The Emergence of West Nile virus in the United States – Implications for Japan

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Background

In summer 1999, a simultaneous outbreak of human encephalitis and an epizootic among American crows occurred in the New York City area. A connection between the two events was not immediately recognized. Based on serological tests, the outbreak of encephalitis was initially diagnosed as St. Louis encephalitis, a flavivirus native to North America, which does not kill North American birds. Subsequent serological testing revealed that the true cause of the outbreak was West Nile virus. This was the first identification of West Nile virus in the Western Hemisphere and subsequent sequencing of the viral genome suggested that the virus had been imported from the Middle East. The initial misdiagnosis resulted from the serological cross-reactivity of the West Nile and St. Louis encephalitis viruses, which both belong to the Japanese encephalitis flavivirus serogroup. After newspaper reports about the human epidemic, it was then realized that the bird epizootic was also caused by the same virus.

While the initial misdiagnosis did not delay control efforts since control methods for St. Louis encephalitis and West Nile outbreaks are similar, considerable negative press resulted. Discussion centered about the lack of information sharing between agencies involved in animal and human health. It was speculated that if information about the bird epizootic were known to the human health authorities, then there would have been further scrutiny about the initial diagnosis of St. Louis encephalitis since it doesn't kill birds.

Subsequently, a surveillance system called ArboNet was established to monitor the spread of West Nile virus. This was the first system in the United States that simultaneously monitored activity of a pathogen in humans and animals, particularly on a real-time basis. A key feature of the ArboNet system was that human and animal data were compiled by state health departments into one standardized data base, which could be transmitted electronically to the federal authorities (CDC). It was noted that this was the first time that state human and veterinary health authorities collaborated extensively to create a common surveillance system as well as a coordinated response to an emerging pathogen.

Because there were no widely available diagnostic tests for West Nile virus in the United States, CDC developed standardized diagnostic tests (both PCR and ELISA), produced and distributed reagents for

these tests, trained health department laboratories on the use of these tests, and initially conducted initial and confirmatory testing as a service to health departments. Over time, the focus of diagnostic testing shifted from CDC to state health departments and then to commercial laboratories.

ArboNet subsequently documented the spread of West Nile virus throughout the United States. Within four years the virus had spread from coast to coast, a distance of approximately 5000 km. While there was some evidence that bird migration dispersed the virus, random bird movements seemed most responsible for the dramatic westward spread of the virus. Dead bird surveillance proved to be the most effective means to monitor initial incursion of the virus into an area, providing advance warning to local health authorities so that local preventive measures could be instituted to help prevent a subsequent human epidemic. It was also observed that human epidemics developed very quickly, and that even short delays in institution of control measures delayed prevention until a considerable number of human cases occurred.

Unfortunately, the West Nile virus epidemic also highlighted several other deficiencies in the United States and Canada. Because for many years it was believed that vector-borne diseases were no longer a public health threat, vector control capacities were lacking in most areas, there were few trained medical entomologists, academic training programs for vector-borne disease specialists had disappeared, diagnostic capacity for vector-borne diseases was limited, there were only two institutions with extensive arbovirus strain collections, and research capacity for vector-borne diseases was minimal. The national West Nile virus response plan thus included recommendations to increase capacities for vector control, training, and research. As a result, training and research programs at universities were funded by the CDC, research at the CDC was greatly expanded, and local vector control capacities were improved.

Lessons learned from West Nile virus in the United States

- An exotic arbovirus can appear anywhere, especially in places of travel, trade, and commerce.
- Once introduced, an exotic arbovirus can spread quickly and extensively and can maintain itself in local enzootic cycles.
- Information sharing and collaboration are required between human and animal health authorities.
- Animal health authorities are often concerned mainly with animals of economic importance and additional emphasis must be placed on wildlife.
- If an exotic arbovirus is introduced, human and animal health data should be coordinated into one surveillance system that preferably functions on a real-time basis.
- Capacities for diagnosis, training, research, and response for arboviral diseases

should be maintained and should be able to be rapidly expanded if a new vector-borne disease problem emerges.

