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Antibody-dependent enhancement (ADE) of dengue (DEN) virus infection in human mononuclear cells in vitro has been standardized using a human promonocytic cell line HL-CZ, purified monoclonal antibodies (MAbs), and select DEN viruses. Characterization of the FC-receptors (FcRs) expressed on HL-CZ cells have indicated that subsets of FcR mediate ADE better than others. Using this standardized system, we have compared the ability of mouse anti-DEN 2 envelope (E) peptides to elicit virus neutralization and ADE. Peptides 1-2, 437 appear to elicit ADE activity in contrast to other peptides that appear to elicit neutralization but not ADE. Though these assays need to be repeated, it appears that differential functions may be attributed to particular E genomic regions. The comparison of the nucleotide sequences of DEN-1 RNA encoding the non-structural proteins to the other DEN sequences has revealed that DEN-4 and DEN-1 share >90% similarity in NS3 and NS4a, 4b genome regions. DEN-3 and DEN-1 have a deletion in NS5 that is conserved in other DEN-1 and DEN-3 isolates. These genomic sequence comparisons indicate that non-structural region differences need to be studied as well for our understanding of DEN replication and pathogenesis.

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FOREWORD

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Principal Investigator's Signature — Date: 4/29/90

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INTRODUCTION

Increased virus replication in dengue infections can lead to the development of severe disease manifestations of vascular permeability and shock (dengue hemorrhagic fever/dengue shock syndrome, DHF/DSS). Different hypotheses have been proposed as the pathogenic mechanism in DHF/DSS. In the same outbreak, DHF/DSS cases have been associated with both primary and secondary infections; thus implicating the infecting virus strain as being the cause of serious disease (6). Alternatively, more DHF/DSS cases are documented in patients with secondary DEN infections suggesting that pre-existing DEN antibodies exacerbate severe disease outcome by mediating the enhanced replication of the second DEN virus (9). Unidentified host genetic differences are also likely to be associated with development of DHF/DSS cases, since not all primary or secondary DEN infections result in severe disease.

<u>Development of standardized antibody-dependent enhancement assay</u>. Antibody-dependent enhancement (ADE) assay is an <u>in vitro</u> system in which DEN virus infection of mononuclear phagocytes in the presence of DEN antibodies is used as laboratory correlates of the sequential infection hypothesis (9). This study uses the principle of ADE to examine the roles of antibody, virus and host cells in DEN replication. In this past year we have developed a standardized ADE assay that permits us to examine the effect of not only antibody on virus replication but, by controlling other variables, examine also the differences in virus strain replication and host susceptibilities.

<u>Rationale for studying pathogenesis of DEN using Caribbean DEN isolates</u>. The development of DHF/DSS is probably not exclusively associated with a single suggested mechanism, but rather is an interplay of several pathogenic factors. Unlike DEN-endemic regions in Southeast Asia, the introduction of DEN-1 CV1636/77 and DEN-2 Jamaica genotypes into the Caribbean region is clearly defined (3,11). In addition, both virus genotypes are temporally associated with the severe DHF/DSS outbreak in Cuba where retrospective epidemiology suggest that DEN-2 DHF/DSS cases were associated with previous exposure to DEN-1 (7).

The nucleotide sequence and deduced amino acid sequence of DEN-2 Jamaica has been completed (4,5). The genomic sequences encoding DEN-1 CV1636/77 proteins is nearly completed (3; Tables 1, 2, 3, and 4). Synthetic peptides consisting of DEN-2 Jamaica E-glycoprotein sequences elicit antibodies that react by ELISA and plaque reduction neutralization (PRNT) to native virus (19).

Chimeric hybrids of DEN-2 E-glycoprotein gene regions have also been constructed and shown to be immunogenic (Chang, unpublished observations). Based on these results, immune sera against synthetic DEN-1 CV1636/77 genomic regions would provide reagents to study if DEN-2 Jamaica replication could be enhanced and thus provide an in vitro explaination for the severe DHF/DSS outbreak as suggested by the epidemiological reports on the Cuba outbreak (7).

ACCOMPLISHEMENTS FOR FY 1990

Selection of a human mononuclear phagocytic cell line for use in ADE assays. Human and monkey peripheral blood leukocytes (PBL) as well as several mononuclear cell lines have been used in ADE assays (2,9,17). We compared the susceptibility of human mononuclear cells to DEN infections using a newly described human promonocytic cell line HL-CZ cells (14, Table 1), K562 (13), U937 (2) cells and cultured human PBLs (Figure 1). DEN-2 16681 virus and 4G2 antibody were added to 3 x 10^5 of U937, K562 or HL-CZ cells and to 1.5 x 10^6 HuPBLs. The cultures were incubated for 4 days and harvested by freezing the samples. The amount of productive virus yield was titrated in the BHK-21 clone 15 cell assay (16). The results in Figure 1 represent the amount of virus yield of samples with antibody - samples without antibody (in thousands of pfu/ml). Both HuPBL and U937 could not be infected with less than MOI of 0.01, comparable to results already published elsewhere (2,17). Infection of K562 and HL-CZ cells at MOI of 0.01 however, resulted in high background of virus growth (>10^3 pfu/ml) thereby masking the level of enhancement. When the MOI's are lowered to 10^{-4}-10^{-5}, background virus levels then do not interfere with enhanced virus replication. Thus compared, HL-CZ cell line is more sensitive to DEN virus replication than U937, K562 or HuPBLs.

Characterization of HL-CZ cells. The underlying presumption of ADE is that the presence of antibody mediates increased virus replication by bringing infectious virus closer to the cell surface via the Fc-receptor (FcR). Because of the differences we observed in comparing virus replication with different cells, we hypothesized that there may be a difference in the number of FcRs expressed on these cell surfaces. A direct visual method was developed using sheep red blood cells (SRBC) (BBL, Becton-Dickinson, Cockeysville, MD) sensitized with rabbit anti-SRBC (sSRBC) (BBL, Becton-Dikinson) to enumerate the number cells that express FcRs on their surfaces. A cell that adsorbs three or more sSRBC after a fixed incubation time was counted as a FcR-bearing cell (rosette), the results are listed in Table 2. The number of FcRs expressed by cultured HuPBL after three days' incubation is 60% of a mixed leukocyte population (T cells, B cells, monocytes, and macrophages). Our assumption was that cloned human cell lines would likely have more uniform expression of FcRs; however, only 50% of U937 cells formed rosettes while K562 and HL-CZ cells expressed >80% rosetting capability. The amount of detectable virus in ADE cultures appears to be associated with the number of FcRs on the cell surfaces and this may likely determine whether a cell may or may not participate in ADE.

There are three types of human immunoglobulin G FcRs: FcRI, FcRII, and FcRIII each identified by monoclonal antibodies 32.2, IV.3, and 3G8 respectively (20). Our next assumption was that there may be qualitative differences in the types of FcRs expressed on each of the cells that participate in ADE. In order to examine those expressed by HL-CZ cells, we pre-blocked cells with the appropriate FcR type-specific monoclonal antibody (Medarex Inc., West Lebanon, NH) then added sSRBC to the blocked cells to determine the FcR-type (Table 3). HL-CZ appears to have all three FcR types with FcRII >FcRI > FcRIII in contrast to K562 cells that have only FcRII expressed on their surfaces. These observations were then confirmed by flow cytometry analysis using FITC-tagged sheep anti-mouse Fab'(Jackson Immunoresearch Labs, West Grove, PA) instead of sSRBC (Figure 2). The results of the forward angle light scatter (FALS) examination of the FITC-stained cells are presented as comparative histograms of equal cell numbers (top left of each panel) and of equal intensity (FALS 1024, top right) between cells pre-incubated with FcR MAbs (open areas) and cells stained with anti-mouse-FITC (closed areas). K562 cells stained with MAb IV.2 (FcRII) is shown as the control (panel A), K562 cells stained with other FcR MAbs were like the control cells (data not shown). HL-CZ cells stained with all three FcR MAbs to varying degrees with FcRII > FcRIII > FcRI, the latter two are different from our rosette-inhibition studies and will need to be resolved by further analyses.

<u>ADE in HL-CZ cells after FcR blocking</u>. We next asked, if by blocking FcR on HL-CZ cells, will we be able to abrogate virus growth? HL-CZ cells were pre-incubated with either one, three, or no (No Ab and 3H5) specific FcR MAbs. The percentage of available FcR remaining on the cell surfaces were enumerated by rosetting (open circle line, Figure 3). The cells were then infected with DEN-2 virus with the addition of 100 ng of 3H5, and the cultures were harvested on days 2 (open bars) and 4 (shaded bars). Background virus growth (without pre-blocking and without 3H5) was significantly lower than that of the positive control samples (no pre-blocking and with 3H5). Cells that were pre-incubated with FcRI MAb (32.2) resulted in less virus growth than the positive control. Where FcRII, FcRIII or all three FcRs are blocked, the enhancing effect of 3H5 was abrogated. These results correlated with the absence of available FcR sites as determined by rosetting, and suggest that selected FcR types may be involved in mediating enhancement.

<u>Antibody preparation for use in a standardized ADE</u>. Three monoclonal antibodies (MAbs) were selected as the antibody standards. These were originally prepared and characterized by the Walter Reed Army Institute of Research (WRAIR) (10). 4G2 is an IgG_{2a} globulin that is flavivirus- and E glycoprotein-specific

reactive. 3H5 is of IgG_1 subclass that reacts with E glycoprotein as well but is specific for DEN-2. 15F3 is directed against the NS1 protein of DEN-1 belonging to IgG_{2a} subclass. Hybridoma culture fluids from these monoclonals were concentrated by 50% ammonium sulfate precipitation, and purified by Protein A column chromatography. Each Ig fraction was standardized by spectrophotmetric protein assay (BioRad, Richmond, CA) to 1 mg/ml and tested for virus reactivity by IFA, ELISA and PRNT.

The specificity of the prepared antibodies were tested in ADE: 4G2, and 3H5 participate in ADE at various concentrations, 15F3 does not mediate ADE of DEN-2 viruses or DEN-1 from Thailand (see following section) and marginally enhances DEN-1 CV1636/77. In previous ADE studies involving DEN infections, it has been pointed out that only antibodies with DEN/flavivirus specificities are inovlved (2). We confirmed this by substituting matched subclass mouse immunoglobulins derived from mineral oil plastocytomas (MOPC 21, UPC 10; Jackson Lab) instead of 4G2 and 3H5 (Figure 4). The lowest limit of our BHK-21 plaque assay is 7 pfu/ml. The addition of 0.001 ug-10 ug/ml of MOPC 21 or UPC 10 did not enhance DEN-2 replication. Both purified 4G2 and 3H5 mediated enhanced virus production at 100-1000 times higher than background. We also observed that the addition of 1.0-10.0 ug/ml of 4G2 induces enhanced virus replication whereas less amounts of 3H5 (100ng-1ug/ml) was needed to mediate ADE. 15F3, though not shown in Figure 4, did not mediate enhanced virus growth.

Selection of DEN viruses as control viruses in ADE. Four virus strains of epidemiological importance were selected to be the control virus strains in our standard ADE (Table 4). The two Thailand strains selected are from endemic DEN regions and are the parental viruses from which attenuated candidates are being tested in vaccine trials (1). In addition, DEN-2 16681 virus is the "prototypic ADE virus strain" since many of the enhancement studies have included this strain (9,17). The Jamaican isolates have been discussed earlier and serve to represent strains from an epidemic region in contrast to the Thailand viruses.

Using a standarized input of virus (MOI = 0.0001; Figure 5) and varying concentrations of purified MAbs, HL-CZ cells were infected, cultured for 4 days, and resulting productive virus assayed. The addition of 4G2 resulted in enhanced replication in every case while 15F3 had minimal effect on DEN-1 CV1636/77 virus replication (>10 fold over background). Regardless of how MAbs affected virus growth, the background growth of each virus and the amount of enhanced growth varied. Jamaican virus strains had a higher background growth than the Thailand viruses, and the enhancement profile of each of the viruses differed. The virus growth yields indicate that each of the DEN viruses, under the same growth conditions, have an intrinsic difference in their ability to replicate in this system. We also noted during these studies that it was important to control the variables carefully if we were to use this system to compare viruses. By varying only the input virus, the enhancement profile changes for DEN-2 Jamaica virus

(Fig. 6). If however at a particular MOI examined that the background virus is already at the threshold level (7 pfu/ml), the enhancement profile does not change, and further diminishing of input virus from that point only results in undetectable virus replication.

