J Microbiol Immunol Infect
2003;36:89-95

Concurrent infections by two dengue virus serotypes among dengue patients in Taiwan

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Received: July 9, 2002 Revised: September 18, 2002 Accepted: September 27, 2002

The co-circulation of multiple dengue virus serotypes in the same region has been reported in several countries in Southeast Asia as well as in Central and South America for decades. Although outbreaks involving more than one serotype of dengue virus have been reported in Taiwan since 1987, concurrent infection in the same individual by multiple serotypes of dengue virus have never been identified. Using a modified multiplex reverse transcription-polymerase chain reaction assay, we detected and determined the serotypes of 21 dengue patients during an outbreak in southern Taiwan in 2000. While either dengue type 2 or type 3 virus was found in most cases, 2 of the 21 cases were concurrent infections by both dengue type 2 and dengue type 3 viruses. This was further confirmed by sequence analysis of the amplified products. This study reports for the first time that concurrent infections occur in Taiwan, and suggests that future virologic surveillance of dengue outbreak in Taiwan should take this into consideration.

Key words: Concurrent infection, dengue virus, reverse transcription-polymerase chain reaction (RT-PCR), serotype

Among the 80 or so arthropod-borne flaviviruses, outbreaks of the 4 serotypes of dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) continue to be a major public health problem in tropical and subtropical areas [1-3]. While most dengue virus infections develop as asymptomatic or mild, self-limited illness, in the form of dengue fever (DF), some patients may develop severe and potentially life-threatening diseases, known as dengue hemorrhagic fever-dengue shock syndrome (DHF/DSS) [2-4].

The global epidemic of dengue has changed dramatically in the past 50 years. The World Health Organization (WHO) recently estimated that approximately 50 million cases of dengue infection occur worldwide every year [1,3,5]. Several factors, including global population growth, unplanned urbanization, lack of mosquito control, and increased air travel, are believed to be responsible for the expanding geographic distribution of mosquito vectors and the 4 dengue virus serotypes. This results in an increase in the countries reported to have dengue cases and an increase in the incidence of severe and fatal cases [1,3,5]. The change was also exemplified by the epidemics of dengue in several countries in the American regions, where no (nonendemic) or only one (hypoendemic) dengue virus was found prior to the 1980s, and multiple serotypes (hyperendemic) of dengue virus in the same country were reported during the 1980s and 1990s [1,3].

In Taiwan, historical epidemics of dengue have been documented in 1902, 1915, and 1922 in Penghu archipelago, in 1927 in southern Taiwan, and in 1931 and 1942 to 1943 island-wide [6-12]. There were no cases reported for 37 years until 1981, when a DEN-2 outbreak occur in Liouchyou Shiang [6,7]. This was followed by the DEN-1 epidemics in 1987 to 1989 in southern Taiwan, particularly in Kaohsuing and Pingtung [8-12]. While the majority of cases were DEN-1, there were 5 DEN-2 and 3 DEN-4 cases in 1987 and 1988, respectively [8-10]. An indigenous DEN-3 case was identified in 1988 [10]. Since then, there have been dengue cases every year and larger outbreaks every 3 years or so. The first DHF case was reported in 1988 [10,11]. Ten years later, the largest DHF epidemic caused by DEN-3 virus occurred in Tainan [11,12].

The co-circulation of multiple serotypes of dengue virus in the same region has been reported for decades in several countries in southeast Asia, and in central and south America [3]. Concurrent infections by

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different serotypes of dengue virus are expected to occur during epidemics involving multiple dengue virus serotypes [3,18]. The first case of dual infection with 2 dengue viral serotypes (DEN-1 and DEN-4) was reported in Puerto Rico in 1982 [13]. There were 6 cases of dual infections (DEN-1 and DEN-3) in New Caledonia in 1989 [14]. Since then, more cases of concurrent infections by multiple dengue virus serotypes have been reported in different countries [15-18]. Although outbreaks involving more than 1 dengue virus serotype have been noted in Taiwan since 1987, cases concurrently infected by 2 or more serotypes of dengue virus have not been documented [9-12]. In this study, we used a modified multiplex reverse transcription-polymerase chain reaction (RT-PCR) assay [19,20] to examine acute plasma samples from suspected dengue patients during an outbreak in southern Taiwan in 2000. Of the 21 confirmed dengue patients, most were infected by either DEN-2 or DEN-3 virus, whereas 2 were concurrent infections of DEN-2 and DEN-3 viruses. We report here that concurrent infections do occur in Taiwan.

