Table 2. Sequences of qPCR primers for CD markers and cytokines.

Target gene	Species	5′-primer sequence -3′[a), b)] Forward	Reverse	Product size (bp)	PCR efficiency	Reference
CD3ε	Cj	GGCTTGCTGCTGCTGGTTTAC	CCGGATGGGCTCATAGTCTG	150	0.865	DQ189218
	Hs	---------------------	--------------------	150	0.848	NM_000733
CD4	Cj	GGAAAACGGGAAAGTTGCATCA	GCCTTCTCCCGCTTAGAGAC	163	0.926	AF452616
	Hs	C------A--------------	--------------C-----	162	0.907	M35160
CD8α	Cj	TCTCCCAAACCAAGTCCAAGG	AGTTTCTCAGGGCCGAGCAG	144	0.940	DQ189217
	Hs	---------A----C------	. ---G---------------	143	0.912	NM_001768
CD20	Cj	GGGCTGTCCAGATTATGAATG	GAGTTTTTCTCCGTTGCTGC	166	0.942	DQ189220
	Hs	---------------------	--------------------	166	1.002	X07203
IL-1β	Cj	TGCACCTGTACGATCCCTGAAC	TTGCACAAAGGACATGGAGAACAC	145	0.806	AB539804
	Hs	---------------A------	---T--------------------	145	0.780	NM_000576
IL-2	Cj	CCCAAGAAGGCCAAAGAATTG	CTTAAGTGAAAGTTTTTGCTTTGAG	104	0.773	DQ826674
	Hs	-------------C----C--	-------------------------	103	0.797	BC070338
IL-4	Cj	CATTGTCACAGAGCAAAAGACTC	CTCAGTTGTGTTCTTGGAGGCA	79	0.910	XM_002744606
	Hs	. GCC----------G-------	----------------------	77	0.878	NM_000589
IL-5	Cj	AATCACCAACTGTGCACTGAAGAA	. TTTGGCGGTCAATGTGTTCCTT	130	0.871	DQ658152
	Hs	------------------------	TT------C--------A--T---	132	0.860	NM_000879
IL-6	Cj	GATTCAATGAGGAGACTTGCC	TGTTCTGGAGGTACTCTAGGTA	81	0.920	DQ658153
	Hs	---------------------	----------------------	81	0.990	NM_00600
IL-10	Cj	CTGCCTCACATGCTTCGAGA	TGGCAACCCAGGTAACCCTTA	134	0.970	DQ658154
	Hs	------A-------------	---------------------	134	0.920	M57627
IL-12β	Cj	. GGACGGCAAGGAGTATGAGTA	TTGAGCTTGTGAACGGCATC	111	0.935	AB539805
	Hs	G----AA---------------	--------------------	112	0.900	M65272
IL-13	Cj	TCCAGCTTGCTTGTCCGAG	CTGCAAATAATGATGCGTT-GATGT	127	0.916	AB571243
	Hs	----------A--------	. --------------T--C--A--	127	0.964	NM_002188
IFN-γ	Cj	GGGTTCTCTTGGCTGTTACTG	TGTCTAAGAAAAGAGTTCCATTATC	116	0.838	FJ598593
	Hs	---------------------	. -C----------------------	115	0.856	NM_000619
TNF-α	Cj	AGCCTGTAGCCCATGTTGTAG	CTCTCAGCTCCACGCCATTG	102	0.887	DQ520835
	Hs	---------------------	--------------------	102	0.817	NM_000594

[a)]Hyphen indicates a nucleotide identical to human sequences.
[b)]Dot indicates a shift nucleotide to marmoset sequences.
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A variety of gene expression stabilities among tissues

To evaluate the expression stability of selected reference genes, we used a publicly available program, *geNorm* applets. *geNorm* provides a ranking of tested genes based on the reference gene stability measure M, which is defined as the average pairwise variation of a particular gene compared with all other control genes. Thus, genes with higher M values have greater variations of expression. In addition, assessment of the pairwise variations ($V_{n/n+1}$) between each combination of sequential normalization factors allows identification of the optimal number of reference genes. In the original publication describing *geNorm* [15], a threshold of 0.15 for pairwise variation was established, below which the inclusion of additional reference genes was not necessary.

geNorm analysis produced line plots indicating the mean expression stability M of the remaining candidate reference genes in each round of the analysis (Figure 2A and 2B), the pairwise variation V (Figure 2C) and ranking of the candidate reference genes from the least stable to the two most stable genes (Figure 3). The stability score M indicated that gene expression in spleen, jejunum and cerebellum were relatively less stable than other tissues (Figure 2A and B). However, all tissues tested exhibited high stabilities, as M values were less than 1.5, which was the default limit even when all eight genes were analyzed. According to pairwise variation V (Figure 2C), the two most stable genes were sufficient for a stable and valid reference for each tissue analyzed by qPCR because V2/3 values were less than 0.15 in all tissues. Jejunum was the most variable tissue with a $V_{2/3}$ value of 0.139. Figure 3 shows ranking of gene expression stability based on M values. *GAPDH*, *ACTB*, *SDHA* and *TBP* had higher stability, while *HPRT*, *rRNA* and *B2M* were variable in most tissues. *TBP* in intestinal segments (jejunum, ileum and colon) and *SDHA* in brain segments (cerebrum, cerebellum and brain stem) were particularly stable. *HPRT* ranked as the worst of the eight genes in the 13 tissues tested.

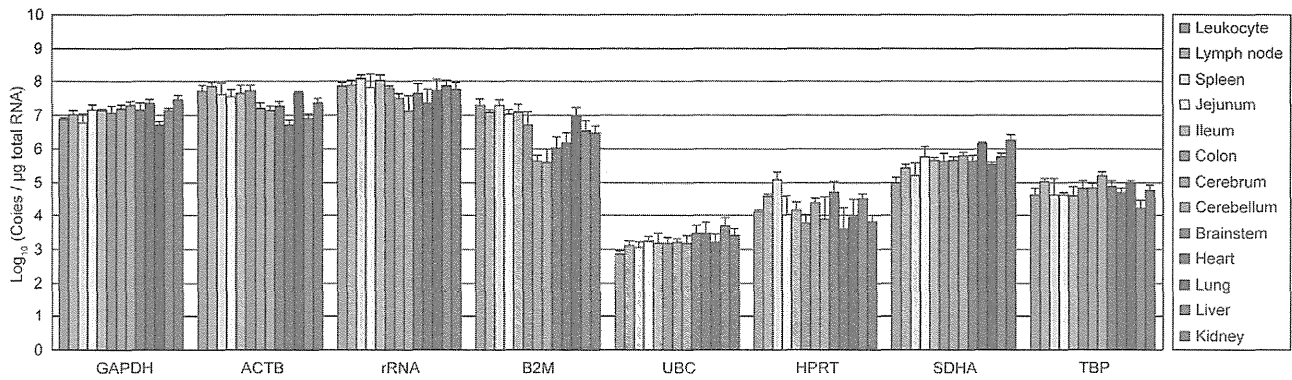

Figure 1. Absolute copy numbers of candidate reference genes. The expression level of each gene in 13 tissues is shown as a logarithmic histogram of absolute copy numbers per µg of total RNA. Means and standard deviations of four individuals are indicated. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; ACTB: actin, beta; rRNA: 18S ribosomal RNA; B2M: beta-2-microglobulin; UBC: ubiquitin C; HPRT: hypoxanthine phosphoribosyltransferase 1; SDHA: succinate dehydrogenase complex, subunit A; TBP: TATA-box binding protein.
doi:10.1371/journal.pone.0056296.g001

Comparison of gene expression levels between human and common marmoset leukocytes

Subsequently, we analyzed gene expression levels of four CD antigens (CD3ε, CD4, CD8α, and CD20) and ten cytokines, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12β, IL-13, interferon (IFN)-γ and tumor necrosis factor (TNF)-α, in peripheral blood leukocytes from humans and common marmosets (Figure 4). The sequences of primers specific for these

