKCGGIDKRTIEKFEKEAQEMGKGSFKYAWVLDKLKAERERGITIDIALWKF  80

EJY57625  81 ETAKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGTGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEP 160
ABF18239  81 ETSKYYVTIIDAPGHRDFIKNMITGTSQADCAVLIVAAGTGEFEAGISKNGQTREHALLAFTLGVKQLIVGVNKMDSTEP 160

EJY57625 161 PYHEARFEEIKKEVSSYIKKIGYNPASVAFVPISGWHGDNMLEPSDKMPWFKGWAIERKEGKAEGKCLIEALDNILPPSR 240
ABF18239 161 PYSESRFEEIKKEVSSYIKKIGYNPAAVAFVPISGWHGDNMLEVSTKMPWFKGWNVERKEGKADGKCLIEALDAILPPTR 240

EJY57625 241 PTDKALRLPLQDVYKIGGIGTVPVGRVETGILKPGMVVVFAPVNITTEVKSVEMHHEALQEALPGDNVGFNVKNVSVKEL 320
ABF18239 241 PTDKALRLPLQDVYKIGGIGTVPVGRVETGVLKPGTVVVFAPVNLTTEVKSVEMHHEALQEAVPGDNVGFNVKNVSVKEL 320

EJY57625 321 RRGYVAGDSKASPPKGAADFTAQVIVLKNHPGQIANGYTPVLDCHTAHIACKFAEIKEKCDRRSGKVTEENPKSIKSGDAA 400
ABF18239 321 RRGYVAGDTKNNPPKGAADFTAQVIVL-NHPGQISNGYTPVLDCHTAHIACKFAEIKEKVDRRSGKSTEENPKSIKSGDAA 400

EJY57625 401 IVNLVPSKPLCVESFQEFPPLGRFAVRDMRQTVAVGVIKSVNFKEATGGKVTKAAEKAQK-KK   462
ABF18239 401 IVNLVPSKPLCVESFQEFPPLGRFAVRDMRQTVAVGVIKSVNFKDASGGKVTKAAEKAQKGKK  463
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Figure 5: Amino acid sequence analysis of two different translation elongation factors EF-1 alpha/Tu. Alignment of these elongation factors (EJY57625 and ABF18239) identified in C6/36 cells, and MGs of *Ae. aegypti* mosquitoes of the IBO-11 and MORI strains is shown. Identified peptides are shown in red color.

the specific phosphorylation on membrane protein receptors and in the regulation of signal transduction mechanisms [70]. Furthermore, beta-ARK also may help virus endocytosis facilitating receptor endocytosis, similarly to beta-ARK reported to directly interact with phosphoinositie-3-kinase (PI3K) promoting its membrane localization, phosphoinositide production, AP-2 adaptor protein recruitment to the receptor, and receptor endocytosis [71]. This protein was identified in C6/36 cells and DMEB, and DS3 *Ae. aegypti* mosquito strains.

The translation elongation factor EF-1 alpha/Tu was the third identified protein in C6/36 cells and MGs of *Ae. aegypti* mosquitoes of the DMEB and IBO-11 strains purified by affinity chromatography using DENV-2 and -4. Previously, this protein was also identified as an NS4 binding protein of DENV and WNV [69]. Furthermore, it has been also reported that DENV envelope protein binds to cadherin [69]. Furthermore, cadherin identified in this work has also been reported to bind to DENV envelope protein [60].

The data in the present paper strongly support that enolase may be a receptor for DENV-2, in MG cells from *Ae. aegypti,* and this protein may correspond to the 57 or 67 kDa protein previously reported [8, 9]. Differences in molecular weight mass may be due to posttranslational modifications, residual protease activity, or association with other molecules as has been formerly reported.

In addition, the procedure described here may be very useful in future studies to determine the proteins that bind to different domains of E protein or to other viral proteins. To the best of our knowledge, this is the first paper that displays a method to purify *Ae. aegypti* MG proteins by affinity chromatography by means of viral particles compared to rE2-DIII and establish the identity of the proteins with apparent molecular weights of 57 and 67 kDa.

5. Conclusions

This study identified enolase, beta-ARK, translation elongation factor EF-1 alpha/Tu, and cadherin mosquito as binding proteins that may play important roles as host factors during viral infection of mosquito cells. Enolase, beta-ARK, and cadherin may serve as DENV receptors, and translation elongation factor EF-1 alpha/Tu may be very important during virus replication. All proteins were identified in C6/36 cells and in the *Ae. aegypti* DS3, DMEB, and IBO-11, and Mori strains that differ in their vector competence for DENV; then we are suggesting that all mosquito strains of *Ae. aegypti* and C6/36 cells from *Ae. albopictus* interact probably with the same protein domain. In addition, the protein with the same apparent molecular weight was bound by DENV-1, -2, and -4 and rE2-DIII. Future studies will be necessary to determine the specific role of each protein in each strain to know how they participate in vector competence.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Clinical Study

Increased Production of Interleukin-4, Interleukin-10, and Granulocyte-Macrophage Colony-Stimulating Factor by Type 2 Diabetes' Mononuclear Cells Infected with Dengue Virus, but Not Increased Intracellular Viral Multiplication

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It has been reported that diabetes mellitus (DM) was an epidemiologically identified risk factor for development of dengue hemorrhagic fever (DHF)/severe dengue in dengue virus (DENV) affected patients, and T helper 2 (Th2) cytokines such as interleukin-4 (IL-4) and IL-10 each plays an important role in the immunopathogenesis of DHF in studies involving general population. To better understand the relationship between these epidemiological and immunological findings, we performed an *in vitro* study evaluating the sequential immunological reactions and viral load in the DENV infected mononuclear cells of adults with type 2 DM (T2DM group, $n = 33$) and normal adults (control group, $n = 29$). We found in the T2DM group significantly higher IL-4 level on the first ($P = 0.049$) and the third ($P = 0.022$) postinfection days, while higher IL-10 ($P = 0.042$) and granulocyte-macrophage colony-stimulating factor (GM-CSF) ($P = 0.009$) were detected on the third postinfection day. No significant difference in DENV viral load between the cultured mononuclear cells from both groups was found on the first and third post-infection days. These data immunologically suggest that patients with T2DM are at higher risk for development of DHF/severe dengue and strengthen the previously epidemiologically identified role of DM being a predictive risk factor for progressing into DHF/severe dengue in DENV-affected patients.

1. Introduction

Dengue is a major medical and public health problem in tropical and subtropical regions. It is estimated that approximately 50 million dengue episodes occur over the globe annually, and more than 2.5 billion people are living in geographic locales where dengue is endemic [1, 2]. There are four dengue virus serotypes (DENV-1, DENV-2, DENV-3, and DENV-4)

[1, 2], and patients infected by any of the DENV serotypes may be asymptomatic or develop a wide array of clinical symptoms/signs ranging from a nonspecific febrile illness, dengue fever (DF) to dengue hemorrhagic fever (DHF). DHF is clinically characterized by bleeding and plasma leak, and a severe DHF leads to hypovolemia and even circulatory collapse in the affected patient, which is known as dengue shock syndrome (DSS) [1, 2]. Well-documented risk factors for DHF include secondary infection caused by a DENV serotype which differs from that responsible for the prior dengue episode [3, 4], the genetic predilection for causing hemorrhage of the culprit DENV [5, 6], the genetic predisposing for hemorrhage of the dengue patient [7], the aging of the host [8, 9], and diabetes mellitus (DM) [10–13]. DM is a multifaceted disease that implicates metabolic derangements and immune dysfunction [14]. The frequently found comorbidities in diabetic patients such as cardiovascular and chronic kidney diseases may add to the altered host responses to infection and clinical outcomes [15, 16]. The immunologic responses of DM patients when suffering from DENV infection have not been fully understood. It was documented that T helper (Th) cells play an important role in the immunopathogenesis of DHF [17]. Based on the types of cytokine production at activation, Th cells are divided into Th1 and Th2 [18, 19]. Activated Th1 produces IFN-γ, IL-2, and IL-12, whereas Th2 produces IL-4, IL-5, IL-10, and IL-13 [18, 19]. Of note, the immunologic responses in a progressive dengue patient were reported to involve a shift from the activated Th1-type cytokine response in DF to the activated Th2-type cytokine response in DHF [17, 20–24]. In general, serum levels of IFN-γ and IL-2 are high in patients suffering from DF, while those of IL-4, IL-6, and IL-10 remarkably upsurge in hosts experiencing severe DHF [17, 20–24]. To better understand the immune responses in dengue individuals with type 2 DM (T2DM), we investigated Th1/Th2 reactions by DENV-infected mononuclear cells of T2DM individuals. The implications of the results will be discussed.

2. Material and Methods

2.1. Ethics Statement. This study was conducted with an informed consent from all participants, which was approved by the Institutional Review Board of Chang Gung Memorial Hospital (Document no. 98-2957B).

2.2. Study Period and Blood Sampling. The study was conducted at Kaohsiung Chang Gung Memorial Hospital, a 2700-bed medical facility serving as a primary care and tertiary referral centre in southern Taiwan, from March through December in 2010. Participants included those with a T2DM and healthy adults, aged between 50 and 60 years. Individuals with T2DM referred to those who have been taking oral hypoglycemic agent(s) for a previously diagnosed DM [14]. Blood specimens sampled from T2DM and healthy individuals were allocated to the study group and the control group, respectively. Eight milliliters of blood were sampled from each participant. The whole blood was immediately separated into plasma and blood cells (i.e., leukocytes and erythrocytes) by centrifugation at 2,500 rpm (150 ×g) for 20 minutes. Plasma was dispensed into several aliquots and kept frozen at −80°C until being used.

2.3. Determination of Past DENV Infection. The serum samples of all participants were tested for dengue virus-specific immunoglobulin M (IgM) and G (IgG) antibodies using a dengue blot detection kit (Gene Labs Diagnostics, Singapore) [25, 26].

2.4. Separation of Mononuclear Cells. Leukocytes were separated from erythrocytes by 4.5% dextran sedimentation. After removal of erythrocytes, leukocytes were further separated into mononuclear cells and polymorphonuclear cells by density gradient centrifugation (350 g/30 min in Ficoll-Paque PLUS, Amersham Biosciences Corp.) according to standard procedures as described elsewhere [26]. Mononuclear cells were suspended in supplemented RPMI 1640 medium (Gibco BRL, Gaithersburg, MD, USA) to yield a final concentration of 1.0×10^6 cells/mL for studies.

2.5. Preparation of DENV-2. DENV-2 (New Guinea C strain) obtained from the Institute of Preventive Medicine, National Defense Medical Center, Taiwan, was propagated in *Aedes albopictus* C6/36 cells as previously described [27] and was used in this study. The DENV-2 viruses were harvested from the C6/36 mosquito cell-culture supernatants after incubation for 5 days. The DENV-2 titers in the supernatants were measured by a standard plaque-forming unit (pfu) assay on baby hamster kidney-21 cells. The virus titers were adjusted to a concentration of 5.0×10^6 pfu/mL in RPMI 1640 medium for studies.

2.6. DENV-2 Infection of Mononuclear Cells. A prior report on multiplicity of infection (MOI) of DENV ranging from 1 to 10 suggested that the higher the MOI, the simultaneously increased the DENV infection and apoptosis in the inoculated mononuclear cells [28]. To achieve a yield of sufficient DENV-infected mononuclear cells while avoiding excessive cellular apoptosis, in this experiment we used the MOI of 5, which was proven appropriate previously [29, 30]. Specifically, mononuclear cells were seeded at a density of 1.0×10^6 cells/mL on 24-well plates. After an overnight incubation, the mononuclear cells were inoculated with DENV-2 from the stock with the MOI of 5 at 37°C for 2 hours [26]. The mononuclear cells were then washed twice in phosphate-buffered saline to remove cell-free viruses, and complete RPMI 1640 medium supplemented with 10% fetal bovine serum was added into each well. The infected cells were cultured in complete RPMI 1640 medium at 37°C in a 5% CO_2 incubator. To demonstrate the innate and adaptive immunity responses *in vitro*, the supernatants and cells were harvested and analyzed on the first and third postinfection days, respectively, for measurement of responsive immune mediators and viral loads.

2.7. Measurement of TNF-α, IL-2, IL-4, IL-10, IL-12, GM-CSF, and MCP-1 Levels. In this study, the innate mediator was

demonstrated by TNF-α [31], the Th1/Th2 reaction by IL-2, IL-4, IL-10, and IL-12 levels [19], vascular leakage mediator by MCP-1 level [32], and activated leukocytes-derived growth factor by the GM-CSF level [33]. The concentrations of TNF-α, IL-2, IL-4, IL-10, IL-4, GM-CSF, and MCP-l in the supernatants from the infected mononuclear cells were measured using the FlowMetrix System (Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions [34].

2.8. Quantitation of Viral Load by Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Viral RNA was extracted from cultured mononuclear cells to assess DENV-2 RNA viral copies by quantitative RT-PCR, as previously described [24]. The forward primer, reverse primer, and nested fluorescent probe sequences for detecting DENV-2 were 50-GGCTTAGCGCTCACATCCA-30, 50-GCTGGCCACCCT-CTCTTCTT-30, and FAM-50-CGCCCACCACTATAG-CTGCCGGA-30-TAMRA, respectively [24].

2.9. Statistical Analysis. Data are presented as mean ± SE. Student's t-test was used to analyze differences in immune mediators (TNF-α, IL-2, IL-4, IL-10, IL-4, GM-CSF, and MCP-l levels) and DENV viral load on the first and third postinfection days between the T2DM group and control groups. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Demographics, Clinical, and Laboratory Information of the Participants. A total of 33 T2DM individuals (mean glycosylated hemoglobin, 7.9 ± 1.6 gm/dL) and 29 healthy individuals were recruited for this study. Participants with and without T2DM were of similar ages (mean age, 55.8 ± 2.4 years versus 54.6 ± 2.6 years; $P = 0.083$). Among the 33 T2DM participants, hypertension (63.6%) was the most common comorbidity, followed by ischemic heart disease (9.1%) and previous stroke (6.1%). All participants were negative for dengue antibody as determined by dengue blot detection kit suggesting that none of them had suffered dengue before participating in this study.

3.2. Comparison of TNF-α, IL-2, IL-4, IL-10, IL-12, GM-CSF, and MCP-1 Levels between T2DM and Control Groups on the First and Third Postinfection Days. On the first postinfection day, no significant difference was found in concentrations of TNF-α, IL-2, IL-10, IL-12, GM-CSF, and MCP-l between the T2DM group and control group, but significantly higher IL-4 level (mean 1.02 ± 0.2 pg/mL versus 0.52 ± 0.13 pg/mL; $P = 0.049$) was detected in the T2DM group (Figure 1).

On the third postinfection day, the T2DM group had significantly higher IL-4 (mean 16.87 ± 4.96 pg/mL versus 4.13 ± 1.23 pg/mL; $P = 0.022$), IL-10 (mean 138.89 ± 34.62 pg/mL versus 55.49 ± 18.7 pg/mL; $P = 0.046$), and GM-CSF (mean 20.8 ± 5.3 pg/mL versus 5.1 ± 1.46 pg/mL; $P = 0.009$) levels than those of the control group. Despite nonstatistical significance, there was a trend suggesting possible higher level of vascular leakage mediator MCP-1 (mean MCP-1, 4739.75 ± 655.6 pg/mL versus 3980.8 ± 639.29 pg/mL; $P = 0.413$) in the T2DM group (Figure 2).

3.3. DENV Viral Load of T2DM and Control Groups. Viral load was not detected in 1 of the specimens in T2DM group on the first and third postinfection days and in 2 and 3 specimens in the control group on the first and third postinfection days, respectively. No significant difference in detectable DENV viral load between the cultured mononuclear cells from both groups was found on the first and third postinfection days (Figure 3).

4. Discussion

DM was found to be one of the many epidemiologically identified risk factors for developing severe dengue/DHF in dengue affected patients [10–13, 35]. Most of the published researches in immunology in dengue involved general population rather than a population with specific underlying disease(s) [36]. When it comes to immunopathogenesis, the role of viral burden has been controversial in DHF [21, 37, 38]. Among the multifaceted mechanism of pathogenesis of dengue, high DENV burden was reported to circumstantially associate with DHF in hosts with secondary infection [37, 38], while the overwhelming activation of Th2 cytokines was documented in the development of DHF in dengue patients of primary and secondary infections alike [17, 20–24]. Specifically, of the Th2 cytokine profile, IL-4 is the most potent cytokine in inducing Th2 cell differentiation, whereas IL-10 is responsible for anti-inflammatory reactions in the host's immune activities [21–23, 31, 36]. A significantly higher level of IL-4 found on the first postinfection day, as well as higher levels of IL-4 and IL-10 detected on the third postinfection day in the T2DM group as compared to the control group, suggests that dengue patients with an underlying T2DM are at higher risk for development of DHF, which was consistent with the same conclusion drawn based on previous epidemiological observations [10–13].

A number of proinflammatory cytokines/chemokines including TNF-α, IL-1 and IL-6, and MCP-1 were well documented to involve in the pathogenesis of diabetes mellitus [39]. GM-CSF stimulates stem cells to produce granulocytes and monocytes and plays an important role in the immune/inflammatory cascade [40]. It was reported that serum GM-CSF was significantly raised in patients with severe dengue as compared to those with mild-form dengue, and the increased GM-CSF level correlated with the development of hypotension in DHF patients [41]. The increased production of the IL-4 and the anti-inflammatory Th2-cytokine IL-10 in DENV-infected mononuclear cells of the diabetes in our study might result from a counterbalance to the comparatively highly produced pro-inflammatory cytokines/chemokines in the diabetic hosts [18, 19].

Limitations of this study are that the included patients were adults with a T2DM suffering from primary dengue infection, and therefore, it is uncertain whether similar immunologic responses occur in pediatric patients, in patients with type 1 DM, and/or in those with secondary dengue infection.

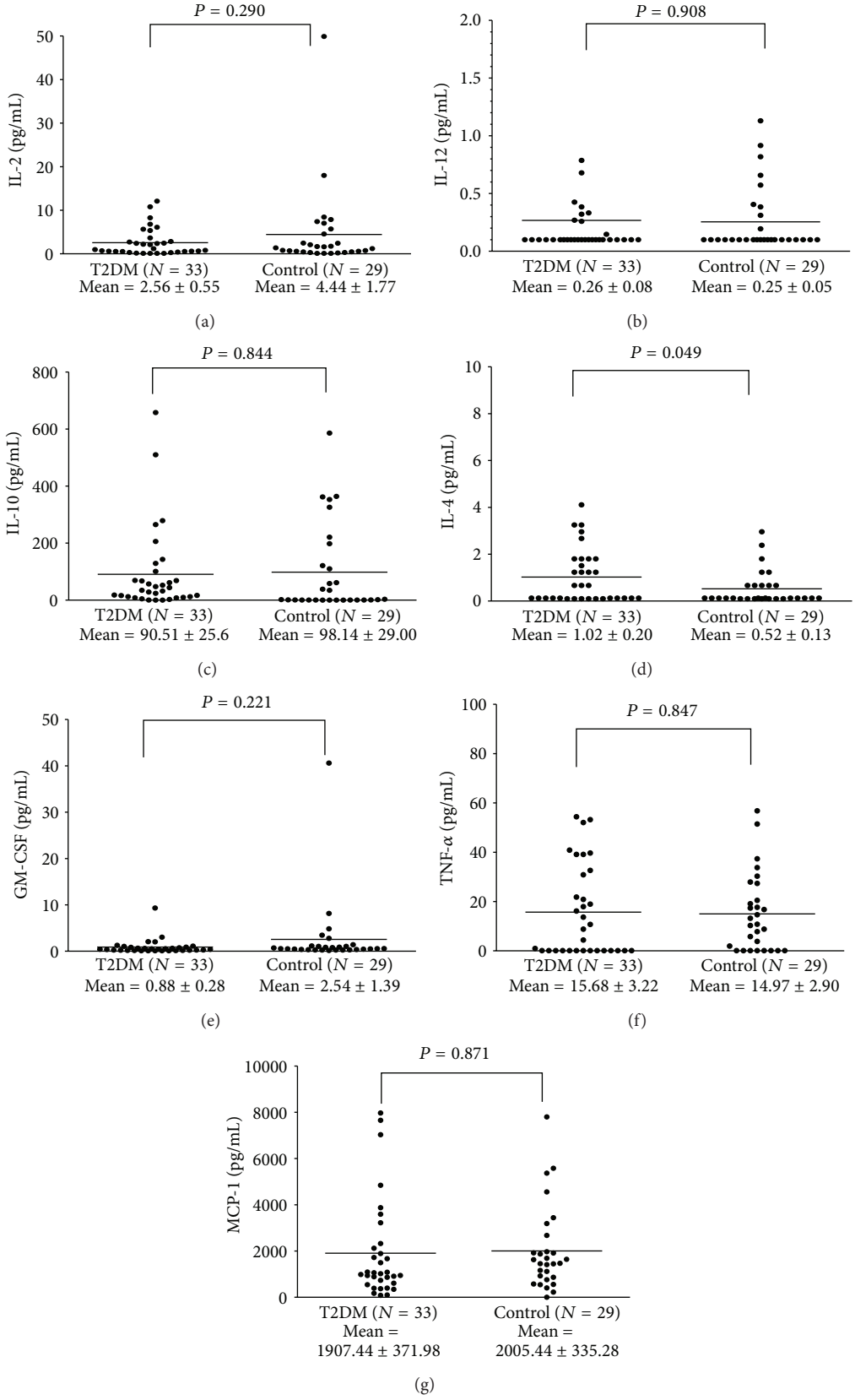

Figure 1: Cytokines/chemokines produced by dengue virus-infected mononuclear cells of T2DM group and control group on the first postinfection day in an *in vitro* infection model.

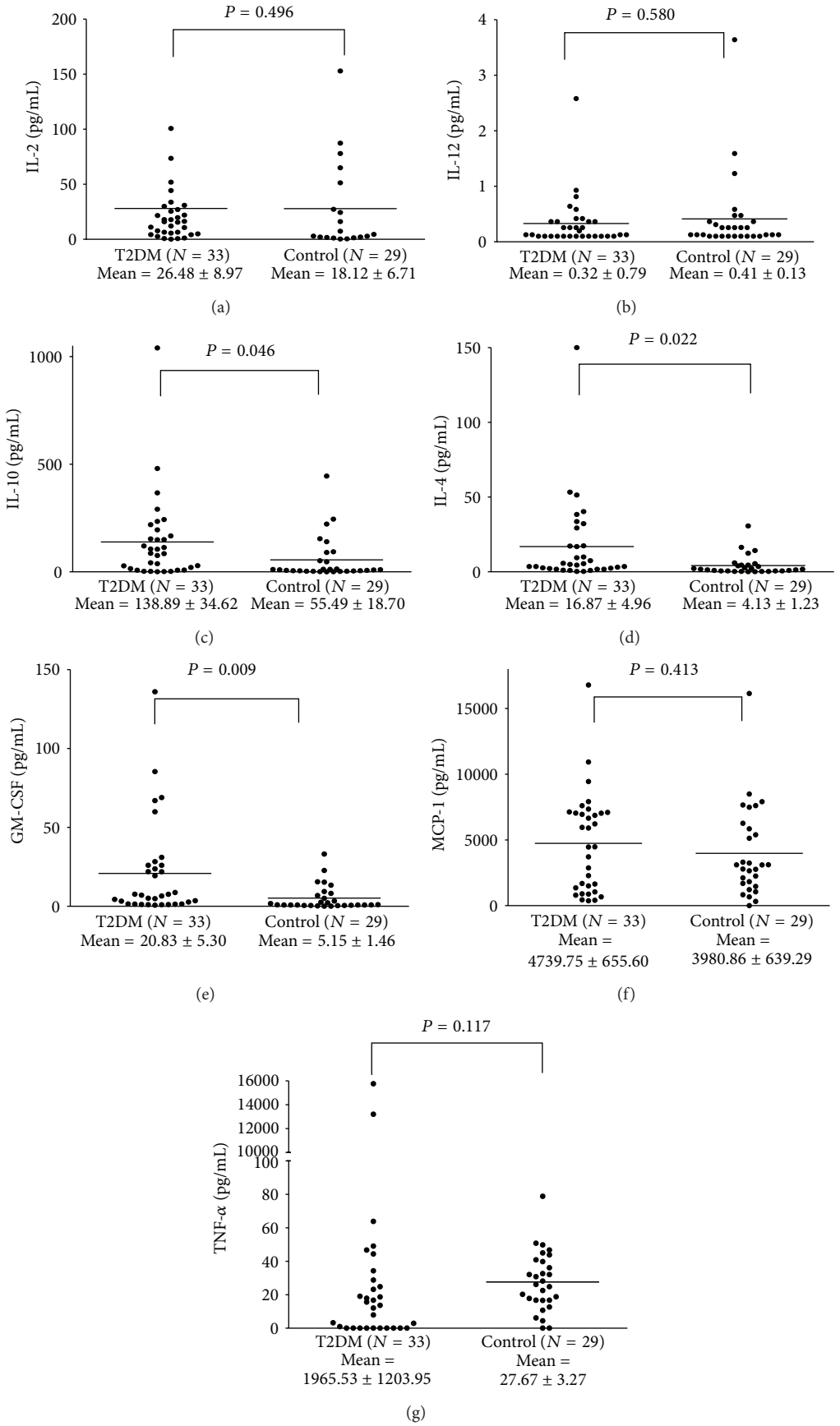

Figure 2: Cytokines/chemokines produced by dengue virus-infected mononuclear cells of T2DM group and control group on the third postinfection day in an *in vitro* infection model.

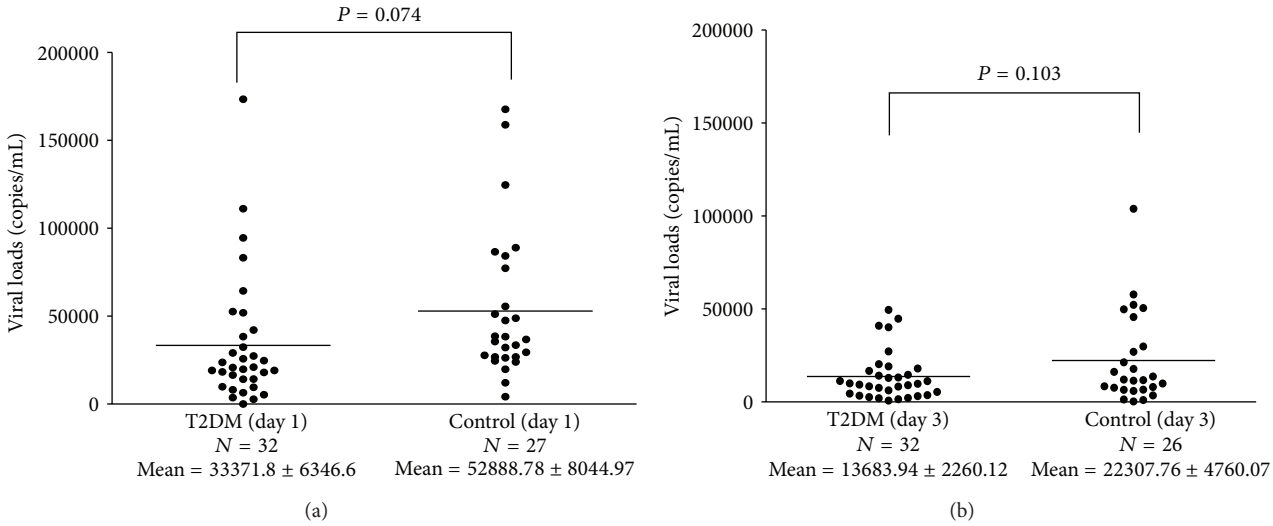

Figure 3: Dengue viral load in the infected mononuclear cells of T2DM group and control group on the first (a) and third (b) postinfection days in an *in vitro* infection model.

In summary, this study explored the immunologic reactions in adults with T2DM experiencing primary dengue infection, and our data give insight into the immunopathogenesis of dengue in this patient population. The immunological findings suggest that patients T2DM are at higher risk for development of DHF and strengthen the previously epidemiologically identified role of DM being a predictive risk factor for progressing into DHF/severe dengue in dengue-affected patients. Stratifications of clinical severity and prediction of the risk for potential clinical deterioration are very important in a large-scale dengue epidemic happening in rural areas where medical resources are deficient, as strict observation is needed for the identified potentially risky patients so that necessary management can be delivered in a timely fashion in case of deterioration.

Acknowledgments

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Review Article

Molecular Mechanisms Involved in the Pathogenesis of Alphavirus-Induced Arthritis

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Arthritogenic alphaviruses, including Ross River virus (RRV), Chikungunya virus (CHIKV), Sindbis virus (SINV), Mayaro virus (MAYV), O'nyong-nyong virus (ONNV), and Barmah Forest virus (BFV), cause incapacitating and long lasting articular disease/myalgia. Outbreaks of viral arthritis and the global distribution of these diseases point to the emergence of arthritogenic alphaviruses as an important public health problem. This review discusses the molecular mechanisms involved in alphavirus-induced arthritis, exploring the recent data obtained with *in vitro* systems and *in vivo* studies using animal models and samples from patients. The factors associated to the extension and persistence of symptoms are highlighted, focusing on (a) virus replication in target cells, and tissues, including macrophages and muscle cells; (b) the inflammatory and immune responses with recruitment and activation of macrophage, NK cells and T lymphocytes to the lesion focus and the increase of inflammatory mediators levels; and (c) the persistence of virus or viral products in joint and muscle tissues. We also discuss the importance of the establishment of novel animal models to test new molecular targets and to develop more efficient and selective drugs to treat these diseases.

1. Introduction

Alphaviruses are enveloped single-stranded positive-sense RNA viruses that belong to the *Togaviridae* family. They are transmitted to humans through the bite of mosquitos from the genera *Culex sp.* and *Aedes (A. albopictus* and *A. aegypti*), in a cycle involving vertebrate reservoir hosts [1, 2]. Alphaviruses are subgrouped accordingly to the prevalence of the clinical symptoms they cause in humans. The encephalitic alphaviruses occur in the Americas and are associated with severe and lethal encephalitis. This group includes the Venezuelan, Eastern, and Western equine encephalitis viruses [3]. The arthritogenic group causes incapacitating and long lasting articular disease/myalgia and comprises the Ross River virus (RRV), Chikungunya virus (CHIKV), Sindbis virus (SINV), Mayaro virus (MAYV), O'nyong-nyong virus (ONNV), and Barmah Forest virus (BFV) [2, 4]. These viruses are globally distributed and are responsible for endemic diseases in some regions (Table 1).