Materials and Methods

Study participants

The diagnoses of DF and DHF followed the WHO clinical definition [4]. Detection of dengue genomic sequences in plasma by a multiplex RT-PCR assay, which was modified from the previously described RT-PCR assays, was the laboratory criteria of confirmation for all cases [4,19,20]. Between June 1 and August 31, 2000, during an outbreak in southern Taiwan, 21 DF patients from 5 hospitals (Kuo General Hospital, Sin-Lau Christian Hospital, Yuan General Hospital, Pingtung Christian Hospital, and Tian-Sheng Memorial Hospital) were confirmed at our laboratory. The day of onset of fever is defined as day 1 of illness. One acute blood sample was collected for each patient in an ethylenediaminetetraacetic acid (EDTA)-containing tube within 8 days of illness. The plasma was prepared within 6 hours of collection and stored at –80°C until use [21].

Isolation of viral RNA

Dengue viral RNA was isolated from the plasma or stock virus using the QIAamp viral RNA mini kit (Qiagen, Germany) as described previously [21]. Plasma samples obtained from 2 hepatitis C virus (HCV) carriers and 2 healthy donors were also subjected to RNA isolation. Stock viruses used in this study included the 4 serotypes of dengue virus, Hawaii (DEN-1), New Guinea (DEN-2), H-87 (DEN-3), and H-241 (DEN-4) strains, with titers of 2.1×10^6 plaque forming units per millimeter (pfu/mL), 1.3×10^6 pfu/mL, 2×10^6 pfu/mL and 1×10^6 pfu/mL, respectively. They were obtained from culture supernatants of infection of mosquito C6/36 cells and then titrated on BHK cells by standard plaque assay. Three Japanese encephalitis virus (JEV) strains (Nakayama vaccine strain, Beijing vaccine strain, and CH1949 Taiwan local strain) with titers ranging from 10^5 to 10^6 pfu/mL were also included in the analysis [21].

Multiplex RT-PCR

The multiplex RT-PCR assay employed in this study was modified from the previously described RT-PCR assays, which can distinguish the 4 dengue serotypes by the size of the products [19,20]. It includes a step of RT-PCR using a highly conserved primer pair, D1 and D2, and a step of second-round PCR using the primer D1 and 4 serotype-specific primers, TS1, TS2, TS3, and TS4 (Fig. 1A). The sequences of the primers are as follows: D1, 5'-TCAATATGCTGAAACGCGCGAG-AAACCG-3' (genome positions 134 to 161 of the DEN-2 Jamaica strain [22]); D2, 5'-TTGCACCAAC-AGTCAATGTCTTCAGGTTC-3' (genome positions 644 to 611 of the DEN-2 Jamaica strain); TS1, 5'-CGTCTCAGTGATCGGGGG-3' (genome positions 586 to 568 of the DEN-1 Hawaii strain [23]); TS2, 5'-CGCCACAAGGGCCATGAAC AG-3' (genome positions 252 to 232 of the DEN-2 Jamaica strain); TS3, 5'-TAACATCATCATGAGACAGAGC-3' (genome positions 421 to 400 of the DEN-3 H-87 strain [24]); and TS4, 5'-CTCTGTTGTCTTAAACAAGAGA-3' (genome positions 527 to 506 of the DEN-4 H-241 strain [25]).

RT-PCR and PCR were performed in a separate room from that used for RNA isolation, and precautions for PCR were followed to avoid contamination [26]. An aliquot (2 μL) of RNA eluate was subjected to RT-PCR using the Superscript one-step RT-PCR kit (Gibco/BRL, Life Technologies, Rockville, MD, US) under the conditions of 52°C for 40 min, and 95°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final step of 72°C for 7 min. The expected size of the RT-PCR products is 511-bp (D1 and D2). An aliquot (2 μL) of the diluted products (1:100) was subjected to the second round PCR using the Taq DNA polymerase (HT Biotechnology, Cambridge, England) under the conditions of 94°C for 5 min, and 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec, and a final step of 72°C for 10 min. The expected sizes of the amplified products are