Anti-peptide sera directed against DEN-2 E glycoprotein regions. The immune serum obtained from mice immunized with a series of DEN-2 Jamaica synthetic peptides were examined in the ADE system. The DEN-2 synthetic peptide antigens used to immunize animals represent continuous and discontinuous E glycoprotein regions (19; Table 5). Mouse anti-peptide sera were diluted from 10^{-3} to 10^{-5} and added to 3 x 10^5 HL-CZ cells with an input of 0.00001 DEN-2 Jamaica virus. These experiments were done in triplicate and repeated three times, the BHK-21 plaque assay results reported as pfu/ml (Table 5). This data suggest that peptides 1-2 and 437 may be involved in eliciting antibody that mediate enhancement. These observations are preliminary and need to be repeated using purified and standardized quantities of anti-peptide sera.

Cloning and sequencing of DEN-1 CV1636/77. The genome encoding the structural genes of DEN-1 CV1636/77 have been previously published (3). To examine if regions other than those representing the E glycoprotein would be important in DEN pathogenesis, we have proceeded to obtain the sequence of the genome coding for the non-structural proteins of DEN-1 CV1636/77. Using the same cloning methods for obtaining structural region clones, cDNA clones that encompass the nucleotide sequence for the non-structural genome were generated (Figure 7). From the many clones generated, 5 overlapping cDNA clones were selected for sequencing (Figure 7, closed arrows); the sequences of portions of one clone and another short clone (Figure 7, open arrow regions) are being determined.

The nucleotide sequences encompassing NS4a, NS4b, and NS5 genomic regions of DEN-1 are presented along with the published sequences of DEN-2 (5), DEN-3 (18), and DEN-4 (15) in Tables 6, 7, and 8 respectively. The nucleotide region for NS1, NS2a, NS2b, NS3 and the final 800 base pair sequence of the 3'-end of the viral RNA has not been finalized. In Figure 8, a diagrammatic representation of the protein similarities between NS4a, NS4b, and NS5 regions of the DEN viruses are presented (narrow bars represent a single amino acid change, whereas wider bars represent from 2 or more to clusters of amino acid changes). In NS4b, DEN-1 and DEN-4 each have 3 amino acid deletions at amino acid positions 21-23 in comparison with DEN-2 and DEN-3 sequences. Additionally, both DEN-1 and DEN-3 have a deletion at amino acid position 176 of the NS5 protein, the deletion at this position has been verified by RNA sequencing of other DEN-1 and DEN-3 viruses as well (Table 9).

The similarity of the each of the gene regions between the 4 DEN viruses are summarized in Table 10. As expected, DEN-1, DEN-2, and DEN-3 share between 63%-82% similarity over each of the non-strucutural gene regions. DEN-1 and DEN-4 however are very similar in NS3, NS4a, and NS4b regions (95%-98%); this is contrasted by a comparison of DEN-1 and DEN-4 over the NS5 region where similarity extends to only 78%. When the combined nucleotide and deduced amino acid sequences over NS3, NS4a, NS4b, and NS5 regions are examined, the similarity of 90% remains between DEN-1 and DEN-4 (Table 11).

CONCLUSIONS

Pathogenic mechanisms by which dengue infections result in serious hemorrhagic manifestations must involve host susceptibility and the infecting virus strain. An animal model in which DHF/DSS can be easily studied does not exist. Therefore *in vitro* studies of DHF/DSS correlates have primarily depended on ADE experiments. Enhanced replication of virus remains a consistent finding in experimental situations and perhapsr reflect *in vivo* observations where patients developing DHF/DSS are highly viremic (8).

Relevance of completed research. The development of a standardized ADE assay provides an useful tool to examine the variables that are involved in enhanced virus replication *in vitro*. We have been able to determine that antibodies of different DEN specificities vary in their ability to mediate enhanced virus growth. We have also determined that viruses have intrinsic differences in their ability to replicate. Our comparison of ADE in different human cell cultures have led to the identification of a cell line that is more analogous to HuPBL in their expresssion of all three FcRs. By using these HL-CZ cells, we have been able to determine that ADE requires the expression of FcRII > FcRIII and probably not as likely to involve FcRI.

Using anti-peptide sera directed against selected DEN-2 E glycoprotein regions, we have been able to demonstrate that some of the E regions will elicit antibody that will mediate enhanced virus replication, neutralization and enhancement, and neutralization alone. Though this series of experiments need to be repeated with purified antibodies, this is the first direct association of specific E-glycoprotein regions with biological functions.

Analyses of the genetic relatedness of DEN viruses. The close relatedness of DEN-1 and DEN-4 in the NS3-NS4a,b regions; the shared deletions of DEN-1 and DEN-3 in NS5 identifies specific genomic regions of interest to study in relating genomic sequence similarity/differences relate to virus replication and antigeneicity.

The comparison of the nucleotide sequences with the other DEN virus strains have been very interesting. It was expected that the similarities between DEN serotypes would extend between 60-80%. The high percentage of shared sequences in NS3, NS4a, NS4b between DEN-1 and DEN-4 suggests two scenarios. The first explaination may be that genomic recombination has occured and the second more likely explaination may be that DEN-1 and DEN-4 have evolved from a common progenitor. Why these relationships have not been detected before is not surprising since the serological tests commonly used to differentiate the viruses are directed toward the structural proteins; alternatively, because the non-structural regions are not expressed, there is little selective pressure to restrain these genomic regions.

OBJECTIVES FOR FY 1991

1. Completion of the sequencing and analyses of DEN-1. The NS1, NS2a, NS2b, and the 3'-end of the DEN-1 sequence will be completed. We will compare the nucleotide sequence and the deduced amino acid sequence with the other DEN serotypes.

2. Identify the genomic regions that elicit antibodies that elicit neutralization/enhancement. Extend our preliminary findings using purified antibodies. Elicit antibodies to new DEN-1 and DEN-2 synthetic antigens, purify the antibodies and examine their reactivities in PRNT and ADE.

3. Examine genomic variation of DEN strains. Determine if DEN virus strains share similar nucleotide sequence over the genomic regions that mediate neutralization/enhancement. We will do primer-directed RNA sequencing of selected DEN strains that are associated with DF or DHF/DSS patients.

4. Confirm the relevance of defined neutralization/enhancement epitopes to human infections. Patient serums will be used to react with the synthetic antigens that define neutralization and/or enhancement activity.

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TABLE 1. HUMAN PROMONOCYTIC CELL LINE
HL-CZ CLONE CCC-5

- 50 YR. OLD MALE WITH ADULT T-CELL LEUKEMIA
- PROMONOCYTIC CELL LINE THAT SHOW EARLY DIFFERENTIATION IN HEMATOPOIESIS
- PHAGOCYTIC, WITH SINGLE, DOUBLE, OR MULTIPLE NUCLEI
- 90% OF THE CELLS POSSESS CD-15 MARKER

- LIU, W.T. et al. (1989) J BIOL MED SCI 4:284

Table 2. DETECTION OF Fc RECEPTORS (FcR) OF HUMAN MONONUCLEAR CELLS

CELLS	KNOWN FcR TYPE (a)	% ROSETTES (b)
HUMAN PBL	I,II,III	60
U937	I,II	50
K562	II	87
HL-CZ	??	87

(a) Unkeless et al., Ann Rev Immunol **6**(1988):251-81.

(b) Sheep red blood cells (SRBC) sensitized with rabbit anti-SRBC (sSRBC). 10^4 human mononuclear cells were incubated with sSRBC at 4C for 12 hours. Percent rosettes are determined by the number of cells with sSRBC/total number of mononuclear cells counted. Total number of mononuclear cells counted = 100 to 400 cells.

TABLE 3. Fc RECEPTOR (FcR) DETECTION BY ROSETTE INHIBITION

Fc CLASS	K562	HL-CZ
FcRI (MAb 32.2)	165/197 (84%)	24/178 (14%)
FcRII (MAb IV.3)	0/165 (0)	1/142 (1%)
FcRIII(MAb 3G8)	186/278 (70%)	70/182 (38%)
NO BLOCKING AB	141/162 (87%)	183/209 (86%)

TABLE 4. ISOLATES FROM DENGUE PATIENTS

STRAIN I.D.				YEAR	
DEN-1	THAILAND	16007	DF	1964	(1)
DEN-2	THAILAND	16681	DHF	1964	(1)
DEN-1	JAMAICA	CV1636/77	DF	1977	(2)
DEN-2	JAMAICA	1409	DF	1983	(3)

(1) Halstead et al. 1970. Yale J Biol Med 42:2
(2) King et al. 1979. PAHO 375:153.
(3) Deubel et al. 1986. Virol 165:234.

Table 5. ACTIVITY OF MOUSE ANTI-DENGUE 2 PEPTIDE IN NEUTRALIZATION AND ADE ASSAYS

PEPTIDE I.D.	COMPRISED OF E AMINO ACID #	PEPTIDE LENGTH	PRNT (a) TITER	ADE (b) TITER
1-2	1-30	30	160	132
35	35-55	22	20	< 7
3-8/2	49-60, 121/140	32	160	< 7
3-8/1	58/73, 106-115, 117/121	31	40	< 7
4-6	72-91, 93-105	33	40	13
79	79-99	21	40	< 7
5-7	90-104, 106-120	30	160	< 7
04	121-140	20	40	< 7
142	142-172	32	40	< 7
142-1	165-172	9	40	< 7
208	208-219	13	160	< 7
06	225-249	26	20	< 7
67	255-274	21	160	< 7
07	302-333	32	20	< 7
16	333-351	22	80	< 7
17	352-368	18	20	< 7
388	388-400	14	20	< 7
437	437-452	17	40	200
CONTROL	3H5 10 NG/ML	NA (c)	--	1466
CONTROL	4G2 100 NG/ML	NA	--	400

(a) PRNT, 70% plaque reduction.
(b) ADE, 0.00001 MOI, HL-CZ cells. Average of triplicate samples expressed as pfu/ml. Negative control background = < 7 pfu/ml.
(c) NA, not applicable.

TABLE 6. NUCLEOTIDE SEQUENCE OF DEN-1 CV1636/77
Nucleotide sequence encoding NS4a genome

		42
DEN-1	AGT ATA ACT CTC GAC ATC CTA ACA GAG ATT GCC AGT TTG CCA	
DEN-2	TC. T.G ..C ..G A.. C.A A.C ... ..A ..G .GT ..G C.A ...	
DEN-3	TCA ..C G.C ..T ..T C.T G.G ..A ..A ..A GGA ..A G.. ..T	
DEN-4	... ...	
		84
DEN-1	ACT TAC CTT TCC TCT AGG GCC AAG CTC GCC CTT GAT AAC ATA	
DEN-2	... .T. A.G A.T CAG .A. ..A .GA GA. ..A ..G ..C ... T..	
DEN-3	T.A C.. T.A G.. CAC ..A A.G .GA AA. ... ..G ..C ..T T.G	
DEN-4	... ...	
		126
DEN-1	GTC ATG CTC CAC ACA ACA GAA AGA GGA GGG AGG GCC TAT CTT	
DEN-2	.CT G.. ..G ..T ..C G.T ..G GC. ..T ..A ... ..G ..C AA.	
DEN-3	..G ... ..G ... ..G T.. ... CAT ..C ..T ... .C AGG	
DEN-4	... ... .AA	
		168
DEN-1	CAC GCC CTG AAC GAA CTT CCG GAG TCA CTG GAA ACA CTC ATG	
DEN-2	..T ..T ..C .GT ... ..G ... ... A.C ... ..G ... ..G C.T	
DEN-3	..T ..A G.. G.G ... ..A ..A ..A ACG A.. ... ... T.A	
DEN-4	... ... A..	
		210
DEN-1	CTT GTA GCT TTA CTA GGT GCT ATG ACA GCA GGT ATC TTC CTG	
DEN-2	T.A C.G A.A C.C ..G .CC A.A G.C ... .G. ..A ... ... T.A	
DEN-3	..C C.G .GA C.G A.G ATC TTG T.A ... .GT ..A GCA A.G ..C	
DEN-4	... ...C	
		252
DEN-1	TTT TTC ATG CAA GGG AAA GGA ATA GGG AAA TTG TCA ATG GGT	
DEN-2	..C ..A ... AGC ..A ... ..T ... .G A.. A.C C.. ..A	
DEN-3	..C ..G ..A TC. ..T ... ..G ..T ..A ..G ACT ... ..A ..A	
DEN-4	... ...	
		294
DEN-1	TTG ATA ACC ATT GCG GTG GCT AGT GGC TTG CTC TGG GTA GCA	
DEN-2	A.. TGT TG. ..A ATC AC. ... ... AT. C.C ..A ... TAT ...	
DEN-3	C.C ..T TGT G.A ATT .CT T.C ..C ... A.. T.A ... ATG ..T	
DEN-4	... ...	
		336
DEN-1	GAA ATT CAA CCC CAG TGG ATA GCG GCC TCA ATC ATA CTA GAG	
DEN-2	C.G ..A ... ..A ..C ... .A ..T ... ..A ... ..G ...	
DEN-3	..T G.C .C. .T. ..A ... ..C ... T.G G.T ..A G.C ..G ...	
DEN-4	... ...	
		378
DEN-1	TTT TTT CGC ATG GTA CTG TTG ATA CCG GAA CCA GAA AAA CAA	
DEN-2	... ... .T. ..A ..T ... C.C ..T ..A ... ...G	
DEN-3	... ... ATG ... ..G T.. C.C ... ..A ... ...G ..G	
DEN-4	... ... .TA	

