



## Phylogenetic Relationships of West Nile Viruses Isolated from Birds and Horses in Israel from 1997 to 2001

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**Abstract.** In November 1997, an outbreak of a neuroparalytic disease caused by West Nile (WN) virus was diagnosed in young goose flocks. Domestic geese were similarly affected in the late summer and fall of 1998, 1999, 2000 and 2001. WN viruses were also isolated from migratory and wild birds and horses in 1998–2001. A 1278 bp sequence of the envelope gene of 24 Israeli WN virus isolates was compared with those of seven isolates from Africa, Europe and New York. As a result, the Israeli isolates could then be grouped into two clusters. The 15 avian and three equine from 1997–2001 in the first cluster of viruses were shown to be identical to WN-NY99, while the second cluster comprised one goose isolate from 1998 and two goose and two pigeon isolates from 2000. These closely resembled the most recent Old World isolates, and indicate that at least two WN genotypes were co-circulating in the region during this time.

**Key words:** domestic and wild birds, envelope gene, horses, phylogeny, West Nile virus

West Nile virus (WNV), an insect-borne member of the Japanese encephalitis serocomplex of the Flavivirus genus, was recognized as a cause of human disease more than 50 years ago [1] and has been isolated infrequently in Israel from wild birds and *Culex* mosquitoes since then [2,3]. WN fever is considered to be endemic in many African and Middle Eastern countries, nevertheless severe epidemics have appeared in recent years in Romania, Russia, and Israel. WN viral disease affecting domestic fowl was never documented in Israel during this period, although sporadic arboviral infections of domestic birds, as exemplified by meningo-encephalitis of turkeys, a closely related flaviviral

disease, have occurred. WN virus was isolated for the first time in November 1997 from four flocks of young domestic geese presenting acute neurological signs of paresis and affected with high morbidity and mortality [4]. The disease then reappeared on goose farms in successive years with 12 flocks affected between August and November in 1998 and 17 young flocks in 1999. In 2000, six flocks of geese were diagnosed with WN virus, the first three in August and the others in September. In 2001, the virus was isolated from sentinel goose at two monitoring sites, once in August and for the second time in September. The affected farms were located throughout the Coastal Plain and the northern and central valleys of Israel. In addition, WN virus was isolated from dead wild birds each year; notably from several white storks and a lappet-faced vulture found in the Arava Rift Valley in southern Israel in 1998. In 1999, WN

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virus was isolated from a paralyzed white-eyed gull from a breeding colony in a university zoo [5]. In 2000, WNV was isolated from 2 sick feral pigeons and a dead crimson rosella found in the vicinity of human habitation and from four horses presenting fever and advanced neurological signs [6].

In this article, we present the partial envelope gene sequences of WN viruses isolated from birds and horses in Israel between 1997 and 2001 and compare them phylogenetically with Old and New World isolates from 1996 to 1999 and Egypt 101, first isolated in 1951.

## Materials and Methods

### *Birds*

Birds were necropsied at the Kimron Veterinary Institute and brains were removed aseptically. Brain extracts were prepared by grinding them in PBS, centrifuged at 3,500 rpm for 5 min and the supernatants filtered (0.22 µm). Samples were kept frozen at  $-70^{\circ}\text{C}$ .

### *Horses*

Heparinized blood samples were taken during the febrile phase of the illness and two weeks later. Buffy coat and plasma were separated by centrifugation and kept frozen at  $-70^{\circ}\text{C}$ .

### *Virus Isolation*

WN virus was isolated by one of three methods. One, 7-day-old chick embryos were inoculated with brain homogenates by the yolk sac route on day 7 of incubation. Two, VERO cell monolayers were infected with brain homogenates and maintained until a cytopathic effect (CPE) was observed. Equine buffy coat cells were inoculated into suckling mice by the intracerebral route (the third method of isolation). WNV was isolated from storks by inoculating suckling mice and VERO cells with mouse brain suspensions. An isolate was considered positive if embryo mortality occurred within 3–4 days post-inoculation, CPE appeared in VERO cells within 3–5 days, and paralysis and in-coordination were seen in the mice 4–7 days post-inoculation. Identification of the virus was determined by immunofluorescence with monoclonal antibodies and was confirmed by RT-PCR.