Epidemiological studies on alphaviruses' infections are restricted due to insufficient surveillance and laboratory diagnostic analyses in most endemic countries, which result in an underestimation of the numbers of cases [5, 6]. Similarities between the clinical manifestations of the diseases caused by alphaviruses and those caused by others virus, such as dengue virus (a member of the *Flaviviridae* family) or Oropouche virus (a member of the *Bunyaviridae* family), also make the diagnosis difficult [7, 8]. This is especially frequent in the case of MAYV infections, in which the limited diagnosis of cases makes the illness largely unknown [6, 8, 9]. Studies on CHIKV infection were also limited before the epidemics at the La Réunion Island, a French territory in the southwest Indian Ocean, where more than 200,000 habitants were infected between 2005 and 2007 [10,

Table 1: Occurrence and geographic distribution of arthritogenic alphaviruses.

Virus	First description	Geographic distribution	Occurrence	References
RRV	1928, in New South Wales, Australia	Australia, Papua New Guinea, Solomon Islands, and the South Pacific Islands	Endemic in Australia and Papua New Guinea, annual epidemics in Australia (~4,000 cases per year). Major epidemics: ~60,000 cases in 1979 in Pacific Islands ~8,000 cases in 1996 in Australia	[2, 4, 23]
SINV	1952, in Sindbis village, near Cairo, Egypt	Europe, Asia, Africa, and Oceania.	Endemic in North Europe; Outbreaks in Finland, Norway, Sweden and Russia (late summer or early autumn)	[2, 4, 21]
CHIKV	1952, in Newala, Tanzania	Africa and Asia (documented cases in Europe, USA, and Oceania)	Sporadic epidemics in Africa and Asia, imported cases reported in Europe and USA. Major epidemics: ~300,000 cases in 2006-2006 in La Réunion (French Indian Ocean territory) ~1.4–6.5 million cases in 2006-2007 in India	[4, 59, 60]
MAYV	1954, in Trinidad and Tobago	Northern South America	Endemic in tropical regions of South America Sporadic outbreaks Pan-Amazonia forest regions	[4, 8, 19]
ONNV	1959, in northern Uganda	Africa	Rare epidemics in Africa (disappeared for 35 years from 1961 to 1996) ~2 million cases in 1959–1961 in East Africa	[2, 4]
BFV	1974, in the Barmah Forest, Australia	Australia	Annual epidemics in Australia (~1,000 cases per year)	[4, 25]

11]. In this outbreak, more than 50% of CHIKV-infected adults presented a severe disease with persistent joint pain [12–14]. After this CHIKV epidemics, several other cases of CHIKV infection were described in many countries and systematic efforts on the investigation of the pathogenesis of CHIKV infection allowed a rapid increase in the knowledge regarding the disease [11, 15, 16]. In contrast, epidemics of ONNV infection, which promote a disease similar to that caused by CHIKV, have been described in Africa since 1959, although ONNV and the pathogenesis of its infection have remained unstudied so far [17, 18]. The outbreaks of RRV, SINV, CHIKV, and some descriptions of MAYV cases are nowadays considered sufficient to point the emergence or reemergence of arthritogenic alphaviruses as an important public health problem with challenges on vector control and development of new strategies to prevent and treat these diseases [19, 20].

In this review, we aimed at discussing the molecular mechanisms that may be associated with exacerbation of muscular/articular damage and with the establishment of arthritis as well as the persistence of symptoms of the alphavirus infection, exploring recent data obtained with *in vitro* systems and *in vivo* studies using animal models and samples from patients.

2. Alphavirus-Induced Arthritis

Arthritogenic alphaviruses usually cause an acute disease, with the onset of symptoms after 3–10 days after infection, and a short (4–7 days) viremia period [18, 21–23]. The clinical manifestations include fever, headache, rash, fatigue, arthritis, arthralgia, and muscular pain [4]. Rash occurs in over 40% of the cases and may appear before, simultaneously or after arthralgia symptoms, lasting 7–10 days [23–26]. Fever can be absent in some cases, mainly in SINV, RRV, and BFV infections [21, 26, 27]. Arthritis is the most prevalent among the symptoms, with the recovery from pain and swelling occurring after some days of infection, although several reports describe the persistence of joint manifestations for months or even years [2, 19, 22, 28–31]. Joint pain and inflammation mainly affect symmetrically the small joints (such as those from fingers, wrists, and tarsus), but eventually occur in the large joints (such as those from knees and shoulders) and may also involve several joints simultaneously (polyarthralgy/polyarthritis) [13, 21, 29, 30]. Besides rash and arthritis, myalgia is a very common symptom during alphaviruses infection, demonstrating also the virus tropism for the muscular tissue [32].

Cellular inflammatory infiltration in joint, muscle, and associated tissues during alphavirus infection has been reported in some mouse models of RRV, SINV, and CHIKV infection, suggesting that muscular and articular damage is an immunopathological inflammatory disorder [33–35]. In RRV and CHIKV infection, the cellular infiltrate reaches synovial tissue, which shows a strong hyperplasia [34, 36, 37]. Monocytes, macrophages, NK cells, and $CD4^+$ and $CD8^+$ T lymphocytes are the main cellular components of the inflammatory infiltrate in animal models, indicating an involvement of these cells in the pathogenesis of the arthritis induced by alphaviruses [34, 36–38]. In agreement with the data obtained in animal models, macrophages and NK cells have been detected in synovial exudates from RRV infected patients [39–41], and a pronounced increase in the plasma levels of inflammatory mediators as well as a high $CD8^+$ T lymphocyte activation were found in CHIKV patients in the acute phase

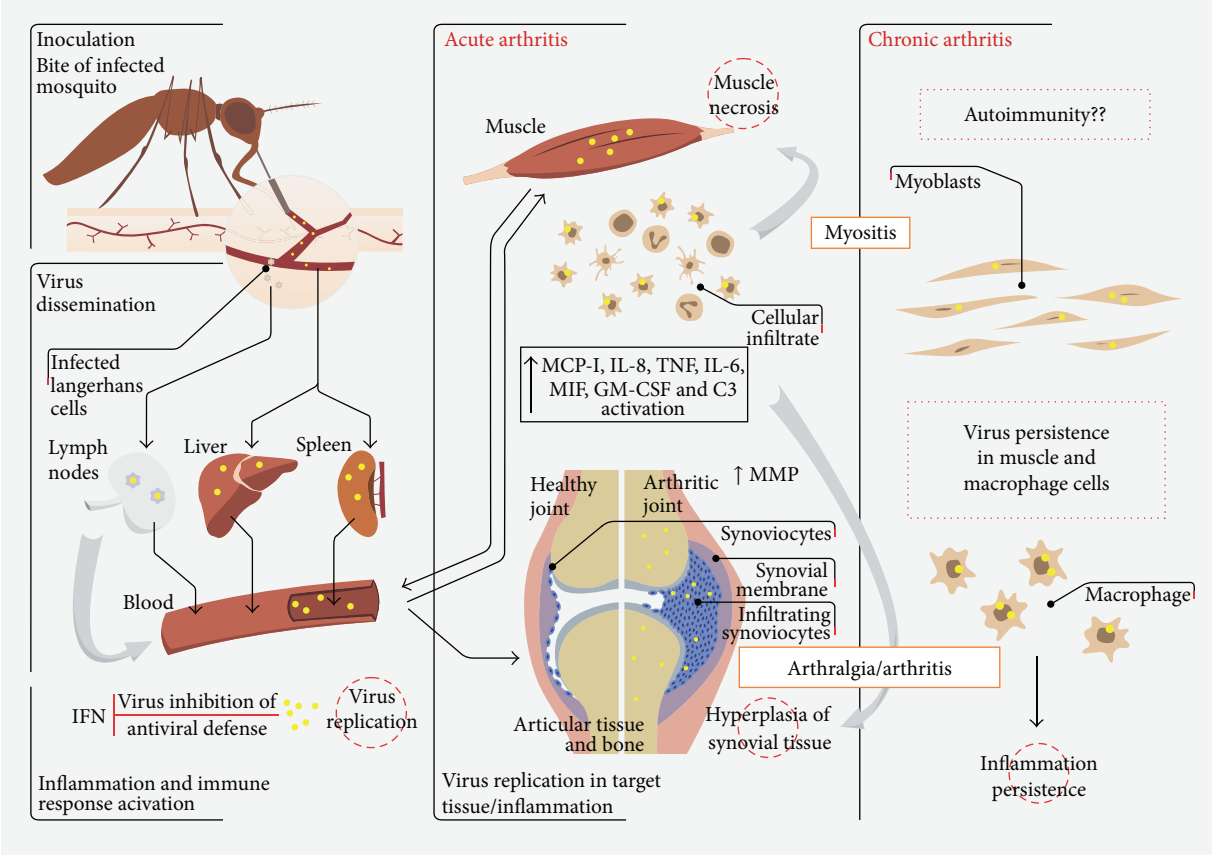

Figure 1: *Pathogenesis of alphavirus-induced arthritis/myositis*. After inoculation through the bite of an infected mosquito in the skin, alphaviruses disseminate in the host organism through the bloodstream. Liver, spleen, muscle, and lymph nodes are sites of primary replication, allowing an efficient virus spread. Langerhans cells facilitate virus delivery to the lymph nodes. Interferon (IFN) program is early activated, but the alphaviruses developed several mechanisms to inhibit this antiviral response. The acute phase of the disease involves virus replication followed by an inflammatory response in the target tissues, which is characterized by an extensive infiltration of lymphocytes, NK cells, neutrophils, and macrophages (the main component). The increase in the levels of several proinflammatory cytokines and chemokines in the site of infection and in the plasma is associated with myositis and arthralgia/arthritis. Also, the secretion of metalloproteinases (MMP) in the joint tissue may contribute to articular damage. Persistence of the symptoms may be related to the persistence of the virus or its products in the target cells with the subsequent accumulation of inflammatory mediators such as IL-6 and GM-CSF. A question that remains open is whether an autoimmune process is associated to the persistence of the inflammatory response, as observed for rheumatoid arthritis.

of infection [42]. Furthermore, an isolated strain of CHIKV from La Réunion epidemics was able to induce a marked swelling of the hind foot in 6-week-old mice 7 days after local subcutaneous injection, which is consistent with the rheumatic symptoms observed in humans [37].

Chronic arthralgia and arthritis due to alphavirus infection cause clinical manifestations ranging from only a restriction of movements with persistence of swelling and pain to a severe and incapacitating disease [14, 28, 29, 43, 44]. Several studies in which patients infected with CHIKV were accompanied for long periods after La Réunion epidemics consistently demonstrated the chronic and severe manifestation of disease [14, 31, 43, 45]. Also, long lasting myalgia, arthralgia, and arthritis occur in about 25–55% of patients infected with RRV, SINV, and CHIKV [14, 30–32, 45–47]. In BFV infection, duration of symptoms seems to be reduced, and MAYV infection is very poorly described in the literature [26]. The causes of the persistence of symptoms remain inconclusive but seem to be associated with the intensity of the inflammatory process, the extension of articular lesion, and the presence of viral products in the joint tissue, as well as due to an autoimmunity process [4, 48].

3. Pathogenesis of the Arthritis Caused by Alphaviruses

After subcutaneous inoculation by the mosquito bite, alphaviruses seem to be disseminated in the host through the lymph nodes route and the microvasculature (Figure 1). Leukopenia in acute phase of the disease is a very common hematologic alteration in alphavirus infection, suggesting a primary replication of the virus in the leukocytes [19, 49, 50]. Liver and spleen are also considered sites of primary viral replication and contribute to virus dissemination [51]. After dissemination, the virus reaches bones, muscles, and articular

tissues, generating the acute phase of the disease, which is strongly associated with a local inflammatory process [34–37, 52]. Host age, the status of the immune system, virus strain virulence, and viral persistence are key determinants for the pathogenesis of alphavirus infection in animals [37, 53, 54]. For example, mice susceptibility to SINV-infection seems to involve age-dependent inflammation associated with stress response to infection [55–58].

Disease severity and persistence of symptoms are associated to the extension of virus replication and the presence of inflammatory mediators in the plasma of patients and in specific tissues of animal models [36]. Interestingly, some cytokines secreted during alphavirus infection are the same of those associated with the progression of rheumatoid arthritis (RA), although inflammation in RA is clearly associated to an autoimmune process, which has not been consistently demonstrated for alphavirus-induced arthritis [48, 61]. Despite particular differences, expression analysis of inflammatory genes in a mouse model of CHIKV infection demonstrated similarities between the induced genes in this model and those induced in RA and collagen-induced arthritis models [61]. Furthermore, specific polymorphisms in human leukocyte antigen (HLA) as well as autoimmunity development, both conditions previously associated to patients' predisposition to rheumatic diseases and RA, were also observed in alphavirus-induced arthritis. The RA-associated alelles HLA-DRB1*01 and HLA-DRB1*04 were identified in CHIKV chronic patients [62]. These patients were later diagnosed for RA, and some of them were positive for autoantibodies, such as the rheumatoid factor (RF), anti-CCP (cyclic citrullinated peptide), and anti-nuclear antibodies, suggesting a role of CHIKV infection in RA initiation [62]. SINV infection also seems to be associated to HLA alleles involved in rheumatic diseases, in particular HLA-DRB1*01 [32, 63]. In addition, SINV-infected patients showed elevated titers of autoantibodies, including anti-nuclear and mitochondrial antibodies, with significant increase in RF three years postinfection [63]. Moreover, HLA-DR7 has been shown to be increased in patients with polyarthritis following RRV infection [64]. Taken together, these observations suggest that RA and alphavirus-induced arthritis share a set of common characteristics that could be useful in the development of therapeutic approaches against viral arthritis.

3.1. Role of the Target Cells for Alphavirus Replication in the Pathogenesis of Arthritis. Articular and nonarticular cells are involved in alphavirus replication and dissemination. Experimental models of alphavirus-induced arthritis suggest that pathogenesis results from a combination of a direct cellular and tissue damage caused by virus replication and an indirect immune response activation in target tissues [34, 37, 65]. Several cell types have been described as targets for arthritogenic alphavirus replication, including cells from joints, bones, and muscles as well as immune cells infiltrated in the synovium and in the infected tissues (Figure 1), highlighting the association between the tissues affected by virus replication and the local inflammatory process in the pathogenesis of alphavirus-induced arthritis.

SINV causes a persistent infection with periodic appearance of cytopathic effects in mouse fibroblasts cultures [66, 67]. In adult mice, SINV replicates in the periosteum, tendons, and endosteum of long bones [35]. Additionally, SINV has been isolated from a muscle biopsy of a patient with chronic myalgia and arthralgia 6 months after onset of the symptoms, indicating virus persistence in muscle cells [32]. This isolated virus was able to replicate in human myoblasts and myotubes cells *in vitro*, confirming virus tropism to muscle cells. Muscle necrosis accompanied by a massive infiltration of inflammatory cells has been observed in mouse models for RRV and CHIKV infection [34, 36, 68, 69]. Furthermore, CHIKV antigens were detected in skeletal muscle progenitor cells in patient biopsies during both the acute phase of CHIKV infection and the late recurrent symptomatic phase of the disease, with muscle necrosis and an inflammatory infiltrate observed in late phase [70]. The long lasting replication of RRV and CHIKV in muscle cells has been also supported by studies *in vitro* using primary mouse and human skeletal muscle cells, respectively [70, 71], reinforcing that viral replication in muscle cells is closely associated with acute and chronic myalgia observed in patients.

Macrophage has been described as the main component of cellular infiltrate observed in the injured tissues after alphavirus infection *in vivo* [34, 51]. The first evidence and the characterization of the central role of macrophage in arthritis pathogenesis have been demonstrated in studies with RRV. RRV antigens were detected in synovial monocytes/macrophages of patients after the beginning of the symptoms onset [47]. Furthermore, lineages of mouse monocytes/macrophages infected with RRV *in vitro* supported a continuous production of viruses for over 50 days after infection with restricted cytopathic effects [33, 72]. Additionally, pharmacological depletion of macrophages in mouse models of RRV and CHIKV infection resulted in lesser extent of muscular/articular damage, demonstrating the importance of macrophages for disease progression [33, 37, 73]. The ability of other alphaviruses besides RRV to replicate and persist in macrophages has also been demonstrated [74–76]. Primary human monocytes and macrophages infected with SINV and CHIKV showed a highly productive viral replication [75, 76]. In an immunocompetent nonhuman primate animal model of CHIKV infection, viral RNA was found 90 days postinfection mainly in spleen and lymph nodes, and macrophages appear to be the primary cells responsible for viral persistence in late stages of infection in this model [51]. Contribution of macrophages to the disease establishment may be due to an association between the maintenance of viral replication and the synthesis of inflammatory mediators in damaged tissue (Figure 1). Additionally, soluble factors secreted from macrophage can amplify the inflammatory process recruiting and activating lymphocytes and NK cells to target tissues [42, 49]. Thus, macrophages seem to be the most suitable candidate for viral reservoirs in affected tissues, playing a central role in alphavirus-induced arthritis.

3.2. Immune Response and Inflammatory Mediators in Alphavirus-Induced Pathology. Several clinical, *in vivo,* and *in*

vitro studies have been carried out to further elucidate the inflammatory process triggered by alphavirus infection and its participation in arthritis pathogenesis.

To investigate the role of cellular immune response during alphavirus infection, several animal models of arthritis induced by RRV, CHIKV, or ONNV were developed. Severe inflammation was observed in bone, joint, and muscle tissues in a mouse model of RRV infection [34], and this inflammatory process was not altered in infected mice deficient in the recombinase activating gene ($RAG^{-/-}$), which lack the functional T and B lymphocytes [34]. Furthermore, a recent study with adult $RAG2^{-/-}$, $CD4^{-/-}$, and $CD8^{-/-}$ CHIKV-infected mice demonstrated that CHIKV-specific $CD4^+$ but not $CD8^+$ T cells are involved in joint swelling [77]. Together, these observations suggest that adaptive immune response has a restricted role in RRV and CHIKV disease pathology. In contrast, pharmacologic depletion of macrophages in mice infected with RRV resulted in the abrogation of disease symptoms and in a lower expression levels of IFN-γ, TNF-α, IL-β, MCP-1 and MIP-1α in muscle and joint tissues when compared to RRV-infected undepleted mice [38, 73]. Moreover, neutralization of IFN-γ, TNF-α, and MCP-1 reduced the clinical score of RRV-infected mice [73]. Similar effects of macrophages depletion was also evident in CHIKV infection, demonstrating a critical role of innate immunity in disease progression [37]. This was reinforced by the observation that CHIKV-infected patients who developed chronic symptoms showed an intense activation of several immune cells in the acute phase of the disease, including the DC, NK, $CD4^+$, and $CD8^+$ cells [31].

Infection by arthritogenic alphaviruses results in the production of a broad range of cytokines and chemokines, which were systematically detected through distinct experimental approaches (Table 2). The profile of these inflammatory mediators has been associated with the severity and persistence of infection. Proinflammatory mediators, such as IL-6, TNF-α, IFN-α/β, and IFN-γ were detected in the sera from RRV-infected and CHIKV-infected mice as well as CHIKV-infected nonhuman primates [37, 51, 73, 79]. The viremia phase was correlated to increased serum levels of several chemokines, such as MCP-1, RANTES, and IP-10, as well as an increase in their mRNA expression in the affected tissues [37, 51, 73, 79]. A strong local activation of the IFN-γ program was also demonstrated in the symptomatic phase of the disease [79]. In agreement with these observations, *in vitro* studies showed an increased expression of IL-8, MCP-1, and GM-CSF in synovial fibroblasts infected with RRV [78]. Consistently, CHIKV infection of a mouse macrophage lineage was associated with an enhanced production of TNF-α, IL-6, and GM-CSF [74]. In addition, primary human osteoblasts were shown to be susceptible to CHIKV infection *in vitro* and infection induced IL-6 and RANKL secretion by these cells with similar kinetics, while osteoprotegerin secretion was gradually inhibited [52]. Thus, infection of osteoblasts by CHIKV and the consequent IL-6 production may contribute to bone loss and to the occurrence of arthralgia and arthritis [52]. Interestingly, a comparison between CHIKV-induced and RA-induced gene expression in mouse models showed a remarkable similarity regarding the immune mediators, including IFNs, IL-4, IL-10, TNF-α, IL-15, GM-CSF, IL-8, and lymphotoxin B [61]. Furthermore, the overlap of gene expression profile between these two diseases increases with severity.

In a clinical study, CHIKV-infected patients in Singapore, the plasma levels of several cytokines and chemokines, including IFN-α, IL-6, IL-12, GM-CSF, IP-10 and MCP-1, correlate with the viral load, and plasma levels of IL-6 and GM-CSF were significantly increased in patients with persistent arthralgia [50]. In similar clinical studies, higher levels of IL-1β, IL-10, and IL-6 were also detected in patient sera, being IL-1β and IL-6 identified as biomarkers of disease severity and persistence [80]. In addition, IL-6 has been associated with the generation of joint pain [83], which reinforces the importance of this cytokine in the progression of disease. Besides cytokines, chemokines such as MCP-1, MIP-1α, and MIP-1β were increased during the chronic phase of CHIKV infection [81]. Elevated levels of MCP-1 were also found in RRV-infected patients [73]. On the other hand, low levels of RANTES were observed in severe and chronic patients [80, 81]. Another clinical study performed during a CHIKV outbreak in Italy showed that IL-6 and the chemokines CXCL9/MIG, MCP-1, and IP-10 were significantly increased in acute phase of disease [82]. In the same work, CXCL9/MIG, IP-10, and high titers of IgG were found in patients with mild and severe symptoms six months after initial infection when compared to recovered patients, suggesting that these factors may be used as disease severity markers [82]. These findings show again a remarkable similarity between alphavirus-induced arthritis and RA, in which CXCL9/MIG and IP-10 are also used as disease markers [84–88]. Also, IgG antibodies seem to be implicated in alphavirus infection as well as in RA, in which these antibodies act through the activation of the mast cells leading to synovial destruction and immune complex formation within the joint [89, 90].

MCP-1 levels are increased in patients in the majority of the clinical studies of alphavirus-induced arthritis [50, 73, 81, 82], suggesting an important role of this chemokine in recruitment of inflammatory cells to injured tissues. In CHIKV-infected patients MCP-1, IL-6, and IL-8 levels were higher in synovial fluids than in the sera, suggesting an active monocyte/macrophage trafficking into the synovial tissue. High levels of matrix metalloproteinase-2 (MMP2) were also found in the synovial tissue of one chronic patient, which would be one of the factors involved in tissue lesion [31]. In agreement, inhibition of MCP-1 action in animal models of RRV and CHIKV infection reduces inflammatory infiltrated, also supporting this hypothesis [91, 92]. MIF, a key cytokine in RA, has also been implicated in the exacerbation of the inflammatory process in RRV and SINV infection [65, 76]. In RA, MIF stimulates synovial macrophages to release several cytokines and the matrix metalloproteinases MPP1 and 3, contributing to tissue destruction in the joints [93, 94]. Likewise, we have demonstrated that SINV replication in human macrophages induced MIF, TNF-α, IL-1β, and IL-6 secretion, followed by an enhancing in the expression of MMP1 and 3, and that cytokine secretion

Table 2: Inflammatory mediators in arthritogenic alphaviruses infection.

Virus	Cell cultures infected *in vitro*	Animal models	Patients		References
			Acute phase	Chronic phase	
RRV	IL-8, GM-CSF, MCP-1	MIF, MCP-1, MIP-1α, TNF-α, IL-1β, IFN-γ	TNF-α, IFN-γ, MCP-1		[65, 73, 78]
CHIKV	IL-6, TNF-α, GM-CSF, MCP-1	IFN-α/β IFN-γ, KC, MCP-1, IP-10, IL-6, IL-10, IL-1β, TNF-α, IL-15, GM-CSF	IL-6, IFN-α, IP-10, IL-12, IL-1Ra, MCP-1, IL-10, IL-15, MIG	IL-6, GM-CSF, IL-1β, IL-8, IL-1Ra, MCP-1, MIP-1α, MIP-1β	[31, 37, 50–52, 61, 74, 79–82]
SINV	IL-6, TNF-α, IL-1β, MIF				[76]

and MMP expression were primarily regulated by MIF [76]. Additionally, RRV infection of MIF-deficient mice caused a mild disease when compared to that developed in wild-type animals, with inflammatory infiltrate reduction accompanied by a lower expression of MCP-1 and IFN-γ in muscle and joints, leading to a decrease in muscle tissue destruction, although the viral titers were similar [65]. As expected, RRV-infected wild-type mice treated with recombinant MIF developed more pronounced disease signs.

3.3. Involvement of the Complement Cascade in the Arthritis Caused by Alphaviruses. Complement activation was detected in the synovial fluids of RRV-infected patients. Levels of C3a, a marker of the central complement system C3 processing, were higher in RRV-infected patients than in patients with noninflammatory osteoarthritis [95]. In agreement with these observations, recent findings obtained using a mouse model of RRV-induced arthritis showed that complement is important to promote inflammatory tissue destruction [95]. Besides the detection of the complement activation products in the serum and in the inflamed joints and muscles of RRV-infected wild-type mice, RRV-infected C3-deficient mice ($C3^{-/-}$) developed a less severe disease and also presented much lower levels of skeletal muscle destruction, despite having similar inflammatory infiltrates than RRV-infected wild-type mice [95].

C3 receptor (CR3 or CD11b/CD18, Mac-1, $\alpha_m\beta_2$) binds several different ligands, including iC3b, a C3 cleavage fragment. As observed for $C3^{-/-}$ mice, RRV-infected CR3-deficient mice ($CD11b^{-/-}$) develop a less severe disease and lower tissue destruction when compared to RRV-infected wild-type mice [96]. CR3 deficiency had no effect on viral replication and inflammatory infiltration, but the expression of the proinflammatory proteins S100A9, S100A8, and IL-6 were significantly reduced in RRV-infected $C3^{-/-}$ and $CD11b^{-/-}$ mice when compared to RRV-infected wild-type mice [96]. In agreement, the levels of heterodimeric complex formed by S100A9 and S100A8 were elevated in the sera of patients with RA or inflammatory muscle diseases, in which the expression of these proteins by macrophages had been associated with muscle fibers degeneration [97–99].

The complement activation pathways that are determinant for the pathogenesis of RRV infection in mice were identified using deficient mice for the key components of the classical ($Clq^{-/-}$), alternative (factor B, $Fb^{-/-}$), or mannose binding lectin ($MBL^{-/-}$) pathways [100]. RRV-infected $MBL^{-/-}$ mice developed less pronounced disease signs, with reduced tissue damage and C3 deposition in muscle tissues. On the other hand, infected $Clq^{-/-}$ and $fB^{-/-}$ mice presented normal disease progression and severity [100]. These observations suggest that RRV infection leads to complement activation through MBL pathway, which contributes to RRV disease severity. In RRV-infected patients, higher MBL levels in both serum and synovial fluid correlated with polyarthritis severity [100], reinforcing the importance of MBL pathway.

3.4. Role of Alphavirus Evasion from Host Antiviral Defense in Pathogenesis. Type I IFN immune response signaling is essential for the control of viral replication and could be the key process in preventing virus dissemination toward the target tissues and the development of alphavirus-induced arthritis. Indeed, IFN-stimulated genes (ISGs) are critical in controlling CHIKV, RRV, SINV, and ONNV replication [17, 101–103]. In a mouse model of ONNV infection, deficiency in STAT, which couples IFN signaling, increases disease lethality [17]. Mice deficient in type I IFN were more susceptible to CHIKV infection, with a broader dissemination of the virus, which reaches the central nervous system besides replicating in liver, muscles, and joints [54]. Viperin, product of an ISG, has been also shown to be critical for host antiviral response to CHIKV infection. Viperin expression, together with type I IFNs and some related ISGs expression, was highly induced in PBMCs of CHIKV-infected patients with a viral load-dependent profile, and CHIKV-infected mice deficient in viperin showed an enhanced viral load and a more severe joint inflammation when compared to infected wild-type mice [104]. Studies using samples from a cohort CHIKV-infected patients showed a tight association between high viral load and an enhanced expression of IFN-α/β and several genes of the type I IFN signaling pathway, such as IRF3, IRF7, and RSAD2 (viperin encoding gene), in patients PBMCs [104]. Furthermore, CHIKV infection activates directly IRF3, inducing the transcription of IFN-β itself and several ISGs through the activation of IPS-1 [105]. In SINV infection, the induction of type I IFN expression was also dependent

on the activation of IRF3, which occurs through the host intracellular pattern recognition receptor (PRR) MDA5 [106]. RRV has been also shown to be recognized by PRR: mice deficient in Myd88 or TLR7 genes infected with RRV develop more extensive tissue damage and higher viral titers than infected wild-type mice [107]. TLR7-deficient mice also produce elevated levels of RRV specific antibodies but with little neutralizing activity and lower epitope affinity when compared to RRV specific antibodies produced by wild-type mice [107]. CHIKV clearance seems to be dependent on both RIG-like receptors and TLRs, which trigger a type I IFN response that acts directly in nonhematopoietic cells, controlling CHIKV replication in the local of infection and preventing virus dissemination [108].

Despite inducing IFN production, arthritogenic alphaviruses are able to antagonize type I IFN response (Figure 1). SINV replication bypasses the need of a functional IFN-induced phosphorylated eiF2α for translation, using an alternative pathway to locate the ribosomes on the initiation codon of the viral RNA [109]. Although CHIKV induces ISGs expression, it promotes a widespread translation shutoff of cellular genes through eiF2α phosphorylation by PKR, while the translation of viral proteins is maintained. [105]. In late infection, CHIKV also induces transcription shutoff of IFN-β and ISGs. In addition, the nonstructural protein nsP1 antagonizes the action of the ISG BST-2 (bone marrow stromal antigen 2, a protein impairs CHIKV particles budding from the infected cells) [110].

Several alphaviruses' virulence factors are involved in viral persistence and evasion from the immune system. Mice deficient in STAT1-dependent IFN response infected with CHIKV developed a much more severe muscoloskeletal pathology with an increased viral replication in joint-associated tissues when compared to infected wild-type mice [111], supporting the hypothesis that alphaviruses' ability to inhibit the IFN-induced JAK/STAT signaling pathway is related to their virulence *in vivo*. Also, infection of adult mice deficient in IRF3 and IRF7 with CHIKV is lethal, and mortality has been associated with an increased virus replication and pathogenesis [112].

