SEROEPIDEMIOLOGY OF ARBOVIRUS INFECTIONS AMONG SOME GROUPS OF THAI HILL TRIBE PATIENTS WITH PYREXIA IN CHIANG MAI PROVINCE

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE (PUBLIC HEALTH)
MAJOR IN INFECTIOUS DISEASES
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2000

ISBN 974-664-205-7

Thesis

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was submitted to the Faculty of Graduate Studies, Mahidol University
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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my major advisor, Associate Professor Dr. Charnchudhi Chanyasanha, Department of Microbiology, Faculty of Public Health for his guidance and meaningful supervision, criticism, valuable advice, encourgement and understanding throughout this study which has enabled me to complete the thesis.

I am very grateful to Assistant Professor Dusit Sujirarat, co-advisor, for his comments about data analysis.

I wish to express my sincere appreciation to Dr. Timothy P. Endy, Chief of Virology Department, my co-advisor and Dr. Ananda Nisalak, Supervisor of Virology Department, Armed Forces Research Institute of Medical Sciences (AFRIMS) for all kindness and suggestion in laboratory and support materials and reagents used in this study.

Greatful acknowledgements are extended to Khun Panor Srisongkram and Khun Somkiat Changnak, Department of Virology, AFRIMS for their kindness, helpful assistance in laboratory training.

Thanks to the Directors of Samoeng Hospital, Mae Taeng Hospital, Phrao Hospital and Chiang Dao Hospital, and special appreciation expressed to the public health personnel who kindly helped me with specimens collection.

Specials thanks to Khun Uraiwon Muktaree and Khun Damrong Chalermsuk for their kind and friendly assistance during the peroid of specimen collection.

My profound gratitude extends to all hill tribe patients who were enrolled in this study.

Finally, I am grateful to my family for their love, kindness, encouragement and understanding throughout this study.

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3936038 PHPH/M : MAJOR:INFECTIOUS DISEASES ; M.Sc. (PUBLIC HEALTH)
KEY WORDS : ARBOVIRUS / RELATED FACTORS / HILL TRIBES

SIRIPORN NASOMJAI : SEROEPIDEMIOLOGY OF ARBOVIRUS INFECTIONS AMONG SOME GROUPS OF THAI HILL TRIBE PATIENTS WITH PYREXIA IN CHIANG MAI PROVINCE. THESIS ADVISORS : CHARNCHUDHI CHANYASANHA, Ph.D., TIMOTHY P. ENDY, M.D., M.P.H., Board Certified Infectious Disease, DUSIT SUJIRARAT, M.Sc. 216 p. ISBN 974-664-205-7.

Dengue hemorrhagic fever, Japanese encephalitis (JE) and Chikungunya infection are arbovirus diseases in Thailand which are transmitted by mosquitoes. The objectives of this study were to know the seroepidemiology of arbovirus infections by serological assay, and to find the factors related to arbovirus infections in hill tribe patients with pyrexia who took treatment at four hospitals in Chiang Mai Province, namely, Samoeng Hospital, Mae Taeng Hospital, Phrao Hospital and Chiang Dao Hospital. A total of 393 cases were studies during May 1997-April 1998. Each patient was interviewed and blood samples were collected (190 cases of single sera and 203 cases of paired sera). The antibody levels were detected by the hemagglutination inhibition (HI) test and the IgM antibody capture ELISA (MAC ELISA). Results showed that 87 cases were positive for arbovirus; 131 cases were negative and 175 cases were uninterpreted. The prevalence of Dengue infection was 25.7% (56/218 cases), Chikungunya virus infection was 11.5% (25/218 cases) and JE virus infection was 7.3% (16/218 cases). In conclusion, prevalence of arbovirus infections was 39.9% (87/218 cases), composed of Alphavirus infection 11.5% (25/218 cases) and Flavivirus infection 37.2% (81/218 cases). The prevalence of arbovirus infections were found to be higher in age group < 15 years old (OR = 3.1), occupation; business men, employees, students and unemployed people (OR = 1.9), income of ≤ 2000 baht per month (OR = 6.7), history of being bitten by mosquitoes 1 week before sickness (OR = 2.3) and having containers or ground water pits for mosquioes breeding place close to the house (OR = 4.4). All factors were found to be significant (p-value < 0.05). The result of this study will be useful for prevention and control the arboviral diseases, planning and vaccination program.

3936038 PHPH / M : สาขาวิชาเอก : โรคติดเชื้อ ; วท.ม. (สาธารณสุขศาสตร์)

ศิริพร นาสมใจ : ระบาดวิทยาการติดเชื้ออาโบไวรัสทางซีโรโลยี ในผู้ป่วยชาวเขาไทยบางกลุ่มที่มีไข้ จังหวัดเชียงใหม่ (SEROEPIDEMIOLOGY OF ARBOVIRUS INFECTIONS AMONG SOME GROUPS OF THAI HILL TRIBE PATIENTS WITH PYREXIA IN CHIANG MAI PROVINCE).คณะกรรมการควบคุมวิทยานิพนธ์ ชาญชุติ จรรยาสัณห์, Ph.D., ทิโมธี เอนดี, M.D., M.P.H., Board Certified Infectious Disease, ดุสิต สุจิรารัตน์, M.Sc.
216 หน้า. ISBN 974-664-205-7

โรคไข้เลือดออก โรคไข้สมองอักเสบเจอี และโรคติดเชื้อชิกุนกุนยา นับเป็นกลุ่มโรคอาโบไวรัสที่สำคัญในประเทศไทยที่มียุงเป็นพาหะนำโรค วัตถุประสงค์การศึกษาเพื่อทราบถึงระบาดวิทยาการติดเชื้ออาโบไวรัสจากการตรวจด้วยวิธีทางซีโรโลยี ตลอดจนหาปัจจัยที่มีความสัมพันธ์กับการติดเชื้ออาโบไวรัส โดยศึกษาในกลุ่มชาวเขาที่มีไข้ และมารับบริการตรวจรักษาที่โรงพยาบาลจังหวัดเชียงใหม่ 4 แห่ง ได้แก่ โรงพยาบาลอำเภอสะเมิง โรงพยาบาลอำเภอแม่แตง โรงพยาบาลอำเภอพร้าว และโรงพยาบาลอำเภอเชียงดาว รวม 393 ราย ระหว่างเดือนพฤษภาคม พ.ศ. 2540 ถึงเดือนเมษายน พ.ศ. 2541 ผู้ป่วยทุกรายจะได้รับการสัมภาษณ์และเจาะโลหิต (เป็นซีรัมเดี่ยว 190 ราย และซีรัมคู่ 203 ราย) ตรวจหาระดับแอนติบอดีโดยวิธี Hemagglutination inhibition (HI) test และโดยวิธี IgM antibody capture ELISA (MAC ELISA) จากผลการตรวจเลือดผู้ป่วยทางห้องปฏิบัติการ และแปรผลการตรวจทั้งสองวิธีร่วมกัน พบว่าผู้ป่วยมีการติดเชื้อเป็นอาโบไวรัส 87 ราย และไม่มีการติดเชื้อ 131 ราย และไม่สามารถแปรผลการวินิจฉัยได้ 175 ราย ความชุกของผู้ป่วยที่มีการติดเชื้อเป็นโรคไข้เลือดออกพบร้อยละ 25.7 (56/218 ราย) โรคติดเชื้อชิกุนกุนยาร้อยละ 11.5 (25/218 ราย) และโรคติดเชื้อไข้สมองอักเสบเจอีร้อยละ 7.3 (16/218 ราย) โดยสรุปพบความชุกของผู้ป่วยที่เป็นโรคอาโบไวรัสร้อยละ 39.9 (87/218 ราย) โดยเป็นอัลฟาไวรัสร้อยละ 11.5 (25/218 ราย) และเป็นฟลาวิไวรัสร้อยละ 37.2 (81/218 ราย) อัตราความชุกโรคอาโบไวรัสพบสูงในกลุ่มอายุน้อยกว่า 15 ปี (OR = 3.1) กลุ่มอาชีพได้แก่ ค้าขาย รับจ้าง นักเรียน และผู้ไม่ได้ทำงาน (OR = 1.9) รายได้ไม่เกิน 2,000 บาทต่อเดือน (OR = 6.7) ผู้ป่วยส่วนใหญ่มีประวัติการถูกยุงกัดก่อนป่วย 1 สัปดาห์ (OR = 2.3) และมีภาชนะหรือแหล่งน้ำเพาะพันธ์ยุงใกล้บ้าน (OR = 4.4) ปัจจัยเหล่านี้มีความสัมพันธ์ต่อการติดเชื้อเป็นโรคอาโบไวรัสอย่างมีนัยสำคัญทางสถิติ (p-value < 0.05) การศึกษาครั้งนี้ประโยชน์เพื่อเป็นแนวทางในการป้องกันและควบคุมโรคติดเชื้อกลุ่มอาโบไวรัส ตลอดจนเพื่อวางแผนการให้ภูมิคุ้มกันโรคต่อไป

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LIST OF ABBREVIATIONS

ABBREVIATION OR SYMBOL	TERM
JE	Japanese encephalitis
DF	dengue fever
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
DEN	Dengue virus
CHIK	Chikungunya virus
HA	hemagglutination
HI	hemagglutination inhibition
ELISA	enzyme linked immunosorbent assay
EIA	enzyme immunoassay
et al.	et alli (Latin), and other
IgM	immunoglobulin M
IgG	immunoglobulin G
MAC	IgM antibody capture enzyme linked immunosorbent assay
GAC	IgG antibody capture enzyme linked immunosorbent assay
° C	degree (s) Celsius

LIST OF ABBREVIATIONS (CONTINUED)

ABBREVIATION OR SYMBOL	TERM
ml	millilitre (s)
µl	microlitre (s)
mg	milligramme (s)
M	mole (s) per litre
l	litre (s)
mM	millimole (s) per litre
SPC	strong positive control
WPC	weak positive control
NC	negative control
OPD	o-phenylene diamine
PBS	phosphate buffer saline
BS	borate saline
BABS	bovine albumin borate saline
PBS-T	0.05% tween 20 in PBS
NHS	normal human serum
HRPO	horse radish peroxidase
OD	optical density

LIST OF ABBREVIATIONS (CONTINUED)

ABBREVIATION OR SYMBOL	TERM
Hr	hour (s)
GRBC	goose red blood cell
NSS	normal saline solution
DGV	dextrose gelatin veronal
VAD	veronal adjusting diluent
S.D.	standard deviation
GMT	geometric mean titers
1°	primary infection
2°	secondary infection
1° or 2°	primary or secondary infection

CHAPTER I

INTRODUCTION

At present, infectious diseases caused by viruses are still an important public health problem in Southeast Asian countries, including Thailand. Some of these serious diseases in those countries are a result of arthropod-borne viruses such as the Dengue viruses, Chikungunya virus and Japanese encephalitis (JE) virus. These viruses are transmitted by mosquitoes and are classified as arboviruses; sharing similar biological and epidemiological characteristics (1). According to the data reported by the Department of Epidemiology, Ministry of Public Health, the morbidity rate and the prevalence trends in diseases caused by these viruses are still rather high (2). This is despite the advancements in medical technology that should help to decrease the severity of the problem.

In Thailand, hemorrhagic and rash fever are caused by Dengue and Chikungunya viruses. Approximately 90-95% of patients with hemorrhagic fever are infected with Dengue virus. Chikungunya virus accounts for approximately 5% of the cases while the remaining percentage of cases (approximately 5%) are a result of co-infection with Dengue and Chikungunya viruses. The World Health Organization (WHO) has classified the clinical severity of Dengue virus infection into dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). The

difference between DF and DHF is that DHF is characterized by plasma leakage (hemorrhage) which results in DSS. Severe hemorrhaging of the body organs may lead to shock and possibly death (3). Severe shock occurs in approximately 36–47% of DHF cases and the subsequent mortality rate is approximately 1–2%.

Dengue hemorrhagic fever was discovered in Thailand in 1949. At that time the mortality rate was relatively high (approximately 17%) due to the etiology, pathology, immunology and treatment of this disease being virtually unknown. In 1958 the first epidemic of DHF occurred in the Thonburi district of Bangkok. There were more than 2,000 cases of which 300 were fatal. In the following year the epidemic of DHF spread throughout the country. The most serious epidemic to hit Thailand was in 1987 when 174,285 cases were reported. At that time, the morbidity rate was 325.18 per 100,000 persons, and 1,008 people died to give a mortality rate of 1.88 per 100,000 persons (2). In the following year the mortality rate was decreased due to advances in medical treatment. Hemorrhagic and rash fevers caused by Chikungunya virus are less severe than those caused by Dengue virus. In 1994, there was a reported epidemic of Dengue and Chikungunya virus co-infections in several provinces in Thailand.

JE virus infection was first reported in Japan in 1871. It was the biggest epidemic in history with approximately 6,000 cases. Later, epidemics of JE were reported in the neighboring countries of Korea and Taiwan. In Thailand, the first reported epidemic of JE occurred in Chiang Mai Province in 1969 which was the same year as a DHF epidemic (4). The trend in the morbidity rates for JE has been

decreasing after 1986 since the government issued the JE vaccination policy in the Expanding Program on Immunization (EPI). However, even though the morbidity rate of JE is low when compared to other diseases, the severity of the disease is very high when it occurs. It is characterized by neuro-pathogenesic features such as sensory nerve disorders, emotional and behavioral changes and a decrease in abilities that require thought processes. As a result, neurological changes are seen in patients who recover from the infection (5).

The annual report by the Department of Epidemiology, Ministry of Public Health, recorded the morbidity and mortality rates for DHF and JE for 1987-1997, as confirmed by the Virus Research Institute, Department of Medical Sciences, Ministry of Public Health. The details of this report are summarized in Table 1.

Dengue hemorrhagic fever and JE can be found in every parts of Thailand. In northern Thailand the incidence rates for both diseases are the highest, with Chiang Mai Province continually rating among the top for the number of epidemics (2, 5-10). Both diseases are found primarily in children aged 5-14 years and are most prevalent during the rainy season (May to October). Climatic conditions, including temperature and humidity, are related to occurrence as these factors affect the breeding cycle of the mosquito vectors for both diseases. At onset, clinical signs and symptoms include acute high fever, weakness and myalgia. The characteristics of arbovirus infection are similar for both diseases (11, 12). However, as hemorrhagic fever due to dengue

Table 1 Number of cases, morbidity rate and case fatality rate of DHF and JE in Thailand, 1987-1997.

Year	Dengue Hemorrhagic Fever			Japanese Encephalitis		
	No. of cases	Morbidity rate/100,000 population	Fatality rate (%)	No. of cases	Morbidity rate/100,000 population	Fatality rate (%)
1987	174,285	325.18	1.88	1,711	3.20	14.60
1988	26,926	49.37	0.66	1,587	2.91	12.35
1989	74,391	133.95	0.39	1,433	2.58	13.60
1990	92,005	163.40	0.45	1,192	2.10	12.70
1991	43,511	76.79	0.30	959	1.69	11.20
1992	41,125	71.16	0.33	929	1.60	12.59
1993	67,017	114.88	0.33	788	1.35	13.83
1994	51,688	87.47	0.27	752	1.27	10.90
1995	60,330	101.46	0.31	584	3.21	0.98
1996	38,109	72.10	0.30	533	1.25	0.99
1997	101,689	167.21	0.23	585	0.96	0.10

Source: Surveillance Report, Department of Epidemiology, Ministry of Public Health, 1987-1997. Laboratory confirmation by hemagglutination inhibition (HI) test (2).

progresses patients may develop a rash with petechiae hemorrhage. Some patient's develop symptoms characterized by plasma leakage and hemorrhage of internal organs. In JE patients, clinical signs develop into encephalomyelitis, headache, nausea, vomiting, convulsions and neck stiffness. Severe cases lead to unconsciousness, coma and ultimately death within 10 days.

Chikungunya virus is widespread in Africa and is also found in Saudi Arabia, Borneo, Malaysia and the Philippines. Outbreaks have occurred in many parts of Africa as well as Thailand, Cambodia, Burma, Sri Lanka and India. The pathology is probably same as Dengue. In Thailand, Chikungunya virus infections are not a serious health threat to the general population; the incidence rate is very low.

Many of the clinical symptoms of arbovirus infections are similar to other diseases such as influenza, measles and malaria (11-13). Therefore, clinical diagnosis of arbovirus infection relies on advanced laboratory procedures. In some areas of Thailand, this is a problem due to a lack of resources and personnel thus making diagnosis difficult without the necessary laboratory confirmation. This may lead to a missed diagnosis and, especially with patients having a fever of more than one week, the infection being classified as pyrexia of unknown origin (PUO).

In Thailand, the number of PUO cases in recent years has been increasing. In 1994, the Department of Medical Science reported that, following laboratory investigation and confirmation of 2,484 cases of PUO, the highest cause was dengue

hemorrhagic fever (309 cases or 12.5%), followed by pneumonia (245 cases or 9.9%) and acute diarrhea (186 cases or 5.5%) (2). The annual report by the Department of Epidemiology, Ministry of Public Health, recorded the morbidity and mortality rate of PUO for 1987-1996, the details are summarized in Table 2.

Table 2. Number of reported cases, morbidity rate and case fatality rate of PUO in Thailand and Chiang Mai Province, 1987-1996.

Year	Thailand			Chiang Mai		
	No. of cases	Morbidity rate/100,000 population	Fatality rate (%)	No. of cases	Morbidity rate/100,000 population	Fatality rate (%)
1987	330,376	616.30	0.07	16,407	1,279.00	0.09
1988	246,685	452.35	0.07	10,959	841.77	0.09
1989	347,083	616.50	0.04	12,153	685.41	0.05
1990	347,083	616.50	0.05	12,153	888.40	0.12
1991	268,576	474.00	0.04	10,554	763.60	0.10
1992	227,171	393.10	0.05	7,534	492.17	0.24
1993	236,721	405.86	0.06	7,729	503.82	0.22
1994	211,973	358.87	0.05	9,088	587.43	0.12
1995	218,977	368.27	0.05	7,014	451.73	0.20
1996	186,916	100.00	0.05	5,367	343.06	0.09

Source: Surveillance Report, Division of Epidemiology, Ministry of Public Health (2).

Chiang Mai Province is the most developed and modern area in northern Thailand. The geographical features include mountains, forests and streams in rural areas. In mountainous areas there are many beautiful and well known places for the travelers. It is one of the provinces which has a large number of hill tribe people. In 1996, the total hill tribe population in Chiang Mai was approximately 208,330 persons (14). Hill tribe people have different lifestyles compared to Thai lowland people, such as historical background, lifestyle, socio-economic structure, religion, culture and language.

The occupations of the majority of the hill tribe people involve agricultural activities including planting and cropping of rice, fruit, vegetables, tea, coffee etc. and labor. They are rather poor and do not have enough income for expenditures. Almost all of them have little knowledge of environmental sanitation, health information and primary healthcare. When they are sick they often use herbal medicines or the traditional healer. Currently, the hill tribe areas are more accessible than previous. However, the general population is still unaware of disease severity and does not take preventative measures. They go to hospital only when they have a very serious illness. For these reasons the morbidity rate for infectious diseases such as dengue hemorrhagic fever and JE are still relatively high. This presents health problems for the government in prevention, control and irradiation of the diseases. As shown from the epidemiological data, in northern Thailand the morbidity and prevalence rates of endemic diseases caused by viruses are rather high, especially for both mentioned diseases when compared with others.

Therefore, this study set out to assay the antibody response to arbovirus infections in patients living in the hill tribes of Chiang Mai Province. The patients are those who come from the hill tribes to have medical treatment at various hospitals in Chiang Mai Province during the epidemic season of these diseases. This study determined the status of antibodies to arboviruses by laboratory assay techniques to confirm the clinical diagnosis made at the hospital. The results of this study indicated the level of missed diagnosis in hospitals which might lead to economic loss and an increase in mortality rates. The study also provided epidemiological data and investigated the factors related to arboviral diseases. The results might be used to assist in the prevention and control of these diseases. In particular, the results might be of relevance to the ongoing JE vaccination program and the planned dengue fever vaccination program. In addition, this study was responding to the policy of the government to assess the health status of people living in hill tribes with respect to communicable disease health problems.

Objectives

General objective

To assay the antibody levels and the factors related to arbovirus infections in hill tribe patients with pyrexia at four selected hospitals in Chiang Mai Province.

Specific objectives

1. To assay antibody levels by laboratory confirmation with the hemagglutination inhibition (HI) test and IgM antibody capture ELISA.
2. To measure the geometric mean titer (GMT) of Dengue (types 1, 2, 3 and 4), Chikungunya and JE virus antibodies in acute and convalescent sera in hill tribe patients.
3. To determine the prevalence rate of arbovirus infections and prevalence rate of arbovirus antibodies exposure (Dengue, Chikungunya and JE) in hill tribe patients.
4. To analyze the demographic, vector and the environmental factors related to the arbovirus infections.

Research hypotheses

1. The geometric mean titers of Dengue (types 1, 2, 3 and 4), Chikungunya and JE viruses in acute and convalescent sera of hill tribe patients are different.
2. The prevalence rate of arbovirus infection and prevalence rate of arbovirus antibodies exposure (Dengue, Chikungunya and JE) are different.
3. The demographic, vector and environmental factors are related to the arbovirus infections.

Scope of research

Hill tribe patients selected for the study had to be greater than one year old and display symptoms of pyrexia. The patients were selected from four hospitals in Chiang Mai Province, namely; Samoeng Hospital, Mae Taeng Hospital, Phrao Hospital and Chiang Dao Hospital.

Definition of terms

1. Arbovirus infection: Patients with pyrexia caused by JE, Dengue and / or Chikungunya virus infections as confirmed by laboratory tests.
2. Dengue virus infection: Patients with pyrexia caused by Dengue virus as confirmed by HI testing (either recent infection or presumptive), and / or by dengue MAC ELISA.
3. Chikungunya infection: Patients with pyrexia caused by Chikungunya virus having laboratory confirmation only by HI testing.
4. JE infection: Patients with pyrexia caused by JE virus having laboratory confirmation by HI testing and / or JE MAC ELISA.
5. Dengue and Chikungunya infections: Patients with pyrexia caused by dual infection with Dengue and Chikungunya virus having laboratory confirmation by HI testing and / or dengue MAC ELISA.
6. Dengue MAC ELISA positive: A patient with dengue virus infection whose serum, which when assayed by dengue MAC ELISA, shows IgM $\geq$ 40 units and / or a units ratio of JE MAC ELISA to dengue MAC ELISA of < 1 (when both dengue MAC ELISA and JE MAC ELISA are $\geq$ 40 units).

7. Dengue primary infection: A patient who has a units ratio of dengue MAC ELISA to dengue GAC ELISA of ≥ 1.8 and/or has a 4-fold rising titer to ≤ 1280 in specimens collected at an interval of ≥ 7 days assayed by HI testing with dengue antigens.
8. Dengue secondary infection: A patient who has a units ratio of dengue MAC ELISA to dengue GAC ELISA of < 1.8 and/or has a 4-fold rising titer to ≤ 2560 in specimens collected at an interval of ≥ 7 days, assayed by HI testing with dengue antigens.
9. Dengue primary or secondary infection: A patient whose paired sera, collected within 7 days of each other, show a 4-fold rising titer to ≤ 1280.
10. JE MAC ELISA positive: A patient with JE virus infection whose serum, which when assayed by MAC ELISA, shows anti-JE IgM units of ≥ 40 and/or a units ratio of JE MAC ELISA to dengue MAC ELISA of < 1 (when both dengue MAC ELISA and JE MAC ELISA are ≥ 40 units).
11. Presumptive secondary flavivirus infection: A patient who has no rising titers or changing titer by HI testing, or any blood specimen showing HI titers ≥ 2560 with dengue and / or JE antigens.
12. Hill tribe patients: Hill tribe members who are aged one year old or older, have a fever of ≥ 38°C and have visited one of the four selected hospitals for treatment.
13. Pyrexia : A patient who had fever of ≥ 38°C for more than one day were studied.
14. Factors related to arbovirus infections: The personal, vector and environmental factors which may be associated with arbovirus infections.

15. The prevalence rate of arbovirus infections: The prevalence rate of recent dengue virus infection and / or prevalence rate of recent Chikungunya virus infection and / or prevalence rate of recent JE virus infection. All infections confirmed and shown to be positive by HI testing (with dengue and / or Chikungunya and / or JE virus antigens) and / or MAC ELISA (with Dengue and / or JE virus antigens). Prevalence rate of infections can be calculated by the formula:

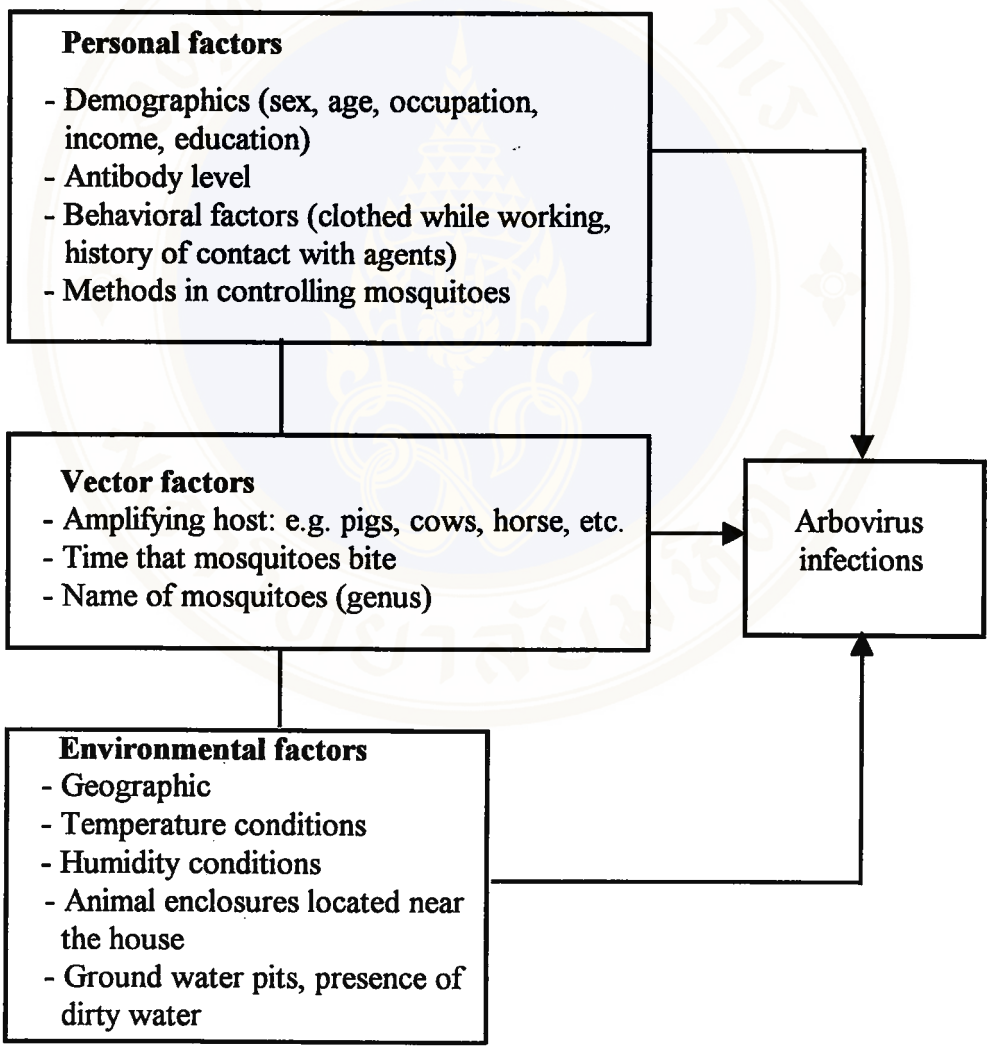
$$\frac{\text{No. of patients with recent arboviruses infection}}{\text{No. of all patients in the study}} \times 100$$

16. The prevalence rate of arbovirus antibodies: The prevalence rate of dengue virus antibodies and / or prevalence rate of Chikungunya virus antibodies and / or prevalence rate JE virus antibodies, which when assayed by HI testing (with Dengue and / or Chikungunya and / or JE virus antigens) produce HI titers ≥ 10. Prevalence rate of antibodies can be calculated by the formula:

$$\frac{\text{No. of patients who have arboviruses antibody titers (HI} \geq 10)}{\text{No. of all patients in the study}} \times 100$$

Conceptual framework

Conceptual framework showing the personal, vector and environmental factors related to arbovirus infections.



CHAPTER II

LITERATURE REVIEW

Arthropod-borne viral infections are still a major public health problem that affects the morbidity and mortality rates of people in the world, especially in Southeast Asian countries including Thailand. Two of these serious arthropod-borne viral diseases are dengue hemorrhagic fever (DHF) and Japanese encephalitis (JE). In 1974, arthropod-borne viruses were classified by an International Committee on Taxonomy of Viruses (ICTV) in the Genus *Flavivirus* of the Family *Togaviridae* (16-17). The Family includes three other genera (Alphavirus, Pestivirus and Rubivirus) and several additional members assigned to the *Togaviridae* by the arthropod-borne virus study group in 1978 (18). Along with their size, as compared to Togaviruses, several fundamental differences have become apparent in regard to Flaviviruses structure, replication strategy, and gene sequence. Based on such criteria, the *Togaviridae* study group proposed the creation of a new Family, the *Flaviviridae,* and this was approved by the ICTV in September 1984 (16). Almost all of the viruses in this Family are classified as arthropod-borne viruses (arboviruses), as they are transmitted by an arthropod vector such as mosquitoes and / or ticks. To qualify as being termed arthropod-borne a virus must multiply in tissues of the vector, including the salivary glands, and then be injected with saliva during biting. This is known as biological transmission. These viruses cause disease in humans when they

are accidentally transmitted by hemophagous insects (19).

The Genus *Flavivirus* includes more than 69 members separated into groups on the basis of serological relatedness (20). They are transmitted to vertebrates by chronically infected mosquitoes distributed worldwide (21-22). The clinical symptoms vary and sometimes include fever and encephalitis. There are several important diseases that are well known and occur in many parts of the world, dengue fever (DF) with its associated dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), Japanese encephalitis (JE) and yellow fever (YF). These three diseases are the entities of major global concern. The other diseases in this family are tick-borne encephalitis (TBE), Kyasanur Forest disease, West Nile encephalitis (WN), St. Louis encephalitis (SLE), Murray Valley encephalitis (MVE), Hepatitis C (Hep C) and other important agents specific to geographically endemic areas (23-25).

Three important diseases in the Family *Flaviviridae* which have been reported in Thailand are DHF, JE and Hep C. However, Hep C is a disease that is not transmitted by arthropods and is not classified in the Flavivirus genus. At present, DHF and JE are the arbovirus infections included in the communicable diseases surveillance control program of the Ministry of Public Health.