TABLE 6. NS4a nucleotide sequence (Page 2)

														420
DEN-1	AGG	ACC	CCA	CAA	GAC	AAT	CAA	TTG	ATC	TAC	GTC	ATA	TTG	ACC
DEN-2	..A	..A	..C	...	...	..C	...	...	.C.	...	..T	G.C	A.A	G..
DEN-3	..A	..T	..C	...	...	..C	...	C.C	GCA	..T	...	G.G	A.A	GG.
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														462
DEN-1	ATT	CTC	ACC	ATC	ATT	GGT	CTA	ATA	GCA	GCC				
DEN-2	..C	...	..A	G.G	G.G	.CC	GC.	.CC	ATG	..A				
DEN-3	..A	..T	..A	T.G	GC.	.CA	A..	G..	..G	...				
DEN-4	...	...	...	...	...	...	...	...	...	...				

TABLE 7. NUCLEOTIDE SEQUENCE OF DEN-1 CV1636/77
Nucleotide sequence encoding the NS4b genome

														42
DEN-1	ACC	GAG	ATG	GGG	CTG	ATT	GAA	AAA	ACA	AAA	ACG	GAT	TTT	GGG
DEN-2	.A.	...	...	..T	T.C	C.G	...	...	..C	..G	.AA	..C	C.C	..A
DEN-3	.AT	..A	...	..A	...	T.G	...	.CT	...	..G	.GA	...	..A	..A
DEN-4	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...
														84
DEN-1	TTT	TAC	CAG	GTA	AAA	ACA				GAA	ACC	ACC	ATC	CTC
DEN-2	..G	GGA	AGC	A.T	.C.	..T	CAG	GAA	TCT	..G	.G.	.A.	...	..G
DEN-3	A.G	.CT	A.A	.A.	CC.	GGT	GTT	GTT	TCT	CC.	...	.G.	TAT	T.G
DEN-4	...	...	...	...	...	...				...	...	...	...	...
														126
DEN-1	GAT	GTG	GAC	TTG	AGA	CCA	GCT	TCA	GCA	TGG	ACG	CTC	TAT	GCA
DEN-2	..C	A.A	..T	C.A	CGT	..T	..A	...	...	...	...	..G	...	..C
DEN-3	...	...	...	...	CAC	...	..A	...	..C	...	..A	T.G	..C	..C
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														168
DEN-1	GTA	GCC	ACC	ACA	ATT	CTG	ACT	CCC	ATG	CTG	AGA	CAC	ACC	ATA
DEN-2	..G	..T	..A	...	T..	G.T	..A	..A	...	T..	...	..T	.G.	..T
DEN-3	..G	...	..A	...	G.A	A.A	..A	..A	...	T..	...	...	...	..A
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														210
DEN-1	GAA	AAC	ACG	TCG	GCC	AAC	CTA	TCT	CTA	GCA	GCC	ATT	GCC	AAC
DEN-2	...	..T	T.C	..A	.TG	..T	G.G	..C	...	A..	...	...	..T	...
DEN-3	..G	..T	T.C	A.A	..A	..T	G.G	..C	..G	..A	...	..A	..T	...
DEN-4	...	...	..T	...	...	...	...	...	...	...	...	...	...	...
														252
DEN-1	CAG	GCA	GCC	GTC	CTA	ATG	GGG	CTT	GGA	AAA	GGA	TGG	CCG	CTC
DEN-2	..A	..C	A.A	..G	...	...	..T	...	..G	...	...	...	..A	T.G
DEN-3	...	...	.TG	...	..G	...	..T	T.A	.AC	...	...	...	...	A.A
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														294
DEN-1	CAC	AGA	ATG	GAC	CTC	GGT	GTG	CCG	CTG	TTA	GCA	ATG	GGA	TGC
DEN-2	TCA	.AG	..C	C..	A..	..A	..T	..C	..C	C.T	..C	..T	...	...
DEN-3	TCG	.A.	...	...	T.G	..C	..A	..A	..A	..G	...	C..	..T	...
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														336
DEN-1	TAT	TCT	CAA	GTG	AAC	CCA	ACA	ACC	TTG	ACA	GCA	TCC	TTA	GGC
DEN-2	..C	..A	..G	..C	...	..T	.T.	..T	C.C	...	...	G.T	C.T	CTT
DEN-3	...	..A	...	...	...	...	CT.	..T	C.T	.T.	..G	G.A	G.A	CTT
DEN-4	...	...	...	...	...	...	...	...	...	G..	...	...	...	.T.
														378
DEN-1	ATG	CTT	TTA	GTC	CAT	TAT	GCA	ATA	ATA	GGC	CCA	GGA	TTG	CAG
DEN-2	T.A	T.G	G..	.CA	...	...	..C	..T	...	..G	...	...	C.T	..A
DEN-3	T..	..A	G.C	ACA	...	...	...	..T	...	..T	...	...	...	...
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														420
DEN-1	GCA	AAA	GCC	ACA	AGA	GAG	GCC	CAG	AAA	AGG	ACA	GCT	GCT	GGG
DEN-2	...	...	..A	..C	...	..A	..T	...	...	..A	G..	..A	..A	..C
DEN-3	...	...	...	..T	C.T	..A	..T	...	...	...	...	...	...	..A
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...

TABLE 7. Nucleotide sequence of NS4b (Page 2)

														462
DEN-1	ATC	ATG	AAA	AAT	CCC	ACA	GTG	GAC	GGG	ATA	ACA	GTA	ATA	GAT
DEN-2	...	...	...	..C	..A	...	..C	..T	..A	...	...	..G	..T	..C
DEN-3	..A	...	..G	...	..A	..G	...	..T	..A	...	.TG	AC.	...	..C
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														504
DEN-1	CTA	GAA	CCA	ATA	TCC	TAT	GAC	CCA	AAA	TTT	GAA	AAG	CAA	TTA
DEN-2	...	..T	...	...	C..	...	..T	...	...	...	...	...	..G	...
DEN-3	...	..T	..T	G..	ATA	...	..T	T..	...	...	...	...	...	C..
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														546
DEN-1	GGG	CAG	GTC	ATG	CTA	CTA	GTC	TTG	TGT	GCT	GGA	CAA	CTA	CTC
DEN-2	..A	..A	..A	...	..C	...	A..	C.C	..C	.TG	ACT	...	G..	T.A
DEN-3	..A	..G	..T	...	..C	..G	..T	C..	...	..A	.TT	...	..T	T.G
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														588
DEN-1	TTG	ATG	AGA	ACA	ACA	TGG	GCT	TTC	TGT	GAA	GTC	TTG	ACT	TTG
DEN-2	A..	...	..G	..T	...	...	...	C.G	...	..G	.CT	C.A	..C	..A
DEN-3	..A	...	...	...	T..	...	..C	..G	...	...	..T	CAT	..C	C.A
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														630
DEN-1	GCC	ACA	GGA	CCA	ATC	TTG	ACC	TTG	TGG	GAG	GGC	AAC	CCG	GGA
DEN-2	..G	..C	..G	..T	...	.CC	..A	C..	...	..A	..A	..T	..A	..G
DEN-3	...	...	...	...	..A	ACA	..A	C.C	...	.AA	..A	TCA	..T	..G
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														672
DEN-1	AGG	TTT	TGG	AAC	ACG	ACC	ATA	GCC	GTA	TCC	ACC	GCC	AAC	ATT
DEN-2	...	...	...	...	..T	...	..T	..A	..G	..A	.TG	..T	...	..C
DEN-3	.A.	..C	...	...	..C	..G	...	..T	..T	...	.TG	..G	...	..C
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
														714
DEN-1	TTC	AGG	GGA	AGT	TAC	TTG	GCG	GGA	GCT	GGA	CTG	GCT	TTT	TCA
DEN-2	..T	..A	..G	..C	...	...	..C	...	...	...	..T	CTC	...	..C
DEN-3	...	..A	..G	..C	..T	..A	..A	...	...	..G	..T	...	C..	..T
DEN-4	...	...	...	...	...	...	...	...	...	...	...	...	...	...
										744				
DEN-1	CTC	ATA	AAG	AAT	GCA	CAA	ACC	CCT	AGG	AGG				
DEN-2	A..	..G	...	..C	A..	AC.	.A.	A.A	..A	..A				
DEN-3	A..	..G	..A	TCA	.TT	GG.	..A	GGA	.A.	..A				
DEN-4	...	...	...	...	...	...	...	...	...	...				

TABLE 8. NUCLEOTIDE SEQUENCE OF DEN-1 CV1636/77
Nucleotide sequence encoding the NS5 genome

