Brief Report

The Distribution of DEN Infected People in Dushan and Xingyi Area of Yunnan-Guizhou Plateau, China

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The dengue viruses (DEN, *genus Flavivirus*, family Flaviviridae) are mosquito borne and have caused 100 million cases of dengue fever each year in most tropical and subtropical areas of the world. However, in the Southwest area of Yunnan-Guizhou Plateau, China, the previous work demonstrated that different geographic strains of *Aedes albopictus* were susceptible to dengue virus. In this study, we collected 456 sera samples from patients with fever and 994 sera samples from healthy population in Dushan and Xingyi area of Yunnan-Guizhou Plateau, China. All sera samples were tested for dengue IgG by enzyme-linked immunosorbent assay (ELISA). Patients' sera samples were tested for dengue IgM and DEN antigen was checked in the sera of 6 from 456 samples with which C6/36 cell in culated by IFA. The results indicate that these patients with fever were infected with DEN-2 and suggest that DEN infection had existed in Dushan and Xingyi area of Yunnan-Guizhou Plateau, China. *Cellular & Molecular Immunology*. 2006;3(6):473-476.

Key Words: dengue virus, DHF, IFA

Introduction

Dengue virus (DEN), belonging to the *genus Flavivirus* (family Flaviviridae), has four distinct antigenic types (serotypes 1 to 4). The main mosquito vector, *Aedes aegypti,* is present in nearly every tropical country, and consequently a third of the world's human population is at risk of infection. Economic disruption and human population migration during the Second World War spread the disease beyond its usual geographical locations and resulted in its reintroduction into some areas. During the second half of the twentieth century, a rapid increase in the numbers of air travelers, further population dislocations and poor public health worsened the situation of *Aedes aegypti* and *Aedes albopictus* (1). Dengue diseases are a major, emerging problem with the cocirculation of different virus serotypes, increased frequency of epidemics, and the introduction of dengue hemorrhagic fever (DHF) in areas where it was not previously known. At present, it is estimated that more than 50-100 million people have contracted dengue each year and that, since 1958, more than 60,000 children have died due to DHF (2-3).

The Xingyi city of Guizhou Province is located in the middle area of the Yunnan-Guizhou Plateau in the Southwest China, and lies in the east longitude of 140°32'-150°11', north latitude 24°38'-25°23'. It has a mid-Asian tropical humid climate, the annual average temperature is 16.1°C, while the average temperature is 4.5°C and 26.8°C for January and July respectively, the annual rainfall 1531.6 mm. It has a population of 670,000, of which the urban population is 124,400, and the population density is 250 persons per square kilometers. Dushan County is 997 metres above sea level. The annual average temperature is about 13.6°C-19.6°C and where rainfall is from 1,100 to 1,400 mm.

Our previous study demonstrated that different geographic strains of Aedes albopictus in Guizhou were susceptible to DEN and a new strain of DEN-2 was isolated from suspension of *Aedes albopictus* collected from Mawei town of Dushan. The dengue virus was detected in mosquitoes (4-8). *Aedes albopictus* in Xingyi is capable of transmitting DEN vertically and after the vertical transmission E gene mutations occurred (9-11). In this research, 456 sera samples were collected from patients with fever in summer and autumn, 994 sera samples from normal people in Dushan and Xingyi to determine whether DEN infection of people have existed in Dushan and Xingyi of

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Abbreviations: PBS, phosphate-buffered saline; DEN, dengue virus; IFA, immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco modified Eagle's medium; DHF, dengue hemorrhagic fever.

Table 1. The schedule of samples for Xingyi area

Age	0-5	6-10	11-15	16-20	21-30	31-40	41-50	≥ 51	Total
Healthy population	59	71	68	58	53	58	51	57	475
Fever patient	15	15	15	28	51	48	51	71	294
Total	74	86	83	86	105	106	102	128	770

Table 2. The schedule of samples for Dushan area

Age	0-5	6-10	11-15	16-20	21-30	31-40	41-50	≥ 51	Total
Healthy population	43	23	58	54	89	97	76	79	519
Fever patient	18	14	18	13	22	20	17	40	162
Total	61	37	76	67	111	117	93	119	681

Yunnan-Guizhou Plateau.