Table 3 Members of the Family *Flaviviridae* (23)

Genus	Group classification	Type member
Flaviviruses	Tick-borne encephalitis (a, b)	Central European encephalitis(TBE-W)
		Far Eastern encephalitis (TBE-FE)
	Rio Bravo (c)	Rio Bravo
	Japanese encephalitis	Japanese encephalitis (JE)
		Kunjin (KUN)
		Murrey Valley encephalitis (MVE)
		St. Louis encephalitis (SLE)
		West Nile (WN)
	Tyuleniy	Tyuleniy
	Ntaya (c)	Ntaya
	Uganda S	Uganda S
	Dengue	Dengue type 1 (DEN-1)
		Dengue type 2 (DEN-2)
		Dengue type 3 (DEN-3)
		Dengue type 4 (DEN-4)
	Modoc	Modoc
	Ungrouped (c)	Yellow fever (YF)
Pestivirus	Bovine viral diarrhea	Bovine viral diarrhea (BVDV)
	Classical swine fever	Hog cholera, or
		Classical swine fever (CSFV) (e)
	Border disease	Border disease (BDV)
Hepatitis C Virus (d)	Hepatitis C	Hepatitis C (HCV)

a. Number of recognized members in each antigenic group from Calisher CH. et al. (20)

b. Arthropod vectors: T = tick; M = mosquito; U = unidentified or no vector.

c. Arthropod vectors for some members of these groups have not been identified. The ungrouped flaviviruses include mosquito and tick transmitted viruses as well as some with no known vector.

d. The Hepatitis C viruses (HCV) include a large number of members that can be divided into several groups or genotypes on the basis of genetic divergence (22). An official name for this genus and a standardized nomenclature for different genotypes has not yet been agreed upon.

e. In the pestivirus literature, HCV has been a common abbreviation for hog cholera virus. More recent publication use CSFV to avoid confusion with the human hepatitis C viruses.

The other member of arboviruses that cause viral infections, and are associated with DHF, is Chikungunya virus, which belongs to Genus *Alphavirus* in the Family *Togaviridae*. The members of *Alphaviruses* are summerized in Table 4. Signs and symptoms of Chikungunya viral infections are similar to other diseases such as dengue fever/dengue hemorrhagic fever, rubella and malaria. Sometimes it is very hard to reach a differential diagnosis without laboratory confirmation (26). Clinicians may simply conclude that the infection was a viral infection or a case of PUO. Hence, data maybe lost concerning quantitative information of Chikungunya virus infections as well as their prevalence rates. In Thailand, the Division of Epidemiology, Ministry of Public Health did not have any information pertaining to cases of Chikungunya virus infections for

several years.

Table 4 Classification of members of the Family *Togaviridae* in the Genus *Alphavirus* (26).

Genus	Subgroup complex	Viral species
Alphavirus	I	Eastern equine encephalitis (EEE)
	II	Venezuelan equine encephalitis (VEE)
	III	Western equine encephalitis (WEE)
		Sindbis
		Semliki Forest (40 others)
	IV	Chikungunya (CHIK)
		Mayaro
	V-VII	Getah (GET) (each subgroup contains a single virus)

There have been several serological studies on arbovirus infections in Thailand which have shown that there are three arboviral diseases that are commonly found. These are DHF, JE and Chikungunya virus infections.

Yamada T et al. (27) studied an epidemic of Japanese encephalitis (JE) in the northern region of Thailand in 1969 and 1970. The group found that the epidemics began

in May, reached their peak in July, and then gradually declined in August, the months of the rainy season in Thailand. The cases of JE were found mostly among patients under twenty years old, and 50% of young healthy people of this age group had no antibody to JE. The JE incidence rate in 1969 was 20.3 per 100,000 persons which had never previously been seen in Thailand.

Gunakasem P et al. (28) surveyed dengue hemorrhagic fever cases in Thailand. Acute and convalescent blood specimens were obtained from patients admitted to 72 provincial hospitals. Samples were assayed by hemagglutination inhibition (HI) testing. Dengue infections were found to be 42, 54 and 52% in 1974, 1975 and 1976, respectively. A few cases were reported in December though February with a peak found from July to August. A high number of cases were from the age group between 1 and 10 years old, and low morbidity found in older age groups. The HI positive reactions for dengue virus infection were highest in ages between 4 and 10 years. There were 42 cases of hemorrhagic fever caused by Chikungunya virus infections from a total of 2,379 patients. It was found in northeast, central and northern Thailand. There were 145 cases who demonstrated no rising antibody titers to Chikungunya virus.

In 1982, Fukunga T et al. (29) surveyed the presence of antibodies among healthy humans at 5 locations in the Chiang Mai area. The prevalence of antibody titers were measured by HI testing against JE and Dengue antigens, and by the enzyme-linked immunosorbent assay (ELISA) against JE antigen. Fang district, in the Mae Kong Valley, showed antibody prevalence rates lower than the other four sampling places in Chiang Mai

Valley. The prevalence of IgM-ELISA antibodies against JE was different among sampling places, indicating a homogeneity of JE virus circulating in the study area.

Bundo K et al. (30), in 1986, studied antibody responses in sera from JE and DHF cases by the indirect micro ELISA, using JE and Dengue virus type 1 antigens. The responses of JE cases in Japan and primary encephalitis cases in Thailand were rather mono-specific to JE antigen, in contrast to DHF patients whose antibody responses were cross-reactive to JE and DEN-1 antigens even in the primary infection.

1. Physical and chemical properties

1.1 *Flaviviruses*

Flavivirus particles consist of a spherical ribonucleoprotein core surrounded by a lipoprotein envelope with small surface projections. Envelope lipids constitute approximately 17 % of virion dry weight. Flavivirions contain three structural proteins; a nucleocapsid or core protein (C) molecular weight (mw) 14 kilodalton (kd), a non-glycosylated membrane protein (M), mw 7 kd, and an envelope protein (E), mw 50 kd, which is usually glycosylated (31-32). The M and E proteins are the major component of the virion surface projections when observed by electron microscopy; they contain the important antigenic determinants subserving hemagglutination and neutralization and thus induce immunological responses in the infected host. The E protein determinants are involved in the binding of virions to call receptors and probably play a role in intra-

endosomal fusion at low pH. These protein constituents are sensitive to enzymatic digestion with trypsin, chymotrypsin, and papain, which render the virus non-infectious but preserves certain antigenic reactivities.

Dengue virus

Mature dengue virions consist of a single stranded, positive sense RNA genome surrounded by an approximately icosahedral or isometric nucleocapsid about 30 nanometers (nm) in diameter. This nucleocapsid is covered by a lipoprotein envelope about 10 nm deep, which contains a major glycoprotein (E protein). The complete virion is about 50 nm in diameter. The virion has a density about 1.23g/cm3 as measured by equilibrium centrifugation in deuterium oxide-sucrose gradients and a sedimentation coefficient of around 210 (33).

It has been proposed that the mature virion contains three structural and seven non-structural proteins. The three structural proteins are the C protein which encapsidates the virion RNA, the M protein (derived by cleavage from prM) and the glycoprotein E, both of which are associated with the lipid envelope. The seven non-structural proteins (NS) are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5. In addition, the immature virion which is mainly an intracellular virus, contains a protein known as preM, a precursor of M protein.

Japanese encephalitis virus (JEV)

The JE virion is an enveloped shericle partical of 381-500 nm in diameter. Nucleocapsid symmetry is probably cubic, but a report showed herical structures in virion preparations. Studies on JE have been highlighted by advances in the molecular aspects. Three structural proteins have been identified for JEV.

M protein, consisting of 75 amino acids with a calculated mw of 8,329. It is hydrophobic and within the lipid envelope (34). The intracellular virions contain only prM protein which is a glycoprotein precursor to the structural protein M, whereas extracellular virions contain predominantly M protein. Glycosylation of prM presumably has an important role in virion assembly and release (35).

Nucleocapsid protein (C), consisting of 136 amino acids with a mw of 13,859, is rich in basic amino acids (20% Lysine and Arginine) and presumably interacts with viral genome RNA to form a nucleocapsid precursor. One Study indicated that the nucleocapsid proteins contained no type-specific or complex-reactive determinants but contained equal amounts of group reactive antigens (36).

Envelope glycoprotein (E) consists of 500 amino acids (mw 53,334) and contains a single potential N-glycosylation site at amino acid number 448. The glycoprotein envelope of St. Louis encephalitis, JE and Dengue viruses were found to contain each of three classes of antigenic determinants. Most of the determinants on the envelope protein

were type-specific, some were complex-reactive, and a small fraction were Flavivirus group-reactive (36). Using anti-JE virus monoclonal antibodies (Mabs) directed against envelope protein of JE virus suggested the existence of at least eight epitopes on the E protein, antigenically closely related to virus of the same subgroup, i.e. Murray Valley encephalitis and West Nile (37).

The presence of complex reactive and type specific antigens on the Flaviviruses glycoprotein explains the serological complexity of the immune response. Neutralizing antibody induced by the Flavivirus glycoprotein is produced to the type antigen and is expressed in the HI reaction. Adsorption of sera with a heterologous antigen makes the HI reaction type-specific (38-41). This indicates that the complex-reactive and type-specific determinants on the enveloped in the Flavivirus HI reaction. Infection with one member of a complex induces complex-reactive HI antibodies which do not provide immunity to infection with a second member of the complex (41-43).

1.2 *Alphaviruses*

Viruses of the Family *Togaviridae* (type-species, Sindbis virus) that are important pathogens of humans or livestock, and that are arthropod-borne, are members of the Genus *Alphavirus* (formerly group A arboviruses). These include eastern equine encephalitis (EEE), western equine encephalitis (WEE), Venezuelan equine encephalitis (VEE), Chikungunya (CHIK), Getah (GET), o'nyong-nyong (ONN), Mayaro (MAY), Ross (RR) and Sindbis (SIN) viruses(19). Alphaviruses are spherical particles, 60-70 nm

in diameter. They contain one segment of single stranded, positive sense RNA and three structural proteins. Two of these are envelope glycoproteins and one is a non-glycosylated capsid protein. Semliki Forest virus pocesses three envelope glycoproteins. At present more than 23 viruses, 22 subtypes and 14 varieties have been assigned to this genus. The envelope proteins are antigenic and hemagglutinate. Antibodies can be detected by HI reactions and neutralization. Nucleocapsid protein can be detected by CF tests. In concert, these tests can be used to determine the antigenic group, antigenic complex and antigenic type of Alphaviruses.

2. Epidemiology of arboviruses

2.1 Geographic distribution

Arboviral infections are found in all temperate and tropical zones, though they are most prevalent in tropical rain forests with their abundance of vertebrate and arthropod species (19,26). The taxonomic diversity of the arboviruses reflects the relative efficiency of virus transmission by blood sucking arthropods. The most important invertebrate hosts are mosquitoes of the genera Aedes and Culex (19) that are responsible for DHF and JE, respectively.

Dengue fever has long been known for the extensive and severe epidemics it causes. It probably originated in 1779 in Southeast Asia in Jakarta as reviewed by Siler et al. (44) but has occurred throughout the inter-tropical zone and even in temperate regions;

as evidenced by the memorable epidemics recorded in Philadelphia, U.S.A. (1789), the West Indies (1872), Hong Kong (1901), Greece (1927-1928) and Japan (1942-1945). Epidemic dengue fever was responsible for hundreds of thousands of cases each year in Southeast Asia where all 4 serotypes of the virus could be found. When Dengue virus type 1 was isolated and identified in the United States (Hawaii) from patients blood, Dengue virus type 2 followed from Papua New Guinea (1950) and Dengue virus types 3 and 4 were discovered in the Philippines (1956) (45). The introduction of single virus serotypes into Central America and the Caribbean basin resulted in large epidemics of dengue fever in 1952, 1963-1964, 1977 and 1981. Subsequently, multiple Dengue virus serotypes have become endemic in most countries of tropical and subtropical America and are regularly associated with disease outbreaks.

Following World War II, new endemic patterns of disease, accompanied by an increased incidence of complicated dengue (DHF and DSS), emerged in Southeast Asia. The features of the epidemiology of DHF/DSS are that (1) the syndrome usually occurs in persons with pre-infection dengue antibody, and (2) the infecting virus precipitating the attack is usually Dengue type 2. The first epidemic of DHF occurred in the Philippines in 1954 and was described by Hammon et al. (46). After a period when DHF was endemo-epidemic in Thailand and the Philippines there was a considerable increase in the number of reported Dengue infections in the years 1971-1975 in various countries of the Southeast Asian and Western Pacific Regions. In 1978 a large outbreak of DF occurred in the southern part of China. This was caused by Dengue virus type 4 and resulted in 122 cases and 14 deaths. In 1979-1980 there was another big outbreak caused by Dengue

virus type 4 in countries in the South Pacific. In 1980, 49,318 cases of DF / DHF (462 deaths) were reported in Vietnam. Later, in 1981, outbreaks of DHF/DSS caused by Dengue virus type 1 were reported to have occurred during the period from 1977 to 1980. A total of 116 143 persons were hospitalized, an estimated 24,000 of these with DHF. Among approximately 10,000 shock cases there were 158 deaths. In 1982, an epidemic of DF/DHF with 3,005 cases (35 deaths) was reported in Malaysia; Dengue virus types 1, 2 and 3 were isolated. An epidemic of DF caused by Dengue virus type 3 occurred in the Solomon Islands, and in towns in North Queenland, Australia. The DF epidemic involved mainly dengue virus type 1. However, types 2 and 3 caused 455 confirmed cases in 1981-1982. Similarly, increasing numbers of DHF have been reported every year in the Caribbean basin since a major epidemic in Cuba. Figure 1 showed the geographical distribution of Dengue virus infection in epidemic areas in 1998 .

DHF/DSS has continued to persist in Burma and Indonesia as well as in Thailand. Although the case fatality rate due to DHF is on the decline, morbidity has been on the increase in all 3 endemic countries. Epidemics of dengue fever; 20,220 cases of dengue in French Polynesia (type 1) (47), 18,000 cases in New Caledonia (type 3) (48), 3,300 cases in Vanuatu (type 1) and 2,230 cases in Wallis and Futana (type 3) were reported in 1988-1989 (49).

In Thailand, before the first large outbreak of DHF/DSS in 1958, approximately 50-100 cases diagnosed as "influenza with hemorrhage" were included in the hospital records of Siriraj Hospital in Bangkok (50). Following the 1958 outbreak in Bangkok

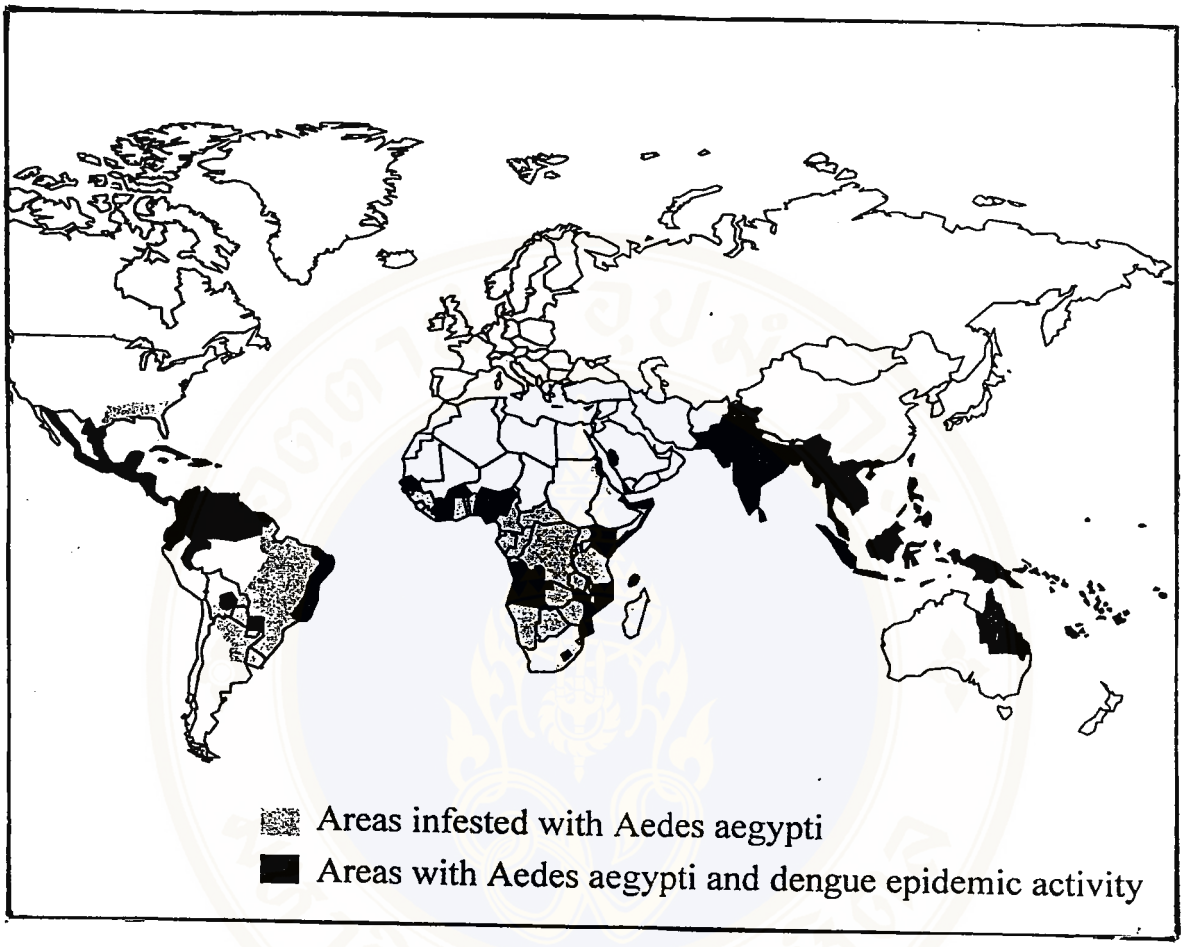


Figure 1 Geographical distribution of Dengue virus infection in epidemic areas in 1998.

Source: Reproduced from the Department of Entomology, London School of Hygiene and Tropical Medicine.

and its suburbs, the disease spread to adjacent provinces in the Central region in 1961 (51). In 1964, a major outbreak occurred in big cities in northern and north-eastern

Thailand (52). The highest record of DHF/DSS (5,403 cases with 216 deaths) was reported. Since 1968 there have been reports of the disease from almost every province in the country. During the first ten year period (1958-1967), the number of patients increased yearly except in 1986. The number of cases recorded was 69,597 in 1984, 80,076 in 1985, 27,837 in 1986 and 174,285 (with 1,007 death) in 1987 (53). Which later was the highest figures were reported in the WHO South-East Asian region. The case fatality rate was approximately 10 % in 1958, but gradually decreased to below 1 % by 1980 (54).

After 1968 the endemic pattern of alternate years changed and became irregular for the whole country. Case reports in Bangkok remained high but did not exceed the 1964 number and followed the country wide pattern. Since 1973 the number of patients in the north-eastern part of the country has increased significantly every year and now comprises almost 50% of the cases for the whole country (55). In the early epidemic years, the number of cases in the dry season was very low (below 100 cases per month) with the most reported cases occurring in Bangkok. In other provinces the number of cases were less than ten per month during the cool dry season (November-February).

Between December and January from 1979 through 1985 the reported number of DHF cases was higher than 100 per province per month in four to five provinces of the central and north-eastern regions.

The total reported number of patients in the cool dry season was, therefore, more than 500 cases per month between November and February. For the whole country, case records reached 2,345 in December 1984 and 1,859 in January 1985. This changing pattern is under investigation.

In Thailand, *Aedes Aegypti* is the main vector of DHF. Isolation of Dengue virus from *Aedes albopictus* was also reported when a small outbreak of DHF occurred in the insular setting of Koh Samui in southern Thailand (56).

Chikungunya virus is widespread in Africa and is also found in Saudi Arabia, Borneo, Malaysia and the Philippines. Clinical outbreaks have occurred in many parts of Africa as well as Thailand, Cambodia, Burma, Sri Lanka and India (Figure 2) (13).

In Thailand, the first reported case of Chikungunya virus infection diagnosed by serology was in 1960 (28) and the last one was in 1991 (57). The disease surveillance system did not specifically include Chikungunya cases during the rainy season of 1995 (June–August). There were at least 2 reported Chikungunya virus outbreaks which might indicate that it is a re-emerging disease in Thailand. However, there is still limited information and knowledge on some aspects of this disease such as clinical manifestations, subclinical cases, duration of illness, complications, transmission, immunity and reservoirs. In 1998, Thaikruea L et al. (58) described the epidemiology of Chikungunya infection based on an outbreak investigations conducted in Khon Kaen

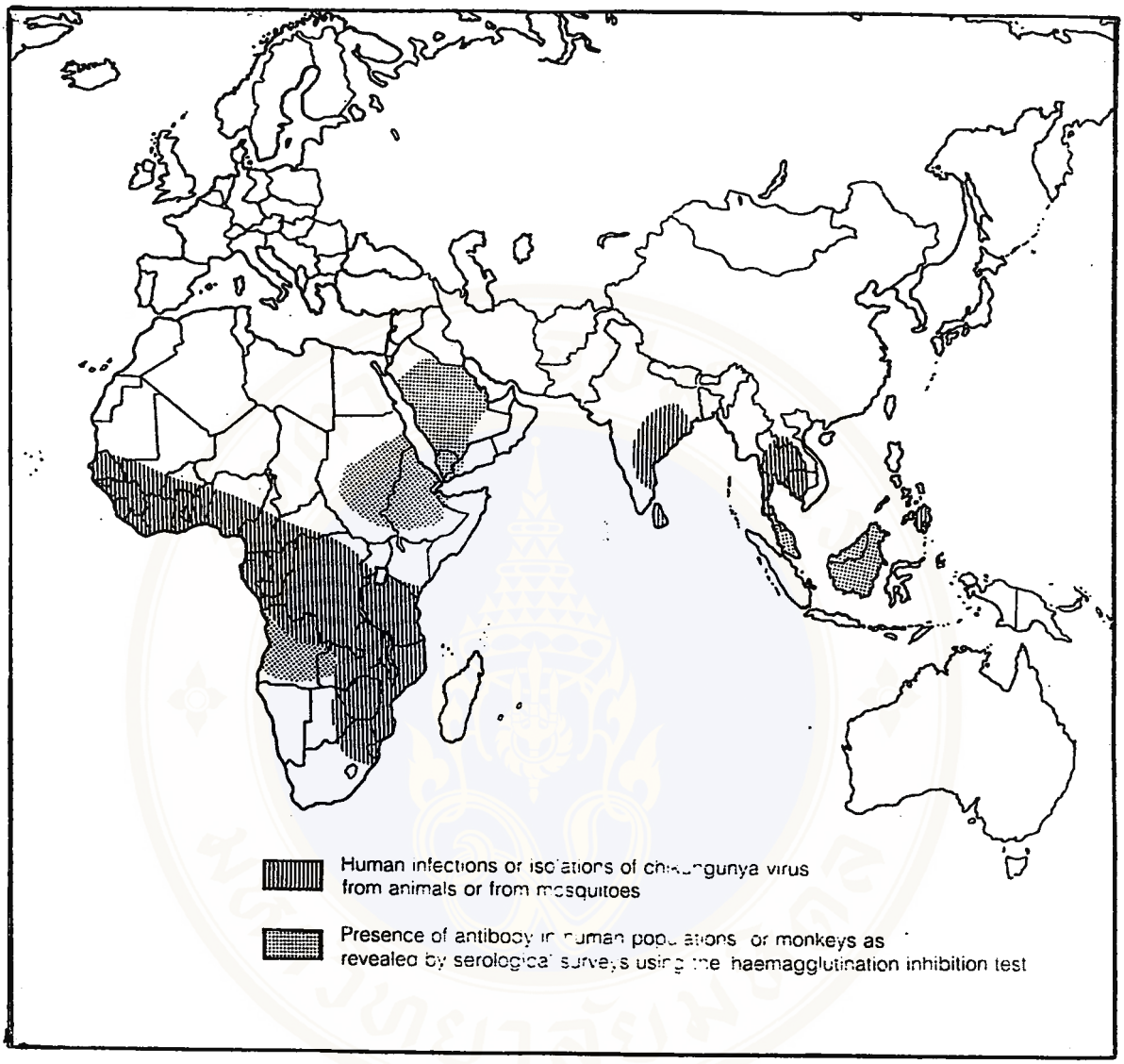


Figure 2 Geographical distribution of Chikungunya virus infection in epidemic areas.

Source: Reproduced from the Department of Entomology, London School of Hygiene and Tropical Medicine.

(July, 1991), Nakorn Sri Thammarat (July, 1995) (59) and Nong Khai (August, 1995) (60) provinces. All three outbreaks occurred during the rainy season. The three most common clinical manifestations were fever, severe arthralgia and a maculopapular rash.

Both genders and all age groups were affected. Serological results were positive for IgM, with a four-fold rise in paired sera, and viral isolation in Nakorn Sri Thammarat and Nong Khai (61). Only in Nong Khai were HI tests conducted and the results were positive. No deaths were reported. The outbreak occurred in rural villages and all three larval indices; Breteau Index (BI), House Index (HI) and Container Index (CI) were very high. The possible vectors in these outbreak were *A. aegypti* and *A. albopictus*. In the Nong Khai outbreak blood specimens were taken on the 3rd-5th day after onset and therefore the proportion of positive results were low. The IgM antibody levels in follow-up cases declined within 3 months.

In JE, the incidence appears to be subsiding in China, Japan and the Republic of Korea but at the same time it has been increasing and spreading over some countries in Asia such as Bangladesh, Myanmar, India, Nepal, Thailand and Vietnam (10). Figure 3 showed the encephalitis cases reported in southeast Asia. The patterns of epidemics appear to be related to climatic and seasonal conditions. In the tropical zone the disease occurs as sporadic cases throughout the year with no seasonal pattern.

Since the routine use of a vaccine in late 1960 in Japan there have not been any epidemics and the background rates have fallen to less than 100 cases per year. This may be due to changes in agricultural and pig rearing practices, increased use of pesticides, and widespread immunization.

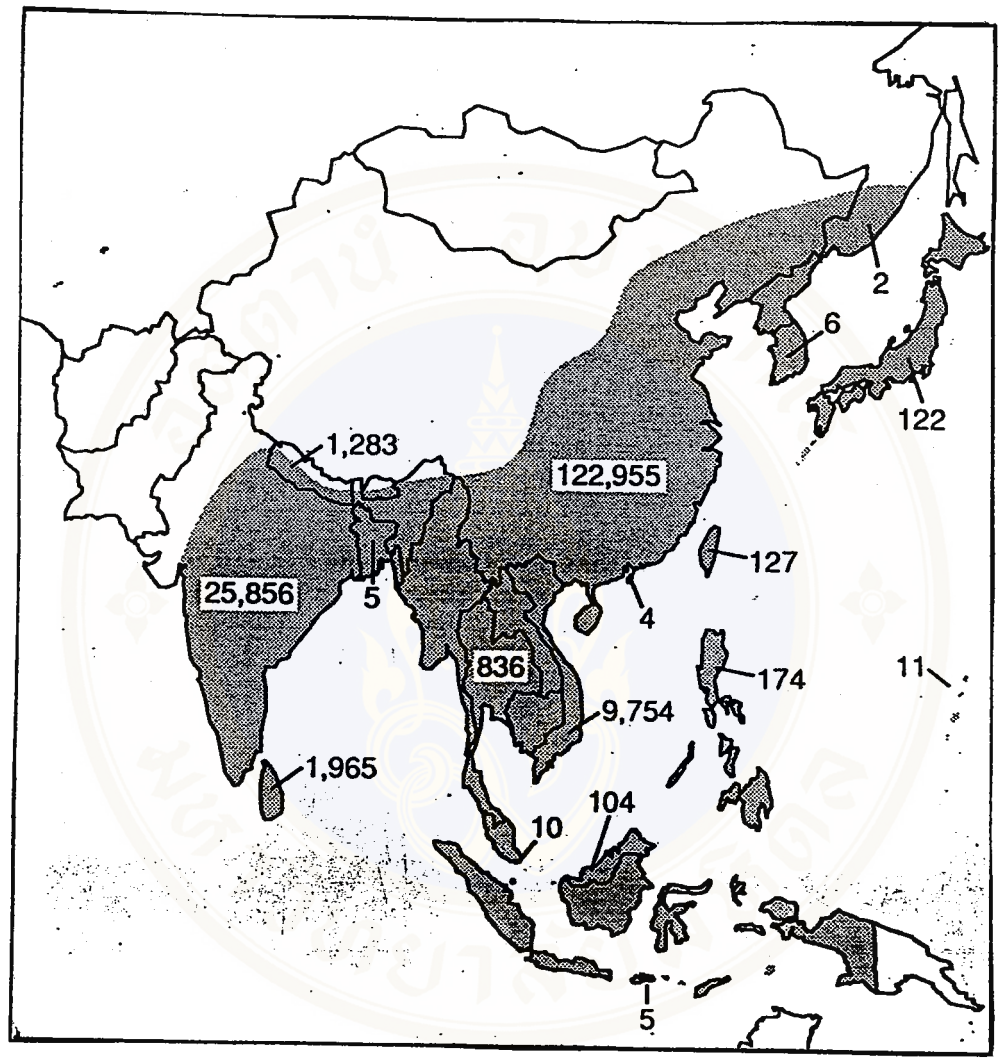


Figure 3 Reported cases of Japanese encephalitis, 1986-1990 and regions of proved or suspected enzootic viral transmission.

Source: Reproduced from the Department of Entomology, London School of Hygiene and Tropical Medicine.

The first outbreak of JE in Thailand was reported in 1969 in Chiang Mai Province (61). Additionally, there was an epidemic of Thai hemorrhagic fever in the same year (27). The epidemics began in May, reached their peak in July and then gradually declined in August. The JE cases were in individuals under twenty years old and 50% of young healthy people of this age group had no antibody for JE. The incidence rate due to JE was 20.3 per 100,000 persons. The strains of virus that were isolated from the brains of fetal cases had very similar characteristics to the JaGAR#01 strain. The results of sero-epidemiological study in the northeast and the south suggested that people in Khon Kaen Province were exposed to group B arbovirus infections (Flaviviruses) more often in childhood and attained high antibody levels more than people in Songkhla Province (62).

Epidemic Japanese encephalitis recurs every year in northern Thailand during the early rainy season. In northeastern and central Thailand it also occurs but with lower attack rates than in the North. Sporadic cases of encephalitis are reported from southern provinces and there is no seasonal peak. This is despite the fact that the population, rice fields, pig densities, annual rainfall and temperature patterns in the South are quite similar to that in the North of Thailand. This may be because the mosquitoes which transmit JE virus to pigs in the South are unable to transmit the virus to man, or prior infections with dengue might be more common in the South and provide partial protection from JE. It is also possible that the vector competence of the strains in southern Thailand is inferior to that of northern stains of the same species (61).

2.2 Population

The age distribution of persons with clinical dengue and JE virus infections varies geographically. In hyperendemic areas of Southeast Asia most infections appear in young children aged below 15 years (63). There are no reported differences in susceptibility to Dengue and JE infection between the sexes but in Southeast Asia there are a slightly higher number of female than male cases (5).

Occupation might also affect the incidence of arboviral infections in some geographical areas. Dengue infections occur mostly in children and adult females who stay indoors during the daytime (64).

2.3 Time

Temporal factors of importance to arbovirus transmission are seasonal changes involving rainfall and daily temperatures. Outbreaks usually begin in May and reach their peaks in July and August before declining in October. The mosquitoes deposit their eggs in peridomestic water holding containers. Periods of increased rainfall, which keep these containers filled with water, result in greater mosquito populations. Higher temperatures also favor the size of mosquito (65-66).

2.4 Vector

Aedes aegypti (*A. aegypti*) is the most efficient of the mosquito vectors in Dengue and Chikungunya infection because of its domestic habits. The female *A. aegypti* can transmit Dengue either immediately, by change of host when blood meals are interrupted, or after an incubation period of 8-10 days during which time the virus multiplies in salivary glands. In areas where *A. aegypti* is absent or scarce *A. polynesiensis* has been found to be a vector in an epidemic that occurred in Futana in 1971 (67). *A. albopictus* is also a major Dengue vector in Southeast Asia. It can also be found in the Solomon Islands, United States and Pacific Islands

Culex Tritaeniorhynchus is the major vector in JE infection. The larvae breed abundantly in rice fields, therefore their ecology is very much influenced by the practice of rice cultivation. The principle culicine vectors lay their rafts of eggs on the surface of water 2 to 3 days after blood feeding (68). A survey of ground water breeding mosquitoes in an area undergoing irrigation development in the Mahaweli Project in Sri Lanka showed that the overall change from uninhabited forest to irrigated rice fields sharply increased the prevalence of *C. Tritaeniorhynchus*. Natural breeding habitats were rainwater pools, marshes and streams (69).