Implications for Japan

Japan's extensive international travel and trade greatly increase the likelihood of the introduction of West Nile virus or another exotic arbovirus, as has occurred with Usutu virus in Austria and West Nile virus in North America. Although it is unknown whether the introduction of West Nile virus would result in establishment of a permanent enzootic cycle in Japan, there are several similarities between Japan and North America that suggest that it could occur and could cause large human outbreaks. First, in both areas, circulation of a Japanese encephalitis serocomplex flavivirus involving birds already exists (St. Louis encephalitis in North America, Japanese encephalitis in Japan). Second, West Nile virus has produced large human outbreaks in the United States and Canada in areas with high summer and low winter temperatures, a situation found in many parts of Japan. Third, large outbreaks have occurred in rice growing areas of California. Fourth, high incidence in the United States and Canada has occurred in rural areas or suburban areas near rural areas. Given the fact that suburban population centers in Japan often occur near rural, rice-growing areas, the potential for large outbreaks in Japan exists. Fifth, competent mosquito vectors and avian hosts for West Nile virus exist in Japan.

The experience with West Nile virus in North America suggests several steps to prepare for the introduction of an exotic arbovirus such as West Nile virus. These include:

- Development and maintenance of vector control capacity in case an outbreak occurs.
- Maintain national capacity to diagnose a wide range of vector-borne pathogens.
- Expansion of an active arbovirus research and pathogen discovery program.
- Maintenance and improvement of communication links and collaborations between human and animal health authorities.
- Further development of medical entomological capacity.

Characterization of dengue viruses prevalent in Indonesia for establishment of the laboratory network for molecular epidemiology of dengue and other mosquito-borne viruses prevalent in Asia

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STUDY BACKGROUND

Advances in technology of gene cloning, nucleotide sequencing, and gene expression have facilitated the understanding of the molecular biology of dengue viruses. Dengue virus genome is composed of approximately 10,600 nucleotides, single stranded positive sense RNA. It contains a single open reading frame that is flanked by two un-translated region; 5` and 3` un-translated region (UTR). The amino acid differences of dengue viruses have been implicated to the pathogenesis of DHF (Mangada and Igarashi, 1998; Pandey &Igarashi, 2000). Dengue virus virion is composed of three structural proteins; Core protein (C), membrane protein (M), and envelope protein (E). Seven non-structural proteins are also present; NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chambers et al., 1990; Rice et al, 1985).

Although dengue epidemic occurres at regular intervals in Indonesia since it was first recognized in Java (Pratana L, 1970), there have been limited reports of the diversity of dengue viruses isolated in Indonesia. Analysis of the viruses isolated from DF/DHF patients in 2004 outbreak in Jakarta demonstrated that all 4 dengue viruses were endemic in Jakarta, but dengue-3 virus (DV-3) was the most predominant (Suwandono, 2006; Cucunawangsih, manuscript in preparation). Dengue 3 virus was also the most frequently isolated virus outside Jakarta and had the widest distribution in Indonesia (Gubler, 1979; Corwin, 2001)

Our recent studies found that DV-3 strains isolated in Indonesia in 1998 belonged to a separate cluster (subtype-2) from those isolated between 1973-1985

(subtype 1) (Raekiansyah, 2005) and the other study has shown that DHF/DSS strains had unique amino acid residues from the DF strains and had ability to propagate to higher level in human monocytes (Dewi, submitted). These findings suggest that the dengue strains circulating in Indonesia changes from time to time, and some of these changes might also followed by changes in phenotypic characteristics.

In the present study, we expand our previous study on the molecular and phenotype characterization to the other dengue serotypes and more strains will be analyzed to get a better picture on the amino acid substitutions from patients with different disease severities. We hope this study can give us better understanding on the molecular epidemiology of dengue viruses and can further contribute to the development of new strategies for control and prevention of dengue infection.

Year 1 plan :

Jan - March 31, 2006

Develop a common protocol for virus isolation, phenotypic and genotypic analyses:

- Intensive communication through e-mails among participating laboratories and clinicians

Establish the research group in Indonesia

- Application of ethical clearance from the Ethical committee Medical Faculty University of Indonesia
- Preparation of materials necessary, including culture cells

- Sample collection, RT-PCR and serology test. of samples.
- Isolation of dengue virus from 40 serum samples
- Identification and type determination of virus isolates by RT-PCR
- cDNA preparation of viral isolates (estimated 10 – 20 isolates)
- Prepare amplification and purification of envelop regions of representatives of DV1, DV2, DV3, DV4 from DF and DHF cases (1 isolates for each)

Implementation up to March 7, 2006.

1. 2006-2007 Research proposal has been written and communicated between Dept. Microbiology FKUI and Dept. Virology I, NIID, Japan. Because of some procedural matter,

the work could be started by end of February.

2. Ethical clearance application had been sent to the ethical committee, FKUI, and the application is now being processed. Thus, works concerning sample collection from patients cannot be started yet.

3. Cell culture :

   For preparation of dengue virus culture, we revitalize our cell culture system. Vero cells are now being propagated.

