Emergence of a New Lineage of Dengue Virus Type 2 Identified in Travelers Entering Western Australia from Indonesia, 2010-2012

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Abstract

Dengue virus (DENV) transmission is ubiquitous throughout the tropics. More than 70% of the current global dengue disease burden is borne by people who live in the Asia-Pacific region. We sequenced the E gene of DENV isolated from travellers entering Western Australia between 2010–2012, most of whom visited Indonesia, and identified a diverse array of DENV1-4, including multiple co-circulating viral lineages. Most viruses were closely related to lineages known to have circulated in Indonesia for some time, indicating that this geographic region serves as a major hub for dengue genetic diversity. Most notably, we identified a new lineage of DENV-2 (Cosmopolitan genotype) that emerged in Bali in 2011–2012. The spread of this lineage should clearly be monitored. Surveillance of symptomatic returned travellers provides important and timely information on circulating DENV serotypes and genotypes, and can reveal the herald wave of dengue and other emerging infectious diseases.

Author Summary

Dengue is currently the most rapidly spreading mosquito-borne viral disease of humans, and is endemic in most tropical and sub-tropical countries. An estimated 390 million infections occur annually, and over 70% of the current global dengue disease burden is borne by people who live in Southeast Asia and the Western Pacific region. DENV serotype and genotype data is lacking in many parts of this region, limiting our attempts to understand the observed patterns of hyperendemicity and disease severity. Many countries in the Southeast Asia and Western Pacific region are popular tourist destinations, and dengue has been identified as a cause of travel-related illness in people returning from endemic countries. We sequenced the E gene of DENV isolated from travellers returning to...
Western Australia from 7 countries throughout Asia between 2010 and 2012. The majority of DENV originated in Indonesia, predominantly Bali, a popular travel destination for Australians. We identified hyperendemic transmission of all four DENV serotypes in Bali in 2010; circulating DENV included dominant local strains which had circulated for several years in Indonesia and Singapore, as well as strains more recently introduced into Bali from other countries in the region. Finally, we show the emergence of a new lineage of DENV2 (Cosmopolitan genotype) in 2011–2012, which should be monitored. Travellers may act as sentinels and provide important information on DENV genotypes and lineages circulating in countries where locally generated detailed genetic data may not be available.

Introduction

Dengue is the most rapidly spreading mosquito-borne viral disease of humans. An estimated 390 million dengue virus infections occur annually, of which approximately one quarter result in clinical disease [1]. Dengue transmission is predicted to be ubiquitous throughout the tropics, with the highest incidence rates in Asia and the Americas. More than 70% of the current global dengue disease burden is borne by people who live in Southeast Asia and the Western Pacific region [2], where dengue incidence has increased consistently since 2000.

Dengue is caused by infection with any one of four antigenically distinct dengue viruses (DENV), single-stranded positive-sense RNA viruses that form their own antigenic complex within the family Flaviviridae. There are four DENV serotypes (DENV1–4), and each DENV serotype can further be subdivided into distinct genotypes, and lineages within the genotypes, largely based on phylogenetic analysis of envelope (E) gene sequences [3–6]. Accordingly, analyses of E gene sequences have revealed that DENV-1 and DENV-2 can be divided into five and six genotypes, respectively, and DENV-3 and DENV-4 into four genotypes, including the sylvatic lineages found in non-human primates.

Previous studies of DENV molecular epidemiology have revealed relatively stable genotypic compositions within individual localities, punctuated by occasional lineage turnover [7]. Although the causes of these lineage replacement events are often opaque, in some cases they have been shown to reflect underlying fitness differences. For example, studies in Vietnam showed that between 1995 and 2009 the dominant Asian /American lineage of DENV-2 was replaced by the Asian I lineage [8]. This displacement was thought to be driven by higher viremia levels in patients infected with the Asian I lineage, potentially leading to increased human-mosquito transmission. In addition, the introduction of new lineages may be associated with higher rates of severe disease, as was shown in the Caribbean in 1981 when the first appearance of the Asian/American [9] lineage of DENV-2 coincided with the first recorded DHF-associated epidemics in the region. As a consequence, it is critical to monitor the appearance and spread of novel lineages, particularly those that are often associated with severe dengue disease.

Dengue transmission in the Asia-Pacific region, including Australia, has been documented since the 1940s, when the four prototype DENV were first identified in Hawaii (DENV-1Hawaii, 1944), New Guinea (DENV-2NGC, 1944), and the Philippines (DENV-3H-87 and DENV-4H-421, 1956) [10–12]. Marked increases in DENV-1–4 transmission have been documented throughout the region over the past three decades and increasing travel within the region has supported introduction of DENV from epidemic areas to countries many thousands of kilometres away [13–16]. Despite the burden of dengue in the Asia-Pacific region, data on which serotypes and genotypes are circulating in many parts of the region are lacking [17], limiting our attempts to understand the mechanisms governing the observed patterns of disease severity and hyperendemicity.
To identify and characterize prevailing serotypes and genotypes in the Asian region we undertook an analysis of DENV in symptomatic infected travellers entering Western Australia, and in doing so identified the emergence of a new lineage of DENV-2 in Bali, Indonesia.

