Arch Virol (2005) 150: 415–425
DOI 10.1007/s00705-004-0445-1

Archives of Virology
Printed in Austria

Dengue virus type 2 in Cuba, 1997: conservation of E gene sequence in isolates obtained at different times during the epidemic

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Received August 11, 2004; accepted October 11, 2004
Published online December 3, 2004

Summary. It was recently reported that disease severity increased during the 1997 Cuban dengue 2 virus epidemic and it was suggested that this might be explained by the appearance of neutralization resistant escape mutants. We investigated these observations and ideas by sequencing 20 dengue 2 virus isolates obtained during the early (low case fatality rate) and the late (high case fatality rate) phases of the outbreak. Our results showed total conservation of the E gene sequence for these isolates suggesting that the selection of envelope gene escape mutants was not the determinant of increased disease severity. Alignment of these sequences with those available in GenBank, followed by Maximum likelihood phylogenetic analysis generated a tree, which indicated that our isolates are closely related to the virus that circulated in Venezuela in 1997/98 and subsequently in Martinique in 1998. This "American/Asian" genotype has therefore gradually dispersed across the Caribbean region during the past 5 years.

Introduction

In terms of morbidity and mortality dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) are the most important

Nucleotide sequence data reported are available in the GenBank database under the accession numbers: AY702040–AY702060.

arthropod-borne viral diseases in the tropical regions of the world. It has been estimated that millions of cases of DF occur annually. There is an average of 2000–3000 deaths reported to WHO each year [8].

The four serotypes in the dengue virus complex (DENV-1 to 4) produce a spectrum of illness that varies from inapparent or mild disease to a severe and occasionally fatal haemorrhagic clinical picture [29, 48]. Several risk factors have been implicated for the development of the severe form of disease [23]. These include virus variation, the vector, host, epidemiological and ecological conditions [3, 7, 15–17, 21, 32, 37].

During the past three decades there have been four major dengue epidemics in Cuba. The first which was widespread throughout Cuba occurred in 1977 and only dengue fever was observed. This epidemic was caused by an American genotype DENV-1 virus [5]. Subsequently, two independent DHF epidemics caused by DENV-2 of Asiatic origin occurred in 1981 throughout Cuba and 1997 in Santiago de Cuba, four and twenty years respectively, after the epidemic caused by DENV-1 [9, 22, 24, 39]. Cuba therefore represented a unique epidemiological setting to investigate whether or not the presence of heterologous antibody mediated antibody dependent enhancement (ADE) 20 years after primary infection [11, 13]. The fourth epidemic occurred in 2001 and it was caused by DENV-3 [30].

During each of the DENV-2 epidemics, severe disease was observed in individuals with serological evidence of infection by DENV-1 in 1977 and DENV-2 either in 1981 or 1997. In both instances, the ratio of DHF/dengue fever cases more than doubled in two months during a peak epidemic period that lasted only three months [10, 13]. The same phenomenon was observed again when a DENV-3 outbreak occurred in Havana during 2001–2002 (Guzman MG, Kouri G unpublished). In the case of the 1981 and 1997 epidemics, we suggested that DENV-2 infections that occurred sequentially in DENV-1 immune individuals may select for mutant viruses that escape heterotypic neutralizing antibodies [10]. Subsequent infection by antibody escape mutant viruses in DENV-1 immune individuals might be subject to the phenomenon of antibody dependent enhancement (ADE). Since the envelope (E) protein of dengue virus is the target for neutralising antibodies and T-cell immune responses and is also the major determinant of flavivirus tropism we considered it appropriate to study this gene in viruses isolated during the 1997 Santiago de Cuba epidemic. Here we report the E gene sequences and maximum likelihood (ML) phylogenetic analysis of three isolates recovered early in the epidemic, fourteen isolated during the middle period and three isolated at the end of the epidemic. For the ML analysis these data were compared with a global selection of DENV-2 sequences.