4. Virus culture

   Since we cannot start to collect samples from patients, we are trying to recover some samples originated from 2004 epidemics in Jakarta (Cucunawangsih, manuscript in preparation). Plasma or PBMC from dengue patients which were stored in –70°C are being inoculated into Vero cells. This work is now in progress. We are planning to passage viral culture 2-3 times in Vero cells to get relatively high titer of virus stock. Virus stock will be stored in –70oC until further analysis.

5. cDNA preparation

   We prepared cDNA from RNA isolated from plasma or PBMC collected in 2004 and stored in -70°C. We used Sensiscript RT (Qiagen) and complementary primers that recognize 3' noncoding region of dengue 1-4, and primers that recognize NS4a region of DV-3. Since at present we don't have random primers or specific primers to amplify the other dengue serotypes, we are now concentrating to work with DV-3. With those 2 above mentioned primers, we expect that almost the whole sequence of DV-3 genome can be covered. We will check the quality of cDNA produced, and if they are good, we will proceed with the amplification of envelop and NS1 region.

   Primers for preparation of cDNA of DV1, DV2, DV4 are now being ordered.

Further plan

1. Finishing target of year 1.
2. Year 2 schedule :

Year II (April 1, 2006 – March 31, 2007)

- Sample collection, RT-PCR and serology test of samples
- Isolation of dengue virus from 80 serum samples
- Identification and type determination of of virus isolates by RT-PCR
- CDNA preparation of 20 - 30 viral isolates which includes representative of all 4 types.
- Sequence envelope regions of 8 isolates from year 1.
- Sequencing of envelope regions of representatives of DV1, DV2, DV3, DV4 from DF and DHF cases (1 isolates for each) from year II
- Sequence NS1 region of DV1, DV2, DV3, DV4 (one each) from year 1 or 2.
- Phenotypic analysis of representatives of DV1, DV2, DV3, DV4 from DF and DHF cases (1 isolates for each)

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Characterization of dengue viruses prevalent in Thailand for establishment of the laboratory network for molecular epidemiology of dengue and other mosquito-borne viruses prevalent in Asia.

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Summary:

A virological and serological study was conducted in six hospitals spread across Thailand from February 2005 to February 2006. All four dengue serotypes were identified, of which DENV-1 and DENV-4 were the most predominant. Among confirmed dengue cases were secondary infection in 92% and primary infection in 8%. Confirm dengue cases was found highest in the 11-15 years age group.

Purpose:

Emergence of pathogenic microorganisms is and increasing concern. Infection by mosquito-borne viruses is a foremost problem in Asia. Understanding the epidemiologic situations of the diseases and the phenotypic and genotypic characteristics of viruses contributes to the development of new strategies for control and prevention. In order to promote communication and exchange of the information of dengue and other mosquito-borne viruses, laboratory network among Asia and Pacific Rim should be developed and strengthened.

Methods:

During February 2005 to February 2006, Blood specimens were collected at acute and convalescent stages from suspected dengue cases who visited Lampang Hospital in Lampang Province (north), Maharat Nakhon Ratchasima Hospital in Nakhon Ratchasima Province (north-east), Pathum Thani Hospital in Pathum Thani Province (central), Chareonkrung Pracharak Hospital in Bangkok, Ratchaburi Hospital in Ratchaburi Province (central) and Hadyai Hospital in Songkhla Province (south). Blood specimens were taken into tubes with EDTA anticoagulant, and centrifuged. Both buffy coat and plasma were obtained at the acute stage, but only plasma was obtain at the convalescent stage. Vials of buffy coat and plasma specimens were stored in a liquid nitrogen tank. All the specimens were transport to Arbovirus laboratory, National Institute of Health, Department of Medical Sciences, Nonthaburi, Thailand.

Virus isolation and determination of serotypes were performed on all buffy coat samples collected at the acute stage. The infected cell were stained by IFA, and dengue virus serotypes were determined. RT-PCR was performed on some acute plasma that almost of them virus isolation were negative. Dengue virus-specific IgM and IgG were measured in all plasma specimens by antibody-capture ELISA. Sequencing of the E-protein gene of DENV-3 were done in 17 plasma samples that collected during 2001 to 2002.

Results:

1538 acute buffy coat specimens were isolated and identify the serotype by IFA (Table 1) and 831 acute plasma specimens were done by RT-PCR techniques (Table 2). From these two techniques 793 positive cases were found. All the four dengue serotypes were detected in the six hospital. When the total number of isolates were analyzed, DENV-1 was the predominant serotype (41.5%) follow by DENV-4 (41.2%) DENV-2 (15.4%) and DENV-3 (1.9%).