Materials and Methods

Samples
Sera from 456 patients with fever and 994 sera samples were collected from normal people in Dushan and Xingyi area of Yunnan-Guizhou Plateau, China. All samples were centrifuged and frozen at -80°C prior to the assay (Tables 1 and 2).

Prepare ELISA antigen and detect DEN antigen with C6/36 cells
C6/36 cells were cultured in DMEM supplemented with 10% fetal calf sera (FCS) and infected with DEN at 28°C in DMEM with 2% FCS for 5 to 6 days. Culture supernatant were harvested and centrifuged and used as the viral antigen in the assays. Control antigen was similarly prepared from uninfected C6/36 cells.

C6/36 cells were inoculated with 456 fever patients' serum samples diluted 1:20 and 1:40, respectively. The inoculum was allowed to adsorb to the cells for 1 h at 28°C, and cells were washed three times with 1.0 ml of PBS to remove residual. Then 1.0 ml of maintenance medium was added to each well and cultured at 28°C in 2% FCS/DMEM for three generations. Control antigen was similarly prepared from uninfected C6/36 cells.

Indirect immunofluorescence
Detected DEN antigen with monoclonal antibody (McAb) against dengue virus types 1-4 by indirect immuno-fluorescence assay (IFA). Infected cells were harvested, centrifuged, suspended in 0.1 M PBS and stored overnight at 4°C. Each microliters of suspension were applied onto each well of 48-well antigen slides, air-dried, fixed in cold acetone for 10 min and stored at -20 °C until used. All samples were detected with IFA used anti-DEN 1-4 McAb. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG at an optimal working dilution was used as the secondary antibody.

ELISA
The tetravalent dengue virus antigen was used at dilutions of 1:50, 1:100, 1:200, 1:400, 1:800, 1:1,000, and 1:1,200, respectively. Sera samples obtained from 13 healthy adults were used as negative controls. Sera samples obtained from 7 healthy children inoculated with encephalitis B vaccine were used as specific controls. Thirteen sera from acute dengue patients were tested in the assays used for DEN IgM detection. 12 sera from convalescent dengue patients were also tested in the assays used for DEN IgG detection.

Statistical analysis
Statistical significance was evaluated with the program SPSS 11.5 for Windows. Chi-square test was employed for comparing groups of samples. Results were considered statistically significant at $p < 0.05$.

Results

DEN IgM and IgG of fever patients were detected by ELISA
In current study, a total of 456 fever patients' sera were tested with dengue specific IgM and IgG antibodies, and 44 (9.63%) and 54 (11.84%) samples were positive for dengue IgM or IgG, respectively. There were no statistically significant difference in positive ratio between male and female. Similarly, no statistically significant difference was found in the two regions (Table 3).

DEN IgG of healthy population was detected by ELISA
Of the 994 normal samples, 129 (12.98%) were positive for dengue specific IgG. There was no statistically significant difference in positive ratio between male and female, Xingyi and Dushan, Han and Buyi nationality (Table 4).

DEN antigen detected with IFA
After inoculated with C6/36 cells and cultured for three generations, the samples were tested for DEN antigen with IFA. Cells were counterstained with Evans blue so that uninfected cells appear red, while infected cells appear

Table 3. The comparison of the positive ratio of the specific anti-DEN2 IgM of fever patients

		n	Positive (%)	Negative (%)	*p*
Area	Xingyi	294	23 (7.82)	271 (92.18)	> 0.05
	Dushan	163	21 (12.88)	142 (87.12)	
Sex	Male	253	26 (10.28)	227 (89.72)	> 0.05
	Female	204	18 (8.82)	186 (91.18)	
Total		456	44 (9.63)	413 (90.37)	

Table 4. The comparison of the positive ratio of the specific IgG against DEN2 in sera of healthy population

		n	Positive (%)	Negative (%)	*p*
Area	Xingyi	475	62 (13.05)	413 (86.95)	> 0.05
	Dusan	519	67 (12.91)	452 (87.09)	
Sex	Male	481	61 (12.68)	420 (87.32)	> 0.05
	Female	513	68 (13.26)	445 (86.74)	
Nationality	Han	526	65 (12.54)	461 (87.46)	> 0.05
	Buyi	269	36 (13.37)	233 (86.63)	
Age	0~	102	11 (10.78)	91 (89.22)	> 0.05
	6~	94	15 (15.96)	79 (84.04)	
	11~	126	21 (16.67)	105 (83.33)	
	16~	112	14 (12.50)	98 (87.50)	
	21~	142	20 (14.08)	122 (85.92)	
	31~	155	17 (10.97)	138 (89.03)	
	41~	127	16 (12.60)	111 (87.40)	
	51~	136	15 (11.03)	121 (88.97)	
Total		994	129 (12.98)	865 (87.02)	

Kelly-green. The results of IFA indicated that some of 356 tested sera, 6 (1.71%) contained DEN-2 antigen (Figure 1).