														42
DEN-1	GGA	ACT	GGG	ACC	ACA	GGA	GAG	ACA	CTG	GGA	GAG	AAA	TGG	AAA
DEN-2	...	...	..C	.A.	.T.	...	...	...	..T	...	..A	...	...	...
DEN-3	...	..A	...	T.A	CA.	..T	..A	..C	T.A	...	..A	..G	...	...
DEN-4	...	...	...	...	...	...	...	...	...	...	...	..G	...	..G
														84
DEN-1	ACA	CAG	TTA	AAC	CAA	CTG	AGC	AAG	TCA	GAA	TTC	AAC	ACC	TAC
DEN-2	.GC	.GA	..G	...	GC.	...	G.A	..A	AGT	...	..T	C.G	.T.	..G
DEN-3	.AG	A.A	...	..T	..G	T.A	TC.	CG.	AA.	..G	..T	G..	CTT	...
DEN-4	.G.	...	C..	...	TC.	T.A	GA.	.GA	AA.	..G	..T	G.A	GAG	..T
														126
DEN-1	AAA	AGG	AGT	GGG	ATT	ATG	GAG	GTG	GAC	AGA	TCC	GAA	GCC	AAA
DEN-2	..G	.AA	...	..A	..C	CA.	..A	...	..G	...	A..	TT.	..A	...
DEN-3	..G	.AA	TCC	..A	..C	.CC	..A	...	..T	...	A.A	..A	...	...
DEN-4	...	..A	...	..A	..A	C.A	..A	...	...	..G	A.T	...	...	..G
														168
DEN-1	GAG	GGA	TTG	AAA	AGA	GGA	GAA	ACA	ACC	AAA	CAT	GCA	GTG	TCG
DEN-2	..A	..C	A.C	...	...	...	...	..G	GA.	C.C	..C	..T	...	...
DEN-3	..A	..G	..A	...	...	...	...	.T.	..A	C.C	...	..C	...	..C
DEN-4	TCT	.CC	C..	...	GAT	..G	TCT	.A.	.TG	..G	...	...	..A	..T
														210
DEN-1	AGA	GGA	ACA	GCC	AAA	CTG	AGG	TGG	TTT	GTG	GAG	AGG	AAC	CTC
DEN-2	C..	..C	T..	..A	...	...	..A	...	..C	..C	...	..A	..T	A.G
DEN-3	...	..C	.GC	..A	...	..T	CAA	...	..C	...	...	..A	...	A.G
DEN-4	...	..G	T.C	GCT	..G	A.C	..A	...	A..	..T	...	..A	GGG	A.G
														252
DEN-1	GTG	AAA	CCA	GAA	GGG	AAA	GTC	ATA	GAC	CTC	GGT	TGT	GGA	AGA
DEN-2	..C	.C.	...	...	...	..G	..G	G.G	...	...	...	..C	..C	...
DEN-3	..C	.TT	..C	...	..A	.G.	...	...	...	T.A	..C	...	...	...
DEN-4	..A	..G	...	A..	...	G..	..T	G..	..T	..T	..C	...	..G	...
														294
DEN-1	GGT	GGC	TGG	TCA	TAT	TAT	TGT	GCT	GGG	CTG	AAG	AAA	GTC	ACT
DEN-2	..G	...	...	...	..C	...	...	.GG	..A	..A	...	..T	..A	.GA
DEN-3	..A	...	...	...	...	..C	...	..A	..A	...	..A	...	..T	..A
DEN-4	..A	..A	...	..T	...	..C	ATG	..G	ACA	..C	...	..C	..G	...
														336
DEN-1	GAA	GTG	AAG	GGA	TAC	ACA	AAA	GGA	GGA	CCT	GGA	CAT	GAG	GAA
DEN-2	...	..C	..A	..C	CTG	...	...	...	...	..A	...	..C	..A	...
DEN-3	...	...	CGA	...	...	.G.	...	..C	..C	..A	...	..C	..A	...
DEN-4	...	...	..A	..G	..T	...	...	...	..T	..A	...	...	..A	...
														378
DEN-1	CCT	ATC	CCA	ATG	GCG	ACC	TAT	GGA	TGG	AAC	CTA	GTA	AAG	CTA
DEN-2	..C	...	..C	...	T.A	..A	...	..G	...	..T	...	..G	CGT	..G
DEN-3	..A	G.A	..T	...	T.T	..A	..C	...	...	...	A..	..C	...	T..
DEN-4	..C	..T	..C	...	..T	..T	...	..T	...	..T	T.G	..C	..A	..C
														420
DEN-1	CAC	TCT	GGA	AAA	GAT	GTA	TTT	TTC	ACA	CCA	CCT	GAG	AAA	TGT
DEN-2	..A	AG.	...	GTT	..C	..T	..C	...	..C	..C	..A	..A	..G	...
DEN-3	ATG	AG.	...	..G	...	C.T	...	.AT	CTG	...	...	..A	..G	...
DEN-4	..T	..A	..G	GTT	..C	T.G	..C	.AC	.A.	..C	A.A	...	C..	GTG

TABLE 8. NS5 nucleotide sequence (Page 2 of 7)

														462
DEN-1	GAT	ACC	CTT	CTG	TGT	GAT	ATT	GGT	GAG	TCC	TCT	CCG	AAT	CCA
DEN-2	...	..A	T.G	T..	...	..C	..A	..G	...	..G	..A	..A	...	..C
DEN-3	...	...	..A	T..	...	..C	...	..A	..A	..T	..A	..A	.GC	...
DEN-4	..C	...	..G	..C	...	...	...	..G	...	..A	...	T.T	...	...
														504
DEN-1	ACT	ATA	GAA	GAA	GGA	AGA	ACG	TTA	CGT	GTT	CTA	AAG	ATG	GTG
DEN-2	..G	...	...	.C.	...	C..	..A	C.C	A.A	..C	..C	..C	T.A	..A
DEN-3	..A	G.G	...	...	A.C	...	..C	A..	A.A	..C	T.G	...	...	..T
DEN-4	..A	...	..G	...	...	...	..A	...	A.A	...	T.G	...	...	...
														546
DEN-1	GAA	CCA	TGG	CTC	AGA	GGA	AAC		CAA	TTC	TGC	ATA	AAA	ATC
DEN-2	...	AAT	...	T.G	.AC	AAT	...	ACC	...	..T	...	...	..G	G.T
DEN-3	...	...	...	..A	.A.	AAC	...		..G	..T	..C	..T	...	G.A
DEN-4	..G	...	...	...	TCT	TC.	..A	CCT	G..	...	..G	..C	...	G..
														588
DEN-1	CTA	AAT	CCT	TAC	ATG	CCA	AGT	GTG	GTA	GAA	ACT	CTG	GAG	CAA
DEN-2	..C	..C	..A	..T	...	..C	TCA	..C	A..	...	.AA	A..	..A	AC.
DEN-3	TTG	..C	..A	...	...	...	.C.	...	A.T	..G	CAC	T.A	..A	AG.
DEN-4	..T	..C	..C	...	...	...	.CA	..C	A..	...	GAG	...	...	A..
														630
DEN-1	ATG	CAA	AGA	AAA	CAT	GGA	GGG	ATG	CTA	GTG	CGA	AAC	CCA	CTC
DEN-2	C.A	...	..G	...	T..	...	..A	GCC	T..	...	A.G	..T	...	...
DEN-3	C.A	...	...	...	...	...	..A	...	..T	...	A..	..T	...	...
DEN-4	C..	..G	...	...	..T	..T	...	.AC	..T	..C	A..	TG.	..G	..G
														672
DEN-1	TCA	AGA	AAT	TCC	ACC	CAT	GAA	ATG	TAC	TGG	GTT	TCA	TGT	GGA
DEN-2	...	C..	..C	...	..A	...	..G	...	...	...	..A	..C	AA.	.C.
DEN-3	...	C..	..C	...	...	..C	...	...	..T	...	A.A	..C	AA.	..T
DEN-4	..C	..G	..C	...	...	...	..G	...	..T	...	..G	...	G.A	.CG
														714
DEN-1	ACA	GGA	AAC	ATT	GTG	TCG	GCA	GTG	AAC	ATG	ACA	TCC	AGA	ATG
DEN-2	T.C	..G	...	..A	...	..A	T..	...	...	...	.TT	..A	..G	...
DEN-3	...	..C	...	..C	..C	..T	T..	..C	...	...	GT.	...	...	T..
DEN-4	T.G	...	...	...	...	AGC	T.T	...	...	.CA	...	..A	.AG	...
														756
DEN-1	TTA	CTG	AAT	CGA	TTC	ACA	ATG	GCT	CAC	AGG	AAG	CCA	ACA	TAT
DEN-2	..G	A.T	..C	A..	...	...	...	AAA	...	.A.	..A	G.C	..C	..C
DEN-3	C..	...	..C	A..	...	...	...	A.A	...	...	.GA	..C	..C	ATA
DEN-4	..G	T..	..C	A.G	...	...	.CA	AGG	..T	...	..A	..C	..T	...
														798
DEN-1	GAA	AGA	GAC	GTG	GAC	TTA	GGC	GCT	GGA	ACA	AGA	CAT	GTG	GCA
DEN-2	..G	.C.	..T	..T	...	C..	..A	AGC	...	..C	C.C	A.C	A.T	.G.
DEN-3	..G	.A.	..T	...	..T	...	..A	..A	...	..C	C..	...	.AC	AAT
DEN-4	..G	.AG	...	..A	..T	C.T	..G	..A	...	..G	...	AG.	..C	T.C
														840
DEN-1	GTG	GAA	CCA	GAG	GTA	GCC	AAC	CTA	GAT	ATC	ATT	GGC	CAG	AGG
DEN-2	A.T	...	AGT	...	A..	C.A	..T	...	..C	..A	..A	..A	A..	..A
DEN-3	.C.	...	...	..A	AC.	C..	...	A.G	...	G..	..G	..G	G.A	..A
DEN-4	ACT	...	A..	A.A	AA.	C.A	G..	A.G	ACA	...	.G.	..G	AGA	...

TABLE 8. NS5 nucleotide sequence (Page 3 of 7)

														882
DEN-1	ATA	GAG	AAT	ATA	AAA	AAT	GAA	CAC	AAG	TCA	ACA	TGG	CAT	TAT
DEN-2	...	...	..A	...	..G	C.A	..G	..T	G.A	A..	T..	...	..C	...
DEN-3	...	A.A	.GG	..C	...	G.G	..G	..T	.GT	...	...	...	..C	...
DEN-4	C.T	C..	CGA	T.G	C..	G.A	..G	...	..A	GA.	..C	...	...	...
														924
DEN-1	GAT	GAA	GAC	AAT	CCA	TAC	AAA	ACA	TGG	GCC	TAT	CAT	GGA	TCA
DEN-2	..C	C..	...	C.C	...	...	...	..G	...	..T	..C	...	..C	AGC
DEN-3	...	..T	..A	...	..T	...	...	..G	...	..T	..C	...	...	..C
DEN-4	...	C.G	..A	..C	..A	...	.G.	..C	...	..G	..T	...	...	AGC
														966
DEN-1	TAT	GAG	GTC	AAG	CCA	TCA	GGA	TCA	GCC	TCA	TCT	ATG	GTG	AAT
DEN-2	...	..A	ACA	..A	.A.	A.T	...	...	..A	...	...	...	...	..C
DEN-3	...	..A	..A	..A	G.C	A..	..C	...	...	..C	..C	...	A.A	...
DEN-4	...	..A	CCT	CCT	T.G	A..	..C	..T	C.A	..C	..C	...	...	..C
														1008
DEN-1	GGC	GTG	GTG	AGA	TTG	CTC	ACA	AAA	CCA	TGG	GAT	GTT	ATC	CCC
DEN-2	..A	...	..C	...	C..	..C	...	...	..C	...	..C	..C	G..	..T
DEN-3	..A	..C	...	.A.	C.C	...	...	...	...	...	...	..G	G.G	..A
DEN-4	..G	..A	..A	.A.	C.C	..A	...	...	..C	...	...	..G	..T	..A
														1050
DEN-1	ATG	GTC	ACA	CAA	ATA	GCT	ATG	ACT	GAT	ACC	ACA	CCC	TTC	GGA
DEN-2	...	..G	...	..G	..G	..A	...	..A	..C	..G	..T	..A	..T	...
DEN-3	...	..G	...	..G	..G	..A	...	..A	...	..A	..T	..A	..T	..C
DEN-4	...	..G	..T	..G	T..	..C	G..	..A	...	G.A	.TC	.AT	.T	..C
														1092
DEN-1	CAA	CAC	AGA	GTG	TTT	AAA	GAG	AAA	GTT	GAC	ACG	CGC	ACA	CCA
DEN-2	...	..A	C.C	..T	..C	...	...	...	..G	...	...	A.A	..C	.A.
DEN-3	..G	..A	...	..T	..	...	...	...	..G	...	..C	A.G	...	..T
DEN-4	...	..A	...	...	..C	...	...	..G	..G	..T	..C	A.A	...	...
														1134
DEN-1	AGA	GCA	AAA	CGA	GGC	ACA	GCA	CAA	ATT	ATG	GAG	GTG	ACA	GCC
DEN-2	GA.	C.G	..G	GA.	...	...	AAG	A..	C.G	...	A.A	A.C	..G	..A
DEN-3	..G	C.C	.TG	.C.	..A	...	AG.	A.G	G..	...	...	A.C	...	..G
DEN-4	CA.	C..	...	.CC	..T	...	CG.	ATG	G..	...	ACC	AC.	...	...
														1176
DEN-1	AAG	TGG	TTA	TGG	GGT	TTC	CTT	TCC	AGA	AAC	AAA	AAA	CCC	AGA
DEN-2	G..	...	C.T	...	AAA	GAA	..A	GGA	.AG	..A	..G	.C.	.T	..G
DEN-3	G..	...	C.T	...	A.G	AC.	..G	GGA	..G	...	...	.G.	...	...
DEN-4	..T	...	C.G	...	.CC	C..	...	GGA	.AG	..G	...	..T	...	...
														1218
DEN-1	ATC	TGC	ACA	AGA	GAG	GAG	TTC	ACA	AGA	AAG	GTT	AGG	TCA	AAC
DEN-2	..G	..T	..C	...	..A	..A	...	...	...	...	..G	..A	AGC	..T
DEN-3	T.A	...	...	..G	...	...	...	...	.AG	...	..C	..A	A.C	...
DEN-4	C.G	...	...	..G	..A	...	...	.TC	TCA	..A	...	..A	...	...
														1260
DEN-1	GCA	GCA	ATA	GGA	GCA	GTG	TTC	GTT	GAT	GAA	AAC	CAA	TGG	AAC
DEN-2	...	..C	T.G	..G	..C	A.A	...	AC.	...	..G	...	A..	...	..A
DEN-3	...	..T	..G	..C	..T	..C	...	ACA	..A	..G	...	...	...	G..
DEN-4	...	..C	..A	..T	CGA	..C	..T	CAG	..A	..A	C.G	GG.	...	.CA