2.5 Animal Reservoir

Dengue: Of several wild and domestic vertebrate species examined only monkeys appear to be involved in the dengue cycle. Examination of monkey's in forest habitats revealed a high prevalence, and significant levels of, dengue antibody similar to those found in man. Dengue is a zoonosis and is maintained by a forest cycle involving wild monkeys and jungle mosquitoes. Such a cycle was demonstrated in Malaysia and appears to be similar to that of jungle yellow fever in Africa except that different local species of monkeys and mosquitoes are involved.

JE: Pigs are important in the epidemiology and clearly are a significant source of mosquito infection. A study in 1970 in Chiang Mai valley showed, by HI testing, the prevalence of JE virus was high in dogs, bovines and indigenous pigs, except for a small group of horses. The geometric mean titer was much higher in pigs than in other mammals. Antibody to Tembusu virus, rather than to JE virus, was more prevalent in domestic fowl (70).

Bird-mosquito cycles are thought to be important in maintaining and amplifying JE virus in the environment (71). The amount of virus circulating in bird blood following experimental infection, either by syringe and needle or by laboratory reared infected mosquitoes, has been found to be sufficiently adequate to infect mosquitoes (72).

Chickens and ducks seem not to be involved in JE virus transmission. JE virus reactive antibody present in chickens was probably the result of infections with Tembusu virus. In suburban Bangkok, chick sero-conversions possibly occurred as a result of large vector populations being forced to seek alternative hosts (73).

Studies in Chiang Mai valley showed that cattle and buffalo were not important amplifying hosts for JE virus and appear to be fairly resistant to JE virus infection, as shown by the relatively high percentage of animals that already had antibody. This fact, combined with the slow rate of turnover of these species, provides evidence that bovines are not important amplifying hosts for JE virus (70), even though identifications of mosquito blood meal sources strongly suggest that *C. Tritaeniorhynchus*, *C. Gelidus* and *C. Fuscocephala* feed mostly on buffalo and cattle rather than pigs or humans (74). Even when calves were inoculated with high titers of JE virus over several days they did not develop viremia. Moreover, *C. Tritaeniorhynchus* mosquitoes which had fed on calves during the first 10 days after a large inoculation of JE virus did not carry viral antigen as determined by an indirect fluorescent antibody test, and did not transmit the virus to susceptible baby chickens by biting. Therefore, cattle do not play a role in the maintenance of JE virus in nature (75). In southern Thailand there is intense transmission of JE virus to pig despite the rare occurrence of human encephalitis (76).

Chikungunya: There is a forest cycle involving monkeys (vervets and baboons) in which the virus is transmitted by A. *africanus* and other mosquitoes. Infected monkeys have a high viremia. Rodents may also be hosts since they show a transient viremia after

being inoculated with the virus (77).

2.6 Method of Transmission

The mosquito-borne arboviruses are maintained in nature by two cycles (1).

2.6.1 Mosquitoes may serve directly as vectors between infected human patients circulating virus in the blood at the time of illness and other susceptible humans (Figure 4). This cycle applies in the case of epidemics of Dengue; the vector is usually *Aedes aegypti*. This mosquito breeds in domestic artifacts such as water pots and water storage tanks, or tins and old motor tires which become filled with rainwater. After an extrinsic incubation period of 10 to 14 days the virus has multiplied within the mosquito and attained a high concentration in the salivary glands. Thus, the mosquito becomes infectious for another susceptible human during a subsequent blood meal.

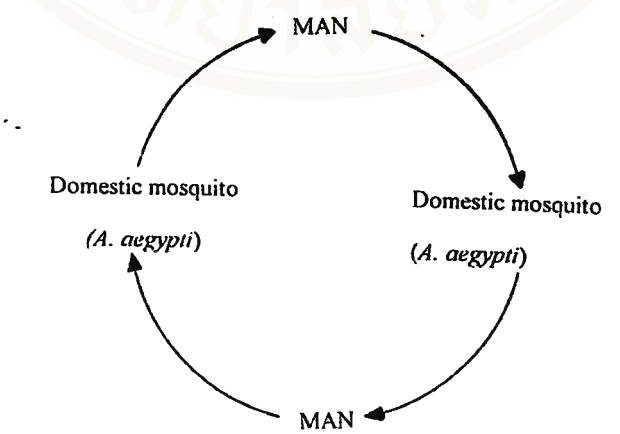


Figure 4 Cycle of infection in Dengue virus

2.6.2 This may be a cycle of infection between wild birds and bush mosquitoes. Some of these bush mosquitoes may invade domestic locations thereby conveying infection to domestic fowls or animals.

A subsidiary cycle of infection may be set up between viremic domestic fowls and domestic mosquitoes. Man may become infected either by the bite of a bush mosquito in areas remote from human habitation, for example, during fishing or hunting, or occasionally by the bite of a domestic mosquito in grounds around the house (Figure 5). This cycle appears in the case of mosquito-borne encephalitis virus.

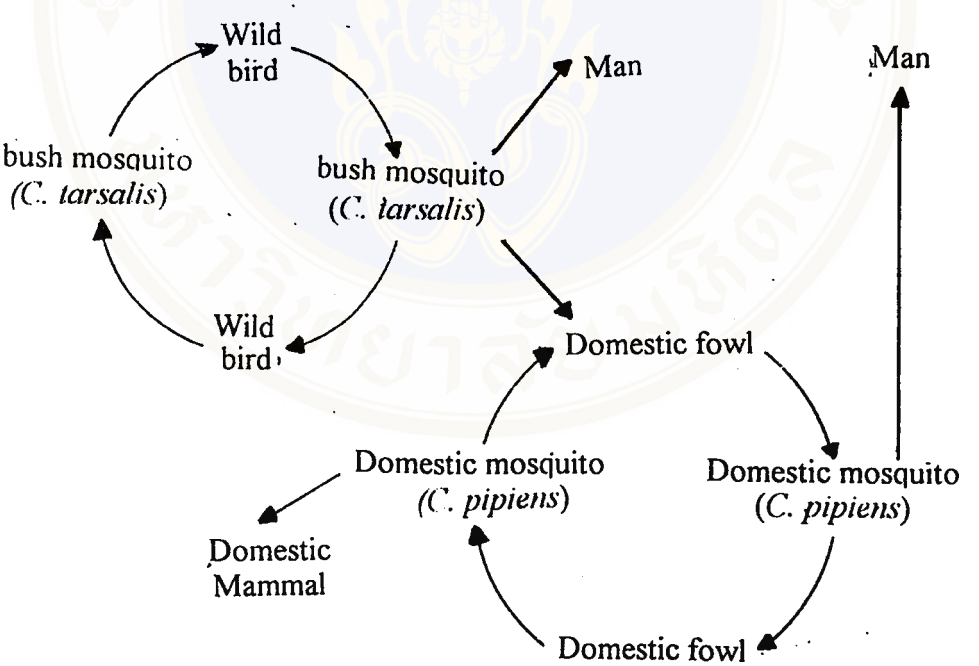


Figure 5 Inter-relationship between rural and domestic cycles of infection with some mosquito-borne encephalitis viruses.

3. Pathogenesis and pathology

The greatest body of information about Flavivirus pathogenesis is derived from experiments on mice and other laboratory rodents.

Dengue

There are 2 main theories proposed for the pathogenesis of DHF and DSS which are the following:

3.1.1 The virulence theory, Rosen (78): It has been suggested that there are two different clinical entities, "normal dengue" on one hand, and "altered dengue" on the other. The life threatening forms in altered dengue are usually associated with severe hemorrage, ypovolemic shock and death. The concept of differences in the virulence of he virus can be explained by the apparent dissemination of dengue hemorrhagic fever from one geographic area to another and its apparent decrease in incidence despite the continued occurrence of dengue infection.

3.1.2 The secondary infection theory; this theory can be divided into two hypotheses.

a) **The hypersensitivity theory, Russel** (79). When a mononuclear phagocyte containing the virus-antibody complexes is attacked by an effector lymphocyte, vascular permeability leading to the leakage of intravascular fluid into tissue and subsequent hemoconcentration can be produced by the dengue virus-antibody complexes which can activate complement by the classical pathway. There is a marked reduction of serum complement C3 during shock phase of the disease (80)

and the degree of reduction correlates well with severity of the disease. When all constituents of complement were measured, it was found that complement depression in DHF involved primary C3, proactivator (C3PA), C4 and C5 with marked depressions in shock cases. These findings indicated that there was complement activation in both the classical and alternative pathways.

b) **The sequential theory, Halstead** (81): The immunological response to dengue infection depends on the individuals past exposure. A person who has had no previous exposure and is infected with dengue will develop a primary serological response to the infecting serotype such that antibody is produced only against that serotype or in greater amounts against the infecting serotype than heterologous types. Dengue viruses replicate in cells of mononuclear phagocyte lineage, and sub-neutralizing concentrations of dengue antibody enhance dengue virus infection in these cells. This antibody dependent enhancement of infection regulates dengue disease in human beings, although disease severity may also be controlled genetically, possibly by permitting and restricting the growth of virus in monocytes. Monoclonal antibodies show heterogeneous distribution of antigenic epitopes on Dengue viruses. These epitopes serve to regulate disease; when antibodies to shared antigens partially neutralize heterotypic virus, infection and disease are dampened enhancing antibodies alone resulting in heightened disease response.

Clinical manifestations

Dengue infection: Dengue viruses cause two forms of clinical syndromes; mild form of DF and severe form of DHF and DSS. Dengue fever is a self limiting disease and represents most cases of dengue infection. In some situations patients infected with Dengue virus develop life threatening complications such as hemorrhagic manifestations and shock (DHF/DSS).

(a) Classical dengue fever (DF)

After an incubation period of 1 to 7 days there is sudden onset with high fever (40°C) lasting 3-5 days accompanied by severe headache, pain behind the eyes, prostration, anorexia and cutaneous hyperesthesia or hyperalgesia (breakbone fever). Rashes may appear in classical dengue as a flush on the face and neck early in the course of illness, or as a fine maculopapular rash as the fever subsides. The latter rash begins on the extremities; the palms and soles. A second phase of the fever, lasting 2-3 days, may occur as the rash desquamates (saddle-back). A positive tourniquet test as well as petechiae and more severe skin hemorrhages may also occur in classical dengue. Thrombocytopenia (platelet count of less than 100,000/mm3) may be observed. Hemorrhagic phenomena such as epitaxis, petechiae, intestinal bleeding, menorrhagia, purpuric lesions are uncommon but may occur at any stage. Although the disease is often incapacitating, death is extremely rare in classical dengue.

(b) Dengue hemorrhagic fever (DHF)

Typical cases of DHF, as seen as in Asian countries, are characterized by four major clinical manifestations: high fever, hemorrhagic phenomena, hepatomegaly and often circulatory failure. Moderate to marked thrombocytopenia with concurrent hemoconcentration is a distinctive clinical laboratory finding. The major pathophysiological change that determines the severity of disease in DHF, and differentiates it from DF, is the leakage of plasma as manifested by a rising hematocrit value and hemoconcentration. The illness commonly begins with sudden rise in temperature which is accompanied by facial flush and other non-specific constitutional symptoms resembling dengue fever, such as anorexia, vomiting, headache and muscle or joint pains. Some patients complain of sore throat and an infected pharynx may be found on examination. The temperature is typically high (40-41°C) and continues for 2-7 days before falling to a normal or subnormal level. Febrile convulsions may occur.

The most common hemorrhagic phenomenon is a positive tourniquet test. Fine petechiae scattered on the extremities, axils, face and soft palate may be been during the early febrile phase. The liver is usually palpable and liver size is not correlated with disease severity. However, hepatomegaly is more frequent in shock cases. Thrombocytopenia and hemoconcentration are a constant finding in DHF. A platelet count of below 100,000/mm3 is usually found between the 3rd and 8th day after onset.

(c) Dengue shock syndrome (DSS)

In severe cases, after fever of a few days duration, the patient's condition suddenly deteriorates. At the time of, or shortly after, the fall in temperature, between the 3rd and the 7th day of the disease, there are signs of circulatory failure. The skin becomes cool, blotchy, and congested. Circumoral cyanosis is frequently observed and the pulse becomes rapid. Shock is characterized by a rapid, weak pulse with narrowing of the pulse pressure (20 mm Hg or less, regardless of the pressure levels) or hypotension, with cold, clammy skin and restlessness. Patients in shock are in danger of dying if appropriate treatment is not promptly given. Patients may pass into a stage of profound shock, both blood pressure and pulse becoming imperceptible. The duration of shock is short; the patient may be die within 12-24 hours or recover rapidly following appropriate antishock therapy.

Alternatively, uncorrected shock may give rise to a more complicated course with metabolic acidosis, severe bleeding from the gastrointestinal tract and various other origins; all associated with a poor prognosis. Patients with or without shock is short and uneventful. Once the shock is overcome, even in cases with profound shock, the surviving patients recover within 2-3 days.

The WHO expert committee on dengue has developed guidelines for the diagnosis of DHF/DSS and the severity of DHF is classified into four grades (82).

Grade I Fever accompanied by non-specific constitutional symptoms; the only hemorrhagic manifestation is a positive tourniquet test.

Grade II Spontaneous bleeding in addition to the manifestations of Grade I patients, usually in the form of skin and/or other hemorrhage.

Grade III Circulatory failure manifested by rapid and weak pulse, narrowing of the pulse pressure (20 mm Hg or less) or hypotension, with the presence of cold clammy skin and restlessness.

Grade IV Profound shock with undetectable blood pressure and pulse.

DHF is all four grades (I to IV), and DSS Grades III and IV. The presence of thrombocytopenia with concurrent hemoconcentration differentiates Grade I and II DHF from dengue fever.

Chikungunya viral infection is an acute, self-limiting febrile disease with a forest and human life-cycle. It has no hemorrhagic or central nervous system complications, although in Thailand it has been associated with haemorrhagic dengue (13).

The incubation period is 2-4 days. The disease is biphasic. Onset is abrupt with severe pain in the joints leaving the patient prostate. After 1-4 days the fever subsides

and there is an afebrile period of 3 days after which the fever returns with an itchy, maculopapular rash on the trunk and extensor surfaces of the limbs. After another 3-6 days the fever subsides and there is complete recovery. In Asia it is associated with mild hemorrhagic features but no shock. There are no chronic sequelae but a crippling arthralgia may occur intermittently for up to 4 months.

In JE, sites of replication and dissemination of virus in the mouse have been described by Huang and Wong (31, 83). During the acute stage, congestion, edema and small hemorrhage are found in the brain. Microscopic lesions include neuronal degeneration and necrosis, neuronophagia, microglial proliferation forming glial nodules and perivascular inflammation. These changes occur in the gray matter and predominantly affect diencephalic, mesencephalic and brain stem structures. Destruction of cerebella Purkinje cells may be prominent. A variety of pathological changes in extraneural tissues have also been noted, including hyperplagia of germinal centers of lymph nodes, enlargement of malpighian bodies in the spleen, interstitial myocarditis, swelling and hyaline changes in hepatic kupffer cells, pulmonary interalveolitis and focal hemorrhages in the kidneys.

In one study of fetal human cases, JE viral antigen was localized to neurons, with no evidence for glial cell infection (84). The highest concentration of infected neurons was in thalamus and brain stem. Among inflammatory cells recruited into perivascular infiltrates. T-cells cells predominated but a minority were T-suppresser/cytotoxic lymphocytes. Macrophages predominated among cells recruited into the brain

parenchyma.

Transplacental infection in swine results in fetal encephalitis, abortion and stillbirth. The virus also produces hypospermia and aspermia in boars (85). Histopathological changes include epididymitis, spermatogenic arrest and inflammation of the tunica testis. Pregnant mice inoculated intraperitoneally also transmit JE virus to the fetus with subsequent abortion (86). A curious feature of this model is that infected mothers, when mated again after 6 months, transmitted virus to the second litter. Latent infections of pregnant mice could be reactivated by cyclophosphamide or subsequent pregnancy. In other studies, latent infections were detected by co-cultivation of mouse spleen cells and virus located principally to lymphocytic cells.

There is also evidence of congenital and persistent infections in humans. In a series of nine pregnant woman infected during an epidemic, four women infected during the first and second trimesters aborted and virus was isolated from fetuses, whereas none of the five women infected in the third trimesters miscarried or had abnormal babies. No other reports of human congenital infection are extant and the frequency of this complication is unknown. Most women of child bearing age in endemic areas of Asia are naturally or artificially immunized during childhood and are therefore not at risk. Evidence for latent infections of humans has been reported. By use of co-cultivation techniques, JE virus was recovered from peripheral blood mononuclear cells of several children who developed recurrent disease as well as in asymptomatic children 9 months after an acute JE infection. Virus has been recovered from cerebrospinal fluid samples as

late as 117 days after onset of clinical symptoms. The frequency with which latent or persistent extraneural or neurological infection occurs, whether such infections are associated with cytopathology or clinically relevant syndromes, and how the immune response modulates such infections remains to be elucidated (86).

After the virus enters the body it reaches the lymphatic system where it multiplies before being released into the blood and thence to the organs affected. Most arbovirus infections are inapparent or mild, diagnosable retrospectively by serological methods. If clinical manifestations arise after infection, they do so after an intrinsic incubation period lasting from a few days to a week or more.

In JE infections, onset is rapid, beginning with a 2-4 days prodromal phase of headache, fever, chills, anorexia, nausea and vomiting, dizziness and drowsiness. In children, abdominal pain and diarrhea may be prominent. These symptoms are followed by the appearance of nuchal rigidity, photophobia, alterated states of consciousness, hyperexcitability, and varying objective neurological signs, including dull, mask-like faces, muscular rigidity, cranial nerve palsies, tremulous eye movements, coarse tremors of the extremities, involuntary movements, generalized and localized paresis, incoordination and pathologic reflexes. Sensory deficits are rare. Paralysis of the upper extremities is more common than paralysis of the legs. Spinal cord involvement may occur and a bulboparetic syndrome has been described. Convulsions are frequent in children but occur in less than 10 % of adult patients. Severe hyperthermia may require specific countermeasures. Death occurs on the fifth to ninth day or during a more

protracted course with cardiopulmonary complications. A poor prognosis is associated with respiratory dysfunction, positive Babinski' sign, frequent or prolong seizures, prolong fever, albuminuria, virus infection of cerebro-spinal fluid (CSF) and low levels of IgM and IgG antibodies in serum and CSF (87).

4. Treatment

There are no specific treatments for Dengue, Chikungunya and JE viral infections. Only supportive treatment such as bed rest, antipyretic and analgesic drugs, fluid and electrolyte replacement. Specific countermeasures should be applied for convulsions and cerebral edema in JE cases. Specific antiviral therapy is clearly needed for JE. One example is interferon-alpha (INF-∝). Studies have shown recombinant INF-∝ (A) at high concentrations is more efficacious in combating the replication of the JE virus in vitro (88). In acute JE patients, high ratio titers of IFN-∝ found in the CSF to those in plasma suggests that INF-∝ is locally synthesized within the CNS (89).

5. Prevention and control

Prevention and control of epidemics of mosquitoes-borne diseases are generally accomplished by reducing vector populations of mosquitoes. Preventative strategies involve long term programs aimed at limiting breeding of mosquitoes through source reduction. Public education programs aimed at protecting against mosquito bites

emphasize use of protective clothing, repellents, window screens and bed nets in houses. If water storage is mandatory, a tight fitting lid or a thin layer of oil in all containers may prevent egg laying or hatching. A larvicide, such as abate, available as 1% sand granule formulation and effective at a concentration of one part per million may be added safely to drinking water. Field trials of organophosphate lavicides and adultcides have proved effective against vectors of dengue and JE. Use of agricultural pesticides in rice growing areas have also reduced populations of *C. tritaeniorhynchus*. Integrated programs have included use of chemical larvicides, larvicidal fish and biological larvicides (*Bacillus thuringiensis, Toxorhynchites splendens*).

6. Vaccine

Dengue vaccine

During 1944 - 1945, Sabin AB and Schlesinger RW (90) prepared and tested the first live, attenuated, Dengue 1 virus vaccine. Vaccinated individuals experienced either no symptoms or a low grade fever, with or without headache and malaise, lasting 24 hours or less. All individuals had maculopapular rashes and petechiae. They fully developed immunity to challenges with unmodified, homolytic Dengue virus.

Later, Dengue viruses attenuated by serial passage through primary dog kidney cells have been demonstrated to be promising vaccine candidates. Vaccine strains produced in this manner are now available for the Dengue 1, Dengue 2 and Dengue 4

viruses. These vaccines are being extensively tested for safety and efficacy (91-92).

In 1985, there was considerable interest in the development of subunit vaccine preparations. Virus nonstructural (NS) protein, NS1, monoclonal antibodies were found to have afforded protection against virus challenge by complement mediated cytolysis and could eliminate infected cells. In addition, antibodies to NS did not bind to virus particles and thus would not be involve in antibody mediated enhancement of virus replication in mononuclear cells which was proposed as pathogenic mechanism in dengue hemorrhagic fever. For this reason, NS protein is an attractive candidate for subunit vaccine development. It was found that NS1 contained both serotype specific and cross reactive antigenic determinants. In 1987, Schlesinge JT et al. (93), showed that vaccinated mice were protected from lethal challenge by immunization with purified Dengue 2 NS1. Extensive work was completed and could demonstrate that both Dengue 4 structural and non-structural protein antigens expressed with the vaccine virus or baculovirus virus vectors were able to protect mice against lethal challenge with Dengue virus (94).

The recombinant approach to production of Flavivirus vaccines has yielded a number of potential candidates. Relevant to the development of efficacious Flavivirus vaccines, studies aimed at defining the antigenic determinants necessary for eliciting protective immunity focused primarily on the structural proteins, in particular the E protein as well as the nonstructural secreted glycoprotein, NS1 (95).

The successful recovery of infectious Flavivirus from cloned cDNA raises the possibility of manipulating these viral genomes as cDNA to construct or propagate candidate live attenuated virus. However, early human administration is required to establish a database of information on human response to the novel antigen preparations (82).

Within Flavivirus endemic areas in Thailand, an effective vaccine is needed for children aged under 15 years to reduce mortality and morbidity rates. Of the WHO programs, the monovalent and bivalent vaccine trials currently underway in Thailand were reported by Bhamarapravati N et al. (96). Of particular interest was the finding that a bivalent vaccine consisting of attenuated Dengue 1 and Dengue 4 virus produced antibodies that cross-reacted with Dengue 2 and Dengue 3 virus, producing levels of circulating antibodies somewhat higher than had been obtained with Dengue 1 alone. Later, clinical trials of a live attenuated, trivalent dengue viral vaccine; Dengue 1 by PDK 13, Dengue 2 by PDK 53 and Dengue 4 by PDK 48, showed neutralizing antibodies to Dengue 1, Dengue 2 and Dengue 4 at days 30 and at day 60. Two cases showed antibody against Dengue 3 on day 30 (97).

Chikungunya vaccine

Levitt NH et al. (1986) (98) developed an attenuated Chikungunya virus (CHIK) clone for production of a live vaccine for human use. CHIK strain 15561 was subjected to 18 plaque passages in MRC-5 cultures before CHIK 181/clone 25 was selected as a

vaccine seed based on homogeneous small plaque size, suckling mouse avirulence, reduced monkey viremia and genetic stability. Oligonucleotide mapping demonstrated differences between parent and clone. Vaccine (pilot-lot production) elicited neutralizing antibody and protected mice and rhesus monkeys against challenge. After challenge, viremias were absent in vaccinated monkeys. Vaccine was then produced and tested in accordance with government regulatory requirements of human use.

Turell MJ and Malinoski FJ (1992) (99) conducted a study to determine the potential for transmission of a live attenuated CHIK vaccine by orally exposed or virus-inoculated mosquitoes. The vaccine (CHIK 181/clone 25) replicated in, and was transmitted by, female *Aedes albopictus* and *Aedes aegypti* after intrathoracic inoculation. Mosquitoes also became infected with the vaccine after ingesting virus from either a blood-soaked cotton pledget or a viremic monkey. However, because of the low viremias produced in inoculated humans, it was unlikely that mosquitoes would become infected by feeding on person inoculated with the live, attenuated CHIK vaccine. Although the vaccine was transmitted by mosquitoes after intrathoracic inoculation, there was no evidence of reversion to a virulent phenotype.

JE vaccine

Vaccines have been used to prevent encephalitis in horses and humans, and for abortion and stillbirth in swine. Vaccination of horses with formalin inactivated vaccines was the first successful application and afforded significant protection during an

epizoonosis in Japan during 1948 and 1949. Since 1972, live attenuated vaccines have been licensed in Japan for use in pigs (100). Although immunization of pigs is a theoretical means of interrupting transmission, and amplification, of JE virus and thereby for preventing human infections, difficulties arise in practice. In many parts of Asia, pigs are only semi-domesticated and wide scale immunization would be difficult. In areas such as Japan, where swine husbandry is highly developed, pigs are born after the summer epidemic period, have maternal antibody for 4 months and are killed at 6 - 8 months of age thereby leaving a very narrow interval for vaccination.

Formalin inactivated vaccines, for use in humans, are prepared from infected adult mouse brains or infected primary hamster kidney cell cultures in Japan and China, respectively. The mouse brain vaccine produced by the Research Foundation for Microbial Diseases (Biken), Osaka, Japan, is purified by protamine sulfate precipitation and ultracentrifugation and has been in wide use since the 1960's. A controlled trial of Biken vaccine in Thailand showed an efficacy of 91% (101). Mass vaccination campaigns have been carried out in Japan, Taiwan and China with children as the target population.

7. Laboratory diagnosis

It is important to determine the specific etiologic agent quickly and accurately in arbovirus infections. Not only will the patient be treated appropriately but public health officials can institute vector control operations to limit further spread of the virus.

Laboratory confirmation of clinical diagnosis for arbovirus infections depends upon virus isolation and serological diagnosis.

7.1 Virus isolation

Traditional methods of virus isolation are still firmly entrenched in many laboratories. Specimens of blood and CSF should be taken within the period of viremia. Suckling mice have been used as laboratory hosts for amplifying virus in diagnostic specimens and from field collected mosquitoes. Mice are inoculated intracranially with clarified suspensions of clinical specimens or with macerated and clarified arthropod pools or animal tissues. Following an incubation period of 5-7 days viral antigen is detected by immunofluorescence and the virus type also identified by immunofluorescence using type-specific monoclonal antibodies.

The development of continuous cell lines of mosquito origin has simplified Dengue virus isolations. The cloned line of Sigh's *A. albopictus*, C6/36, was developed especially for its ability to grow Dengue virus and other arboviruses to high titer (102). In addition, mosquito cell cultures, particularly AP-61 (*Aedes pseudoscutellaris*) and TR-248 (*Toxorhychites amboninensis*), are being increasingly used for virus isolation.

It was found that by using antibody mediated infection enhancement of Dengue viruses in a mouse macrophage cell lines, virus isolations were obtained from more than 80% of clinically and serologically confirmed Dengue patients.

In JE, virus can rarely, if ever, be isolated from the peripheral blood during the acute illness in humans. Virus can be isolated from the CSF early in the course of acute encephalitis; successful isolation is an ominous prognostic sign. If an adequate sample of fresh brain tissue is obtained at autopsy the virus can be isolated from virtually every case and intraneuronal viral antigens are readily detected (103).

Identification of virus

Serotype identification techniques have been the plaque reduction neutralization test (PRNT) using LLC-MK2 cells and the complement fixation (CF) test using antigen prepared in mosquitoes. Both tests use polyclonal antibodies, usually produced as ascitic fluids in mice. In spite of being laboratory and time consuming the PRNT assay has long been considered the standard for virus typing.

Henchal EA et al. (104) developed type-specific monoclonal antibodies prepared against the four dengue virus serotypes to evaluate their ability to identify low passage human and mosquito isolates. The results obtained using the immunofluorescence assay were consistent with virus identifications obtained using the more classical, but costly and time consuming, plaque reduction neutralization test. More viral isolates and higher virus yields were obtained using the C6/36 clone of *Aedes albopictus* cells rather than LLC-MK2 (monkey kidney) cells. Dengue type-specific monoclonal antibodies detected prototype viral antigens 24-48 hours post-infection in C6/36 cells. This is the first time that monoclonal antibodies have been used to serotype low passage Flavivirus isolates.

7.2 Antibody detection

The classical techniques for serological diagnosis include the hemagglutination inhibition test (HI), complement fixation test (CF), neutralization test (NT), an adaptation of the enzyme-linked immunosorbent assay (ELISA) and an indirect fluorescent antibody test (IFA). The acceptable standard has been a 4-fold or greater increase in antibody titers determined by these methods.

For JE virus diagnosis the HI test has been widely used since 1953 because it is relatively sensitive, simple and rapid. JE patients seem to develop higher HI antibody titers than persons with inapparent infection. In primary cases the HI test can distinguish JE virus from Dengue virus infection but it has limitations in secondary cases.

HI testing is the most sensitive and accurate indicator of inapparent infection with JE virus in the early post-infection phase (105), but the test cannot be used with as great an accuracy as the NT test to discriminate between the presence or absence of past JE virus infections in individual humans. Wide cross-reactivity at high titer levels between JE virus and Dengue virus in the HI test has been noticed in acute and convalescent specimens of almost all of the hemorrhagic fever cases and in some of the encephalitis cases (106). The IgG antibodies have greater cross-reactivity than do IgM antibodies in reactions with Flaviviruses (107). The HI titer correlated much better with the IgG than the IgM and rises in the HI titer correlates better with rises in IgG anti-JE activity than with rises in IgM anti-JE activity (108). However, HI testing is most widely used because

it is relatively sensitive, simple and rapid.

Bundo K et al. (109) showed that IgG-ELISA titers in endemic areas were distributed over a broad range more than non-epidemic areas. IgM-ELISA titers in healthy inhabitants were rather low even in endemic areas.

The adaptation of the ELISA has attracted attention for use as diagnostic test since Westaway et al. (110), in 1974, reported that the IgM antibodies were more specific than total immunoglobin in Flavivirus infections. In 1979, Edelman R and Pariyanonda A (111) reported the measuring of IgM class of HI antibodies after separation by sucrose gradient sedimentation could be used to differentiate JE from other Flavivirus infections. In the same year, Dittmar D et al. (112) studied ELISA by the use of specific antiglobulin enzyme for conjugate and found that the ELISA was specific to human IgM antibody. Subsequently, there were the various reports of the adaptation of the ELISA for rapid diagnosis of dengue hemorrhagic fever.

A number of neutralization tests have been described for Dengue viruses. In primary infections, relatively monotypic neutralizing antibodies are detected during early convalescence. In secondary infections, high titer neutralizing antibody is produced against two to four Dengue types. The highest titer in convalescent serum of previously infected patients is called "original antigenic sin".

In 1987, Lam SK et al. (113) reported the development of a modification of the

IgM capture ELISA for dengue infection using a dengue monoclonal antibody for enzyme conjugated. This eliminated the use of normal human serum in the diluent and reduces the background reading when compared the HI test. A positive to negative (P:N) ratio of greater than or equal to 2.0 was considered positive. As a result, all 36 convalescent samples tested were positive, giving 100% correlation with HI results. In addition, 11 of 23 (47.82%) acute sera from secondary infections were also positive for dengue IgM. The specificity was 100%.