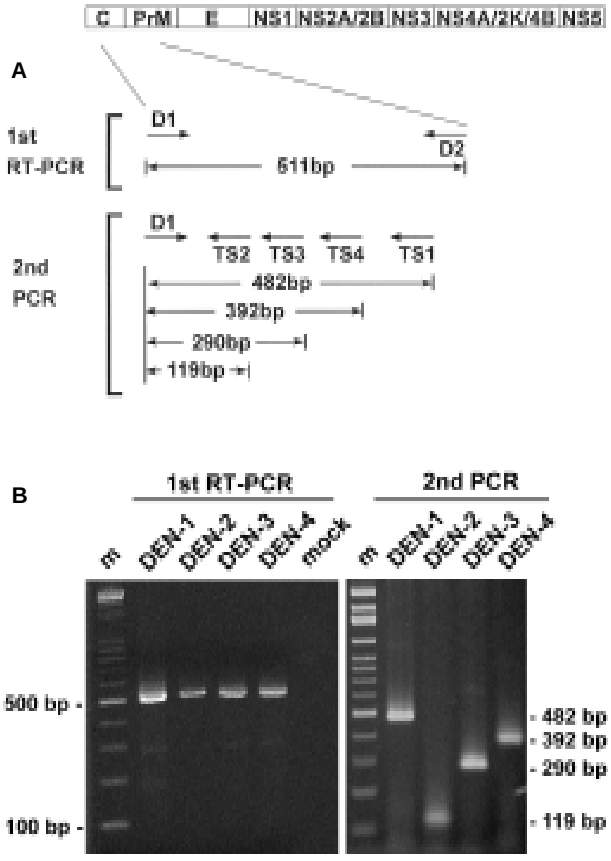

Fig. 1. (A) Schematic diagram of the dengue virus genome and the primers used in the modified multiplex RT-PCR assay. The predicted sizes of the products are shown. **(B)** The ethidium bromide stained gel of the products of the first round RT-PCR and second round PCR, using RNA derived from the 4 dengue virus serotypes, DEN-1 (Hawaii), DEN-2 (New Guinea), DEN-3 (H-87), and DEN-4 (H-241). Mock = no RNA template added; m = molecular size marker.

482-bp (D1 and TS1 for DEN-1 virus), 119-bp (D1 and TS2 for DEN-2 virus), 290-bp (D1 and TS3 for DEN-3 virus), and 392-bp (D1 and TS4 for DEN-4 virus). The products were electrophoresed through 2% agarose gel, stained with ethidium bromide, and examined under ultraviolet light using a digital gel documentation system as described previously [21].

Sequencing of the amplification products

The products of predicted size from the second round were cut from the gel and purified with the GFX^{TM} PCR DNA and gel band purification kit (Amersham Pharmacia, Piscataway, NJ, US). The purified products were sequenced using the BigDye terminator cycle sequencing kit under conditions recommended by the manufacturer (PE Applied Biosystems, Foster City, CA, US). Samples were loaded onto 4.75% polyacrylamide gel of ABI automated sequencers (Applied Biosystems ABI-377) as described previously [21].

Sequence analysis

The nucleotide sequences of a fragment of the 119-bp products and those of the 290-bp products were aligned with sequences of the corresponding regions of the 4 dengue virus serotypes, including the prototype DEN-1 (45AZ5), DEN-2 (New Guinea), DEN-3 (H-87), and DEN-4 (H-241) viruses, as well as of the JEV SA14wt strain, using the program Dnaman Version 4.15 (Lynnon Biosoft, Canada). The nucleotide sequence similarity of each comparison was calculated. A phylogenetic tree was generated by the program MEGA version 2.1 (Molecular Evolutionary Genetics Analysis, Pennsylvania State University, PA, US) using the neighbor-joining method.