**Materials and Methods**

**Viruses**

DENV was isolated from de-identified acute phase serum samples submitted to Pathwest Laboratory Medicine WA for diagnostic testing. These serum samples were stored at -20°C until used. Virus isolation was attempted on 313 serum samples. Briefly, 100\(\mu\)l of NS1 antigen-positive serum (Platelia Dengue NS1 Antigen ELISA kit; Bio-Rad, Australia) was inoculated onto Vero cells in 5.5ml cell culture tubes and cell culture supernatants were harvested after 2 or 3 blind passages. Briefly, 100\(\mu\)l of serum was inoculated onto a Vero cell monolayer in minimal media and incubated overnight. On the following day the inoculum was removed and 3ml of DMEM with 2% FBS was added to the cells, and the culture was maintained for 7 days at 37°C with 5% CO₂. Successful virus isolation was identified by NS1 antigen ELISA. A total of 86 viruses (27.5%) were successfully isolated, from seven different countries: 74 isolates from Indonesia, of which 66 originated in Bali; two from Thailand; two from India, and one each from Laos, Philippines, Timor-Leste, and Vietnam (an additional four viruses were from unspecified countries). One person reported travel to Bali and Japan. Fifty-one viruses were isolated from samples submitted in 2010, six from 2011, and 29 from 2012 (Table 1; Fig. 1). Sample data were analyzed anonymously. This project was approved by the University of Western Australia Human Research Ethics Committee.

**RNA extraction and sequencing**

Viral RNA was extracted from 140 \(\mu\)l of culture supernatant using QIAmp viral RNA Mini kits (Qiagen), according to the manufacturer’s instructions. cDNA was synthesized from extracted RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) as per the manufacturer’s instructions. DENV serotype was confirmed by RT-PCR using serotype-specific primers (Table 2), and the LongRange PCR Kit (Qiagen) (thermocycling conditions are available on request). To remove excess oligonucleotide primers and dNTPs, PCR products were prepared for sequencing using Exo-SAP-IT following the manufacturer’s protocol (USB Corporation, Cleveland USA). The nucleotide sequences were determined with template-specific primers (Table 2) using fluorescence-based cycle sequencing conditions (BigDye Terminator v3.1 Cycle Sequencing kit, AB, Foster City USA). All incubation steps were completed in thermal cyclers (AB2720 thermal cycler, AB, Foster City USA). Unincorporated dye terminators were removed from sequencing reactions using gel-filtration following the manufacturer’s protocol (DyeEx 2.0 Spin Kit, GmbH, Germany). Sequencing was performed on a 16-capillary genetic analyzer (3130xl Genetic Analyzer, AB, Foster City USA). Chromatogram editing (Chromas v2.3, Technelysium Pty Ltd) and sequence alignment (Bioedit, TA Hall software, and the Lasergene software of DNA Star (DNASTAR, Madison USA)) were undertaken by comparison to sequence information in GenBank. All sequences have been deposited in GenBank and assigned accession numbers KM216664-KM216747 and KM222447-KM222448.

**Phylogenetic analysis**

To put the evolution of the dengue viruses circulating in travellers returning to Australia into the context of those circulating more widely, and particularly in Asian countries, all E gene sequences of human DENV-1 to DENV-4 were downloaded from GenBank and utilized as a
Table 1. Origin and phylogenetic placement of DENV sequences from travelers entering Western Australia.