### *Immunofluorescence*

Following the appearance of CPE, a glass cover slip was placed in the dish receiving the succeeding passage. Three days after inoculation of the monolayer the cover slip was removed and the cells fixed with cold acetone. Monoclonal antibodies to flaviviruses were reacted with the monolayer and then incubated with FITC-labeled anti-mouse immunoglobulin. The flavivirus mAbs included 2B4, 6B8 and 6E12 from N.K. Blackburn (National Institute for Virology, South Africa), 5F10, 1C9 and 1B4 from B.E. Lachmi (Israel Institute for Biological Research), F7/101 from the Centre for Environmental Health, Oxford, UK, 3H6 and 813 from TropBio Pty Ltd, Townsville, Australia. Counter-staining was performed with Evans blue. Slides were observed in a UV microscope.

### *RNA Extraction and RT-PCR*

Identity of the WN isolates was also confirmed by RT-PCR [7]. Four types of tissue were used as a source of RNA: culture medium of VERO cells, homogenates of dead embryos and mouse and avian brains (Table 1). The RNA was extracted with the QIAamp Viral RNA kit (Qiagen) according to the manufacturer's protocol and re-suspended in 60 µl of RNase-free water. The primer pair wn132/240 (5' ends at genome positions 1402 and 1645, respectively) was used to synthesize a 255 bp product in the E gene region. The resulting DNA fragment was visualized on 1.5% agarose gel stained with ethidium bromide.

### *Sequence and Phylogenetic Analysis*

A total of 23 Israeli WN virus isolates, comprising 15 goose (one from 1997, seven from 1998, one from 1999, four from 2000 and two from 2001), one white stork (1998), one white-eyed gull (1999), two feral pigeons (2000), one crimson rosella (2000) and three horses (2000), were selected for analysis. The isolation histories and GenBank accession numbers of each isolate are given in Table 1. Two additional RT-PCRs using the primer pairs, WN240-Kun848 (5' ends at genome positions 848 and 1645, respectively) and Kun1653-Kun2234c (5' ends at genome positions 1653 and 2234, respectively) were

Table 1. Provenances of the sequenced WNV isolates compared by phylogenetic analysis