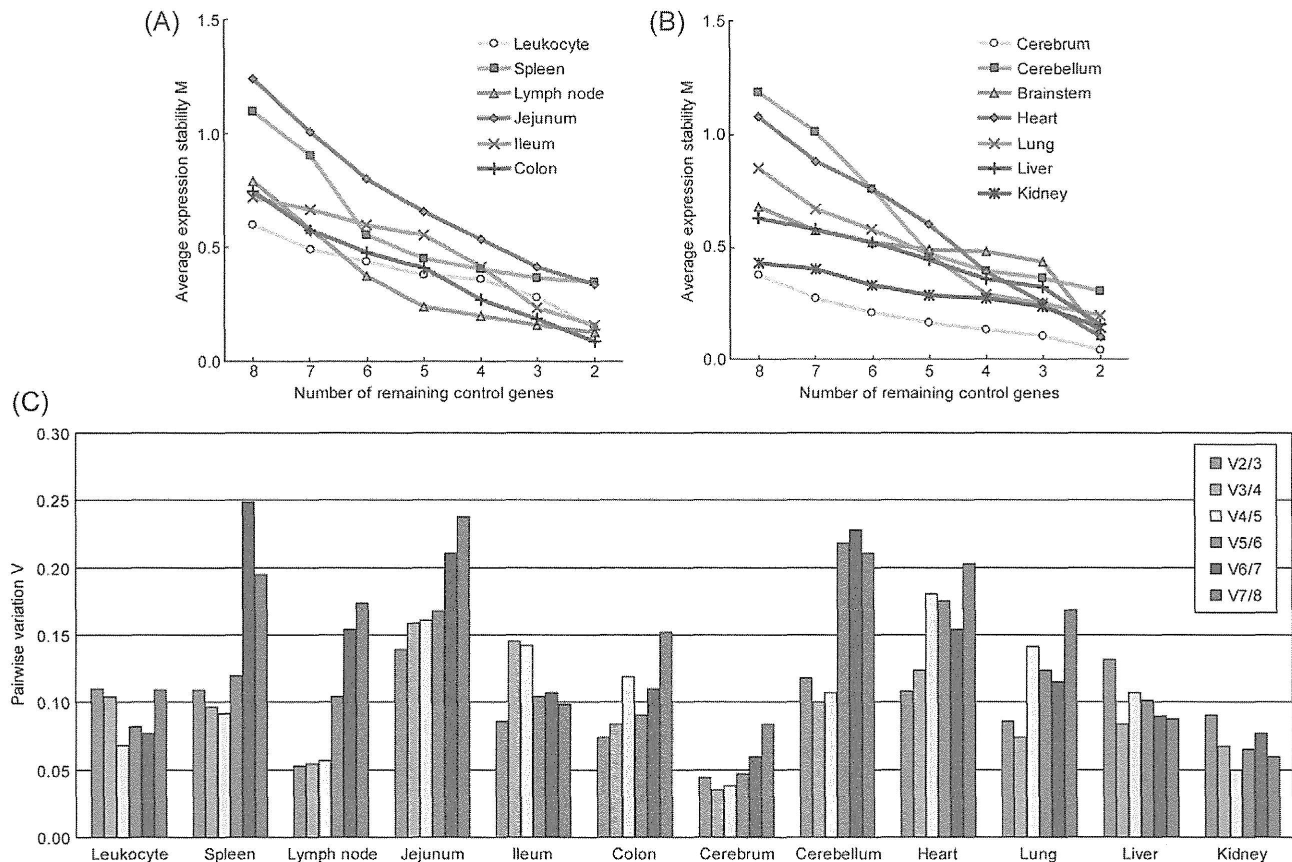

Figure 2. Gene expression stability and pairwise variation of candidate reference genes using *geNorm* analysis. (A) and (B): Average gene expression stability values M of the remaining reference genes during stepwise exclusion of the least stable gene in the different tissue panels are shown. Data are divided into two figures to avoid closely-packed lines. See also figure 3 for the ranking of genes according to their expression stability. (C) Pairwise variation analysis was used to determine the optimal number of reference genes for use in qPCR data normalization. The recommended limit for V value is 0.15, the point at which it is unnecessary to include additional genes in a normalization strategy.
doi:10.1371/journal.pone.0056296.g002

Stability	High						Low
Leukocyte	GAPDH-UBC	ACTB	SDHA	TBP	B2M	rRNA	HPRT
Spleen	GAPDH-SDHA	ACTB	TBP	UBC	rRNA	B2M	HPRT
Lymph node	rRNA-TBP	ACTB	SDHA	UBC	GAPDH	B2M	HPRT
Jejunum	UBC-TBP	GAPDH	B2M	SDHA	ACTB	rRNA	HPRT
Ileum	B2M-TBP	UBC	ACTB	HPRT	SDHA	GAPDH	rRNA
Colon	ACTB-TBP	SDHA	GAPDH	rRNA	UBC	HPRT	B2M
Cerebrum	GAPDH-SDHA	rRNA	ACTB	HPRT	UBC	TBP	B2M
Cerebellum	SDHA-TBP	GAPDH	ACTB	UBC	rRNA	B2M	HPRT
Brainstem	ACTB-SDHA	UBC	rRNA	HPRT	GAPDH	TBP	B2M
Heart	SDHA-TBP	GAPDH	ACTB	UBC	B2M	rRNA	HPRT
Lung	SDHA-TBP	ACTB	GAPDH	UBC	B2M	rRNA	HPRT
Liver	GAPDH-SDHA	HPRT	rRNA	ACTB	TBP	UBC	B2M
Kidney	GAPDH-SDHA	TBP	UBC	ACTB	B2M	rRNA	HPRT

Figure 3. Ranking of gene expression stability of candidate reference genes using *geNorm* analysis. Candidate reference genes are ranked in order of stability for each tissue with the two most stable genes at the left and the least stable at the right.
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immune-related genes are shown in Table 2. The normalization factor for common marmoset leukocytes was calculated using *GAPDH* and *UBC* based on the *geNorm* analysis as described above. For human leukocytes, we found that the expression of all eight genes were stable (M value = 0.363), of which *ACTB* and *HPRT* had the best score (M value = 0.163, $V_{2/3}$ = 0.062) and were selected for use. The expression levels of CD4 and IL-4 were significantly lower in common marmosets than in humans while those of IL-10, IL-12β and IFN-γ were significantly higher in common marmosets compared with humans. Of interest, the expression level of IL-4 was notably lower in common marmosets than humans, and was close to the detection limit. There was no statistical difference in the expression levels of the other genes tested between common marmosets and humans.

Difference of CD4/CD8 ratio between humans and common marmosets

We calculated ratios of the expression levels of CD4 to CD8 (CD4/CD8 ratio) in human and common marmoset leukocytes (Figure 5, left panel). CD4/CD8 ratios were significantly higher in human leukocytes compared with common marmoset leukocytes (mean ± sd, 0.59±0.22 vs. −0.49±0.41, $P<0.01$). To confirm the difference in CD4/CD8 ratios, we examined the proportion of $CD4^+$ and $CD8^+$ in $CD3^+$ T cells by flow cytometric analysis. As shown in Figure 6, the rates of $CD3^+$ cells in the lymphocyte gate were similar between common marmosets (30%) and humans (38%). However, the rates of $CD4^+/CD3^+$ cells and $CD8^+/CD3^+$ cells was 36% and 61% in common marmosets, respectively, and 75% and 21% in humans, respectively. Similarly, the CD4/CD8 ratio was markedly different between common marmosets and humans (mean ± sd, 0.56±0.08 vs. 3.22±0.35, $P<0.01$) by qPCR. This indicated a good correlation between the results from FACS analysis and that of qPCR analysis. To examine whether the CD4/CD8 ratio is affected by age, we further performed FACS analyses with PBMCs from young and old marmosets (Table 3). The result showed that the inverted CD4/CD8 ratio was fairly constant among individuals and over ages.

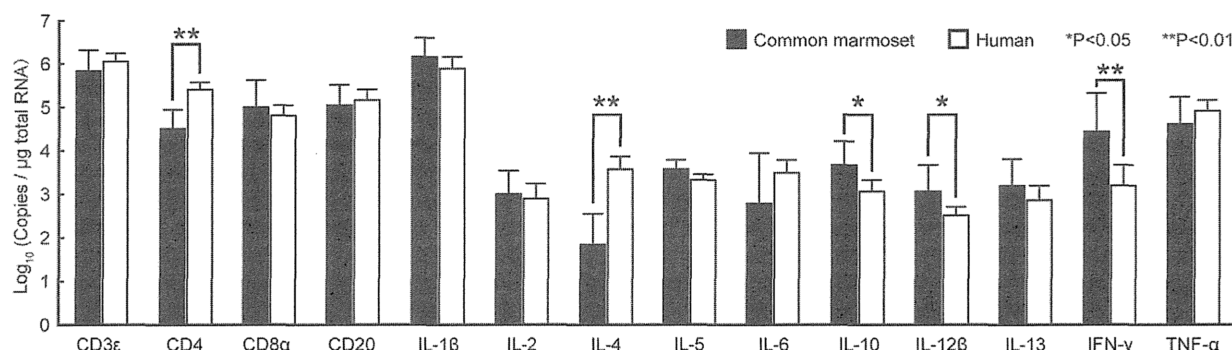

Figure 4. The expression levels of CD antigens and cytokine genes in common marmoset and human leukocytes. The expression level of each gene is shown as a logarithmic histogram of absolute copy numbers per μg of total RNA. Means and standard deviations of eight individuals are indicated. Asterisk indicates statistically significant differences between marmosets and humans by Student's *t*-test (**P* value<0.05, ***P* value<0.01).
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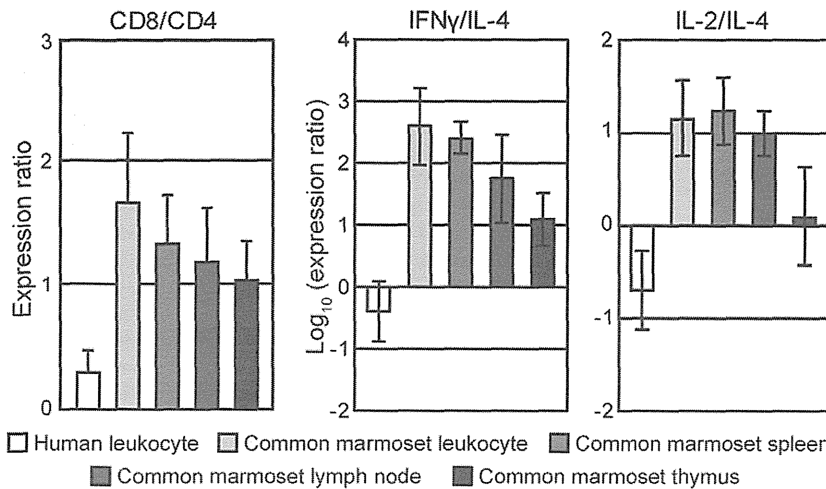

Figure 5. The expression ratios of CD8 to CD4 (CD8:CD4) and Th1-related genes to Th2-related genes. The ratio of CD8:CD4 (left panel), IFN-γ:IL-4 (middle panel) and IL-2:IL-4 (right panel) in human and common marmoset leukocytes, spleen, lymph node and thymus are shown. Significant differences in the CD8:CD4, IFN-γ:IL-4 and IL-2:IL-4 ratios were found between human leukocytes and common marmoset tissues (*$P<0.05$).
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Difference in T helper 1 (Th1)/T helper 2 (Th2) balance between humans and common marmosets

We compared the ratios of expression levels of Th1-related genes (IFN-γ or IL-2) and Th2-related genes (IL-4) (IFN-γ:IL-4 or IL-2:IL-4 ratio) (Figure 5, middle and right panels). Both logarithmic values of the IFN-γ:IL-4 and IL-2:IL-4 ratios were negative in human leukocytes whereas those of common marmoset leukocytes, spleen, lymph node and thymus indicated positive values, showing a clear difference in the Th1/Th2 balance between humans and common marmosets.