Genetic determinants in viral nonstructural proteins nsP1 and nsP2 were also associated to the modulation of STAT activation and to the virulence in SINV and RRV [113, 114]. Additionally, SINV nsP2 has been implicated in the development of the cytopathic effect induced by infection [115]. Furthermore, small-plaque mutant RRV (with mutations in E2 and nsP regions) showed increased resistance to IFNα/β antiviral response compared to the parental strain, which allows high virus titers in mice, leading to an increase in the severity of hind limb disease, myositis, and mortality [116].

The induction of type I IFN response by RRV is also dependent on whether the virus is produced by mammalian or mosquitos cells. The mosquito cell-derived virus fails to induce IFNα/β due to the lack of complex carbohydrates on virus particle, and it seems that N-linked glycans in E2 glycoprotein from the mammalian-cell-derived virus are needed for a strong IFN response [117, 118].

Altogether, these findings suggest that viremia control in alphavirus infection depends on different factors such as the presence of strain virulence determinants in nsP1 and nsP2, the extent of the induction of type I IFN response during infection as well as the virus ability to evade from this response. Since IFN response is activated early in the disease, viral persistence in affected tissues during chronic phase of arthritis might be seen as a failure in this early response.

4. Concluding Remarks

Even with the recent advances in the understanding of the pathogenesis of joint damage associated with alphavirus infection, many gaps remain and need to be explored. Most of the studies are currently focused on CHIKV infection and therefore the differences and similarities among the mechanisms involved in arthropathy induction by the distinct alphaviruses still cannot be pointed out. Improvements in the diagnostic of new cases as well as in the generation of animal models for the study of the arthritis induced by SINV and MAYV consist in a key challenge for the progress in a broader understanding of the mechanisms involved in alphavirus-induced arthritis.

The data accumulated so far indicate that the pathogenesis involved in alphavirus-induced joint damage is determined by host inflammatory response as well as by virus persistence and virulence. Inflammatory response includes the production of cytokines, chemokines, and other inflammatory mediators that are involved in macrophage, NK, and T cells recruitment to the sites of viral replication (Figure 1). Viral persistence could occur in target tissue, as muscles and joint connective tissues, but macrophages seem to be the main viral reservoirs and may play an important role in virus dissemination to the target tissues. Chronic infection of host cells is also closely related to the chronic disease establishment and the long lasting of the symptoms. Furthermore, differences in alphavirus genetic determinants promote virulence and evasion from the cellular antiviral response, which may contribute to disease development.

Some efforts have been made toward the development of therapeutic approaches against alphavirus-induced arthritis. Drugs used to control inflammation in patients with RA have been used as supportive therapy to joint symptoms in patients infected with RRV and CHIKV, but the results were limited and variable [28, 69, 119]. Mouse models for RRV and CHIKV infections have been useful to test drugs that control host inflammatory response, such as bindarit, an inhibitor of MCP-1 receptor [91, 92]. Nonetheless, the understanding of the mechanisms involved in the pathogenesis of alphavirus-induced arthritis as well as the establishment of novel animal models are essential steps to the development and characterization of new molecular targets and more efficient and selective drugs to treat these diseases.

Authors' Contribution

Iranaia Assunção-Miranda and Christine Cruz-Oliveira contributed equally to this work.

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Review Article

Possible Future Monoclonal Antibody (mAb)-Based Therapy against Arbovirus Infections

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More than 150 arboviruses belonging to different families are known to infect humans, causing endemic infections as well as epidemic outbreaks. Effective vaccines to limit the occurrence of some of these infections have been licensed, while for the others several new immunogens are under development mostly for their improvements concerning safety and effectiveness profiles. On the other hand, specific and effective antiviral drugs are not yet available, posing an urgent medical need in particular for emergency cases. Neutralizing monoclonal antibodies (mAbs) have been demonstrated to be effective in the treatment of several infectious diseases as well as in preliminary *in vitro* and *in vivo* models of arbovirus-related infections. Given their specific antiviral activity as well-tolerated molecules with limited side effects, mAbs could represent a new therapeutic approach for the development of an effective treatment, as well as useful tools in the study of the host-virus interplay and in the development of more effective immunogens. However, before their use as candidate therapeutics, possible hurdles (e.g., Ab-dependent enhancement of infection, occurrence of viral escape variants) must be carefully evaluated. In this review are described the main arboviruses infecting humans and candidate mAbs to be possibly used in a future passive immunotherapy.

1. Introduction

Arthropod-borne virus (arbovirus) infections are increasingly becoming an emerging medical problem mostly affecting endemic areas such as developing countries or upcoming economies (like China and India). In particular, the major outbreak source of arbovirus-related diseases in endemic areas is mostly related to the presence of the viruses in an animal reservoir and a following expansion in humans. Moreover, epidemic episodes, which occur mainly during seasons with increased disease activity or outbreaks (e.g., because of climate variations), have also been described. In addition, increasing traveling to exotic and medically high-risk locations has enlarged this problem also to previously non-endemic areas, due to the global rise of travelers and movement of large populations [1].

Of the over 545 suspected arbovirus species, the most known virus-transmitting arthropods (vectors) are mosquitoes (mostly female *Aedes aegypti* and *Aedes albopictus*), ticks, midges, and sandflies. Humans are usually dead-end hosts, as they do not develop the high viremia required to infect arthropods that is sustained by vertebrate animal reservoirs [1].

Although several arboviruses of clinical significance in humans are known (more than 150), only a restricted group of them is globally diffused, the majority of which are zoonotic and belong to the Flaviviridae, Bunyaviridae, or Togaviridae families, with a small number belonging to Reoviridae and Orthomyxoviridae [1]. Highly effective vaccines for several of them are available, including tick-borne encephalitis (TBEV) [2], yellow fever (YFV) [3], and Japanese encephalitis (JEV) viruses [4], but for no one of them a specific antiviral drug is currently approved for clinical use. In the course of viral infections, neutralizing monoclonal antibody (mAb)-based therapy represents a promising and safe alternative strategy, in particular when a specific and efficacious treatment is not yet available [5–11]. At present, human mAb-based passive immunotherapies for arbovirosis are at very early stage of development. However, previous studies in mice have shown that passive transfer of either monoclonal or polyclonal Abs

can be protective against homologous or heterologous dengue virus (DENV) challenge as well as against other *flaviviruses* and human arboviruses. Moreover, engineering rendering mAbs capable of crossing the blood-brain barrier in order to limit viral dissemination within CNS may be considered.

Finally, a possible administration of mAbs in those subjects that could be at risk of exposure to arbovirus infections, such as travelers in endemic areas, could reduce the possible incidence and consequent augmented risk of epidemic episodes.

In this review, we describe the major clinical relevant and worldwide diffused arboviruses infecting humans and the recently major described mAbs to be possibly used in a future passive immunotherapy.

2. *Flaviviruses*

The *Flavivirus* genus, including more than 70 viruses, is the only one within the Flaviviridae family which holds arboviruses that are responsible for significant morbidity and mortality worldwide [12].

About 2.5 billion people are at risk of infection in tropical and subtropical countries, mainly South-East and South Asia, Central and South America, and the Caribbean. In addition, multiple *Flavivirus* infections have been reported in the same areas, complicating early diagnosis and identification [13].

Flavivirus infections can cause fever, encephalitis, hemorrhagic disease, flaccid paralysis, and death in humans. However, the immunopathogenesis of these viruses is not fully understood. In the last decade, the *flaviviruses* have reemerged as aggressive human pathogens [13].

The human *flaviviruses* includes 53 recognized species. However, five of them are considered clinically important like DENV, YFV, JEV, TBEV, and West Nile virus (WNV).

The genome of all the members of the Flaviviridae family consists of a 9.5–12.5 kb positive-sense, single-stranded RNA. They are enveloped small virions (40–60 nm in diameter) with two or more species of envelope glycoproteins (e.g., M and E proteins), which are involved in the binding and fusion processes. In particular, the precursor of the mature M protein (prM) interacts with E glycoproteins, acting as a chaperone and preventing the fusion of the virus with the membrane in the cell during egress through acidic compartments of the secretory pathway. Then, cleavage of prM by the cellular protease furin during transit through the Golgi network is a required step in the viral lifecycle that defines the transition from an immature non-infectious virus particle into an infectious form. However, immature infectious virions retaining some uncleaved prM molecules could be released [14].

The M and E glycoproteins constitute an icosahedral scaffold surrounding a nucleocapsid, which consists of the viral genome complexed with a core of approximately 30 nm composed of multiple copies of a small, basic capsid (C) protein. Binding, uptake and fusion by target cells are believed to involve clathrin-mediated and low-pH-induced endocytosis [14].

Flaviviruses can utilize multiple receptors for different cell types and host species. They are thought to firstly interact with dendritic cells through DC-SIGN and L-SIGN binding of glycans on E glycoprotein dimers. In addition, highly sulfated glycosaminoglycans (e.g., heparan sulfate) have been demonstrated to play an important role in the initial attachment of several *flaviviruses* to their target cells. Other molecules identified as possible receptors are integrins, mannose-binding receptor on macrophages, laminin-binding protein, GRP78 (BiP), and CD14 [15].

Uncoating and replication of the viral genome, through a minus-strand RNA intermediate, occurs in the cytoplasmic replication complexes associated with perinuclear membranes, where viral proteins are produced as part of a single long polyprotein of more than 3,000 amino acids, generating three structural (C, prM, and E) and seven non-structural (NS1, NS2A, NS2B, the viral protease NS3, NS4A, NS4B, and the RNA-dependent RNA polymerase NS5) proteins by cleavage of host and viral proteases, respectively. Progeny virions are thought to assemble by budding into an intracellular membrane compartment, probably the endoplasmic reticulum, then transited through the host secretory pathway, and released at the cell surface [16].

Efforts to develop effective prophylactic approaches for several clinically important *flaviviruses* are underway [17]. The crucial role of the humoral immune response against *Flavivirus* infections is well established, as infection with one serotype provides life-long protective immunity to the homologous infecting serotype and cross-protection in the first few months against the other serotypes. Conversely, individuals experiencing a secondary infection with a distinct serotype are at greater risk of severe complications, as discussed later [18].

Only a limited number of *Flavivirus* vaccines are available today; however, no approved antiviral drugs are yet available for their clinical use. Thus, giving the lack of vaccines as well as specific antiviral drugs, broadly cross-neutralizing mAbs, could be helpful in the development of an effective therapeutic strategy against these infections as well as in the progress towards effective immunogens.

In the next paragraphs, we describe the molecular targets of *flaviviruses*, including DENV, JEV, TBEV, WNV, YFV, and St. Louis encephalitis virus (SLEV) followed by a description of the therapeutic candidate mAbs directed against them.

2.1. Domains and Functions of the Surface Envelope (E) Glycoprotein of Flaviviruses . The major target of the host humoral immune response and of neutralizing Abs against *flaviviruses* is represented by the envelope (E) glycoprotein, which is a 56-kDa protein and the major represented antigen on the surface of virions [19]. However, Ab response towards other structural and non-structural proteins, such as prM and NS1, respectively, has also been described [20, 21]. However, prM-specific Abs display limited neutralizing activity, while anti-NS1 Abs have been detected only during the convalescent period of a primary DENV infection but were strongly identified during the acute phase of a secondary DENV infection, suggesting that both Abs could contribute to the pathogenesis of the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [22].

The crystallographic structure of the E protein of TBEV has been proposed as a model for the envelope protein of the *flaviviruses* [23].

Further studies performed through X-ray crystallography have revealed that the E protein is a type II viral fusion protein with three β-barrel domains (D): DI, DII, and DIII (corresponding to the antigenic domains C, A, and B, resp.) connected to the viral membrane by a helical structure called the stem anchor [24]. In its native form the E protein folds as an homodimer with an antiparallel structure and an unusual herringbone pseudoicosaedral symmetry pattern with the M protein located centrally within the symmetry, consistent with a head-to-tail configuration lying parallel to the envelope lipid bilayer. The DIII of the E glycoprotein (amino acid residues 295–395) has an immunoglobulin-like fold, contains seven β-strands as well as type- and subcomplex-specific neutralizing B-cell epitopes and is the proposed receptor-binding domain through four peptide loops on the solvent exposed face. Directly linked to DIII, there is the stem/transmembrane region, spanning amino acid residues 401–495 and containing regions important for oligomerization with prM protein (amino acid residues 431–449 and 450–472) [25]. DII has a long, finger-like structure and contains two extended loops that project from DI and the highly conserved and hydrophobic glycine-rich fusion loop at its tip (amino acid residues 98–110), interacting with the stem region and the endosomal membrane of the cell during the fusion process [26]. In the trimeric conformation, the hydrophobic fusion peptide is exposed in DII to mediate the fusion with the membranes of the cell. DI is a ~120 amino acid central domain, formed by a discontinuous hinge region consisting in a central β-barrel of eight strands that connects DII and DIII and plays a crucial role in the structural rearrangement of E from a homodimer to a trimer, which occurs on exposure to low pH of endosomes that is required for the membrane fusion process. In some *flaviviruses*, an N-linked glycosylation site is present at amino acid position 154–156 of the E protein and does not appear to be necessary for E function, but it has been associated with the increased neuroinvasiveness of WNV lineage I outbreak strains from the USA and a pH-sensitive decrease in stability of the non-glycosylated strains [27].

2.2. Flavivirus Neutralizing B-Cell Epitopes. As previously described, Abs are a significant component of the host's protective response against *Flavivirus* infections. However, virus-specific Abs have been implicated in the pathogenesis of severe clinical manifestations following a secondary DENV as well as YFV and WNV infections.

Moreover, cross-reactivity between *Flavivirus* serogroups could complicate the interpretation of diagnostic assays but could be of interest in isolating cross-reactive and cross-neutralizing mAbs. However, Ab-dependent enhancement (ADE) of infection has been described for this genus as it represents the major risk of complications when reinfection occurs. Thus, great care must be taken in evaluating neutralizing activity and possible Ab-mediated infection enhancement in the characterization of future possible therapeutic mAbs against these viruses [28].

However, prophylactic and therapeutic use of neutralizing mAbs for *Flavivirus* infections has been shown to be effective in animal models as reported later.

Neutralizing epitopes were found to be located in each of the three domains of the E protein and have been confirmed to be surface exposed in the high-resolution X-ray crystal structure of the pre-fusion dimer of DENV2 E glycoprotein [29].

Most potent mAbs against *flaviviruses* are directed toward epitopes on DIII. For this reason, DIII-based immunogens are under evaluation as promising subunit *Flavivirus* candidate vaccines [30, 31]. However, several cross-reactive mAbs that bind to residues from the AB loop were found to be poorly neutralizing as this loop projects toward the lipid bilayer in the mature viral particle. Conversely, lateral ridge region of DIII (e.g., BC, DE, and FG loops) is targeted by strong, serotype-specific neutralizing mAbs [32–35]. Cross-reactive mAbs specific for DII have also been described but with less neutralizing and variable profiles. However, few of them, whose epitope encompasses the fusion loop, are cross-reactive and neutralizing [36].

2.3. Ab-Dependent Enhancement (ADE) of Infection. *In vitro* and *in vivo* studies have demonstrated that increased disease severity, causing DHF/DSS, upon reinfection with a different DENV serotype, is mostly due to the phenomenon of ADE of infection, determined by cross-reacting but poorly or non-neutralizing Abs, generated during the primary infection, that facilitate virus entry through Fc-γ or complement receptors present on cells, such as monocytes/macrophages (Figure 1(a)). Additionally, in 6 to 9 month-old children of DENV immune mothers, severe disease is associated with primary infection, possibly because of declining levels of neutralizing maternal Abs [64].

These cross-reactive Abs are mainly directed against the E and prM glycoproteins. In particular, experimental lines of evidence have suggested that cross-reactive Abs against DII of E protein can, under certain conditions, enhance infectivity of WNV *in vitro* [65]. However, the phenomenon of ADE could reflect the presence of non-neutralizing concentrations of virus-reactive Abs and has been observed for several *flaviviruses in vitro* and *in vivo*, such as WNV, TBEV, JEV, and YFV as well as other Flaviviridae members [28, 66–68]. In particular, it has been described that ADE of infection occurs at virus-bounded Ab concentrations in the upper limit by the stoichiometric threshold for neutralization, and at lower concentrations by the minimal number of Abs that allow attachment of the virion to cells. Furthermore, Abs that recognize infrequently displayed epitopes that do not support neutralization may enhance infection even at saturation [69].

Thus, the ADE of infection mechanism may pose a threat for the development of a safe and efficacious vaccine as well as a possible immunotherapy against Flaviviridae.

Indeed, cross-neutralizing mAbs, as candidate therapeutics for these infections, may be considered after having well ascertained their broadly cross-neutralizing activity and the absence of possible mAb-mediated ADE of infection mechanisms. Alternatively, the risk of ADE of infection could be overtaken by the removal of the Ab heavy chain (i.e., the CH2

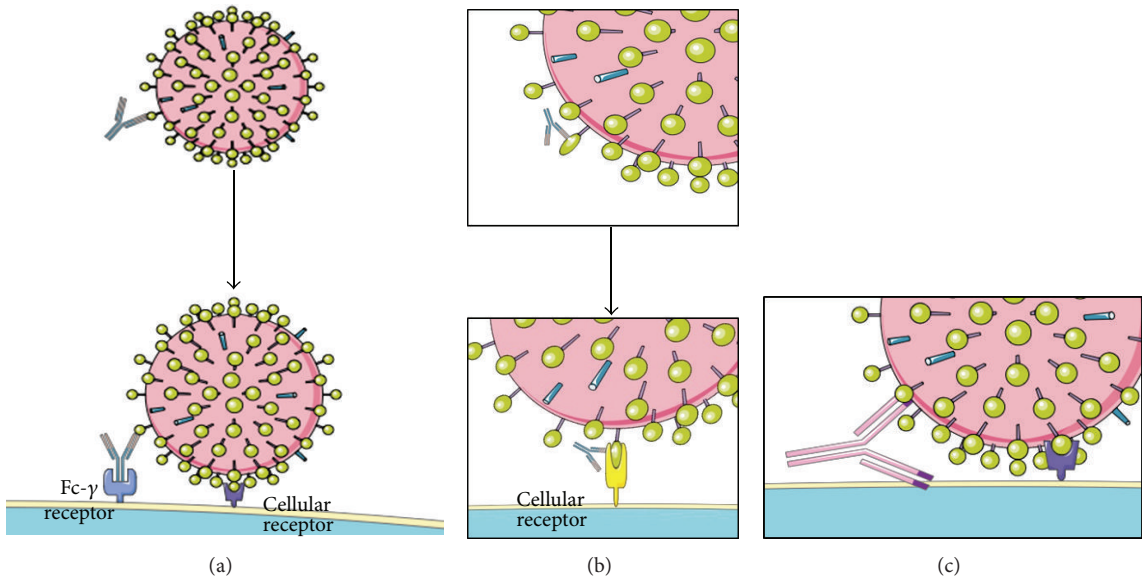

Figure 1: Mechanisms of antibody-dependent enhancement (ADE) of infection. (a) After binding to the viral epitope, the Ab is recognized by a cellular Fc-γ receptor, bringing the viral particle in proximity of the target cell; (b) the binding of the Ab induces conformational changes within the structure of the viral target protein. These changes improve the affinity for the cellular receptor; (c) molecular mimicry by a viral motif of cellular membrane components leads an autoreactive Ab to bind both the viral and the cellular target, bringing the virus in proximity of the target cell.

and CH3 portions or direct expression of the mAb as a Fab fragment) and/or deletion of the N-linked sugars on IgG molecules that are both required for interactions with Fc-γ receptors (Fc-γR), or eventually, by the blocking of Fc-γR engagement with anti-Fc-γR Abs [70]. Similarly, the identification and detailed examination of cross-neutralizing epitopes that do not promote ADE of infection may define novel targets for vaccine development.

However, in some instances, ADE of infection may occur when Ab molecules cross-react with both viral and cellular antigens (Figure 1(c)). In this regard, Fc-γ receptor-independent mechanism of infection, in particular for DENV prM-specific Abs able to bind simultaneously to the virus and target cells, has been described. Indeed, other members of the Flaviviridae belonging to another genus, like hepatitis C virus (HCV), have been shown to elicit polyreactive Abs that are probably responsible for an ADE of infection mechanism as well as of secondary clinical manifestations. In connection with this, several studies, employing both polyclonal and monoclonal Abs, suggested the presence of common epitopes containing particular short motifs on NS1 and the three domains of the E protein as well as human proteins that may play a role in ADE of infection mechanisms and thus in DHF/DSS pathogenesis [70]. Interestingly, these common motifs have been found to be additionally represented in DENV2 strains which have greater human pathogenic capacities [22].

Moreover, another molecular mechanism of ADE of infection could consist in the facilitation of conformational changes of the targeted surface protein that are required for virus entry, as described and hypothesized for other viruses (e.g., HIV, HCV, and HSV) (Figure 1(b)) [71, 72].

Finally, Fc-γR engagement could result also in "intrinsic" ADE of infection mechanisms involving signaling events, such as inhibition of antiviral response and increased viral replication. Disrupted signaling events include the RIG-I/MDA5 cascade and type I interferon (IFN) response as well as induction and suppression of anti-inflammatory (IL-10) and proinflammatory (IL-12 and TNF-α) cytokines, respectively [73].

2.4. Dengue Virus (DENV). Dengue virus (DENV) is responsible for 50–100 million symptomatic infections each year, resulting in 500,000 hospitalizations and over 20,000 deaths which occurs mostly in tropical and subtropical regions of the world. Although malaria remains the most important cause for systemic febrile disease in travelers, chikungunya virus (CHIKV) and DENV are increasingly diagnosed, with dengue currently being the second most important cause for febrile disease in travelers.

Decreases in mosquito control efforts during the end of the 90s, coupled with societal factors (e.g., globalization, migrations, and dense urbanization) have contributed to the reemergence of *flaviviruses* such as DENV in South and Central America. Development of effective DENV vaccines that exhibit cross-protection, thought to be important for preventing subsequent dengue-associated immunopathogenesis, is proving to be particularly challenging [1].

A vaccine for this infection is not yet available despite considerable public and private efforts. This difficulty is mainly due to the perceived need to simultaneously protect the four known serotypes of DENV (DENV1–4), which share about 70% of sequence homology, while genotypes can vary up to 3%. Moreover, as anticipated and discussed previously,

non-protective Abs may contribute to more severe clinical outcomes in vaccinated people [74].

2.4.1. Murine versus Human Anti-DENV Humoral Immune Response. It is well ascertained that anti-E DIII-directed Abs are virtually absent from the naïve human repertoire as they are directed away from this domain, probably against weakly neutralizing and immunodominant regions. Moreover, it has been demonstrated that over than 90% of the human Ab response of primary DENV infected patients is able to bind only native DENV virus particles instead of a recombinant form of the E protein, posing difficulties in the cloning strategies of neutralizing mAbs [39]. Thus, it can be concluded that *in vivo* only a small fraction of DENV-specific Abs are responsible for neutralization.

Conversely, murine mAbs that recognize all the three domains of E have been identified. Epitope-mapping studies using murine serotype-specific mAbs have demonstrated that loops located in the lateral ridge region of DIII constitute the strongest neutralizing B-cell target. In this region, sequence diversity between serotype is high while cross-reactive murine mAbs that recognize another loop showed generally a weaker neutralization profile. In particular, type-specific mAbs with neutralizing activity against DENV2 localized to the BC, DE, and FG loops on the lateral ridge of DIII, whereas subcomplex-specific mAbs recognized an adjacent epitope centered on the connecting A strand of DIII at residues K305, K307, and K310 [32–35].

Cross-reactive mAbs directed against prM and NS1 have also been described, but, as anticipated, they feature weakly neutralizing or absent as well as ADE of infection activity. In the following paragraphs, we report the better characterized neutralizing mouse and human mAbs, describing their molecular features further summarized in Table 1.

2.4.2. Anti-DENV Murine mAbs. Murine mAbs directed against all the three domains of the E protein and endowed of different neutralizing and binding characteristics have been described. However, only DIII and DII are classically recognized by neutralizing Abs. In this regard, Sukupolvi-Petty et al. isolated twenty-four anti-DENV2 mouse mAbs with moderate or strong neutralizing activity against the homologous virus in cell culture assays. Binding sites were mapped for the majority of these in distinct epitopes in regions located in DI (lateral ridge), DII (dimer interface, lateral ridge, and fusion loop), and DIII (lateral ridge, CC′ loop, and A strand). Moreover, 16 of the neutralizing mAbs were tested in mice, with most of them being protective when given as prophylaxis. Seven of these had post-exposure therapeutic activity when administered as a single dose by intraperitoneal route even 3 days after intracranial infection. For the mAbs with the greatest therapeutic potential, protection was confirmed with an Ab-enhanced vascular leakage mouse model of DENV2 infection [35].

Recently, Deng et al. described a cross-reactive murine mAb, named 2A10G6, that is able to recognize DENV1–4, YFV, WNV, JEV and TBEV, to potently cross-neutralize DENV1–4, YFV and to a lesser extent WNV. This mAb recognizes a highly conserved motif (amino acid residues 98–101) located within the fusion loop of the *Flavivirus* DII of the E glycoprotein. Moreover, this mAb exerts its neutralizing activity in a post-attachment step during the virus entry process, as demonstrated by kinetic neutralization tests performed *in vitro*. Additionally, protection experiments performed in mouse models showed that treatment with 100 μg/mL of 2A10G6 conferred full protection against lethal DENV2 challenge, and 20 μg/mL and 4 μg/mL of 2A10G6 protected 89% and 40% of infected mice from lethal challenge, respectively. For infection with DENV1, 3, and 4, use of 100 μg/mL of 2A10G6 conferred partial protection, and 53%, 77%, and 73% of the infected mice survived after challenge, respectively. Finally, the protection profile of 2A10G6 against WNV showed that prophylactic administration with a single dose of 200 mg of 2A10G6 conferred 80% protection in mice. Most importantly, 3 of 8 (37.5%) mice survived when 2A10G6 was administered one day after WNV challenge. Similarly, another mouse mAb, 4G2, is able to recognize the fusion loop at the extremity of DII from all four DENV serotypes and to prevent syncytia formation [36].

Rajamanonmani et al. described a mouse mAb, 9F12, raised against the DIII of DENV2 E protein, that is able to recognize DENV1–4 serotypes as well as WNV and to neutralize five DENV strains representative of all DENV serotypes [41]. Similarly, Cockburn et al. reported a comparative, high-resolution crystallographic analysis of an A-strand DIII murine mAb, 4E11, in complex with its target domain of the E protein from the four DENV serotypes. MAb 4E11 is capable of recognizing and neutralizing all four serotypes with IC50 values varying between 1 and 100 nM. The structures reported also highlight the mechanism by which anti-A-strand mAbs disrupt the architecture of the mature virion, inducing dimer dissociation, premature fusion-loop exposure, and concomitant particle inactivation [40]. Also, Midgley et al. functionally characterized another murine mAb, 2H12, raised in mice against DIII of E protein. Similarly to the previously described mAbs; 2H12 is able to bind all the four DENV serotypes in a epitope encompassing the conserved ^{314}ETQH^{317} motif. However, the neutralizing potential of 2H12 is lower than a number of other anti-DIII mAbs, with IC50 values ranging from 0.56 to 145 nM for DENV1, 2, and 4. On the other hand, it showed no ADE of infection activity [38].

Austin et al. isolated a murine mAb, named E111, which recognize a novel CC′-loop epitope on DIII of the E protein from two different DENV1 genotypes. Docking of the mAb structure onto the available cryoelectron microscopy models of DENV virions revealed that the E111 epitope was inaccessible, suggesting that this mAb recognizes an uncharacterized virus conformation. While the affinity of binding between E111 and DIII varied by genotype, a limited correlation with neutralizing activity was observed. These data support the conclusion that potent neutralization depends on genotype-dependent exposure of the CC′ loop epitope [49]. In fact, as previously described elsewhere, binding of some E reactive Abs depends on the dynamic movement of protein molecules "breathing" in the virion particle leading to transient exposure of hidden epitopes. For instance, optimal binding of mouse mAb 1A1D-2 to EDIII requires incubation at 37°C [75].