Isolate				Sequenced DNA		
Name	Strain	Source	Year	Origin	No. Pass.	GenBank No.
Goose97	ISR97-Goo1	<i>Anser anser</i>	1997	VERO cells	2	AF380663
GooseTA98	ISR98-GooTA	<i>Anser anser</i>	1998	Goose brain	0	AY052408
GooseKha98	ISR98-GooKha	<i>Anser anser</i>	1998	Mouse brain	1	AY052409
GooseAzr98	ISR98-GooAzr	<i>Anser anser</i>	1998	Mouse brain	1	AY052410
GooseMth98	ISR98-GooMth	<i>Anser anser</i>	1998	Mouse brain	1	AY052411
GooseKY98	ISR98-GooKY	<i>Anser anser</i>	1998	Goose brain	0	AY052412
GooseAza98	ISR98-GooAza	<i>Anser anser</i>	1998	Mouse brain	1	AY052413
Goose98	ISR98-Gool	<i>Anser anser</i>	1998	VERO cells	2	AY033388
Stork98	ISR98-ST1	<i>Ciconia ciconia</i>	1998	VERO cells	3	AY033389
Goose99	ISR99-Goo	<i>Anser anser</i>	1999	VERO cells	2	AY033391
Gull99	ISR99-Gull	<i>Larus leucophthalmus</i>	1999	VERO cells	2	AY033390
GooseMe100	ISR00-GooM	<i>Anser anser</i>	2000	Goose brain	0	AF380664
GooseMaV00	ISR00-GooMaV	<i>Anser anser</i>	2000	Chicken embryo	1	AF380666
GooseMaS00	ISR00-GooMaS	<i>Anser anser</i>	2000	Goose brain	0	AF380667
GooseNah00	ISR00-GooN	<i>Anser anser</i>	2000	Chicken embryo	1	AF380665
Rosella00	ISR00-Ros	<i>Platycercus elegans</i>	2000	VERO cells	1	AF380668
PigeonC00	ISR00-PigC	<i>Streptopelia turtur</i>	2000	VERO cells	1	AF380671
PigeonT00	ISR00-PigT	<i>Streptopelia turtur</i>	2000	VERO cells	3	AF380670
Horse70	ISR00-Eq1	<i>Equus equus</i>	2000	Mouse brain	1	AF380669
Horse81	ISR00-Eq2	<i>Equus equus</i>	2000	Mouse brain	1	AY052407
Horse82	ISR00-Eq3	<i>Equus equus</i>	2000	Mouse brain	1	AY052406
GooseMhS01	ISR01-GooMhS	<i>Anser anser</i>	2001	Mouse brain	1	AY184820
GooseMiz01	ISR01-GooMiz	<i>Anser anser</i>	2001	Goose brain	0	AY184821
NY99eq	NY99-eqhs	<i>Equus equus</i>	1999			AF260967
Volgograd99	VLG-4	<i>Human brain</i>	1999			AF317203
Romania96m	R097-50	<i>Culex pipiens</i>	1996			AF130362
Romania96h	96-1030	<i>Human CSF</i>	1996			AF130363
Kenya98	KN3829	<i>Culex univittatus</i>	1998			AF146082
Eg101	Eg101	<i>Human</i>	1951			AF260968
Italy98		<i>Equus equus</i>	1998			AF404757
Isr00h	WNV-hISR2000	<i>Human brain</i>	2000			AF394217

performed on the E gene yielding 800 and 580 bp products respectively [8]. The PCR products were extracted from the agarose gel with the QIAquick gel extraction kit (QIAGEN) according to manufacturer's protocol. Sequencing was performed with the BigDye terminator cycle sequencing kit (ABI) yielding a 1,278 bp product for further analysis.

Sequences of the 23 Israeli isolates were aligned using ClustalX 1.5 b alignment software [9] with eight other isolates: New York 1999 equine, two Romanian 1996 (one human and one mosquito), one Kenya 1998 mosquito, one Volgograd 1999 human, one Italy 1998 equine, one Egypt 1951 human and one Israel 2000 human isolate (cf. Table 1). Phylograms for the 1,278 bp sequence and for the 426 amino acids were constructed with the MEGA package, version 2.0 [10]. With MEGA, we determined the genetic

distance by the proportional distance method, Kimura's two parameters, Jukes-Cantor and the Tajima-Nei method (with equal weighting for both transition and transversion for all three codon positions). For tree building, various genetic distance matrices were estimated by the neighbor-joining method with calculated bootstrap confidence intervals of 1,000 heuristic search replicates.

## Results

### Nucleotide Sequences

The neighbor-joining tree with bootstrap analysis of the nucleotide sequences of the 31 WN viruses is shown in Fig. 1 and is derived in part from that