Materials and methods

Viruses

Twenty DENV-2 isolates obtained during the Santiago de Cuba epidemic, 1997 were studied. Eighteen viruses were recovered from acute phase sera and two from spleen tissue obtained at autopsy (Table 1). Viruses were recovered in C6/36 continuous *Aedes albopictus* cell cultures

Table 1. Data of the DENV-2 Cuban isolates sequenced in this study

Isolates	Date of fever onset	Passage histories	Clinical classification	Dengue infection
13/97	30/1/97	2P C6/36	DF	primary
58a/97	5/2/97	2P C6/36	DF	primary
70a/97	5/2/97	1P C6/36	DF	secondary
23/97	25/5/97	2P C6/36	DF	secondary
28/97	25/5/97	2P C6/36	DF	–
30/97	25/5/97	1P C6/36	DHF	secondary
32/97	25/5/97	2P C6/36	DF	–
46/97	3/6/97	1P C6/36	DF	primary
89/97	**1/6/97**	**2P C6/36**	**DHF/DSS**	**secondary**
115/97	10/6/97	2P C6/36	DF	–
118/97	10/6/97	1P C6/36	DF*	secondary
120/97	10/6/97	1P C6/36	DF*	secondary
137/97	12/6/97	1P C6/36	DHF	secondary
133/97	12/6/97	1P C6/36	DF*	secondary
163/97	12/6/97	1P C6/36	DF*	secondary
165/97	12/6/97	1P C6/36	DHF	secondary
166/97	12/6/97	1P C6/36	DHF	secondary
187/97	27/6/97	1P C6/36	DF	–
188/97	27/6/97	1P C6/36	DF	–
205/97	**1/7/97**	**1P C6/36**	**DHF/DSS**	**secondary**

*Dengue fever with haemorrhagic manifestations. Fatal cases are in bold

by centrifuging the inocula onto cell monolayers as described previously [34]. The presence and identification of virus in infected cells was determined by fluorescence microscopy using type-specific monoclonal antibodies [18]. Passage histories of the isolates and clinical classification are given in Table 1. The case definitions for DF and DHF follow the PAHO classification [29].

RNA extraction, RT-PCR and sequencing

Viral RNA was extracted from 200 µl of supernatant medium of virus-infected cells using the RNAgents Total RNA Isolation system (Promega Corporation, Madison, WI, USA). First strand cDNA synthesis was carried out in a volume of 30 µl. Eleven microliters of RNA and 5 µl of primer Den-2-3utr-1r 10 pmol/µl were mixed and heated at 95 °C for 2 min. The mixture was then chilled and 3 µl of dNTP (10 mM), 3 µl of DTT (0.1 mM), 1 µl of RNAsin (40 units), 6 µl of 5× buffer and 1 µl of reverse transcriptase Superscript II were added (Invitrogen Corporation, Carlsbad, CA, USA). The mixture was incubated at 43 °C for 3 hours and 65 °C for 10 min. Nucleotides from position 701 in PrM to 2545 in the non-structural NS1 encoding gene were amplified using PCR. A sample of 3 µl of the cDNA from the RT reaction was used for PCR amplification by 30 cycles of denaturation at 94 °C (40 s), annealing at 55 °C (1 min) and extension at 72 °C (1 min). A final extension step was carried out at 72 °C for 10 min. Taq DNA polymerase (Sigma) was used. The primers used for amplification and/or sequencing were designed on the basis of published DENV-2 sequences and are listed in Table 2.