1,599 acute plasma specimen and 892 convalescent plasma specimens were determined by Antibody capture ELISA 40 cases were primary infection, 457 cases were secondary infection, 380 cases were probably secondary infection, 134 cases were either primary or secondary infection,

67 cases were not dengue infection and 521 cases were uninterpretable (Table 4) Positive dengue cases were found in male 49.9% and in female 50.1%. The ratio between DF and DHF cases were approximately 1:1.4, 1:2.6, 1:1.2 and 1:1.1 in patients infected with DENV-1, DENV-2, DENV-3 and DENV-4 respectively (Table 5). In DHF cases, The primary infection accounted for 13.6, 0, 0, 3.3% in patients infected with DENV-1, DENV-2, DENV-3 and DENV-4 respectively (Table 6). Confirmed dengue cases was found highest in the 11-15 years age group follow by 6-10 and 1-5 years age group. Age of distribution of all the DF and DHF cases are shown in Table 7. The envelope gene of 17 DENV-3 plasma samples that collected in the year 2001 and 2004 have been sequenced.

Discussion:

All four dengue serotypes circulate continuously in Thailand, with one serotype emerging as the cause of each periodic moderate to severe epidemic. Each dengue virus serotype has characteristics that influence the nature of dengue epidemic and disease severity. In the past DENV-4 was found few cases each year in Thailand but in this year study we found the DENV-1 DENV-4 is the predominant and DENV-3 was found few cases. In the future we will sequence from isolated dengue viruses in every serotypes.

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Table 1 Virus isolation results.

Hospital	Total	D1	D2	D3	D4	Neg
Lampang	105	10	2	0	3	90
Nakhon-Ratchasima	171	9	6	2	10	144
Pathum Thani	247	34	4	0	24	185
Bangkok	50	10	0	2	6	32
Ratchaburi	731	92	23	0	95	521
Songkhla	234	36	8	0	9	181
Total	1538	191	43	4	147	1153

Table 2 PCR results.

Hospital	Total	DENV-1	DENV-2	DENV-3	DENV-4	Neg
Lampang	92	20	5	5	8	54
Nakhon-Ratchasima	96	8	11	2	47	28
Pathum Thani	144	37	7	0	16	84
Bangkok	30	11	2	2	6	9
Ratchaburi	317	30	22	0	73	192
Songkhla	157	32	32	2	30	56
Total	831	138	79	11	180	423

Table 3 Area distribution of dengue serotype in Thailand, Feb 2005 - February 2006

		DENV-1		DENV-2		DENV-3		DENV-4	
Hospital	Total	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Lampang	53	30	56.6	7	13.2	5	9.4	11	20.8
Nakhon-Ratchasima	95	17	17.9	17	17.9	4	4.2	57	60.0
Pathum Thani	122	71	58.2	11	9	0	0	40	32.8
Bangkok	39	21	53.8	2	5.1	4	10.3	12	30.8
Ratchaburi	335	122	36.4	45	23.4	0	0	168	50.1
Songkhla	149	68	45.6	40	2.7	2	1.3	39	26.2
Total	793	329	41.5	122	15.4	15	1.9	327	41.2

Table 4 Types of infection

Types of infection	No.
Primary	40
Secondary	457
Pri or Sec	134
Probably 2	380
Not dengue inf.	67
Uninterpretable	521
Total	1599

Table 5 Distribution of dengue positive case by sex.

Sex	No. Case	%
Male	388	49.9
Female	398	50.1
Total	777	

Table 6 Ratio between DF and DHF cases caused by each of four dengue virus serotypes

Diagnosis	Serotype								Total
	DENV-1		DENV-2		DENV-3		DENV-4		
	No.	%	No.	%	No.	%	No.	%	
DF	111	41.4	24	27.9	5	45.5	109	48.0	249
DHF	157	58.6	62	72.1	6	45.5	118	52.0	343
- DHF Grade 1, 2	139	51.9	49	57.0	3	45.5	102	44.9	293
- DHF Grade 3, 4	18	6.7	13	15.1	3	45.5	16	7.0	50
Total	268		86		11		227		592

Note : Information were not available for other 201 case

Table 7 Ratio between primary and secondary infection among DF and DHF cases caused by each of four dengue virus serotypes.