Table 1: Schematic summary of the best characterized mAbs against arboviral pathogens.

mAb	Origin	Reactivity	Target protein	Epitope	Cloning strategy	Format/isotype	Neutralizing activity	Reference
4.8A D11C 1.6D	Human	DENV1–4	E	DII	EBV transformation	IgG	IC50 (μg/mL): DENV1: 1.5 DENV2: >40 DENV3: 2.4 DENV4: >40 IC50 (μg/mL): DENV1: 1.5 DENV2: 1.0 DENV3: 10.2 DENV4: 1.6 IC50 (μg/mL): DENV1: 1.5 DENV2: 0.2 DENV3: 0.5 DENV4: 2.7	[37]
2H12	Murine	DENV1–4	E	DIII (AB loop: aa 314–317)	Hybridoma from BALB/c mouse immunized with DENV2 E/DIII	IgG2b	IC50 nM: DENV1: 0.56–54 DENV3: 29 DENV4: 145	[38]
C9	Murine/chimeric	DENV2	E	DIII	Phage display of a chimeric murine hybridoma library	VH1/Vκ1	PRNT50 850 μg/mL	[39]
4E11	Murine	DENV1–4	E	DIII (strand A: 308, 312 and strand G: 387, 389, 391)	Hybridoma	IgG2a/κ	IC50 (μg/mL): DENV10.16 DENV2: 0.13 DENV-3: 8 DENV-4: 15	[40]
4G2	Murine	DENV1–4	E	DII (fusion loop)	Hybridoma from DENV2 E immunized mice	IgG	PRNT50 (μg/mL): DENV2: 15	[39]
9F12	Murine	DENV1–4, WNV	E	DIII (aa 305, 307, 310, 330; A strand and BC loop)	Hybridoma from BALB/c mouse immunized with DENV-2 E/DIII	IgG1k	DENV1–4PRNT50: $2 \cdot 10^{-8}$–$2 \cdot 10^{-7}$ M	[41]
2A10G6	Murine	DENV1–4, YFV, WNV, JEV, TBEV	E	DII (Fusion loop: aa 98–101)	Hybridoma from BALB/c mouse immunized with inactivated DENV2	IgG1	PRNT50 (μg/mL): DENV1: 2 DENV2: 1.5 DENV3: 2.1 DENV4: 1.8 YNF: 3.6 WNV: 46	[36]
mAb11	Human	DENV1–4, WNV, SLEV, YFV, JEV MVEV	E	DII (fusion loop)	Phage display of human scFv	Fusion protein scFv-Fc	PRNT80 (μg/mL): WNV: 1.25 DENV2: 6.25	[42, 43]

Table 1: Continued.

mAb	Origin	Reactivity	Target protein	Epitope	Cloning strategy	Format/isotype	Neutralizing activity	Reference
E16 (MGAWN1)	Murine	WNV	E	DIII (LR; aa 302–309)	Hybridoma from immunized mice with WNV E	IgG2b/humanized	PRNT50: 4–18 ng PRNT90: 53–297 ng	[44, 45]
1A1D-2	Murine	DENV1–3	E	DIII (A strand: aa 307, 310 and 312)	Hybridoma from immunized mice with different pH-treated virus	IgG2a	DENV2 PRNT50: 2.1 nM	[46, 47]
1F4	Human	DENV1	E (virion)	DI-DII	Electrofusion of infected memory B cells from DENV-immune subjects with EBV	IgG	DENV1 IC50 0.11 μg/mL	[48]
2D22		DENV2		DIII			DENV2 IC50 0.08 μg/mL	
5J7		DENV3		DI-DII			DENV3 IC50 0.10 μg/mL	
E105	Murine	DENV1	E	DIII (BC loop: G328, T329 and D330; DE loop: K361E and E362K; FG loop: K385)	Hybridomas derived from C57BL/6 IFN-$\alpha\beta R^{-/-}$ mice infected with DENV1	IgG	PRNT50 DENV-1: 0.5–59.2 ng/mL	[32]
E106				DIII (BC loop: G328, T329 and D330; DE loop: K361E and E362K; FG loop: K385; A strand: S305, K307, E309, K310, and E311)			PRNT50 DENV-1: 0.6–59.2 ng/mL	
E111				DIII (CC′ loop)			PRNT50: 3.8–25 μg/mL	[49]
CR4374	Human	WNV	E	DIII	Phage display of scFv IgG library	VH2-05/VL1e	PRNT50: 0.18 μg/mL PRNT90: 0.95 μg/mL	[50]
CR4353						VH3-30/Vk3-A27	PRNT50: 0.026 μg/mL PRNT90 36.4 μg/mL	
1A5	Chimpanzee	DENV1–4, WNV, JEV, LGTV	E	DII (aa G106)	Phage display of Fab library from DENV1–4 infected chimpanzees	Humanized IgG1	PRNT50 (μg/mL): DENV1: 0.48 DENV2: 0.95 DENV3: 3.2 DENV4: 4.3 WNV/DENV4: 3.8 JEV: 21 LGTV: 28	[51]

Table 1: Continued.

mAb	Origin	Reactivity	Target protein	Epitope	Cloning strategy	Format/isotype	Neutralizing activity	Reference
mAb11	Human	DENV1–4, WNV	E	DII (fusion loop, W101, G104, G106)	scFv library	scFv-Fc	PRNT80 (μg/mL): DENV2: 6.25 WNV: 1.25	[42, 43]
3B4C-4	Murine	SLEV	E	1a	Hybridoma	IgG	SLEV PRNT: <1.7	[52, 53]
1B2C-5		SLEV		1b			SLEV PRNT: <1.7	
6B5A-2		SLEV		1c			SLEV PRNT: 4.8	
4A4C-4		SLEV		1d			SLEV PRNT: 2.9	
1B5D-1		SLEV, JEV		2			SLEV PRNT: <1.7	
2B5B-3		SLEV, JEV, MVEV, WNV, YFV		3			SLEV PRNT: 2.3	
2B6B-2		All *Flavivirus*		4a			SLEV PRNT: <1.7	
6B6C-1		All *Flavivirus*		4b (in DII)			SLEV PRNT: 2.3	
A3	Chimpanzee	JEV	E2	DI aa. K179	Phage display	Humanized	PRNT50 0.04–0.2 nM	[54]
B2				DII aa. I126	Phage display		PRNT50 0.02–2 nM	
E3				DIII aa. G132	Phage display		PRNT50 0.14–0.93 nM	
FabTJE12B02	Human	JEV	E	N/A	Phage display	Fab	FRNT50 50.2 μg/mL	[55]
5F10	Human	CHIKV	E2	Domain B	EBV transformation	IgG1λ2	IC50 < 100 ng/mL	[56]
8B10	Human	CHIKV	E1-E2	E2 Domain A	EBV transformation	IgG1k	IC50 < 100 ng/mL	
CHK-152	Murine	CHIKV	E2	aa 59	Hybridoma	IgG2c	IC50 1–3 ng/mL	[57]
11E7	Murine	CCHFV	Gn	C-ter	Hybridoma	IgG	PRNT80 diluted 1/2560	[58]
4-39-CC	Murine	RVFV	G2	Domain IV	Hybridoma	IgG	PRNT80 diluted 1/20480–1/81920	[59]
2C9	Murine	YFV	E		Hybridoma	IgG2a	PRNT90: $1/10^4$ 17D $1/10^{5.2}$ Asibi	[60]
5A 7A R3(27)	Human	YFV	E	DI-DII	Phage display	ScFv	PRNT90: 0.1–0.3 μg/mL	[61]
13D6	Murine	TBEV	E	DIII	Hybridoma	IgG/chimeric	PRNT50: 1.9 μg/mL FRNT50: 4.5 μg/mL IC50 1.9–16.7 μg/mL	[62]
3B4C-4 (Hy4-26C)	Murine	VEEV	E2	aa 182–209	Hybridoma	IgG/humanized	PRNT70 (μg/mL): 39.4–125	[63]

The structure of the 1A1D-2 bound to EDIII indicates that the mAb binds to sites that are transiently exposed during viral "breathing" at 37°C and block infection during attachment of the virion to the cell. In particular, this mAb neutralizes DENV1, 2, and 3 serotypes and residues K307 and K310 are the most critical residues for binding of 1A1D-2 mAb [46, 75].

Finally, Shrestha et al. immunized mice with a genotype 2 strain of DENV1 virus and generated 79 new mAbs 16 of which strongly inhibited infection by the homologous virus and localized to DIII of E protein. Surprisingly, only two mAbs, E105 and E106, retained strong binding and neutralizing activity against all five DENV1 genotypes as well as being protective in immunocompromised infected mice. Moreover, E105 and E106 exhibited therapeutic activity even when administered as a single dose four days after inoculation with a heterologous genotype 4 strain of DENV1 in the same mouse model [32].

2.4.3. Other Non-Human mAbs. Other non-human and non-murine as well as humanized mAbs directed against the E glycoprotein have been described. In this regard, Goncalvez et al. isolated a large panel of anti-E Fab fragments from chimpanzees infected with all four DENV serotypes. However, only a limited number of them displayed a cross-neutralizing activity against DENV1 and 2 and to a lesser extent against DENV3 and 4. In particular, the authors calculated that, among them, the 1A5 mAb that was further humanized, neutralized DENV1–4 at a PRNT50 titer of 0.48, 0.95, 3.2, and 4.3 μg/mL, respectively. Interestingly, the humanized 1A5 was also tested for binding and neutralization against the WNV/DENV4 chimera, JEV strain SA14-14-2, and Langat virus (LGTV) strain TP 21, giving a PRNT50 titer of 3.8, 21, and 28 μg/mL, respectively. Moreover, the authors calculated that when administered in a dose of 2 mg per kg of body weight 1A5 would give a serum titer of approximately 40 and 20 50% reduction in plaque reduction neutralization test (PRNT50) against DENV1 and DENV2, respectively [76]. Furthermore, epitope mapping of 1A5 mAb localized to G106 within the *Flavivirus*-conserved fusion loop in DII of DENV2 E protein [51].

2.4.4. Anti-DENV Human mAbs. As anticipated, attempts to isolate neutralizing human mAbs have been more challenging due to the restricted elicited Ab-repertoire which recognizes DIII of E glycoprotein in naïve as well as in infected patients [77, 78]. However, several groups reported the isolation of neutralizing mAbs from infected subjects. In this regard, Setthapramote et al. isolated a total of 136 human hybridoma clones producing specific mAbs against DENV, obtained using PBMCs from nine blood samples from four acute-phase patients secondarily infected with DENV2 and five convalescent-phase patients. Interestingly, the authors found that most of the acute-phase mAb clones were cross-reactive with all four DENV serotypes, with most of them recognizing the E protein and endowing of neutralizing activity against all DENV serotypes, compared to those derived from convalescent-phase patients. In particular, from the acute-phase PBMCs, 81.8% were anti-E, 6.6% were anti-prM, and 3.3% were anti-NS1, while 13.3% anti-E, 13.3% anti-prM, and 53.3% anti-NS1 mAbs clones were obtained from convalescent-phase PBMCs [79]. Previous studies confirmed the data obtained by Setthapramote et al. on convalescent-phase patients, but this group firstly reported the efficient preparation of human mAbs with strong neutralizing activity titers against all four DENV serotypes using PBMCs from acute-phase patients secondarily infected with DENV [80, 81].

The group of de Alwis, through immunoglobulin depletion studies, reported that a substantial fraction of DENV-reactive Abs in human immune sera, including type-specific neutralizing Abs, bound to the intact virion but not to recombinant E protein. The authors confirmed these observations also isolating human neutralizing mAbs and proposed that humans produce Abs that neutralize DENV infection by binding a complex, quaternary structure epitope that is expressed only when E proteins are assembled on a virus particle [48]. Similar findings have been described for several other viruses, including WNV and HIV [82, 83]. Mapping studies indicate that this epitope has a footprint that spans adjacent E protein dimers and includes residues at the hinge between domains I and II of E protein [48].

Recently, Costin et al. isolated three broadly neutralizing anti-DENV human mAbs named 4.8A, D11C, and 1.6D. These mAbs were isolated from three different convalescent patients with distinct histories of DENV infection. All three mAbs recognized the E glycoprotein with high affinity, neutralized all four serotypes of DENV but mediated ADE of infection in Fc receptor-bearing cells at subneutralizing concentrations. Mapping studies revealed that all three mAbs bind discontinuous epitopes within the highly conserved fusion-loop region of DII (contacting residues W101, L107, and/or G109) [37].

2.5. Yellow Fever Virus (YFV). The WHO estimates that there are 200,000 cases of yellow fever virus (YFV) infections and 30,000 related deaths every year especially in Sub-Saharan Africa. In fact, different to the majority of human arboviruses and similarly to DENV and CHIKV, YFV has expanded its host range to include humans as an amplifying host [1]. Symptoms occurring from 3 to 6 days after infection with YFV consist mostly in fever, chills, anorexia, lumbosacral pain, nausea, and vomiting. This syndrome lasts about 3 days in most cases and is sometimes followed by a one-day lasting period of remission. Fever will reoccur together with dark hematemesis, melena, petechiae, and other hemorrhagic symptoms. Convalescence is characterized by deep asthenia lasting up to two weeks. Rarely, prognosis can be fatal. An effective live-attenuated vaccine (17D) derived from the Asibi strain by serial passages in chicken embryos is available [84].

2.5.1. mAbs against YFV. In 1983, Schlesinger et al. produced a battery of mAbs after immunization of BALB/c mice by injection of 17D YFV vaccine strain [84]. Among them, the specificity towards the E and NS1 proteins was described, even though anti-NS1 Abs were not able to show neutralizing activity. The 13 IgG and 1 IgM anti-E protein mAbs were classified in five groups according to their specificity. Group A, consisting of the only IgM produced in this experiment,

could neutralize 17D YFV virus only; group B 17D and Asibi strains only; group C Asibi strain only; group D could only neutralize Asibi but not 17D strain, showing also reactivity against DENV2, Zika, or Banzi viruses. All of the four mAbs belonging to this group were able to cross-react with Zika and/or Banzi viruses, and two (4E11 and 5H3) neutralized DENV2 virus, with 4E11 neutralizing also Banzi virus. The 3E9 mAb, the only component of the group E, could not neutralize 17D nor Asibi strains.

All of the IgG mAbs resulted able to protect both prophylactically and therapeutically BALB/c or CD-1 mice from lethal intracerebral challenge with 17D-204 strain [85]. The chimeric form of the group B mAb 2C9 IgG (2C9-cIgG) was able to provide AG129 mice a 72% survival when administered 24 hours before infection with 17D-204 strain. Its murine form could provide the survival of the 95% of the mice. Appreciable results were also obtained when both murine 2C9 and 2C9-cIgG were administered 24 hours post-infection, with the survival of 70% and 20% of mice, respectively. Viral presence was not detected in surviving mice [60].

In 2005, Daffis and colleagues constructed two Ab-phage libraries by cloning the repertoire of YFV-infected patients. Panning was then performed with YFV-17D virions. The scFv-5A, 7A and R3(27) showed a neutralizing activity spanning from 50% to 100% in PRNT assays against both YFV 17D-204-WHO and Asibi strains. In further tests, reactivity was observed against wild-type YFV strains of West Africa genotype I and II (Nigeria 1987 and Asibi strains, resp.), and East/Central Africa (CAR 1986, Ethiopia 1961 strains). A concentration from 0.1 to 0.3 μg/mL could yield a 90% plaque reduction. Reactivity was also observed against the strain Senegal 1990 in a lesser extent. Production of escape variants could demonstrate that scFv-5A, 7A and R3(27) epitopes are built up by residues extensively separated in the monomers of E glycoprotein, that however result in closed proximity when the homodimeric form of E is constituted [61].

2.6. West Nile Virus (WNV). The West Nile virus (WNV) is an epidemic neurotropic virus estimated to be responsible of about 36,000 cases and 1,500 deaths registered in the United States between 1992 and 2012 [86]. WNV antigenicity allows its classification into the Japanese encephalitis virus (JEV) serocomplex. Genomic analysis has revealed two main genetic lineages of WNV: lineage I viruses, circulating in the USA, Europe, the Middle East, Africa, India, and Australia, and lineage II viruses, isolated from Sub-Saharan Africa and Madagascar [1].

WNV was firstly identified in 1937 and is endemic in many countries of Africa, Middle East and West Asia. However, after 1990 frequent outbreaks of WNV infections were reported in Romania, Israel and later in North America and across the USA in 1999 and recently, in 2012 [87].

Most of WNV infected individuals do not develop symptoms while about 20% develop a self-limiting illness called West Nile fever. Acute symptoms include fever, tiredness and swollen lymph glands but in a minority of cases also encephalitis with long-term deficits in cognitive function and motor skills have been reported. However, in less than 1% of infected patients, WNV having crossed the blood-brain barrier, is responsible of neuroinvasive and potentially lethal form of the disease. In these cases, degeneration and apoptosis upon infection of neurons and the consequent inflammatory response can occur. Related symptoms include high fever, coma, muscle weakness, and paralysis. Immunodepression and advanced age have been correlated with an augment risk to develop a severe disease [87]. There are no specific treatment options or licensed vaccines for humans [88].

WNV is transmitted by infected mosquitos and initial replication is thought to occur primarily in dendritic cells in the skin, which migrate to secondary lymphoid tissues where the replicating virus enters the circulation. To date, only efficacious WNV veterinarian vaccines have been licensed, while there are no licensed vaccines for protection against WNV in humans [88].

2.6.1. mAbs against WNV. Broad-spectrum antivirals, such as type I IFN-α, ribavirin, mycophenolic acid, in WNV infection showed ineffective results *in vivo* despite *in vitro* they showed some activity [89].

However, experiments in murine models, extrapolation of clinical data as well as passive administration of pooled immune-γ-globulins (OmriGam), containing a significant titer of neutralizing Abs, before and after infection, showed an important role of the humoral immune response in controlling viremia and prevent viral dissemination. Indeed, the development of a neutralizing mAb-based therapy seems to be encouraging for a possible treatment of infected patients [90–92].

Similarly to what has been observed for DENV, only anti-E Abs have been identified as neutralizing and protective. Also anti-NS1 Abs have shown a protective role, however, their mechanism of protection has not yet been elucidated, as the NS1 protein is secreted from infected cells and not present on the virions [93–95].

In this regard, similarly to what has been described for DENV, studies performed using a naïve human scFv library for panning with purified WNV E protein, support the hypothesis that no Abs against the neutralizing DIII can be isolated. However, DIII specific Abs were isolated in a subsequent study using immunoglobulin libraries obtained from three WNV infected patients for biopanning on purified inactivated virus, virus-like particles consisting of prM and E proteins or recombinant E glycoprotein. Although the proportion of DIII-specific mAbs was low (8%) compared with anti-DII mAbs (47%). Two out of the four anti-DIII mAbs were potently neutralizing and protective *in vivo*, whereas only three out of the 24 anti-DII were weakly neutralizing *in vitro* and non-protective *in vivo*. In particular, residues that are critical for neutralization lies on the regions spanning amino acids 305–312, 330–333, and 365 that are located on adjacent exposed loops of DIII. However, sequence alignment of E protein of different *flaviviruses*, such as DENV and JEV, revealed a considerable variation compared to the whole E protein. This observation further suggests that differently from broadly cross-reactive anti-DII Abs, anti-DIII neutralizing Abs are virus-type specific. However, of

these mAbs, two (CR4374 and CR4353) protected mice from lethal WNV challenge at 50% protective doses of 12.9 and 357 μg/kg of body weight, respectively [50].

Sánchez et al., using three different immunization strategies (i.e., inactivated virus, naked DNA, and recombinant protein), isolated nine murine mAbs, most of which bound to conformation-dependent epitopes in DIII of the E protein. In particular, neutralizing mAbs, named 8B10, 11C2, 10C5, and 17C8, were obtained from mice immunized with inactivated virus alone or in combination with a DNA plasmid and bound to the same region of DIII with high affinity. In contrast, mAbs obtained by immunization with a soluble version of the E glycoprotein did not exhibit neutralizing activity. These non-neutralizing mAbs were cross-reactive with several other *flaviviruses*, including SLEV, JEV, YFV, and Powassan virus, confirming the conserved nature of *Flavivirus* non-neutralizing epitopes [96].

Gould et al., isolated 11 unique human single-chain variable region Ab fragments (scFvs) that bind the E protein of WNV. Among them, a human mAb, named mAb11, expressed as a scFv-Fc fusion protein was further characterized. It recognizes the fusion loop, at the distal end of DII of the WNV E protein and cross-reacts with all four DENV serotypes, and provides protection against DENV2 and 4 as well as WNV [42]. Moreover, therapeutic studies of this mAb in WNV-infection model mice provided substantial protection when administered after 5 days post-infection. Interestingly, a neutralization escape variant of this mAb failed to cause lethal encephalitis (at higher infectious doses) or induce the inflammatory responses associated with blood-brain barrier permeability in mice, compared to the parental WNV, suggesting an important role for the fusion loop in viral pathogenesis [43].

Oliphant et al., isolated an anti-DIII E protein mAb, named E16, from hybridomas obtained after immunization of mice with recombinant WNV E protein, which neutralized all WNV strains with PRNT50 values of 4 to 18 ng and PRNT90 values of 53 to 297 ng. One hundred micrograms of mAb protected greater than 90% of mice from lethal infection and even a single 4 μg treatment of E16 on day 2 after infection prevented mortality. Moreover, humanization of this mAb confirmed as therapeutically effective in mice [44]. Subsequent studies revealed that mAb E16 neutralization is mediated by engagement of four discontinuous segments of DIII including the amino-terminal region (amino acid residues 302–309) and the three connecting loops BC (amino acid residues 330–333), DE (amino acid residues 365–368), and FG (amino acid residues 389–391). Moreover, no ADE of infection was detected when E16 mAb was used at saturating concentrations [45, 47].

Furthermore, results of a Phase I safety study of the humanized E16 mAb (designated MGAWN1) have been reported and suggested that doses of up to 30 mg/kg were well tolerated with few mild adverse events and would provide an excess of virus neutralizing activity for 3-4 weeks after treatment. However, a case of anti-MGAWN1 Ab elicitation occurred with the consequent increased rate of clearance and indeed impacting efficacy. A Phase II safety and efficacy study of MGAWN1 is ongoing [97]. Furthermore, the possibility of preventing or treating WNV-induced memory deficits was recently investigated. In this study, hamsters were treated intraperitoneally with 32 mg/kg of MGAWN1 mAb at 4.5 days after subcutaneously challenging with WNV. Interestingly, MGAWN1 prevented mortality, weight loss and improved food consumption of WNV-infected hamsters compared to controls [98].

Recently, Lelli et al. isolated six anti-E mAbs from inactivated-WNV immunized mice. In particular, three of them (3B2, 3D6 and 4D3) neutralized lineage I and II WNV, with the first two recognizing the same epitopes located on the distal lateral surface of DIII (critical amino acid residue K307). Conversely, 4D3 mAb recognized a novel neutralizing epitope on DII (critical residues S276 and T278). Indeed, further protective and therapeutic studies are needed to ascertain their neutralizing activity *in vivo* [99].

Finally, to conclude, like DENV infection, Abs directed against the M protein are not protective and neutralizing. Moreover, in humans, a skewed humoral immune response against DII has been frequently observed and confirmed also with the hybridoma technology [50]. The isolation and elicitation of neutralizing Abs directed against the fusion loop and DIII of the E protein represent thus the most challenging and promising goal for the development of new effective therapeutic inhibitors and immunogens, respectively.

2.7. Tick-Borne Encephalitis Virus (TBEV). Tick-borne encephalitis virus (TBEV) is one of the most dangerous agents causing human neuroinfections, occurring mostly in Europe and Asia, and with a potential fatal prognosis [100]. TBEV is believed to cause 3,500–10,000 human cases of encephalitis in Europe per year, with a high morbidity in Russia, Czech Republic, Austria, and Germany. In particular, between 1990 and 2007, an average of 8,755 cases of TBE was reported per year in Europe and Russia. Despite the fact that Russia is the country with most infections registered annually, Czech Republic incidence is among the highest in Europe, with 400–1,000 clinical cases reported every year [101].

Three subtypes of TBEV are classified, namely, European, Siberian, and Far-Eastern, sharing most of the genetic and antigenic features [102]. In fact, a high degree of antigenic homogeneity between different strains of TBEV has been described [103].

Clinical manifestations vary among the subtypes, but usually they start with a short febrile period of 7–14 days after the tick bite. Fatigue, headache, and pain in the neck, shoulders, and lower back, together with high fever and vomiting may be present [100]. These manifestations are often followed by an asymptomatic phase lasting from 2 to 10 days after remission from the fever, with possible progression to neurological disease. Neurological symptoms include meningitis, encephalitis, myelitis, and radiculitis. Mortality occurs in 1-2% of the European subtype-infected patients, but fatal prognosis can occur in up to 20–40% of the Far-Eastern subtype-infected patients [104]. Mortality rates of the Siberian subtype are similar to those observed for the European [105].

Effective and safe vaccines against TBEV produced from inactivated virus have been developed and licensed for their

use in humans. However, an emergency therapy in the absence of a mass immunization is needed as no effective treatments are yet available [106].

2.7.1. mAbs against TBEV. Levanov et al. described the chimerization of two murine mAbs (13D6 and 10C2) directed against the DIII and DII, respectively, of the TBEV E glycoprotein. The chimeric mAbs present binding characteristics similar to the parental mAbs. Moreover, as the parental mAbs, only the chimeric mAb 13D6 was able to neutralize TBEV infectivity *in vitro*. In particular, neutralization studies with the murine, chimeric, and scFv forms of mAb 13D6 were performed. In particular, murine 13D6 showed an IC50 titer of 11.5 μg/mL in Focus Reduction Neutralization Tests (FRNT) against TBEV strain 205 and of 2.9 μg/mL in PRNT against TBEV strain Softjin. Chimeric 13D6 showed an IC50 titer of 4.5 μg/mL in FRNT against TBEV strain 205 and of 1.9 μg/mL in PRNT against TBEV strain Softjin. ScFv 13D6 showed an IC50 titer of 16.7 μg/mL in FRNT against TBEV strain 205 and of 11.2 μg/mL in PRNT against TBEV strain Softjin [62].

2.8. Japanese Encephalitis Virus (JEV). Japanese encephalitis virus (JEV) is a mosquito-borne *flavivirus* that over the past few decades has caused several outbreaks throughout China, Southeast Asia, Australia, and Papua New Guinea with a prevalence recently estimated to be of about 70,000 cases/year [107–109]. A 40% mortality was recorded in some of the JEV-affected areas. Moreover, many survivors face some neurological problems and complications [108]. Since 1995, the disease has also emerged in Non-Asian regions such as Northern Australia [110, 111]. The situation in Southeast Asia, however, is further complicated by the overlapping epidemics of JEV and DENV as well as sporadic cases of WNV infections detected in some of the affected areas particularly in India [112].

Effective vaccines, both live-attenuated and inactivated strains of JEV, have been developed and licensed in major countries. However, as for the other arthropod-borne *Flavivirus* members, no specific antiviral drugs are currently available [4].

2.8.1. mAbs against JEV. As previously described for the other *Flavivirus* members, many groups isolated and characterized anti-JEV mAbs showing different neutralizing and protective properties. In this regard, Gupta et al. used combinations of anti-E JEV mAbs (Hs1-4) in mice protection experiments. In particular, they found that the singularly mAb protection ranged from 45 to 65% when 100 μg of mAb were administered, while equimolar combinations of two or three mAbs gave 85–90% or 100% protection, respectively [113]. In similar experiments, Lee et al. demonstrated that a *Flavivirus* anti-NS1 mAb, named 16NS1, cross-reacted with JEV as well as WNV and exhibited protective activity against WNV as well as a lethal JEV infection. However, no neutralizing activity was observed using this mAb against both WNV and JEV in *in vitro* experiments, suggesting the participation of other Ab-mediated mechanisms *in vivo*. In particular, 95% of mice were protected when 500 μg of mAb were administered intraperitoneally and concomitantly to intramuscular injection of JEV.

Overlapping peptide mapping analysis combined with site-specific mutations identified the ^{116}KAWGKSILFA^{125} region and critical amino acid residues, W118 and I122, as 16NS1 mAb epitope, highly conserved in WNV and JEV strains [114].

Arakawa et al. isolated from a combinatorial human Fab library constructed from peripheral blood lymphocytes obtained from JEV hyperimmune volunteers. Among 188 randomly selected clones, FabTJE12B02 showed the best 50% focus reduction endpoint at the concentration of 50.2 μg/mL against the JEV strain Nakayama [55].

Goncalvez et al. isolated three mAbs, named Fabs A3, B2, and E3, by repertoire cloning from chimpanzees initially immunized with inactivated JE-VAX and then boosted with attenuated JEV SA14-14-2. In particular, these mAbs reacted with epitopes in three different E domains: in DI (amino acid residue K179), in DII (I126), and in DIII (G132) for Fabs A3, B2, and E3, respectively. Moreover, these Fabs as well as the derived humanized counterpart mAbs exhibited high neutralizing activities against a broad spectrum of JEV genotype strains. Moreover, these mAbs exhibited a 50% protective dose of 0.84 μg (B2), 5.8 μg (A3), and 24.7 μg (E3) in mouse models. Finally, administration of 200 μg/mouse of mAb B2 one day after otherwise lethal JEV infection protected 50% of mice and significantly prolonged the average survival time compared to that of mice in the unprotected group [54].

2.9. St. Louis Encephalitis Virus (SLEV). St. Louis encephalitis virus (SLEV) was first discovered as the mosquito-borne agent responsible for over 1,000 cases of encephalitis during a 1933 summer outbreak in St. Louis (Missouri) and now is a reemerging human pathogen widely distributed in the American continent, causing several human encephalitis outbreaks over the last 80 years. Additional epidemics have indeed occurred from 1964 to 2006 in the Americas, ranging from the US to Argentina and Brazil. As a member of the *Flavivirus* genus, the SLEV E glycoprotein ectodomain is 68% identical to serocomplex-related JEV E but only 46% and 40% identical to those of DENV2 and TBEV, viruses from different serocomplexes [115].

2.9.1. mAbs against SLEV. In 1983, Roehrig et al. isolated twenty-one hybridomas producing murine mAbs specific for the E glycoprotein of SLEV, strain MSI-7. Serologic reactivities were initially determined by cross-reactivity indirect immunofluorescence assays using 22 strains of SLEV and 8 other related *flaviviruses*. Four groups demonstrating type-, subcomplex-, supercomplex-, and group-specific reactivity patterns were identified. Analysis of hemagglutination-inhibition and virus neutralization subdivided the cross-reactivity groups into eight epitopes (E-1a, b, c, d, E-2, E-3, and E-4a, b), one of them following localized in DII (4b) [52, 53].

Moreover, among the previously described isolated anti-WNV mAbs by Gould et al., the chimeric scFv-Fc 79, effectively neutralized also SLEV, resulting in >80% of PRNT80 when used at 5 μg/mL [42].

3. *Alphaviruses*

Major human arboviruses in the Togaviridae family, Chikungunya (CHIKV) and Venezuelan equine encephalomyelitis (VEEV) viruses, belong to the *Alphavirus* genus, which is composed of viruses with an icosahedral nucleocapsid surrounded by a lipid envelope and glycoprotein spikes. The structural proteins of *alphaviruses* arise through co- and post-translational processing of a polyprotein encoded by a single, positive-stranded RNA producing the capsid (C), PE2, 6 K, and E1. The PE2 glycoprotein is a precursor containing the E3 glycoprotein fused to the amino terminus of the E2 envelope glycoprotein. The PE2 glycoprotein is followed by 6 K, a small membrane-associated protein, and E1, the second polypeptide component of glycoprotein spikes.

Trimerized heterodimers of the E1 and E2 viral glycoproteins form the surface spikes and contain determinants of viral tropism and virulence. The E3 glycoprotein acts as a signal for transport of PE2 across the membranes of the rough endoplasmic reticulum and may promote the formation and intracellular transport of E1-PE2 heterodimers to the cell surface. During transport to the cell surface, PE2 undergoes a maturational cleavage event by a furin-like protease to produce E2 and E3 [116].