Table 2. Oligonucleotide primers used for amplification and sequence

Reaction	Primer name*	Sequence (5′→3′)
RT	Den2-3′utr-1r	AGAACCTGTTGATTC
PCR	Den2-PrM-1f	GAGAAAAAAGATCAGTGGCACTCG
PCR	Den2-ns1-1r	GTTTTGAAGGGGATTCTGGTTGGAACTT
Sequencing	Den2s-1f	AGTTGTTAGTCTACGTGGAC
Sequencing	Den2s-351f	GAAAGAGATTGGAAGGATGC
Sequencing	Den2s-701f	GAGAAAAAAGATCAGTGGCA
Sequencing	Den2s-1051f	CCAACATTGGATTTTGAACTG
Sequencing	Den2s-1401f	AGGAAAACATGGCAAGGAAA
Sequencing	Den2s-1751f	TGTCATCAGGAAACTTACTG
Sequencing	Den2s-2101f	CTCAACTGGTTTAAGAAAGG
Sequencing	Den2s-2451f	TAAAGAACTGAAATGTGGCAG
Sequencing	Den2s-2674r	TTACCTCATTTTCTGATAGAAT
Sequencing	Den2s-2324r	ATGATGACTCCTATGAGGAT
Sequencing	Den2s-1974r	GTGTCTTTTTTCCAAATCCAT
Sequencing	Den2s-1624r	TTGATCCTTGTGTGTCCGC
Sequencing	Den2s-1274r	ATGCCTCCCTTTCCAAATAA
Sequencing	Den2s-924r	AGCGACAGCTGTCAGTAAG
Sequencing	Den2s-574r	CACACAATTCACCAAGATCT
Sequencing	Den2s-224r	GGTCCTCGTCCTTGCAG

*Primers names with an f indicate a viral-sense orientation; names with r indicate a complementary and reverse orientation. Nucleotide numbering is based on the sequence of Jamaica 1409/83

PCR products were directly sequenced after purification using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA, USA). Direct sequencing of the E gene was carried out using the Applied Biosystems ABI Prism automated DNA sequencing kit and ABI377 automated sequencer according to the manufacture's protocol. Briefly, for each sequencing reaction, 2 μl of the purified PCR products were mixed with 1 pmol of primer, 5 μl of water and 2 μl of the reaction mixture containing the four dye-labelled dideoxynucleotide terminators. Cycle sequencing was performed as follows: 25 cycles at 96 °C (30 s), 50 °C (60 s) and 60 °C (4 min). The sequencing reaction was purified using Centri-Sep Spin columns (Applied Biosystems, Foster City, USA). DNA was dried using vacuum centrifugation before sequencing. All sequences determined in this study have been deposited in GenBank (accession numbers AY702040–AY702060).

Phylogenetic analysis

The E gene sequences obtained from 20 Cuban DENV-2 isolates were compared with a global selection of available published E gene DENV-2 sequences deposited in Genbank (Table 3). The total data set in our phylogenetic analysis comprised 56 sequences each of 1620 bp. The DENV-2 sequences used to root the phylogenetic tree were: P8-1407 from a sentinel monkey of Malaysian origin, DAKARa578 isolated from *Aegypti tailory sensu lato* on the Ivory Coast, the sylvatic PM33 974 strain from Guinea and the sylvatic DAKHD10674 strain from Senegal. Phylogenetic trees were estimated using an ML method. The starting tree in this analysis used neighbor-joining analyses and this was followed by successive rounds of TBR branch-swapping, identifying the ML substitution parameters at each stage,