Diagnosis	DENV-1		DENV-2		DENV-3		DENV-4		Total
	No.	%	No.	%	No.	%	No.	%	
DF									
Primary	8	19.5	0	0	1	50	0	0	9
Secondary	41	83.7	3	100	2	66.7	36	100	82
Total	49		3		3		36		91
DHF									
Primary	9	13.6	0	0	0	0	1	3.3	10
Secondary	57	86.4	15	100	2	100	29	96.7	103
Total	66		15		2		30		113
DF+DHF									
Primary	17	14.8	0	0	1	20	1	1.5	19
Secondary	98	85.2	18	100	4	80	65	98.5	185
Total	115		18		5		66		204

Note : Information of clinical diagnosis and antibody response were not available for other 589 case

Table 8 Age distribution of all the DF and DHF cases

Age group	Primary				Secondary				Total
	DF		DHF		DF		DHF		
	No.	%	No.	%	No.	%	No.	%	
< 1	2	13.3	7	35.0	0	0	1	0.4	10
1-5	4	26.7	5	25.0	22	12.9	21	8.8	52
6-10	5	33.3	4	20.0	61	35.9	79	32.9	149
11-15	3	20.0	3	15.0	71	41.8	112	46.7	189
16-25	1	6.7	1	5.0	11	6.5	16	6.7	29
26-35	0	0	0	0	4	2.4	3	1.3	7
36-45	0	0	0	0	0	0	5	2.1	5
46-55	0	0	0	0	0	0	2	0.8	2
56-65	0	0	0	0	0	0	0	0	0
66 up	0	0	0	0	0	0	0	0	0
Total	15		20		169		239		443

Note : Information of age and antibody response were not available for other 495 cases

CLINICAL CHARACTERIZATION, EPIDEMIOLOGY, AND SEROPREVALENCE OF DENGUE INFECTIONS IN THE PHILIPPINES

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REVIEW OF RELATED LITERATURE

Epidemiology

Arboviral disease worldwide has been predominantly caused by dengue viruses, single stranded RNA viruses from the Flaviviridae family [1]. They are endemic to tropical regions causing an estimated 50-100 million cases annually, with over 250,000 – 500,000 cases of dengue hemorrhagic fever, and 24,000 deaths [2]. In the Philippines, figures have been steadily rising, from 7413 cases reported in 1995 [3] to 28,807 last year, a 28% increase from 2004 figures, by the third quarter [4].

Dengue and dengue hemorrhagic fever are caused by four closely related, but antigenically distinct serotypes, DEN-1, DEN-2, DEN-3, and DEN-4 [5,6]. In the Philippines, several studies have shown that the prevalent serotypes have been DEN-1, DEN-2, and DEN-3, with DEN-2 occurring most frequently, and DEN-1 having more severe clinical manifestations [7,8]. A retrospective study done at the Research Institute for Tropical Medicine in 1997 again showed these three serotypes to be circulating, with DEN-2 being most frequent. Secondary infections showed a slight predominance, at 51% [9]. A prospective surveillance study was conducted in the same year, enrolling 143 patients with dengue-like illness, 67 of whom were lab-confirmed by virus isolation and serology. Only DEN-1 and DEN-2 serotypes were found to occur [10]. A limited laboratory investigation of a large outbreak in 2005 in Quezon City showed circulation of DEN-2 and DEN-3 serotypes [NEC, 2005].

In September 2001, a laboratory-based surveillance was initiated by the Department of Health, involving sentinel hospitals in the top four regions reporting cases of dengue virus infection, specifically the National Capital Region (NCR), Region II, Region VII, and Region XI. Results showed 796 (53.49%) laboratory confirmed cases (IgM Capture ELISA), out of 1488 clinical cases presenting as dengue-like illness (DLI). Serotyping, however, was not performed and analysis of epidemiologic factors is still being completed [NEC, 2001].

Virology and Pathogenesis

The four dengue virus serotypes, DEN-1 to DEN-4, belong to the genus *Flavivirus*, family *Flaviviridae* [6].

Flavivirus particles appear to be spherical, 40-60 nm in diameter, containing an electron dense core

(about 30 nm in diameter) surrounded by a lipid bilayer. Because of the lipid envelope, flaviviruses are readily inactivated by organic solvents and detergents [11]. The flavivirus genome is approximately 11,000 bases long and is made up of three structural and seven nonstructural proteins [6]. The virions are associated with 3 viral proteins: the **E** (envelope) **M** (membrane), and **C** (capsid) proteins. The E protein is the major surface protein of the viral particle, probably interacts with viral receptors and mediates virus-cell membrane fusion [11].

All flaviviruses have common group epitopes on the E protein resulting in extensive cross-reactions in serological tests. This is particularly true of the four dengue serotypes [6]. Infection with one serotype elicits lifelong homotypic immunity [11]. Cross protection is not provided by infection with one of these serotypes thus, theoretically, resulting in four possible dengue infections in one's lifetime. Sequential or secondary dengue infections, however, have been observed to cause more severe disease on the basis of possible immune enhancement [6, 13].