Discussion

In the present study, we evaluated the expression stability of common marmoset housekeeping genes in various tissues. To the best of our knowledge, this is the first report of a systematic evaluation of potential reference genes in common marmosets. We chose eight commonly used classical housekeeping genes. Of all genes tested, *rRNA* showed the most abundant expression and *UBC* showed the lowest expression. The *UBC* gene contains multiple directly repeated ubiquitin coding sequences (i.e., polyubiquitin precursor protein) [17]. However, the primer set we used enabled amplification of the unrepeated sequence at the 5′ region of the *UBC* gene only. Thus, low *UBC* expression in our data does not reflect the amount of ubiquitin C protein. *B2M* expression levels were markedly lower in brains and hearts than in other tissues. Resident brain cells normally express few or no MHC class I and B2M molecules [18–20]. In addition, *B2M* expression is upregulated by infection or autoimmune disease [21–23]. Therefore, in disorders with cellular infiltration such as inflammation (especially encephalitis) or cancer cell invasion, *B2M* expression levels may be significantly varied compared with normal tissue.

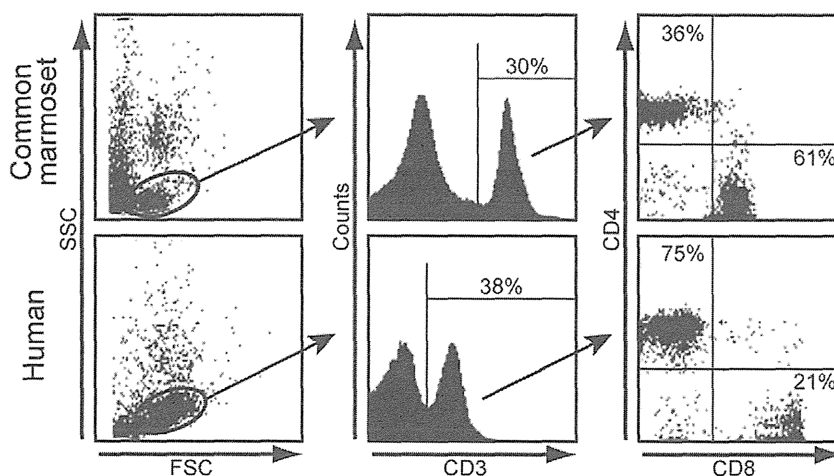

Figure 6. The ratio of CD4^{+} to CD8^{+} cells in common marmoset and human peripheral blood mononuclear cells (PBMCs) by flow cytometry. Representative scattered plots of FSC and SSC are shown in the left panels. Middle panels represent a histogram of CD3 analyzed in the lymphocyte gate. Gated CD3^{+} cells were analyzed for CD4 and CD8 expression (right panels).
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Table 3. CD8/CD4 ratio in PBMCs from young and old marmosets.

Age	Sex	% positive		CD8/CD4 ratio
		CD8	CD4	
3 month*	male	58.3	38.4	1.52
1.5 year	female	60.7	36.1	1.68
1.5 year*	male	55.1	41.5	1.33
2.0 year	male	52.7	44.6	1.18
10 year*	female	58.6	37.8	1.55
Mean ± sd		57.1±3.2	39.7±3.4	1.45±0.20

*Only FACS analysis, but not qPCR, was done with PBMCs from these three marmosets.

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Thus, we predict that *B2M* may be unsuitable as a reference gene in many cases.

We assessed gene expression stability using the *geNorm* applet. As shown in Figure 2, *geNorm* analysis indicated that all tested genes were stable in each tissue. However, there were some trends in the stability ranking (Figure 3). For example, *TBP* in intestine segments and *SDHA* in brain segments represented prominently high stabilities. *GAPDH*, *ACTB*, *SDHA* and *TBP* were generally ranked high followed by *UBC*. In contrast, the stability of *rRNA* was generally low. This suggests the amount of mRNA is not always proportional to that of total RNA as reported by other studies [24,25]. In addition, *HPRT*, *rRNA* and *B2M* varied widely among tissues and rarely ranked high.

We analyzed the expression levels of CD antigens and cytokines by qPCR to compare the characteristics of peripheral blood leukocytes between common marmosets and humans (Figure 4). We observed that the expression levels of CD4 and IL-4 were lower in common marmosets than in humans. In contrast, the expression levels of IL-10, IL-12β and IFN-γ were higher in common marmosets. We calculated PCR efficiency of each primer set and found there was no great difference between primers for common marmosets and those for humans (Tables 1 and 2). Thus, the differences in the gene expression levels between common marmosets and humans are not attributable to the differences in PCR efficiency.

We also observed that the CD4:CD8 ratio and Th1/Th2 balance were inverted in common marmosets by qPCR analysis (Figure 5). In particular, we confirmed the inverted CD4:CD8 ratio by flow cytometric analysis (Figure 6 and Table 3). The inverted CD4:CD8 ratio was stable over age. Of interest, we noted that the Th1/Th2 balance is different between common marmosets and humans, although we can only speculate on the cause of the difference. First, intestinal parasite infections may affect the Th1/Th2 balance by regulating expression of genes encoding cytokines [26–28]. In particular, protozoan parasites are potent stimulators of IFN-γ expression and Th1 responses [29]. Moreover, humans living in poor hygienic conditions in developing countries had higher Th1 cytokine levels compared with people in developed countries [30]. Although the common marmosets used in this study were maintained in specific pathogen-free conditions, we cannot rule out that such infectious agents may be one of a number of factors responsible for the difference in Th1/Th2 balance.

A second possible reason may be a difference in the number of cells producing the respective cytokines. As shown in Figure 6, the ratio of $CD4^+$ to $CD8^+$ cells were markedly different in total leukocytes from common marmosets and humans. Since IL-4 is mainly produced by $CD4^+$ T cells [31,32], its expression level may be influenced by the CD4:CD8 ratio. However, this is not true for all the cytokines tested. For example, the expression levels of IL-2, IL-5 and IL-13, largely produced by T cells, were not significantly different between common marmosets and humans. Therefore, we suggest that the CD4:CD8 ratio has little effect on Th1/Th2 balance. IL-10 is produced by T cells and monocytes [33] and IL-12β is naturally produced by dendritic cells and macrophages [34,35]. However, we could not verify these cell numbers in the common marmoset. Further studies are required to determine whether the numbers of cytokine-producing cells influence the expression levels of IL-10 and IL-12β.

Another possibility is genetic variation. Bostik et al., reported distinct sequence differences in the promoter region or the proximal region of cytokine genes including IL-4, IL-10, IL-12β and TNF-γ among humans, macaque and mangabey monkeys, which affected regulation of cytokine synthesis [36]. Jeong et al., reported that the expression level of IL-4 was lower in monkeys (baboon and macaque) than in hominoids (human and chimpanzee) while the expression levels of IL-12β and the IFN-γ were higher in monkeys [37]. It is likely that Th1 dominant expression is common to primates other than hominoids and the difference in Th1/Th2 balance may be caused by genetic differences between common marmosets and humans.

The use of common marmoset is growing in popularity as a non-human primate model in many fields including autoimmune disease and infectious disease. In this study, we presented data regarding gene expression stabilities of common marmoset housekeeping genes and differences in the Th1/Th2 balance between common marmosets and humans. This difference may affect host defense and/or disease susceptibility, which should be carefully considered in biomedical research using common marmoset as an experimental model. We believe our data will contribute to future investigations using common marmoset models of various diseases.

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Author Contributions

Conceived and designed the experiments: YF TM K. Kitaura TS YH IK RS. Performed the experiments: YF K. Kitaura KS SS TT YK ST HK. Analyzed the data: YF RS. Contributed reagents/materials/analysis tools: K. Kumagai KS. Wrote the paper: TM K. Kitaura TS YH IK RS.