| Sequence ID     | Entering WA From | Date of Collection | Genotype, Lineage |
|-----------------|------------------|--------------------|-------------------|
| **DENV-1**      |                  |                    |                   |
| D1/IDN/Bali_004/2010 | Bali             | 10/28/2010         | Genotype II, Lineage 2 |
| D1/IDN/Bali_005/2010 | Bali             | 11/18/2010         | Genotype II, Lineage 2 |
| D1/IDN/Bali_009/2010 | Bali             | 4/27/2010          | Genotype I, Lineage 14 |
| D1/IND/Delhi_011/2010 | Delhi         | 10/21/2010         | Genotype III, Lineage 1 |
| D1/IDN/Bali_012/2010 | Bali             | 9/19/2010          | Genotype II, Lineage 6 |
| D1/IDN/Bali_013/2010 | Bali             | 11/2/2010          | Genotype II, Lineage 2 |
| D1/IDN/Bali_014/2010 | Bali             | 5/5/2010           | Genotype I, Lineage 13 |
| D1/IDN/Bali_015/2010 | Bali             | 8/6/2010           | Genotype II, Lineage 2 |
| D1/IND/022/2010    | India             | 9/24/2010          | Genotype III, Lineage 1 |
| D1/IDN/Java_023/2010 | Java             | 11/16/2010         | Genotype II, Lineage 2 |
| D1/IDN/Bali_024/2010 | Bali             | 8/12/2010          | Genotype I, Lineage 14 |
| D1/IDN/Bali_030/2010 | Bali             | 12/22/2010         | Genotype II, Lineage 2 |
| D1/031/2010        | N/A               | 9/6/2010           | Genotype I, Lineage 10 |
| D1/IDN/Bali_032/2010 | Bali             | 12/21/2010         | Genotype II, Lineage 2 |
| D1/IDN/Bali_033/2010 | Bali             | 5/27/2010          | Genotype I, Lineage 14 |
| D1/IDN/Bali_034/2010 | Bali             | 4/28/2010          | Genotype II, Lineage 4 |
| D1/IDN/Bali_037/2010 | Bali             | 8/19/2010          | Genotype I, Lineage 12 |
| D1/IDN/Bali_038/2010 | Bali             | 10/18/2010         | Genotype I, Lineage 13 |
| D1/IDN/Bali_039/2010 | Bali             | 12/29/2010         | Genotype II, Lineage 3 |
| D1/040/2010        | N/A               | 6/9/2010           | Genotype I, Lineage 8 |
| D1/THA/41/2010     | Thailand          | 12/16/2010         | Genotype I, Lineage 9 |
| D1/VNM/042/2010    | Vietnam           | 5/8/2010           | Genotype II, Lineage 6 |
| D1/IDN/Jakarta_048/2010 | Jakarta      | 8/25/2010          | Genotype II, Lineage 5 |
| D1/IDN/Bali_055/2010 | Bali             | 4/22/2010          | Genotype II, Lineage 4 |
| D1/IDN/Bali_068/2011 | Bali             | 8/15/2011          | Genotype I, Lineage 14 |
| D1/IDN/Bali_071/2011 | Bali             | 3/1/2011           | Genotype I, Lineage 13 |
| D1/IDN/Bali_072/2011 | Bali             | 3/19/2011          | Genotype I, Lineage 11 |
| D1/IDN/Bali_073/2011 | Bali             | 4/8/2011           | Genotype I, Lineage 11 |
| **DENV-2**        |                  |                    |                   |
| D2/IDN/Bali_010/2010 | Bali             | 8/5/2010           | Cosmopolitan, Lineage 1 |
| D2/IDN/Bali_020/2010 | Bali             | 10/16/2010         | Cosmopolitan, Lineage 5 |
| D2/IDN/Bali_021/2010 | Bali             | 8/6/2010           | Cosmopolitan, Lineage 1 |
| D2/IDN/Bali_025/2010 | Bali             | 10/16/2010         | Cosmopolitan, Lineage 5 |
| D2/IDN/Bali_035/2010 | Bali             | 12/26/2010         | Cosmopolitan, Lineage 8 |
| D2/LAO/043/2010    | Laos              | 10/8/2010          | Asian I, Lineage 9 |
| D2/IDN/044/2010    | Indonesia         | 6/10/2010          | Cosmopolitan, Lineage 1 |
| D2/IDN/045/2010    | Indonesia         | 6/6/2010           | Cosmopolitan, Lineage 3 |
| D2/046/2010        | N/A               | 5/29/2010          | Cosmopolitan, Lineage 7 |
| D2/IDN/Bali_047/2010 | Bali             | 5/7/2010           | Cosmopolitan, Lineage 3 |
| D2/IDN/Bali_049/2010 | Bali             | 9/3/2010           | Cosmopolitan, Lineage 5 |
| D2/IDN/Bali_050/2010 | Bali             | 6/10/2010          | Cosmopolitan, Lineage 5 |
| D2/IDN/Bali_051/2010 | Bali             | 4/22/2010          | Cosmopolitan, Lineage 1 |
| D2/IDN/Bali_052/2010 | Bali             | 8/6/2010           | Cosmopolitan, Lineage 6 |
| D2/IDN/Bali_057/2010 | Bali             | 4/19/2010          | Cosmopolitan, Lineage 2 |
| D2/IDN/Bali_059/2010 | Bali             | 11/2/2010          | Cosmopolitan, Lineage 7 |