Table 3. DENV-2 E gene sequences used in this study

Strain	GenBank accession number	Strain	GenBank accession number
Tonga/74	X54319	CTD113/97	AF410358
Mexico131/92	AF100469	Philippines/83	L10045
IQT1797/95	AF100467	Taiwan/87	L10052
Puerto Rico/69	L10046	SLMC-451	AF297009
1421Mexico/83	D45394	DOH-005	AF295697
India/57	L10043	CI-15	AF295696
Trinidad/53	L10053	DOH-077	AF295699
Jamaica1409/83	M20558	Burkina Faso/83	L10042
Brazil/90	L10041	Somalia/84	L10051
BR64022	AF489932	Torres/96	AF004019
PTCOl96/96	AF163096	Indonesi/76	L10044
Peru-6658/01	AY079423	CookIs/97	AF004020
Martinique/98	AF208496	CAMR4/91	AF410379
Ven1693/97	AF363077	CAMR14/98	AF410377
Ven1657/97	AF363072	FJ-10	AF276619
CTD226/98	AF410367	CAMR8/91	AF410373
China04/85	AF119661	CAMR11/93	AF410375
D91-533/91	AF195040	SL714/89	L10055
MalayM1/87	X15434	SL767	M24449
MalayM2/86	X15433	SL206/91	L10049
P7-863/69	AF231716	Malay P8-377/69	AF231715
ThNh-p11/93	U31952	Mal70-P8-1407/70	AF231717
CO167//96	AF100464	IC80-DAKAr578/80	AF231718
D83-307/83	AF195035	Guinea81-PM33974/81	AF231719
PUO-312/80	AF264053	Sen70-DAKHD10674/70	AF231720
16681/64	U87411	New Guinea C/44	AF038403
TH-36/58	D10514	SL77	M24450

until the tree of highest likelihood was found. All these analyses were undertaken using PAUP* package [41].

Results

Nucleotide sequences of the E gene of 20 DENV-2 isolates obtained from DF and DHF patients from the early (low case fatality rate), the intermediate and the late (high case fatality rate) period of the outbreak that occurred in Santiago de Cuba, 1997, were determined. Comparison of the deduced amino acid (aa) sequences revealed 100% identity in the E gene for all the isolates.

Nucleotide sequence alignment of the isolates combined with a global selection of available published E gene sequences of DENV-2 isolates deposited in Genbank, followed by ML Phylogenetic analysis generated a tree that is presented as Fig. 1. The tree illustrates that the Cuban isolates group with the "American/Asian" genotype as defined by Twiddy et al., 2002 [44]. On the basis of