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Article

Presence of Viral Genome in Urine and Development of Hematuria and Pathological Changes in Kidneys in Common Marmoset (*Callithrix jacchus*) after Inoculation with Dengue Virus

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Abstract: Common marmosets (*Callithrix jacchus*) developed high levels of viremia, clinical signs including fever, weight loss, a decrease in activity and hematuria upon inoculation with dengue virus (DENV). Presence of DENV genome in urine samples and pathological changes in kidneys were examined in the present study. Levels of DENV genome were determined in 228 urine samples from 20 primary DENV-inoculated

marmosets and in 56 urine samples from four secondary DENV-inoculated marmosets. DENV genome was detected in 75% (15/20) of marmosets after primary DENV infection. No DENV genome was detected in urine samples from the marmosets with secondary infection with homologous DENV (0%, 0/4). Two marmosets demonstrated hematuria. Pathological analysis of the kidneys demonstrated non-suppressive interstitial nephritis with renal tubular regeneration. DENV antigen-positive cells were detected in kidneys. In human dengue virus infections, some patients present renal symptoms. The results indicate that marmosets recapitulate some aspects of the involvement of kidneys in human DENV infection, and suggest that marmosets are potentially useful for the studies of the pathogenesis of DENV infection, including kidneys.

Keywords: dengue virus; animal model; marmoset; urine; hematuria; kidney

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1. Introduction

Dengue fever is a serious global health problem. In dengue fever patients, urine samples contain dengue virus (DENV) genomes and virus antigens were present in renal biopsies. However, the association between disease symptoms from appearance of DENV genome in urine, renal injury (occurrence rate of 2.9–13.3% of dengue patients) and haemolytic uraemic syndrome in the pathogenesis of dengue fever is unclear [1,2]. Poor outcome, including severe dengue and mortality, correlated with poor renal function [1]. To elucidate disease pathogenesis, it is important to establish an animal model that exhibits clinical signs which are comparable to those of human DENV infection. Marmosets develop high levels of viremia and demonstrate changes in biochemical and hematological parameters upon DENV inoculation [3,4]. In the present study, we constantly detected DENV genome in urine samples from DENV-inoculated marmosets. These marmosets also exhibited hematuria and pathological changes in the kidneys. The marmoset DENV infection model appears to recapitulate some aspects of DENV infection, and thus, offer the possibility of use in pathogenesis studies of DENV infection.

2. Results and Discussion

A total of 228 urine samples were obtained on days 1–14 from 20 marmosets after primary infection and 56 urine samples from four marmosets after homologous secondary infection (Table 1). DENV genome was detected in 18 of the 20 primary DENV-inoculated marmosets (Table 1, experiments 1–5). DENV genome was first detected three days after inoculation, and detected up to day 14. The levels of viral genome ranged from 3.8×10^3 to 8.4×10^7 copies/ml. The positive detection rates were 10% (8/82) on days 1–5, 20% (16/79) on days 6–10 and 27% (18/67) on days 11–14 after primary inoculation (Table 2). No DENV genome was detected in 56 urine samples (0%, 0/56) from 4 marmosets re-inoculated with the same serotype (Table 2, experiment 6, marmosets D2-2, D2-3, D2-4 and D2-5) on days 1–14 after inoculation. The detection rates in urine samples were 6% on day 3, the rates ranged from 17 to 31% (mean percentage = 22 ± 4%) on days 4–13, and 35% on day 14. In comparison to detection of viral genome in urine samples, DENV genome was detected on days 2–7 in serum samples (Table 2).

Table 1. Levels of dengue viral genome in urine samples from marmosets inoculated with dengue virus (DENV).

Animal ID	Virus strain	Dengue vRNA copy numbers (copies/ml)														
		pfu/dose	Days after inoculation													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
Primary inoculation																
Experiment 1																
D1-1	02-17/1	3.5×10^7	-	NT	NT	NT	7.5×10^4	-	NT	-	-	NT	-	-	-	-
D2-1	DHF0663	6.7×10^7	-	NT	NT	NT	3.8×10^3	2.5×10^4	7.0×10^4	4.0×10^4	5.0×10^4	3.7×10^4	6.5×10^4	4.2×10^3	1.0×10^4	-
D3-1	DSS1403	4.5×10^6	-	NT	NT	NT	-	-	NT	4.8×10^4	-	-	NT	8.5×10^4	-	-
D4-1	05-40/1	1.5×10^6	-	NT	NT	NT	-	-	NT	-	-	NT	-	-	-	-
Experiment-2																
D2-2	DHF0663	4.4×10^7	-	NT	8.3×10^4	-	-	-	2.9×10^5	1.8×10^5	-	-	-	8.1×10^4	-	-
D2-3			-	NT	-	7.3×10^4	-	7.1×10^4	1.5×10^5	-	4.8×10^5	-	-	-	-	-
D2-4		1.8×10^5	-	NT	-	-	1.4×10^5	-	-	-	-	-	-	-	-	2.0×10^5
D2-5			-	NT	-	-	-	-	-	-	-	-	-	-	-	-
Experiment-3																
D2-6	DHF0663	1.8×10^4	-	-	-	-	-	-	-	-	-	1.0×10^5	-	-	-	-
D2-7			-	-	-	-	-	-	-	-	-	-	-	-	-	8.5×10^4
D2-8		1.8×10^3	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3×10^4
D2-9			-	-	-	-	-	-	-	-	-	-	1.9×10^4	-	-	-
Experiment-4																
D2-10	Jam/77/07	1.2×10^5	-	-	-	-	-	-	-	-	-	-	-	3.0×10^5	-	-
D2-11			-	-	-	6.7×10^3	-	-	-	-	-	-	-	-	-	4.2×10^5
D2-12	Mal/77/08	1.9×10^5	-	-	-	-	-	-	-	NT	-	5.9×10^6	1.7×10^5	-	7.8×10^5	-
D2-13			-	-	-	-	-	-	-	-	1.3×10^6	-	4.4×10^5	-	-	8.1×10^5
Experiment-5																
D2-14	DHF0663	6.7×10^7	-	-	-	*	*	*	*	*	*	*	*	*	*	*
D2-15			-	-	-	-	-	*	*	*	*	*	*	*	*	*
D2-16			-	-	-	-	-	-	*	*	*	*	*	*	*	*
D2-17			-	-	-	2.7×10^5	2.6×10^5	4.4×10^6	-	-	NT	8.4×10^7	5.9×10^7	-	1.9×10^7	8.2×10^6

- indicates viral RNA below limit of detection using RT-PCR, NT indicates not tested, * indicates that the marmosets were sacrificed and no samples were collected.

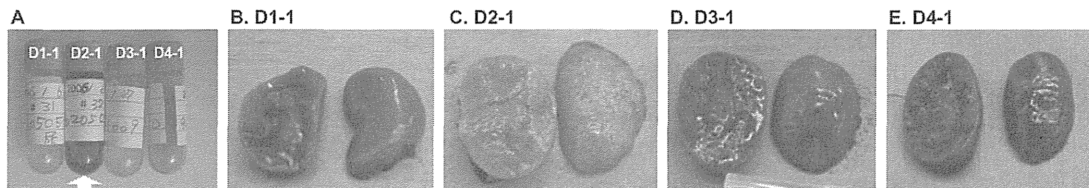
Table 2. Positive rates in the detection of DENV genome in urine samples on each of the 14 days following inoculation with DENV, in comparison with the previously reported data of serum samples [3].

Days after inoculation	Number of serum samples positive by RT-PCR (% positive, total tested)	
	Urine	Sera *
Day 1	0/20 (0)	NT †
Day 2	0/12 (0)	12/12
Day 3	1/16 (6)	7/8
Day 4	3/15 (20)	11/11
Day 5	4/19 (21)	4/5
Days 1–5	**8/82 (10)**	**34/36 (94)**
Day 6	3/18 (17)	NT
Day 7	3/14 (21)	11/18 (61)
Day 8	3/16 (19)	0/1 (0)
Day 9	3/16 (19)	NT
Day 10	4/15 (27)	0/4 (0)
Days 6–10	**16/79 (20)**	**11/23 (48)**
Day 11	5/16 (31)	NT
Day 12	4/17 (24)	NT
Day 13	3/17 (18)	NT
Day 14	6/17 (35)	0/17 (0)
Day 11–14	**18/67 (27)**	**0/17 (0)**
Total	**42/228 (18)**	**45/76 (59)**

* The DENV genome levels in serum samples were previously reported [3], † NT: indicates test not done.

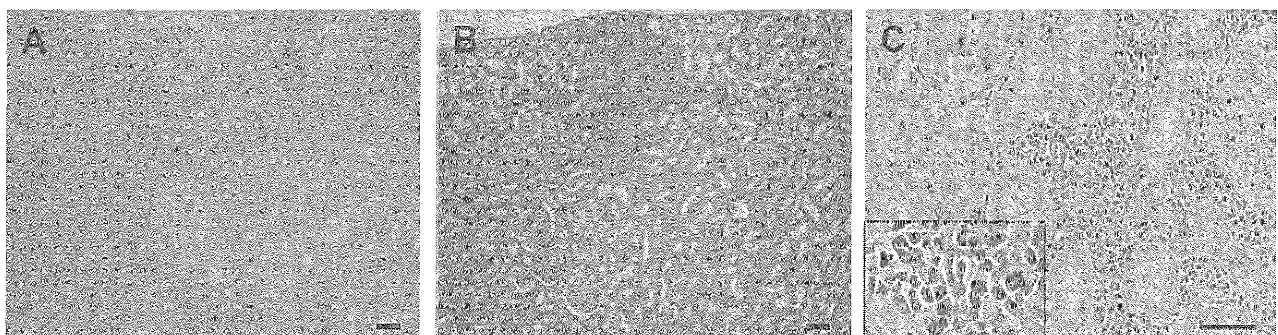
In human dengue patients, DENV genome was detected in 30% (9/30) of urine samples as compared to 79% (34/43) in serum samples from days 1–5 after onset of disease, and 61% of urine samples as compared to serum samples (11%) on days 10–16 after onset of disease [5]. In marmosets, the rate of DENV genome-positive urine samples (10%, 8/82, Table 2) was lower as compared those of serum samples (94%, 34/36) on days 1–5 after DENV inoculation, and DENV genome was detected at a higher rate in urine samples (27%, 18/67) as compared to serum samples (0%, 0/17) on days 11–14 after infection (Table 2) [3]. Thus, the kinetics of viral genome clearance in urine and serum samples in marmosets were similar to those of human DENV patients. It is of interest that for both DENV patients and DENV-inoculated marmosets, infectious DENV was not detected in urine samples even when DENV genome was detected [5]. Limitations of our study include lack of data on the presence or absence of infectious virus in kidneys. Although DENV genome and viral antigen were present in kidneys, the antigens could represent reabsorbed immune complexes after clearance, and may suggest mechanisms other than viral replication in renal tissue, are involved in renal dysfunction during DENV infection.