Viremia usually peaks at the time of or shortly after onset of illness and may remain detectable from 2 to 12 days, depending on virus strain and immune status of the infected individual [6]. Onset of viremia has been found to be within 6 to 18 hours prior to onset of clinical illness [13]. Levels of viremia have been found to be higher for primary infections [6]. In secondary infections, it has been hypothesized that the virus is recognized by a preexisting heterologous dengue antibody and forms an antigen-antibody complex which is bound to and internalized by immunoglobulin Fc receptors on leukocyte cell membranes, especially macrophages. As the antibody is not heterologous, the virus is not neutralized and is free to replicate once inside the macrophage. This antibody-dependent enhancement (ADE), can serve to increase the number of antigen-presenting cells infected during secondary dengue, leading to activation of pre-existing cross-reactive dengue virus-specific T lympohocytes from the primary flavivirus infection. Cytokines and vasoactive mediators are then released, resulting in increased vascular permeability, leading to hypovolemia and shock [6, 13].

It is also accepted that high rates of mutation affect the RNA genomes of dengue viruses culminating in their genetic variability and appearance of certain strains with heightened virulence [14].

Other factors postulated to contribute to the pathogenesis of dengue virus infection include: specific virulence genotypes replicating at high levels resulting in an enhanced immune response and disease; genetic predisposition to a severe clinical picture; other risk factors such as age and nutrition [13]. A higher level of viremia early in the course of infection seems to be crucial and is likewise associated with a more severe presentation [13].

Clinical Features

The incubation period of dengue virus infections, after a bite from an infective mosquito is typically 4-7 days (range 3-14) [6, 15]. The clinical

manifestations may range from being entirely asymptomatic to severe and fatal hemorrhagic disease [6, 15-16]. In areas with high endemicity, the illness may be clinically nonspecific, especially in children [6]. Identified risk factors for severe illness include strain and serotype of the virus, immune status, age, and genetic background of the infected individual [6].

Most infections are mild consisting of an undifferentiated fever with or without a rash, usually among infants and young children. Upper respiratory infections, especially pharyngitis, are common [1]. Classic dengue fever (DF) is usually seen among older children and adults and these cases are less likely to be asymptomatic [1]. Onset is generally abrupt heralded by high grade fever, severe headache, myalgia, arthralgia, retro-orbital pain, nausea and vomiting, and maculopapular rash [1,16]. Other signs and symptoms include flushed facies, sore throat, cough, cutaneous hyperesthesia, and taste aberrations [1]. Dengue fever is generally self-limiting and is rarely fatal. Recovery or the convalescent phase may be prolonged for weeks and include depression [1, 16].

Differences in clinical presentation, according to serotype, was demonstrated in a retrospective review of dengue cases in Thailand in 2000. DEN-1 was shown to present with the mildest clinical presentation. DEN-2 infections were predominantly secondary cases and presented with more severe disease, with greater degree of plasma leakage, shock, and complications of fluid overload. DEN-3 and DEN-4 infections, on the other hand, demonstrated greater liver involvement [17].

Dengue hemorrhagic fever (DHF) is predominantly seen among children less than 15 years of age in hyperendemic areas. It is characterized by increased capillary permeability and hemostatic changes [1]. Plasma leakage and thrombocytopenia are critical features found to distinguish dengue fever (DF) from dengue hemorrhagic fever (DHF) [12]. If a major plasma leak occurs, it usually develops 24 hours before to 24 hours after defervescence. Patients may develop effusion and ascites with a variable amount of bleeding [1,6]. Vaughn *et al* [13] found that pleural effusion can occur in up to 90% of DHF cases. Hepatic enlargement and tenderness has been seen in up to 40% of cases. Mortality can be as high as 10-20% (>40% if shock occurs) in the absence of timely management. Signs of impending shock include sustained abdominal pain, persistent vomiting, change in sensorium, and sudden shift from fever to hypothermia, and a sudden drop in platelet count [1].

It is during the acute febrile period, between 2 to 10 days, that dengue viruses may be found circulating in the blood. Other vectors which bite the individual during this period of viremia may be infected and subsequently transmit the virus to other uninfected individuals, after an extrinsic incubation period of 8 to 12 days [6].