(Continued)
| Sequence ID          | Entering WA From | Date of Collection | Genotype, Lineage         |
|---------------------|------------------|--------------------|--------------------------|
| D2/IDN/Jakarta_060/2010 | Jakarta          | 10/18/2010         | Cosmopolitan, Lineage 5   |
| D2/IDN/Bali_075/2011  | Bali             | 12/14/2011         | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_076/2012  | Bali             | 1/16/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_077/2012  | Bali             | 1/17/2012          | Cosmopolitan, Lineage 4   |
| D2/TLS/Timor_078/2012 | Timor Leste      | 1/21/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_079/2012  | Bali             | 1/25/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_080/2012  | Bali             | 1/25/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_081/2012  | Bali             | 1/27/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_082/2012  | Bali             | 1/29/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_084/2012  | Bali             | 1/30/2012          | Cosmopolitan, Lineage 4   |
| D2/THA/086/2012      | Thailand         | 1/31/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Lombok_087/2012| Lombok           | 2/1/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_088/2012  | Bali             | 2/2/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_089/2012  | Bali             | 2/2/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_090/2012  | Bali             | 2/3/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Lombok_091/2012| Lombok           | 2/3/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_092/2012  | Bali             | 2/6/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_094/2012  | Bali             | 2/10/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_095/2012  | Bali             | 2/13/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_096/2012  | Bali             | 2/13/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_097/2012  | Bali             | 2/13/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_098/2012  | Bali             | 2/14/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_100/2012  | Bali             | 2/15/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_102/2012  | Bali             | 2/24/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_103/2012  | Bali/Japan       | 2/24/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_104/2012  | Bali             | 2/27/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_105/2012  | Bali             | 2/28/2012          | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_106/2012  | Bali             | 3/5/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_107/2012  | Bali             | 3/6/2012           | Cosmopolitan, Lineage 4   |
| D2/IDN/Bali_108/2012  | Bali             | 2/6/2012           | Cosmopolitan, Lineage 4   |
| DENV-3               |                  |                    |                          |
| D3/IDN/Bali_007/2010  | Bali             | 12/30/2010         | Genotype I, Lineage 3     |
| D3/IDN/Bali_054/2010  | Bali             | 3/6/2010           | Genotype I, Lineage 1     |
| D3/IDN/Bali_056/2010  | Bali             | 9/21/2010          | Genotype I, Lineage 2     |
| DENV-4               |                  |                    |                          |
| D4/IDN/Java_002/2010  | Java             | 10/7/2010          | Genotype III, Lineage 4   |
| D4/PHL/006/2010      | Philippines      | 2/8/2010           | Genotype III, Lineage 5   |
| D4/IDN/Bali_036/2010  | Bali             | 3/3/2010           | Genotype III, Lineage 1   |
| D4/IDN/Bali_062/2010  | Bali             | 11/13/2010         | Genotype III, Lineage 2   |
| D4/IDN/Bali_063/2010  | Bali             | 11/11/2010         | Genotype III, Lineage 2   |
| D4/IDN/Bali_064/2010  | Bali             | 11/8/2010          | Genotype III, Lineage 3   |
| D4/IDN/Bali_065/2010  | Bali             | 10/18/2010         | Genotype III, Lineage 3   |
| D4/066/2010          | N/A              | 5/4/2010           | Genotype III, Lineage 2   |
| D4/IDN/Bali_069/2011  | Bali             | 9/8/2011           | Genotype III, Lineage 2   |

doi:10.1371/journal.pntd.0003442.t001
comparison data set. DENV sequences associated with non-human primates (i.e. from the sylvatic cycle), those with excessively long branches such that they are likely to reflect sequencing error, and obvious laboratory contaminants (i.e. where there is little measurable evolution over multiple years, such as multiple copies of the DENV-2 strain New Guinea C) were excluded. Combined with the viruses isolated from travellers to Australia in the present study, this resulted in data sets of the following size that could easily be visually aligned and subjected to phylogenetic analysis: DENV-1 = 2289 taxa, 1485 nt; DENV-2 = 2263 taxa, 1485 nt, DENV-3 = 1256 taxa, 1479 nt, DENV-4 = 670 taxa, 1485 nt.

Phylogenetic trees for each of the four serotypes were estimated using the maximum likelihood (ML) method available in the RAxML program [18] and assuming the GTR+Gamma substitution model (i.e. the general time-reversible nucleotide substitution model), combined with a rapid bootstrap (100 replicates) procedure. The resultant phylogenies were visualized in

Figure 1. Origin and diversity of dengue viruses isolated from travelers entering Western Australia during 2010–2012. (A) Geographic origins of all imported DENV; (B) Geographic origin of individual DENV serotypes.

doi:10.1371/journal.pntd.0003442.g001
FigTree (kindly provided by Dr. Andrew Rambaut; http://tree.bio.ed.ac.uk/software/figtree/).

The number of phylogenetically distinct lineages of viruses sampled from Australian travellers were identified by a combination of visual inspection (i.e. the detection of phylogenetically distinct groups of sequences associated with travellers) and by the level of bootstrap support, with bootstrap >70% indicative of strong support for groupings of viruses sampled from travellers.