Results

Typing of dengue viruses by a modified multiplex RT-PCR assay

To determine the dengue virus serotypes of plasma samples, a multiplex RT-PCR assay was modified from the previously described RT-PCR assays [19,20]. This includes a step of RT-PCR using a highly conserved primer pair, D1 and D2, and a step of second-round PCR using the primer D1 and 4 serotype-specific primers, TS1, TS2, TS3, and TS4 (Fig. 1A). An aliquot of RNA eluates derived from stock viruses of the 4 dengue serotypes, Hawaii (DEN-1), New Guinea (DEN-2), H-87 (DEN-3), and H-241 (DEN-4) strains, was subjected to RT-PCR first. As shown in Fig. 1B left, RT-PCR products of the predicted size of 511-bp were seen in reactions containing the RNA templates derived from the 4 dengue viruses but not in the reaction containing no template. An aliquot of the diluted products was then subjected to the second-round PCR. Amplified products of the expected sizes of 482-bp, 119-bp, 290-bp, and 392-bp were seen in the reactions of DEN-1, DEN-2, DEN-3, and DEN-4 viruses, respectively (Fig. 1B right). RNA templates derived from other flaviviruses prevalent in Taiwan, including 3 JEV and 2 HCV, as well as from the plasma of 2 healthy donors were also subjected to the multiplex RT-PCR assay. None of these resulted in amplified products of expected size in the first-round RT-PCR or in the second-round PCR (data not shown).

Determination of dengue viral serotypes of confirmed dengue patients

Between June 1 and August 31, 2000, 49 acute blood

samples of 49 suspected dengue cases from 5 hospitals in southern Taiwan were sent to the laboratory. RNA isolated from plasma was subjected to the modified multiplex RT-PCR assay to detect and type dengue virus serotypes. Among the 49 samples, 21 were confirmed dengue cases based on the presence of amplified products of predicted sizes. The results of some samples are shown in Fig. 2. While most cases were found to have products of 119-bp, the predicted size of DEN-2 virus, some had products of 290-bp, the predicted size of DEN-3 virus, or 392-bp, that of DEN-4 virus. Interestingly, 2 different size RT-PCR products, 119-bp and 290-bp, were seen in 2 cases (patients 660 and 671), suggesting that they were concurrently infected by 2 dengue viruses, DEN-2 and DEN-3 (Fig. 2). The serotypes of the 21 confirmed dengue patients were summarized in Table 1. There were 15 DEN-2 cases, 3 DEN-3 cases, 1 DEN-4 case, and 2 dual infections by DEN-2 and DEN-3 viruses.

Sequence analysis

To confirm the identities of the amplified products of the 2 dual infected patients, the 119-bp bands and 290-bp bands derived from both cases were purified from the gel and subjected to direct DNA sequencing. The sequences of a 124-nucleotide region of the 290-bp products from patients 660 and 671 were aligned with sequences of the corresponding region of the 4 prototype dengue viruses, DEN-1 (45AZ5), DEN-2 (New Guinea), DEN-3 (H-87), and DEN-4 (H-241), and the similarity of the nucleotide sequences was analyzed. As shown in Table 2, the nucleotide sequences of the 290-bp products from patients 660 and 671 had higher degrees of similarity (93.5% and 96.0%, respectively) to that of DEN-3 virus than to those of other serotypes,

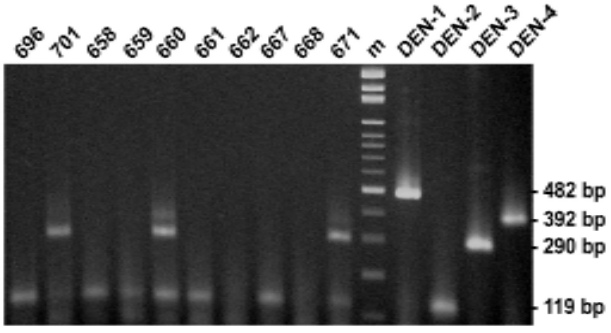

Fig. 2. Determination of dengue virus serotypes of plasma samples by the modified multiplex RT-PCR assay. The patients' ID is shown at the top. The predicted sizes of the amplified products of the four dengue viruses, DEN-1 (Hawaii), DEN-2 (New Guinea), DEN-3 (H-87), and DEN-4 (H-241), are shown at the right. m = molecular size marker.

Table 1. Serotypes of dengue patients determined by the modified multiplex RT-PCR assay

Serotype	No. of cases
DEN-2	15
DEN-3	3
DEN-4	1
DEN-2 and DEN-3	2
Total	21

suggesting that these were DEN-3 sequences. Phylogenetic analysis was also performed and revealed a clustering of the sequences from both patients with that of DEN-3 virus, indicating that these were indeed DEN-3 sequences (Fig. 3A).