Bundo K et al. (114), in 1988, reported on the IgM class of antibody titers in paired sera from 19 JE and DHF patients in Thailand and 42 JE patients in Japan as measured by the antibody capture ELISA. They found that 41 of 42 DHF patients in Japan and 11 of 19 encephalitis patient in Thailand could be diagnosed as having JE, while 2 of 19 encephalitis and 26 of 44 DHF patients in Thailand could be diagnosed as having Dengue virus infections.

In 1989, Chan YC et al. (115) performed DENGUE BLOT for the diagnosis of recent dengue infections using the HI test as the gold standard. The results showed that DENGUE BLOT had a 25.9% sensitivity rate in detecting recent primary dengue infections and 100% in detecting secondary (including presumptive) infection when paired sera were tested. The specificity of DENGUE BLOT determined in patients with non-dengue illness and in healthy adults were 100% and 95.6%, respectively. It was concluded that DENGUE BLOT was as sensitive as the HI test in diagnosing a recent secondary dengue virus infection, using either single or paired sera, but not as sensitive

for primary dengue infection.

In the same year, Chunge E et al. (116) compared an immunoglobulin G enzyme-linked immunosorbent assay (IgG-ELISA) with the HI test for the detection of antibodies against 4 types of Dengue antigen. They found a sensitivity of 83.9 to 96.7%, specificity of 95.8 to 100% and an agreement rate of 89.2 to 96.4%. The test uses a small volume of serum (10 μl), and crude antigens were advantageous for the IgG-ELISA. In addition, IgG-ELISA was rapid, easy to perform and suitable for large scale studies.

Innis BL et al. (117), in 1989, studied the diagnostic sensitivity and specificity of detection of anti-dengue IgM by antibody capture ELISA in a variety of clinical settings. Sera from uninfected controls were uniformly negative. Serial specimens from experimental and natural infections showed that viremia and fever terminated as anti-dengue IgM became detectable. Anti-dengue IgM appeared in most cases by the third febrile day of illness and declined to undetectable levels after 30-60 days. Assay sensitivity was 78% in admission sera and 97% in paired sera thus exceeding or matching the performance of the HI test. Measurement of anti-dengue IgM to anti-JE IgM ratio correctly identified all sera from 112 patients with strictly defined JE and 98% of sera from patients whose dengue infections were confirmed by virus isolation. Dengue infections could be classified as primary or secondary by determining the ratio of units of dengue IgM to IgG antibody (> 0.78) in acute serum.

Petchclai B et al. (118), in 1990, developed a gold immunoblot technique

employing colloidal gold conjugate for detection of dengue antibody. Dengue HA antigen was dotted onto nitrocellulose paper, blocked with protein, and then reacted with test sera and protein A-gold conjugate in rapid succession. Positive results were displayed as a pink dot against a white background. The test was compared with HI using 126 sera from suspected cases of dengue fever and 110 sera from blood donors. Comparison of titers between the two tests generally showed good correlation. This method inherits the limitation in interpretation present in HI, but the simplicity and rapidity will make this HI based test more useful for the diagnosis of Dengue.

Kuno G et al. (119), in 1991, developed a rapid test which employed a single specimen for classification of serological response in dengue infections based on the ratio of IgM and IgG-ELISA. When serum specimens were tested as pairs, using HI as the standard test, concordant results with ELISA were obtained in 81% and 95% of primary and secondary infections, respectively. Tested as single specimens the diagnoses by ELISA and HI agreed in 41% and 52% of acute specimens, of primary and secondary infection, respectively. On the other hand, diagnoses by ELISA and HI agreed in 79% and 95% of primary and secondary infections when single convalescent specimens were used. Analysis of the discordant results between the two tests revealed that the interpretation by IgG and IgM ratio agreed better in HI classifications practiced by some investigators than it did with the WHO.

IgM-ELISA indicated that antibody responses after primary vaccination were relatively low as did the IgG-ELISA. However, IgG-ELISA detected a marked response

in booster immunizations and other results seemed to indicate that the NT test was most sensitive to detect sero-conversion by the vaccination (120). Studies in 85 medical students aged 18 to 25, who received two doses of JE vaccination by three different schedules, showed that the ELISA results could not demonstrate differences between antibody titers before and after vaccination and that an ELISA system could not be used for demonstration of the immune status (121).

The IgM capture ELISA was compared with the HI test for establishing a laboratory diagnosis of acute JE virus infection using specimens of dried blood eluted from filter paper strips. During non-epidemic periods the proportions diagnosed as JE by MAC ELISA and HI were 26% and 33%, respectively. Detection by MAC ELISA is superior to the HI method for establishing a diagnosis of acute JE using dried blood specimens. There were no significant difference in test specimens collected as serum or eluted from dried blood on filter paper strips to determine the anti-dengue antibodies by micro-neutralization test (122).

Thein S et al. (123), in 1992, studied 1,548 children who were admitted to the Rangoon Children's Hospital in Myanmar with hemorrhagic fever in 1984. No evidence of recent dengue infection was found in 577 of the 803 children from whom paired sera were obtained, raising the possibility of reappearance of Chikungunya virus infection in Myanmar. An ELISA for the detection of anti-Chikungunya virus IgM antibody was prepared and standardized using only reagents which were commercially available or which could be prepared without the use of sophisticated equipment. While there was

90% agreement between HI and the IgM-ELISA in the diagnosis of acute Chikungunya virus infections, 12 additional patients with stationary anti-Chikungunya virus HI antibody titers could be identified as having acute Chikungunya infection using ELISA. Furthermore, the ELISA could identify twice as many patients (31/103), at the time of admission to hospital, as HI (15/103). There were no false positive IgM reactions with the ELISA which could be attributed to the presence of rheumatoid factor. Using the test, samples from 163 children who presented to the Rangoon Children's Hospital with fever/hemorrhagic fever were assayed. 103 were diagnosed as having Chikungunya virus infection, 4 had possible dual Chikungunya and Dengue infections, 16 had Dengue, 30 had neither Chikungunya nor Dengue infections, and a definitive diagnosis could not be made for 10 patients. Routine use of the ELISA would alert to future outbreaks of Chikungunya virus infection and avoid admission to hospital of patients with a non life-threatening viral disease.

7.3 Genome detection

Serological methods are not able to identify the infecting serotype of the virus. This can only be achieved virus isolation or viral genome detection. A protocol was developed for highly sensitive detection of viral RNA in blood specimens by reverse transcription coupled with a nested polymerase chain reaction (PCR). Using JE virus as a model, the optimized reverse transcription (RT-PCR) detects as few as 3-5 virions in 0.1 ml of whole blood specimens.

Morita K et al. (124), using PCR, identified and typed Dengue virus isolates. Four primer pairs were selected on the basis of published sequence data of 4 Dengue serotypes so that each unique target sequence could be amplified for each serotype by PCR. The procedure consisted of (1) RNA preparation (2) reverse transcription and (3) PCR. All steps could be completed within 2 hours in a single tube per specimen. The amplified sequence size revealed by ethidium bromide stained gel electrophoresis was unique for each serotype using infected culture fluid of prototype viruses, thus enabling both identification and typing of field isolates.

In 1992, Lanciotti RS et al. (125) developed and applied RT-PCR for detecting and typing Dengue viruses from viremic human serum specimens. Oligonucleotide consensus primers were designed to anneal to any of the four Dengue virus types and amplify a 511 base pairs (bp) product in a RT-PCR. No cross-reactivity was detected between the type specific primers and heterologous Dengue virus types. The assay demonstrates sensitivities of 94 % with Dengue type 1 virus, 93 % with Dengue type 2 virus and 100 % with Dengue type 3 and 4 viruses, compared with virus isolation.

Subsequently, Chen HS et al. (126) designed and synthesized a pair of 20 mer oligonucleotides based on a conserved sequence block of Dengue 2 virus (DEN-2) strains isolated from different geographical areas. RNA samples were prepared from two DEN-2 strains, prototype New Guinea (NGC) and a local isolate Hainan 98 (HN98). The reverse trancription step was performed for cDNA synthesis before the standard PCR procedures. The amplified products were fragments about 476 bp in length, corresponing

to the upper one third of DEN-2 envelope gene (E1 to E476nt). Specificity of the amplification products was confirmed by "nested" PCR using the internal primers and by Southern and dot-blot hybridization to cloned DEN-2 cDNA probes following agarose gel electrophoresis. Further improvement and the potential application of the methods in study of Dengue virus RNA and discussed.

Chanyasanha C et al. (127), in 1995, reported viral isolation and viral genome detection by RT-PCR. The technique was used on serum specimens collected from a total 162 patients, consisting mostly of Dengue, admitted to a Childrens Hospital from May to September 1993. In total, 13 Dengue virus strains were isolated (isolation rate: 8.02%), consisting of two Dengue type 1, two Dengue type type 3, and nine Dengue type 4 viruses. Dengue viral genome was detected by RT-PCR in 7.4% of the serum specimens after RNA extraction, in contrast to 6.17% when direct rapid RT-PCR was applied without RNA extraction. The virus isolation and genome detection rate decreased according to the day after onset of the diseases from 50% on day 3 to 2.5% on day 6.

Meiyu F et al. (128), in 1997, used a universal primer set designed to match the sequence of the NS1 gene of Flaviviruses. The virus RNA of Dengue virus, Japanese encephalitis virus, powassan and langat Flavivirus were successfully amplified by PCR via cDNA and with different internal primers. The serotypes of Dengue viruses were identified in 78 clinically diagnosed dengue fever patients; 18 patients were positive for DEN-1, 48 patients for DEN-2 and 8 patients concurrently infected with DEN-4. Of the 48 patients admitted with Japanese encephalitis, 45 patients were determined to be JEV

infections. By nested PCR they completed the identification within 2 days. The results show that seven primers have a potential value for rapid clinical diagnosis of Flavivirus infections.

Pfeffer M et al. (129), in 1997, developed a RT-PCR for genus-specific detection of Alphaviruses. Based on the available published sequences, degenerate primers were designed to ensure hybridization to a conserved region within the non-structural protein 1 gene of all Alphavirus species. The expected 434 bp cDNA fragment was amplified from 27 Alphavirus species by using RNA extracted from 200 ml of infected cell culture supernatant. In addition, eight strains of Venezuelan equine encephalitis(VEE) virus and 10 strains of Sinbis virus were amplified. The viral origin of the amplicons was confirmed by restriction enzyme analysis and comparison with the expected cleavage pattern based on published sequence data. The PCR products of Alphaviruses, with so far unknown nucleotide compositions, were sequenced. About 120 nucleotides downstream of the forward primer a region shown sufficiently hemologous for the design of another forward primer was found and used in a semi-nested PCR. The expected 310 bp semi-nested fragment was demonstrated for all viruses investigated. The sensitivity of the RT-PCR was about 1,200 plaque-forming units(PFU) for VEE virus reference strain from a Trinidad monkey. The detection limit after the semi-nested PCR was 1.2 PFU. The sensitivity was not hampered by the presence of human serum thus making this test suitable for an application in viremic individuals. Chikungunya virus RNA was amplified from infected mouse brain tissue by the described RT-PCR assay. The data suggest that the semi-nested RT-PCR may be applied as a highly sensitive alternative to virus

isolation in the rapid screening and diagnosis of Alphavirus infections, including post-mortem diagnosis. Phylogenetic analysis of the amplicon sequence data identified six genotypes within the Alphavirus genus.

Harris E et al. (130), in 1998, described rapid detection and typing of Dengue virus in clinical samples and mosquitoes. Assays based on RT-PCR amplification of Dengue viral RNA can offer a rapid, sensitive and specific approach to the typing of Dengue virus. They have reduced a 2-step nested RT-PCR protocol to single tube reaction with sensitivity equivalent to that of the two step protocol (1 to 50 PFU) in order to maximize simplicity and minimize the risk of sample cross-contamination. This assay was also optimized for use with a thermostable RT-polymerase. They designed a plasmid based internal control that produces uniquely sized products and can be used to control for both reverse transcription or the amplification step without the risk of generating false positive results. This single tube RT-PCR procedure was used to type Dengue viruses during the 1995 and 1997-1998 outbreaks in Nicaragua. In addition, an extraction procedure that permits the sensitive detection of viral RNA in pools of up to 50 mosquitoes without PCR inhibition or RNA degradation was developed. This assay should serve as a practical tool for use in countries where dengue fever is endemic, in conjunction with classical methods for surveillance and epidemiology of Dengue viruses.

Paranjpe S, Banerjce K (131), in 1998, described a simple and rapid RT-PCR for detection of Japanese encephalitis virus envelope(E) gene sequences in various biological samplcs. The assay successfully amplified E gene sequences from infected cell cultures,

Aedes aegypti larvae, mosquitoes and mouse blood. The sensitivity of the assay detected 1 ng of JEV RNA and could be increased up to 1 pg on the background of 1 microgram of cellular RNA by biotinylation of the PCR product.

Waterman SH et al. (65), in 1985, reported a study of Dengue transmission in two Puerto Rican communities that occurred in 1982. Paired serological, entomological and environmental surveys were performed in Salinas and Manati, in the summer and fall of 1982. Paired samples on 434 persons in Salinas and 324 persons in Manati showed recent Dengue infection rates of 35 and 26%, respectively. *A. Aegypti* larval indices were higher in Salinas than in Manati but were relatively high throughout both communities. Breteau indices in neighborhoods ranged from 43 to 173 and infection rates in the neighborhoods were 22-45%. They analyzed possible associations of environmental variables with dengue incidence and prevalence of dengue antibody. Wood constructed housing and low socioeconomic status were among the variables significantly associated with Dengue incidence. Predictors of dengue antibody prevalence included socioeconomic levels, tree height, shade window and door screens. Recent dengue infections clustered within the sampled members of households (p <0.05). An estimated 85% of Dengue infections were symptomatic and no serious illnesses were reported.

Linnette RF et al. (132), in 1995, documented risk factors for Dengue infection during an outbreak in Yanes, Puerto Rico in 1991. They performed epidemiological and serological surveys linked to an earlier entomological study in a community of 426 houses in Yanes (Florida), Puerto Rica. They obtained a household response rate of 95% (98 of

103) and blood samples from 84% (345 of 410) of the participants. Dengue incidence as volunteered by the respondents was 5% (210 of 410) but serological diagnosis using IgM and IgG ELISA indicated a recent infection rate of 18% (59 of 337). The presence of anti-dengue antibodies was detected in 277 (84%) of 331 persons tested. In their final sample of 65 households and 111 persons, they analyzed the association of 12 entomological, environmental and behavioral variables with the proportion of household members with laboratory confirmed recent dengue. The number of female *A. Aegypti* per person was the only significant (p= 0.02) household risk factor. The results of this study underscores the importance of intradomicitiary mosquito populations in Dengue transmission and may serve as a guide for mosquito control efforts.

Swaddiwudhipong W et al. (133), in 1992, evaluated the value of a health education program on the prevention and control of dengue hemorrhagic fever (DHF) in the municipality of Mae Sot, Tak Province. A survey of adult residents, mainly housewives, was conducted in late April 1990, to assess their knowledge of DHF and practice of prevention methods. A total of 417 respondents from 417 households selected by a systematic cluster sampling method were interviewed. More than 90% of them knew that the disease is transmitted by Aedes mosquitoes and indicated water jars and water retention units in the house as common breeding places. However, the other two common breeding places, ant traps and cement baths, were less frequently mentioned. This finding was consistent with the greater proportion of respondents who reported no larval control methods for these two types of containers than for the others. Covering water containers was the most common practice to prevent mosquito breeding in drinking-water containers

whereas addition of abate (temphos sand granules) or changing stored water was commonly used for non-drinking water storage. Larval control for ant-traps was mainly accomplished by the addition of chemicals which included abate, salt, oil and detergent. Health education efforts in this area could induce the majority of respondents to accept themselves as responsible for the Aedes control program. Health education by health personnel played an important role in disseminating DHF information and prevention methods. Radio and television were the main effective mass media for public health education on DHF in this area.

Emilio HB et al. (134), in 1992, reported an outbreak of classical dengue fever from March to August in 1988 in the city of Taxco, Guerrero State in Mexico. Taxco is at an elevation of 1,700 meters above sea level and this study documents the highest altitude at which an outbreak of dengue fever has occurred. An investigation was conducted to obtain serological confirmation of Dengue infection, determine the extent of the outbreak and identify risk factors for dengue illness Toxorhynchites cell lines were used for viral isolation and hemagglutination inhibition was used to measure anti-dengue antibody titers. DEN-1 was isolated from five acute cases. Of 1,686 people living in the infected area, 42% (715) met the case definition. Large water containers (200 liters) were significantly associated with infection (relative risk = 1.7, 95% CI 1.5-1.9). The effect of altitude on epidemic transmission is most likely modulated by seasonal temperatures. The epidemiological and serological confirmation of a Dengue outbreak at 1,700 meters above sea level illustrates the capability of *A. aegypti* to adapt to new environments and shows the potential for epidemic spread in cities at comparable altitudes or higher.

Sharp TW et al. (135) studied dengue fever among U.S. troops during "operation restore hope" in Somalia during 1992 and 1993. The study included hospitalized troops with fever in addition to a sero-epidemiological survey of 530 troops. Among 289 febrile hospitalized troops 129 (45%) did not have an identified cause of fever. Dengue virus was recovered from 41 of 96 (43%) of these patients by inoculation of admission sera into Cb/3b cell cultures.39 (41%) of the isolates were identified as DEN-2 and 2 (2%) as DEN-3 by an indirect immunofluorescent antibody assay. An additional 18 of 37 (49%) culture-negative cases were shown, by IgM antibody capture ELISA to have anti-dengue virus antibody. Failure to use bed nets was the only identified risk factor for infection (adjusted odds ratio = 2.2, 95% CI =1.4-3.0). Other potential risk factors which included; age, rank, race, guard duty along the river, keeping sleeves rolled down at all times, regular use of topical insect repellant or the use of repellant/insecticide applied to clothing, were not associated with dengue virus infection.

Rosenbaum J et al. (136), in 1995, found that the priorities of public health planners were often at variance with a communities own environmental sanitation priorities and perspectives. Public opinion about individual, collective and governmental responsibilities in addressing these issues and priorities is of particular importance when designing community based programs. In a study conducted in Trinidad and Tobago on knowledge, attitudes and practice regarding Dengue prevention and control, a high level of awareness about Dengue and its etiology was evident. However, there was poor understanding of the symptoms and hence little concern about the health risks associated with it. The most important household pest problem identified by the respondents related

to mosquito nuisance, particularly from night-biting mosquitoes. Rodents were also a major concern perceived as being responsible for economic losses, spoiled foodstuffs and a health hazard. Unreliable water supply, a factor associated with *A. aegypti* abundance was an environmental issue of major importance to householders in rural areas. No correlation was found between knowledge of dengue and numbers of *A. aegypti* as measured by larval surveys of respondents premises. The study gave a clear indication of the need for broad-based environmental sanitation strategies when planning community-based vector control initiatives for the prevention and control of Dengue in Trinidad and Tobago.

Hayes CG et al. (137), in 1996, studied the epidemiology of Dengue virus infection in the Amazon region of Peru. Blood specimens and data on demographic, environment and medical history factors were collected from volunteers in an urban sector of Iquitos, rural area on the outskirts of Iquitos and three nearby jungle communities. Follow-up blood specimens were collected approximately one year later from a sample of subjects. Sera were tested for dengue IgG antibody by ELISA and specificity was verified using a plaque-reduction neutralization test. The prevalence of dengue antibody was; 66% in the urban population, 26% in 1the rural population and 32-67% in the three jungle areas. A significant association was found between age and antibody prevalence, with a steady increase in prevalence from 18% among subjects less than 5 years of age to greater than 90% for subjects greater than 50 years of age. Increased antibody prevalence was also associated with urban and jungle residence and with a piped source of drinking water. Sero-conversions were documented in 4 of the five communities surveyed.

Patiyol P et al. (138) studied environmental issues related to the spread of JE in a non-endemic area, including human behavior, breeding places of mosquito vectors, and vertebrates which facilitate, or were reservoir hosts of JE in Nakornsrithammarat Province. Studied sites representing different geographical areas of Thasala, Lansaka and Chainyai districts. Results indicated that Nakornsrithammarat Province has three geographical areas; plains, mountains and coastal areas. Round with the highest prevalence in the age group of under 15 years old, 78% of the studied population had primary school education, 59.2% of households studied less than 1 Km from the paddy fields and 30.2% had water holes around their houses. Major domestic animals were chicken, oxen and pigs. Most of them (80.6%) had no knowledge of JE. Only 10.6% knew the mode of disease transmission. Very few people knew methods of prevention, and 92 % had personal activities in the early evening. Among the several methods used to prevent mosquito bites, 95% of the population slept using a mosquito net as protection. In conclusion, the environment, behavior and peoples knowledge in Nakornsrithammarat Province, an area with low endemicity, was no different from that in an area with a high incidence of JE.

Chen WJ et al. (139), in 1996, studied IgM antibody to Dengue virus from a total of 3,099 serum samples collected in southern Taiwan. of 1,232 sera collected from a junior high school and from elementary schools in Liu Chiu, 36 were IgM positive and 2 of 192 adults in the local community were positive. The IgM-positive subjects tended to be aggregated around a port. Fishing boats that stopped in neighboring endemic countries were presumed to have introduced the virus periodically. Causing a low level of

inapparent infections. In the Kuohsinang area, 2 of the 108 suspected clinical cases and 4 of 642 community based sera were IgM positive. Rapid urbanization has provided appropriate circumstances for vector breeding in this area and the high population density has also increased contact frequency between human and mosquito vectors. This has, in turn, increased the possibility of silent transmission of the Dengue virus via either intermittent reintroduction of the virus or continuation of inapparent infections, or both. Establishment of an early warning system using the IgM antibody-capture ELISA was suggested for effective monitoring of disease.

CHARTER III

MATERIALS AND METHODS

1. Research design and data collection

The cross-sectional study was conducted to determine HI antibody titers and IgM and IgG antibody units. Acute serum was collected within a day of hospital admission. Convalescent serum was collected shortly before discharge from the hospital, and if possible, 7-14 days following admission. In some cases a house visit was necessary when follow up appointments were not met. Blood samples (3-5 ml) were collected from each patient by venepuncture. Samples were allowed to stand for 30 minutes for blood clotting, after which time the serum was separated and preserved at –20°C until use. An abbreviated case history, including physical examination and laboratory findings, were recorded on a form. Questionnaires were completed which detailed the demographic characteristics and the factors which were possibly related to arbovirus infections.

2. Serum sample and sample size

The samples from the study group were specifically from from hill tribe patients. Serum specimens were collected from patients aged more than one year old

who showed clinical symptoms with fever or pyrexia of unknown origin (PUO). These patients were selected from outpatients and admitted patients from four selected hospitals: Samoeng Hospital, Mae Taeng Hospital, Phrao Hospital, and Chaing Dao Hospital. Samples were collected from April 1997 to April 1998. Sample size was evaluated by proportion of population.

$$n = Z^2PQ / D^2$$

n = Sample size

P = The proportions expected in the population. For JE infection the prevalence rate was 57%, and 67% for Dengue infections, Igarashi A *et al* (9).

Z = Normal standard deviation, equal to 1.96

D = An accepted error deviated from actual value

Q = 1 - P

JE: $n = (1.96)^2(0.57)(0.43) / (0.05)^2$

$= 376$

Dengue : $n = (1.96)^2(0.67)(0.33) / (0.05)^2$

$= 340$

The three main arbovirus infections found in Thailand are caused by Dengue, JE and Chikungunya viruses. However, dengue and JE viruses represent a more serious health threat to the population in Thailand. As the prevalence rate of Chikungunya infection is very low, approximately 1.76% (140), a large sample size would be

required for it to be statistically representative. The sample size would extend beyond the allocated budget. Additionally, Chikungunya virus infections are not considered a highly significant health problem. Therefore, the sample sizes required for this study were calculated by using the prevalence rates for only Dengue and JE virus infections.

Blood samples were collected from 393 patients who were also interviewed using the structured questionnaire. Paired sera were collected from 203 patients and single serum samples were collected from 190 patients.

3. Laboratory methods

All sera were assayed for antibody detection by 2 methods. Firstly, detection by enzyme immuno assay (EIA); either by the IgM antibody capture enzyme-linked immunosorbent assay (MAC ELISA) or the IgG antibody capture enzyme-linked immunosorbent assay (GAC ELISA), using JE and Dengue virus antigens (serotypes 1, 2, 3 and 4 mixed together). Secondly, by the hemagglutination inhibition (HI) test; with JE, Dengue (individual serotypes; 1, 2, 3 or 4) and Chikungunya virus antigens.

3.1 Antibody capture ELISA

(a) Antigens

JE and Dengue antigens were prepared by sucrose acetone extraction from infected suckling mice brain as described by Clarke and Casal (141). These antigens were prepare1d from seed virus prototype strains which were in the following: DEN-1 (Hawaii strain), DEN-2 (New Guinea C strain), DEN-3 (H-87 strain), DEN-4 (H-241 strain) and JE (Nakayama strain). The antigens were distributed into vial and kept at -70°C until use for antibody assay.

(b) Positive and negative control sera for ELISA

Strong positive controls (SPC), weak positive controls (WPC) and negative controls (NC) for EIA against JE and Dengue, either MAC ELISA or GAC ELISA, were kindly provided by the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS), US component, Bangkok, Thailand. All control sera were kept at -20°C until use.

(c) Preparation of 20% normal human serum (NHS)

Normal human serum (NHS)

The normal human sera was obtained from healthy persons who did not have an antibody titer for flavivirus infections. These sera were confirmed by HI testing with Dengue and JE antigens; the HI titer being less than 1:10. The sera were provided by the Department of Virology, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand. Sera were pooled and frozen at -20°C until the extraction step. The NHS was extracted in 20% acetone and then dissolved in phosphate buffered saline (PBS) (pH 7.4).

Acetone extraction procedure

The NHS was inactivated in a water bath at 56°C for 30 minutes. 1000 ml of chilled acetone was poured into a 2000 ml beaker with a stirrer. 10 ml of NHS was added dropwise and stirred for 15 minutes. After the precipitate was allowed to settle for 2 days at room temperature, the clear solution was decanted, a further 1000 ml of chilled acetone added, stirred and left overnight at room temperature. After decanting most of the acetone, the deposit (approximately 4-5 ml) was transferred into a 50 ml tube and centrifuged at 1800 rpm for 10 minutes at 4°C. All the acetone was removed and the precipitate was spread by gently rolling the tube in order to make a thin sheet on the inner surface. The precipitate was dried using a vacuum pump for 2 hours or by

leaving at room temperature overnight. The precipitate was reconstituted with 50 ml of PBS (pH 7.4) to make a final solution equivalent to 20% NHS in PBS. This was mixed and flushed with a pipette and allowed to stand for 30 minutes before being centrifuged at 1800 rmp for 10 min at 4°C. 40 ml of the supernatant was transferred to a separate tube which was stored at -20°C until use.

(c) Procedure of Antibody Capture ELISA

MAC ELISA and GAC ELISA were performed by the technique as described by Innis B *et al.* (117). The procedure was performed in 7 basic steps. All stages were carried out in a humidified box and phosphate buffered saline with Tween 20 (PBS-T) was used as the wash solution.

Step 1: Sensitization

A 96-well micro-immuno plate (Linbro/Titertex, Biomedicals Inc., Horsham, PA) was sensitized with a 1:1500 dilution of goat anti-human IgM antibody or goat anti-human IgG antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). 6.7 µl of goat anti-human IgM, or 10 µl of anti-human IgG, mixed with 10 ml of 0.006 M bicarbonate buffer (pH 9.0) was used. 100 µl of the mixture was dispensed into each well and the plate incubated for 24 hours at 4°C. If the plate was not to be used at the end of the incubation period the solution was removed and the plate wrapped in foil and stored at -20°C. Plates stored in this way can be kept for up to 1 month with no apparent loss of activity.

Step 2: Antibody

The SPC, WPC, NC and serum specimens were diluted 1:100 in PBS (pH 7.4) (10 µl sample in 1 ml diluent). The sensitized plates were washed with 0.05 % Tween-20 in PBS (pH 7.4) six times and the plates tap-dried several times over clean tissues to remove excess fluid. Each serum was tested in 4 separate assays; JE MAC ELISA, JE GAC ELISA, DEN MAC ELISA and DEN GAC ELISA. 50 µl of diluted control serum and each serum sample were placed in the appropriate wells according to the document chart. All sera were tested in duplicate except for the WPC which was run in quadruplicate. Once the plate was prepared it was incubated overnight at 4°C in a humidified box or for 2 hours at room temperature. At the end of the incubation period the plate was washed once more.

Step 3: Antigen

Antigens were diluted in 20% acetone extracted NHS to the appropriate concentrations which were the following; JE antigen at 50 HA units, dengue antigens (serotypes 1, 2 and 3) at 16 HA units and Dengue serotype 4 at 8 HA units. 50 µl of tetravalent Dengue antigen solution was dispensed into all wells for dengue MAC ELISA or dengue GAC ELISA, and JE antigen dispensed into wells for JE MAC ELISA or JE GAC ELISA. The plates were incubated in humidified boxes overnight at 4°C or for at least 2 hours at room temperature. At the end of the incubation period the plates were washed once more.

Step 4: Conjugate

The conjugates of anti-flavivirus IgG-HRPO were prepared from high titer patients sera (as per HI test), from which IgG was extracted and labeled with horseradish peroxidase (HRPO). The conjugate was kindly prepared and titrated for the optimal dilution by AFRIMS. The appropriated dilution of conjugate showed the yield, at an absorbance reading at 492 nm, equal to 0.3-0.4 for WPC and < 0.1 for NC.

Conjugate was diluted in 20% acetone extracted NHS containing 0.5% bovine albumin. 25 µl of diluted conjugate was dispensed into each well and the plate incubated in a humidified box at 35-37°C for 1 hour, after which the plate was washed again.

Step 5: Substrate

5 mg of O-phenylenediamine tetrahydrochloride (Kodak, SIGMA Chemical Co., St.Louis, USA.) was dissolved in 10 ml of citrate phosphate buffer (pH 5.0) and 33 µl of fresh 3% hydrogen peroxide (H_2O_2). 100 µl was dispensed into each well and the plate incubated at room temperature for 30 minutes in a dark place.

Step 6: Stopping the reaction and reading the result

The reaction was stopped by the addition of 50 µl of 4M sulphuric acid (H_2SO_4). Color development in each well was read by spectrophotometer for the optical density (O.D.) value measured at a wavelength of 492 nm.

Step 7: Calculation and interpretation for DEN MAC ELISA and JE MAC ELISA

The WPC is defined as 100 units. A binding index (BI), in units, may be calculated by using the following formula :

$$BI = \frac{[\text{O.D. (Test sample)} - \text{O.D. (NC)}]}{[\text{O.D. (WPC)} - \text{O.D. (NC)}]} \times 100$$

An interpretation of cut-off level considered positive for IgM antibody was ≥ 40 units, either in DEN MAC ELISA or JE MAC ELISA. On occasions that both were positive a units ratio value between DEN MAC ELISA and JE MAC ELISA of ≥ 1.0 was defined as Dengue infection, while a units ratio value of < 1.0 was defined as JE infection.

For interpretation of the sequence of Dengue infection a units ratio value between DEN MAC ELISA and DEN GAC ELISA of ≥ 1.8 was defined as a primary infection, while a units ratio value of < 1.8 was defined as a secondary dengue infection. This units ratio is not valid for determining if JE occurred in a dengue-immune host.

Single serum samples positive only by JE MAC ELISA should be described as "recent JE virus infection". Samples positive by DEN MAC ELISA, at a value of ≥ 40 units, should be described as "recent dengue virus infection". The IgM antibody will usually have disappeared within 60 days of the infection.