Table 2. Primers used in PCR amplification and sequencing reactions.

| DENV serotype | Primer* | Primer pair | Fragment size (bp) | Primer sequence (5'-3') |
|---------------|---------|-------------|--------------------|------------------------|
| 1             | D1–760F† | 1           | 840               | AAC GTG GAT GTC CTC TGA AGG |
|               | D1–1600R† |             |                   | GGA GGT CCA AGG CAG TG   |
|               | D1–1418F† | 2           | 1182              | GCA ACC ATA ACA CCT CAA  |
|               | D1–2600R† |             |                   | TGG CTG ATC GAA TTC CAC AC|
|               | D1–1220F‡ |             |                   | TTT GTG GAC AGA GGC TGG G |
|               | D1–1559F‡ |             |                   | CAC AAA CAA TGG TTT CTA GAC TT A C |
|               | D1–1948F‡ |             |                   | GAC CCA AGA TGA GAA AGG AGT|
|               | D1–1240R‡ |             |                   | TGC CCC AGC TCT TGT CCA C |
|               | D1–1424R‡ |             |                   | GAC GTA GGA GCT TGA GTG GTT AT |
|               | D1–1868R‡ |             |                   | CAT GCT GGG TCT CAG CCA C |
| 2             | D2–789F† | 1           | 1131              | GAA ACA TGC CCA GAG AAT TGA AAC T |
|               | D2–1920R† |             |                   | CCC TTC ATA TTG TAC TCT GAT AAC AT T TGT TTC |
|               | D2–1547F† | 2           | 852               | AAG CTG GTA TGG TGC ACA GGC AAT GGT T |
|               | D2–2537R† |             |                   | GGG GAT TCT GTG TGG AAC TTG TAT TGT TCT GCC C |
|               | D2–1136F‡ |             |                   | ACA CAA CAA CAG CAT CTC GCT |
|               | D2–1499R‡ |             |                   | CTC GGA GAG CAT TCC ATC GT |
| 3             | D3–291F† | 1           | 1528              | TGG CTA GAT GGG GTA CCT TC |
|               | D3–1819R† |             |                   | CAT CCC TTT GAG TTT CAA TTT GTC CAT |
|               | D3–1685F† | 2           | 865               | CTA GGA GCT CAA GAA GGA GCA ATG CA |
|               | D3–2550R† |             |                   | ATG GCT GTC GCC ACT CTT GGG GGA |
|               | D3–875F‡ |             |                   | GGG AAG CGG TTG TGG TTT GT |
|               | D3–1277R‡ |             |                   | GGG AAG CTC TTT CTG GCC CCA GGT T |
| 4             | D4–742F† | 1           | 1096              | TGG GAT TGG AAA CAA GAG CTG AGA CAT GGA TGT C |
|               | D4–1838R† |             |                   | CGT GTA GCA TAT TCC CTT GAT TCT CAA TTT CTC CA |
|               | D4–1569F‡ | 2           | 970               | CAA TGG TTT TTG GAC CTA CCT CTA CCA TGG |
|               | D4–2539R‡ |             |                   | GGG GAC TCT GTG TGA AAT TTG TAC TCT GTC CTA |

*F indicates forward primer orientation, R indicates reverse primer orientation.
†Primer used for both PCR and sequencing reactions (26)
‡Sequencing primer [27]
§Sequencing primer (designed by Dr. Glenys Chidlow)

Results

Origin of imported DENV

To obtain a snapshot of the diversity of DENV circulating in the South-East Asian region and being imported into Australia we assessed the genetic diversity of DENV isolated from travelers entering Western Australia between March 2010 and March 2012. Accordingly, we noted that the majority of DENV originated in Indonesia, predominantly Bali. This was not unexpected as approximately 80% of dengue cases notified to the Communicable Disease Division of the
Western Australian Department of Health in 2010–2012 originated in Bali [19]. Due to the high number of imported cases, Western Australia notifies the most dengue cases among the eight Australian States and Territories despite having only 10% of the Australian population and no endogenous dengue transmission, due to absence of the mosquito vectors.

**DENV diversity**

Our phylogenetic analysis of the four DENV serotypes revealed a remarkable diversity of viruses in Australian travellers, including the co-circulation of multiple genetically diverse viral lineages (Table 1; Figs. 1–5). With a few interesting exceptions, the viruses sampled from travelers to Australia were generally closely related to those previously circulating in regions of Indonesia, particularly Bali. We now describe each serotype in turn.

**DENV-1**

Our analysis of DENV-1 comprised a total of 28 sequences from travelers entering Western Australia with clinical dengue who were sampled between April 2010 and August 2011. These viruses fell into 14 phylogenetically distinct lineages in three DENV-1 genotypes (Fig. 2), making this the most genetically diverse of all the four serotypes found in this study. As expected, the majority of these sequences were closely related to those previously documented to be circulating in Bali and Indonesia, falling into genotypes I and II of DENV-1 that are common in South-East Asia, and which suggests that these lineages are the dominant regional variants.