The E2 glycoprotein promotes specificity of virus binding to the host cell surface and is a target of the humoral immune response. The E1 glycoprotein mediates fusion of the virion envelope with the membranes of acidified endosomes, allowing release of the nucleocapsid into the cytoplasm and the onset of viral replication. *Alphavirus* E1 shares no appreciable sequence identity to *Flavivirus* E protein. Despite differences in their amino acid sequences and arrangements on the viral particle, the structures of E and E1 are remarkably similar. Indeed, the conservation of three domains has been well documented in crystal structures of *flavivirus* E ectodomains from TBEV, DENV, JEV, and WNV, as well as *Alphavirus* E1 from Semliki Forest virus (SFV) and CHIKV. Abs to the E1 glycoprotein do not typically neutralize virus infectivity *in vitro* but can protect against lethal challenge in animals.

By analogy with the prM protein of dengue *Flavivirus*, furin cleavage of PE2 may begin after transit of an acidic late component of the Golgi body, where E3 is thought to suppress the acid pH-triggered activation of glycoprotein E1 fusion capability. Protective anti-E3 mouse mAbs have also been described [117].

3.1. Chikungunya Virus (CHIKV). The chikungunya virus (CHIKV) belongs to the Semliki Forest clade and was firstly isolated in 1953 in Tanzania during an epidemic outbreak, following occurred also in Asia and Africa. In the last five decades, several CHIKV outbreaks have been described both in Africa and Asia separated by gaps lasting from two to twenty years. In 2005-2006, about 300,000 cases out of 785,000 inhabitants were reported in La Réunion island, with a fatal prognosis for 237 of the patients [117]. Neither Europe nor the Americas have had outbreaks of CHIKV so far, except for imported isolated cases. Three genotypes of CHIKV are described: Asian, East/Central/South African (ECSA), and West African, with an amino acid identity spanning from 95.2% to 99.8%. Recent epidemics in Africa and Indian subcontinent were caused by strains belonging to the ECSA genotype. Transmitted by *Aedes* mosquitoes, CHIKV is maintained in the human population by human-mosquito-human transmission [1]. The disease is characterized by dengue-like symptoms such as chills, high fever, headache, and persistent myalgia and a further incapacitating arthralgia (from which the name chikungunya) which affects 40% of infected subjects. However, prognosis is rarely fatal [116].

There is currently no commercial vaccine and antiviral treatment for CHIKV, although some candidate vaccines have been tested in humans. In this regard, in 2000 US Army performed a Phase II clinical trial testing a live-attenuated CHIKV vaccine (TSI-GSD-218) derived from a 1962 strain (15561) of an outbreak in Thailand. Out of 58, every patient developed neutralizing Abs, and 5 lamented mild to moderate joint pain [117]. A phase III trial of this candidate vaccine is ongoing. Furthermore, a new formulation using virus-like particles was able to induce neutralizing Abs in macaques against different CHIKV strains [117, 118]. Indeed, it has been described that infection seems to elicit long-lasting protective immunity and cross-protection among CHIKV and other *alphaviruses*.

CHIKV entry into host cell is demonstrated to be mediated by envelope glycoproteins E1 and E2, which allow virus fusion to cell membrane in low pH conditions and recognition of an unknown cellular receptor, respectively [116, 119–121]. Even though the genome replication relies on an error-prone RNA-dependent RNA-polymerasis, CHIKV strains S27 and 05.115 Reunion showed in E1 and E2 a low amino acid variation of 0.68% and 3.3%, respectively, and such a low variability may be needed for an effective replication in two phylogenetically distant hosts [116, 122]. Recently, the crystal structure of E1 and E2 heterodimer has been resolved both at low pH and natural conditions, shedding light on E1 and E2 ectodomains' structure. In particular, similarly to *flaviviruses* E protein, E1 ectodomain is made of the N-terminal DI, the DII containing the fusion loop, and the DIII at the C-terminal. On the other hand, E2 ectodomain comprises the N-terminal domain A, the domain B supposed to interact with the host cell's unknown receptor, and domain C at the C-terminal. E1 DIII and E2 domain C are located close to the viral membrane [123, 124].

3.1.1. mAbs against CHIKV. Warter et al. described 5F10 and 8B10, two human mAbs which strongly neutralized several CHIKV isolates *in vitro*, without cross-reactivity against other *alphaviruses* but to Onyong-nyong virus [56]. Mixed preparation of 5F10 and 8F10 did not show neither synergistic nor addictive effect *in vitro*, and studies upon escape mutants demonstrated that 5F10 mAb binds at the tip of the E2 domain B, while 8B10 recognizes residues close to E1 fusion loop and amino acids within E2 domain A, which form a transitional epitope under low pH conditions. The authors speculated that 5F10 and 8B10 may inhibit CHIKV entry and fusion to the cell membrane, respectively. It is worth noting that the previously mentioned escape mutants displayed mutations associated with reduced viral fitness *in vitro* even after 13 neutralization/amplification rounds. In the same

study, cell-to-cell transmission was firstly demonstrated as an escape mechanism, enhanced by the E2 mutation R82G [122].

In further *in vivo* studies, 5F10 and 8B10 significantly delayed CHIKV-caused death of AGR129 mice both in prophylactic and therapeutic tests. Interestingly, in therapeutic treatment, these mAbs showed a synergistic effect when administered in combination, with the total amount of mAbs injected being the same of the single-mAbs. Possibly, these treatment did not allow the mice to survive CHIKV challenge due to Ig clearing and 6–10 days half-life usually described for human mAbs [125].

Recently, Pal et al. cloned thirty-six murine mAbs able to neutralize the ECSA La Reunion 2006 OPY-1 strain of CHIKV (CHIKV-LR), the majority of which also neutralize infection of other strains corresponding to the Asian and West African genotypes. In particular, mAb CHK-152 showed the highest and broadest neutralizing activity, with FRNT values indicating an IC50 value of 1 to 3 ng/mL depending on the viral strain used. Among the thirty-six mAbs, four (CHK-102, CHK-152, and CHK-166, CHK-263) could prevent lethality of immunodeficient *Ifnar*−/− C57BL/6 mice when administered one day before exposition, with CHK-152 and CHK-263 showing the ability to protect mice at the lowest dose (10 μg). Therapeutic studies were performed on these mAbs administering a single dose of 100 μg 24 hours after infection. The highest activity was described for CHK-166, able to protect 63% of the mice. Testing the combined activity of the mAbs in therapeutic studies, administration of CHK-166 plus CHK-152 in a dose of 250 μg each turned out to be the most effective, protecting 71% of the *Ifnar*−/− C57BL/6 mice 60 hours after infection [57].

3.2. Venezuelan Equine Encephalomyelitis Virus (VEEV). Venezuelan equine encephalomyelitis virus (VEEV) is maintained in a natural transmission cycle between mosquitoes and small rodents. The first documented outbreaks occurred in the 1930s, and several epidemics have been reported so far in Latin-American countries such as Venezuela, Colombia, Peru, Ecuador, Bolivia, Honduras, Mexico, and Panama with hundreds of thousands of human cases being reported and a case-fatality rate up to 1% during the 1969 Ecuador outbreak. Clinical manifestations of VEE are indistinguishable from Dengue [126]. Six serogroups (I–VI) are currently recognized within the VEEV complex. VEEV caused human and equine outbreaks in the Americas for nearly a century. Equine epizootics have high mortality (38–83%) leading to a high viremia followed by a lethal encephalitis and often to human epidemics involving thousands of cases and hundreds of deaths. Indeed, VEEV is infectious for humans by the airborne route and has been responsible for a number of laboratory infections. In humans, the disease is usually self-limiting with a febrile illness with 1–4% of cases progressing to severe encephalitis. It is a hazard to laboratory workers, has been developed as a biological weapon, and is a potential bioterrorist agent [127]. There are no antiviral drugs and vaccines licensed for the treatment of VEEV infection in humans. Indeed, although experimental live-attenuated vaccines have been developed (e.g., TC-83) with good levels of protection in equine and mice, in humans they may fail to give protection in the majority of cases [128]. Considering the protective role exerted by humoral immune response in animal models, it could be concluded that treatment with specific IgG may have a beneficial antiviral effect in human airborne infections with pathogenic strains of VEEV.

3.2.1. mAbs against VEEV. Several works support that mAbs may protect against airborne VEEV as well as after airborne exposure to VEEV. Ab reactivity to both surface glycoproteins (E1 and E2) is associated with neutralization of the virus *in vitro* and passive protection against virus challenge. A series of anti-E1 and anti-E2 mouse and humanized mAbs have been described recently. Among them, Phillpotts et al. examined two anti-E2 murine mAbs, named 1A4A-1 and 1A3A-9, both having potent protective activity against subcutaneous VEEV challenge in mice. Both mAbs had a similar half-life (5.8 and 10.0 days) in mouse serum after a single intraperitoneal dose, suggesting that mAbs, delivered by this route at or around the time of VEEV infection, persists at high levels in the blood as well as secretions in respiratory transudation throughout the clinical course of the disease. In particular both mAbs, administered 24 h prior to airborne challenges had a substantial protective effect (90–100%). Treatment of mice, 2 or 24 h after airborne infection, with a single intraperitoneal dose of 100 μg of 1A3A-9 mAb, led to approximately 50% survival. There was no beneficial effect when mAb treatment was delayed to 72 h. Moreover, there was evidence of synergy *in vitro* in PRNT, between 1A3A-9 and 1A4A-1, as has been demonstrated for other viruses and mAb pairs. However, no synergy was found in mouse protection when mAbs were delivered intraperitoneally as a mixture in equal parts, to animals challenged 24 h previously with VEEV [129].

In a subsequent study Phillpotts et al. reported two other protective mAbs. The 3B2A-9 mAb protected against all the serogroup I strains while 1A3B-7 mAb protected well against challenge with all of the viruses tested. Both 3B2A-9 and 1A3B-7 protected against airborne exposure to the IA/B serogroup virus strain Trinidad donkey (TrD). However, there was no evidence of synergistic protection when these mAbs were combined in equal proportions. An intraperitoneal dose of 10 μg was sufficient to protect 50% of the mice with either mAb [130]. According to other data, the mechanism of protection did not appear to depend upon neutralization [131]. Similar results were obtained by O'Brien et al., which described a non-neutralizing mAb (IgG2a), named CUF37-2a, able to protect 50% of mice from a subcutaneous VEEV challenge when a dose of 9.15 μg of mAb was administered 24 h prior to challenge [132].

In a following study the 1A3B-7 mAb was humanized (and following reported as Hu1A3B-7) maintaining the same features of its murine counterpart. In particular, evaluation of *in vitro* studies indicated that Hu1A3B-7 retained both broad specificity and neutralizing activity. Furthermore, *in vivo* experiments showed that Hu1A3B-7 successfully protected mice against lethal subcutaneous and aerosol challenges with VEEV strain TrD. Moreover, the effectiveness of the humanization process was determined by assessing proliferation responses in human T-cells to peptides derived from the

murine and humanized versions of the VH and VL domains. This analysis showed that the number of human T-cell epitopes within the humanized Ab had been substantially reduced, indicating that Hu1A3B-7 may have low immunogenicity *in vivo* [133].

Similarly, Hunt et al. described a humanized murine mAb derived from the 3B4C-4 mAb, recognizing the E2c epitope (amino acid residues 182–209) and following named Hy4-26C, showing neutralizing activity similar to the murine mAb. Moreover, it was protective (70–100% of survival) at 100 ng dose in mice animal model intraperitoneal inoculated and at 500 μg for intranasal challenge (80%). Therapeutic studies in mice revealed that Hy4-26C was able to cure mice up to 24 h following infection at 10 μg, on the contrary the mouse mAb when given 1 hour after virus challenge [63].

In another work, Hunt et al. isolated two neutralizing humanized Fabs, F5 and L1A7, employing a blocking strategy [134, 135]. The anti-E2 specific F5 IgG had a 70% PRNT endpoint of 10 ng/mL, equivalent to that described for the most effective neutralizing anti-VEEV E2 mAbs. In particular, the E1-specific hFab L1A7 had a PRNT endpoint of 3 μg/mL, 300-fold lower than F5.

Further studies revealed that F5 epitope is located in the 115–199 amino acid region. Moreover F5 IgG had potent ability to protect mice from infection by either route when administered 24 h before exposure; however, mice treated 24 h and 48 h after aerosol exposure developed central nervous system infections but exhibited no clinical signs of disease [135].

Hu et al. reported that passive immunization with the humanized chimeric mouse mAb hu1A4A1IgG1-2A in mice at 50 μg 24 h before or after virulent VEEV challenge provided complete protection, indicating that hu1A4A1IgG1-2A has potent prophylactic and therapeutic effects against VEEV infection [136].

Anti-VEEV mAbs isolated from a non-human primate gene library has also been reported. In particular the humanized chimeric scFv-Fc ToR67-3B4 recognized viable as well as formalin and β-propiolactone inactivated virus particles. It detected specifically the viral E1 envelope protein of VEEV but did not react with reduced viral glycoprotein preparations suggesting that recognition depends upon conformational epitopes. The recombinant Ab was able to detect multiple VEEV subtypes and displayed only marginal cross-reactivity to other *Alphavirus* species except for Eastern equine encephalitis virus (EEEV). In addition, the scFv-Fc fusion described here might be of therapeutic use since it successfully inactivated VEEV in a murine disease model. In particular, when the recombinant Ab was administered 6 hours after challenge, 80% to 100% of mice survived lethal VEEV IA/B or IE infection. Forty to sixty percent of mice survived when scFv-Fc ToR67-3B4 was applied 6 hours after challenge with VEEV subtypes II and former IIIA [137].

4. Bunyaviridae

The Bunyaviridae family contains human arboviruses belonging to the *Orthobunyavirus*, *Phlebovirus*, and *Nairovirus* genera. Bunyaviridae includes enveloped viruses with a fragmented, single-stranded RNA genome of negative polarity. Their tripartite genome consists of a small (S), a medium (M), and a large (L) fragment and the envelope glycoproteins Gn and Gc that are cleaved out of a polyprotein synthesized by the M fragment. These glycoproteins have been proved to mediate the formation of the virus particle, to play a role in the interaction with cell surface receptors, to mediate the entry of the virus into cells, and to serve as targets for the majority of neutralizing Abs described so far.

Orthobunyavirus, transmitted through mosquitoes or midges vectors, are divided in 18 serogroups, based on cross-titrations in haemagglutination inhibition assays and neutralization assays, and correlating with main vector preferences. However, the most clinically important viruses belong only to two serogroups, the California encephalitis and the Simbu serogroups.

Crimean-Congo hemorrhagic fever virus (CCHFV) is considered the only one of clinical relevance within the *Nairovirus* genus, which uses tick as main vector and is divided in seven serogroups, while Toscana virus and Rift Valley fever virus (RVFV), which are transmitted by sandflies and mosquitoes, respectively, belong to the *Phlebovirus* genus [138].

MAbs against the *Nairovirus* CCHFV and the *Phlebovirus* RVFV have been described.

4.1. Crimean-Congo Hemorrhagic Fever Virus (CCHFV). Crimean-Congo Hemorrhagic Fever Virus (CCHFV) distribution covers the greatest geographic range of any tick-borne virus known, as viral isolation and/or disease has been reported from more than 30 countries in Southeastern Europe, Africa, Asia, and Middle East [139, 140]. Namely, from 1953 to 2010, about 6,000 human cases were reported in Southeastern Europe. CCHFV causes sporadic outbreaks with mortality rates ranging from 10 to 80% [141, 142]. A significant variation in the time of incubation has been described [139]. Prehemorrhagic symptoms include high fever, chills, headache, photophobia, and back and abdominal pains. Among other symptoms, neuropsychiatric changes have been reported in some patients. In severe cases, 3–6 days after the onset of the disease, hemorrhagic symptoms occur with petechiae, ecchymosis, bleeding in the form of melena, hematemesis, epistaxis [139].

In comparison with the nucleocapsid proteins, in CCHFV Gn and Gc show a higher degree of antigenic variability probably due to their exposition to the host immune system, hypothesis corroborated in the description by Hewson et al. in 2004 of four M segment phylogenic groups, namely, M1, M2, M3, and M4 [141, 142].

4.1.1. mAbs against CCHFV. Blackburn et al. firstly described in 1987 murine anti-CCHFV mAbs able to recognize nucleocapsid proteins, but no neutralizing activity has been described thus far [143].

On the other hand, Bertolotti-Ciarlet et al. produced a panel of murine mAbs recognizing conformational epitopes

within the Gn and Gc glycoproteins expressed by the CCHFV strain IbAr10200. The effectiveness of the mAbs was studied by performing PRNT80 and none of the anti-Gn mAbs showed neutralizing activity, although many of the anti-Gc mAbs neutralized IbAr10200 strain *in vitro* with clones 8A1, 5E3, 12A9, 6C2, and 9H3 showing activity at a >5120 dilution. MAbs 11E7 and 30F7 exhibited neutralizing activity at 2560 dilution. A suckling mice protection test was therefore performed for these mAbs. The previously mentioned anti-Gc Abs were capable to protect mice to an appreciable degree when applied 24 hours before and, in a weaker manner, 24 hours after virus challenge. Many of the anti-Gn mAbs were able to confer significant protection to IbAr10200 both 24 hours before and after virus administration. The relevant effectiveness of the anti-Gn mAbs 6B12, 10E11, 13G8, and 10G4 suggests that these mAbs may possess some neutralizing activity due to Ab-based effector mechanisms (e.g., ADCC) [58].

In further studies, the same group characterized the broadly cross-reactive neutralizing activity of the previously described murine mAb 11E7, able to bind conformational epitopes within the C-terminal region of the Gn glycoprotein of all CCHFV M groups [58, 141].

4.2. Rift Valley Fever Virus (RVFV). Similarly to CCHFV, Rift Valley Fever Virus (RVFV) causes sporadic outbreaks. The largest epidemic occurred in Egypt in 1977, with an estimated 200,000 infections, 18,000 patients manifesting symptoms and 600 fatal prognoses [144]. Symptoms include hemorrhagic episodes, fever, encephalitis, and blindness [145].

The major target of the humoral immune response is the RVFV glycoproteins Gc (G1) and Gn (G2), ranging from nucleotides 480–2090 and 2091–3614, respectively [144].

4.2.1. mAbs against RVFV. Within G2 glycoprotein are described three neutralizing antigenic sites, namely, epitope I: nucleotides 792–893 (amino acid residues 258–291); epitope II: nucleotides 1164–1196 (amino acid residues 382–392); and epitope IV: nucleotides 858–917 (amino acid residues 280–299) [59, 145].

Despite the high degree of variability observed in CCHFV glycoproteins, RVFV G2 presented an unexpected high conservation among 22 isolates [59]. In the same study, only the LUNYO and 900060 isolates were resistant to *in vitro* neutralization by murine mAb 4D4 (recognizing epitope II). All other isolates were neutralized with PRNT80 titers from 10,240 to >81,920. On the other hand, only SNS isolate showed reduced sensitivity to 4–39-CC (epitope IV). All other isolates were neutralized with PRNT80 titers from 20,480 to >81,920. Protection studies on mice would be of interest for the development of a passive immunization prophylaxis [59].

Protection studies from lethal South African RVFV AN1830 strain infection were successfully performed on a battery of anti-G1 and anti-G2 mAbs produced by Besselaar and Blackburn, with the strongly neutralizing anti-G1 mAb 3E5 and anti-G2 mAb 9C4 being the most effective [146]. The 9C4 antigenic area maps within G2 epitope I, and a broadly neutralizing activity may be investigated.

5. Conclusions

Arbovirus infections have acquired increasing interest given the augmented globalization and tourism movement all over the world that could be at the basis of epidemic events. Furthermore, the same vectors can sometimes transmit several arboviruses concomitantly, complicating the diagnosis as well as the therapy. For example, confusing mixed epidemics have occasionally been described, such as YFV plus CHIKV, DENV plus CHIKV, or more recently *Plasmodium falciparum* malaria plus DENV1 and CHIKV on Madagascar's east coast [1].

In the last decade, several efforts have been employed in the development of effective immunogens and therapeutics giving the current absence of specific antiviral drugs as well as effective vaccines for the most diffused arbovirus infections (i.e., DENV and WNV).

However, at the moment, mosquito control is the best available method for preventing arbovirus infections. Thus, to control the emerging public health problem of arbovirus infections, new antiviral therapeutic strategies that provide potent, and broadly cross-protective immunity (especially for *flavivirus* infections) are an urgent globally medical need.

As described in this review the majority of highly neutralizing mAbs are of murine origin and indeed their utility in the treatment of these infections is limited. In fact, their employment can elicit an immunogenic response (i.e., human anti-mouse Abs, HAMA), the therapeutic efficacy could be reduced by a relatively faster clearance in humans (compared to human Abs), potentially exacerbated by the HAMA response. Finally, murine Abs exhibit relatively weak effector functions (e.g., ADCC) compared to human Abs. Indeed, chimerization, humanization, or better, the isolation and characterization of fully human broadly neutralizing mAbs would be the best choice for their possible use in a future mAb-based therapy against arbovirus infections [147].

Finally, possible ADE of infection mechanisms should be evaluated before considering a mAb as a possible candidate therapeutic in a post-exposure setting as well as in the development of new Ab-eliciting immunogens.

Conflict of Interests

The authors declare no conflict of interests, in particular, with OmriGam and any other cited Ab-based preparation mentioned in the paper.

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Review Article

Understanding the Dengue Viruses and Progress towards Their Control

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Traditionally, the four dengue virus serotypes have been associated with fever, rash, and the more severe forms, haemorrhagic fever and shock syndrome. As our knowledge as well as understanding of these viruses increases, we now recognise not only that they are causing increasing numbers of human infections but also that they may cause neurological and other clinical complications, with sequelae or fatal consequences. In this review we attempt to highlight some of these features in the context of dengue virus pathogenesis. We also examine some of the efforts currently underway to control this "scourge" of the tropical and subtropical world.

1. Introduction

Dengue fever is a mosquito-borne virus disease of humans. In terms of numbers of individuals infected, it is by far the most devastating of all the recognised arthropod-transmitted virus diseases. It is estimated that more than 3 billion humans live in dengue endemic regions of the world, and currently, more than 50 million infections occur annually with at least 500,000 individuals requiring hospitalisation [1]. Of these, tens of thousands have a high risk of developing haemorrhagic disease, potentially with fatal consequences depending to a large extent on the quality of the available medical services.

The dengue viruses are positive stranded RNA viruses in the genus *Flavivirus*, family *Flaviviridae* [2]. There are four distinct dengue virus (DENV) serotypes that share antigenic relationships (DENV-1, DENV-2, DENV-3, and DENV-4), and although infection with one serotype confers lifelong protection against that serotype, it does not necessarily protect against a secondary infection with a heterologous serotype. Indeed, nonprotective but cross-reactive antibodies may enhance disease severity [3]. Currently, there are no effective vaccines or antiviral drugs against these viruses. This problem is being addressed as a matter of urgency as failure to develop effective DENV control strategies will inevitably result in a further increase in the number of infected humans, as predicted more than a decade ago [4]. This problem is also exacerbated by the continuing dispersal of these viruses to new geographic regions.

This review therefore focuses on our current understanding of dengue virus pathology, epidemiology, pathogenesis, evolution, biogeography, and disease control.

2. Dengue Fever/Haemorrhagic Fever/Shock Syndrome

2.1. Clinical Picture. In most cases asymptomatic or relatively mild disease follows infection with dengue virus. However, to take into account the increasing number of clinical cases, the World Health Organization (WHO) produced guidelines [5]

in which they identified the clinical pictures resulting from infection with dengue virus. The first, known as dengue fever (DF), is characterised by an abrupt onset of fever accompanied by frontal headache and retroorbital pain, followed by a variety of possible clinical symptoms such as myalgia, arthralgia, vomiting, and weakness. A generalised maculopapular rash appears one or two days after fever defervescence. Minor haemorrhagic manifestations such as petechiae may be observed in some patients. DF is generally self-limiting and rarely fatal. Most patients recover without complications around ten days after the onset of illness.

The second clinical picture, dengue haemorrhagic fever (DHF), is a more severe form of the disease and occurs in up to 5% of dengue cases. It is initially characterized by the same variety of clinical symptoms as are seen in DF. The critical period in DHF starts at the moment of defervescence but haemorrhagic manifestations may occur 24 hours earlier. A positive tourniquet test indicates that the patient has increased capillary fragility. Petechiae, bleeding at venepuncture sites, epistaxis, gum bleeding, and haematemesis may also be observed. High fever, haemorrhagic manifestations, thrombocytopenia (platelet count 100 000/mm^3 or less), and haemoconcentration (>20% difference) characterize DHF. Plasma leakage is the most significant pathophysiological event in determining the severity of the disease. Signs of circulatory failure such as irritability, cold clammy extremities, flushed face, and restlessness may be observed. This crisis usually persists for 24–36 hours. With appropriate supportive medicine and carefully monitored intravenous isotonic crystalloid therapy, to ensure adequate fluid replacement, most patients recover. However, during this critical period, it is essential to look for characteristic warning signs of worse to come. Patients progressing to shock (dengue shock syndrome—DSS) show intense abdominal pain or tenderness, persistent vomiting, weak pulse, and hypotension. If increased vascular permeability progresses to vascular collapse the outcome is usually fatal as a result of irreversible DSS. In addition to DF, DHF, and DSS, it is now recognised that other clinical manifestations can be associated with infection by dengue virus, for example, encephalitis, myocarditis, hepatitis, cholecystitis, myelitis, and acute colitis [6–11].

Despite the rigour of the DF/DHF/DSS classification and its intrinsic worth in clinical case management, in recent years, a growing number of clinicians and authors have argued that the 1997 WHO scheme for dengue clinical classification should be reassessed [12–14] because it distinguishes strictly between DF, DHF, and DSS, whereas it is now recognised that the point of transition between DF and DHF is not easily defined. The requirements for the WHO definition of DHF (fever, haemorrhage, thrombocytopenia, and signs of plasma leakage) are not always satisfied; severe thrombocytopenia may be observed in uncomplicated as well as severe cases associated with "unusual manifestations" and therefore may not be consistent with the DHF/DSS classification [15]. A WHO/TDR-supported prospective clinical multicentre study across dengue-endemic regions was established to collect and coordinate specific criteria for classifying clinical cases into levels of severity [16]. The study confirmed that, by using a set of clinical and/or laboratory parameters, one sees a clear-cut difference between patients with severe dengue fever and those with nonsevere dengue fever. However, for practical reasons it was considered necessary to split the large group of patients with nonsevere dengue into two subgroups: patients with warning signs (abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleeding, lethargy, restlessness, liver enlargement >2 cm, and increase in hematocrit concurrent with rapid decrease in platelet count) and those without these warning signs. On the other hand, the criteria for severe dengue fever include extensive plasma leakage, severe bleeding, or severe organ impairment.

The third and most recent edition of the WHO/TDR dengue guidelines for diagnosis, treatment, prevention, and control includes a new clinical classification [17]. This publication serves as an authoritative reference source for health workers and researchers. These new guidelines provide a revised case classification which is intended to facilitate effective triage and patient management and collection of improved comparative surveillance data [18]. However, due to the recommendation that cases of dengue fever with warning signs and also cases of severe dengue fever should be admitted to hospital, there is concern that this could result in overadmission of patients to hospitals during epidemics, inevitably reducing the efficiency of patient triage and adversely affecting the quality of clinical case management [19, 20]. Furthermore there is additional concern that the WHO/TDR classification may impact significantly on dengue pathogenesis research since it requires the identification and study of distinct dengue syndromes. Because the 2009 WHO case definitions do not require laboratory tests for the diagnosis of severe dengue, it is considered that retrospective identification of patients with clinically significant vascular permeability, from data on hospital charts, may be difficult if not impossible [21]. The previous discussion highlights the difficulties of designing a totally acceptable classification scheme for dengue pathogenesis.

2.2. History of DF and DHF. Clinically diagnosed DF was widespread during the 18th and 19th centuries in North and South America, the Caribbean Basin, Asia, and Australia. In the Americas, this was largely due to the repeated introduction from Africa of *Stegomyia (St.) aegypti* (formerly *Aedes aegypti*) [22–24]. Moreover, together with yellow fever virus (YFV), DENV-infected humans and mosquitoes were introduced via the slave ships and other commercial vessels that crossed the Atlantic Ocean from Africa during the past five or more centuries [25–31].

It is also important to note that disease clinically compatible with the more severe and often fatal syndromes, DHF and DSS, was sporadically reported from 1780 onwards [32] although it is not clear if the more severe cases were confined to individuals of European descent.

DF thus became endemic in Latin America and the Caribbean region, periodically causing epidemics. At the same time, YFV was also causing epidemics in South

America, prompting the Pan American Health Organisation (PAHO) to introduce a mosquito-eradication programme which lasted from 1946 until the late 1970s. Since both DENV and YFV are transmitted to humans via *St. aegypti* this eradication campaign in South America also resulted in a lower incidence of DF in South America. Thus, DF was confined mainly to the Caribbean basin [33, 34]. Subsequently, the gradual decline of mosquito control measures and increasing introduction and dispersal of mosquitoes via transportation for commercial and military purposes led to the reemergence of dengue as a major health problem during the mid and later parts of the 20th Century. The incidence of dengue fever increased dramatically in Southeast Asia during World War II and continued to intensify with increased geographic spread of the viruses and the principal mosquito vector, *St. aegypti*. In addition to the major influence of increased shipping and air-traffic globally, other major factors for the reemergence of dengue fever include ecological and demographic changes in the tropical zones [2, 34–39].

During the 1980s and 1990s, rapidly expanding populations of *St. aegypti* in Brazil resulted in successive epidemics due to DENV-1, DENV-2, and DENV-3. In Brazil, these infections presented mostly as DF, with surprisingly few cases of DHF. This contrasts with Asia where the proportion of DHF cases was significantly higher during DF epidemics. These differences have been partly attributed to the widespread presence of dengue virus resistance genes in Latin Americans with African ancestry [33, 40, 41]. The differences may also be partly explained by the high levels of antibody against the American DENV-2 genotype and antigenically cross-reactive DENV-1, both of which had been endemic in Latin America for many years.

Today, all four DENV serotypes circulate in Africa, South and Southeast Asia, the Western Pacific region, the Caribbean basin, and Central and South America [39, 42–44]. Frequent introductions into the Southern states of North America are also regularly recorded although to date they have not resulted in epidemic outbreaks in the USA; DF has the potential to become reestablished as an endemic disease in this country. In fact, sustained transmission of dengue has occurred in Florida during recent years. Conditions exist that could facilitate sustained dengue transmission, including environmental factors, competent mosquito vectors, limited vector and dengue surveillance, increased domestic outdoor daytime activities in warmer months, and low public awareness of the disease [45]. Indeed, dengue continues to spread more widely as demonstrated in 2010 by the first recorded cases of autochthonous dengue fever in southern France [46] and Croatia [47].