1. Anti-human IgM coated well (▬)

2. Tested serum and serum control adding (▯)

3. Antigen adding (JE or Dengue antigens) (▬)

4. Conjugate adding (human anti-Flavivirus IgG-HRPO conjugate) (◠)

5. Substrate adding (OPD) (o)

6. Stop reaction with 4 M H_2SO_4

Figure 6 Basic step of antibody capture ELISA

3.2 Hemagglutination inhibition test (HI)

HI testing was performed according to the Clarke and Casal method (141) by the Department of Virology, AFRIMS. This test was performed using Dengue, JE and Chikungunya antigens.

(a) Preparation of Erythrocytes

Goose red blood cells (GRBC) were kindly provided by the Department of Veterinary Medicine, AFRIMS, Bangkok, Thailand. They were collected from the adult male, white domestic goose (*Anser cinereus*). Alsever's solution was used for blood anticoagulant in the ratio of 20 ml whole blood to 5 ml of Alsever's solution. The cells were stored at 4°C.

Preparation of packed GRBC

The GRBC in Alsever's solution was centrifuged at 1800 rpm for 10 minutes at 4 °C and the packed GRBC collected. The GRBC were washed 3 times with 0.9% normal saline solution (NSS) by gentle mixing and centrifugation as above. The NSS was discarded and a GRBC "pellet" obtained following the final spin. The packed GRBC were used to treat sera in order to remove natural agglutinin and for the preparation 8% GRBC.

Preparation of 8% GRBC

The 8% GRBC were prepared freshly before tests. Packed GRBC, after being washed 3 times with NSS as described above, were washed a further 3 times with dextrose gelatin veronal (DGV) solution in ratio of GRBC to DGV equal to 1:3.5. This solution was then centrifuged at 1800 rpm for 10 minutes at 4°C. After the supernatant was removed the GRBC were used to prepare 8% GRBC in DGV at ratio of GRBC to DGV equal to 2:23 before being stored at 4°C. These 8% GRBC were used for HI testing by diluting in various pH solutions (pH 6.0, 6.2, 6.4, 6.6) of veronal adjusting diluent (VAD) in a ratio of 1:24.

(b) Preparation of serum specimens

Patients sera were extracted with acetone to remove non-specific inhibitors and then absorbed with packed GRBC to remove natural agglutinin. For the acetone extraction, 100 µl of serum was placed in a 15 ml centrifuge tube and inactivated in water bath at 56°C for 30 minutes. After 30 minutes 10 ml of chilled acetone was added (stored at -20°C), mixed and centrifuged at 1800 rpm for 10 minutes at 4°C. The acetone was then decanted from the pellet and the extraction repeated once more. Each serum sample was subjected to two extraction steps.

Pellets were made into thin sheets on the inner surface of tubes by gently rolling the tube and allowing them to dry on a vacuum pump for 2 hours or by leaving at room

temperature overnight. The sheets were dissolved with 500 µl of borate saline (BS, pH 9.0) then mixed gently. Since the original volume of serum was 100 µl and the final volume of this step was 500 µl a 1:5 dilution has been made. One drop of packed GRBC was added using an 18G needle, mixed gently and placed in an ice bath for 20 minutes, with occasional shaking, in order to absorb natural agglutinin. The mixture was then centrifuged at 1800 rpm for 10 minutes at 4°C. The supernatant was decanted, diluted 1:5 and stored at 4 °C until the HI test.

(c) Titration of antigen by hemagglutination (HA)

All antigens; Dengue (each type), JE and Chikungunya were prepared by the same method and used for either HI testing or capture ELISA. Following each antigen extraction the HA titration was performed to determine the end-point titer. The antigens (Dengue antigen types 1 to 4, JE and Chikungunya virus) were titrated by HA in a 18 x 10-well lucite plate (Rockefeller Foundation).

Briefly, each of the antigens were diluted 1:10 in 0.4% bovine albumin borate saline (BABS, pH 9.0) and maintained in an ice bath. 0.4 ml of 0.4 % BABS was placed into every well as a diluent. 0.4 ml of the 1:10 dilution of each antigen was then added to the first well of the appropriate row. Serial 2-fold dilutions were made using pipette droppers resulting in a dilution series from 1:20 to 1:10240. The last well of each row of antigen was used as a control for GRBC plus diluent without any antigen. 0.4 ml of GRBC, in their optimal pH solution (8% GRBC in VAD diluent), were

transferred into each well of each row of antigen and their controls. Usually, the optimal pH was pH 6.2 for DEN-1 and Chikungunya antigens, pH 6.4 for DEN-2 antigen, pH6.6 for DEN-3 and JE antigens, and a pH of 6.8 for DEN-4 antigen. The plate was gently agitated, either manually or using a shaker, in order to facilitate mixing of antigen and GRBC. Plates were then incubated for 1 hour at room temperature for DEN-1, DEN-2, DEN-3 and DEN-4 antigens, or at 35-37°C for JE and Chikungunya antigens. The HA results of each antigen were read and the titer which showed the highest dilution of antigen hemagglutination with GRBC was recorded. Hemagglutination can be recognized by the uniform pink appearance of the well after the plate has been left to stand for 30 minutes.

The results were recorded as follows:

+ = complete agglutination

⊕ = partial agglutination

± = trace agglutination

O = no agglutination

The highest dilution of antigen preparation, at optimal pH, that showed complete agglutination was considered to be the end-point and equal to 1 HA unit. Therefore, each antigen was read and the value of their HA units calculated by counting backwards from the last well (i.e. 1, 2, 4 ,8, 16, 32, 64 ,128...) until the first well. The dilution which contained 16 HA units per 0.05 ml of antigen (or 8 HA units per 0.025 ml of antigen) was used for the HI test. Thus, for each antigen, a dilution factor could be calculated that resulted in a working concentration of 8 HA units. To determine the

dilution that contained 8 HA units, primary titration was done again by HA, and result showed 4 dilution counted back from the last dilution which showed complete agglutination (1, 2, 4, 8 HA units).

(d) Hemagglutination inhibition test (HI)

The HI test was also performed in microtiter V-well plate. 0.025 ml of 0.4% BABS diluent (pH 9.0) was dispensed into each well. A 1:5 dilution of serum was added to the first well and mixed with the diluent. 2-fold serial dilutions were made using a 0.025 ml loop to mix and transfer the serum, resulting in a dilution series from 1:10 to 1:10240. 25 µl (4 HA units) of antigen was added to each well, mixed and incubated overnight at 4°C. The plates were stacked according to test antigen and covered with a blank plate.

Following incubatuon the test plates were removed from the refrigerator and allowed to reach room temperature. GRBC were suspended 1:24 in VAD, at the optimal pH, for each antigen just before they were added to the plate. 0.05 ml of the goose red cell suspension was added and mixed. The plates were then held for 1 hour at room temperature for DEN-1, 2, 3 and 4, or at 37°C for JE and Chikungunya, before being examined for pattern formation. Inhibition of agglutination (HI) was defined by a negative reaction (button of cells). The HI titer is taken as the highest dilution of each serum which causes complete, or almost complete, inhibition of agglutination. This is expressed as the serum dilution.

(e) Interpretation

HI antibody titers obtained from laboratory assays were interpreted according to the criteria of the World Health Organization (WHO), either for dengue or JE infection (142). The interpretation criteria are shown detail in below (Table 5).

Table 5 Interpretation of Dengue HI antibody responses

Antibody responses	S1-S2 interval	Convalescent Titer (any Dengue Antigen)	Interpretation
≥ 4x rise	≥ 7 day	≤ 1 : 1280	Definite infection, primary
≥ 4x rise	any specimen	≥ 1 : 2560	Definite infection, secondary
≥ 4x rise	< 7 days	≤ 1 : 1280	Definite infection, possible primary or secondary
no change	any specimen	≥ 1 : 2560	Presumed infection, secondary
no change	≥ 7 days	≤ 1 : 1280	Not dengue
no change	< 7 days	≤ 1 : 1280	Uninterpretable
-	one specimen only	≤1 : 1280	Uninterpretable

For laboratory diagnosis by HI testing paired sera collected at least 7 days apart should be tested simultaneously. Single samples are less useful for serological diagnosis using this test.

Because of the cross-reactivity within the flaviviruses a serological test can never provide identification of the infecting virus, especially with respect to Dengue and JE. Clinical signs and symptoms were used to assist interpretation.

Interpretation of JE HI antibody responses

1. Primary antibody response (paired sera):

Acute serum titer less than 1:20. There is a 4-fold or greater increase in titer in convalescent specimens (2-4 weeks after onset). The response is usually monotypic, JE primary infection.

2. Secondary antibody response (paired sera):

A 4-fold or greater rise in titer to JE antigen, in combination with a typical clinical history of encephalitis, is presumptive evidence for secondary JE.

3. Single serum:

A definitive diagnosis with the HI test is not possible with a single serum specimen. If the serological reaction in a convalescent serum is monotypic for JE antigen, in combination with a typical history of encephalitis, a presumptive diagnosis of JE may be made, particularly if the anti-JE titer is 1:320, or greater.

Interpretation for Chikungunya virus infection

Only equal, or a 4-fold rising titer in paired sera, taken at least 7 days apart, was used for interpretation of Chikungunya virus infection by HI testing. Single serum samples were less useful for diagnosis by the HI test.

4. Data analysis

The SPSS/PC for Windows was used for data analysis.

The mean antibody titer for Dengue (DEN-1, DEN-2, DEN-3 and DEN-4), JE and Chikungunya virus infections were calculated by geometric mean titers (GMT).

Prevalence of antibody and recent infection rates for each arbovirus infection was calculated by percentages.

Difference of geometric mean titers (GMT) for Dengue (type 1, 2, 3 and 4), Chikungunya and JE viruses in acute and convalescent serum were analyzed by Friedman test ($\alpha < 0.05$).

Difference of prevalence rate infection and prevalence rate antibodies exposure for arboviruses were analyzed by Cochran Q test ($\alpha < 0.05$).

Factors related to arbovirus infections were analyzed by Chi-square test, Fisher exact test and logistic regression ($\alpha < 0.05$).

CHAPTER IV

RESULTS

General characteristics of study group

Blood samples were collected from three hundred and ninety three hill tribe patients in this study at four selected hospitals in Chiang Mai Province. The blood samples were composed of 190 cases of single serum and 203 cases of paired sera. All of these patients had clinical symptoms with pyrexia. Seven hill tribe groups who came for treatment at four selected hospitals contributed to the number of patients. Karen was the highest hill tribe group of patients in this study (35.6%), and either single or paired sera groups found that one third of blood samples were collected at Samoeng Hospital. The details are shown in Table 6 and Table 7.

It was found that the sex ratio of the patients were slightly in the same proportion. The ratio of male to female was 1.1:1 (203 / 190 cases). The age groups were classified and found that number of samples in this study was highest in the age group 15 - 19 years old (18.1 %), and lowest in 1 - 4 years old (0.5 %). There were no infant group (< 1 year old) in this study. The mean and standard deviation (SD) of the age was 25.9 ± 15.8 years old. When classified by the hospital where patients came for treatment, the highest number of samples were collected from Samoeng Hospital

and Chiang Dao Hospital (32.8 and 31.8 % respectively), followed by Mae Taeng Hospital and Phrao Hospital (17.8 and 17.6 % respectively). Nearly half of these patients did not have prior education (46.8 %), while in the educated group, the majority graduated level at Prathom 1 - 6 (45.6 %), which is not a high educational standard. Half of the patients's occupation were agriculture (50.9%). The family incomes were rather low, one third of them earned 1 - 1,000 baht per month (33.1%), while the other third had no income (32.8%). More than half of their religions were Buddhist (62.6%). The details of general demographic data are shown in Table 8.

Table 6 Number of hill tribe patients visiting at four selected hospitals, classified by hill tribe group.

Hill tribe groups	Total No. (%)	No. of patients in hospital (%)			
		Samoeng (S)	Mae Taeng (M)	Phrao (P)	Chiang Dao (C)
Karen	140 (35.6)	105 (81.4)	6 (8.5)	21 (30.4)	8 (6.4)
Meo	62 (15.8)	13 (10.1)	31 (44.3)	7 (10.1)	11 (8.8)
Lahu	87 (22.1)	2 (1.6)	16 (22.9)	18 (26.1)	51 (40.8)
Lisu	79 (20.1)	6 (4.6)	16 (22.9)	20 (29.0)	37 (29.6)
Akha	9 (2.3)	-	-	2 (2.9)	7 (5.6)
Lua	9 (2.3)	3 (2.3)	1 (1.4)	1 (1.5)	4 (3.2)
Palong	7 (1.8)	-	-	-	7 (5.6)
Total	393 (100.0)	129 (100.0)	70 (100.0)	69 (100.0)	125 (100.0)

Table 7 Number of hill tribe patients in single serum group and paired serum group at four selected hospitals, classified by hill tribe group.

Hill tribe groups	Total	No. of single serum				Total	No. of paired serum			
		S	M	P	C		S	M	P	C
Karen	57	35	6	9	7	83	70	1	11	1
Meo	33	12	11	4	6	29	1	21	3	4
Lahu	48	1	4	9	34	39	2	10	9	18
Lisu	36	4	7	11	14	43	1	9	10	23
Akha	5	-	-	1	4	4	-	-	1	3
Lua	7	2	1	1	3	2	1	-	-	1
Palong	4	-	-	-	4	3	-	-	-	3
Total	190	54	29	35	72	203	75	41	34	53

S = Samoeng Hospital

M = Mae Taeng Hospital

P = Phrao Hospital

C = Chiang Dao Hospital

Table 8 Number of the hill tribe patients at four selected hospitals in Chiang Mai Province according to their general demographic data.

General demographic data	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
(1) Sex			
Male	203 (51.6)	98 (51.6)	105 (51.7)
Female	190 (48.4)	92 (48.4)	98 (48.3)
(2) Age group (years old)			
1 - 4	2 (0.5)	2 (1.1)	-
5 - 9	45 (11.4)	23 (12.1)	22 (10.8)
10 – 14	57 (14.5)	22 (11.6)	35 (17.3)
15 - 19	71 (18.1)	34 (17.9)	37 (18.2)
20 - 24	45 (11.4)	21 (11.0)	24 (11.8)
25 - 29	35 (8.9)	14 (7.4)	21 (10.4)
30 - 34	30 (7.7)	17 (8.9)	13 (6.4)
35 - 39	42 (10.7)	17 (8.9)	25 (12.3)
≥ 40	66 (16.8)	40 (21.1)	26 (12.8)
Mean ± SD	25.9 ± 15.8	27.8 ± 16.9	24.5 ± 14.8

Table 8 Number of the hill tribe patients at four selected hospitals in Chiang Mai Province according to theirs general demographic data. (continued)

General demographic data	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
(3) Hospital treatment			
Samoeng	129 (32.8)	54 (28.4)	75 (36.9)
Mae Taeng	70 (17.8)	29 (15.3)	41 (20.2)
Phrao	69 (17.6)	35 (18.4)	34 (16.8)
Chiang Dao	125 (31.8)	72 (37.9)	53 (26.1)
(4) Marital status			
Single	185 (47.1)	82 (43.2)	103 (50.7)
Married	202 (51.4)	106 (55.8)	96 (47.3)
Widow	6 (1.5)	2 (1.0)	4 (2.0)
(5) Religion			
Buddhist	246 (62.6)	113 (59.5)	133 (65.5)
Christian	144 (36.6)	75 (39.5)	69 (34.0)
Islam	1 (0.3)	1 (0.5)	-
Other	2 (0.5)	1 (0.5)	1 (0.5)
(6) Education			
None	184 (46.8)	99 (52.1)	85 (41.9)
Prathom 1-6	179 (45.5)	77 (40.5)	102 (50.2)

Table 8 Number of the hill tribe patients at four selected hospitals in Chiang Mai Province according to their general demographic data. (continued)

General demographic data	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
Mathayom 1-3	21 (5.3)	10 (5.3)	11 (5.4)
Mathayom 4-6	7 (1.8)	2 (1.1)	5 (2.5)
Vocational	1 (0.3)	1 (0.5)	-
Bachelor's degree	1 (0.3)	1 (0.5)	-
(7) Occupation			
Agriculture	200 (50.9)	97 (51.1)	103 (50.7)
Farmer	19 (4.8)	5 (2.6)	14 (6.9)
Forest item gatherer	1 (0.3)	1 (0.5)	-
Employee	45 (11.4)	20 (10.5)	25 (12.3)
Business	1 (0.3)	1 (0.5)	-
Student	109 (27.7)	53 (27.9)	56 (27.6)
Unemployed	18 (4.6)	13 (6.9)	5 (2.5)
(8) Income (Baht per month)			
None	129 (32.8)	63 (33.2)	66 (32.5)
1 - 1000	130 (33.1)	66 (34.7)	64 (34.4)
1001 - 2000	76 (19.4)	39 (20.5)	37 (18.2)
2001 - 3000	20 (5.1)	12 (6.3)	8 (3.9)

Table 8 Number of the hill tribe patients at four selected hospitals in Chiang Mai Province according to their general demographic data. (continued)

General demographic data	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
3001 – 4000	21 (5.3)	3 (1.6)	18 (8.9)
4001 – 5000	11 (2.8)	4 (2.1)	7 (3.5)
> 5000	6 (1.5)	3 (1.6)	3 (1.5)
Mean ± SD	836. 9 ± 21.4	905.8 ± 22.6	756.4 ± 19.8
(9) Migration			
Yes	71 (18.1)	40 (21.1)	31 (15.3)
No	322 (81.9)	150 (78.9)	172 (84.7)
(9.1) Cause of migration	(n = 71)	(n = 40)	(n= 31)
To change place for agriculture	56 (78.9)	33 (82.5)	23 (74.2)
To employee	13 (18.3)	6 (15.0)	7 (22.6)
To escape natural dangers	2 (2.8)	1 (2.5)	1 (3.2)

Informations about present illness of hill tribe patients

The designed questionnaires were included with the information of their present illness with signs and symptoms. The highest percent of clinical signs and symptoms often found were fever (100.0%), malaise (64.6%) and muscle pain (42.2%), while a few cases showed unconscious and convulsions (0.5%).

When they have been sick, half of them (50.4%) did not do anything. On the other hand, one third (31.5%) of them went for treatment at primary health care clinics. Some of cases (9.2%) bought the medicine from drug stores, of which 83.3% were analgesics and antipyretics, 13.9% were antibiotics and 2.8% were herbal traditional drugs. Almost all of the patients (92.1%) never received JE vaccination, and had never been sick with DHF (96.4%). They were bitten by *Aedes. spp.* mosquitoes (30.0%), and the time that mosquitoes often bit them was night time (58.3%) more than day time (41.7%). The data are shown in Table 9.

Table 9 Present illness of the hill tribe patients who came for treatment at four selected hospitals in Chiang Mai Province.

Present illness	Total	Single serum group	Paired serum group
	No. of case (%) (n = 393)	No. of case (%) (n = 190)	No. of case (%) (n = 203)
(1) Before sickness for 1 week, had you ever had any symptoms (the answer may more than one)			
Malaise	254 (64.6)	113 (59.5)	141 (69.4)
Fever	393 (100.0)	190 (100.0)	203 (100.0)
Chills	148 (37.7)	79 (41.6)	69 (34.0)
Eye pit pain	27 (6.9)	13 (6.8)	14 (6.9)
Muscle pain	166 (42.2)	78 (41.1)	88 (43.3)
Epitaxis / malena / hematemesis	6 (1.5)	1 (0.5)	5 (2.5)
Anorexia	268 (68.2)	126 (66.3)	142 (69.9)
Nausea	163 (41.5)	85 (44.7)	78 (38.4)
Vomiting	141 (35.9)	71 (37.4)	70 (34.5)
Abdominal pain	67 (17.1)	29 (15.3)	38 (18.7)
Diarrhea	70 (17.8)	27 (14.2)	43 (21.2)
Right costal margin pain	20 (5.1)	8 (4.2)	12 (5.4)
Stiffness of neck and back	3 (0.8)	1 (0.5)	2 (0.9)

Table 9 Present illness of the hill tribe patients who came for treatment at four selected hospitals in Chiang Mai Province. (continued)

Present illness	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
Unconsciousness / convulsion	2 (0.5)	1 (0.5)	1 (0.5)
(2) While you have been sick, what were your clinical signs ? (the answer may more than one)	(n= 91)	(n= 42)	(n= 49)
Conjunctivitis	13 (14.3)	6 (14.3)	7 (14.3)
Rash on face / extremities / body	18 (19.8)	8 (19.0)	10 (20.4)
Jaundice	60 (65.9)	28 (66.7)	32 (65.3)
(3) When you have been sick before this admission, what did you do?			
Nothing	198 (50.4)	99 (52.1)	99 (48.8)
Went to clinic	7 (1.8)	1 (0.5)	6 (2.9)
Went to primary health care service	124 (31.5)	66 (34.8)	58 (28.6)

Table 9 Present illness of the hill tribe patients who came for treatment at four selected hospitals in Chiang Mai Province. (continued)

Present illness	Total	Single serum group	Paired serum group
	No. of case (%) (n = 393)	No. of case (%) (n = 190)	No. of case (%) (n = 203)
Bought some medicine from drug store	36 (9.2)	12 (6.3)	24 (11.8)
Went to hospital	9 (2.3)	4 (2.1)	5 (2.5)
Traditional treatment	10 (2.5)	5 (2.6)	5 (2.5)
Other (magic)	9 (2.3)	3 (1.6)	6 (2.9)
(3.1) If you bought medicine from drug store, what kind of medicine?			
	(n = 36)	(n = 12)	(n = 24)
Antibiotics	5 (13.9)	4 (33.3)	1 (4.2)
Analgesic and antipyretic	30 (83.3)	8 (66.7)	22 (91.6)
Herbal	1 (2.8)	-	1 (4.2)
(4) Have you ever been immunized with JE vaccine?			
Yes	31 (7.9)	14 (7.4)	17 (8.4)
No	362 (92.1)	176 (92.6)	186 (91.6)
(5) Have you ever been sick with dengue haemorrhagic fever?			
Yes	14 (3.6)	7 (3.7)	7 (3.4)
No	379 (96.4)	183 (96.3)	196 (96.6)

Table 9 Present illness of the hill tribe patients who came for treatment at four selected hospitals in Chiang Mai Province. (continued)

Present illness	Total No. of case (%) (n = 393)	Single serum group No. of case (%) (n = 190)	Paired serum group No. of case (%) (n = 203)
(6) One week before the onset of illness, were you bitten by mosquitoes?			
No	59 (15.0)	28 (14.7)	31 (15.3)
Yes, by *Aedes spp.*	118 (30.0)	60 (31.6)	58 (28.6)
Yes, by *Culex spp.*	17 (4.3)	10 (5.3)	7 (3.4)
Yes, by *Anopheles spp.*	6 (1.6)	3 (1.6)	3 (1.5)
Don't know	101 (25.7)	44 (23.1)	57 (28.1)
Not sure	92 (23.4)	45 (23.7)	47 (23.1)
(7) If you were bitten by mosquitoes, what time did mosquitoes often bite you ?			
Day time	164 (41.7)	82 (43.2)	82 (40.4)
Night time	229 (58.3)	105 (56.8)	121 (59.6)

Background knowledges about arboviral diseases in all hill tribe patients

A knowledge part was included in the questionnaires for assessing the hill tribe patients' knowledge about arboviral diseases. Most patients didn't know about the causes leading illness with DHF and JE (65.6% and 94.9%), didn't know what was the peak season in which DHF and JE mostly occurred (57.0%), and didn't know the signs and symptoms of DHF (90.1%) and JE (96.2%). Details are shown in Table 10.

Table 10 Background knowledge about DHF and JE of all the hill tribe patients at four selected hospitals in Chiang Mai Province.

Knowledge questions	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(1) Do you know that when you are bitten by mosquitoes (*Aedes spp.*), you may become sick with DHF ?			
Yes	135 (34.4)	58 (30.5)	77 (37.9)
No	258 (65.6)	132 (69.5)	126 (62.1)
(2) Do you know that when you are bitten by mosquitoes (*Culex spp.*) you may become sick with JE ?			
Yes	20 (5.1)	12 (6.3)	8 (3.9)
No	373 (94.9)	178 (93.7)	195 (96.1)

Table 10 Background knowledge about DHF and JE of all the hill tribe patients at four selected hospitals in Chiang Mai Province. (continued)

Knowledge background	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(3) Have you ever heard about mosquitoes *Aedes spp.* and *Culex spp.*?			
Know both mosquitoes	45 (11.4)	21 (11.0)	24 (11.8)
Know *Aedes spp.*, but don't know *Culex spp.*	107 (27.2)	52 (27.4)	55 (27.1)
Know *Culex spp.*, but don't know *Aedes spp.*	1 (0.3)	-	1 (0.5)
Don't know both mosquitoes	240(61.1)	117 (61.6)	123 (60.6)
(4) What season DHF and JE frequently occurred ?			
Summer	5 (1.3)	3 (1.6)	2 (1.0)
Rainy season	164 (41.7)	71 (37.4)	93 (45.8)
Winter	-	-	-
Don't know	224 (57.0)	116 (61.0)	108 (53.2)
(5) Do you know that DHF patients experience fever, headache, anorexia, nausea, vomiting and petechiae haemorrhage ?			
Yes	39 (9.9)	20 (10.5)	19 (9.4)
No	354 (90.1)	170 (89.5)	184 (90.6)

Table 10 Background knowledge about DHF and JE of all the hill tribe patients at four selected hospitals in Chiang Mai Province. (continued)

Knowledge background	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(6) Do you know that JE patients experience fever, headache, nausia, vomitting, neck and back stiffness, convulsions and unconciousness?			
Yes	15 (3.8)	9 (4.7)	6 (3.0)
No	378 (96.2)	181 (95.3)	197 (97.0)

Personal behaviors and environmental information of the hill tribe patients

The working time of the majority of hill tribe patients were from 8.00 – 16.00 (85.3%). More than half of them wear covered clothing while working (57.5%). They choose various methods for protection from mosquitoes bite, while most of them did not choose any methods for removing the breeding places of mosquitoes (77.4 %). The data are shown in Table 11.

More than half of their working area were in the urban area (57.5%). In the working area or 100 meters away of their house, there were several kind of animals feeding. Most of them have containers and ground water pits close to their house (84.7%). They found mosquitoes but the majority didn't know what type of

mosquitoes in their house (71.5%), and mosquitoes often found in the general areas (75.1%). The data are shown in Table 12.

Table 11 Personal behaviors of the hill tribe patients visiting at four selected hospitals in Chiang Mai Province.

Personal behavior	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(1) Working time	(n = 266)	(n = 124)	(n = 142)
8.00 – 16.00	227 (85.3)	106 (85.5)	121 (85.2)
8.00 – 20.00	39 (14.7)	18 (14.5)	21 (14.8)
(2) Clothing while working	(n = 266)	(n = 124)	(n = 142)
Covered clothes	153 (57.5)	79 (63.7)	74 (52.1)
Open clothes	113 (42.5)	45 (36.3)	68 (47.9)
(3) What time did the mosquitoes often bite you ?			
Day time	164 (41.7)	82 (43.2)	82 (40.4)
Night time	229 (58.3)	108 (56.8)	121 (59.6)

Table 11 Personal behaviors of the hill tribe patients visiting at four selected hospitals in Chiang Mai Province. (continued)

Personal behavior	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(4) Which methods did you choose for protection from mosquitoes ?			
Sleep under mosquitoes net	75 (19.1)	37 (19.5)	38 (18.7)
Use mosquitoes coil	105 (26.7)	50 (26.3)	55 (27.1)
Use herbicide	13 (3.3)	6 (3.2)	7 (3.4)
Use repellent	6 (1.5)	-	6 (3.0)
Other (smoke)	194 (49.4)	97 (51.0)	97 (47.8)
(5) Which methods do you choose for removing breeding places of mosquitoes ?			
Remove garbage	23 (5.8)	15 (7.9)	8 (3.9)
Put abate sand in water	10 (2.6)	4 (2.1)	6 (3.0)
Feed *Poccillia spp.* fish	-	-	-
Use chemical insecticides	56 (14.2)	19 (10.0)	37 (18.2)
None	304 (77.4)	152 (80.0)	152 (74.9)

Table 12 The environmental informations of the hill tribe patients visiting at four selected hospitals in Chiang Mai Province.

Enviromental information	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
(1) Environment of working area	(n = 266)	(n = 124)	(n = 142)
Farm and forest	113 (42.5)	54 (43.5)	59 (41.5)
In urban area	153 (57.5)	70 (56.5)	83 (58.5)
(2) Within 100 meters of your house or your working area do you have animals feeding ? (the answer may more than one)			
None	90 (22.9)	46 (24.2)	44 (21.7)
Pig	202 (51.4)	94 (49.5)	108 (53.2)
Dog	169 (43.0)	84 (44.2)	85 (41.9)
Bird	205 (52.2)	99 (52.1)	106 (52.2)
Cow, buffalo	27 (6.9)	9 (4.7)	18 (8.9)
Not in category	57 (14.5)	26 (13.7)	31 (15.3)
(3) Are there containers or ground water pits close to your house ?			
No	60 (15.3)	33 (17.4)	27 (13.3)
Big jar	256 (65.1)	119 (62.6)	137 (67.5)

Table 12 The environmental informations of the hill tribe patients visiting at four selected hospitals in Chiang Mai Province. (continued)

Environmental information	Total case (%) (n = 393)	Single serum group case (%) (n = 190)	Paired serum group case (%) (n = 203)
Coconut bark, can, bottle	56 (14.3)	32 (16.8)	24 (11.8)
Water drainage tap	21 (5.3)	6 (3.2)	15 (7.4)
(4) What types of mosquitoes are often found in your house ?			
Aedes spp.	94 (23.9)	40 (21.1)	54 (26.6)
Culex spp.	12 (3.1)	5 (2.6)	7 (3.4)
Anopheles spp.	6 (1.5)	4 (2.1)	2 (1.0)
Don't know	281 (71.5)	141 (74.2)	140 (69.0)
(5) Where did you often find mosquitoes in your house ?			
Bathroom	48 (12.2)	22 (11.6)	26 (12.8)
Under floor of house	10 (2.5)	5 (2.6)	5 (2.5)
Corner of room and closet	40 (10.2)	23 (12.1)	17 (8.4)
General areas	295 (75.1)	140 (73.7)	155 (76.3)

Three hundred and ninety three cases of patients were assayed by HI test against four serotypes of Dengue (DEN-1, DEN-2, DEN-3 DEN-4), JE and Chikungunya antigens. According to WHO criteria for laboratory diagnosis, 40 cases were interpreted as definitely positive Dengue infection (2 cases primary and 38 cases secondary infection), 4 cases as positive primary JE infection, 23 cases with positive rising titers to either Dengue or JE (22 cases primary and 1 case secondary infection) and 25 cases positive for Chikungunya infection. The results are summarized in Table 13.

Furthermore, when these 393 patients cases were assayed by MAC ELISA for Flaviviruses diagnosis, 54 cases were definitely interpreted as Dengue infection, while 13 cases as JE infection. Details are summarized in Table 14.

When co-interpreted by either HI test or MAC ELISA for Flaviviruses diagnosis in each case of all patients, 56 cases were interpreted as definite positive Dengue infection (7 cases primary, 39 cases secondary and 10 cases possible primary or secondary), 16 cases were definitely JE infection (12 cases primary and 4 cases possible primary or secondary), and 9 cases were Flaviviruses infection.