Clinical Diagnosis

Early clinical diagnosis may be difficult as clinical and laboratory criteria may not be present in the first few days of the illness. In the study done by

Kalayanarooj *et al* [12] on children presenting with febrile illness, symptoms of anorexia, nausea, and vomiting plus a positive tourniquet test and laboratory findings of leukopenia, neutropenia, monocytopenia, and elevation in plasma AST were found to be more likely among dengue cases rather than other febrile illnesses [12]. A flushed face without coryza and a positive tourniquet test were reported as early predictors of DHF among febrile patients [18]. Definitive clinical diagnosis of DHF may be made with only with the development of thrombocytopenia and plasma leakage, which usually occurs 1-2 days before the onset of shock [12].

The clinical definition of DHF set by the WHO is based on the presence of high, continuous fever, hemorrhagic manifestations (including at least a positive tourniquet test), Hepatomegaly, thrombocytopenia (platelet count ≤ 100,000/mm ^{3}), and hemoconcentration (hematocrit increased by ≥20% above baseline value). DHF is further subdivided into four grades on the basis of the presence of spontaneous bleeding and the presence and severity of shock [19].

Laboratory Diagnosis

Although clinical features are essential for diagnosis of dengue fever and DHF, laboratory tests are essential for confirmation. Detection of the virus by culture is the definitive diagnostic test. Its use in practice, however, is limited primarily because of the time-consuming and cumbersome nature of the test. Mosquito cell lines are commonly used for inoculation (C6/36 or AP-61). The virus may also be inoculated into larval or adult mosquitoes, vertebrate cell lines (e.g VERO, LLC-MK_2), and in intracerebrally inoculated newborn mice. Once the virus is isolated, serotype-specific anti-dengue monoclonal antibodies are used which are subsequently revealed by a second-labeled antibody [20].

Several serological tests have been used for the diagnosis of dengue infection: hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), IgM capture enzyme linked immunosorbent assay (MAC-ELISA), and IgG-ELISA. Hemagglutination inhibition has been utilized as a standard method in diagnosis and is of value in seroepidemiology and in the differentiation between primary and secondary infections [19,20]. It is sensitive, easy to perform, requires minimal equipment, and is reliable if properly done. The test is based on the fact that dengue viruses, under controlled conditions of pH and temperature, can agglutinate goose red blood cells, and this effect can be inhibited by specific antibodies [20]. The major disadvantage of the HI, however, is its lack of specificity, making it unrealiable for identifying the infecting virus serotype [19-21].

The MAC-ELISA is a simple, rapid test base on detecting the dengue-specific IgM antibodies in test serum by capturing them out of solution using antihuman IgM that was previously bound to the solid phase [20,22]. MAC-ELISA is slightly less sensitive than the HI test for diagnosing dengue infection. It has become an invaluable tool for surveillance of dengue virus infections. In areas where dengue is not endemic, it can be used in clinical

surveillance for viral illness or for random, population-based serosurveys, with the certainty that any positives detected are recent infections [21,22].

The indirect IgG-ELISA can also be used to differentiate primary and secondary dengue infections. It is, however, very non-specific and exhibits the same broad cross-reactivity among flaviviruses as the HI test. It cannot be used to identify the infecting dengue serotype [22].

Dengue type-specific RT-PCR assay is a highly sensitive and specific tool which could correctly identify the dengue virus serotype. It generally yields positive results in dengue viremic sera collected within 2 to 5 days of the fever [13]. When samples are collected during the febrile phase of the illness, RT-PCR has been found to be a reliable diagnostic test in both secondary and primary dengue virus infections and its use in combination with IgM and IgG ELISA increases the accuracy and sensitivity of the laboratory diagnosis of dengue virus infection, especially in secondary dengue virus infection [23].

SIGNIFICANCE

There has been a paucity of data on the characterization of the dengue virus and dengue virus infections in the Philippines over the past few years. Although earlier cited local cross-sectional studies have been able to determine circulating serotypes, a comprehensive characterization of the infection here has yet to be performed. Identification of differences in clinical presentation of the different serotypes and perhaps among different patient sets may provide information in terms of planning for case management particularly in the setting of outbreaks. A comprehensive serologic, virologic epidemiologic surveillance is a necessary step to establish baseline data subsequently leading to a molecular characterization of circulating serotypes in the country, in comparison with other dengue-endemic countries in the region.

OBJECTIVES

The general objectives of this proposal are as follows:

- To establish baseline seroprevalence of dengue virus infection among cases admitted for dengue-like illness.
- To describe the clinical features of laboratory-confirmed cases of dengue virus infection, according to serotype.