Many countries in the tropics and subtropical regions show cocirculation of at least two DENV serotypes [36], and increasingly, cocirculation of all 4 serotypes is being recorded in individual countries. Taken together with the ecological and demographic changes, this partly explains why the pattern of epidemics is gradually increasing from a frequency of outbreaks every 3–5 years to approximately every 2 years [48]. Additional explanations for this increased incidence include the possibility that more highly pathogenic strains of DENV are also emerging [44, 49–51]. Greater awareness of this disease, as the result of more extensive monitoring, is also impacting on our understanding of and the apparent increased periodicity of dengue virus epidemiology.

Comparison of disease incidence in Asia and Latin America reveals a distinct difference in the age distribution of DF and DHF. In Asia, hospitalizations principally involve children, whereas in the Americas, they tend to involve a greater proportion of adults [33]. The reasons for this apparent difference have not been adequately defined. However, to complicate this issue, a recent epidemic in the State of Rio de Janeiro revealed that the incidence of DHF in children was significantly higher than in previous epidemics in Brazil [52, 53].

2.3. Risk Factors Associated with the Development of Severe Dengue. The principal vector associated with all 4 DENV serotypes is the African mosquito *St. aegypti* an urban-dwelling anthropophilic mosquito. However, *St. albopicta* the Asian "Tiger" mosquito is also competent to reproduce and transmit DENV between humans. In contrast with *St. aegypti, St. albopicta* is peridomestic, with a preference for the rural environment. In some parts of Asia and Africa, *St. albopicta* has displaced *St. aegypti* [54, 55]. A possible scenario of this changing pattern of mosquito distribution and the continuing dispersal of *St. albopicta* is that the dengue viruses will disperse even more widely, gradually establishing in the warmer regions of the temperate zones, including Europe [46, 47, 56, 57], the southern regions of North America [58], and more northern regions of Asia [59].

The pathogenetic basis for DHF has been a subject of study for decades, and whilst significant progress has been made in understanding the most important risk factors involved, the precise biochemical and immunological pathways have not yet been defined [60–62]. Amongst the several possibilities that have been identified, there is compelling evidence that secondary infection with a heterologous DENV serotype, or primary infection in infants born to dengue-immune mothers, is an important individual risk factor for DHF/DSS [63–70]. During secondary infection with a different serotype, the presence of low levels of heterotypic neutralizing antibodies may reduce disease severity. Alternatively, in the absence of such neutralizing antibodies, heterotypic cross-reactive antibodies may form complexes with the virus and the Fc-receptors on these complexed antibodies may attach to mononuclear phagocytes, thus enhancing the efficiency of infection and thereby increasing the number of infected mononuclear phagocytes [71–74]. This phenomenon is known as antibody dependent enhancement (ADE) [75]. Humans infected with one serotype maintain a life-long protective immunity to infection by the homologous virus, but protective immunity to infection with heterologous serotypes is relatively short-lived [76]. The precise mechanism by which DENV replication is amplified in the infected cells remains unclear. One possibility is that there is a relationship between DENV-ADE infection, suppression of nitric oxide during the innate immune response, and the corresponding cytokine-expression pattern in THP-1 cells [77]. Recent evidence suggests that viral susceptibility or resistance to nitric oxide may be regulated by the viral NS5 protein [78].

It has also been argued that strain differences in virulence may contribute to disease severity [51, 79–84]. However, the fact that severe dengue disease is identified most consistently following secondary dengue infections supports the view that virulence must be defined in a two-infection context [39]. Host risk factors such as gender, ethnicity, the presence of chronic disease (bronchial asthma, diabetes mellitus, and sickle cell anaemia) [85–87] and also the genetic characteristics of the individual are also likely predisposing factors for severe illness. Human leukocyte antigen (HLA), FcγR, tumor necrosis factor- (TNF-) α, and dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), among other genes, have been associated with the pathogenesis of dengue [41, 66, 86, 88–93]. In addition, T-cell mediated immune mechanisms, involving skewed cytokine responses resulting in plasma leakage, are also risk factors for DHF [62]. It has been proposed that an inappropriate immune response to the secondary virus infection, that in turn induces reactivation of cross-memory T cells specific for the first rather than the secondary DENV infection, results in delayed viral clearance [94, 95]. More recently, it was suggested that the presence of an effective antiviral inflammatory response in the presence of adequate immune regulation could be associated with protection during dengue secondary infection [96]. However, as highlighted in a recent review on dengue pathogenesis, it is worth noting that other infectious diseases and inflammatory disorders result in elevated cytokines without the attendant increased vascular permeability seen in severe dengue [97]. Indeed, one of the major challenges in dengue, magnified due to absence of good animal models of disease, is to dissect those elements of the host immune response that are causally linked to capillary permeability from those that simply reflect the normal host immune response to a pathogen [97].

Independent of this, antibodies specific for the NS1 viral protein may form immune complexes with the NS1 protein in the circulation and on the surface of infected cells leading to complement activation [98]. An additional risk factor for DHF is believed to be dependent on autoimmune responses against cross-reactive viral components. For example, antibodies specific for dengue virus NS1 protein may induce platelet lysis and/or nitric oxide-mediated apoptosis of endothelial cells, contributing to thrombocytopenia and vascular damage [99–104].

The serious complications observed during dengue infection occur as plasma viremia is resolving. This is thought to be immunologically mediated. Tam et al. performed a randomised trial to verify the effects of short-course oral corticosteroid therapy in early dengue infection. No association between treatment allocation and any of the predefined clinical, hematological, or virological endpoints was found [105]. Unexpectedly, the steroid doses administered were not immunosuppressive. Based on these observations it was suggested that rather than dengue-mediated vascular permeability being T-cell mediated, an alternative pathogenetic mechanism could involve the dengue soluble complement fixing antigen or the viral NS1 protein. Indeed, it was recently proposed that during the late stages of clinically apparent dengue infection, secreted DENV NS1 protein may bind to prothrombin and inhibit its activation, which in turn could contribute to the prolongation of activated partial thromboplastin time and haemorrhage in DHF patients [106]. Also, previous studies on the virological course of dengue infections in monkeys have shown that the peak of cellular infection occurs at the end of the viremic phase. Accordingly, it was proposed that dengue vascular permeability syndrome could be the equivalent of a viral toxicosis induced by circulating NS1 protein [107].

Whilst higher levels of viremia and circulating NS1 protein have been associated with dengue disease severity [108], collective results arising from different epidemiological settings are inconclusive, showing variations depending on the infecting serotype and patient immune status [109–112]. Therefore, the usefulness of these markers for the recognition of patients with increased risk of progression towards the more severe forms of dengue is still limited.

2.4. Underlying Basis for the Emergence of DHF in Particular Epidemiological Settings. Taking into account many of the factors described earlier and based on observations made during the 1981 Cuban epidemic of DF/DHF, Kouri and coworkers presented an integral hypothesis in which the association of different factors, such as immunological status, genetic background, host condition, viral strain, and epidemiological and ecological conditions, determines whether or not and to what extent, DHF will be involved in any particular epidemic [113]. Research conducted during the past 20 years strongly supports this unifying view of the situation [3, 39, 48, 114].

With the exception of Chile, Uruguay, and Cuba, that experience occasional epidemics, resulting from introduced virus, DF is endemic in Latin America and the Caribbean region. Cuba is a relatively small island with a well-integrated medical health and research infrastructure. When combined with the epidemiological history of DF in Cuba, this situation has provided a unique opportunity to investigate the specific risk factors for severe illness in detail [48]. Firstly, it is important to realise that from the end of World War II until 1977, dengue virus was not evident in Cuba. This was supported by a national seroepidemiological survey in 1975, which identified only 2.6% of the population with DENV hemagglutination-inhibition (HI) antibodies. Importantly, most of the positives were individuals older than 45 years [115, 116]. However, in 1977, based on serological evidence [117] it was estimated that up to 44.5% of all Cubans became infected by an introduced Asian strain of DENV-1. Nevertheless, no cases of DHF were recorded. These results demonstrate that in the absence of heterologous immunity, primary DENV-1 infections did not result in cases of DHF and, bearing in mind that subsequent DHF epidemics in Cuba all involved secondary infections (see later), the results strongly support the contention that secondary infections by heterologous serotypes are a very important risk factor for DHF as proposed previously [118].

During the past 28 years, three DENV-epidemics involving DHF have occurred in Cuba. The first epidemic started in 1981 [113], the second was seen in 1997 [65], and

the third occurred in 2001 [63]. During each epidemic, secondary infection was demonstrated as the most important host risk factor for DHF. Additionally, specific sequential virus serotypes were associated with severe disease, independent of the time-gap between the primary and secondary infection. For example, in Cuba, two epidemics of DHF have been associated with primary infections due to an Asian strain of DENV-1 and secondary infections due to DENV-2 (i.e., DENV-1/DENV-2) [119, 120]. Significantly, during the 1997 Cuban epidemic, it was demonstrated for the first time that there was a higher risk of DHF in DENV-1/DENV-2 individuals when the average time gap between primary and secondary infections was about 20 years as opposed to 4 years, a more commonly reported timespan [64]. Moreover, comparison of attack rates and case fatality rates, in the same age groups, revealed that during the 1997 epidemic the rate in patients older than 15 years of age was 40 times higher than during the 1981 epidemic [121].

Subsequently, another Cuban epidemic, caused by DENV-3 and involving cases of DHF, occurred in 2001. Thus, DHF occurred in DENV-3-infected Cubans 24 years after primary exposure to DENV-1 infection [122]. Interestingly, Cubans infected sequentially with DENV-1/DENV-3 were associated with severe disease whilst those infected sequentially with DENV-2/DENV-3 were associated with milder disease or asymptomatic infections [63, 123]. Additionally, the DENV-3 immune individuals, infected during the 2001 Cuban epidemic, revealed differences in the neutralization capability of their sera to different DENV-3 strains belonging to different genotypes [124]. This observation might be anticipated taking into account that differences in neutralisation capability have been found using different genotypes of DENV-2. However, it was highly significant to find differences in neutralisation against strains belonging to the same genotype [125]. Moreover, the strains involved in the Cuban 1981, 1997, and 2001 epidemics [80, 125, 126] had previously been associated with severe epidemics and therefore had the potential to produce DHF. Nevertheless, in all of these epidemics, an extremely high number of primarily infected individuals were asymptomatic [64].

Using human volunteers, Dr. Albert Sabin was the first scientist to demonstrate that heterotypic immunity can prevent disease induced by a different dengue virus serotype [127]. Whilst DENV-1 immunity did not appear to prevent DENV-2 infections, partial immunity may have downregulated infections, thus reducing severity to mild disease during secondary dengue infections. It has been postulated that if virological factors are involved in determining disease severity, they may reflect common antigenic determinants shared between the first and second infecting viruses [39]. An exceptional illustration of this phenomenon is the neutralization of American genotype DENV-2 by human antibodies to DENV-1 [81]. These results suggest that the apparent lower virulence of American genotype DENV-2 results from a DENV-1 like surface epitope on the DENV-2 that permits partial neutralization (and downregulation of disease) by DENV-1 antibodies [81]. In contrast, Asian genotypes DENV-2 are poorly neutralised by human antibodies to DENV-1 [128]. Furthermore, a significant increase in the mean titre of homologous DENV-1 neutralizing antibodies and a significant decrease in heterologous antibodies to DENV-2 American genotype were reported in a long-term study in Cuba [128]. This finding may reflect time-dependent changes in severity of disease observed following secondary dengue infection.

On the other hand, case fatality rates were observed to increase month by month during epidemics that were studied in Cuba. Taking into account that DENV-2 epidemics occurred in 1981 and 1997, Guzmán and coworkers proposed a neutralisation escape mutant hypothesis based on the association of severe disease with dengue secondary infection [129]. Furthermore, during the DENV-3 epidemic that occurred in Havana in 2001, the same sequential increase in case fatality rates was observed [48, 85, 122].

Although specific viral factors alone probably do not determine the severity of dengue infection in individual cases, the demonstrated increasing severity of infection with time during a single epidemic strongly argues that significant changes occur in the virus causing the epidemic. Indeed, host factors do not appear to explain this observation of increasing severity with time, because it is not logical to assume that the most susceptible individuals would all be infected towards the end of the epidemic.

The 1997 Cuban epidemic was the most severe reported in Cuba to date. Nevertheless, a search for evidence of the appearance of neutralisation-escape mutants proved negative. The structural gene sequences were highly conserved in viruses isolated at different times during the epidemic [126]. However, nucleotide substitutions were found in the nonstructural genes and in general they correlated with the time of sampling, showing a clear pattern of virus evolution during the epidemic [130]. Therefore, at least in this study, antibody-driven selection of escape mutants in the structural genes was not the key selective force. On the other hand, cytotoxic T-lymphocytes (CTLs) play a crucial role in controlling infection in RNA viruses, including dengue viruses [62, 131]. Variation in the epitopes recognized by CTL is common and frequently offers potential escape routes for mutant virus. Forthcoming studies will assess whether or not the reported mutations in NS1 and NS5 proteins [130] are represented in antibody inducing or CTL epitopes.

Regardless of which mechanism, that is, natural selection or genetic drift, is operating, it is likely that a fitter virus could be selected during the period of high transmission in individuals that have experienced secondary infections. However, it is a very difficult task to study dengue epidemiology because it is not only endemic in most tropical countries but there are four serotypes and many different genotypes often cocirculating. Nevertheless, Cuba represents a unique epidemiological setting for this kind of research because epidemics caused by only one serotype have occurred providing the opportunity for carefully defined epidemiological studies.

Mutations in the nonstructural genes of DENV-2, isolated during the Santiago de Cuba epidemic, may correlate with increased efficiency of virus replication. Variation in nonstructural proteins has been also associated with increasing severity in epidemiological settings corresponding to endemic/epidemic transmission [132–136]. However,

the specific relevance of these types of mutation has not yet been investigated thoroughly. This is in large part due to the lack of suitable animal models with which to study dengue virus "virulence" [51, 137].

During the most recent and severe DENV-4 epidemic in Puerto Rico in 1998, viruses were distinguished by three amino acid replacements in the NS2A protein (I14V, V54T, and P101T), which were fixed more rapidly than would have been expected by drift alone. This study demonstrates the significance of viral genetic turnover within a focal population and the potential importance of adaptive evolution during viral epidemic expansion [132]. In contrast, a retrospective phylogenetic study of events on the Southern Pacific islands three decades ago, where severe dengue was described in patients infected with the DENV-2 American genotype, recorded attenuation of this virus following a series of outbreaks involving nonsynonymous mutations, also in the NS2A gene [138].

Similarly, study of the population structure of dengue viruses transmitted in Aragua, Venezuela, during the period 2006-2007, under hyperendemic conditions also suggested that the nonstructural proteins could play an important role in DENV evolution. According to this particular epidemiological setting, changes in NS1, NS2A, and NS4B proteins were either favourable or adverse in terms of viral fitness. The authors argued that specific mutations could be associated with severe disease but some could be associated with mild disease due to the appearance of naturally attenuated strains [139].

The flavivirus NS2A protein is a small, hydrophobic, multifunctional membrane-associated protein involved in RNA replication [140, 141], host-antiviral interferon response [142–145], and assembly/secretion of virus particles [146–148]. In addition, the NS2A and the NS4B proteins may participate in the modulation of vector competence [149]. According to previous reports, changes in NS1 and NS4 proteins could be involved in viral attenuation [150, 151]. On the contrary, recent studies have demonstrated that mutations in the NS4B protein may increase the efficiency of DENV replication. In addition, it has been suggested that mutations in this protein may also be involved in species tropism of DENV and may even modulate the balance of efficient replication in mosquito and mammalian cells [152]. Moreover, it has been shown that a single amino acid in the nonstructural NS4B protein namely, L52F, confers virulence on DENV-2 in AG129 mice through enhancement of viral RNA synthesis [153].

Whilst these results suggest a possible role for the NS genes in determining viral fitness, the importance of the structural genes should not be overlooked. The sequences compared in the cited studies represent a consensus of those observed within each patient and may not necessarily represent the dominant variant present in the original clinical sample. For example, virus isolation using mosquito cell lines [154] is known to perturb the distribution of variants in the original clinical sample. This is particularly important given that studies of dengue virus populations sampled from individual humans or mosquitoes have revealed significant sequence variation [155]. Consequently, a greater focus on studies of viral population variation during epidemics is needed and the data should be obtained directly from clinical samples.

The demonstration of long-term transmission of defective dengue virus in humans and mosquitoes has added a new dimension to the study of dengue evolution. The increased frequency of the "stop-codon" strain was concomitant with a major reduction in DENV-1 prevalence in Myanmar. The authors suggested that complementation between defective variants might provide a mechanism for the survival of "hyperparasites," and this process of viral complementation could impact on pathogen transmission and virulence [156].

Obviously, more comprehensive approaches, including sequencing of larger numbers of viral genomes obtained directly from diverse clinical samples corresponding to longitudinal studies, are needed to examine how the genetic structure of dengue virus is influenced by heterotypic antibodies.

Data obtained in two carefully planned clinical studies of dengue in Nicaragua demonstrate that the complex interplay between viral genetics and serotype-specific immunity determines the risk of severe dengue disease. Indeed, these data provide insights into viral evolution and the interaction between viral and immunological determinants of fitness and virulence. The abrupt increase in disease severity across several epidemic seasons of DENV-2 transmission coincided with clade replacement events. Interestingly, DENV-2 strains corresponding to NI-1 clade caused severe disease specifically in children who were immune to DENV-1, whereas DENV-3 immunity was associated with more severe disease among NI-2B infections, signifying that mutations altering the neutralization profile of some DENV strains can lead to increased viral fitness [157].

Most dengue virus genomic studies have been directed at identifying the origin and genetic relatedness of the viruses causing epidemics. Other studies have focussed on identifying genetic markers associated with severe disease and comparing viruses isolated from DF and DHF/DSS cases within the same epidemiological setting. However, genetic variations have not been consistently associated with differences in clinical outcome. Conversely, introduction of new genotypes/serotypes with replacement/displacement of the existing viruses or changes in viral populations during an interepidemic period, extinction events, and sustained transmission of dengue virus due to repeated introductions have all been related to changes in the severity pattern of local epidemics [134, 158–161].

Recently, in Vietnam, the introduction of the Asian 1 genotype of DENV-2 led to the complete replacement of the resident Asian/American genotype of DENV-2. The transmission fitness advantage of Asian 1 viruses was attributed to this virus attaining higher viremia levels in humans [162]. However, there are multiple factors implicated in the transmission dynamic of DENVs that remain unclear. Epidemiological data have suggested that fitness is always context dependent and that as the immunological landscape changes, viral lineages that evade cross-immunity will be at a selective advantage [163].

Clearly, there is still wide scope for research on the molecular basis of dengue virus epidemiology and pathogenesis. We need to know whether or not a circulating dengue virus

that produces an asymptomatic infection in one host differs in sequence from the same virus that causes a fatal infection in another host. We also need to know if different tissues [164] within a single host house the same dominant dengue virus strain. Similarly, does the virus that circulates in a single epidemic have the same sequence in individuals with different histories of dengue infection?

2.5. Intrahost Genetic Variation. The population genetics and evolutionary epidemiology of RNA viruses have been reviewed [165]. The authors describe migration or gene flow as a factor to consider during RNA virus evolution. Accordingly, they advocate that migration must not only be understood at a macroscopic level (i.e., among hosts within a population, among populations, or between host species), but also within a single infected individual. From the site of inoculation, viruses can be transported to several tissues, generating intrahost spatial variation. This has been studied in Hepatitis C virus, family *Flaviviridae* [166]. However, the effect of a nonhomogeneous population distribution on the spread, fitness, and variability of virus populations has not been studied extensively. Nevertheless, a positive correlation between migration rate and average fitness of the population has been observed [167]. The outcome of acute Hepatitis C infection has been attributed to the evolution of viral quasispecies [168]. Large-scale sequencing of complete viral RNA genomes obtained directly from clinical samples is needed to investigate the role of variation in viral populations on dengue pathogenesis. The purifying selection that phylogenies have revealed [169] thus far may be misleading because most of the sequences analysed over the years were obtained from tissue culture viral isolates. Additionally, an intrinsic inadequacy of utilising consensus sequences to make inferences concerning the fitness of viral populations is that consensus sequences only reflect the majority nucleotide at any given position of the viral genome. Consequently, low frequency variants will remain undetected.

Examination of the viral population structure in mosquitoes and patients has revealed that the sequences of the major variants are the same but the extent of sequence variation seen with the mosquitoes is generally lower than that seen with the patients, suggesting that the mosquito contributes to the evolutionary conservation of dengue virus by maintaining a more homogenous viral population and a dominant variant during transmission [170]. In addition, by studying the evolutionary relationships of DENV-1 viruses that have circulated in French Polynesia and the viral intrahost genetic diversity according to clinical presentation, Descloux and coworkers suggested for the first time that clinical outcome may correlate with intrahost genetic diversity [171]. On the other hand, a recent study in Vietnam showed no relationship between the extent and pattern of DENV-1 genetic diversity and disease severity, immune status, or level of viremia. Interestingly, despite the high sequence conservation observed, clear evidence for mixed infection with the presence of multiple phylogenetically distinct lineages present within the same host was demonstrated [172].

Indeed, most attempts to investigate intrahost genetic variation in DENV characterised only a few viral genes or a limited number of full-length genomes. A new study in Nicaragua using a whole-genome amplification approach coupled with deep sequencing to capture intrahost diversity across the entire coding region of DENV-2 showed significant genetic diversity among genes [173]. However, the extension of that diversity was less than expected, suggesting strong purifying selection across transmission events as have been proposed previously [174–176].

Another point of view is that there is no reason to ignore vector-driven selection [177]. However, the interaction between virus and vector has been less extensively explored. By comparing the ability of DENV-1 isolates from Thailand, spanning a 24-year period, to infect and be transmitted by *St. aegypti*, Lambrechts et al. found that a major clade replacement event in the mid-1990s was associated with a higher transmission potential of the isolates belonging to the new clade. Higher transmissibility was mainly due to a higher infectious titre of virus in the vector's haemocoel, which is predicted to result in a higher probability of transmission. This finding supports the hypothesis that major clade replacement events can be driven by natural selection and emphasizes the potentially important role of vector-virus interactions in DENV evolution [178].

Since the 1900s, the extrinsic incubation period (EIP), that is the time taken for the viremic bloodmeal to be amplified in the mosquito and then transmitted to a new host, had been recognized as an important component of DENV transmission dynamics. The DENV EIP is generally considered to be between 8 and 12 days [17]. Nonetheless, different factors can induce variations in EIP. For example, considerable degrees of variation in EIP have been shown to vary depending on the specific DENV strain studied. Mosquitoes feeding on humans infected with an unadapted strain of DENV-1 had shorter EIPs (14 days) than mosquitoes feeding on humans infected with strains at low mouse passage levels, where the EIP was 22 days [127]. In addition, long EIPs have been observed with dengue virus attenuated strains [179, 180]. Likewise, highly controlled laboratory studies have demonstrated the effect of distinct genotypes, serotypes, and mosquito population on the EIP [181, 182]. Taking into account the advanced technologies available in molecular biology, new avenues for the study of virus-vector interactions should reveal new mechanisms involved in dengue virus transmission dynamics.

3. Disease Control Strategies

Increasing human and associated mosquito population densities and mobility of humans and commercial goods are the main factors that have determined the very successful reemergence of DF and DHF during recent decades. In contrast with YFV, which exploits the same mosquito species (*St. aegypti*) to infect humans, the dengue viruses have evolved to become independent of the need for a reservoir sylvatic environment with which to sustain their epidemicity. Thus, in the absence of effective control strategies we are

faced with the prospect of further increases in morbidity and mortality due to the dengue viruses. Currently, several different approaches (reviewed after) are being developed in the future hope of alleviating this "scourge" of modern times.

3.1. Vector Control. History has shown that vector control measures can be effective in reducing arthropod-borne virus diseases [120, 183–186]. However, many developing countries do not have the necessary resources and infrastructure for successful eradication measures to be implemented and sustainable in the manner that has been achieved in Singapore and Cuba [187]. This situation is exacerbated by the emergence of resistance to insecticides and the environmental issues arising from the use of potentially toxic chemicals [188]. Today, dissemination of insecticide resistance throughout vector populations is much faster than the rate of development of new insecticides. In addition, the existence of cross-resistance, based on the activation of general detoxifying mechanisms in the vector, can shorten the useful lifespan of alternative insecticides or even prevent their implementation [189].

New approaches to vector eradication including the use of naturally occurring plant insecticides/larvicides [190] and vaccines that induce antibodies to impair vital functions in mosquitoes [191] are being considered but such approaches are unlikely to provide effective vector control measures in the near future.

Primary prevention of dengue is largely dependent on larval and adult mosquito control. *St. aegypti* surveillance has relied heavily upon larval indices. However, this has been strongly criticised as they provide little information to determine the risk of DENV transmission. The studies of Bisset Lazcano and colleagues used pupal surveillance for the *St. aegypti* control programme in Cuba and focused on the most productive mosquito water containers [192]. In urban areas, *St. aegypti* breed on water that collects in artificial containers such as plastic cups, used tyres, broken bottles, flowerpots, and other water traps. Elimination of these containers is the most effective way of reducing the mosquito breeding grounds. The use of insect repellents, mosquito traps, and mosquito nets in the home can also be moderately effective in reducing the number of bites due to mosquitoes.

Novel alternative approaches have also been investigated. In Vietnam, trials were conducted in which children and local communities were encouraged to place, a known mosquito predator, the crustacean *Mesocyclops* [193], in water tanks and discarded containers where *St. aegypti* are known to thrive [194]. The concept exploited the principle that this procedure might be more cost-effective and environmentally friendly than the use of pesticides. Over a period of years and in defined rural provinces of Viet Nam a reduction in mosquitoes and dengue fever was observed [195, 196]. However, such approaches are only likely to be successful in regions of countries with the organisational infrastructure and appropriate community attitudes. A community education strategy is utilised to promote participation in dengue prevention in Cuba, resulting in reduced mosquito vector infestation levels. The main principle has been to increase community participation in decision-making and strengthening the competence of the medical teams and community working groups [197]. Whether or not a similar approach could be successfully applied to major cities and urban areas in other countries remains to be seen.

An alternative approach involves infecting *St. aegypti* with the bacterium Wolbachia [189]. Early studies suggest that this reduces the adult lifespan of the mosquito by 50% [198]. This is important because the adult female mosquito is the primary vector of the virus. Insects infected by Wolbachia transmit them transovarially to the next generation. Thus, by reducing the lifespan of the mosquito, virus-vector competence, and virus transmission efficiency, a significant reduction of *St. aegypti* should be observed. Another important feature of Wolbachia is its ability to induce resistance to a variety of pathogens, including DENV, in its insect hosts [199]. In the transinfected *St. aegypti*, all the three different types of Wolbachia, wAlbB, wMelPop-CLA, and wMel, induce significant inhibition of DENV replication and dissemination, resulting in either complete or partial block of virus transmission [200–202]. Recent studies also show that Wolbachia induces production of reactive-oxygen species which then activate the Toll-pathway to induce expression of antiviral effectors [203]. Interestingly, it was recently demonstrated that native Wolbachia symbionts limit transmission of DENV in *St. albopictus* by restricting the delivery of infectious viral particles from the mosquito saliva when biting. These results might therefore explain the low vector competence of *St. albopictus* for dengue and thus its relatively weak contribution as an epidemic dengue vector [204].

Understanding how Wolbachia density is regulated by mosquito hosts and how the Wolbachia machinery controls its replication will facilitate the current effort to eliminate dengue through Wolbachia-based population replacement [205].

The genetic structure of *St. aegypti* populations and its implications for potential mosquito releases have been studied in Queensland, Australia [206], and Tri Nguyen village, Vietnam [207, 208]. Populations of *St. aegypti* artificially infected with strains of *Wolbachia pipientis* that interfere with its vector competence are being backcrossed into wild mosquito genetic backgrounds from north Queensland and assessed as potential candidates for release [209]. In addition, a pilot release—http://www.eliminatedengue.com/project/vietnem/progress —of infected mosquitoes has been authorised to take place from April 2013 on Tri Nguyen village (611 households) on Hon Mieu Island in central Vietnam. Subject to satisfactory results larger scale studies could be launched within five years [210].

Promisingly, studies related with the effect of Wolbachia on insecticide susceptibility in lines of *St. aegypti* have demonstrated that spreading Wolbachia infections are unlikely to affect the efficacy of traditional chemical methods of controlling mosquito outbreaks [211].

3.2. Development of Vaccines against Dengue Viruses. It is generally agreed that vaccination can provide an effective method with which to control virus diseases. In the case of the dengue viruses, the four serotypes are sufficiently antigenically different that it is considered necessary to produce four monovalent vaccines, which will then be mixed to produce a tetravalent immunological response. This is a logical approach that has previously been employed successfully with the three monovalent poliovirus vaccines. However, as discussed earlier, the dengue viruses also present the problem of antibody mediated enhancement of disease severity [75, 118, 212]. Long-term protection is essential as severe dengue has been observed in individuals secondarily infected more than 20 years after the primary infection [63, 85]. As it is virtually impossible to test whether or not the tetravalent formula would overcome this potential problem, an element of uncertainty might prevail following the introduction of the vaccines in Asia and/or Latin America where dengue viruses are most prevalent. In addition, although the protective role of neutralizing antibodies is recognised, correlates of protection need to be defined [213].

Several different approaches are being employed to develop dengue virus vaccines. The vaccine pipeline includes live empirically attenuated vaccines, newer live attenuated vaccines developed using infectious clone technology, genetic vaccines using virus and plasmid vectors, and many recombinant subunit vaccine candidates [214].

Potential vaccines already progressing through clinical trials include a live attenuated tetravalent vaccine, produced via serial subculture in primary dog kidney cells [215–217]. Another approach involves the use of genetic modification of dengue viruses to attenuate their virulence [218–222]. Although vaccine candidates based on infectious virus have shown the greatest progress amongst different dengue vaccine approaches, there are safety concerns associated with their use based on potential reactogenicity, interference amongst the viruses, possible reversion to native virus, and possible increase of virus infectivity and/or virulence via antibody dependent enhancement [223].