In conclusion, of 393 cases of patients having laboratory diagnosis for Flaviviruses infection (HI test and MAC ELISA) and for Alphavirus infection (HI test) it was found that 87 cases were positive arbovirus infections (43 cases were only Dengue infection, 13 cases were both Dengue and Chikungunya co-infections, 11 cases were only JE infection, 5 cases were both JE and Chikungunya co-infections, 8

cases were only Flaviviruses infection, 1 case both Flaviviruses and Chikungunya co-infections and 6 cases were only Chikungunya infection). Details are summaried in Table 15 and Table 16.

The antibody exposure of two groups arboviruses, the Flaviviruses and the Alphavirus, are summarized by HI test using cut off titers of ≥ 1:10 as a positive value. It was found that positive arboviruses antibody exposure were found in 293 cases, which are shown in Table 17.

Table 13 Laboratory interpretation of hill tribe patients by HI test.

Serum group	Flaviviruses (DEN & JE)											Alphavirus (CHIK)		
	Positive									Negative	Uninter-preted	Positive	Negative	Uninter-preted
	DEN			JE			DEN or JE							
	1°	2°	1°or 2°	1°	2°	1°or 2°	1°	2°	1°or 2°					
Single serum	-	2	-	-	-	-	-	1	-	-	187	-	-	190
Paired serum	2	36 (13)	-	4 (1)	-	-	22 (5)	-	-	139 (6)	-	25	178	-
Total	2	38 (13)	-	4 (1)	-	-	22 (5)	1	-	139 (6)	187	25 (19)	178	190

Note : ( ) cases of co-infection with Dengue and Chikungunya or JE and Chikungunya cases

1° : primary infection

2° : secondary infection

Table 14 Laboratory interpretation of hill tribe patients by DEN MAC ELISA and JE MAC ELISA

Serum group	Total	Positive (≥ 40 units)				Negative (< 40 units)
		DEN	JE	DEN or JE		
	No. (%)	No. (%)	No. (%)	DEN/JE ≥1 No. (%)	DEN/JE <1 No. (%)	No. (%)
Single serum	190	11 (5.8)	4 (2.1)	-	-	175 (92.1)
Paired serum	203	43 (21.2)	9 (4.4)	1 (0.5)	-	150 (73.9)
Total	393	54 (13.7)	13 (3.3)	1 (0.3)	-	325 (82.7)

Table 15 Laboratory diagnosis of *Flaviviruses* and *Alphavirus* infections in hill tribe patients co-interpreted by HI test and MAC ELISA

Serum group	Flaviviruses (DEN & JE)											Alphavirus (CHIK)		
	Positive									Neg	Uninter	Positive	Negative	Uninter-
	DEN			JE			DEN or JE				preted			preted
	1°	2°	1°or 2°	1°	2°	1°or 2°	1°	2°	1°or 2°					
Single serum	-	3	8	-	-	4	-	-	-	-	175	-	-	190
Paired serum	7	36	2	12	-	-	9	-	-	137	-	25	178	-
		(13)		(5)			(1)			(6)				
Total	7	39	10	12	-	4	9	-	-	137	175	25	178	190
		(13)		(5)			(1)			(6)		(19)		

Note: ( ) cases of co-infections with Dengue and Chikungunya or JE and Chikungunya, or Flaviviruses and Chikungunya, or negative for Flaviviruses and Chikungunya.

1° : primary infection

2° : secondary infection

Table 16 Summary of the laboratory diagnosis of arboviruses infections in hill tribe patients co-interpreted by HI test and MAC ELISA.

Serum group	Total	Arboviruses infections	
		Positive DEN, JE and CHIK (%)	Negative DEN, JE and CHIK (%)
Single serum	15	15 (100.0)	-
Paired serum	203	72 (35.5)	131 (64.5)
Total	218	87 (39.9)	131 (60.1)

Table 17 Summary of the laboratory diagnosis of Arboviruses, *Flaviviruses* and *Alphavirus* antibody exposure (HI titers ≥1:10) in hill tribe patients interpreted by HI test

Serum group	Total examined	Arboviruses (DEN & JE & CHIK)		Flaviviruses (DEN & JE)		Alphavirus (CHIK)	
		Positive (≥1:10) (%)	Negative (< 1:10) (%)	Positive (≥1:10) (%)	Negative (< 1:10) (%)	Positive (≥1:10) (%)	Negative (< 1:10) (%)
Single serum	190	136 (71.6)	54 (28.4)	127 (66.8)	63 (33.2)	148 (77.9)	42 (22.1)
Paired serum	203	157 (77.3)	46 (22.7)	143 (70.4)	60 (29.6)	123 (60.6)	80 (39.4)
Total	393	293 (74.6)	100 (25.4)	270 (68.7)	123 (31.3)	271 (39.0)	122 (31.0)

The prevalence rate of arbovirus infections interpreted by MAC ELISA and HI test according to the criteria of WHO and prevalence of antibodies exposure interpreted by HI test (HI titers ≥ 1:10), the results showed that prevalence rate of arbovirus infections was 39.9% (87/218 cases) and arbovirus antibodies exposure was 74.6% (293/393 cases). When comparison prevalence of arbovirus infections and prevalence of antibodies exposure analyzed by Cochran Q test the result showed a significant difference (p-value < 0.05). Data are shown in Table 18.

Table 18 Comparison of prevalence of arbovirus infections and prevalence of antibodies exposure.

	Arbovirus infections			Arbovirus antibodies exposure		
Serum group	Total	Positive	Negative	Total	Positive	Negative
Single serum	15	15 (100.0)	-	190	136 (71.6)	54 (28.4)
Paired serum	203	72 (35.5)	131 (64.5)	203	157 (77.3)	46 (22.7)
Total	218	87 (39.9)	131 (60.1)	393	293 (74.6)	100 (25.4)
p-value		< 0.001*				

(exclude uninterpreted cases of arbovirus infections)

Validity of acute phase sera interpretation

The validity of co-interpretation by HI test and MAC ELISA was performed to find out if a possible single sera group could be used for analyzing the factors related to arbovirus infections. The acute phase interpretation of the paired sera group were used to compare with acute-convalescent phase interpretation as a gold standard. It was found that the sensitivity, when interpreted only acute phase, was 20.8%, while specificity was 100.0%, as shown in table 19.

Since acute phase sensitivity was very low, so that the single sera group can not be used to analyze the related factors. Hence, only the paired sera group and some positive cases from the single sera group were combined for further analysis.

Table 19 Validity of co-interpretation (HI and MAC ELISA) in acute phase compared with acute-convalescent phases of paired sera group as a gold standard

Paired serum group	Acute-convalescent phase interpretation (cases)		Total
Acute phase Interpretation (cases)	Positive	Negative	
Positive	15 (a)	- (b)	15 (a+b)
Negative	57 (c)	131 (d)	188 (c+d)
Total (cases)	72 (a+c)	131 (b+d)	203 (a+b+c+d)

Sensitivity of test = a / a+c = 15 / 72 = 20.8%

Specificity of test = d/ b+d = 131 /131 = 100.0%

Accuracy of test = a+d / a+b+c+d = 146 / 203 = 71.9%

Positive Predictive Value (PPV) = a / a+b = 15 / 15 = 100.0%

Negative Predictive Value (NPV) = d / c+d = 131 / 188 = 69.7%

Prevalence of arbovirus infections and arbovirus antibodies exposure

The prevalence rate of arbovirus infections was 39.9% (87/218 cases) and arbovirus antibodies exposure was 74.6% (293/393 cases) (Table 18). Female patients had a higher prevalence rate higher than males both in infection (42.2% and 37.9%) and exposure (76.3% and 72.9%). The age groups were classified and found that the highest prevalence infection rate was in the age group 1-4 years old (100.0%). On the other hand, this age group had the lowest antibodies exposure prevalence rate. Figure 5 showed percentage in each age group of arbovirus infections and arbovirus antibodies exposure. When classified by the hospital where the patients came for treatment, the highest infection prevalence rate and antibodies exposure rate was found in Phrao Hospital (42.5% and 79.7%). Karen was the group of hill tribe patients found to have the highest infection prevalence rate (46.1%) when compared with the others, while Palong had the highest antibodies exposure prevalence rate (85.7%). A high prevalence of infection was found in student groups (61.3%), where as the forest item gatherer and business groups had high antibodies exposure (100.0%). All details are shown in Table 20.

Table 20 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data.

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(1) Sex						
Male	44 (37.9)	72 (62.1)	116	148 (72.9)	55 (27.1)	203
Female	43 (42.2)	59 (57.8)	102	145 (76.3)	45 (23.7)	190
(2) Age group (years old)						
1- 4	1(100.0)	-	1	1 (50.0)	1 (50.0)	2
5- 9	11 (47.8)	12 (52.2)	23	34 (75.6)	11 (24.4)	45
10-14	25 (65.8)	13 (34.2)	38	42 (73.7)	15 (26.3)	57
15-19	17 (43.6)	22 (56.4)	39	55 (77.5)	16 (22.5)	71
20-24	6 (24.0)	19 (76.0)	25	35 (77.8)	10 (22.2)	45
25-29	5 (23.8)	16 (12.2)	21	25 (71.4)	10 (28.6)	35
30-34	6 (37.5)	10 (62.5)	16	21 (70.0)	9 (30.0)	30
35-39	9 (33.3)	18 (66.7)	27	31 (73.8)	11 (26.2)	42
≥ 40	7 (25.0)	21 (75.0)	28	49 (74.2)	17 (25.8)	66

Table 20 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data. (continued)

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(3) Hospital treatment						
Samoeng	35 (44.3)	44 (55.7)	79	95 (73.6)	34 (26.4)	129
Mae Taeng	15 (34.9)	28 (65.1)	43	50 (71.4)	20 (28.6)	70
Phrao	17 (47.2)	19 (52.8)	36	55 (79.7)	14 (20.3)	69
Chiang Dao	20 (33.3)	40 (66.7)	60	93 (74.4)	32 (25.6)	125
(4) Hill tribe group						
Karen	41 (46.1)	48 (53.9)	89	101 (72.1)	39 (27.9)	140
Meo	11 (36.7)	19 (63.3)	30	48 (77.4)	14 (22.6)	62
Lehu	-	4(100.0)	4	67 (77.0)	20 (23.0)	87
Lisu	20 (45.5)	24 (54.5)	44	57 (72.2)	22 (27.8)	79
Akha	15 (32.6)	31 (67.4)	46	7 (77.8)	2 (22.2)	9
Lua	-	2(100.0)	2	7 (77.8)	2 (22.2)	9
Palong	-	3(100.0)	3	6 (85.7)	1 (14.3)	7

Table 20 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data. (continued)

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(5) Marital status						
Single	56 (50.5)	55 (49.5)	111	134 (72.4)	51 (27.6)	185
Married	29 (28.4)	73 (71.6)	102	153 (75.7)	49 (24.3)	202
Widow	2 (40.0)	3 (60.0)	5	6(100.0)	-	6
(6) Religion						
Buddhist	52 (37.1)	88 (62.9)	140	179 (72.8)	67 (27.2)	246
Christian	34 (44.7)	42 (55.3)	76	112 (77.8)	32 (22.2)	144
Islam	-	-	-	1(100.0)	-	1
Other	1 (50.0)	1 (50.0)	2	1 (50.0)	1 (50.0)	2
(7) Education						
None	25 (27.5)	66 (72.5)	91	142 (77.2)	42 (22.8)	184
Prathom 1-6	50 (45.5)	60 (54.5)	110	125 (69.8)	54 (30.2)	179
Mathayom 1-3	8 (66.7)	4 (33.3)	12	20 (95.2)	1 (4.8)	21
Mathayom 4-6	4 (80.0)	1 (20.0)	5	5 (71.4)	2 (28.6)	7

Table 20 Prevalence of arbovirus infection and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data. (continued)

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
Vacational	-	-	-	1(100.0)	-	1
Bachelor's degree	-	-	-	-	1 (100.0)	1
(8) Occupation						
Agriculture	33 (30.6)	75 (69.4)	108	149 (74.5)	51 (25.5)	200
Farmers	7 (50.0)	7 (50.0)	14	11 (57.9)	8 (42.1)	19
Forest item-gatherer	-	-	-	1(100.0)	-	1
Employee	7 (25.0)	21 (75.0)	28	32 (71.1)	13 (28.9)	45
Business	-	-		1(100.0)	-	1
Student	38 (61.3)	24 (38.7)	62	85 (78.0)	24 (22.0)	109
Unemployed	2 (33.3)	4 (66.7)	2	14 (77.8)	4 (22.2)	18

Table 20 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data. (continued)

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(9) Income (Baht per month)						
None	44 (60.3)	29 (39.7)	73	98 (76.0)	31 (24.0)	129
1-1000	25 (36.8)	43 (63.2)	68	92 (70.8)	38 (29.2)	130
1001-2000	14 (34.1)	27 (65.9)	41	57 (75.0)	19 (25.0)	76
2001-3000	1 (12.5)	7 (87.5)	8	16 (80.0)	4 (20.0)	20
3001-4000	3 (16.7)	15 (83.3)	18	16 (76.2)	5 (23.8)	21
4001-5000	-	7 (100.3)	7	8 (72.7)	3 (27.3)	11
≥ 5001	-	3 (10.0)	3	6(100.0)	-	6
(10) Migration						
Yes	9 (29.0)	22 (71.0)	31	52 (73.2)	19 (26.8)	71
No	78 (89.7)	109 (58.3)	187	241 (74.8)	81 (25.2)	322

Table 20 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by general demographic data. (continued)

General demographic data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(10.1) Cause of migration						
	(n=9)	(n=23)	(n=32)	(n=52)	(n=19)	(n=71)
To change place for agriculture	4 (16.0)	21 (84.0)	25	40 (71.4)	16 (28.6)	56
To employee	5 (71.4)	2 (28.6)	7	10 (76.9)	3 (23.1)	13
To escape natural danger	-	-	-	2(100.0)	-	2

The information about present illness of arbovirus infections and arbovirus antibodies exposure found that patients had fever 100.0%, most common symptoms were anorexia, malaise and nausea (65.5%, 64.4%, 46.0% and 67.0%, 66.2%, 41.3%, respectively). Signs of jaundice were found in infection (68.0%). Almost all of them had traditional treatment before coming to hospital (both equally 80.0%). They never had JE vaccination before (38.2% and 74.3%, respectively), and no history of sickness

with DHF (38.6% and 74.1%, respectively). They had a prior history of being bitten by *Aedes spp.* mosquitoes (54.7% and 85.6% respectively). The details are shown in Table 21.

Table 21 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present illness.

Present illness	Arbovirus infection			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(1) Sypmtoms (the answer may more than one)						
Malaise	56 (64.4)	91 (69.5)	147	194 (66.2)	60 (60.0)	254
Fever	87(100.0)	131(100.0)	218	293(100.0)	100(100.0)	393
Chills	29 (33.3)	45 (34.4)	74	108 (36.9)	40 (40.0)	148
Eye pit pain	6 (6.9)	10 (7.6)	16	21 (7.2)	6 (6.0)	27
Muscle pain	32 (36.8)	62 (47.3)	94	122 (41.6)	44 (44.0)	166
Expistaxis, hematemesis, malena	4 (4.6)	1 (0.8)	5	5 (1.7)	1 (1.0)	6
Anorexia	57 (65.5)	94 (71.8)	151	205 (67.0)	63 (63.0)	268
Nausea	40 (46.0)	46 (35.1)	86	121 (41.3)	42 (42.0)	163
Vomiting	37 (42.5)	41 (31.3)	78	107 (36.5)	34 (34.0)	141

Table 21 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present illness. (continued)

Present illness	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
Abdominal pain	18 (20.7)	24 (18.3)	42	51 (17.4)	16 (16.0)	67
Diarrhea	21 (24.1)	26 (19.8)	47	52 (17.8)	18 (18.0)	70
Right costal margin pain	6 (6.9)	8 (6.1)	14	15 (5.1)	5 (5.0)	20
Stiffness of neck and back	1 (1.2)	1 (0.8)	2	2 (0.7)	1 (1.0)	3
Unconsciousness, convulsion	1 (1.2)	-	1	2 (0.7)	-	2
(2) Signs	(n=25)	(n=28)	(n=53)	(n=41)	(n=9)	(n=91)
Conjunctivitis	3 (12.0)	5 (17.9)	8	9 (69.2)	4 (44.4)	13
Rash on face / extremities body	5 (20.0)	6 (21.4)	11	17 (41.5)	1 (11.2)	18
Jaundice	17 (68.0)	17 (60.7)	34	15 (36.6)	4 (44.4)	19

Table 21 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present illness. (continued)

Present illness	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(3) Activity before came to hospital						
Nothing	44 (42.3)	60 (57.7)	104	154 (77.8)	44 (22.2)	198
Went to clinic	2 (33.3)	4 (66.7)	6	4 (57.1)	3 (42.9)	7
Went to primary health care service	27 (40.9)	39 (59.1)	66	92 (74.2)	32 (25.8)	124
Bought some medicine from drug store	8 (30.8)	18 (69.2)	26	23 (63.9)	13 (36.1)	36
Went to hospital	1 (20.0)	4 (80.0)	5	7 (77.8)	2 (22.2)	9
Traditional treatment	4 (80.0)	1 (20.0)	5	8 (80.0)	2 (20.0)	10
Other	1 (16.7)	5 (83.3)	6	5 (55.6)	4 (44.4)	9

Table 21 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present illness. (continued)

Present illness	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(4) Type of medicine bought						
	(n=8)	(n=18)	(n=26)	(n=23)	(n=13)	(n=36)
Antibiotics	-	1 (100.0)	1	4 (80.0)	1 (20.0)	5
Analgesic antipyretics	8 (33.3)	16 (66.7)	24	18 (60.0)	12 (40.0)	30
Herbal	-	1 (100.0)	1	1(100.0)	-	1
(5) History of immunized with JE vaccine						
Yes	11 (57.9)	8 (42.1)	19	24 (77.4)	7 (22.6)	31
No	76 (38.2)	123 (61.8)	199	269 (74.3)	93 (25.7)	362
(6) History of been sick with DHF						
Yes	6 (75.0)	2 (25.0)	8	12 (85.7)	2 (14.3)	14
No	81 (38.6)	129 (61.4)	210	281 (74.1)	98 (25.9)	379

Table 21 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present illness. (continued)

Present illness	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(7) History of being bitten by mosquitoes (1 week before sickness)						
No	9 (27.3)	24 (72.7)	33	39 (66.1)	20 (33.9)	59
Aedes spp.	35 (54.7)	29 (45.3)	64	101 (85.6)	17 (14.4)	118
Culex spp.	3 (42.9)	4 (57.1)	7	13 (76.5)	4 (23.5)	17
Anopheles spp.	2 (66.7)	1 (33.3)	3	4 (66.7)	2 (33.3)	6
Don't know	22 (36.1)	39 (63.9)	63	74 (73.3)	27 (26.7)	101
Not sure	16 (32.0)	34 (68.0)	50	62 (67.4)	30 (32.6)	92
(8) Time that mosquitoes often bite						
Day time	37 (42.0)	51 (58.0)	88	129 (78.7)	35 (21.3)	164
Night time	50 (38.5)	80 (61.5)	130	164 (71.6)	65 (28.4)	229

Personal behavior were asked about in the questionnaire and found that the prevalence of infection and the prevalence of antibody exposure during their working times from 8.00-20.00 were 33.3% and 79.5%, respectively. The prevalence rate in both groups working in urban area were 36.0% and 53.2%, respectively (Table 22).

Pig, bird and dog were the popular animals feeding. The majority of positive cases had coconut bark, can, bottle or water drainage tap close to their house (50.0% and 81.0%, respectively). Patients informed that *Aedes spp.* mosquitoes were often found in their house (57.4% and 85.1%, respectively) (Table 23).

Table 22 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present behaviors.

Present behaviors	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(1) Working time	(n=47)	(n=103)	(n=150)	(n=194)	(n=72)	(n=6)
8.00 – 16.00	40 (31.0)	89 (69.0)	129	163 (71.8)	64 (28.2)	227
8.00 – 20.00	7 (33.3)	14 (66.7)	21	31 (79.5)	8 (20.5)	39
(2) Clothed while working						
Covered clothing	24 (29.6)	57 (70.4)	81	112 (73.2)	41 (26.8)	153
Open clothing	23 (33.3)	46 (66.7)	69	82 (72.6)	31 (27.4)	113
(3) Methods of protection from mosquitoes						
Sleep under mosquito net	17 (44.7)	21 (55.3)	38	59 (78.7)	16 (21.3)	75
Use mosquito coil	24 (40.0)	36 (60.0)	60	71 (73.3)	28 (28.3)	99

Table 22 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by present behaviors. (continued)

Present behaviors	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
Use herbicide	3 (33.3)	6 (66.7)	9	11 (84.6)	2 (15.4)	13
Use repellent	2 (33.3)	4 (66.7)	6	4 (66.7)	2 (33.3)	6
Smoke	41 (39.0)	64 (61.0)	105	142 (73.2)	52 (26.8)	194
(4) Methods of removing breeding places of mosquitoes						
Remove garbage	5 (62.5)	3 (37.5)	8	21 (91.3)	2 (8.7)	23
Put abate sand in water	5 (71.4)	2 (28.6)	7	7 (70.0)	3 (30.0)	10
Use chemical insecticides	14 (34.1)	27 (65.9)	41	42 (75.0)	14 (25.0)	56
None	63 (38.9)	99 (61.1)	162	223 (73.4)	81 (26.2)	304

Table 23 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by environmental data.

Environmental data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
	(n=47)	(n=103)	(n=150)	(n=194)	(n=72)	(n=266)
(1) Environment of working area						
Farm and forest	15 (24.6)	46 (75.4)	61	82 (72.6)	31 (27.4)	113
In urban area	32 (36.0)	57 (64.0)	89	112 (53.2)	41 (26.8)	153
(2) Have animals feeding within 100 meters of house or working area (the answer may more than one)						
None	15 (32.6)	31 (67.4)	46	64 (71.1)	26 (28.9)	90
Pig	57 (48.7)	60 (51.3)	117	161 (79.7)	41 (20.3)	202
Dog	33 (35.5)	60 (64.5)	93	131 (77.5)	38 (22.5)	169
Bird	52 (44.4)	65 (55.6)	117	151 (73.7)	54 (26.3)	205
Cows, buffalo	6 (31.6)	13 (68.4)	19	14 (51.9)	13 (48.1)	27
Not in category	10 (31.3)	22 (68.7)	32	39 (68.4)	18 (31.6)	57
(3) There are containers or ground water pits close to house						
No	4 (14.8)	23 (85.2)	27	42 (70.0)	18 (30.0)	60
Big jar	64 (43.0)	85 (57.0)	149	190 (74.2)	66 (25.8)	256

Table 23 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by environmental data. (continued)

Environmental data	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
Coconut bark, can, bottle	13 (50.0)	13 (50.0)	26	44 (78.6)	12 (21.4)	56
Water drainage tap	6 (37.5)	10 (62.5)	16	17 (81.0)	4 (19.0)	21
(4) Type of mosquitoes often found in house						
Aedes spp.	27 (57.4)	30 (52.6)	57	80 (85.1)	14 (14.9)	94
Culex spp.	3 (42.9)	4 (57.1)	7	8 (66.7)	4 (33.3)	12
Anopheles spp.	1 (50.0)	1 (50.0)	2	4 (66.7)	2 (33.3)	6
Don't know	56 (36.8)	96 (63.2)	152	201 (71.5)	80 (28.5)	281
(5) Places to find mosquitoes in house						
Bathroom	10 (35.7)	18 (64.3)	28	34 (70.8)	14 (29.2)	48
Under floor of house	3 (60.0)	2 (40.0)	5	9 (90.0)	1 (10.0)	10
Corner of room and closet	7 (38.9)	11 (61.1)	18	29 (72.5)	11 (27.5)	40
General area	67 (40.1)	100(59.9)	167	221 (74.9)	74 (25.1)	295

Table 24 showed the background knowledge of hill tribe patients in this study. They did not know the cause of DHF (39.0% and 74.8%, respectively), the cause of JE (40.0% and 74.5%, respectively), the vector borne mosquitoes (39.8% and 75.0%, respectively), did not know the signs and symptoms of DHF (38.9% and 74.6%, respectively) and did not know the signs and symptoms of JE (39.6% and 74.3%, respectively).

Table 24 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by knowledge.

Items of knowledge	Arbovirus infections			Arbovirus antibodies expossure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(1) Know cause of DHF is *Aedes spp.* mosquitoes						
Yes	34 (41.5)	48 (58.5)	82	100 (74.1)	35 (25.9)	135
No	53 (39.0)	83 (61.0)	136	193 (74.8)	65 (25.2)	258
(2) Know cause of JE is *Culex spp* mosquitoes						
Yes	3 (37.5)	5 (62.5)	8	15 (75.0)	5 (25.0)	20
No	84 (40.0)	126 (60.0)	210	278 (74.5)	95 (25.5)	373
(3) Know *Aedes spp.*and *Culex spp.*						
Know both mosquitoes	14 (51.9)	13 (48.1)	27	35 (77.8)	10 (22.2)	45

Table 24 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by knowledge. (continued)

Items of knowledge	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
Know *Aedes spp.* but don't know *Culex spp.*	20 (35.1)	37 (64.9)	57	78 (72.9)	29 (27.1)	107
Know *Culex spp* but don't know *Aedes spp.*	-	1(100.0)	1	-	1 (100.0)	1
Didn't know both mosquitoes	53 (39.8)	80 (60.2)	133	180 (75.0)	60 (25.0)	240
(4) Know what season DHF and JE frequently occured						
Summer	-	2(100.0)	2	4 (80.0)	1 (20.0)	5
Rainy season	43 (43.9)	55 (56.1)	98	120 (73.2)	44 (26.8)	164
Winter	-	-	-	-	-	-
Don't know	44 (37.3)	74 (62.7)	118	169 (75.4)	55 (24.6)	224
(5) Know signs and symptoms of DHF						
Yes	10 (50.0)	10 (50.0)	20	29 (74.4)	10 (25.6)	39
No	77 (38.9)	121 (61.1)	198	264 (74.6)	90 (25.4)	354

Table 24 Prevalence of arbovirus infections and prevalence of arbovirus antibodies exposure in hill tribe patients, classified by knowledge. (continued)

Items of knowledge	Arbovirus infections			Arbovirus antibodies exposure		
	Positive case (%) (n=87)	Negative case (%) (n=131)	Total case (n=218)	Positive case (%) (n=293)	Negative case (%) (n=100)	Total case (n=393)
(6) To know signs and symptoms of JE						
Yes	3 (50.0)	3 (50.0)	6	12 (80.0)	3 (20.0)	15
No	84 (39.6)	128 (60.4)	212	281 (74.3)	97 (25.7)	378

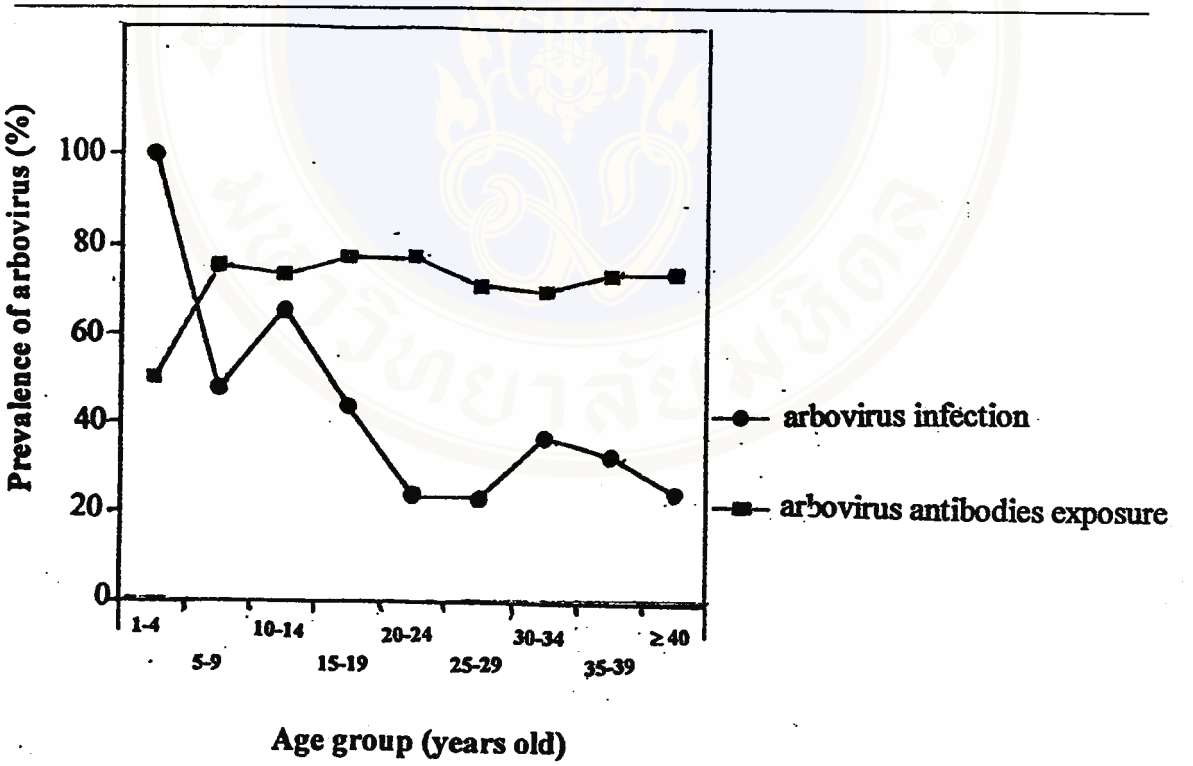


Figure 7 Prevalence of arbovirus infections and arbovirus antibodies exposure in each age group.

Geometric mean titers (GMT) of acute and convalescent serum

The geometric mean titers (GMT) and standard deviation (SD) of HI antibodies against DEN-1, DEN-2, DEN-3, DEN-4, JE and Chikungunya antigens in 393 acute and 203 convalescent sera in studied subjects were classified by age group.

In either acute or convalescent sera, the GMT ± SD of all age groups were found highest in DEN-3, and showed titers of 20.3 ± 4.5 and 38.9 ± 10.5, respectively. When classified by age group, GMT ± SD of acute sera were not very different according to each antigen, while GMT ± SD of convalescent sera were found highest for all antigens in 10-14 years old. Details are shown in Table 25.

The GMT ± SD of sex and hospitals treatment were analyzed and showed that there were no difference between males and females, either in acute or convalescent antigen. Hospital treatment center showed little difference in GMT ± SD, except at Phrao Hospital, where the GMT was higher than the others when compared by both phases of sera for each antigen. Details are shown in Table 26 and Table 27.

Table 25 Geometric mean titers (GMT) and standard deviation (SD) against arbovirus antigens by HI test in the hill tribe patients, classified by age group and phase of serum.