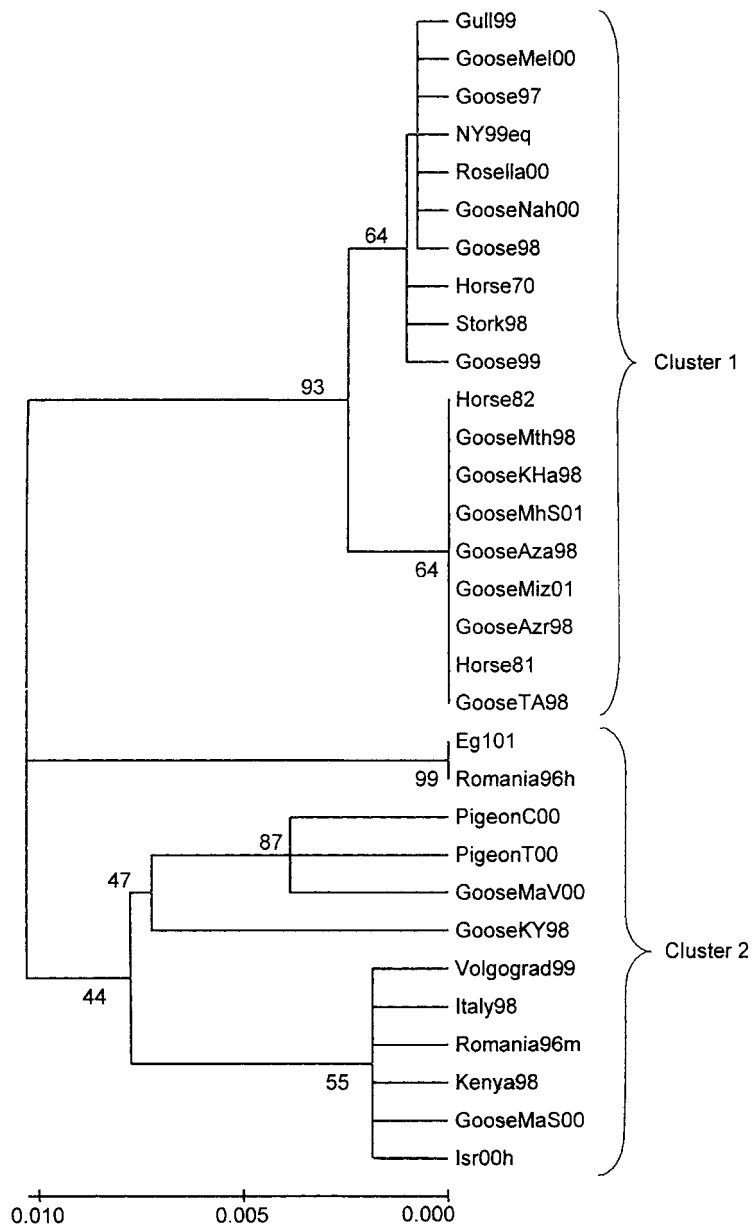


Fig. 1. Phylogenetic tree of the Israeli, New York, Volgograd, Kenya, Romania, Italy and Egypt West Nile virus isolates based on neighbor-joining analysis of 1,278 bp of the E gene by Kimura-2 parameters method. Numbers for each node indicate the percentage of the 1000 bootstrap replicates.

published [11]. Genetic distances estimated by all methods yielded very similar placements of the 31 strains in each tree. An expanded segment emphasizing the relationships among the recently isolated Israeli WN viruses is shown. These fell into two clusters; the first included 12 out of 15 goose isolates, the stork and gull from 1998 and 1999, respectively,

and the three equine and the rosella isolates from 2000. All 18 sequences were very closely related to the NY-99 equine isolate. The second cluster included the other 3 geese (MaV00, MaS00 and KY98), two pigeons (C00 and T00) and the human isolate from 2000 and resembled more closely the most recent Old World isolates: Romania96m,

Kenya98, Italy98 and Volgograd99 sequences. A total of 140 changes (10.9%) were observed, of these 89 (6.9%) are common to at least two isolates. The percentage nucleotide difference between the two clusters was of the order of 8%.