The sequence of a 47-nucleotide region of the 119-bp product from patient 660 was also aligned with the corresponding sequences of the 4 dengue viruses and subjected to similar analysis. The 119-bp product from patient 671 was not included in this analysis, as the yield was too low for direct DNA sequencing. The nucleotide sequence of the 119-bp product from patient 660 was closer to that of DEN-2 virus (similarity, 93.6%) than to those of other serotypes, suggesting that this was DEN-2 sequence (Table 2). This was further supported by the clustering of this sequence with that of DEN-2 virus in the phylogenetic analysis (Fig. 3B). Taken together, the sequence analysis of the amplified

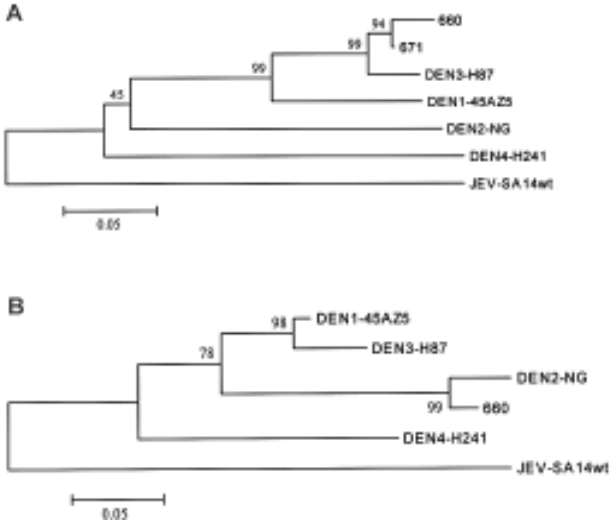

FIG. 3. Phylogenetic analysis of the sequences of the amplified products of 290-bp **(A)** and 119-bp **(B)** from two dual infected cases, patients 660 and 671, and the sequences of the four prototype dengue viruses, DEN-1 (45AZ5), DEN-2 (New Guinea), DEN-3 (H-87), and DEN-4 (H-241), using the program MEGA. The sequence of JEV strain Sa14wt was also included in the analysis. Numbers at nodes represent bootstrap values.

Table 2. Nucleotide sequence (%) similarity between the amplified products from dual infected patients and the 4 dengue virus serotypes

	The 290-bp product[a]		The 119-bp product[b]
	Patient 660	Patient 671	Patient 660
DEN-1[c]	83.1	85.5	80.9
DEN-2[c]	67.7	69.4	93.6
DEN-3[c]	93.5	96.0	74.5
DEN-4[c]	63.7	62.9	63.8

[a]A 124-nucleotide region of the PCR product was sequenced and analyzed.
[b]A 47-nucleotide region of the PCR product was sequenced and analyzed.
[c]The 4 dengue viruses used in the analysis are 45AZ5 (DEN-1), New Guinea (DEN-2), H-87 (DEN-3), and H241 (DEN-4).

products from these 2 patients confirmed that they were concurrently infected by 2 dengue viruses, DEN-2 and DEN-3.

Discussion

It is generally believed that concurrent infections by different serotypes of dengue virus will occur during epidemics in which multiple dengue virus serotypes are being transmitted. Although co-circulation of multiple serotypes of dengue virus in the same region has been recognized in several countries for decades [3], it was not until 1982 that the first case of concurrent infections with 2 dengue virus serotypes was reported [13]. The detection of concurrent infections could be attributed to the common use of serotype-specific monoclonal antibodies in the immunofluorescence assay to detect different dengue virus serotypes during routine virus isolation [13,27-29]. In addition, incorporation of serotype-specific primers in the RT-PCR assays further facilitates the detection of concurrent infections [14-18]. In this study, we developed a modified multiplex RT-PCR assay to examine acute plasma samples from dengue patients during an outbreak in southern Taiwan in 2000. To our knowledge, this study reports for the first time that concurrent infections by 2 serotypes of dengue virus occur in Taiwan.

Compared with the RT-PCR assay described previously by Lanciotti *et al* [19], which required 4 separate reactions to type dengue viruses, our modified multiplex RT-PCR assay was simpler and more convenient. The multiplex RT-PCR assay reported by Harris *et al* combined the steps of RT and PCR in 1 single tube, and therefore had only 1 round of PCR amplification [20]. Our multiplex RT-PCR assay employs 2 rounds of amplification (the first round RT-PCR step and the second round PCR step) as the original RT-PCR assay by Lanciotti *et al*, of which the sensitivity was reported to be similar to that of the virus isolation or immunofluorescence assay system [18,19,29]. With regard to the specificity, our multiplex RT-PCR assay uses the same primers as those of the RT-PCR assay by Lanciotti *et al*, which has been shown to be specific for dengue virus [20]. Consistent with this, the present assay can detect the 4 dengue virus serotypes, but not other flaviviruses prevalent in Taiwan, including JEV and HCV (data not shown).