Age group	Serum phase	GMT ± SD by HI test					
		DEN- 1	DEN-2	DEN-3	DEN– 4	JE	CHIK
1-4	Acute	28.3 ±11.6	20.0 ± 7.1	28.3 ±11.6	20.0 ± 7.1	28.2 ±11.5	19.9 ± 7.1
	Convalescent	-	-	-	-	-	-
5-9	Acute	15.4 ± 3.6	16.4 ± 3.5	20.6 ± 3.7	16.4 ± 3.8	18.8 ± 3.7	7.0 ± 1.7
	Convalescent	45.3 ± 8.3	41.3 ± 8.9	45.4 ± 8.9	49.9 ± 9.9	66.2 ± 9.3	10.6 ± 2.8
10-14	Acute	21.2 ± 4.6	21.5 ± 4.8	24.3 ± 5.5	22.0 ± 4.6	24.0 ± 4.2	8.1 ± 2.1
	Convalescent	121.2 ±15.8	119.0 ±15.4	136.6 ±17.9	123.8 ±15.4	95.6 ±10.2	13.2 ± 3.0
15-19	Acute	19.4 ± 4.4	20.4 ± 4.1	25.5 ± 4.8	22.9 ± 4.5	24.8 ± 3.9	6.4 ± 1.7
	Convalescent	48.3 ±13.8	44.8 ±12.3	50.0 ±12.4	51.1 ±12.8	40.0 ± 5.8	8.4 ± 2.2
20-24	Acute	16.6 ± 4.2	16.9 ± 4.0	22.6 ± 4.5	20.0 ± 4.3	18.8 ± 3.0	7.6 ± 2.3
	Convalescent	19.4 ± 8.7	21.8 ± 7.8	23.1 ± 8.3	17.4 ± 6.9	18.9 ± 3.9	10.0 ± 2.9

Table 25 Geometric mean titers (GMT) and standard deviation (SD) against arbovirus antigens by HI test in the hill tribe patients, classified by age group and phase of serum.(continued)

Age group	Serum phase	GMT ± SD by HI test					
		DEN- 1	DEN-2	DEN-3	DEN– 4	JE	CHIK
25-29	Acute	10.0 ± 2.6	9.8 ± 2.5	12.9 ± 3.1	11.5 ± 2.8	14.6 ± 3.1	6.3 ± 1.7
	Convalescent	14.9 ± 4.5	13.5 ± 4.2	18.1 ± 4.8	17.0 ± 4.1	24.4 ± 4.1	7.7 ± 2.5
30-34	Acute	18.2 ± 5.3	14.8 ± 4.5	21.0 ± 5.4	18.2 ± 5.6	15.2 ± 3.3	7.8 ± 2.0
	Convalescent	13.8 ± 5.5	12.4 ± 4.9	15.3 ± 6.1	15.3 ± 6.1	16.2 ± 4.9	5.9 ± 1.5
35-39	Acute	13.9 ± 3.9	13.9 ± 3.6	15.6 ± 4.3	14.9 ± 4.1	16.7 ± 3.3	7.9 ± 2.4
	Convalescent	27.9 ± 7.4	28.7 ± 6.1	38.9 ± 8.7	35.8 ± 8.0	36.8 ± 4.6	7.4 ± 1.9
≥40	Acute	15.4 ± 4.1	15.9 ± 4.4	18.4 ± 4.2	17.1 ± 4.7	17.3 ± 3.3	6.6 ± 1.7
	Convalescent	17.2 ± 7.2	17.5 ± 6.7	19.5 ± 7.3	21.1 ± 9.0	21.7 ± 5.0	8.5 ± 3.0
Total GMT	Acute	16.4 ± 4.1	16.5 ± 4.0	20.3 ± 4.5	18.1 ± 4.3	19.2 ± 3.6	7.2 ± 2.0
± SD	Convalescent	33.9 ±10.2	32.9 ± 9.5	38.9 ±10.5	36.8 ±10.2	36.0 ± 6.5	9.1 ± 2.6

Table 26 Geometric mean titers (GMT) and standard deviation (SD) against arbovirus antigens by HI test in the hill tribe patients, classified by sex and phase of serum.

Sex	Serum phase	GMT ± SD by HI test					
		DEN- 1	DEN-2	DEN-3	DEN– 4	JE	CHIK
Male	Acute	15.7 ± 4.1	15.4 ± 4.1	19.5 ± 4.7	17.8 ± 4.4	19.1 ± 3.6	7.4 ± 2.1
	Convalescent	29.9 ± 9.9	28.0 ± 8.6	33.7 ±10.2	33.0 ± 9.9	35.3 ± 6.7	8.3 ± 2.3
Female	Acute	17.2 ± 4.1	17.7 ± 4.0	21.2 ± 4.3	18.5 ± 4.2	19.3 ± 3.6	7.0 ± 1.9
	Convalescent	38.9 ±10.7	39.2 ±10.5	45.4 ±10.8	41.2 ±10.6	38.6 ± 6.2	10.1 ± 2.9
Total GMT ± SD	Acute	16.4 ± 4.1	16.5 ± 4.0	20.3 ± 4.5	18.1 ± 4.3	19.2 ± 3.6	7.2 ± 2.0
	Convalescent	33.9 ±10.2	32.9 ± 9.5	38.9 ±10.5	36.8 ±10.2	36.8 ± 6.5	9.1 ± 2.6

Table 27 Geometric mean titers (GMT) and standard deviation (SD) against arbovirus antigens by HI test in hill tribe patients, classified by hospital treatment and phase of serum.

Hospital	Serum phase	GMT ± SD by HI test					
		DEN- 1	DEN-2	DEN-3	DEN– 4	JE	CHIK
Samoeng	Acute	15.1 ± 4.3	14.7 ± 3.9	17.3 ± 4.5	16.4 ± 4.3	17.0 ± 3.5	7.4 ± 2.1
	Convalescent	38.5 ±11.9	36.1 ±11.2	42.7 ±11.8	41.1 ±11.4	37.5 ± 7.2	9.2 ± 2.6
Mae Taeng	Acute	13.9 ± 3.8	14.9 ± 3.9	17.1 ± 4.4	14.7 ± 3.9	18.5 ± 3.5	6.9 ± 1.9
	Convalescent	22.9 ± 7.8	24.5 ± 9.2	27.6 ± 9.9	24.9 ± 8.3	32.1 ± 6.4	10.5 ± 3.3
Phrao	Acute	23.2 ± 4.4	24.5 ± 4.0	33.7 ± 4.4	26.8 ± 4.5	24.2 ± 4.1	6.8 ± 1.7
	Convalescent	66.6 ±14.1	59.0 ±11.3	81.7 ±13.6	66.9 ±14.1	51.1 ± 7.7	8.2 ± 2.0
Chiang Dao	Acute	16.2 ± 3.9	15.8 ± 4.1	20.0 ± 4.4	18.2 ± 4.3	19.6 ± 3.4	7.3 ± 2.0
	Convalescent	24.9 ± 7.5	25.0 ± 6.5	27.7 ± 7.0	28.5 ± 7.6	32.4 ± 5.0	8.5 ± 2.4
Total GMT	Acute	16.4 ± 4.1	16.5 ± 4.0	20.3 ± 4.5	18.1 ± 4.3	19.2 ± 3.6	7.2 ± 2.0
± SD	Convalescent	33.9 ±10.2	32.9 ± 9.5	38.9 ±10.5	36.8 ±10.2	36.8 ± 6.5	9.1 ± 2.6

Comparison of geometric mean titers (GMT) of arbovirus antigens

Comparison of GMT in acute and convalescent serum for each antigens (DEN-1, DEN-2, DEN-3, DEN-4, JE and Chikungunya) by Friedman test showed the statistically significant difference between each antigen of Dengue (type 1, 2, 3, 4) and JE virus (*p*-value < 0.05) while in Chikungunya virus showed a statistically not significant in either acute or convalestcent serum. Data shown in Table 28.

Table 28 Comparison of GMT in acute and convalescent serum for arbovirus antigens

	GMT ±SD					
Serum phase	DEN-1	DEN-2	DEN-3	DEN-4	JE	CHIK
Acute	16.4± 4.1	16.5±4.0	20.3±4.5	18.1± 4.3	19.2±3.6	7.2±2.0
Convalescent	33.9±10.2	32.9±9.5	38.9±10.5	36.8±10.2	36.8±6.5	9.1±2.6
p-value	< 0.001*	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.130

All 393 acute and 203 convalescent patients sera, were assayed by MAC ELISA and GAC ELISA for Dengue and JE antigens, and were calculated for X ± SD units. When classified by sex, hospital treatment and age group the mean of antibody units against Dengue and JE antigens, either assayed by dengue MAC ELISA and dengue GAC ELISA or JE MAC ELISA and JE GAC ELISA, found that all convalescent sera showed higher unit mean titers than in acute sera. Details are shown in Table 29-31.

Table 29 Mean and standard deviation (SD) of *Flaviviruses* antibody units assayed by MAC ELISA and GAC ELISA, classified by sex.

Sex	Serum phase	DENGUE X ± SD		JE X ± SD	
		MAC ELISA	GAC ELISA	MAC ELISA	GAC ELISA
Male	Acute	9.4 ± 2.9	7.5 ± 2.6	7.2 ± 2.8	9.8 ± 2.8
	Convalescent	12.5 ± 4.1	14.6 ± 4.4	8.9 ± 3.2	12.5 ± 3.5
Female	Acute	9.3 ± 3.2	8.2 ± 2.6	7.7 ± 2.9	10.3 ± 2.5
	Convalescent	14.3 ± 4.3	13.0 ± 4.0	10.7 ± 3.1	11.4 ± 3.1
Total	Acute	9.3 ± 3.0	7.8 ± 2.6	7.4 ± 2.8	10.0 ± 2.6
Mean ± SD	Convalescent	13.4 ± 4.2	13.8 ± 4.2	9.7 ± 3.2	11.9 ± 3.3

Table 30 Mean and standard deviation (SD) of *Flaviviruses* antibody units assayed by MAC ELISA and GAC ELISA, classified by hospital treatment.

		DENGUE X ± SD		JE X ± SD	
Hospital	Serum phase	MAC ELISA	GAC ELISA	MAC ELISA	GAC ELISA
Samoeng	Acute	9.4 ± 3.3	7.1 ± 2.6	7.5 ± 2.8	9.8 ± 2.8
	Convalescent	13.6 ± 4.4	12.5 ± 4.3	9.1 ± 3.6	11.2 ± 3.1
Mae Taeng	Acute	9.1 ± 3.0	10.8 ± 2.1	9.1 ± 2.9	10.1 ± 2.7
	Convalescent	12.6 ± 4.8	15.2 ± 4.0	9.8 ± 2.9	12.2 ± 3.0
Phrao	Acute	9.5 ± 3.1	9.4 ± 2.4	6.9 ± 3.0	11.2 ± 2.4
	Convalescent	19.2 ± 3.5	24.7 ± 4.7	11.4 ± 2.9	24.2 ± 3.7
Chiang Dao	Acute	9.3 ± 2.8	6.7 ± 2.9	6.9 ± 2.7	9.6 ± 2.6
	Convalescent	10.6 ± 3.9	10.4 ± 3.7	9.7 ± 3.0	8.5 ± 3.1
Total	Acute	9.3 ± 3.0	7.8 ± 2.6	7.4 ± 2.8	10.0 ± 2.6
Mean ± SD	Convalescent	13.4 ± 4.2	13.8 ± 4.2	9.7 ± 3.2	11.9 ± 3.3

Table 31 Mean and standard deviation (SD) of *Flaviviruses* antibody units assayed by MAC ELISA and GAC ELISA, classified by age group.

Age group	Serum phase	DENGUE X ± SD		JE X ± SD	
		MAC ELISA	GAC ELISA	MAC ELISA	GAC ELISA
1-4	Acute	16.0 ± 3.0	-	7.2 ±16.3	4.2 ± 1.6
	Convalescent	-	-	-	-
5-9	Acute	10.7 ± 3.0	8.8 ± 2.7	8.4 ± 3.4	9.1 ± 2.8
	Convalescent	14.2 ± 4.1	16.4 ± 3.1	13.0 ± 4.6	14.9 ± 3.1
10-14	Acute	10.6 ± 3.3	8.4 ± 2.5	8.7 ± 2.9	9.3 ± 2.3
	Convalescent	25.5 ± 4.4	21.1 ± 5.6	15.9 ± 3.1	21.9 ± 3.2
15-19	Acute	11.1 ± 3.2	8.9 ± 2.4	8.9 ± 2.5	9.4 ± 2.8
	Convalescent	13.4 ± 5.7	23.8 ± 3.6	9.4 ± 3.0	12.9 ± 3.0
20-24	Acute	8.7 ± 2.8	7.3 ± 2.1	7.1 ± 3.2	10.4 ± 2.1
	Convalescent	8.5 ± 3.7	7.4 ± 3.6	12.1 ± 1.8	8.4 ± 2.8
25-29	Acute	5.6 ± 3.1	5.0 ± 2.7	6.5 ± 2.5	8.7 ± 2.7
	Convalescent	9.8 ± 3.0	6.6 ± 2.6	6.7 ± 3.8	6.6 ± 3.2
30-34	Acute	10.3 ± 2.8	8.1 ± 2.5	5.6 ± 2.4	11.2 ± 2.5
	Convalescent	11.5 ± 2.5	5.1 ± 3.3	5.6 ± 2.4	6.0 ± 1.8
35-39	Acute	8.4 ± 3.0	5.2 ± 3.2	6.5 ± 2.8	12.5 ±2.4
	Convalescent	11.8 ± 3.7	18.1 ± 2.9	8.7 ± 2.5	14.7 ± 3.4
≥40	Acute	8.5 ± 2.9	9.7 ± 2.7	6.8 ± 2.9	11.2 ± 3.1
	Convalescent	9.9 ± 4.1	14.6 ± 5.5	6.2 ± 3.3	10.0 ± 4.0
Total Mean ± SD	Acute	9.3 ± 3.0	7.8 ± 2.6	7.4 ± 2.8	10.0 ± 2.6
	Convalescent	13.4 ± 4.2	13.8 ± 4.2	9.9 ± 3.2	11.9 ± 3.3

Factors related to arbovirus infections

Factors related to arbovirus infections were analyzed by Chi-square test, Fisher exact test and logistic regression. The results are shown in Table 32, the factors related to arbovirus infections with statistical significant difference (p-value < 0.05) among hill tribe patients were age, occupation, income, history of being bitten by mosquitoes before sickness and having containers or ground water pits close to house.

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals.

Factors	Arbovirus infections		χ^2	p-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(1) Sex						
Male	44 (37.9)	72 (62.1)	0.404	0.525		
Female	43 (42.2)	59 (57.8)			0.7-2.1	1.2
(2) Age (years old)						
< 15	37 (59.7)	25 (40.3)	14.120	< 0.001*	1.7-5.8	3.1
≥ 15	50 (32.1)	106 (67.9)				

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections		χ^2	*p*-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(3) Hill tribe group						
Karen	41 (46.1)	48 (53.9)	12.279**	0.371	1.0-11.1	1.5
Meo	11 (36.7)	19 (63.3)	df = 6			
Lahu	-	4 (100.0)				
Lisu	20 (45.5)	24 (54.5)				
Akha	15 (32.6)	31 (67.4)				
Lua	-	2 (100.0)				
Palong	-	3 (100.0)				
(4) Hospital treatment						
Samoeng	35 (44.3)	44 (55.7)	2.985**	0.313	0.9-1.6	1.5
Mae Taeng	15 (34.9)	28 (65.1)	df = 3			
Phrao	17 (47.2)	19 (52.8)				
Chiang Dao	20 (33.3)	40 (66.7)				

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections		χ^2	*p*-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(5) Occupation						
Agriculture, farmer, forest item gatherer	40 (32.8)	82 (67.2)	5.859	0.015*		
Business, employee student, unemployed	47 (49.0)	49 (51.0)			1.1-3.4	1.9
(6) Income (Baht)						
≤ 2000	83 (45.6)	99 (54.4)	14.910	< 0.001*	2.3-19.6	6.7
> 2000	4 (11.1)	32 (88.9)				
(7) Migration						
Yes	9 (29.0)	22 (71.0)	1.782	0.182		
No	78 (89.7)	109 (58.3)			1.3-4.0	1.7
(8) Work time	(n= 47)	(n=103)				
8.00 – 16.00	40 (31.0)	89 (69.0)	0.045	0.831		
8.00 – 20.00	7 (33.3)	14 (66.7)			0.4-2.9	1.1

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections		χ^2	*p*-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(9) Clothed while working	(n=47)	(n=103)				
Covered cloth	24 (29.6)	57 (70.4)	0.2380	0.626		
Open cloth	23 (33.3)	46 (66.7)			0.6-2.4	1.2
(10) History of immunized with JE vaccine						
Yes	11 (57.9)	8 (42.1)	2.808	0.094	0.9-5.8	2.2
No	76 (38.2)	123 (61.8)				
(11) History of been sick with DHF						
Yes	6 (75.0)	2 (25.0)	f	0.062	0.9-24.2	4.8
No	81 (38.6)	129 (61.4)				
(12) History of being bitten by mosquitoes (1 week before sickness)						
Yes (*Aedes spp.* *Culex spp.* and *Anopheles spp.*)	40 (54.1)	34 (45.9)	9.348	0.002*	1.4-4.3	2.3
Other (Not sure, don't know)	47 (32.6)	97 (67.4)				

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections		χ^2	*p*-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(13) Time that mosquitoes bite						
Day time	37 (42.0)	51 (58.0)	0.281	0.596	0.7-2.0	1.2
Night time	50 (38.5)	80 (61.5)				
(14) Having animals feeding within working area or house						
Yes	72 (41.9)	100 (58.1)	2.910	0.061	0.9-3.8	1.5
No	15 (32.6)	31 (67.4)				
(15) Methods of protection from mosquitoes						
Sleep under mosquito net	17 (44.7)	21 (55.3)	0.447	0.503	1.1-2.6	1.3
Use mosquito coil, use herbicide, use repellant, smoke	70 (38.9)	110 (61.1)				

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections		χ^2	*p*-value	95%CI	OR
	Positive case (%) (n= 87)	Negative case (%) (n= 131)				
(16) Methods of get rid of breeding mosquitoes place						
Yes (Get rid garbage and mosquitoes, breeding place, put abate sand, use chemical insecticides)	24 (42.9)	32 (57.1)	0.128	0.768	0.6-2.1	1.2
No	63 (38.9)	99 (61.1)				
(17) There are ground water pits close to house						
Yes (Big jar, coconut bark can, bottle, water drainage tap	83 (43.5)	108 (56.5)	8.091	0.004*	1.5-13.3	4.4
No	4 (14.8)	23 (85.2)				
(18) Know cause of DHF						
Yes	34 (41.5)	48 (58.5)	0.132	0.716	0.6-1.9	1.1
No	53 (39.0)	83 (61.0)				

Table 32 Factors related to arbovirus infections among hill tribe patients at four selected hospitals. (continued)

Factors	Arbovirus infections: Positive case (%) (n= 87)	Arbovirus infections: Negative case (%) (n= 131)	χ^2	*p*-value	95%CI	OR
(19) Know cause of JE						
Yes	3 (37.5)	5 (62.5)	f	1.000		
No	84 (40.0)	126(60.0)			0.3 -4.8	1.1
(20) Know *Aedes spp.* and *Culex spp.*						
Know (each or both mosquitoes)	34 (40.0)	51 (60.0)	0.025	0.982	0.6- 1.8	1.0
Don't know	53 (39.8)	80 (60.2)				
(21) Know what season DHF and JE mostly frenquently occurred						
Rainy season	43 (43.9)	55 (56.1)	1.170	0.279	0.8-2.3	1.4
Others	44 (36.7)	76 (63.3)				
(22) Know signs and symptoms of DHF						
Yes	10 (50.0)	10 (50.0)	0.935	0.333	0.6-3.9	1.6
No	77 (38.9)	121(61.1)				
(23) Know signs and symptoms of JE						
Yes	3 (50.0)	3 (50.0)	f	0.685	0.3-7.7	1.5
No	84 (39.6)	128 (60.4)				

df = 1 f = Fisher exact test ** = Logistic regression

* = Significant at p-value < 0.05

Day of blood samples collection

In either acute or convalescent single serum group and paired serum group, IgM positive antibodies were found in 54 cases (85 sera) for dengue infection, and 13 cases (17 sera) for JE infection, The data is shown in Table 33.

Most of the acute sera were collected on the 3rd day after onset of the disease (29.5%), followed by day 2 (24.9%) and day 4 (12.4%), while most of the convalescent sera were collected on day 16-30 (24.7%), day 14 (13.0%) and day 15 (12.5%), respectively. The duration of blood sample collection in acute sera was 1-30 days, while in convalescent sera was 2->121 days after the onset of the disease. The number of IgM antibody positive cases and sera for dengue and JE infection in acute sera, according to the day after the onset of disease, were detected in some cases at days 7 to 8, while in convalescent sera were detected in some cases at day 150. The majority were found to be positive during day 1 to day 30. All data is shown in detail in Table 34.

Table 33 Number of cases and sera for IgM positive in Dengue and JE, classified by group of serum.

Serum group	No. of positive IgM DEN		No. of positive IgM JE	
	cases	serum	cases	serum
Single serum group				
Acute +	11	11	4	4
Paired serum group				
Acute + , Convalescent -	3	3	-	-
Acute - , Convalescent +	9	9	5	5
Acute + , Convalescent +	31	62	4	8
Total	54	85	13	17

Table 34 Number of patients examined and positive IgM antibodies to Dengue and JE infection in acute and convalescent sera, classified by the days after the onset of the disease.

Days after the onset of the disease	No. of cases examined		No. of positive IgM antibodies serum			
	Acute serum cases (%)	Covalescent serum cases (%)	DEN		JE	
			Acute	Convalescent	Acute	Convalescent
1	21 (5.3)	-	1	-	-	-
2	98 (24.9)	1 (0.5)	13	-	4	-
3	116 (29.5)	2 (0.9)	16	-	2	-
4	49 (12.4)	7 (3.3)	4	2	-	-
5	44 (11.2)	7 (3.3)	5	2	1	-
6	14 (3.6)	2 (0.9)	3	-	-	-
7	24 (6.1)	4 (1.9)	2	1	1	-
8	9 (2.3)	-	1	-	-	-
9	2 (0.5)	-	-	-	-	-
10	3 (0.8)	2 (0.9)	-	-	-	-
11	2 (0.5)	9 (4.2)	-	-	-	-
12	3 (0.8)	13 (6.0)	-	2	-	2
13	1 (0.3)	13 (6.0)	-	3	-	-
14	4 (1.0)	28 (13.0)	-	5	-	2
15	1 (0.3)	27 (12.5)	-	4	-	1
16-30	2 (2.3)	53 (24.7)	-	5	-	3

Table 34 Number of patients examined and positive IgM antibodies to Dengue and JE infection in acute and convalescent sera, classified by the days after the onset of the disease. (continued)

Days after the onset of the disease	No. of cases examined		No. of positive IgM antibody serum			
	Acute serum cases (%)	Covalescent serum cases (%)	DEN		JE	
			Acute	Convalescent	Acute	Convalescent
31- 45	-	14 (6.5)	-	3	-	-
46- 60	-	4 (1.9)	-	1	-	-
61- 75	-	5 (2.3)	-	1	-	-
76- 90	-	5 (2.3)	-	1	-	-
91-105	-	3 (1.4)	-	1	-	-
106-120	-	6 (2.8)	-	3	-	-
≥ 121	-	10 (4.7)	-	6	-	1
Total	393(100.0)	215(100.0)	45	40	8	9

Isolation and identification of extra - sera for epidemiological study

One hundred suspected dengue virus infection patients who had fever during the study period were collected for isolation in C6/36 tissue culture cell, and identification by monoclonal antibody ELISA (DEN –1, DEN –2, DEN –3, DEN –4, JE and Flaviviruses). Isolation and identification for epidemic serotyping were kindly done by Associate Professor Dr. Charnchudhi Chanyasanha and using facilities supported by Department. of Virology, AFRIMS, Bangkok.

It was found that 3 cases were identified as DEN –1 infection and 1 case was identified as Flavivirus infections. (data not shown)

Table 35 showed the distribution of arbovirus infections cases during a period of specimen collection, May 1997 to April 1998 , the results found that cases gradually increased to a peak August and October (21 cases), November (18 cases) and September (10 cases), respectively which related to the rainfall data. No positive cases in May, February to April. The rainfall data at four selected studied hospitals during studied period and number of cases positive also shown in Figure 8.

Table 35 Number of arboviruses examined, positive cases and rainfall data during May 1997 to April 1998, classified by month.

Month	No. of case examined	No. of case positive	% of positive to cases examined	Number of rainy days in each hospital area*				Total rain volume per month (mm.) in each hospital area*			
				S	M	P	C	S	M	P	C
May	1	-	-	6	5	10	10	38.8	73.2	79.1	91.1
June	15	5	33.3	11	8	9	13	73.8	73.4	39.3	66.0
July	32	5	15.6	16	21	15	22	166.5	308.8	293.3	279.0
August	71	21	29.6	19	15	22	18	216.5	415.5	237.6	230.7
September	63	10	15.9	18	8	17	17	143.2	283.8	368.6	320.7
October	90	21	23.3	8	4	9	6	160.0	96.1	97.5	137.6
November	63	18	28.6	2	2	3	3	5.9	5.5	119.5	24.3
December	24	5	20.8	-	-	-	-	-	-	-	-
January	6	2	33.3	-	-	-	-	-	-	-	-

Table 35 Number of arboviruses examined, positive cases and rainfall data during May 1997 to April 1998, classified by month.

(continued)

Month	No. of case examined	No. of case positive	% of positive to cases examined	Number of rainy day in each hospital area*				Total rain volume per month (mm.) in each hospital area*			
				S	M	P	C	S	M	P	C
February	10	-	-	-	-	-	-	-	-	-	-
March	16	-	-	1	1	-	1	30.5	65.0	-	8.7
April	2	-	-	2	1	-	2	8.0	9.1	12.7	6.5
Total	393	87	22.1	83	65	88	92	843.2	1330.4	1247.6	1164.6

* Source : Computer Section, Climatology Division, Meteorogical Department, Bangkok, Thailand

S = Samoeng Hospital M= Mae Taeng Hospital

P = Phrao Hospital C = Chiang Dao Hospital

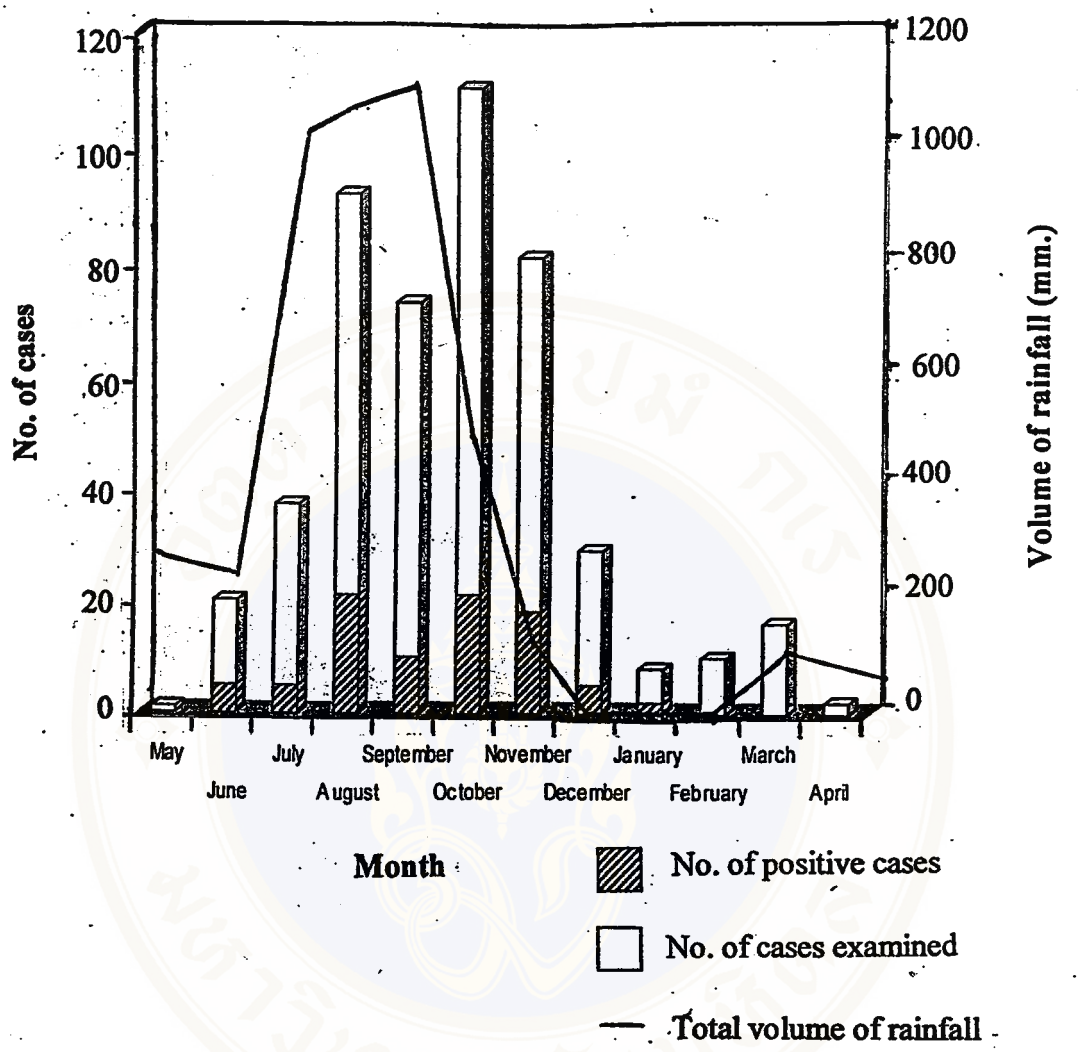


Figure 8 Number of positive cases, cases examined and total volume of rainfall at four selected hospital, clssified by month (May 1997-April 1998)

CHAPTER V

DISCUSSION

Dengue and dengue hemorrhagic fever, JE and Chikungunya infections are the mosquito borne viral diseases found in Thailand. These three diseases are also the well known arboviral diseases of the Pacific and Southeast Asia (143). The hill tribe patients in this study found that Karen was the highest hill tribe group, and one third of them were collected from Samoeng Hospital (Table 6-7). This may be because Karen villages settle nearer to the low lands when compared with other hill tribe groups. Road construction makes for easy access to their villages which allows them to go for treatment at this hospital when they have been sick, while the other hill tribe groups may take several hours to travel to the hospital in vehicles unappropriate for the terrain.

The majority of these patients had no education (46.8%) or had little education (45.5%), no income (32.8%) or very low income (33.1%) (Table 8). Their background knowledge and personnal behavior about arbovirus infections showed a below standard life quality (Table 10-11). Low knowledge and low income were direct problems affecting their health status, which possibly lead to poor hygenic conditions and exposure several infectious diseases such as AIDS, STD and parasitic infections (144-146). Nevertheless, arbovirus infections which occurred in the last two decades

in hill tribe groups (147) are still one of the major public health problems in Chiang Mai Province. Present illnesses of hill tribe patients in this study showed that 91.6% of them had never been vaccinated with JE vaccine, and 96.4% had never been sick with dengue hemorrhagic fever which is higher than the studied in Yanes, Puerto Rico in 1991 which found 80% of patients had never been infected with Dengue (132).

Even though this study tried to collect all convalescent phase sera, 190 cases were missed (Table 7). There were several problems identified during blood sample collection, an inadequate interval between acute and convalescent samplings since the majority of patients were discharged in less than one week, and patients' inability to return to the hospital for their check up. Hence, convalescent sera could not be obtained. Some cases had convalescent sera collected during home visits.

Laboratory diagnosis by co-interpretation with HI test and MAC ELISA showed the number of arboviruses positive cases to be higher than when interpreted by only one test (Table 13-15). Co-interpretation with both assays, gave laboratory confirmation and a much more accurate and correct diagnosis than interpretation by single assay, as shown by some negative and uninterpreted cases with HI test which were positive by MAC ELISA. In fact, MAC ELISA helped to screen more positive cases from the single serum group samples, especially in cases when HI titers were less than 1:2560, or in the paired sera group samples in which an interval of sera collection was less than 7 days and had no evidence of four fold rising titers. One reference showed that MAC ELISA helped to characterize dengue infections where that place had both Dengue and JE circulating (117).