*Amino Acids*

The deduced amino acid sequence yielded an alignment composed of 426 sites (shown in Fig. 2). A total of 19 location changes (4.5%) was observed, of these 14 substitutions occurred in individual isolates and one was shared by two isolates (Eg101 and

Romania96h). Of the remaining four informative changes (details showed in Fig. 3), two were shared by all the isolates of the second cluster: AA.126 (isoleucine replaced by threonine) and AA.159 (valine replaced by isoleucine except Volgograd99 in which valine was replaced by methionine). At position 90 (phenylalanine replaced by tyrosine) was shared only by three Israeli 2000 isolates: PigeonT00, PigeonC00 and GooseMaV00, while changes at position 93 (arginine replaced by lysine) were shared by the same three Israeli isolates and Eg101 and Romania96h. The construction of the phylogenetic tree of the 31 isolates showed a first amino acid

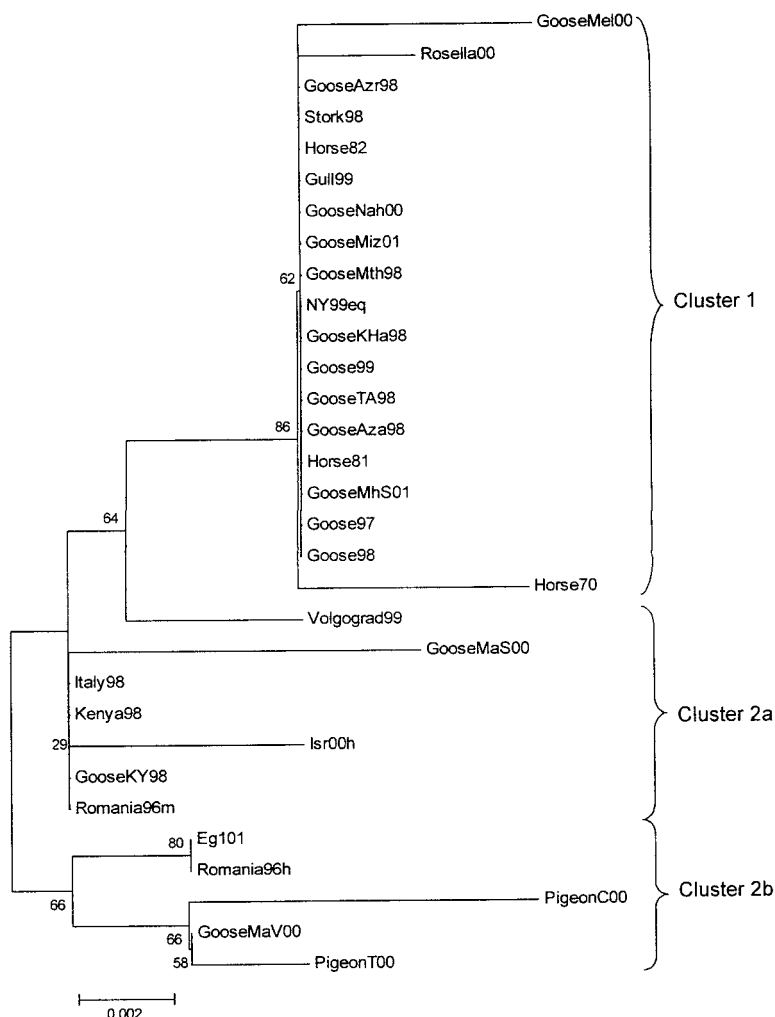


Fig. 2. Phylogenetic tree of the Israeli, New York, Volgograd, Kenya, Romania, Italy and Egypt West Nile virus isolates based on neighbor-joining analysis of 426 amino acids deduced from the partial sequence of the E gene by pairwise distance method. Numbers at each node indicate the percentage of the 1,000 bootstrap replicates.

	81							160								
NY99eq	HNDKRADPAF	VCRQG	VVDRG	WGNCG	GLFGK	GSIDT	CAKFA	CSTKA	IGRTI	LKENIK	YEVA	IFVHG	PPTVE	SHGNY	STQVG	
Goose97	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Goose98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Goose99	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Gull199	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseNah00	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseAza98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseTA98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Horse81	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Horse82	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseMhS01	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Stork98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseAzr98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseMth98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseKHa98	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseMiz01	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Rosella00	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
GooseMel00	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Horse70	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	
Volgograd99	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	K.....M.	
GooseMaS00	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
Isr00h	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	E.....	.....	.....	.....	.....	I.
Kenya98	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
GooseKY98	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
Romania96m	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
Italy98	.....	.....	.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
Eg101	.....	.....	K.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	P.....I.
Romania96h	.....	.....	K.....	.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	P.....I.
PigeonC00	.....	.....	Y.....	K.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
GooseMaV00	.....	.....	Y.....	K.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.
PigeonT00	.....	.....	Y.....	K.....	.....	.....	.....	.....	T.....	.....	.....	.....	.....	.....	.....	I.