TABLE 8. NS5 nucleotide sequence (Page 4 of 7)

														1302
DEN-1	TCA	GCA	AAA	GAA	GCA	GTG	GAA	GAC	GAA	AGG	TTT	TGG	GAT	CTC
DEN-2	..G	...	CGT	..G	..T	..T	...	..T	AGT	...	...	...	..G	..G
DEN-3	AGT	..G	.G.	.CT	..T	..T	..G	..C	...	GAA	...	...	A.A	..T
DEN-4	...	..C	.GT	...	..T	...	A.T	...	AGC	C..	...	...	..A	..G
														1344
DEN-1	GTG	CAC	AGA	GAG	AGG	GAC	CTT	CAT	AAA	CAG	GGA	AAA	TGT	GCC
DEN-2	..T	G..	..G	..A	..A	A.T	..C	...	CTT	G.A	...	..G	...	..A
DEN-3	...	G..	...	..A	C.T	..A	..C	..C	...	.T.	..C	..G	...	.GA
DEN-4	..T	G..	.A.	..A	...	.CC	..A	..C	CAG	G.A	..G	...	...	.AA
														1386
DEN-1	ACG	TGT	GTC	TAC	AAC	ATG	ATG	GGG	AAG	AGA	GAG	AAA	AAA	TTA
DEN-2	..A	...	..G	...	...	...	...	..A	..A	...	...	..G	...	C..
DEN-3	.GC	..C	..T	...	...	...	...	..C	..G	...	...	...	...	C.T
DEN-4	T..	...	...	..T	...	...	...	..A	..A	C.T	...	...	..G	...
														1428
DEN-1	GGA	GAG	TTT	GGA	AAG	GCA	AAA	GGA	AGT	CGT	GCA	ATA	TGG	TAC
DEN-2	..G	...	..C	..C	...	...	...	..T	..C	A.A	..C	...	...	..G
DEN-3	...	...	...	..T	..A	...	...	..C	...	A.G	..T	...	...	...
DEN-4	...	...	...	..C	.GA	..C	..G	...	..C	..A	...	..C	...	...
														1470
DEN-1	ATG	TGG	CTG	GGA	GCA	CGC	TTT	CTA	GAG	TTC	GAA	GCC	CTT	GGT
DEN-2	...	...	..T	...	...	...	..C	T..	...	..T	...	...	..A	..A
DEN-3	...	...	T..	...	..C	A.G	.AC	..T	...	..C	..G	..G	..C	..A
DEN-4	...	...	...	...	..G	..G	...	..G	..A	..T	...	...	..G	...
														1512
DEN-1	TTC	ATG	AAT	GAA	GAT	CAC	TGG	TTC	AGT	AGA	GAG	AAT	TCA	CTC
DEN-2	...	T..	...	...	...	...	...	...	TCC	...	..G	..C	..C	..G
DEN-3	...	C.C	...	...	..C	...	...	...	TCG	C.T	..A	..C	..T	TA.
DEN-4	...	T..	...	...	...	...	...	...	G.C	...	..A	...	...	TGG
														1554
DEN-1	AGT	GGA	GTG	GAA	GGA	GAA	GGA	CTG	CAC	AAA	CTT	GGA	TAC	ATA
DEN-2	...	...	...	...	...	...	..G	...	...	..G	..A	..C	...	..T
DEN-3	...	...	..A	...	...	...	..A	...	...	..G	..G	...	...	..C
DEN-4	...	...	...	...	..G	...	..T	...	...	.G.	T.G	...	..T	..C
														1596
DEN-1	CTC	AGA	GAC	ATA	TCA	AAG	ATT	CCG	GGG	GGA	AAT	ATG	TAT	GCA
DEN-2	T.A	...	...	G.G	AGC	...	.AG	GAA	...	...	GCA	...	..C	..C
DEN-3	TTG	...	..T	..T	..C	...	..A	..C	..A	...	GCC	...	...	..T
DEN-4	..G	GAG	..G	...	GAC	...	.AG	GAT	..A	.AC	CTA	...	...	..T
														1638
DEN-1	GAT	GAT	ACA	GCC	GGA	TGG	GAC	ACA	AGA	ATA	ACA	GAG	GAT	GAT
DEN-2	...	..C	...	..A	...	...	...	...	...	..C	...	CTA	..A	..C
DEN-3	...	..C	...	...	..T	...	...	...	...	...	...	..A	...	..C
DEN-4	...	..C	...	..A	..C	...	...	...	...	..C	..T	...	...	..C
														1680
DEN-1	CTT	CAG	AAT	GAG	GCT	AAA	ATC	ACT	GAC	ATC	ATG	GAG	CCT	GAA
DEN-2	T.A	A.A	...	..A	.AA	.TG	GTA	..A	A..	CA.	...	..A	CGA	...
DEN-3	..G	..C	...	...	.AA	...	...	..A	C.G	CAG	...	..C	...	...
DEN-4	...	..A	...	...	.AC	CTG	...	..G	..A	CAG	...	.CT	..C	CAC

TABLE 8. NS5 nucleotide sequence (Page 5 of 7)

														1722
DEN-1	CAT	GCT	CTA	TTG	GCT	ACG	TCA	ATT	TTT	AAG	CTG	ACC	TAC	CAA
DEN-2	..C	AAG	AA.	C.A	..C	GA.	G.C	..A	..C	..A	T.A	..G	...	...
DEN-3	..C	AGG	CAG	C.A	..G	.AC	G.T	..A	..C	...	..C	..A	...	...
DEN-4	..C	AAG	ATC	C.A	..C	.AA	G.C	...	..C	..A	..A	...	..T	...
														1764
DEN-1	AAC	AAG	GTG	GTG	ACC	CTG	CAA	AGA	CCA	GCA	AAA	AAT	GGA	ACC
DEN-2	...	...	...	...	CGT	G..	...	...	...	A..	CC.	.GA	..C	..A
DEN-3	...	..A	...	..C	.AA	G.C	...	C..	...	A.T	CC.	..G	..C	..G
DEN-4	...	..A	...	...	TTT	G.C	CTC	...	..C	A..	CCG	.GA	...	G.G
														1806
DEN-1	GTG	ATG	GAT	GTT	ATA	TCC	AGA	CGT	GAC	CAG	AGA	GGA	AGT	GGA
DEN-2	..A	...	...	A.C	...	..G	...	A.A	...	..A	...	..C	...	..G
DEN-3	..A	...	..C	A.C	...	..T	..G	AAA	...	..A	...	..C	...	...
DEN-4	..A	...	...	A.C	...	...	..G	AAA	..A	..A	...	..T	...	...
														1848
DEN-1	CAG	GTC	GGA	ACT	TAT	GGC	TTA	AAT	ACT	TTC	ACC	AAT	ATG	GAG
DEN-2	..A	...	..C	..C	...	...	C.T	...	..A	...	...	..C	...	..A
DEN-3	...	..G	...	...	...	..T	C.G	..C	..A	...	...	..C	...	..A
DEN-4	..A	..T	...	..A	...	..T	..G	..C	..A	...	...	..C	...	..A
														1890
DEN-1	GTC	CAA	CTA	ATA	AGA	CAA	ATG	GAG	TCT	GAA	GGA	GTC	ATC	ACA
DEN-2	.CT	...	T..	..T	...	..G	...	...	GGA	...	...	A..	T..	.A.
DEN-3	.C.	..G	...	..C	...	...	...	..A	GGA	...	..C	..G	T.G	T..
DEN-4	..T	...	..C	.GC	C.C	...	...	..A	G..	...	...	...	...	...
														1932
DEN-1	CAA	GAT	GAC	ATG	CAG	AAC	CCA	AAA	GGT	TTG	AAA	GAA	AGA	GTT
DEN-2	AGC	AT.	C.G	CAC	.T.	.CA	GTC	.C.	.AA	GAA	.TC	.CT	GT.	CAG
DEN-3	A.G	.CA	...	C.C	G..	...	..C	C.T	CCG	C.A	G.G	A.G	.A.	A..
DEN-4	...	...	...	...	...	...	...	...	..G	...	...	...	...	...
														1974
DEN-1	GAG	AAA	TGG	TCG	AAA	GAG	TGT	GGT	GTC	GAC	AGG	CTG	AAA	AGA
DEN-2	A.C		...	.TA	GC.	AGA	GTG	..G	CGT	..A	...	..A	TC.	...
DEN-3	ACA	C..	...	.T.	G..	ACT	AAA	..A	..G	..G	...	T.A	...	...
DEN-4	...	...	...	CT.	...	...	...	...	...	...	...	T.A	..G	..G
														2016
DEN-1	ATG	GCA	ATT	AGC	GGA	GAT	GAT	TGT	GTG	GTG	AAA	CCA	ATT	AGT
DEN-2	...	..C	..C	..T	...	...	...	...	..T	..A	...	..T	T.A	GA.
DEN-3	...	..C	..C	...	..G	...	...	..C	..A	...	...	...	..C	GAC
DEN-4	...	...	..C	..T	...	..C	...	..C	...	...	..G	..C	C.A	GA.
														2058
DEN-1	GAC	AGG	TTC	GCA	ACA	GCC	TTA	ATA	GCT	CTG	AAT	GAC	ATG	GGA
DEN-2	...	..A	..T	...	.GT	..T	...	.C.	...	..A	...	...	...	...
DEN-3	...	..A	...	..C	.AT	...	CTG	C.T	..C	...	..C	..T	...	...
DEN-4	..G	...	..T	.GC	..T	T..	C.C	C.C	TTC	T..	..C	C.G	...	...
														2100
DEN-1	AAA	GTA	AGA	AAA	GAC	ATA	CCG	CAG	TGG	GAA	CCT	TCA	AAA	GGA
DEN-2	...	..T	..G	...	..T	...	.AA	..A	...	...	...	...	.G.	...
DEN-3	..G	..T	..G	..G	...	...	..T	..A	...	C.G	..A	...	..G	...
DEN-4	..G	..G	..G	...	...	..T	...	...	...	...	..A	..T	..G	...