Discussion

Dengue is the most prevalent mosquito-borne and currently disease in human and it was estimated that there are 50-100 million cases of dengue fever (DF) per annum worldwide, 500,000 of which result in the severe forms of the disease, DHF and dengue shock syndrome (DSS) (1). The main mosquito vector, *Aedes aegypti*, is present in nearly every tropical country, and consequently a third of the world's population is at risk of infection.

The specific IgM antibody is tended to appear early during the course of disease and allows for a provisional diagnosis to be made from a single sera sample. Detection of dengue specific IgM antibodies is an easier method of diagnosing DF as compared to other classical serological methods like as haemagglutination inhibition, neutralization and complement fixation tests, etc. In this study, the results showed that 9.63% (44/456) and 11.84% (54/456) fever

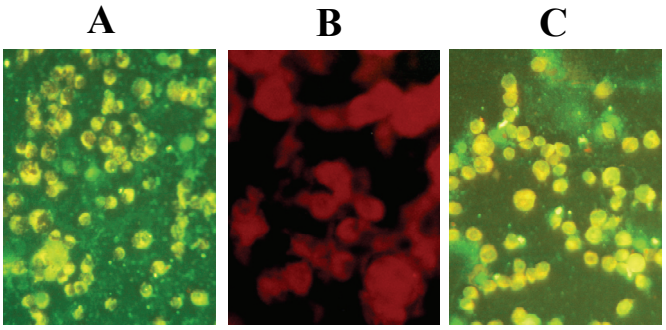

Figure 1. The identification of DEN antigen by IFA. (A) positive control by C6/36 cells infected with DEN-2, (B) negative control of C6/36 cells infected by sera without DEN-2, (C) representative image of detected samples (400×).

patients' sera were tested for dengue specific IgM or IgG antibody, respectively. And DEN antigen was detected in the sera of 6 from 356 (1.71%) sera samples with which C6/36 cell by IFA. It was suggested that some fever patients infected with DEN in summer and autumn had existed in Dushan and Xingyi area of Yunnan-Guizhou Plateau, China.

We also present evidence that 12.98% (129/994) healthy sera were tested for dengue specific IgG antibody, the difference in percentage positivity in females compared with males was not statistically significant, between the overall difference of the dengue IgG positive rates in Han and Buyi nationality was also not statistically significant. When the samples were divided into age according to eight groups, the observed dengue IgG positivity percentage in comparison to the age did not reveal a definitive relativity.

Economic disruption and human population migration during and after the Second World War promoted the disease spreading beyond its usual geographical locations and resulted in its reintroduction into some areas. During the second half of the twentieth century, a rapid increase in the numbers of air travelers, further population dislocations and poor public health measures worsened the situation. Using an empirical model of the effect of population and climate change on the global distribution of dengue fever, we conclude that predicted changes in humidity will increase the areas with a climate suitable for dengue transmission (12, 13).

The increasing global trend in international travel also facilitates the dissemination of virus serotypes and strains in vulnerable population (15), genetic variability is another element to be considered, the genetic diversity of the viruses is increasing, with some genotypes associated with severe disease (16). Recombination has been demonstrated in all four serotypes of dengue virus, but the implications in terms of pathogenesis are unknown. In addition to recombination, mutations, gene flow, and other factors could further influence the genetic diversity and selection of virulent strains. At the same time, in addition to initial observations of the higher risk of DHF in Caucasian than in those of African descent, a few reports associate some human leukocyte

antigen alleles with disease severity (17). The sequence of infecting viruses and, more recently, the longer interval between primary and secondary infection as a risk factor for DHF, add a new perspective to the problem (18).

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