Fig. 3. Partial alignment of amino acids from AA 81 to AA 160 of the E gene of the West Nile virus isolates. Identical = .

cluster identical with that of the nucleotides and two additional clusters that corresponded to the second nucleotides cluster. The allocation of the second cluster into two distinct groups corresponded with the amino acid changes. Cluster 2a included Italy98, Kenya98, Israel 2000 human, Romania96m and the two Israeli goose: KY98 and MaS00 isolates. It comprised isolates not having changes at positions 90 and 93. Cluster 2b contained Eg101, Romania96h, Israeli goose MaV00 and two pigeon isolates. It included isolates having a change at position 93.

## Discussion

According to the sequence data presented here, the predominant isolate from wild birds and geese during 1997–2001 resembled most closely WN-NY99eq. Apparently, this strain has now been circulating in Israel in an endemic form for the last 5 years and may even be overwintering in the area. In 1998 and 2000, however, a second strain was isolated from Israeli geese and pigeons that closely resembled strains

isolated in Kenya and Europe and may have been introduced into Israel by migrating birds. All the isolates from the second cluster showed differences at amino acids 126 and 159. It is reported that phenotypic markers have been associated with changes at or near these two sites [12]. Amino acid 126 region is one of two primary domain II-binding sites for virus neutralization, while amino acid 159 has been associated with flavivirus attenuation and could explain their reduced virulence and the sporadic success of isolation.

The presence of two co-circulating WN virus phenotypes in Israel was recently corroborated by Hindiyeh et al. [13] who have isolated four viruses from sick viremic humans during the 2000 Israel epidemic. Based on sequencing and phylogenetic analysis of a 1648 bp fragment spanning the preM, M and the 5' terminus of the E gene, two of their isolates were related to the NY99 prototype while the other two resembled the Romanian96 and Volgograd99 sequences. An antigenic comparison of the two clusters has not been attempted although this would be worthwhile in view of the report by

Lvov et al. [14] of concurrent circulation of two antigenic WN virus strains in Volgograd during the 1999 epidemic.

Within an endemic regional background one might expect that a second, more pathogenic isolate would appear, presumably by bird migration, rather than by micro-evolution of an existing circulating endemic strain. In such a scenario, factors favoring the emergence of a new epidemic would be satisfied. We have recently identified migrating white storks as carriers of WN virus between Europe and Africa with the Middle East serving as a stopover during their spring and fall migrations [5]. This migratory route was recently alluded to by Miller [15] in their discussion of the role of migratory birds in re-circulation of WN viruses between Africa and Europe. They showed that a strain of WN virus isolated from a mosquito in Kenya in 1998 closely resembled an isolate from the 1996 Bucharest epidemic. Our analysis indicates that the second cluster of the 2000 Israeli isolates resembles more closely the Egyptian 101 strain. It is possible that this endemic prototype strain has been circulating in the Middle East, Africa and more recently, Russia and Romania for at least 50 years.

The viruses isolated from geese and migrating storks in September 1998 were phylogenetically similar to those re-isolated in the following year from geese and gulls. One feature of the NY99 isolate is its pathogenicity for many species of birds, including geese [16]. Unusual mortality of wild or domestic birds has not been recorded in the recent French, Italian, Romanian or Russian outbreaks nor have crows been affected during the Israeli epizootic [17]. No direct correlation between bird and horse mortality and human epidemics is discernable at present except to remark that the circulating virus has apparently undergone a major shift in its biological affinity.

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