Figure 1. Gross appearance of kidney and urine specimens of DENV inoculated marmosets. Urine samples for D1-1, D3-1 and D4-1 were pale yellow and clear (Figure 1A). Hematuria was detected in urine from DENV-2 inoculated marmoset D2-1 (indicated by a white arrow). Gross appearance of kidneys from marmosets inoculated with DENV-1 (marmoset D1-1, Figure 1B), DENV-2 (marmoset D2-1, Figure 1C), DENV-3 (marmoset D3-1, Figure 1D) and DENV-4 (marmoset D4-1, Figure 1E). The kidney from DENV-2 inoculated marmoset (D2-1) was swollen and fawn-colored (Figure 1C).



In comparison to the clear appearance of urine samples from DENV-1, DENV-3 and DENV-4 inoculated marmosets (D1-1, D3-1 and D4-1), marmoset D2-1 demonstrated apparent hematuria (Figure 1A). Microscopic hematuria was detected in marmoset D2-17 on days 5 and 8 after DENV inoculation but was not detected in other 7 marmosets tested (D2-6, D2-7, D2-8, D2-9, D2-14, D2-15, and D2-16) on days 1 to 14. Marmosets D1-1, D2-1 and D4-1 exhibited signs of ascitis formation (data not shown). Kidneys of both sides from marmoset D2-1 were swollen and fawn-colored (Figure 1C).

Figure 2. Histopathology associated with DENV-2 inoculation in marmosets. Severe non-suppressive interstitial nephritis with renal tubular degeneration was detected in kidney of DENV-2 inoculated marmoset (marmoset D2-1, Figure 2A). DENV-2 inoculated marmosets also exhibited moderate interstitial nephritis, and segmental glomerulosclerosis and renal tubular atrophy (D2-1, Figure 2B). Dengue viral antigen was detected in cells that morphologically correspond to lymphocytes and macrophages in the kidney from marmoset D2-17 (Figure 2C). Scale bar = 50 μm.



Upon histopathological examination, the kidney from marmoset D2-1 demonstrated significant renal lesions (Figure 2A,B). Despite the limited numbers of marmosets evaluated, levels of DENV genome in urine samples of two marmosets (D2-1 and D2-17) which demonstrated hematuria and significant renal lesions (mean virus titer = 3.7 log10 copies/ml) were higher as compared to urine samples from 18 marmosets which did not demonstrate hematuria (mean virus titer = 0.5 log10 copies/mL, $P < 0.01$; two-tailed Student's t-test). Mean genome positive days of urine samples for

marmosets D2-1 and D2-17 was 8.0 days as compared to 1.4 days for marmosets that did not demonstrate hematuria.

Renal dysfunction, occurs in 2.9–13.3% of human dengue patients, and is associated with disease severity [1,2]. Severity of renal dysfunction was also associated with higher percentages of severe dengue and mortality in humans. Although the number of marmosets used in the present study was limited, two DENV-inoculated marmosets (2/18, 11%) demonstrated clinical signs of the renal system. In addition to viremia and biochemical changes, development of these clinical signs in marmosets, suggests the feasibility of marmosets as an animal model to elucidate the pathogenesis of DENV infection, including the renal system.

3. Experimental Section

A total of 20 male common marmosets (*Callithrix jacchus*) were used in accordance with "Guides for animal experiments according to institutional guidelines (Approval no. 608011, 609014 and Approval no. 20-003, 21-013). The marmosets were inoculated subcutaneously in the back with either DENV-1, DENV-2, DENV-3 or DENV-4. Four marmosets (D2-2, D2-3, D2-4 and D2-5) were inoculated with the same serotype (DENV-2) at 33 weeks after primary inoculation [3]. DENV type 1 (DENV-1), 02-17/1 strain, DENV-2 DHF0663 strain (Accession no. AB189122), DENV-2 Jam/77/07 strain, DENV-2 Mal/77/08 strain, DENV-3 DSS1403 strain (Accession no. AB189125), and DENV-4 05-40/1 strain, were used for inoculation studies. Day 0 was defined as the day of virus inoculation. Urine samples were examined for gross hematuria for all 20 marmosets and a urine dipstick (Bayer Urine Dipstick, IN, USA) for marmosets D2-6, D2-7, D2-8, D2-9, D2-14, D2-15, D2-16 and D2-17). For histological analyses, paraffin-embedded tissues sections (4μm sections) were deparaffinized and stained with HE stain. For immunohistochemical analyses, sections were stained using HRP-conjugated marmoset polyclonal anti-DENV antibody. For viral RNA isolation, High Pure Viral RNA Kit (Roche Diagnostics) was used. To determine the levels of dengue viral RNA, each sample was assayed in a 25 μL reaction containing 5 μL of sample RNA, 0.9 μM of each forward and reverse DENV-serotype-specific primer, RT/RNAse Inhibitor Mix, 0.2 μM TaqMan DENV-serotype-specific probe, and TaqMan Universal Master Mix (Invitrogen). The thermal conditions were (1) reverse transcription at 48 °C for 30 minutes, (2) *Taq* polymerase activation at 95 °C for 10 minutes, (3) forty cycles of PCR consisting of denaturing at 95 °C for 15 seconds and annealing at 57 °C for 1 min [3]. RNA standards with RNA copies of 10^8 to 10^4 were used to quantify the dengue viral RNA. All TaqMan RT-PCR assays were performed in duplicate. Replicate variability threshold was set at 0.5, the RT-PCR detection limit of this study ranges from 3.1×10^2 to 4.4×10^4 copies/ ml for four DENV serotypes. Data analysis was done using Microsoft Excel and GraphPad Prism Statistical Package (Graphpad Software, CA, USA). Percentage of genome positive days was calculated using the formula (number of days with positive viral genome detection/ number of sampling days) × 100%.

4. Conclusions

Common marmosets demonstrate DENV genome in urine, hematuria and pathological changes of the kidneys upon DENV infection. The recapitulation of these clinical aspects of DENV infection,

including the involvement of kidneys, suggests the feasibility of the use of marmosets for studies on the pathogenesis of DENV infection.

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Conflict of Interest

The authors declare no conflict of interest.

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Original article

First Isolation of Dengue Virus from the 2010 Epidemic in Nepal

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Abstract: Dengue is an emerging disease in Nepal and was first observed as an outbreak in nine lowland districts in 2006. In 2010, however, a large epidemic of dengue occurred with 4,529 suspected and 917 serologically-confirmed cases and five deaths reported in government hospitals in Nepal. The collection of demographic information was performed along with an entomological survey and clinical evaluation of the patients. A total of 280 serum samples were collected from suspected dengue patients. These samples were subjected to routine laboratory investigations and IgM-capture ELISA for dengue serological identification, and 160 acute serum samples were used for virus isolation, RT-PCR, sequencing and phylogenetic analysis. The results showed that affected patients were predominately adults, and that 10% of the cases were classified as dengue haemorrhagic fever/ dengue shock syndrome. The genetic characterization of dengue viruses isolated from patients in four major outbreak areas of Nepal suggests that the DENV-1 strain was responsible for the 2010 epidemic. Entomological studies identified *Aedes aegypti* in all epidemic areas. All viruses belonged to a monophyletic single clade which is phylogenetically close to Indian viruses. The dengue epidemic started in the lowlands and expanded to the highland areas. To our knowledge, this is the first dengue isolation and genetic characterization reported from Nepal.
Key words: Dengue fever, dengue 1 virus, epidemiology, Nepal

INTRODUCTION

Dengue fever (DF) is considered to be the most important of the arthorpod-borne viral disease in humans, and more than half of the world's population in over 100 countries is at risk of infection [1]. Dengue hemorrhagic fever (DHF), the severe form of the disease, is endemic and frequently intensifies into epidemics in the countries of Southeast Asia (Laos, Cambodia, Vietnam, Singapore, Indonesia and Thailand), resulting in more cases and deaths [1, 2]. Dengue is also endemic in India and Pakistan and has recently emerged in Bangladesh (2000) and in Bhutan (2004) [3, 4].