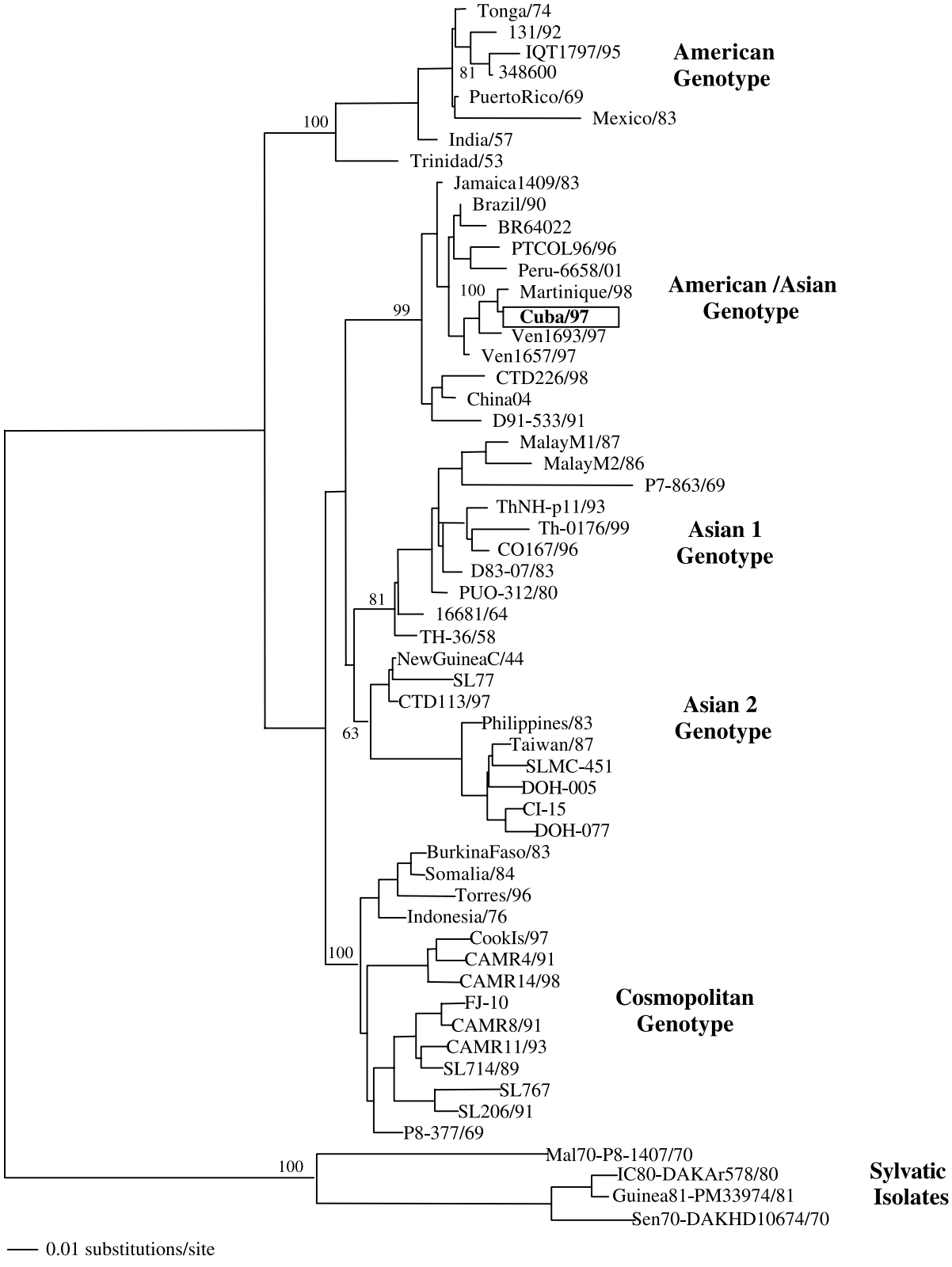

Fig. 1. Phylogenetic tree generated by ML analysis of envelope (E) gene sequences from 56 strains of DENV-2. The tree is rooted by the sylvatic DENV-2 strains. All horizontal branch lengths are drawn to scale. Bootstrap values are shown for key nodes

these results the Cuban isolates are closely related to the DENV-2 that circulated in Venezuela in 1997/98 and subsequently circulated in Martinique in 1998. The Venezuelan strain 1693/97 showed 98.6% similarity to the Cuban strain in nucleotide sequences and 99.79% identity in the amino acid sequence analysis. The Martinique strain showed 99.46% similarity to the Cuban/97 strain in nucleotide sequence and 100% amino acid identity. The tree shows that the closely related Cuban/Venezuelan/Martinique strains also cluster with other American strains from Brazil and Peru. Nevertheless, all of these viruses appear to have their genetic origin in Asia.

In order to study the impact of nucleotide changes on the E protein, the deduced amino acid sequences of all strains used for the Phylogenetic analysis representing each DENV-2 genotype, were aligned and compared with the Cuban isolates. Within the American/Asian genotype the strain Jamaica 1409, isolated in 1983 and the Cuban strain isolated in 1997 showed 98.25% similarity in nucleotide sequence and 99.39% amino acid identity. Only two non-conservative amino acid substitutions were observed, Leucine (L)/Glutamine (Q) at position E131 and Methionine(M)/Threonine(T) at position E340. It is noticeable that the substitution at E340 located in the B domain region of this protein is unique to the Venezuelan, Cuban and Martinique isolates. Additionally, it is manifest that the Cuban isolates conserved an Asparagine (N) at position E390 like many viruses of Asiatic origin.