An alternative approach is based on the use of the live attenuated YFV 17D vaccine as a backbone for the production of four chimaeric live attenuated viruses in which the prM and E genes of the 17D vaccine virus are replaced by the corresponding genes of the four dengue virus serotypes [224–226]. Preclinical studies demonstrated that the tetravalent vaccine is genetically and phenotypically stable, nonhepatotropic, less neurovirulent than the tried and tested YFV 17D vaccine, and does not infect mosquitoes by the oral route. Vaccine reactogenicity, viremia induction, and antibody responses have been investigated in phase 1 trials in the USA, the Philippines, and Mexico. Preclinical and clinical trials showed favourable immunogenicity and short-term safety of this vaccine [227]. Relatively favourable results of phase 2 trials were published recently. A surprising lack of efficacy against DENV2 was observed, and the fact that DENV2 was the prevalent serotype during the study diminished the overall vaccine efficacy in this setting [228].

Several possible causes of this apparent failure have been proposed including significant genetic differences between the circulating DENV-2 genotype and the strain incorporated in the yellow fever chimaeric vaccine, imbalanced viraemias, or immune responses due to interference. Nevertheless, Sanofi's CYD dengue vaccine has been discussed as a potential "75% solution" referring to the vaccine's efficacy towards three of the four DENVs in the context of potential antibody-dependent enhancement. The general view seems to be that this approach would be inappropriate [229]. Probably, the most relevant issue is related to the long-term safety of such vaccines. DENV-2 has been associated with severe disease in several epidemiological settings. In fact, studies in Cuba demonstrated that disease severity increased notably when infection with DENV-2 follows infection with DENV-1 at an interval of 20 years [64], probably due to a significant decrease in mean titre of heterologous neutralizing antibodies [128]. Thus, there is justified concern that CYD vaccinated individuals could develop severe disease if infection with DENV-2 occurs after a relatively long interval of time. On the other hand, depending on the DENV-2 genotype that might subsequently circulate, it cannot be ruled out that heterologous neutralisation might lead to a satisfactory immune outcome, as occurred in Cuba during the 2001-2002 epidemic caused by DENV-3, where most DENV-2 immune individuals (infected in 1981) developed asymptomatic DENV-3 infections whilst a high proportion of the DENV-1 immune cases suffered overt disease [63, 230]. In summary our limited understanding of the underlying processes of the immunopathological response to primary and successive infections with the four DENV largely determines our inability to predict the clinical outcome [231, 232]. Nevertheless, ongoing large-scale phase 3 studies in more than 30,000 volunteers in ten countries in Latin America and Asia should provide critical data with which to overcome initial problems identified with the CYD dengue vaccine candidate.

A similar approach is being developed based on the attenuated DENV-2 virus, DENV-2 PDK-53, and three chimaeric viruses containing the prM and E genes of DENV-1, DENV-3, and DENV-4 virus in the genetic backbone of the DENV-2 PDK-53 virus (termed DENVax). Based on the safety, immunogenicity, and efficacy in preclinical studies in animal models, phase 1 clinical testing of tetravalent DENVax has been initiated [233–235]. This candidate might have advantages as the DENV backbone could reduce the risk of unanticipated effects due to the YFV NS proteins present in the Sanofi vaccine candidate [228].

Potential vaccines not yet progressing through clinical trials include the development of subunit vaccines based on domain III of the dengue virus envelope protein. Recombinant fusion proteins formed by domain III and P64k protein from Neisseria meningitides expressed in *E. coli* induce functional and protective immunity in mice and nonhuman primate models inducing highly serotype specific immune responses [236–238]. Additionally, the domains of each serotype have been engineered in tandem in a yeast expression system [239]. A recombinant adenovirus system has been utilised to express the DENV NS1 proteins [240]. The paediatric measles vaccine has been modified to express a fragment of the DENV-1 M protein together with

domain III of the envelope protein [241]. More recently, the evaluation in mice of a novel domain III-capsid chimaeric protein expressed in *E. coli* provides additional evidence for a crucial role of cell-mediated immunity in protection against dengue virus [242–245]. Finally, a novel single-dose lipidated consensus dengue virus envelope protein domain III (LcEDIII) subunit vaccine was shown to induce humoral and cellular immune responses in mice [246]. This group also evaluated the efficacy of the newly developed water-in-oil-in-water multiphase emulsion system, termed PELC plus CpG oligodeoxynucleotides, in potentiating the protective capacity of dengue-1 envelope protein domain III concluding that it could be a promising adjuvant for recombinant protein candidates [247].

Whilst subunit vaccines may have some advantages in terms of type-specific neutralisation with a low potential for inducing ADE via cross-reactive antibodies and low reactogenicity, multiple doses are usually needed to ensure long-term immune protection. However, it has been suggested that recombinant domain III vaccine could function as a booster if used in combination with live vaccine [223].

It is anticipated that within the next five years, increased understanding of the basis for dengue pathogenesis [248] and the protective immune response to DENV will improve our ability to develop safer and more effective vaccines [223].

Critical issues in dengue vaccine development have been reviewed [249]. Of relevance is the potential impact of vaccination on the evolution of naturally occurring DENVs. Vaccination could ultimately produce an environment where relatively low transmission of natural DENV occurs. This is especially relevant if vaccination is focused on a selected portion of the population, thereby increasing stochastic events that will allow new DENV genotypes to emerge possibly with greater virulence. Furthermore, dengue vaccination may produce a background of low titres of enhancing antibody to specific DENV serotypes, resulting in the emergence of specific serotypes in a population. Recent studies suggest that strain diversity may limit the efficacy of monoclonal antibody therapy or tetravalent vaccines against DENV as neutralization potency generally correlates with a narrowed genotype specificity [250]. Consequently, a better understanding of dengue immunopathogenesis will assist not only development of therapeutic interventions but also the understanding of dengue vaccine efficacy or vaccine adverse events [97]. Therefore, laboratory surveillance of dengue needs to be improved considerably to increase our knowledge of the circulating viruses at the molecular level, preferably before the introduction of a vaccine on a large-scale.

3.3. Development of Antivirals against Dengue Viruses. Whilst no approved antiviral therapeutic agents are available to treat individuals presenting with symptoms of DF/DHF, several potential virus inhibitors are under consideration for further development. The dengue viruses provide a variety of potential targets for inhibitors of infection/replication. As Hepatitis C virus (HCV) is also a member of the *Flaviviridae*, antivirals under development to control disease due to HCV may also prove to be effective against the dengue viruses. One of the major problems likely to be encountered is drug resistance. Consequently, the discovery and development of at least two antivirals that attack different viral targets should be a minimum goal. Another major hurdle for dengue viruses is the lack of availability of a validated animal model that faithfully reflects DHF and DSS observed in patients.

The NS5 viral RNA dependent RNA polymerase (RdRp) and the methyl transferase, as well as the NS3 protease and helicase, are considered good targets for inhibitors of dengue infection, because they are all major components of the replicative viral complex [251]. The viral envelope (E) protein is also a good target for antivirals. The use of E protein-specific monoclonal antibodies has been shown to have some potential in this context [252]. The NS1, prM, and capsid protein of the dengue viruses have not been studied at the same level of intensity, and thus there are few if any potential antivirals against these targets.

One approach that appears quite successful both *in vitro* and *in vivo* has been the demonstration that specific antisense morpholino oligomers can inhibit dengue virus replication [253, 254]. However, major efforts are required to reduce the risk of toxicity and to provide safe and effective delivery systems for these oligomers [252, 255].

Other approaches are being utilised to identify flavivirus chemotherapeutic agents, including screening known inhibitors of other viruses, rational design based on protein crystal structures or secondary viral RNA structures, optimization of known viral inhibitors, use of humanized antibodies, use of immunoglobulins, and nucleic acid-based therapy [256].

Polyoxotungstates and sulphated polysaccharides show some potential as viral inhibitors. They impair flavivirus adsorption and entry into host cells *in vitro*, apparently by binding to the cell surface [257, 258]. Sulphated galactomannans protected mice from lethal YFV infection when inoculated simultaneously with the virus [259].

The licensed drug Ribavirin has been used to treat a number of RNA viral infections. It functions as an RNA cap analogue and mutagen, causing errors in synthetic pathways [260–262]. However, the *in vitro* and *in vivo* activity of Ribavirin against YFV and DENV was poor [263–265]. Prophylactic Ribavirin treatment of rhesus monkeys infected with DENV had little effect on viremia [266] and in mice, intraperitoneal administration of Ribavirin had no effect on survival following intracerebral inoculation with DENV. However, treatment with Ribavirin-2′,3′,5′-triacetate, a prodrug of Ribavirin, resulted in a significantly increased survival time and rate, possibly due to its higher ability to cross the blood-brain barrier [267].

Nucleoside analogues, characterized for chemotherapeutic use against HIV and Hepatitis B virus, show inhibitory activity in cell culture against YFV, DENV, and West Nile virus (WNV) [268]. Rather than blocking RNA replication, some analogues inhibit flaviviruses by inhibiting nucleoside triphosphate synthesis in host cells. For example, 6-azauridine acetate, pyrazofurin, and 2 thio-azauridine inhibit orotidine monophosphate decarboxylase (OMPDC). In contrast, mycophenolic acid and Ribavirin-2′,3′,5′-triacetate inhibit inosine monophosphate dehydrogenase (IMPDH)

and block viral RNA synthesis [269, 270]. Carbamate prodrugs have also been recommended as IMPDH inhibitors since they show *in vivo* activity [271]. Recently a uracil-based multifunctional compound was shown to have strong activity against dengue virus. It is likely that the mechanism of action of the antiviral activity of this compound is through inhibition of the enzyme, IMPDH [272].

An alternative strategy in the search for effective antivirals that potentially reduces the lead-time for their development is to identify drugs already licensed for use to control diseases other than those caused by the target virus. For example, the aminoglycoside, Geneticin (G418), was recently shown to have antiviral activity against bovine viral diarrhea virus (BVDV). Since BVDV, DENV, and YFV all belong to the virus family *Flaviviridae*, it seems possible that a common step in their life cycle might be affected by this aminoglycoside. Geneticin prevented the cytopathic effect resulting from DENV-2 infection of BHK cells, in a dose-dependent manner [273]. However, Geneticin had no detectable effect on YFV in BHK cells.

Ivermectin, a broadly used antihelmintic drug, displays specific inhibitory unwinding activity against helicases from several flaviviruses, including YFV, DENV, and WNV with the half maximal inhibitory concentration (IC_{50}) values in the submicromolar range [274]. Preliminary studies indicate higher binding efficiency with YFV than with DENV. Nevertheless, disappointingly, Ivermectin did not protect hamsters against infection with YFV. Structure-based optimization may result in analogues exerting potent activity against flaviviruses both *in vitro* and *in vivo*.

Doxorubicin is an antineoplastic antibiotic obtained from *Streptomyces peucetius*. This antibiotic exhibits *in vitro* antiviral activity against the YFV17D vaccine strain and the DENV-2 NGC strain. Doxorubicin proved to be cytotoxic in uninfected host cells. However, a novel derivative of doxorubicin, SA-17, showed excellent antiviral activity against DENV and markedly reduced cytopathogenicity [275]. Dose-dependent anti-DENV activity was confirmed using a dengue reporter virus. Time-of-drug addition studies indicated that SA-17 acts at an early stage of the replication cycle. It does not inhibit the replication of the replicon and thus does not work at the level of the viral replication machinery. Further studies revealed that SA-17 exerts it activity *via* a virucidal effect, even when using very high titres of the virus as the inoculum.

A large number of small molecules derived by computer modelling of known enzyme domains were screened for inhibitory activity against DENV-2 virus. Two of these molecules, ARDP0006 and ARDP0009, inhibited DENV-2 with high efficiency. ARDP0009 had no apparent toxicity at the concentrations tested. Selectivity indices calculated for ARDP0006 and ARDP0009 were comparable to those calculated for Ribavirin which has demonstrated inhibition of the DENV-2-O-methyltransferase NS5 [276] and HCV replication when used in combination with interferon. Antiviral activity, *in vitro,* of 3′,5′ di-*O*-trityluridine has also been identified. The compound inhibits DENV and YFV replication by targeting the elongation process of the viral NS5 RNA-dependent RNA polymerase. A nucleoside analogue (T-705) which is a substituted pyrazine compound that has been used in clinical trials for the treatment of human influenza virus infection is an analogue of T-1106, a known HCV polymerase inhibitor [277]. T-705 significantly improved survival and disease parameters in YFV-infected hamsters despite the lack of good *in vitro* antiviral activity. These studies highlight the possibility that nucleoside analogues could potentially be developed for flavivirus therapy although more potent compounds with reduced toxic effects on the host cells will need to be generated.

Flaviviral inhibitory activity has also been observed with plant extracts. Boesenbergia rotunda (L.) Mansf. Kulturpfl. (BR) is a common spice belonging to a member of the ginger family (Zingiberaceae). Some of the BR compounds, such as flavanoids and chalcones, have been shown to be pharmaceutically active. The chalcone, cardamonin, isolated from BR, was recently reported to exhibit appreciable anti-HIV-1 protease inhibition [278]. Moreover, inhibitory activity by six compounds isolated from BR has also been demonstrated on DENV-2 virus NS3 protease activity.

In conclusion, whilst mosquito control strategies have been shown to be successful in reducing the incidence of dengue infections, such methods are most effective in those tropical/subtropical countries that have well-developed human and environmental health infrastructures. Clearly, there is a real need for more effort to understand the complex epidemiology and pathogenesis of the dengue viruses to expedite the development of suitable vaccines and/or antiviral therapies. Although some vaccine candidates appear promising, as yet none has been licensed. Due to the presence of the four DENV serotypes, these viruses present a different situation from YFV, tick-borne encephalitis virus, and Japanese encephalitis virus. The question of whether or not deteriorating antibody levels will leave vaccinated people liable to the development of DHF via ADE will need to be addressed. Moreover, the relationships between the presence of neutralizing antibodies, the level of protection afforded and the duration of protection by each of the four serotypes will need to be critically assessed. There is a pressing need for global collective efforts to develop antiviral therapeutics with which to combat dengue viruses. The current trend of expanding our efforts on antiviral drug discovery is encouraging in this respect.

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Review Article

Dengue in the United States of America: A Worsening Scenario?

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Dengue is a febrile illness caused by any of the four dengue virus types (DENV-1 to -4, genus *Flavivirus*, family Flaviviridae) mainly transmitted by the mosquito *Aedes aegypti*. DENV can be transmitted by blood transfusion. Dengue has been historically present in the continental United States (US), in the state of Hawaii, and in the US insular territories in the Caribbean and the Pacific. During the second half of the 20th century, most of the cases reported in the US were imported cases brought to the country by travelers. Since 2009, cases of autochthonous dengue have been recognized in the state of Florida after 75 years of absence, followed by intensification of transmission in endemic places including the US territories of US Virgin Islands and Puerto Rico, which experienced a large dengue epidemic in 2010. The widespread distribution of dengue mosquito vectors, deficient mosquito control measures and increased frequency of DENV-infected visitors to the US coming from dengue-endemic locations or places experiencing epidemics appear to be jointly responsible for the emergence and reemergence of dengue in the US and its territories.

1. Introduction

Dengue, the most prevalent arthropod-borne viral disease in the world, is an acute, febrile disease caused by any of the four dengue virus types (DENV-1 to -4, genus *Flavivirus*, family Flaviviridae) [1]. DENV is naturally transmitted by mosquitoes from the genus *Aedes,* mainly by the urban species *Aedes aegypti,* and in some geographical regions by *Aedes albopictus*. Infection by dengue viruses can be asymptomatic or cause disease of variable degree of severity. Dengue ranges from a mild, influenza-like illness known as dengue fever (DF) to a severe and potentially life-threatening condition called dengue hemorrhagic fever (DHF). DHF can ultimately evolve to hypovolemic shock (dengue shock syndrome (DSS)) and death [2]. DHF and DSS are classified as severe dengue in the newest World Health Organization (WHO) dengue clinical classification [3].

DENV can also be transmitted by transfusion of blood and blood components and by solid organ transplants containing infectious virus [4–6]. Dengue is endemic in most countries of tropical America, the Caribbean and Southeast Asia, and causes episodic epidemics in islands of the Pacific and in Africa [7, 8]. Many DENV endemic regions are hyperendemic, which is defined by the circulation of all DENV types in a specific geographical area. This may increase the opportunity for occurrence of secondary infections with increased clinical severity via antibody-dependent enhancement of the infection by sub-neutralizing concentrations of anti-dengue antibodies in individuals previously infected with a different dengue type (heterologous infection) [9].

Approximately, 50–100 million cases of dengue were calculated to occur each year around the globe [3]. However, the newest estimates raise these numbers to about 390 million cases per year (range 284–528), from which approximately 96 (range 67–136) will present clinical manifestations of any severity [10]. Despite its low lethality rates, dengue causes severe economic and social disruption and has profound impact on the welfare of regions affected by the disease since it is endemic mostly in developing countries where healthcare systems have limited resources [11–13]. After many years of continuous efforts, there are still neither effective vaccines nor specific antiviral treatments available against DENV [14].

The mosquitoes that serve as DENV vectors have been able to reach sub-tropical and temperate regions, including North America and countries in Europe [15–17]. In addition, DENV brought in by infected travelers has been able to establish autochthonous infection cycles in some of these countries. DENV is considered the most common cause of

febrile illness in travelers returning to the USA from destinations in the Americas and Asia [18].

The scope of this review is to provide an analysis of the epidemiology of dengue in the United States and its territories, with emphasis on the changes in dengue activity in the last decade and on aspects on the molecular epidemiology of currently circulating DENV.

2. Dengue in the United States

Dengue is thought to have been present in the USA since the end of the 18th century, when Dr. Benjamin Rush, a physician and signatory of the US Declaration of Independence, described a disease resembling dengue fever in Philadelphia during 1780 [27]. During the first half of the 20th century, a number of dengue outbreaks were reported in the continental USA, especially in the gulf and southeastern states (i.e., Alabama, Florida, Georgia, Louisiana, Mississippi and Texas), in the state of Hawaii, and in the USA Territories in the Caribbean and the Pacific Ocean (Table 1).

The continental USA comprises the 48 contiguous states and the District of Columbia, and the non-contiguous states of Alaska in North America and Hawaii in the Pacific Ocean and a number of unincorporated territories including Puerto Rico and the US Virgin Islands (USVI) in the Caribbean and American Samoa, Guam, and the Northern Mariana Islands in the Pacific Ocean [19].

Autochthonous dengue has been reported sporadically in the Mexico-Texas border, where indigenous cases of dengue reappeared in 1980 after more than 30 years of absence. Since then, most dengue cases reported in the USA have been imported, brought by infected travelers returning from visits to endemic countries or places experiencing dengue epidemics. Dengue has also been present in the state of Hawaii, the US territories in the Pacific (American Samoa, Guam, and Northern Mariana Islands) and the territories of Puerto Rico and USVI. In 2009, after over 70 years of absence, autochthonous dengue reappeared in the state of Florida (Figure 1).

In all those instances, dengue outbreaks were facilitated by the presence of the mosquito vector, the favorable climatic conditions for its subsistence, and the presence of susceptible individuals. The dengue mosquito vectors *Aedes aegypti* and *Aedes albopictus* have been reported to be present in several counties in the USA, especially in the Southern and Southeastern regions of the country [28] (Figure 2).

2.1. Dengue in the Mexico-Texas Border. The first autochthonous case of dengue reported in the USA since 1945 occurred in Brownsville, Texas in 1980 when DENV-1 was isolated from a 5-year-old girl that did not have history of travel outside Brownsville (Table 1). Brownsville is located across the Rio Grande from the city of Matamoros in the state of Tamaulipas, Mexico where many years of dengue activity had been observed [21].

Subsequently, surveillance studies reported 63 additional dengue cases (all caused by DENV-1) that were laboratory confirmed in Texas, 52 of which occurred in counties contiguous to the Mexico-Texas border [30]. Six years later, autochthonous transmission of DENV-1 was again reported in at least 9 individuals in Texas [22]. The Mexico-Texas border region is at risk for dengue endemicity due to the presence of the competent mosquito vectors *Aedes aegypti* and *Aedes albopictus* and to the circulation of all DENV types (DENV-1 to -4) in recent years [31]. In sum, dengue has been detected in residents of seven Texas counties: Bee, Cameron (where Brownsville is located), Hidalgo, Maverick, Nueces, Travis, and Webb [23, 30, 32].

In 2005, a woman who had not traveled to Mexico in the months before the onset of dengue symptoms developed DHF, and this was the first case of autochthonous DHF reported in Texas. During that year, a large dengue epidemic developed in the neighboring Mexican state of Tamaulipas with more than 1,200 dengue cases of which 223 (18%) were classified as DHF. Meanwhile, 25 dengue cases were reported in Brownsville, of which at least three were locally acquired [32]. The disparities in the number of cases observed on different sides of the border have been explained by the higher standard of living in the USA including the use of air-conditioners and the presence of nets in doors and windows, which remain closed most of the time reducing the density of indoor mosquitoes thus minimizing the risk of mosquito bites [33].

2.2. Dengue in Florida. After 75 years without reports of dengue activity in Florida, a case was identified in 2009 in a New York patient who had traveled to Key West (Table 1). This individual had not traveled to any other place either in the USA or abroad before the onset of symptoms, and the case was laboratory-confirmed by the Centers for Disease Control and Prevention (CDC) using RT-PCR. A serosurvey conducted in 2009 revealed that at least 5.4% of the residents of Key West had serological evidence of recent dengue infection [24].

According to the Florida State Department of Health, between 2009 and 2012, a total of 103 autochthonous dengue cases have been reported in Florida including those from the outbreak of 2009. Of these, 27 were reported in 2009 in Key West (Monroe county), 65 during 2010 (Broward, Miami-Dade, and Monroe counties), 7 in 2011 (Hillsborough, Martin, Miami-Dade, and Palm Beach counties) and 4 in 2012 (Miami-Dade, Seminole, and Osceola counties) [25, 26], (Table 2).

DENV-1 was detected in mosquito pools collected in Key West [49] and in a blood donor from Key West during 2010 [50]. The strains isolated from these two specimens were sequenced, and subsequently, a number of DENV-1 strains obtained from samples from dengue cases from Key West/Monroe county (8 patients) and one strain each from patients from Broward, Miami-Dade, Orange, and Pinellas counties were sequenced [51]. All DENV-1 strains from Florida from 2009-2010 sequenced to date (obtained from mosquito pools, dengue cases, and a blood donor) belong to the genotype V of the virus and have phylogenetic relationships with Central American strains; however, all but one of the strains sequenced from Key West grouped together in the phylogenetic trees, suggesting that *in situ* microevolution has occurred and that this Key West sublineage was transmitted

Table 1: Dengue activity in the continental USA between 1780 and 2013.

Year(s)	Activity reported	References
1780	Dengue suspected in Philadelphia, PA	[19]
1826	USA ports report 1st dengue outbreak	[20]
1827-1828	Epidemic in Southern USA	[20]
1845	Dengue reported in St. Louis, MO	[20]
1850-1851	1st report of dengue epidemic inland (including GA and MS), epidemic in Southern USA, New Orleans, LA, and along South Coast	[20]
1870–1872	Epidemic in Southern USA	[20]
1873	Dengue reported in LA, AL, and MS; ≈40,000 cases reported in New Orleans	[20]
1879-1880	Epidemic in Southern USA	[20]
1885-1886	Dengue in gulf ports of TX, dengue in Austin, 16,000 estimated cases of 22,000 inhabitants	[20]
1897–1903	Epidemic in Southern USA, TX most heavily affected	[20]
1904	Dengue reported in FL and TX	[20]
1916	Fatal case of possible DHF reported in TX	[20]
1922	Dengue epidemic, 500,000 to 600,000 cases in TX, 30,000 in Galveston, and 7,561 in LA	[20]
1923	1,376 dengue infections reported in LA	[20]
1924	1 dengue case in LA	[20]
1941–1944	Texas and gulf states involved in epidemic	[20]
1945	Last continental epidemic of dengue reported in LA	[20]
1980	1st indigenous dengue cases in USA since 1945 (Brownsville, TX), DENV-1 isolated	[21]
1981	DENV-4 cases reported, 1st isolation of DENV-4 in the USA	[20]
1982	1st reports of DENV-2 in the USA	[20]
1983	1st reports of DENV-3 in the USA	[20]
1986	DENV-1 reported in TX	[22]
1987	Autochthonous dengue reported in TX	[20]
1990	DENV-1, -2, and -3 isolated in the USA, reports of 102 dengue cases	[20]
1991	DENV-1 and DENV-3 isolated in the USA, 25 dengue cases reported	[20]
1994	91 cases of dengue, DENV-2 and -3 isolated in the USA	[20]
2005	First case of autochthonous DHF case reported in TX	[23]
2009–2011	Autochthonous dengue transmission in FL, DENV-1 isolated. DENV-1 isolated from a blood donor from Key West, FL in 2010	[24–26]
2012	4 DENV cases reported in FL, 2 of them in Miami-Dade	[26]
2013*	No indigenous dengue cases reported	[25]

Adapted from [20].
AL: Alabama, FL: Florida, GA: Georgia, LA: Louisiana, MO: Missouri, MS: Mississippi, PA: Pennsylvania, and TX: Texas.
* As of May 10, 2013.

during 2009 and 2010 (Figure 3) [50, 51]. Sequences of DENV-1 strains obtained from cases acquired in other counties in Florida clustered in a separate phylogenetic group, more closely related to Central American strains (i.e., from Costa Rica, Mexico, and Nicaragua). Only one DENV-1 strain from Key West was found clustering outside the Key West lineage, which suggests that introduction of at least two different DENV-1 lineages occurred in Key West during 2009-2010 epidemics [51] (Figure 3).

The dengue outbreak of 2009-2010 has been linked to *Aedes aegypti*, which is prevalent in Monroe county where Key West is located. Although *Aedes albopictus* is present in Florida, it has not become established in Monroe county. Mosquitoes from both *Aedes* species obtained from colonies in the state of Florida were experimentally infected with a DENV-1 strain isolated from Key West, and vector competence for this virus was assessed. There were no significant differences in the rates of infection, dissemination, and transmission between these two mosquito species occurring in Florida. The entire genome of the DENV-1 specimen utilized in this vector competence study has been sequenced [16] and shown to cluster within the Key West lineage (Figure 3).

In addition to the autochthonous cases, an important number of dengue imported cases (392) from travelers to countries in Central and South America, the Caribbean, Africa, the Middle East, and South and Southeast Asia have

Table 2: Imported and autochthonous cases of dengue reported in the state of Florida, USA, 2009–2013 (as of April 27, 2013).

Year	Number of imported cases	Countries visited* (number of cases)	Number of autochthonous cases	Florida counties (number of cases)
2009	36	BO (2), BR, CO (2), DO (3), GT (2), HT (10), HN (2), IN (3), MY, MX, NI, PA (3), PH, PR (3), SR	27	Monroe (27)
2010	133	BD, BR, KY, CO (8), CR (4), CU, DO (13), EC, SV, GH, GD (4), GT (2), HT (6), HN (6), JM (5), MQ (2), MV, MX, NI (13), PK, PH, PR (36), TH, TT, VE (16), VI (3), MY/AE/BD**, PA/VE**	65	Broward (1), Miami-Dade (1), and Monroe (63)
2011	61	AW, BS (14), BD (3), BR (3), CO, CR, CU (5), DO, GD, GY, HT (2), IN, JM (2), NI (2), PK, PA (2), PR (11), LC (2), TT (4), TC, VE, VN	7	Hillsborough (1), Martin (1), Miami-Dade (3), and Palm Beach (2)
2012	135	BR, CO, CU (29), DO (17), EC (4), SV (2), GH, GY (2), HT (17), HN, IN, JM (23), MX (2), NI (2), PA, PH (4), PT, PR (16), ZA, LK, VC, SR, TT (4), TC, VI	4	Miami-Dade (2), Seminole (1), and Osceola (1)
2013	27	AO, BB, BR, CO (2), DO (3), GT, HT, ID, JM (3), NG, PH, PR (8), MF	0	—
Total	392		103	

*AE: United Arab Emirates, AO: Angola, AW: Aruba, BB: Barbados, BD: Bangladesh, BO: Bolivia, BR: Brazil, BS: Bahamas, CO: Colombia, CR: Costa Rica, CU: Cuba, DO: Dominican Republic, EC: Ecuador, GD: Grenada, GH: Ghana, GT: Guatemala, GY: Guyana, HT: Haiti, HN: Honduras, ID: Indonesia, IN: India, JM: Jamaica, KY: Cayman Islands, LC: Saint Lucia, LK: Sri Lanka, MF: Saint Martin, MQ: Martinique, MY: Malaysia, MX: Mexico, MV: Maldives, NG: Nigeria, NI: Nicaragua, PA: Panama, PH: The Philippines, PK: Pakistan, PR: Puerto Rico, PT: Portugal, SR: Suriname, SV: El Salvador, TC: Turks and Caicos Islands, TH: Thailand, TT: Trinidad and Tobago, VC: St. Vincent and the Grenadines, VE: Venezuela, VI: US Virgin Islands, VN: Vietnam, and ZA: South Africa.
**Travel to more than one country.
Source: Florida State Department of Health [25, 26].

Table 3: Dengue activity in the state of Hawaii and the Territories of American Samoa, Guam, and Northern Mariana Islands, 1840s–2010.