In the Himalayan country of Nepal, the first case of dengue was reported in 2004 [5]. In 2006, an outbreak of dengue occurred there with 32 laboratory-confirmed cases. All four dengue virus (DENV) serotypes DENV-1, DENV-2, DENV-3 and DENV-4 were identified in nine districts of the lowland Terai region, but unfortunately without information on sequence analysis [6, 7]. From 2007, a few sporadic clinical cases were reported. Entomological studies carried out in 2009 identified larvae of *Aedes aegypti* (*Ae. aegypti*) in the Kathmandu and Lalitpur districts in Nepal [8]. *Ae. aegypti* is the primary vector of dengue, and it is likely that this vector is being introduced in this zone seasonally. In 2010, Nepal suffered its largest dengue epidemic. There had been no dengue virus isolation from a native Nepalese patient in the past. The main objective of the present study is to isolate the virus from Nepalese patients and to determine the origin of these viruses.

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Disclaimers: None

METHODS

Patients

In 2010, a total of 280 suspected dengue patients from various district hospitals in Nepal were referred to the Sukraraj Tropical and Infectious Disease Hospital (STIDH) in Kathmandu, the capital city of Nepal. Clinical information was collected from patients who were admitted to the STIDH and their specific dengue disease, if present, was clinically identified as dengue fever (DF) or dengue haemorrhagic fever (DHF) based on the WHO classification (WHO, 1997). Initially, a patient was suspected to have DF when he or she had an acute onset of high fever lasting for 2–7 days and exhibited at least two of the following features: rashes, headache, arthralgia and leucopenia. A patient was suspected to have DHF when haemorrhagic manifestations such as ecchymosis, mucosal bleeding and plasma leakage were observed in addition to the features of DF. The suspected DF and DHF patients underwent further examinations for confirmation of the illness. A case was labeled as "probable" if the patient was found positive for the presence of IgM antibodies against dengue, and a case was labeled as "confirmed" when found positive by RT-PCR or virus isolation. Patients also underwent chest radiography and abdominal ultrasound tests in addition to hemoglobin count, hematocrit, and total blood and platelet monitoring as part of the clinical assessment.

Blood samples

Blood specimens were collected from patients, kept at 4°C for less than 24 hours and stored at –70°C in Kathmandu. These samples were transported in dry ice from Nepal to the Institute of Tropical Medicine in Nagasaki, Japan for further processing. If a serum sample had been taken from a patient less than 7 days from the onset of fever, it was subjected to virus isolation. If the sample came from a patient who had fever of more than 7 days, it was tested for the presence of anti-dengue IgM antibodies by in-house IgM-capture ELISA. To rule out the presence of IgM antibodies against JE, the sample was also tested using the JE-DEN IgM combination ELISA kit (Panbio, Australia).

IgM-Capture ELISA

Serum samples obtained after the centrifugation of blood specimens were subjected to in-house IgM-capture ELISA for the detection of IgM antibodies against dengue following the procedures described previously [9, 10]. These samples were also examined to rule out the presence of IgM antibodies against Japanese encephalitis virus (JEV) using the Panbio JE-DEN combination ELISA kit (Panbio, Australia). Panbio units were computed for each sample according to the protocol of the manufacturer. If the value was greater than 11 JE or 11 DEN Panbio units, the sample was considered positive for IgM antibodies against JE or dengue, respectively and if less than 9 for either JE or dengue, the sample was considered negative for these antibodies.

Virus isolation

A volume of 10 μl from each acute serum sample was inoculated in a culture tube containing 70% confluent C6/36 cells. The cells were maintained in MEM with 2% FCS, incubated at 28°C for 7 days and observed daily for cytopathic effects. Infected culture fluid from each tube was collected and clarified by centrifugation, and the supernatant was stored at –80°C until use in the detection of dengue virus RNA by RT-PCR.

RNA extraction and RT-PCR

QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany) was used to extract the genomic viral RNA from 140 μl of infected culture supernatants following the manufacturers' protocol. RNA was eluted from the QIAspin columns in a final volume of 100 μl of elution buffer and stored at –70°C until use. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) and random hexamers or gene specific primer, DenV1NR. PCR was conducted in a reaction mixture containing TaKaRa LA Taq DNA polymerase (Takara Bio Inc., Otsu, Japan) and appropriate primers. The following primers were used: DENV consensus primers to detect the presence of dengue virus [11], serotype-specific primers [11] to identify the specific serotype if sample was found positive for dengue virus, and primers (Table 1) to amplify the part that contained the E protein-coding region and the NS5-3'-UTR (10125–10428, 304 bp). PCR was then carried out and the amplicons were analyzed by gel electrophoresis on a 2.0% agarose gel (Dotite) containing ethidium bromide (0.5 μg/ml). Millipore Ultrafree®-DA (MA, USA) was used to purify the amplicons to be used as a template for sequence analysis.

DNA sequencing and phylogenetic analysis

For phylogenetic analysis, the purified amplicons containing the E protein-coding and NS5-3'-UTR-spanning regions of the virus genome were sequenced using certain primers (Table 1) and the Big Dye Terminator Cycle Sequencing System. The products were analyzed using a 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). MAFFT program version 6.864 [12] was used to align sequence data. PHYLIP package, version 3.69 [13] was used to construct the neighbor-joining phylogenetic tree [14] based on 2283 E region sequences. For NS5-

Table 1. Primer used for gene amplification and sequencing.

	Name of Primers	Sequence
E-region		
For Amplification	DEN1-E1-F	ACATGCCATAGGAACATCCA
	DEN1-E2-R	TCATTGGTGACAAAAATGCC
For Sequencing	Den1Seq04F01429	CGTCGGAAATACAGCTGACC
	DEN1-E2-F	ATGGCTAGTCCACAAACAATGG
	Den1Seq05F01862	GACCCAGCATGGAACTGTTT
	DEN1-E1-R	GTGATCCTAATACGACTACTTCCTG
	Den1Seq05R02439	GTTCTCTGCCCTTCCAGTTG
NS5-3'UTR region		
For Amplification	Den1Seq24F09950	CCATCACCAATGGATGACAA
	Den1Seq25R10672	TGCCTGGAATGATGCTGTAG
For Sequencing	Den1Seq25F10372	AAAATGAAGTCAGGCCGAAA
	Den1Seq24R10457	CAGCCTCCCAGGTTTTACA
For Reverse transcription		
	Name of Polynucleotide	Sequence
	DenV1NR	AGAACCTGTTGATTCAACAGCACCATTCCA

3'-UTR region, Bayesian MCC tree was constructed using BEAST software version 1.6.2 [15]. Models were selected with the use of MrModeltest version 2.3 [16] and PAUP* version 4.0 beta 10 [17]. The trees were drawn using FigTree software, version 1.3.1 [18].

Vector collection

House-to-house surveys were conducted on a total of 256 houses in the pre-monsoon season, 825 in the monsoon season, and 545 for screening of larval breeding places, covering residential areas, hotels, market, industrial areas of Kathmandu and Lalitpur district from April 2009 to March 2010. For each house, the number and category of potential larval habitats with and without water were noted. Surveys were conducted by searching all accessible artificial containers containing water in and around the houses for the presence of *Aedes aegypti* and *Ae. albopictus* larvae and/or pupae. Altogether nine different types of habitat were investigated: discarded tires, plastic drums, metal drums, metal containers, plastic buckets, flower pots, mud pots, cement tanks, and plastic pots. The larvae and pupae of mosquitoes were collected by the dipper method. Aspiration collections of adults were attempted in all outdoor dwellings with the help of flash-lights in order to record species during the morning hours (0700–0900 hrs) in those breeding habitats. A different collection bottle was used for each category of container in each locality, and the larvae were brought to the laboratory and reared [19] until the emergence of adults and identified on the basis of morphological characteristics using published taxonomic keys [20]. Altogether, 1,876 wet containers in 892 households were found in Kathmandu district, and 1,807 wet containers in 734 households were found in Lalitpur, Dhading and Tanahu districts as breeding habitats for *Ae. albopictus* larvae.

Statistical Analysis

Statistical analysis was conducted using the software SSPS 13.0 version.

Ethical Statement

The study did not require approval from an institutional review board because the blood samples came from patients undergoing medical treatment at Sukraraj Tropical and Infectious Diseases Hospital under the auspicious of the Ministry of Health and Population, Nepal. Through this hospital, the government not only helps in providing medical care for patients, but also gathers data from patients as part of health surveillance duties.

For the entomological study, permission from the owners/residents was obtained before mosquito specimens were collected from their premises or residences.

RESULTS

Geographical distribution and seasonality

Dengue is an emerging disease in Nepal. A small outbreak was reported in this country in 2006, and sporadic cases were reported from 2007 to 2009. From August to December 2010, an unusual increase in the number of febrile patients was observed. The 2010 outbreak started at

the beginning of August from Damauli city (altitude of 360 meters) in the Tanahu district and Dhangadi city in the Kailali district (altitude of 190 meters) (Fig. 1) [21]. It expanded immediately to Bharatpur city, Chitawan district. The number of cases increased rapidly during the first week of September, and in October the outbreak spread even further to neighboring districts of Nawalparashi and Butwal, and to the headquarters of Rupendehi district. Finally, by the end of October the disease appeared in the highland districts of Dhading and Kathmandu (altitude of 1090 and 1350 meters, respectively), previously known as "dengue-free" zones (Fig. 1). The outbreak peaked in November (after the rainy season had finished) and subsided during the dry winter season around the second week of December.