TABLE 8. NS5 nucleotide sequence (Page 6 of 7)

														2142
DEN-1	TGG	AAT	GAC	TGG	CAG	CAA	GTG	CCT	TTC	TGT	TCA	CAC	CAT	TTC
DEN-2	...	..	..T	...	ACA	...	...	...	...	...	...	...	...	..T
DEN-3	...	C..	..T	...	..A	..G	..C	...	...	..C	..C	...	..C	..T
DEN-4	...	..A	A..	...	..A	G.G	..T	...	..T	..C	G.C	...	..C	C.T
														2184
DEN-1	CAC	CAG	CTG	ATC	ATG	AAG	GAT	GGG	AGG	GAA	ATA	GTG	GTG	CCA
DEN-2	..T	G..	T.A	G..	...	..A	...	..T	C.C	.TG	C.C	..A	..C	...
DEN-3	..T	G.A	T..	...	...	..A	...	..A	..A	A.G	T.G	..A	..T	..C
DEN-4	...	A..	ACC	T.T	...	...	...	..C	C.C	TC.	C..	..T	..T	...
														2226
DEN-1	TGC	CGC	AAC	CAA	GAT	GAA	CTT	GTG	GCA	AGG	GCT	AGA	GTA	TCA
DEN-2	...	A.A	...	...	...	...	..G	A.T	.GT	..A	..C	C..	A.T	..C
DEN-3	...	A.A	CC.	..G	..C	...	..A	A.A	.G.	..A	..G	...	A.C	..T
DEN-4	..T	A.A	...	..G	...	...	..G	A.A	.G.	..A	..C	...	A.C	..G
														2268
DEN-1	CAA	GGC	GCC	GGA	TGG	AGC	CTG	AGA	GAA	ACT	GCT	TGC	CTA	GGC
DEN-2	..G	..A	...	..G	...	TCT	T..	.AG	..G	..G	..C	..T	T.G	..G
DEN-3	...	..A	..A	.A.	...	...	..T	...	...	...	..A	..T	...	..G
DEN-4	..G	..A	..T	...	...	...	T.A	...	...	..A	..C	..C	..G	...
														2310
DEN-1	AAG	TCA	TAT	GCA	CAA	ATG	TGG	CAG	CTG	ATG	TAC	TTC	CAC	AGG
DEN-2	...	..T	..C	..C	...	...	...	ACC	...	...	...	...	...	..A
DEN-3	..A	G.C	..C	..T	...	...	...	ACT	..C	...	..T	..T	...	..A
DEN-4	..A	G.T	..C	..C	..G	...	...	TC.	..T	...	...	...	...	..A
														2352
DEN-1	AGA	GAC	CTG	AGA	CTA	GCG	GCT	AAT	GCT	ATC	TGT	TCA	GCC	GTC
DEN-2	C.T	...	..C	...	..G	...	..A	...	...	..T	..C	..G	..A	...
DEN-3	...	..T	..T	...	..A	..A	T.C	..C	..C	..A	...	...	..A	..A
DEN-4	.AG	..T	...	C.T	T..	..C	T.C	.TG	..C	..A	..C	...	..A	..T
														2394
DEN-1	CCA	GTT	GAT	TGG	GTC	CCA	ACC	AGC	CGC	ACA	ACC	TGG	TCA	ATC
DEN-2	..G	TCA	C..	...	..T	...	..A	..T	C.A	...	...	...	..C	..A
DEN-3	...	..C	C..	...	...	..C	..G	...	A.A	..G	..A	...	..T	..T
DEN-4	...	ACG	..A	...	T.T	...	..A	..C	A.A	...	..A	...	...	...
														2436
DEN-1	CAT	GCC	CAC	CAC	CAA	TGG	ATG	ACA	ACA	GAA	GAC	ATG	TTA	TCA
DEN-2	..C	..T	A.G	..T	G..	...	...	..G	..G	...	...	...	C.G	G..
DEN-3	...	..T	...	..T	..G	...	...	..T	...	...	...	...	C.T	ACT
DEN-4	..C	..T	..T	...	..G	...	...	..C	..T	...	..T	...	C.C	AA.
														2478
DEN-1	GTG	TGG	AAT	AGG	GTT	TGG	ATA	GAG	GAA	AAC	CCA	TGG	ATG	
DEN-2	..C	...	...	...	..C	...	..C	C.A	...	...	..G	...	...	GAA
DEN-3	..C	...	..C	...	..G	...	...	...	..C	..T	...	...	...	GAA
DEN-4	...	...	..C	..A	..G	...	...	..A	..C	...	..T	AAT	...	ACT
														2520
DEN-1														
DEN-2	GAC	AAA	ACT	CCA	GTG	GAA	TCA	TGG	GAA	GAA	GTC	CCA	TAC	TTG
DEN-3	GAC	AAA	ACT	CCA	GTC	ACA	ACT	TGG	GAA	GAT	GTT	CCA	TAT	CTA
DEN-4	GAC	AAG	ACT	CCA	GTC	CAT	TCG	TGG	GAA	GAT	ATA	CCT	TAC	CTA

TABLE 8. NS5 nucleotide sequence (Page 7 of 7)

	2562
DEN-1	
DEN-2	GGA AAA AGA GAA GAC CAA TGG TGC GGC TCA TTG ATT GGG CTG
DEN-3	GGA AAG AGA GAA GAC CAA TGG TGC GGA TCA CTC ATA GGT CTC
DEN-4	GGG AAA AGA GAG GAT TTG TGG TGT GGA TCC CTG ATT GAA CTT
	2604
DEN-1	
DEN-2	ACA AGC AGG GCT ACC TGG GCA AAG AAC ATC CAA ACA GCA ATA
DEN-3	ACT TCC AGA GCA ACC TGG GCC CAG AAC ATA CTC ACA GCA ATC
DEN-4	TCT TCC AGA GCC ACC TGG GCG AAG AAC ATT CAC ACG GCC ATA
	2646
DEN-1	
DEN-2	AAT CAA GTC AGA TCC CTT ATA GGC AAT GAG GAA TAC ACA GAC
DEN-3	CAA CAG GTG AGA AGC CTC ATA GGC AAT GAA GAG TTT CTG GAC
DEN-4	ACC CAG GTC AGG AAC CTG ATC GGA AAA GAG GAA TAC GTG GAT
	2688
DEN-1	
DEN-2	TAC ATG CCA TCC ATG AAG AGA TTC AGA AGG GAA GAG GAA GAG
DEN-3	TAC ATG CCT TCG ATG AAG AGA TTC AGG AAG GAG GAG GAG TCA
DEN-4	TAC ATG CCA GTA ATG AAA AGA TAC AGT GCT CCT TCA GAG AGT
	2703
DEN-1	
DEN-2	GCA GGT GTC CTG TGG
DEN-3	GAG GGA GCC ATT TGG
DEN-4	GAA GGA GTT CTG

TABLE 9. VERIFICATION OF THE DELETION IN THE NS5 1055–1065 REGION

	NUCLEOTIDE SEQUENCES						
DEN–1	AGA	GGA	AAC	---	CAA	TTC	TGC
DEN–2	AAC	AAT	AAC	AAC	CAA	TTT	TGC
DEN–3	AAA	AAC	AAC	---	CAG	TTT	TGC
DEN–4	TCT	TCA	AAA	CCT	GAA	TTC	TGG
	PRIMER–DIRECTED RNA SEQUENCES						
CV1636	AGA	GGA	AAC	---	CAA	UUC	UGC
454–1	AGA	GGA	AAC	---	CAA	UUC	UGC
16007	AGA	GGA	AAC	---	CAA	UUC	UGC
155–3	AAA	AAC	AAC	---	CAG	U	
035–3	AAA	AAC	AAC	---	CAG	U	

TABLE 10.

COMPARISON OF DENGUE NON-STRUCTURAL PROTEINS

	DEN-2 JAMAICA	DEN-3 H87	DEN-4 DOMINICA
DEN-1 CV1636/77			
NS3	63%	72%	95%
NS4a	65%	60%	98%
NS4b	80%	79%	98%
NS5	77%	82%	78%

TABLE 11.

SIMILARITY BETWEEN ALIGNED NUCLEOTIDE AND AMINO ACID SEQUENCES OF THE NS3, NS4a, NS4b, AND NS5 REGIONS OF DEN

		NUCLEOTIDE			
		D1	D2	D3	D4
AA	D1	—	70%	69%	90%
	D2	74%	—	63%	66%
	D3	73%	70%	—	68%
	D4	91%	74%	78%	—

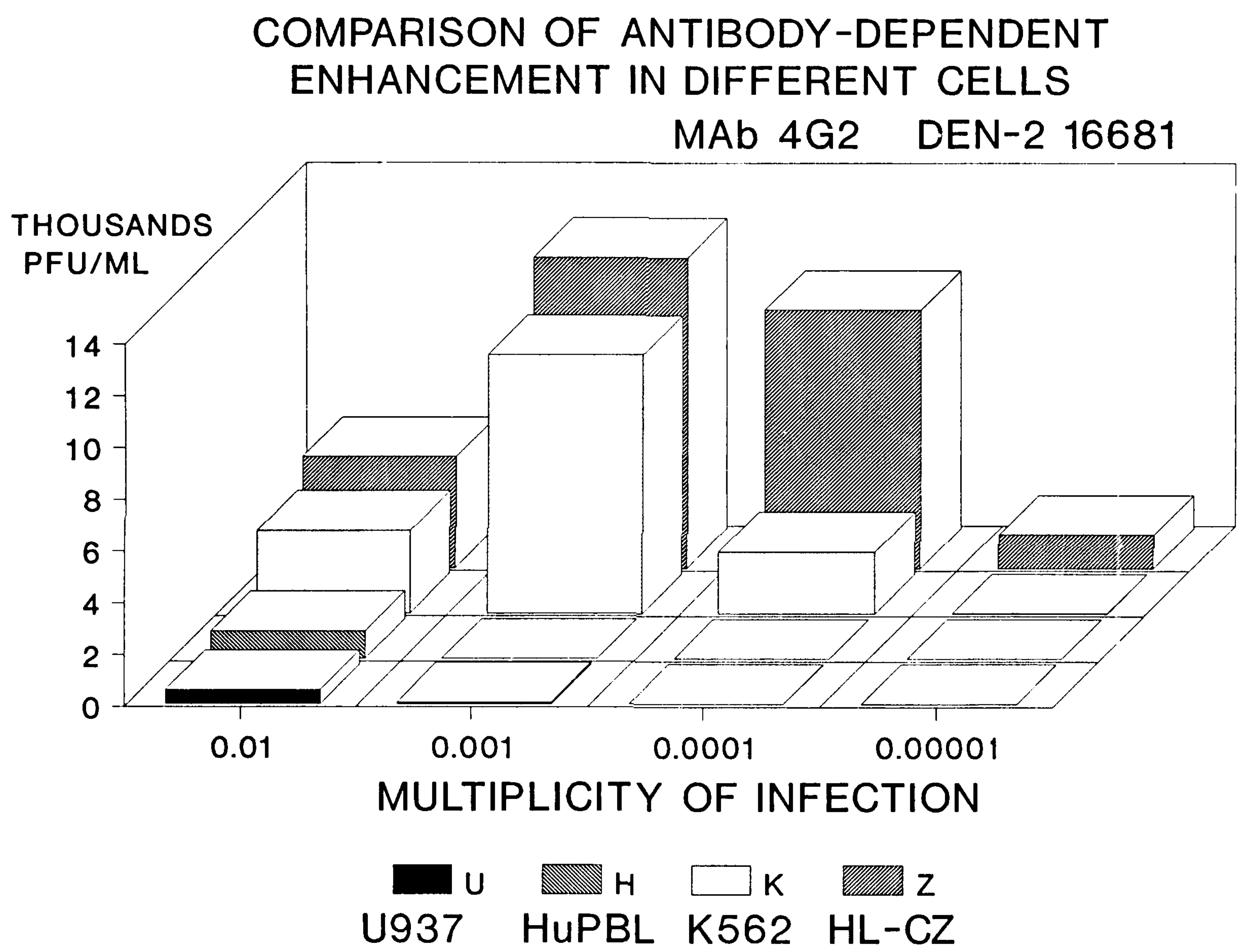
COMPARISON OF ANTIBODY-DEPENDENT
ENHANCEMENT IN DIFFERENT CELLS
MAb 4G2 DEN-2 16681
THOUSANDS
PFU/ML
14
12
10
8
6
4
2
0
0.01
0.001
0.0001
0.00001
MULTIPLICITY OF INFECTION
U
H
K
Z
U937
HuPBL
K562
HL-CZ

FIGURE 1.