Year(s)	Activity reported	References
Late 1840s	First large dengue epidemic recorded in HI, associated with *Aedes aegypti*	[34]
1903	Large dengue epidemic in HI, ≈30,000 cases, associated with *Aedes aegypti*	[35]
1943-1944	DENV-1 epidemic in HI, ≈1,500 cases	[34, 36]
1944	*Aedes aegypti* reported to be eradicated from GU. *Aedes albopictus* reported to be present in the island	[37]
<1950	Dengue cases were reported in AS and GU before 1950, no dengue epidemics reported in GU in recent times	[38]
1995	Possible dengue infection in German visitors to HI	[39]
1972	Dengue epidemic in AS (DENV-2)	[40]
1975	Dengue epidemic in AS (DENV-1)	[38, 40]
1995-1996	Dengue epidemic in AS (DENV-3)	[38]
1997	Reports of dengue cases in AS	[41]
1998	Dengue seropositive individuals reported in Saipan (MP) during 1998, DENV-2 implicated in epidemic activity	[42, 43]
2001	More than 1,600 dengue cases reported in AS (DENV-1), 3 deaths	[44]
	Dengue outbreak in the MP, >1,400 cases reported	[45]
2001-2002	Autochthonous transmission of dengue in HI, 122 confirmed cases, DENV-1 isolated, and *Aedes albopictus* was the implicated vector	[46]
2007	63 dengue cases confirmed in AS, 23 cases hospitalized	[47]
2008	Dengue activity reported in AS	[48]
2009	Outbreak of ≈400 confirmed cases in AS	[48]
2010	Dengue cases reported in AS. Serosurvey conducted in 2010 revealed >95% of the tested individuals as seropositive for dengue	[8, 48]

AS: American Samoa, GU: Guam, HI: Hawaii, and MP: Northern Mariana Islands.

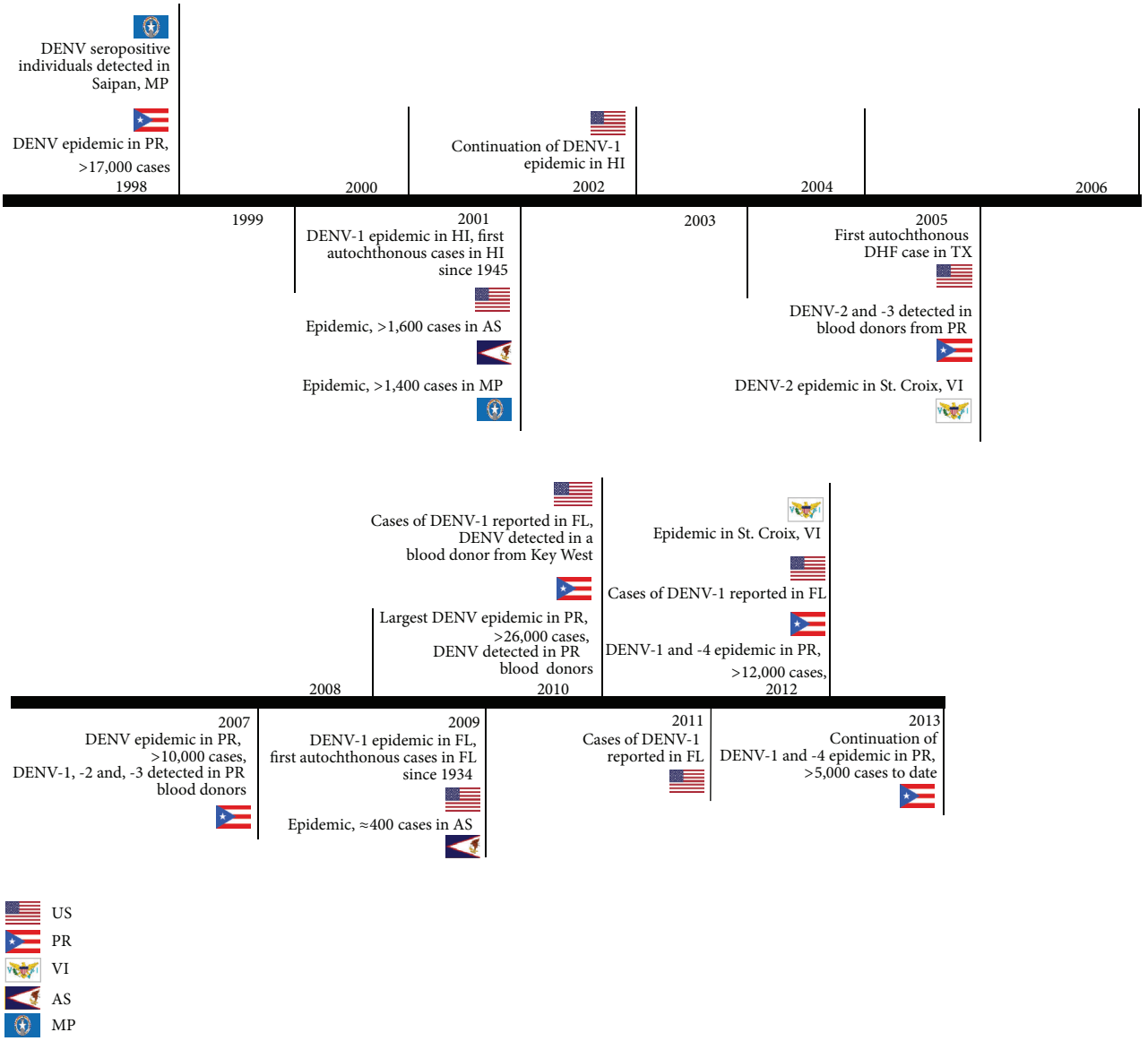

Figure 1: Timeline of selected recent dengue activity in the U.S. and its territories, 1998–2013. U.S.: United States; representing the states of Florida (FL), Hawaii (HI) and Texas (TX), P.R.: Puerto Rico, V.I.: U.S. Virgin Islands, A.S.: American Samoa, M.P.: Northern Mariana Islands. Numbers shown represent dengue reported cases.

been reported in Florida since 2009 [25, 26] (Table 2). The wide geographical area of origin of dengue imported cases in Florida reflects the importance of the Miami International Airport as a gateway to the USA and a possible route of entry for these and other pathogens.

2.3. Dengue in Hawaii. The first large dengue epidemic on record occurred in Hawaii in the mid-19th century. Another large epidemic occurred in 1903 with approximately 30,000 cases reported [34, 35]. *Aedes aegypti* was the vector implicated in those dengue outbreaks. But at a certain point during the early 20th century, *Aedes albopictus* was introduced in these islands and displaced *Aedes aegypti* [52].

A DENV-1 epidemic occurred in Hawaii from 1943 to 1944, with almost 1,500 cases reported [36, 53]. As in the continental USA no autochthonous cases of dengue were reported after 1945 and imported dengue cases were reported at a low frequency in Hawaii [54]. In 1995, two German travelers developed symptoms compatible with dengue infection after a trip to Hawaii [46] (Table 3).

However, in 2001, following 56 years without reports of autochthonous dengue cases, an epidemic occurred in the island of Maui. A total of 122 laboratory-confirmed dengue cases were reported between 2001 and 2002, 92 of which occurred in Maui, 26 in Oahu, and 4 in Kauai. DENV-1 was the type identified in viral isolates obtained from 15 cases from Hawaii, and the mosquito vector implicated in this epidemic was *Aedes albopictus* [54].

Molecular epidemiologic studies suggest that at least two distinct DENV-1 strains were introduced in Hawaii during

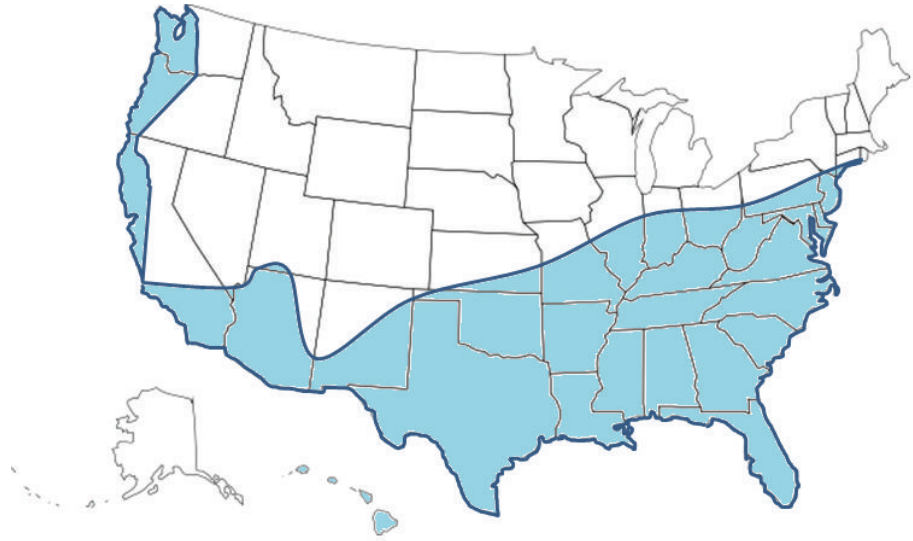

Figure 2: Map of the U.S. showing the areas at risk of dengue outbreaks, based on the approximate distribution of dengue mosquito vectors *Aedes aegypti* and *Aedes albopictus*. Map adapted from [28, 29]. The delimited area represents the approximate geographical area in which either dengue mosquito vector (*Aedes aegypti* and/or *Aedes albopictus*) have been found present in the USA and are therefore considered to be at risk for the establishment of dengue outbreaks. The noncontiguous states of Alaska and Hawaii are not shown at scale. U.S. territories are not shown.

the 2001-2002 epidemics, although most of the isolates analyzed belong to the "Pacific subtype" (genotype IV) of DENV-1, and cluster together with Tahitian DENV-1 strains, suggesting that Tahiti was the source of these strains. In contrast, the same study reported a single DENV-1 isolate from Hawaii obtained during 2001 from a traveler to Samoa that clustered outside the Tahitian cluster and that closely associated with a DENV-1 isolate previously obtained from another traveler to Samoa [39] (Figure 3).

2.4. Dengue in American Samoa, Guam and Northern Mariana Islands. Dengue was reported in American Samoa and Guam before 1950 [38] (Table 3). The main vector, *Aedes aegypti,* had been considered as eradicated from Guam since 1944. However, *Aedes albopictus* is abundantly present in the island [37]. Guam has not had dengue outbreaks in recent times, but it is at risk of dengue epidemics due to the presence of *Aedes albopictus* mosquitoes and the arrival of infected travelers from neighboring islands experiencing dengue activity.

Conversely, American Samoa had dengue epidemics in 1972 (DENV-2), 1975 (DENV-1), and 1995-1996 (DENV-3) [38, 40, 71, 72], while some dengue cases were reported in 1997 [41]. In 2001, more than 1,600 dengue suspected cases caused by DENV-1 were reported [42, 47], with 237 hospitalizations and three deaths due to DHF. In 2007, at least 63 dengue cases were confirmed, 23 of which were hospitalized [47]. Dengue activity was reported in 2008-2009; in 2009, an important outbreak of about 400 confirmed cases (for an incidence of 644/100,000 inhabitants) was registered [48].

In 2010, less than 100 dengue cases were reported for an incidence of 77/100,000 inhabitants [8, 48]. Epidemiological surveillance performed in 794 serum samples collected in three islands of American Samoa in 2010 revealed that 759 (95.6%) of the tested individuals were positive for IgG antibodies to dengue [44]. The dengue vectors, *Aedes aegypti* and *Aedes albopictus*, are present in American Samoa [73].

There are reports indicating that individuals from the Northern Mariana Islands, without travel history, were seropositive for DENV (IgM and IgG) in 1998 in the island of Saipan [43], where DENV-2 was the DENV type implicated in those infections [42]. Furthermore, these islands suffered a dengue outbreak in 2001 with more than 1,400 cases reported [45]. Since then, no other dengue cases have been reported.

2.5. Dengue in US Virgin Islands. Dengue has been endemic in the USVI since at least 1924, when the first documented dengue epidemic in the Caribbean is thought to have started in the USVI [20]. These islands are located in close proximity to Puerto Rico, and include the four inhabited islands of St. Croix, St. Thomas, St. John and Water Island. Dengue outbreaks occurred in 1978 (DENV-1) and in 1990, this time involving DENV-1, DENV-2 and DENV-4; 1990 was the year of the first occurrence of DENV-4 in the USVI [20]. The sequence of a DENV-4 strain isolated in 1994 from St. Croix closely associated with strains that were circulating in Puerto Rico around the same time (Figure 4).

Forty dengue cases were reported in the island of St. Thomas in 2004. In 2005, a DENV-2 epidemic was reported in St. Croix, with 331 suspected cases of which 37% were laboratory-confirmed [65]. The sequence of a DENV-2 strain from the 2005 epidemic clustered within the clade IA of the American/Asian genotype, together with a number of strains from the Caribbean and South America [74].

During November 2012, 27 dengue cases were reported in the island of St. Croix, some of which were later laboratory

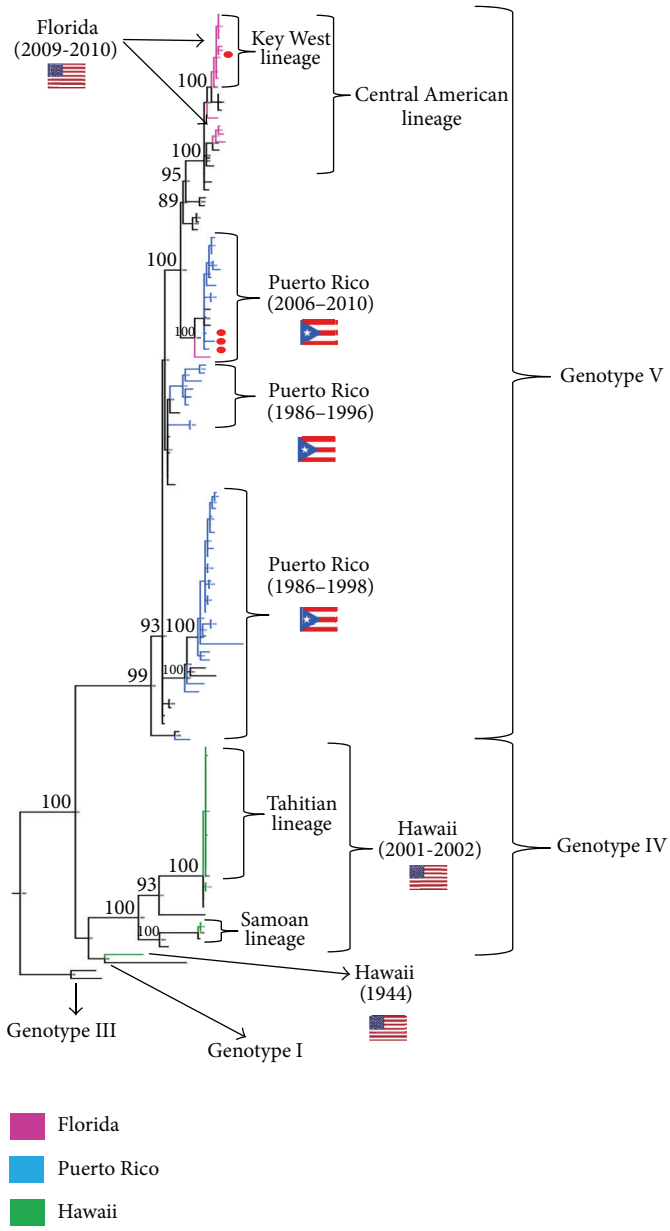

Figure 3: Phylogeny of DENV-1 in the USA and Puerto Rico. A consensus phylogenetic tree (50% majority rule) was obtained by Bayesian phylogenetic analysis (Mr. Bayes, v. 3.2.) based on the envelope protein gene. Analysis included sequences of strains from Hawaii (2001-2002) ($n = 21$), Florida (2009-2010) ($n = 15$), and Puerto Rico (1986–2010) ($n = 45$) available in the GenBank database and representative sequences from DENV-1 genotypes I, III–V ($n = 44$). DENV-2, DENV-3, and DENV-4 were used to root the tree (not shown). Bayesian posterior probability values (>80) are shown for the principal nodes. Taxa are highlighted according to its geographical origin: Hawaii (green), Florida (pink), and Puerto Rico (light blue). A red dot identifies sequences obtained from blood donors.

confirmed. A serosurvey found that around 20% of the students and staff from a school in the island were positive for IgM antibodies to dengue; four students were positive for DENV-1 or DENV-4 RNA by PCR [69]. There is no available genetic information about the DENV strains circulating in St. Croix in 2012.

2.6. Dengue in Puerto Rico. Dengue is endemic in Puerto Rico, and cases have been reported every year for more than 50 years (Table 4). The public health definition of a dengue epidemic in Puerto Rico is based on a historical average: the event is called an epidemic when the number of cases detected by the passive surveillance system is higher than the 75th percentile of the distribution of cases for the same epidemiological week in previous years [75].

A large epidemic of DENV-3 involving approximately 27,000 individuals was reported in 1963-1964 [55, 56]. Another epidemic caused by DENV-2 occurred in 1969 [57]. Subsequently, sporadic dengue cases caused by DENV-2 were reported during the early 1970s [57]. In 1975, a case of

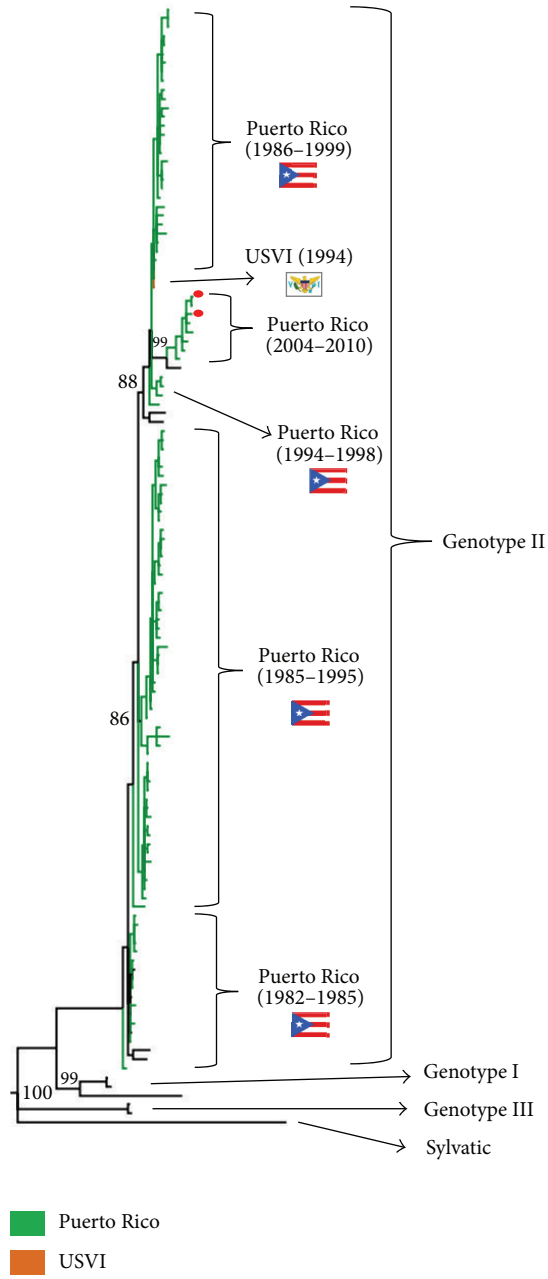

Figure 4: Phylogeny of DENV-4 in Puerto Rico and the US Virgin Islands (USVI). A consensus phylogenetic tree (50% majority-rule) was obtained by Bayesian phylogenetic analysis (Mr. Bayes, v. 3.2.) based on the envelope protein gene. Analysis included sequences of strains from USVI (1994) ($n = 1$) and Puerto Rico (1982–2010) ($n = 115$) available in the GenBank database, and representative sequences from DENV-4 genotypes I–III and Sylvatic ($n = 12$). DENV-1, DENV-2, and DENV-3 were used to root the tree (not shown). Bayesian posterior probability values (>80) are shown for the principal nodes. Taxa are highlighted according to its geographical origin: Puerto Rico (green) and USVI (orange). A red dot identifies sequences obtained from blood donors.

DHF (associated with DENV-2) was reported for the first time in Puerto Rico and in the Western Hemisphere [20]. The outbreak in 1977 was caused by DENV-2, DENV-3 and DENV-1 (DENV types appear in order of frequency of detection), and that was the first documented occurrence of DENV-1 in the island [58].

The following year (1978), another dengue outbreak was reported, this time caused primarily by DENV-1 [59]. In 1981, an outbreak caused by DENV-4, and DENV-1 (in order of frequency) was reported and that was the first report of DENV-4 in the Americas [60]. Additional outbreaks followed in 1982 (DENV-4) [60, 61] and 1985-1986 (DENV-4, DENV-1 and DENV-2, in order of frequency of detection), when a dengue epidemic caused 31 DHF cases and 3 deaths [62] (Table 4).

In 1994-1995, approximately 24,000 cases of dengue were reported in Puerto Rico, with circulation of DENV-2, DENV-4 and DENV-1 (in order of frequency of detection)

Table 4: Dengue activity in Puerto Rico and the USA Virgin Islands, 1915–2013.

Year(s)	Activity reported	References
1915	Dengue epidemic reported in PR	[20]
1924	1st recorded epidemic of dengue in the Caribbean-Gulf-Atlantic region begun in the VI	[20]
1941–1946	Dengue epidemic reported in PR	[20]
1963	Epidemic of ≈27,000 cases (DENV-3) in PR	[55, 56]
1968-1969	DENV-2 (only) epidemic, 1st report of DENV-2 in PR, 16,665 cases	[57]
1970–1974	Sporadic DENV-2 cases reported in PR	[20, 57]
1975	DHF suspected among 3 serologically confirmed dengue cases, shock seen in 1 patient in PR - DHF described for the 1st time in the Western Hemisphere	[20, 57]
1977-1978	DENV-1 outbreak in PR, ≈12,700 cases, 1st report of DENV-1 in PR, after DENV-2 and DENV-3 reports from earlier during that year	[58, 59]
1978	DENV-1 outbreaks in VI	[20]
1981–1983	DENV-1 and DENV-4 outbreaks in PR, 1st reports of DENV-4 in both PR and the Americas	[60, 61]
1985	2 DHF cases associated with DENV-4 in PR	[20, 62]
1986	Dengue epidemic in PR associated with DENV-4, 10,659 cases, 31 DHF cases, 3 deaths	[62]
1987	17 DHF cases in PR, 1 death	[20]
1988	8 DHF cases in PR	[20]
1989	DENV-1, -2, and -4 cases reported in PR, including 12 DHF cases, 5 deaths	[20]
	Dengue cases reported in the VI	[20]
1990	6 DHF cases in PR, 1 death	[20]
	Dengue cases reported, DENV-1, -2, and -4 involved in outbreaks, 1st report of DENV-4 in the VI	[20]
1991	14 DHF cases in PR, 1 death	[20]
1994	≈24,700 cases of dengue reported in PR	[63]
1998	>17,000 dengue cases reported in PR, 173 DHF cases, 9 deaths, all 4 serotypes isolated	[64]
1999	All 4 serotypes reported present in PR, 34 DHF cases (6 deaths), 4,993 dengue cases	[20]
2000	DENV-1, -2 and -3, isolated in PR, 24 DHF cases, 2,433 dengue cases	[20]
2005	Dengue reported in blood donors from PR, DENV-2 and DENV-3 isolated	[4]
	Dengue epidemic reported in St. Croix, VI	[65]
2007	Epidemic caused by DENV-3, -2, -1, and -4 (in order of frequency) in PR, more than 10,000 cases, 227 DHF cases, 40 deaths. Dengue reported in blood donors from PR	[66]
2010	Largest epidemic in PR history, DENV-1, -4, -2, and -3 isolated (in order of frequency), 26,766 cases reported, 448 DHF cases, 128 deaths. Dengue reported in blood donors from PR, DENV-1, DENV-4, and DENV-2 isolated	[67]
2012	Dengue epidemic in PR, 12,877 cases reported, DENV-1 and -4 isolated	[68]
	Dengue epidemic in the VI	[69]
2013*	Dengue epidemic in PR, >5,000 cases reported, DENV-1 and -4 isolated	[70]

Adapted from [20]. PR: Puerto Rico, VI: USA Virgin Islands.
* As of May 20, 2013.

[63]. In 1998, over 17,000 dengue cases were reported, and for the first time the co-circulation of all four DENV types was observed. During this outbreak, DENV-3 was detected in the island after an absence of 20 years [64] (Table 4).

In 1999, all four DENV types co-circulated in the island resulting in 4,993 reported cases. The 2000 epidemic had co-circulation of DENV-1, DENV-2, and DENV-3 and resulted in 2,433 dengue cases reported [20]. DENV-3 was the predominant type in the inter-epidemic period between 1999 and 2003, to be later displaced by DENV-2 as the predominantly DENV type detected during the inter-epidemic period spanning from 2004 to 2006 [66].

In 2007, a large epidemic (10,508 suspected dengue cases, with 227 fulfilling criteria for DHF) occurred after an inter-epidemic period of almost ten years; this epidemic was the second outbreak to have co-circulation of all DENV types, after the 1998 epidemic. During the 2007 dengue epidemic, 2,175 individuals tested positive for DENV RNA, of which 62% was infected with DENV-3, 31% with DENV-2, 6% with DENV-1, and 1% with DENV-4. DENV-1, and DENV-4 reappeared after approximately 9 years of absence (Table 4). Overall, an increased incidence of severe disease was reported in the 2007 epidemic when compared with previous outbreaks, resulting either from more efficient reporting of

severe cases or from a true increase in the incidence of severe dengue [66].

The largest epidemic of dengue in Puerto Rico's history occurred during 2010, with almost 27,000 suspected dengue cases, of which more than 12,000 individuals (about 47% of tested cases) were laboratory-positive; more than 1,300 cases were classified as severe dengue, and there were 40 dengue-associated deaths (Table 4). The DENV types implicated in this epidemic were in order of frequency DENV-1 (69%), DENV-4 (23.7%), and DENV-2 (7.3%), with only two DENV-3 cases reported (<0.1%). The 2010 epidemic is considered the longest-lasting dengue outbreak ever registered in Puerto Rico, with cases starting to appear during the first week of 2010, peaking around August and returning to levels below the historical average in December of that year [67].

In 2012, an epidemic resulted in 12,877 suspected cases reported, of which 5,652 (44%) were laboratory confirmed. DENV-1 and DENV-4 were the predominant types detected, similar to the 2010 epidemic [68], (Figure 1). This outbreak has continued into 2013, although with a declining trend. Up to April 22, 2013, 5,251 dengue suspected cases had been reported, 2,573 (49%) of which were laboratory-confirmed, and similar to the observed during the 2012 epidemic, DENV-1 (78%) and DENV-4 (21%) were the DENV types predominantly reported [70], (Figure 1).

2.6.1. Epidemiological Data Obtained from Asymptomatic Infections in Blood Donors. Because DENV can be transmitted by blood transfusion [4, 6] and infection can be asymptomatic [1–3], there is great concern about DENV activity among blood donors in endemic places in the absence of overt epidemic and during epidemic times. Prevalence studies in blood donors have been performed in various areas around the world where DENV is endemic [76].

A study performed in asymptomatic blood donors from Puerto Rico during the inter-epidemic year 2005 reported that 0.7% (12 out of 16,521) blood donations tested positive for DENV RNA. Of these, three donors were identified by TaqMan as infected with DENV-2 and one with DENV-3, which were the DENV types circulating in the island during that year (Figure 1). Two of the DENV-2 and the DENV-3 strains were isolated in cell culture or by mosquito inoculation [4]. Another study conducted among blood donors during the epidemic year of 2007 found that 29 out of 15,350 donations tested positive for DENV RNA using an assay that detects all four DENV types but does not discriminate between DENV types. Discriminatory real-time PCR detected 12 positive samples: one was identified as DENV-1, four as DENV-2, and seven as DENV-3 (Figure 1). All these 12 samples were infectious in C6/36 cell cultures [6].

Study of DENV strains circulating in 2010 performed in blood donors [50] and in symptomatic cases [67] showed that these DENV-1 strains belong to the genotype V (the only one found to circulate in the Americas to date) but were from a lineage different from those that circulated in Puerto Rico during and before 1998 (Figure 3). Likewise, the sequenced DENV-4 strains from 2010 were found to belong to the genotype II (also the only one circulating in the Americas) and are distinct from those that circulated in Puerto Rico in the 1990s, although with less genetic variation than that observed in the newest DENV-1 strains circulating in the island (Figure 4). The results suggest that overall, a clade replacement for DENV-1 and DENV-4 may have occurred at some point in Puerto Rico during the inter-epidemic period between 1999 and 2006 [50, 67].

3. Conclusions

Dengue has emerged and re-emerged in many locations around the world, including countries in Europe (e.g., France and Croatia) [77, 78] and in North America [24] during the last two decades. Dengue has reemerged and caused epidemics in the continental USA for the first time after several decades of absence, and a worrisome panorama is expected if the trend of transmission continues. Several articles expressing these concerns have been published before and after the re-emergence of dengue in Florida in 2009 [79, 80]. Such concern is augmented by evolving climatic and ecological conditions that favor vector sustainability and by high travel activity with subsequent importation of cases. In fact, cases of dengue in returning travelers to the USA have been on the rise [81], and cases of dengue in countries in Central America, South America, and the Caribbean, very popular destinations for American tourists, have also been on the rise in the past decades [82].

Although the primary mosquito vector for dengue is *Aedes aegypti,* a highly domesticated urban mosquito, the virus can be also transmitted by *Aedes albopictus,* albeit in a less efficient manner. *Aedes albopictus* is probably responsible for the maintenance of dengue in rural/sylvatic cycles in endemic countries [83], and future research should address the biological and epidemiological implications of the displacement and replacement of *Aedes* species in the context of dengue epidemics.

In the case of the USA, both dengue vector species are widely distributed in the southern parts of the country (Figure 2), and one or another dengue vector is present in all US insular territories [28, 29]. Travel, especially by air, has been considered an important risk factor for the rapid dissemination of pathogens and their vectors in an efficient and rapid manner [84–87]. New promising vector control strategies based on the release of Wolbachia-infected *Aedes aegypti* mosquitoes have been tested in regions of Australia with potential for the occurrence of dengue epidemics and if deemed suitable, this approach could be utilized in endemic regions from Asia and the Americas [88].

Many large cities in the US are important hubs for air travel and therefore receive a high number of individuals potentially infected with pathogens that cause asymptomatic disease, including several arboviruses (e.g., dengue viruses, Japanese Encephalitis virus and Chikungunya virus). Thus, there is an increasing risk of introduction of these "exotic" pathogens to urban conglomerates where mosquito vectors are present or have the potential to become established (e.g., Miami, Atlanta, Baltimore/Washington, D.C., and New York City in the East Coast, and Los Angeles and San Francisco in the West Coast).

Despite the current economic and budgetary constraints, strict mosquito control policies and activities, that may include both traditional and biological vector control strategies, must be implemented and maintained in localities that have the potential to become the port of entry for these viruses and become the focus for another dengue epidemic in the USA.

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