Validity studies were performed and compared with an interpretation of the acute phase and acute-convalescent phases (Table 19). Sensitivity and specificity when interpreted using only acute phase sera showed 20.8% and 100.0%, respectively. Low sensitivity and high specificity in acute phase were found from this studied indicated that negative cases were not exactly negative, while positive cases were truly positive. A study found that even though MAC ELISA was used, only 60.5% of the positive results were obtained by using acute sera (148). Validity calculation supported the decision to exclude all of 175 uninterpreted cases in the single serum group in this study (Table 15) for analysis of factors related to arbovirus infections.

The majority of positive arboviruses infection cases found that 69.6% were a secondary infection (39/56 cases). This finding was in agreement with a surveillance of Dengue infection at a Children's Hospital in Bangkok (149), a study in Metro Manila, Philippines and in Havana, Cuba (150-151). Halstead proposed that dengue hemorrhagic fever is due to a self destructive host response and some persons are sensitized by the first infection, hence the course of a secondary infection with a different serotype may be related to an adverse immune response (152).

Although Chikungunya virus infection cases in Thailand are rare, 6.4% (25/393 cases) positive Chikungunya infection were found in this study, which is higher than the four years (1976-1979) studied on the infectivity rate of Chikungunya virus infection in Chanthaburi Province; 3.0% positive (140). A studied in 72 provinces in Thailand reported result of 1.8 %, Chikungunya infection, indicating that this virus was not a significant in the clinical and laboratory study in the surveillance

program (28). During the outbreaks of severe hemorrhagic disease caused by dengue viruses of multiple serotypes reported in the Philippines, Thailand, Malaysia, Vietnam and eastern India, this virus was simultaneously the cause of a similar, but probably milder, disease. The most severe form of Chikungunya infection, observed in Thailand, has been a febrile illness with minor hemorrhagic manifestations and not a life threatening disease (153).

Prevalence rate in females were higher than males in this study, either in arboviruses infection (42.2:37.9) or in arbovirus antibodies exposure (76.3:72.9) (Table 20). In addition, positive dengue virus infections were found higher than Japanese encephalitis cases (Table 15). The National surveillance report from the Department of Epidemiology, Ministry of Public Health for dengue hemorrhagic fever and JE found a higher ratio of males to females (2).

When classified by age group, prevalence of infection rates were rather high between 1-14 years old, which indicate that dengue infection is still a disease of children under 14 years old. At present, the trend of arboviral diseases, including those in this study found a high prevalence shift to the adult children, especially in a age group 10–14 years old, when compared with the 1987 peak in ages of 5-9 year (154). Surveillance in Metro Manila reported that highest incidence of dengue infection occurred among older children and young adults (150), while ten years observations of dengue cases in Jakarta, Indonesia reported an increasing proportion occurring in adults (155). This perhaps could be attributed to the transmission of

multiple Dengue serotypes at relative low rates of infection whereby previously uninfected adults could become susceptible to dengue infection.

A prevalence of antibody exposure showed little difference in each age group, except for 1-4 years olds. It could be that the majority of them had primary infections with arboviruses when less than 1 year old and above this age almost all were infected as secondary infection. These was same findings as reported on the immune respondes of Dengue virus infection (152).

A rash on face, extrimities and body was found in 20.0% (5/25 cases) of arbovirus infections cases (Table 21), which is less than a study of dengue fever in American military personnel in the Philippines during the 1984 epidemic which found 54.2% (13/24 cases) (156). A study of 25 American troops with confirmed dengue infections in Ubon Ratchathanee Province, Thailand, found no petechiae (52). A study of dengue hemorrhagic fever in Irian Jaya, Indonesia, found one of the predominant complaints was fever (100.0%) (157), similar to this study, and indicates that fever is one of a cause of viral infection. In the last decade, atypical manifestations were reported from dengue laboratory confirmed cases. The observations of fatal Dengue infections in Jarkarta, Indonesia found encephalitic signs such as convulsion, coma / semi-coma, stiff neck (158). One case was investigated for virus isolation from CSF but the result was negative. However, the encephalitic symptoms such as neck stiffness was found in one case in this study, but its possible this case was co-infected with JE as an asymptomatic infection. A prospective observational study was conducted to identify early indicators of acute dengue virus

infection. The study reported that it was the simple clinical and laboratory parameters such as duration of fever, hematocrit, platelet counts helped to identify children with Dengue infection (159).

In this study, several factors were found related to arboviruses infection with statistically significant difference (p-value < 0.05), which were age, occupation, income, history of being bitten by mosquitoes before sickness, and having containers or ground water pits close to the house (Table 32). Recently, several study had been reported on the factors related to dengue virus infection. The study from Mexico in 1988 found that large waters containers were significantly associated with infection with a 1.7 relative risk (134). Furthermore, a reported from Yanes, Puerto Rico in 1991 found that increasing age was significantly associated with recent dengue infection and there was a significant interaction between age and use of mosquito nets (132). A significant association was found between age and dengue antibody prevalence, and the increased antibody prevalence was also associated with urban and jungle residence including a piped source of household drinking water (160). Wood constructed housing and low socioeconomic status were the variables found significantly associated with dengue incidence in dengue transmission (65). Another study of dengue fever, which was a cause of febrile illness in US troops deployed to Somalia during Operation Restore Hope in 1992-1993, found that failure to use bed nets was the only identified risk factor for Dengue infection, with a 2.2 odds ratio (135). A surveillance of Dengue in Texas, Puerto Rico in 1995 found that several of infections in people who were lived in homes with intact screens and some living in air-conditioned homes (161), which may not protect against Dengue due to it being

transmitted by day biting mosquitoes. This study suggested that daytime risk factors might include certain outdoor occupations or activities such as yard work, gardening, and walking. Recently, five major factors responsible for the increased incidence DF and DHF for the global emergence were summarized (162). First, was the unprecedented global population growth. Second, the associated unplanned and uncontrolled urbanization, especially in tropical developing countries. Third, the lack of effective mosquito control in areas where dengue is endemic. Fourth, the global emergence of Dengue and DHF with increased air travel, which provides the ideal mechanism for the transport of dengue and other urban pathogens between population centers of the world. Fifth, a lack of resources has led to a critical shortage of trained specialists who understand and can develop effective prevention and control programs for vector borne diseases.

One of hypotheses for the pathogenesis of dengue hemorrhagic fever (DHF) is the secondary infection or sequence response (152). In severe dengue infection, named dengue shock syndrome (DSS), risk factors studied from Rayong Province, Thailand found secondary infections with DEN-2 which followed primary infections with DEN-1, DEN-3 or DEN-4 (164). However, the data from the Western Pacific and from Thailand indicate that serious DHF and even fatal DSS may occur with primary infection (164-165).

The GMT of each Flaviviruses antigens response (DEN 1-4, JE) by HI test in acute sera and in convalescent sera according to theirs age group, sex, hospital treatment, seem not to be different (Table 25-27). The HI test showed high cross-

reaction with all flaviviruses antigens due to the group specific property. Even though cross-reactions were found in MAC ELISA, HI testing showed a higher cross-reactivity.

Positive arboviruses IgM antibody was detected in 67 cases or 102 sera, which were comprised of 85 and 17 sera positive with dengue and JE (Table 33). When classified, the duration between day of blood collection and the day after the onset of the disease, 83.3% (85/102 sera) of all IgM positive sera was detected during the first 30 days (Table 34). Recently, a study of dengue fever in Haiti, measured by the ELISA, found that the IgM titers increased faster and higher than the IgG titers, and the IgM response lasted at least 50 days (166). In addition, some studies found IgM appeared on the 4^{th} day of disease and IgM was not detected in sera obtained after the 60^{th} day (167), while another study reported that IgM can last up to 252 days after onset of illness (168). All of these reports, including this study, support that, in general, the IgM antibodies appear in 1 to 2 weeks, peak in 3 to 6 weeks, and decline to undetectable levels over the following few months (169).

During the same epidemic, and at the same time of this study, 100 additional samples were isolated in *A. albopictus* mosquitoes cell line, and identified DEN-1 as the serotype predominant in the hill tribes of Chiang Mai Province during the study period in 1997-1998. In an outbreak of classical dengue fever in Guerrero State, Mexico in 1988, several cases were isolated as DEN-1 (134), while during an outbreak in Yanes, Pueto Rico in 1991, DEN-2 was the predominant serotype (132). In Cuba, the epidemic in 1977 was caused by DEN-1, while in 1981 was caused by DEN-2

(170). During the first epidemic of DHF in French Guina in 1991-1992, DEN-2 was identified as being responsible for most cases (171). Several isolated strains are useful to study for the molecular evolution and the distribution in nature of the virus. Recently, five genotypic groups of DEN-1 were classified by comparing relatively short nucleotide sequences, which were useful in understanding Dengue virus epidemiology (172).

At present, genome detection by PCR is used to detect dengue viruses as well as other arboviruses in serum specimens. It is a rapid test, and takes only a few hours for a result. Reactions can also be performed in mosquito pools as studies on virological surveillance in the fields serve as an early warning monitoring system for dengue outbreak (173).

The number of positive cases and their prevalence rates were found to peak during the rainy season beginning in May, reaching a high in August and then declining, which was in agreement with the rainfall volume reported form Division of Climatology, Meteorological Department (Table 35). This study is in agreement with the epidemic of dengue fever reported in Karnataka State of India, which reached its peak by mid-August and then started declining (174).

Even though Ma Taeng Hospital had the highest hill tribe patients coming for treatment when compared with the other hospitals, the highest prevalence rate was found in Phrao Hospital (Table 20). It might be possible that this area had a high population of arboviruses infected mosquitoes. However, when compared with the

rainfall volume (Table 35), high rainfall was also found in Phrao District, and may be suitable for breeding places of mosquitoes. The study in Puerto Rico indicated that mosquitoes density were positively correlated with rainfall. The watering pans and automobile tires were the most important mosquito breeding sites (175).

CHAPTER VI

CONCLUSION

Arbovirus infections were studied in 393 cases of pyrexia hill tribe patients (203 cases of paired serum and 190 cases of single serum) from four selected hospitals in Chaing Mai Province, namely: Samoeng Hospital, Mae Taeng Hospital, Phrao Hospital and Chaing Dao Hospital.

The blood specimens were collected and patients interviewed by questionnaire. The study was done during May 1997 to April 1998.

The male to female ratio of all patients was 1: 1.1. The mean and standard deviation of age was 25.9 ±15.8. Most of patients were the 15-19 years old age group.

The number of days between onset of disease and blood collection was calculated for each serum sample. The majority of acute sera were collected on day 2 (24.9%) or day 3 (29.5%) after onset of illness, while convalescent sera were on day 14 (13.8%) or days 16-30 (26.1%). The duration between acute and convalescent were collection were 2 to 120 days.

The clinical manifestaions found were fever (100.0%), anorexia (68.2%), malaise (64.6%) and muscle pain (42.2%).

All sera were tested by MAC ELISA / GAC ELISA against JE and Dengue antigens and by HI test against four serotypes of Dengue, JE and Chikungunya antigens. Result interpretation was performed according to the criteria

for laboratory diagnosis of WHO. Results showed that the prevalence rate of arbovirus infections was 39.9% (87/218 cases) and prevalence of arbovirus antibodies exposure was 74.6% (293/393 cases). Positive arbovirus infections rates were found higher in female more than male patients (42.2% and 37.9%), and were found highest in the Karen group when compared with the others. The highest positive rate was in the 1-4 years old age group, and the main occupations rate were student. Phrao Hospital had the highest rate of arbovirus infections.

Comparison the prevalence rate of arbovirus infections and prevalence rate of antibodies exposure by Cochran Q test the results showed a statistically significant diffrence (p-value < 0.05).

The geometric mean titers (GMT) and standard deviation (SD) of HI antibodies against Dengue, JE and Chikungunya antigen in 393 acute and 203 convalescent sera were found to be highest in DEN-3 when compared with the others, and the GMT ± SD found in acute and convalescent sera were 20.3 ± 4.5 and 38.9 ± 10.5, respectively. The acute sera GMT ± SD in each age group were not very different according to each antigen, while in convalescent sera, the GMT ± SD to be high for all antigens in the 10-14 years old age group. The GMT ± SD according to sex and hospital treatment were no different either in acute or convalescence phases in each antigen, except at Phrao Hospital.

Comparison of GMT in acute and in convalescent phases of sera for each antigens (DEN-1, DEN-2, DEN-3, DEN-4, JE and Chikungunya) by Friedman test (p-value < 0.05) results showed a statistically significant difference in each antigens excepted Chikungunya virus.

Factors related to arbovirus infections with statistical difference (p-value < 0.05) analyzed by Chi-square test, Fisher exact test and logistic regression were age, occupation, income, history being bitten by the mosquitoes 1 week before sickness, and having containers or ground water pits close to the house.

SUGGESTION

(a) For prevention and control of diseases

Long-term prevention and control should be based on health education activities aimed at increasing community participation in the control of arbovirus vectors. Health personnel who were responsible for control programs should collaborate in providing technical guidance in simple and understandable language to community leaders, primary health care personnel and others, such as teachers, who often have day-to-day contact with the community. Special attention should be directed towards the instruction of members of communities, especially students and their parents, in prevention and control of disease.

(b) For future research

1. Acute serum (S1) should be collected as soon as possible after the onset of illness, while convalescent sera (S2) should be collected if possible 7 days after disease onset or before discharge from the hospital. Failure to leave an interval of 7 days between S1 and S2 may prevent a serological diagnosis or the ability to differentiate primary infection.

2. Future laboratory studies should include the viral isolation from studied serum samples, to confirm the serotypes of Dengue infection.
3. Study should be done either on the vectors or the reservoir host of the JE disease.
4. Disribution of study subjects should equally represent every group; sex, age, occupation, hill tribe group and hospital treatment center.
5. More intensive studies of knowledge, attitude and practices related to arbovirus infections among hill tribe patients, as well as qualitative studies, are need.

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APPENDIX A

REAGENTS

1. Antibody capture enzyme linked immunosorbent assay

1.1 0.006 M carbonate–bicarbonate buffer pH 9.0

Solution A	Na_2CO_3	0.64 gm / 1,000 ml.
Solution B	$NaHCO_3$	0.50 gm / 1,000 ml.

1.2 Phosphate buffered saline (PBS) pH 7.4 (10X PBS formula)

NaCl	160.00 gm.
KCl	4.00 gm.
$NaH_2PO_4.H_2O$	2.80 gm.
Na_2HPO_4	20.00 gm.

Add distilled water to 2,000 ml, adjust pH to 7.4 by NaOH

1.3 0.05% Tween–20 in PBS pH 7.4 (PBS–T)

PBS (10X)	200.00 ml.
Distilled water	1,800.00 ml.
Tween–20	1.00 ml.

1.4 0.1 M citrate phosphate buffer pH 5.0

Solution A 0.1 M citric acid 19.21 gm / 1,000 ml.

Solution B 0.2 M sodium phosphate dibasic, anhydrous 28.40 gm / 1,000 ml.

Mix 24.3 ml. of solution A with 25.7 ml. of solution B and make up to 90 ml., adjust pH to 5.0 by adding solution A or solution B ; bring to final of volume 100 ml.

1.5 Substrate solution

O–phenylene diamine (Kodak)	5.00 ml.
Citrate phosphate buffer	10.00 ml.
Fresh 3 % H_2O_2	33.00 ml.

Dissolve 5 mg O–phenylene diamine (Kodak) in 10 ml. of citrate phosphate buffer and add 33 µl of fresh 3 % H_2O_2

1.6 4 M H_2SO_4 for stop reaction

Conc. H_2SO_4 (95-97 %)	22.00 ml
Distilled water	78.00 ml.

2. Hemagglutination inhibition test (HI)

2.1 0.4 % Bovine albumin pH 9.0 (0.4 % BABS)

Bovine albumin	2.00 gm.
Borate saline pH 9.0	500.00 ml.

2.2 Borate saline pH 9.0

2.2.1 1.5 M NaCl

2.2.2 NaCl	87.75 gm.
Distilled water to	1,000.00 ml.

2.2.3 0.5 M H_3BO_3

H_3BO_3	31.00 gm.
Distilled water to	1,000.00 ml.

2.2.4 1.0 N NaOH

NaOH	80.00 gm.
Distilled water to	2,000.00 ml.

Borate saline pH 9.0

1.5 M NaCl	160.00 ml.
0.5 M H_3BO_3	200.00 ml.
1.0 N NaOH	48.00 ml.

Distilled water to 2,000 ml., adjust pH to 9.0

2.3 0.9 % NaCl

NaCl	9.00 gm.
Distilled water	1,000.00 ml.

2.4 Dextrose gelation veronal solution (DGV)

Veronal (Barbital)	0.58 gm.
Gelatin	0.60 gm.
Sodium Veronal (Sodium barbital)	0.38 gm.
$CaCl_2$ (anhydrous)	0.02 gm.
$MgSO_4 .7 H_2O$	0.12 gm.
NaCl	8.50 gm.
Dextrose	10.00 gm.
Distilled water to	1,000.00 ml.

Veronal and gelatin dissolved in hot water. The mixture can be sterilized by autoclave for 10 minutes at 10 lbs.

2.5 Veronal adjusting diluent (VAD)

Make up solution A and B then mix the amounts indicated below, pH of mixtures should approximate the predicted pH

Solution A

1.5 M NaCl	100.00 ml.
0.5 M Na_2HPO_4	400.00 ml.
Distilled water to	1,000.00 ml.

Solution B

1.5 M NaCl	100.00 ml.
1.0 M $NaH_2PO_4.HO_2$	200.00 ml.
Distilled water to	1,000.00 ml.

Antigen	Final pH	Solution A	Solution B
Den – 1	6.2	22.00 ml.	78.00 ml.
Den – 2	6.4	32.00 ml.	68.00 ml.
Den – 3 and JE	6.6	45.00 ml.	55.00 ml.
Den – 4	6.8	55.00 ml.	45.00 ml.
CHIK	6.2	22.00 ml.	78.00 ml.

2.6 Alserver's solution

Dextrose	2.05 gm.
Sodium citrate ($Na_3C_6H_5O_7.H_2O$)	0.80 gm.
Citric acid	0.055gm.
Sodium chloride	0.42 gm.
Distilled water to	100.00 ml.

Sterilized by autoclave to 10 minutes 10 lbs.

APPENDIX B

Questionnaire

Questionnaire for interviewing the hill tribe patients at 4 selected hospitals in Chiang Mai Province and factors related to arboviruses infections

Name of patient .....

Date of inteview .....

Hospital number (HN) .....

Name of hospital .....

Part 1 Demographic data

1. Age ..... years old

2. Sex

   1) Male 2) Female

3 Religion

   1) Buddhist 2) Christian

   3) Islam 4) Others (specify .....)

4 Hill tribe group

   1) Karen 2) Meo 3) Lahu

   4) Lisu 5) Yao 6) Akha

   7) Lua 8) Others (specify .....)

5 Marital status

1) Single 2) Married 3) Divorced

4) Widow 5) Separated

6 Occupation

1) Agriculture (specify .....)

2) Farmer

3) Forest item gatherer

4) Government official (specify .....)

5) Employee (specify .....)

6) Business

7) Others (specify .....)

7 Average legitimate income per month ..... Baht

8 Level of education

1) None

2) Prathom 1 – 6

3) Mathayom 1 – 3

4) Mathayom 4 – 6

5) Vocational education certificate

6) Bachelor's degree

7) Others (specify .....)

9 Present address .....

10 Previous address .....

Part 2 Information about present sickness

11 Before admission, have you ever had any of the following signs and symptoms ?

(The answer may be more than one)

1) Fever for ..... days.
2) Headache
3) Malaise
4) Anorexia, nausea and vomiting
5) Right costal margin pain
6) Abdominal pain
7) Rash on face / extremities / body
8) Epistaxis, malena, hematemesis
9) Stiffness of neck and back
10) Unconciousness, convulsion

12 Clinical feature (The answer may be more than one)

1) Conjunctivitis
2) Rash
3) Lymphatic gland enlargement
4) Jaundice

13 Before visiting this hospital, what did you do ?

1) Nothing
2) Went to clinic
3) Went to primary health care clinic
4) Bought some medicine from drug store
5) Went to hospital

6) Traditional treatment

7) Others (specify .....)

14 If you bought medicine from drug store, what kind of medicine ?

1) Antibiotics

2) Analgesics, antipyretics

3) Herbal

4) Don't know

5) Others (specify .....)

15 Have you ever been immunized with JE vaccine ?

1) No

2) Yes (when .....)

16 Have you ever been sick with dengue haemorrhagic fever ?

1) No

2) Yes (when .....)

17 One week before the onset of illness, were you bitten by mosquitoes ?

1) No

2) Yes, by *Aedes spp.*

3) Yes, by *Culex spp.*

4) Yes, by *Anopheles spp.*

5) Don't know

6) Not sure

18 If you were bitten by mosquitoes, what time did most of the mosquitoes bite you ?

1) Day time

2) At night

Part 3 Behavioral information

19 What time do you working ?

1) 8.00 – 16.00

2) 8.00 – 20.00

20 Style of clothes while you are working

_____

_____

21 Which methods do you choose for protection from mosquitoes ?

1) Sleep under mosquito net

2) Use mosquito coil

3) Use herbicide

4) Use repellent

5) Others (specify .....)

22 Which methods do you choose for removing the breeding places of mosquitoes ?

1) Get rid of garbage and mosquioes breeding place

2) Put abate sand in water

3) Feed *Poccillia spp.* fishes

4) Use chemical insecticides

5) None

6) Others (specify .....)

Part 4 Information about environmental factors

23 Within 100 meters of your house or your working area, do you have aminals feeding?

1) None

2) Yes (specify .....)

1.1 Pig

1.2 Dog

1.3 Bird

1.4 Cow, buffalo

1.5 Not in category (specify .....)

24 Are there ground water pits close to your house ?

1) None

2) Yes (specify .....)

2.1 Big jar

2.2 Coconut bark, can, bottle

2.3 Water drainage tap

2.4 Rubber tire

2.5 Others (specify .....)

25 What types of mosquitoes are found in your house ?

1) *Aedes spp.* mosquitoes

2) *Culex spp.* . mosquitoes

3) *Anopheles spp.* . mosquitoes

4) Don't know

26 Where did you often find mosquitoes in your house ?

1) Bathroom

2) Under floor of house

3) Corner of room and closet

4) General areas

Part 5 Knowledge about dengue haemorrhagic fever and JE

27 Do you know that when you are bitten by mosquitoes (*Aedes spp.*) you may become sick with DHF ?

1) No

2) Yes

28 Do you know that when you are bitten by mosquitoes (*Culex spp.*) you may become sick with JE ?

1) No

2) Yes

29 Have you ever heard about mosquitoes *Aedes spp.* and *Culex spp.* ?

1) Know both mosquitoes

2) Know *Aedes spp.*, but didn't know *Culex spp.*

3) Know *Culex spp.*, but didn't know *Aedes spp.*

4) Didn't know about both mosquitoes

30 Do you know what seasons dengue haemorrhagic fever and JE mostly frequently occur ?

1) Summer

2) Rainy season

3) Winter

4) Didn't know

31 Do you know how to prevent DHF and JE ?

1) No

2) Yes (How, specify .....)

32 Do you know that DHF patients experience fever, headache, anorexia, nausea, vomiting and petechiae haemorrhage ?

1) No

2) Yes

33 Do you know that JE patients experience fever, headache, nausea, vomiting, neck and back stiffness, convulsion and unconciousness ?

1) No

2) Yes

APPENDIX C

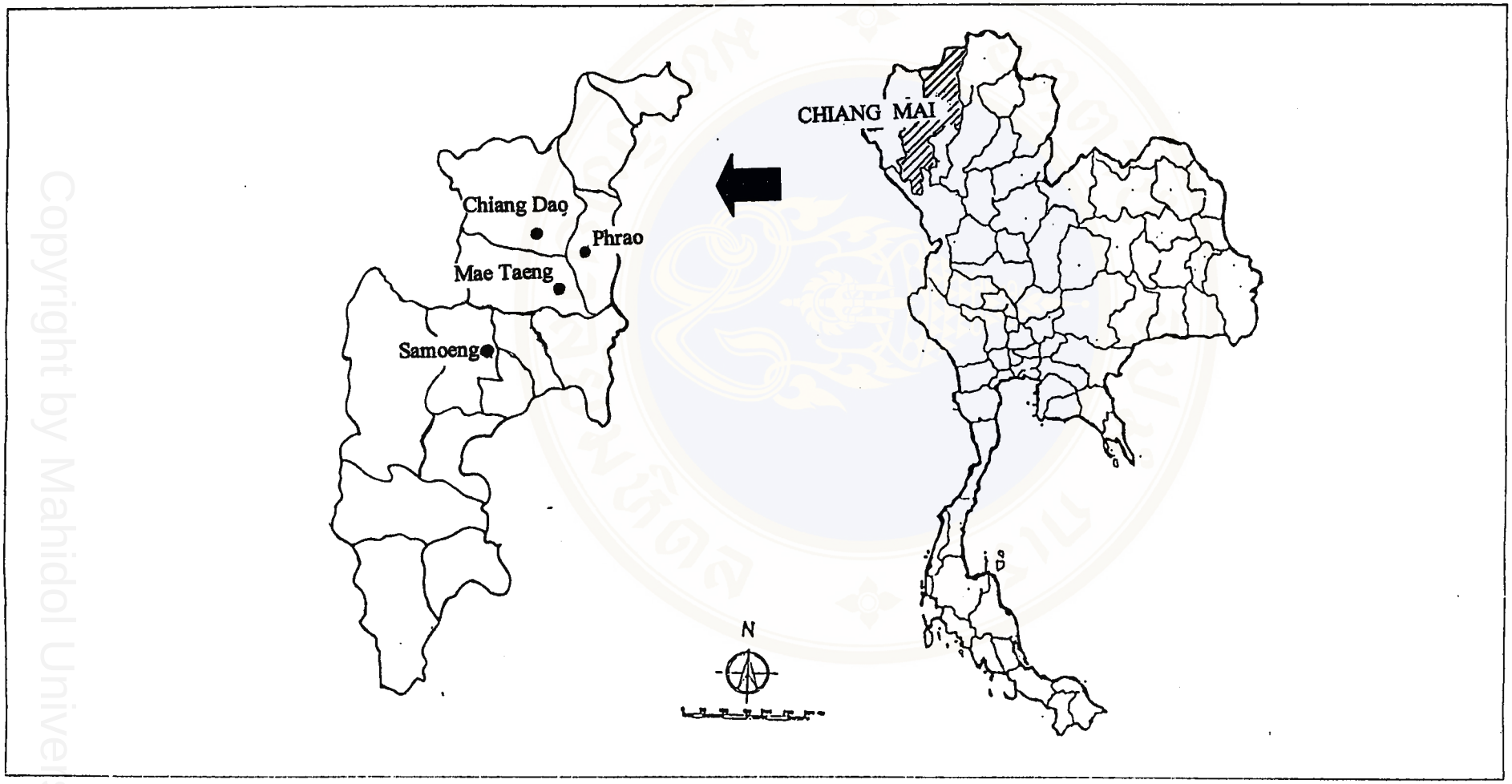
Table A The number of villages, households and hill tribe population in Chiang Mai Province, 1996.

Hill tribes group	No. of villages	No. of households	No. of persons
1. Karen	803	23,649	123,630
2. Lahu	176	6,510	34,220
3. Meo	69	2,354	20,228
4. Lisu	70	2,473	14,464
5. Lua	31	1,989	9,774
6. Akha	23	760	4,001
7. Yao	9	230	1,631
8. Htin	1	62	380
9. Khamu	1	2	2
Total	1,183	38,029	208,330

Source: Tribal population summary in Thailand Report, Data processing and Analysis, Tribal Research Institute, Chiang Mai, Thailand (14).

APPENDIX D

Figure 9 The map of Chiang Mai Province



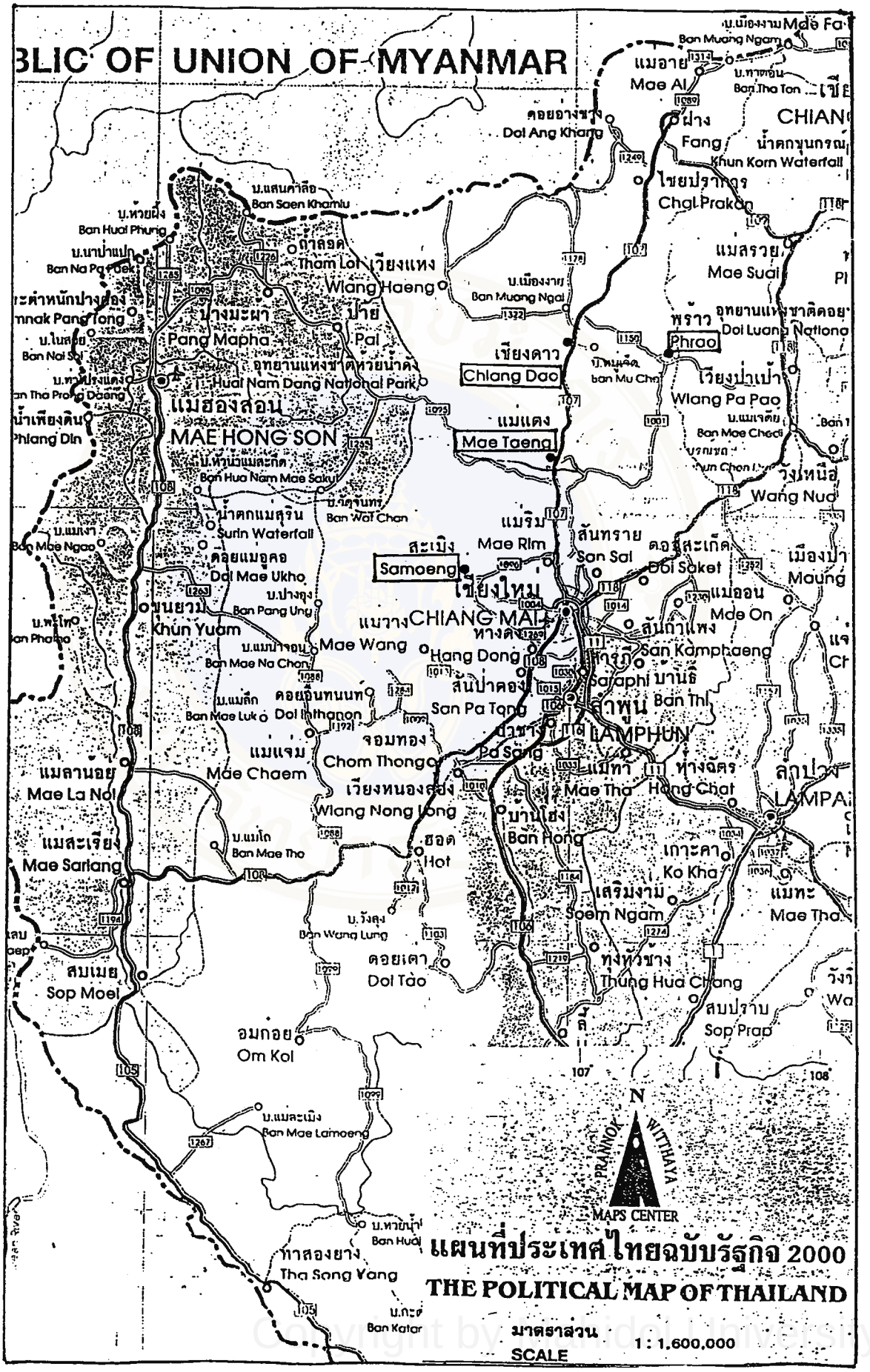


Figure 9 The map of Chiang Mai Province (continued)

BIOGRAPHY

Name	Miss Siriporn Nasomjai
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