Discussion

Secondary infection by DENV has been shown to be an important risk factor in dengue disease severity. In the context of the current study it is important to remember that the second Cuban DENV-2 epidemic occurred 20 years after a DENV-1 epidemic. Therefore the primary DENV-1 exposure was believed to be responsible for the severe disease in adults receiving a secondary DENV-2 infection [11, 13]. We have tried to explain the increasing severity observed in the DENV-2 Cuban epidemics by postulating that more virulent strains appeared as the result of escape from heterotypic neutralising antibodies Guzman et al., 2000 [10]. However, our 20 DENV-2 isolates, obtained during one epidemic and in the same geographic area, showed complete conservation of the E protein. This suggests that if mutants did arise during the epidemic, a study of the full genome would be needed to identify whether or not other regions of the genome have mutated. Whilst the E protein is the target for neutralising antibodies and T-cell immune responses and it is known as the major determinant of flavivirus tropism, our results suggest that the E gene is not directly related with the phenomenon of intra-epidemic disease severity increase at least in this particular situation.

On the other hand, our phylogenetic analysis confirms that the DENV-2 epidemic that occurred in Santiago de Cuba, 1997 was caused by a strain of Asiatic origin that has been circulating in the Latin American region, associated with high virulence potential. As the tree suggests, the virus causing the epidemic was

probably introduced from nearby Venezuela. However, taking into account the frequent travel and trade between Cuba and the neighbouring countries, more sequence data from regional strains is required to establish the most likely source.

The amino acid alignment obtained using available strains representing each DENV-2 genotype, showed a substitution M/T at position E340 that is specific for Cuba-Venezuela-Martinique. This non-conserved substitution is located in an important antigenic region that contains multiple T- and B-cell epitopes. Peptides from the region encompassing amino acid 333–368 elicit virus-binding antibody and stimulate T-cell proliferation in mice [35, 36]. In the same region, similar amino acid changes have been observed by Lewis et al., 1993 who compared the amino acid sequences of strains PR159 and the S1 vaccine derivative. They suggested that these changes might be important in attenuation of the S1 vaccine strain and may have altered its immunogenicity [28].

Only one more non-conservative change (L/Q) was observed when Jamaica/83 and Cuba/97 isolates were compared. Therefore after fourteen years of circulation in Latin America only two non-conservative substitutions in the E gene were observed in the Cuban strain, indicating that there has been very little *in situ* evolution of this strain since it was introduced from Asia. Consequently, the Cuban isolates have maintained the presence of N at position 390, predicted to be a determinant associated with the ability to cause severe disease [6, 27, 31, 38].

Despite the fact that the Santiago de Cuba outbreak of DENV-2 was very severe, seroepidemiological studies have shown that the virus was also being transmitted silently amongst the population [11]. Such silent transmission was previously reported in Taiwan [4] and more recently in Brazil [42]. This confirms that even in the presence of a "virulent" strain the immune status of the population and even more the host genetic characteristics will play an important role in determining disease severity as has been extensively reported [2, 12, 14, 23, 25, 26, 33, 40, 47]. Future epidemiological studies should pay attention to positive dengue IgM results in asymptomatic persons in order to determine some of the factors associated with non-severe disease.

In addition to mutation, there is evidence that recombination is also responsible for the generation of genetic diversity amongst the dengue viruses [1, 19–21, 43, 45, 46, 49]. Cuba is unique because after the DENV-2 epidemic of 1981 no cases of DF/DHF/DSS were identified until 1997. This can largely be explained by the extraordinary measures that were taken to reduce the *Aedes* spp. population in Cuba. Therefore, when a new epidemic arose in 1997, it is reasonable to suppose that a single strain was circulating. Since, secondary infection was demonstrated in most of the severe cases during the 1997 epidemic, mutants could have been selected due to the pressure of heterotypic antibodies. However, recombinants based on different strains of virus are unlikely to have arisen during this period in Cuba as there was not the opportunity for mixed infections. Taking all of these factors into account we believe it will be necessary to determine the complete genome sequences of the 20 isolates described in this paper in order to identify any changes in the virus that may determine the difference in virulence for humans.

Acknowledgements

We thank Dr. Edward C. Holmes at the Department of Zoology, University of Oxford, UK for his invaluable contribution to the Phylogenetic Analysis.

This research was supported by The Wellcome Trust grant No. 062752/Z/00/Z.

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