Clinical features

Of the 280 patients who were referred to and sought medical help at STIDH, 242 were admitted. Almost all of these patients manifested features of classical DF such as fever, headache and myalgia. Hemorrhagic manifestations characteristic of DHF were also noted in the form of ecchymosis, epistaxis, gum bleeding and gastrointestinal (GI) bleeding. Based on the clinical data, 20% (48/242) of the patients experienced GI bleeding, abdominal pain, vomiting and difficulty in movement in addition to the features of DF. Twelve percent (28/242) of the patients suffered coughs and difficulty in breathing. Unusual manifestations such as splenomegaly were also observed in 4% (9/242) of patients with DHF. Abdominal ultrasound showed that 15% (36/242) of the patients had hepatomegaly, while 8% (19/242) had mild to moderate ascitis. Some of the patients showed acalculus cholelithiasis and thickening of the gall bladder. Four percent (9/242) of the patients showed hepatosplenomegaly. One patient showed signs of acute pancreatitis. On chest X-

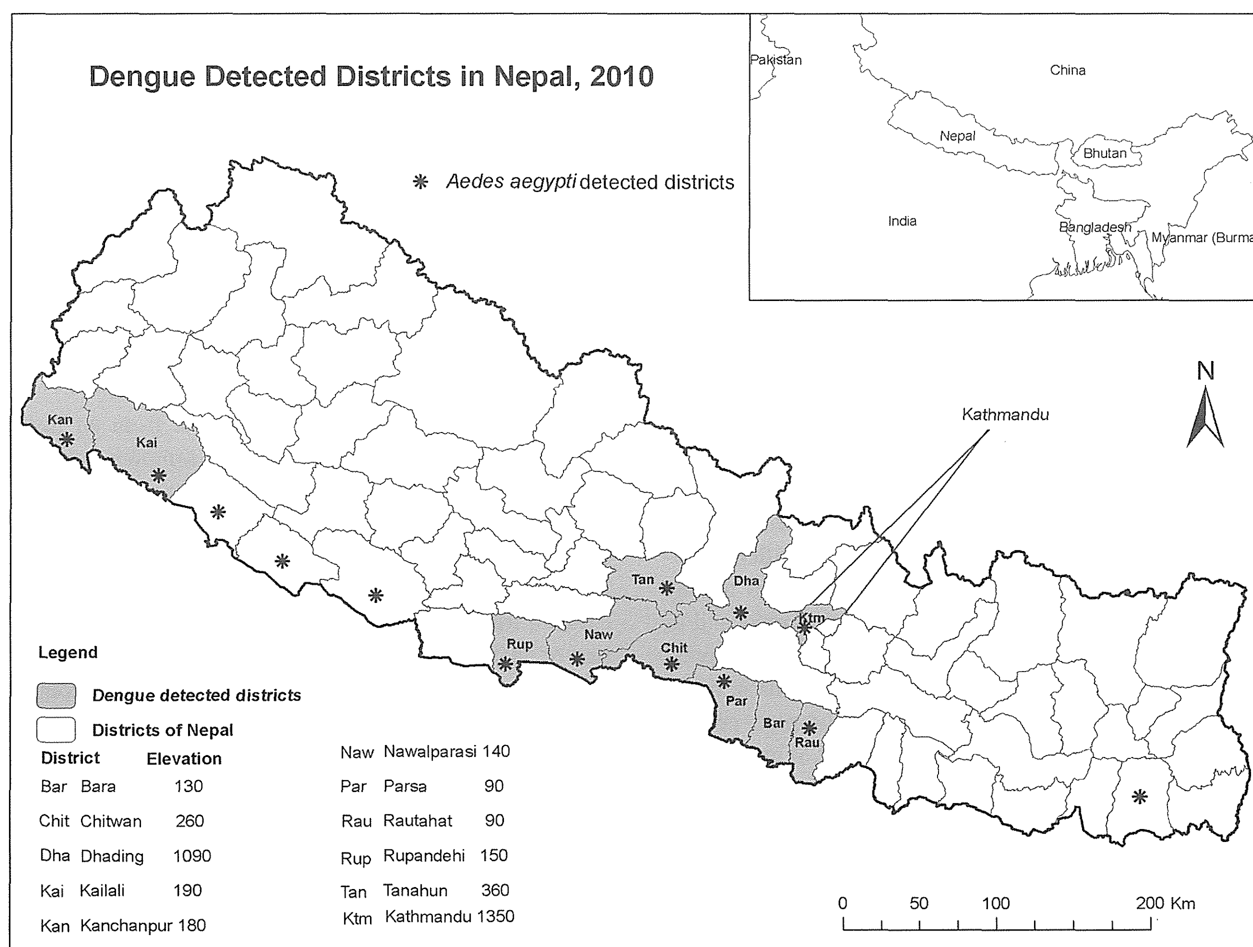

Fig. 1. Dengue and *Aedes aegypti* confirmed districts in Nepal, 2010.
(*) indicates *Aedes aegypti* positive districts and shaded areas indicate dengue positive districts. Numbers indicate the elevation (meter) of each district capital.

ray, 4% of the patients with DHF showed pleural effusion that was mostly unilateral.

Incidence

The Epidemiology Disease Control Division reported 4,529 suspected cases, 917 serologically-confirmed cases and five official deaths by the end of December. However, these figures were only from government hospitals across Nepal (Fig. 2). It is noted that the affected patients were predominately adults, the child adult ratio being 0.2:1. Most of the patients were diagnosed clinically as DF. But 10% of the patients in the present study admitted at STIDH were diagnosed clinically as DHF and were treated with blood and platelet transfusions.

Serological confirmation

A total of 120 serum samples collected after 7 days of fever were investigated using IgM-capture ELISA for dengue, and 35% were found to be positive for dengue. These samples were also investigated for JE, and all were found to be negative for anti-JE IgM.

Virus isolation and serotyping

Only 160 acute serum samples were available for virus isolation in C6/36 cells. Twenty one (13%) of these samples were found to be positive for dengue after the culture fluids of the cells inoculated with these samples were subjected to RT-PCR in the presence of dengue consensus primers. Our genetic characterization of dengue viruses (all DENV-1, Table 2) isolated from patients in four major outbreak areas

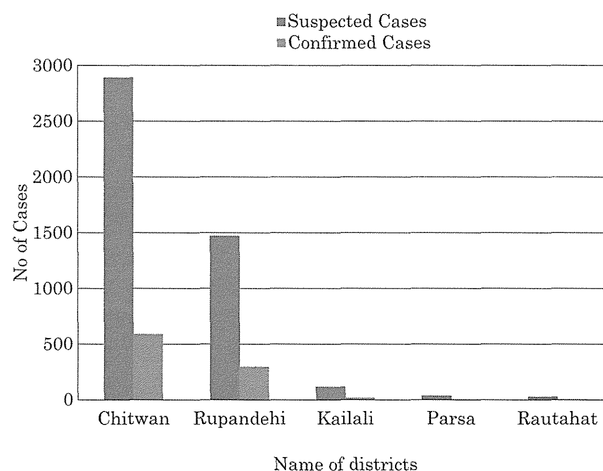

Fig. 2. Epidemiological curve showing the distribution of dengue cases in 2010.

Table 2. List of isolates with clinical information of patients and GeneBank accession numbers of registered nucleotide sequences of E and NS5-3'UTR regions.

	ID	Age/Sex	Onset	Serotype	City/District	E region	NS5-3'UTR
1	NDaH14-10	12/F	11/08/2010	D1	Damauli/Tanahu		
2	NDaH15-10	11/M	11/08/2010	D1	Damauli/Tanahu		
3	NDaH16-10	8/M	11/08/2010	D1	Damauli/Tanahu	JF754983	JF754994
4	NDaH17-10	15/M	11/08/2010	D1	Damauli/Tanahu		
5	NDaH19-10	21/F	11/08/2010	D1	Damauli/Tanahu	JF754984	JF754995
6	NDaH23-10	5/F	11/08/2010	D1	Damauli/Tanahu	JF754985	JF754996
7	NDaH24-10	36/F	11/08/2010	D1	Damauli/Tanahu	JF754986	JF754997
8	NDaH25-10	24/F	11/08/2010	D1	Damauli/Tanahu	JF754987	JF754990
9	NDaH26-10	56/F	11/08/2010	D1	Damauli/Tanahu	JF754988	JF754998
10	NDaH41-10	67/F	11/08/2010	D1	Damauli/Tanahu		JF754999
11	NDaH44-10	8/F	12/08/2010	D1	Damauli/Tanahu	JF754989	JF755000
12	NBpH12-10	23/F	22/09/2010	D1	Nawalparashi/Chitwan	JF754982	JF754993
13	NBpH13-10	26/F	22/09/2010	D1	Nawalparashi/Chitwan		
14	NBH12-10	17/F	13/10/2010	D1	Bharatpur/Chitwan	JF754980	JF754991
15	NBH29-10	30/F	13/10/2010	D1	Bharatpur/Chitwan	JF754981	JF754992
16	NBH33-10	19/M	13/10/2010	D1	Bharatpur/Chitwan		
17	NBH23-10	26/M	13/10/2010	D1	Bharatpur/Chitwan		
18	NBH36-10	38/M	13/10/2010	D1	Bharatpur/Chitwan		
19	NBH15-10	20/M	14/10/2010	D1	Bharatpur/Chitwan		
20	NBH44-10	29/M	14/10/2010	D1	Bharatpur/Chitwan		
21	NDbH20-10	68/M	22/10/2010	D1	Dhadingbeshi/Dhading	JF800928	JF800929

of Nepal suggests that the DENV-1 strains responsible for the 2010 epidemic originated from India. We read sequences of eleven strains. Fig. 3 and Fig. 4 show the phylogenetic tree drawn from Nepalese strains representing major epidemic districts.