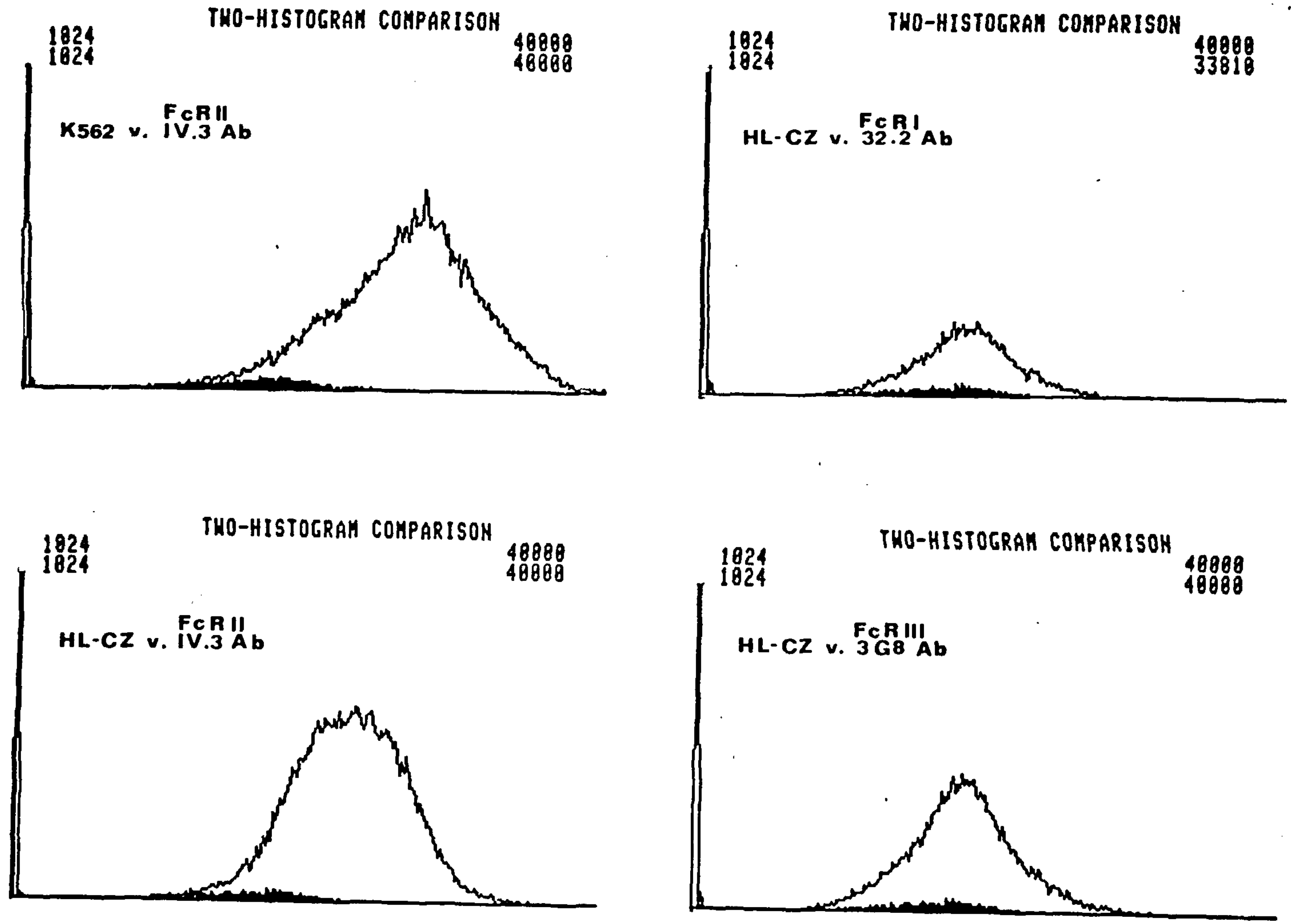

FIGURE 2. ○ CELLS & TEST Ab ● CELLS & CONTROL Ab

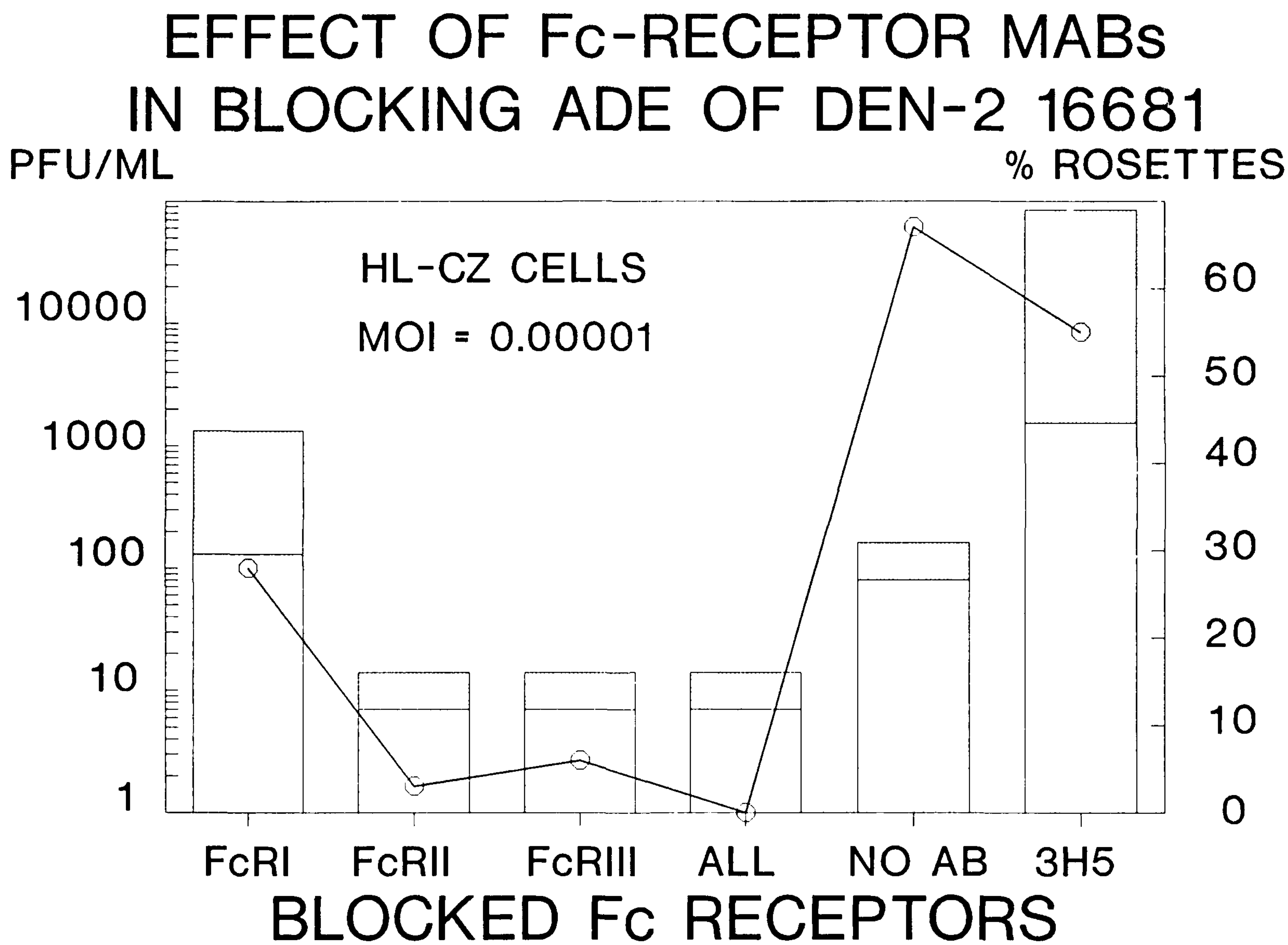

FIGURE 3. DAY 2 Harvest (open area), DAY 4 Harvest (shaded area)

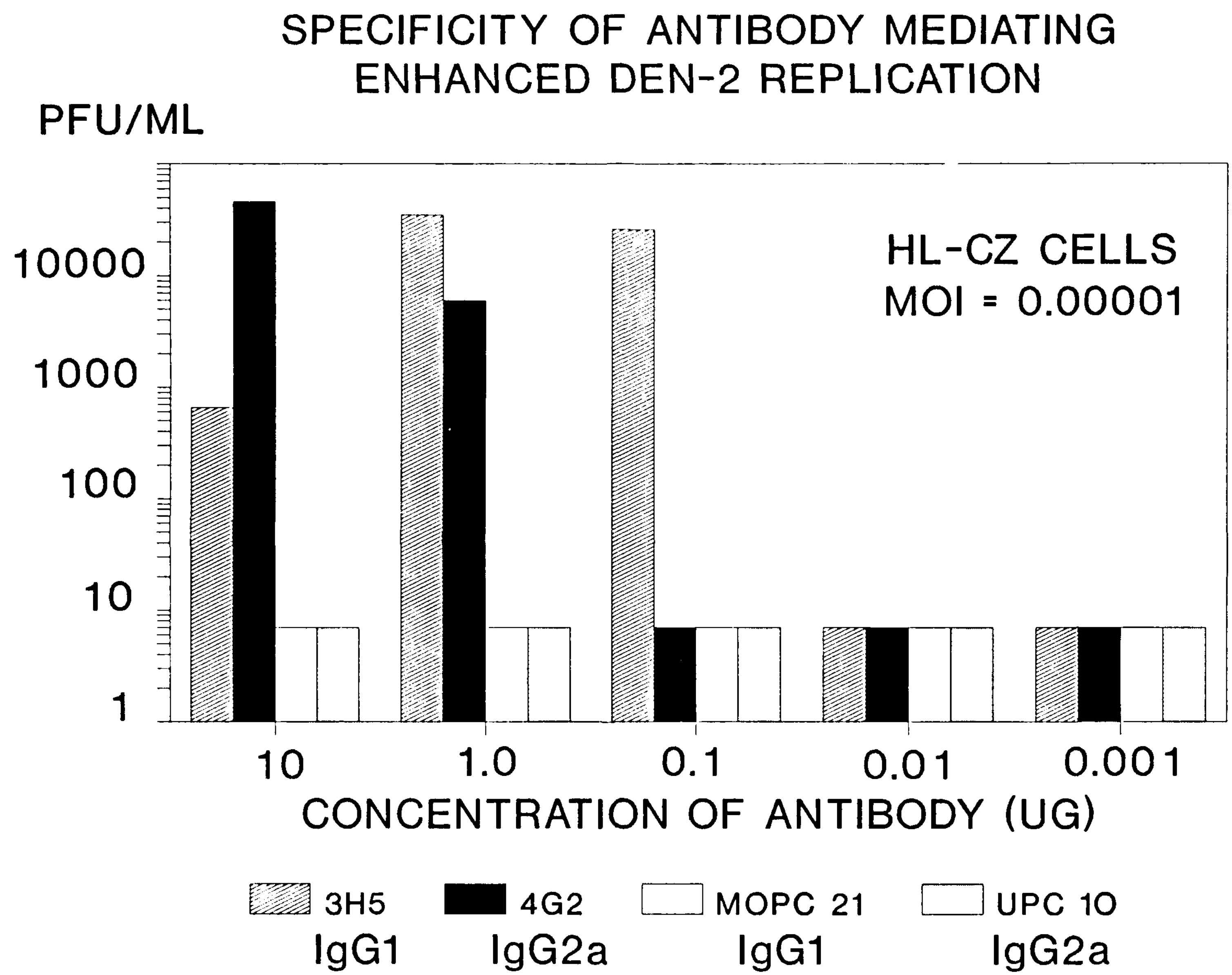
SPECIFICITY OF ANTIBODY MEDIATING
ENHANCED DEN-2 REPLICATION
PFU/ML
10000
1000
100
10
1
HL-CZ CELLS
MOI = 0.00001
10
1.0
0.1
0.01
0.001
CONCENTRATION OF ANTIBODY (UG)
3H5
IgG1
4G2
IgG2a
MOPC 21
IgG1
UPC 10
IgG2a

FIGURE 4.