The only exceptions were two travellers returning from India who, notably, possessed genotype III strains most closely related to viruses circulating in China (lineage 1). Similarly, the lineage 2 (genotype II) viruses of Balinese origin were most closely related to viruses from China (and which share identical E gene sequences). These phylogenetic patterns are compatible with viral traffic between China, India and Bali that merits further investigation. Finally, it was also notable that a number of lineages co-circulated in the Balinese source population. Specifically, lineages 2, 6, 13 and 14 co-circulated for a period during 2010, while lineages 11, 13 and 14 co-circulated during 2011. Indeed, it is striking that lineage 14 persisted for over a year (April 2010–August 2011).

**DENV-2**

The biggest data set (n = 46) of traveler sequences available here were from DENV-2. With the exception of one sequence, all these viruses were from the Cosmopolitan genotype that has been sampled from a wide range of geographic localities including those in Africa, the Americas, Asia (including Indonesia, Malaysia, Myanmar, Thailand, Singapore) and Oceania (Fig. 3). The single exception (lineage 9) was sampled from Laos and fell within the Asian I genotype, and closely related to a number of other viruses from Laos. Hence, these data provide some support for the existence of a major phylogeographic split, with the Asian I genotype largely sampled in the northern part of South-Asia (including Myanmar, Thailand, Vietnam), and the Cosmopolitan genotype being sampled in more southerly regions. Including the single sequence from Laos, our phylogenetic analysis provided evidence for nine separate lineages, which likely represent separate introductions, into Australian travelers. Again, these introductions largely seem to have their origins in Bali, as they were all closely related to viral lineages previously circulating in either Bali or other regions of Indonesia. Perhaps the most notable phylogenetic pattern was the presence of a major cluster of closely related sequences in Bali (lineage 4) found in patients sampled between December 2011 and March 2012, and which was the most recently circulating lineage detected in this study. Such clustering is indicative of a major local outbreak in Bali which has also affected other parts of Indonesia (Lombok),...
Figure 2. Phylogenetic tree of 2289 DENV-1 E sequences including 28 from travelers to Australia. Because of the very large numbers of sequences involved, where possible phylogenetic clusters have been ‘collapsed’ (such they are they depicted as triangles) to reveal the positions of the Australian viruses in more detail. Major genotypes and geographic locations of sequences are indicated. Those viruses sampled from travellers to Australia are colored.
Timor-Leste and Thailand as the cluster contains sequences sampled from travelers entering from these localities. In addition, given the very close evolutionary relationships among these sequences, many of which are identical, it is possible that there was direct viral transmission among some of the travelers.

**DENV-3**

In the case of DENV-3 only three sequences were obtained from travelers entering Western Australia, all of which fell into genotype I that has been described in a variety of locations across South-East Asia (Fig. 4). However, all these viruses were singletons representing phylogenetically distinct lineages within the genetic diversity of DENV-3, such that they represent three independent introductions in 2010. In all cases, the travelers in question were entering from Bali, and the closest sequences matches were viruses from Indonesia, again suggesting that the travelers have been infected by locally circulating lineages.

**DENV-4**

Our sample of DENV-4 comprised nine sequences from travelers entering Western Australia, and which provided evidence for five phylogenetically distinct lineages, and hence introductions (Fig. 5). All these DENV-4 sequences fell as relatively closely related lineages within genotype III that is commonly sampled within South-East Asia. In addition, all the sequences from travelers to Western Australia were most closely related to background viruses sampled from Bali and other locations within Indonesia, indicating that these are local variants that likely evolved in situ. Also of note were the observations that lineage 2, which comprised four sequences, was sampled between May 2010-September 2011 such that it likely persisted within Bali for over a year, and that lineage 3 (sampled between October-November 2010) spread to both Bali and Java.

**Discussion**

Dengue is endemic throughout Southeast Asia, a region where the incidence of both dengue fever and severe dengue has increased dramatically since the 1950s when the hemorrhagic form of the disease was first recognized. We analyzed DENV derived from travelers entering Western Australia after visiting seven countries throughout Asia between 2010–2012 and identified a diverse range of DENV genotypes and lineages within all four serotypes. Importantly, one of these lineages (DENV-2, Cosmopolitan genotype, lineage 4) appears to have emerged during a major outbreak in Bali in 2011–2012, and hence should be closely monitored.