In this study, we identified 2 cases of concurrent infection by the RT-PCR assay, which is one of the laboratory criteria for confirmation of dengue cases according to the WHO clinical definition [4]. Since only acute plasma samples were available, serological tests could not be used for confirmation. Sequence analysis was therefore conducted to confirm the identities of the amplified products of the 2 dually infected patients. Due to technical limitation in sequencing DNA directly from PCR products, only a 47-nucleotide fragment of the 119-bp band was sequenced. Comparison of the nucleotide sequence of this region of patient 660 with those of the 4 dengue serotypes revealed that it had a similarity of 93.6% to DEN-2 virus, which is within the range of nucleotide similarity between dengue viruses of the same serotype in this region (Table 2 and data not shown). The nucleotide similarities of this region to those of DEN-1, DEN-3, and DEN-4 viruses were from 63.8 % to 80.9%, which were in the range of the nucleotide similarity between dengue viruses of different serotypes (Table 2 and data not shown) [30]. These results indicate that this was indeed the DEN-2 sequence, which was also confirmed by phylogenetic analysis (Fig. 3B). Similarly, comparison of the nucleotide sequences of a 124-nucleotide region of the 290-bp products from patients 660 and 671 with those of the 4 dengue virus serotypes, together with the phylogenetic analysis, demonstrated that the 290-bp products from both patients were DEN-3 sequences (Table 2 and Fig. 3A). It should be noted that the possibility of cross-contamination between samples was remote, since the nucleotide distance of the 124-nucleotide capsid region between patients 660, 671, and the DEN-3 virus (H87 strain) used in this study was

from 2.4% to 6.5%, a range higher than that of sequence diversity of the capsid gene of DEN-3 virus within the same patient (mean pairwise p-distance 0.12% to 1.02%) [31].

With the dramatic changes in the global epidemiology of dengue virus infection during the past 50 years, there are not only increases in the number of countries reported to have dengue cases but also in the number of severe and fatal cases [1,3,5]. The change in the epidemiological patterns from hypoendemicity (only 1 serotype) to hyperendemicity (multiple serotypes) has been seen in many countries [1,3]. Using a modified multiplex RT-PCR assay, this study demonstrated that concurrent infections also occur in Taiwan. The frequency of concurrent infection in this study, 9.5% (2/21) was close to the frequencies reported by Lorono-Pino *et al* (11% in Indonesia and 5.5% in Mexico, Puerto Rico, and Indonesia together) [18]. Common occurrence of concurrent infections by multiple dengue virus serotypes has been recognized recently and is expected to be seen in the future [18]. Whether concurrent infections are associated with more severe diseases remain to be investigated. Although the epidemiological pattern of dengue in Taiwan, like those in other parts of the world, has changed in the past 20 years, active surveillance, epidemiological investigation, effective mosquito control, and public health infrastructure all together are believed to contribute to the reduction in the number of cases during each outbreak [12]. The epidemiology of dengue in Taiwan is very different from that in other southeast Asian countries in that it contains imported cases every year and has not been considered yet as a hyperendemic region [12]. The present findings, however, indicate that one of the features commonly seen in hyperendemic areas is also found in southern Taiwan. Obviously, the future virologic surveillance of dengue outbreaks in Taiwan should be conducted from a new perspective.

Acknowledgments

We thank Shu-Mei Chang at the Yuan's General Hospital, Hsiu-O Kuo at the Pingtung Christian Hospital, Chiang-Chyuan Su at the Tian-Sheng Memorial Hospital, Shih-Ting Ho at the Sin Lau Christian Hospital, and Shih-Chung Lin at the Kuo General Hospital for kindly providing clinical samples, and Tzu-Ling Sung, Yu-Chen Tsai, and Tsai-Yu Lin for technical assistance. We thank Dr. DJ Gubler for the DEN-2 New Guinea strain, and the DEN-3 H87 strain. We also thank Dr. Chuan-Liang Kao for providing the four stock viruses. This work was supported by the National Health Research Institute (NHRI-CN-CL8903P), and in part by the National Science Council (NSC90-2320-B-002-150), Taiwan, Republic of China.

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