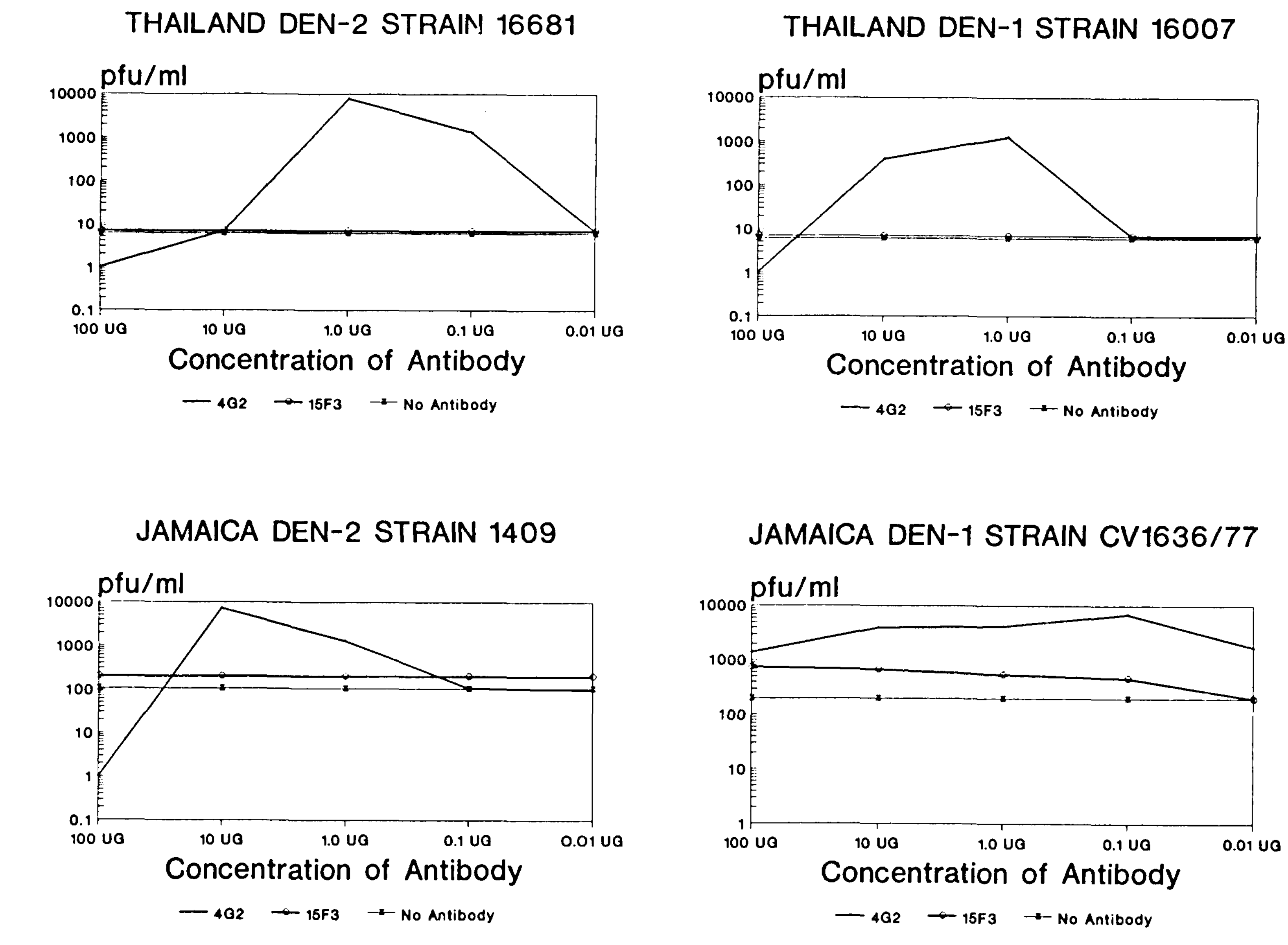
THAILAND DEN-2 STRAIN 16681
pfu/ml
10000
1000
100
10
1
0.1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody
THAILAND DEN-1 STRAIN 16007
pfu/ml
10000
1000
100
10
1
0.1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody
JAMAICA DEN-2 STRAIN 1409
pfu/ml
10000
1000
100
10
1
0.1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody
JAMAICA DEN-1 STRAIN CV1636/77
pfu/ml
10000
1000
100
10
1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody
MOI = 0.0001

FIGURE 5.

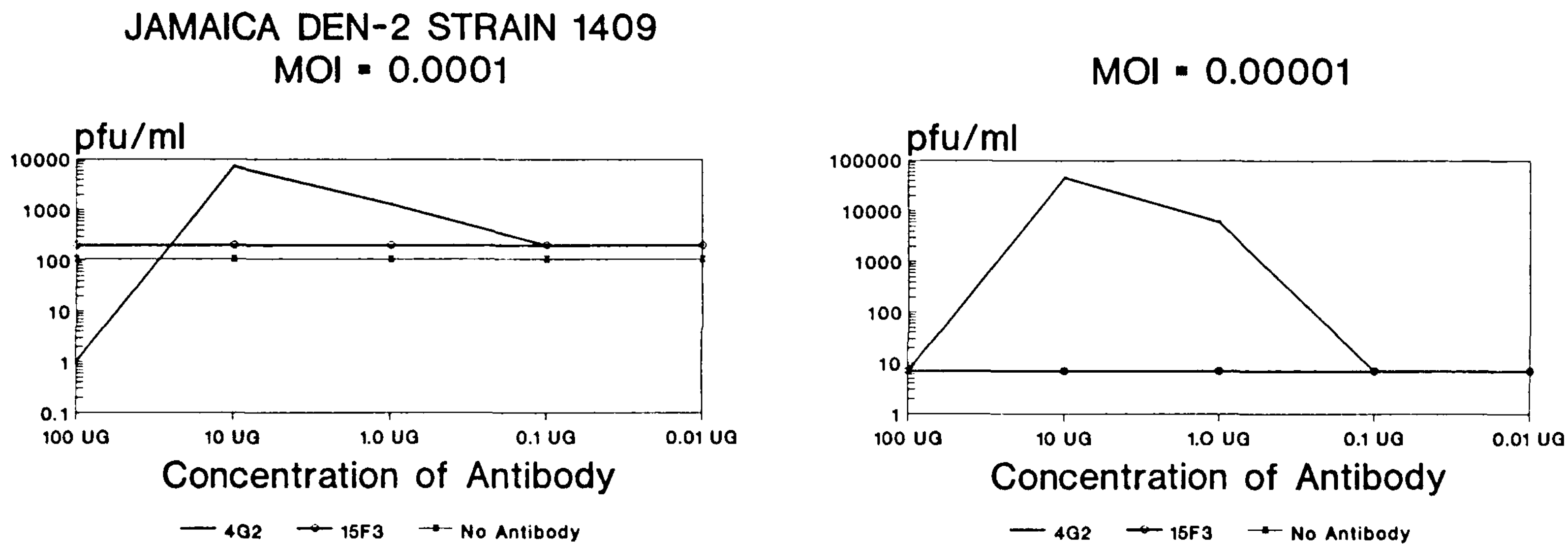
JAMAICA DEN-2 STRAIN 1409
MOI = 0.0001
pfu/ml
10000
1000
100
10
1
0.1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody
MOI = 0.00001
pfu/ml
100000
10000
1000
100
10
1
100 UG
10 UG
1.0 UG
0.1 UG
0.01 UG
Concentration of Antibody
4G2
15F3
No Antibody

FIGURE 6.

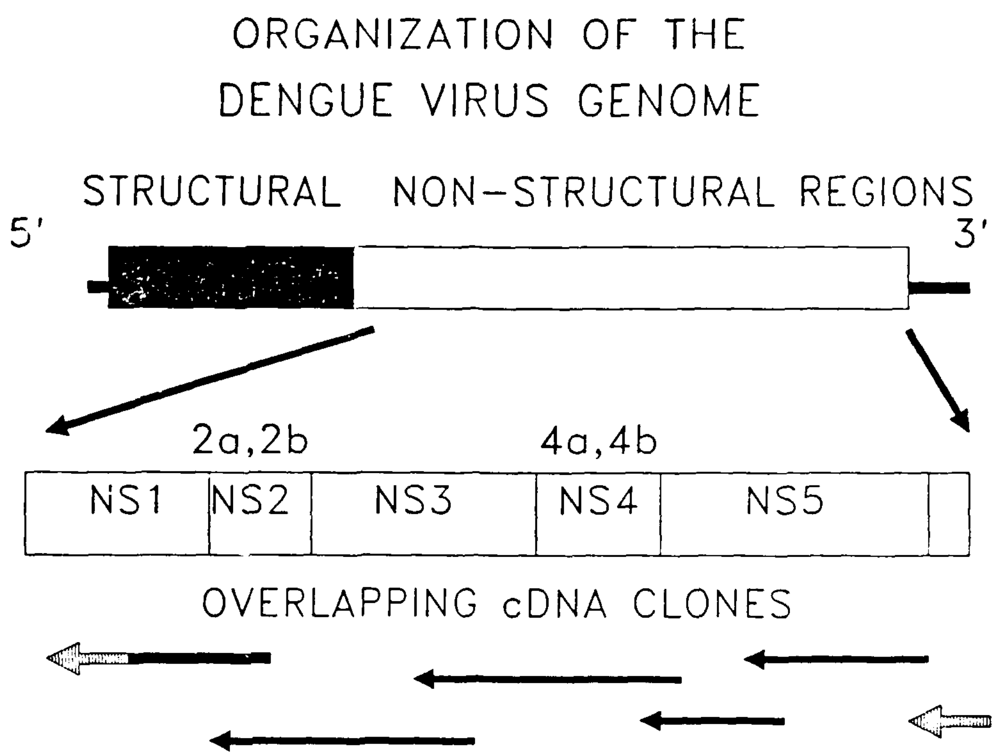
ORGANIZATION OF THE
DENGUE VIRUS GENOME
STRUCTURAL
NON-STRUCTURAL REGIONS
5'
3'
2a,2b
4a,4b
NS1
NS2
NS3
NS4
NS5
OVERLAPPING cDNA CLONES

FIGURE 7.

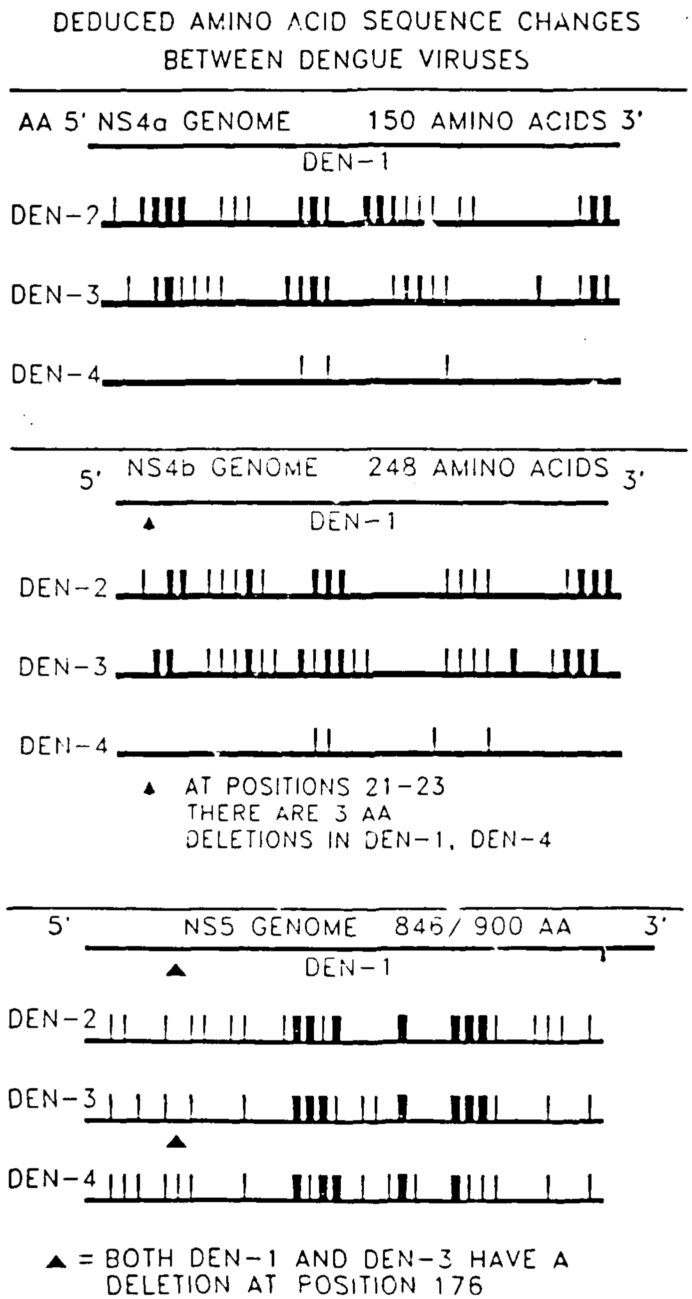
DEDUCED AMINO ACID SEQUENCE CHANGES
BETWEEN DENGUE VIRUSES
AA 5' NS4a GENOME 150 AMINO ACIDS 3'
DEN-1
DEN-2
DEN-3
DEN-4
5' NS4b GENOME 248 AMINO ACIDS 3'
DEN-1
DEN-2
DEN-3
DEN-4
▲ AT POSITIONS 21-23
THERE ARE 3 AA
DELETIONS IN DEN-1, DEN-4
5' NS5 GENOME 846/900 AA 3'
DEN-1
DEN-2
DEN-3
DEN-4
▲ = BOTH DEN-1 AND DEN-3 HAVE A
DELETION AT POSITION 176

FIGURE 8.