Most of the travelers studied here had entered from Bali, Indonesia, a popular holiday destination for residents of Western Australia. In recent years new budget airlines began offering affordable Bali package holidays and travel increased 300-fold between 2006 and 2010, coinciding with a sharp increase in dengue cases notified to the Communicable Diseases Division of the WA Department of Health [20]. Epidemic dengue hemorrhagic fever was first described in Indonesia (Java) in 1968 [21], and transmission of all four DENV serotypes was described during an epidemic of severe dengue focused in Jakarta (Java) in 2004, with DENV-3 the predominant serotype [22]. Genome sequence analysis of a subset of isolates showed that the DENV circulating in Indonesia were local strains with strong epidemic potential which had circulated in the region for decades [23]. In our analysis, many of the DENV1–4 sampled from
Figure 3. Phylogenetic tree of 2263 DENV-2 E sequences including 46 from travellers to Australia. Other features are as they are described in Fig. 2.

doi:10.1371/journal.pntd.0003442.g003
Figure 4. Phylogenetic tree of 1256 DENV-3 E sequences including three from travellers to Australia. Other features are as they are described in Fig. 2.

doi:10.1371/journal.pntd.0003442.g004
Figure 5. Phylogenetic tree of 670 DENV-4 E sequences including nine from travellers to Australia. Other features are as they are described in Fig. 2.

doi:10.1371/journal.pntd.0003442.g005
Indonesia during 2010–2012 also appeared to be local regional variants, although with clear lineage importation including the presence of two DENV-1 Genotype II viruses sampled from travelers entering WA from Bali but which were most closely related to viruses circulating in China.

Bali is a major travel destination and attracts visitors from many countries, predominantly from the Asia-Pacific and ASEAN regions where dengue burden is the highest globally. The continuing annual growth increase in visitor arrivals into Bali indicates there is a high likelihood that novel DENV lineages will continue to be introduced into Bali. Official surveillance data for Bali are not immediately available. However, local newspapers reported that the Bali Health Agency recorded almost 6,000 cases in 2013 (a prevalence of 145 per 100,000 population) with a mortality rate of 1%, for a population of 4.1 million, and that there was a large number of dengue cases in 2010 with a prevalence of 365 persons per 100,000 population [24]. Our analysis indicates that there was hyperendemic transmission of all four serotypes in Bali during 2010, and circulating DENV included dominant local strains which had circulated for several years, as well as strains more recently introduced into Bali from other countries in the region. Hence, these data suggest that Bali is a melting pot of substantial DENV diversity, and therefore may in turn serve as a hub for dengue transmission and mixing. Indeed DENV traffic into other countries via travelers returning from Bali has been described [25,26].

Systematic surveillance in acutely ill travelers entering Australia from endemic Asia-Pacific countries, which are popular travel destinations, provides valuable and timely information on DENV lineages circulating in the region. We identified an emerging lineage of DENV-2 that appeared to be associated with a major outbreak in Bali in 2012, and which was also identified in travelers entering from Timor-Leste and Thailand. It is unclear how long this lineage has been present in the region as there is a dearth of publicly available contemporaneous DENV sequence data. Additional DENV-2 isolates from 2010–2014 are currently being sequenced to determine if this virus was present in other countries prior to 2012, and to confirm continued transmission after this time.

More generally, this study demonstrates that surveillance of returned travelers may provide important information on DENV genotypes and lineages circulating in dengue endemic countries where locally-generated detailed genetic data may not be available.

Acknowledgments

We thank Carolien Giele and Paul Effler of the Communicable Disease Division, Western Australia Department of Health, for advice and helpful discussions. We thank the staff of PathWest Laboratory Medicine WA for preparing patient sera and for diagnostic testing.

Author Contributions

Conceived and designed the experiments: AI DWS. Performed the experiments: TE SM GC DLS ECH. Analyzed the data: AI TE SM GC ECH DWS. Contributed reagents/materials/analysis tools: GC ECH DWS. Wrote the paper: AI TE ECH DWS.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, et al. (2103) The global distribution and burden of dengue. Nature 496:504–7.
2. World Health Organization Western Pacific Region (2014) Dengue fact sheet. Available at http://www.wpro.who.int/mediacentre/factsheets/fs_09032012_Dengue/en/. Accessed 13 October 2014.
3. Rico-Hesse R. (1990) Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. Virology 174:479–93. PMID:2129562
4. Holmes E, Twiddy S. (2003) The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3:19–28. PMID: 12797969

5. Weaver SC, Vasilakis N. (2009) Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol 9:523–40. doi: 10.1016/j.meegid.2009.02.003 PMID: 19460319

6. Twiddy SS, Farrar JJ, Vinh Chau N, Wills B, Gould EA, et al. (2002) Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. Virology 298:63–72. PMID: 12093174

7. Bennett SN, Holmes EC, Chirivella M, Rodriguez DM, Beltran M, et al. (2003) Selection-driven evolution of emergent dengue virus. Mol Biol Evol 20:1650–58. PMID: 12832629

8. Vu TT, Holmes EC, Duong V, Nguyen TQ, Tran TH, et al. (2010) Emergence of the Asian 1 genotype of dengue virus serotype 2 in Viet Nam: in vivo fitness advantage and lineage replacement in South-East Asia. PLoS Negl Trop Dis 4:e757. doi: 10.1371/journal.pntd.0000757 PMID: 20651932