Nucleotide sequence and phylogenetic analysis

DNA sequencing of the E-coding region and the NS5-3'UTR was done on 11 DENV-1 isolates representing the major epidemic districts in Nepal (Table 2). The nucleotide sequences of the E region and the NS5-3'UTR were compared with other sequences published in GenBank. Phylogenetic trees generated for the E region and the NS5-3'UTR are shown in Figure 4, respectively. The phylogenetic tree on E region shows that all the eleven Nepalese strains formed a monophyletic clade and that they belonged to genotype V, a cosmopolitan genotype containing American, West African and Asian strains. In the phylogenetic tree for

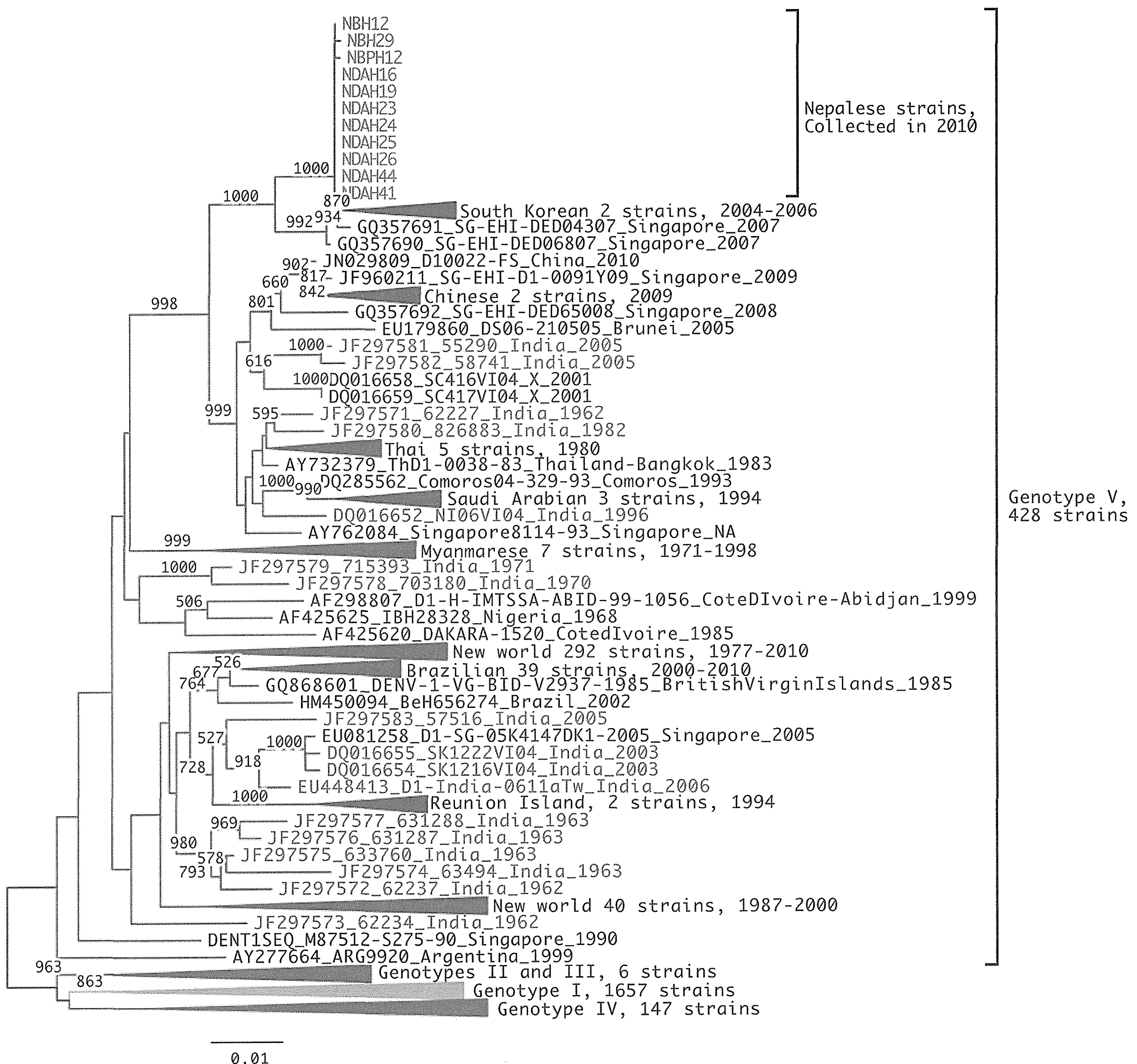

Fig. 3. Phylogenetic tree of DENV-1 E region. Representative analysis of E region, 1,485 bps, 2,238 strains. Red and blue indicate Nepalese strains and Indian strains, respectively. The tree was constructed after 1,000 replicates of bootstrap analysis using neighbor joining method. Bootstrap values (%) greater than 50% are shown above branches. Labels of strains conform to the following format: (GenBank accession nos)_(Strain name)_(Country-region)_(Year of isolation). "NA" means that the information is not available.

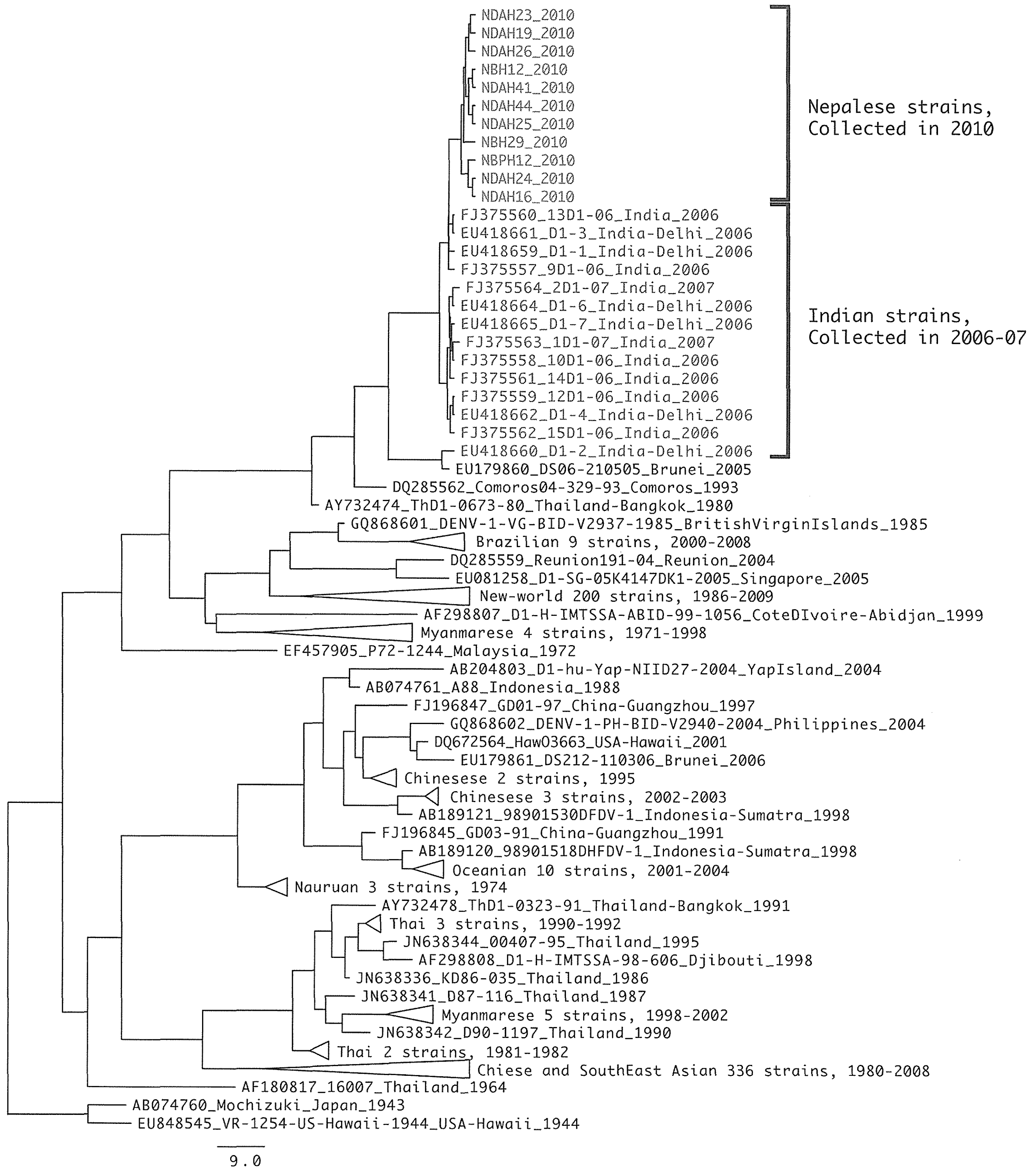

Fig. 4. Phylogenetic tree of DENV-1 E NS5-3'UTR spanning region. Maximum clade credibility (MCC) tree of NS5-3'UTR spanning region, 304 bps, of 579 strains with uncorrelated relaxed clock, GTR+G+I model.

the NS5-3'-UTR region, Nepalese strains are shown to form a subcluster with strains isolated from India between 2006 and 2007.

Vector

Aedes aegypti has been found in most of the epidemic sites (Fig. 1), suggesting that this mosquito species is the