MATERIALS AND METHODS

SUBJECT ENROLLMENT. Subjects presenting in three tertiary hospitals, 2 government facilities (Philippine Children's Medical Center and the National Children's Hospital) and one private hospital (to be determined), during the months of peak dengue activity, June to October (NEC, 2005) will be assessed for eligibility. Entry criteria will include the following: cases ≥ 2 years of age meeting the surveillance definition of dengue like illness (DLI). Cases with a specific identifiable cause of fever will be excluded. Cases will also be

excluded if there is anemia, malnutrition, or any history of chronic medical illness.

STUDY PROTOCOL. Clinically suspected cases of dengue-like illness meeting the clinical case definition, seen/admitted at any of the three abovementioned hospitals at the National Capital Region, will be included in the study.

A case of dengue fever (DF) will be defined as an acute febrile illness of 2 to 7 days duration with **ANY TWO OR MORE** of the following: headache, retro-orbital pain, myalgia/arthrlagia, rash, hemorrhagic manifestations (petechiae and positive tourniquet test), and leucopenia [22].

A clinical case of dengue hemorrhagic fever (DHF) will be defined by the presence of **ALL** of the following: acute febrile illness, thrombocytopenia (platelet count of 100,000 mm^3 or less); hemorrhagic tendencies/manifestations as evidence by at least ONE of the following: (positive tourniquet test; petechiae, ecchymoses, or purpura; bleeding from the mucosa, gastrointestinal tract, injection sites, or others); and plasma leakage due to increased capillary permeability manifested by at least ONE of the following: Hematocrit on presentation that is >20% above average for that age and population, >20% drop in hematocrit following treatment, and commonly associated signs of plasma leakage (ascites, hypoproteinemia, pleural effusion) [22].

A clinical case of dengue shock syndrome (DSS)will be defined as the presence of **ALL** four criteria for dengue hemorrhagic fever **PLUS** evidence of circulatory failure manifested by all of the following: rapid and weak pulse; narrow pulse pressure (20 mm Hg or less) or hypotension for age; and cold clammy skin and altered mental status [22].

Data collection will be recorded separately and the following information will be obtained:

1. Demographic data
2. Date of onset of illness and fever
3. Date of fever lysis, if applicable
4. Recorded temperature(s) prior to presentation
5. Character of fever
6. Immunization status
7. Previous hospitalizations
8. History of dengue infection
9. History of any recent travel
10. Signs and symptoms eliciting the following (at a minimum): appearance of a rash (description), malaise/anorexia, arthralgia, myalgia, vomiting, abdominal pain, headache, sensorial changes, cough, rhinorhea, epistaxis, gum bleeding, hemoptysis, abdominal pain, melena, hematochezia, vaginal bleeding

Acute serum specimens will be extracted from identified subjects and the following laboratory tests will be performed: virus isolation, dengue IgM/IgG immunoassay, and nested RT-PCR.

Two to ten milliliters of blood (in EDTA containing tubes) will be obtained from these cases on

admission/presentation and immediately placed in ice. Specimens will be transported to the Research Institute for Tropical Medicine virology laboratory.

Dengue virus isolation will be attempted on acute specimens using the C6/36 (*Aedes albopictus*) cell line. Virus isolates will be identified by serotype by indirect immunofluorescence assay by use of serotype-specific anti-dengue antibodies

Dengue IgM capture and IgG ELISA will likewise be performed on acute specimens. JEV IgM capture ELISA will likewise be run in parallel to facilitate distinction between the two diseases. A second (convalescent) serum specimen will be collected upon discharge or 14 to 21 days after admission, whichever comes first and IgM/IgG titers will again be determined.

Acute serum samples obtained will be assayed for DEN virus RNA by a nested RT-PCR. RT-PCR will also be used to identify the dengue virus serotype.

A confirmed case of dengue virus infection will be diagnosed with a clinical presentation of dengue-like illness plus **ANY** of the following: a fourfold or greater rise in dengue virus specific antibody (IgM/IgG) in paired sera (acute and convalescent); isolation of the dengue virus from serum or plasma; detection of the viral genomic sequence in serum samples by RT-PCR.

Clinical and laboratory data which will be collected will include:

1. Type and duration of clinical symptoms
2. Physical examination findings
3. Dengue hemorrhagic fever grading (according to WHO Criteria)
4. Any transfusion(s) given
5. Baseline complete blood count
6. Hospital course (bleeding episodes, complications)
7. Replacement Fluids

Outcomes to be measured will be all-cause mortality.

ETHICAL CONSIDERATIONS

Informed consent will be obtained from all subjects enrolled in the study. Laboratory results will likewise be disclosed to all investigators and collaborators.

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