9. Rico-Hesse R, Harrison LM, Salas RA, Trovar D, Nisalak A, et al. (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230:244–51. PMID: 9143280

10. Hota S, Kimura R. (1952) Experimental studies on dengue 1. Isolation, identification, and modification. Commun Dis Intell Q Rep. Mar 31; 37(1):E55. doi: 10.1016/j.meegid.2009.02.003 PMID: 19460319

11. Sabin A. (1952) Research on dengue during World War II. Am J Trop Med Hyg 1:30–50. PMID: 14903434

12. Hammon WMD, Rudnick A, Sather G, Rogers KD, Morse LJ. (1960) New hemorrhagic fevers of children in the Philippines and Thailand. Trans Assoc Am Physicians 73:140–155 PMID: 13711073

13. A-Nuegoonpipat A, Berlioz-Arthaud A, Chow V, Endy T, Lowry K, et al. (2004) Sustained transmission of dengue virus type 1 in the Pacific due to repeated introductions of different Asian strains. Virology 329:505–12. PMID: 15518627

14. Effler PV, Pang L, Kitsutani P, Vorndam V, Nakata M, et al. (2005) Dengue fever, Hawaii, 2001–2002. Emerg Infect Dis 11:742–9. PMID: 15890132

15. Imrie A, Roche C, Zhao Z, Bennett S, Laille M, et al. (2010) Homology of complete genome sequences for dengue virus type-1, from dengue-fever- and dengue-haemorrhagic-fever-associated epidemics in Hawaii and French Polynesia. Ann Trop Med Parasitol 104:225–35. doi: 10.1179/136465910X12647085215570 PMID: 20507696

16. Imrie A, Zhao Z, Bennett SN, Kitsutani P, Laille M, et al. (2006) Molecular epidemiology of dengue in the Pacific: introduction of two distinct strains of dengue virus type-1 [corrected] into Hawaii. Ann Trop Med Parasitol 100:327–36. PMID: 16762119

17. Messina JP, Brady OJ, Scott TW, Zou C, Pigott DM, et al. (2014) Global spread of dengue virus types: mapping the 70 year history. Trends Microbiol 22:138–46. doi: 10.1016/j.timm.2013.12.011 PMID: 24468533

18. Stamatakis A, Hoover P, Rougemont J. (2008) A rapid bootstrap algorithm for the RAxML Web servers. Syst Biol. 57:758–71. doi: 10.1086/510954 PMID: 18853362

19. Knope K, National Arbovirus and Malaria Advisory Committee, Giele C. (2013) Commun Dis Intell Q Rep. Mar 31; 37(1):E55–9. PMID: 23692160

20. Department of health (2012) Bali travel behind spike in dengue fever. Available at http://www.health.wa.gov.au/diseasewatch/2012_03/bali_travel.cfm. Accessed 25 June 2014.

21. Nathin MS, Harun SR, Sumarmo. (1988) Dengue hemorrhagic fever and Japanese B encephalitis in Indonesia. Southeast Asian J Trop Med Public Health 19:474–81.

22. Suwandono A, Kosashi H, Nurhayati, Kusriastuti R, Harun S, et al. (2006) Four dengue virus serotypes found circulating during an outbreak of dengue fever and dengue haemorrhagic fever in Jakarta, Indonesia, during 2004. Trans R Soc Trop Med Hyg 100:855–62. PMID: 16507313

23. Ong SH, Yip JT, Chen YL, Liu W, Harun S, et al. (2008) Periodic re-emergence of endemic strains with strong epidemic potential—a proposed explanation for the 2004 Indonesian dengue epidemic. Infect Genet Evol 8:191–204. doi: 10.1016/j.meegid.2007.12.005 PMID: 18243816

24. The Bali Daily (2013) Bali has highest number of dengue cases. Available at http://www.thebalidaily.com/2013-12-30/bali-has-highest-number-dengue-cases.html. Accessed 25 June 2014.

25. Huang J-H, Su C-L, Yang C-F, Liao T-L, Hsu T-C, et al. (2012) Molecular characterization and phylogenetic analysis of dengue viruses imported into Taiwan during 2008–2010. Am J Trop Med Hyg 87:349–58. doi: 10.4269/ajtmh.2012.11-0666 PMID: 22855770

26. Warrillow D, Northill JA, Pyke AT. (2012) Sources of dengue viruses imported into Queensland, Australia, 2002–2010. Emerg Infect Dis 18:1850–57 doi: 10.3201/eid1811.120014 PMID: 23092682

27. Gonzalvez AP, Escalante AA, Pujol FH, Ludert JE, Tovar D, et al. (2002) Diversity and evolution of the envelope gene of dengue virus type 1. Virology 303:110–9. PMID: 12482662