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Pathogenicity evaluation of twelve West Nile virus strains belonging to four lineages from five continents in a mouse model: discrimination between three pathogenicity categories

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Abstract

Rodent models have been used extensively to study West Nile virus (WNV) infection because they develop severe neurological symptoms similar to those observed in human WNV neuroinvasive disease. Most of this research has focused on old lineage (L) 1 strains, while information about pathogenicity is lacking for the most recent L1 and L2 strains, as well as for newly defined lineages. In this study, 4-week-old Swiss mice were inoculated with a collection of 12 WNV isolates, comprising 10 old and recent L1 and L2 strains, the putative L6 strain from Malaysia and the proposed L7 strain Koutango (KOU). The intraperitoneal inoculation of 10-fold dilutions of each strain allowed the characterization of the isolates in terms of LD_{50}, median survival times, ID_{50}, replication in neural and extraneural tissues and antibody production. Based on these results, we classified the isolates in three groups: high virulence (all L1a strains, recent L2 strains and KOU), moderate virulence (B956 strain) and low virulence (Kunjin and Malaysian isolates). We determined that the inoculation of a single dose of 1000 p.f.u. would be sufficient to classify WNV strains by pathotype. We confirmed the enhanced virulence of the KOU strain with a high capacity to cause rapid systemic infection. We also corroborated that differences in pathogenicity among strains do not correlate with phylogenetic lineage or geographic origin, and confirmed that recent European and African WNV strains belonging to L1 and L2 are highly virulent and do not differ in their pathotype profile compared to the prototype NY99 strain.

INTRODUCTION

West Nile virus (WNV, *Flaviviridae*, *Flavivirus*) is an emerging zoonotic arbovirus (arthropod-borne virus) that is widely distributed throughout the world, and it has a considerable impact on public and animal health [1].

WNV is transmitted by mosquitoes, with a wide variety of bird species acting as natural reservoir hosts, amplifying the virus. WNV can also affect a range of vertebrate species, including amphibians, reptiles and mammals. It is particularly pathogenic in humans and horses, which act as 'dead-end hosts' as they do not develop viraemia levels sufficiently high to efficiently transmit the virus to a new vector [2, 3].

Two main genetic variants or lineages can be distinguished, namely lineages 1 and 2 (L1 and L2). L1 includes most of the strains isolated in Africa, Europe, Asia, Oceania (Kunjin isolates) and North America. L2 was mainly restricted to Africa, but it has recently been introduced in Central and Eastern Europe [4, 5], and extended further into Southern Europe [6, 7].

Other viral variants presenting 20–30 % genetic divergence from L1 and L2 have been isolated in different parts of the world and have been proposed as different lineages [8]. L3 corresponds to the 'Rabensburg' strain isolated in the Czech Republic in 1997 from *Culex pipiens* [9], and L4 consists of the 'Krasnodar' strain isolated in ticks in southern Russia [10]. WNV strains found in humans and mosquitoes in India form L5 [11], while the putative L6, L7 and L8 correspond, respectively, to the KUN MP502–66 ('Sarawak') strain from Malaysia [12], the Koutango (KOU) isolate

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Abbreviations: d.p.i., days post infection; HV, high virulence; L, lineage; LV, low virulence; MST, median survival time; MV, moderate virulence; WNV, West Nile virus; IP, intraperitoneal; p.f.u., plaque-forming unit; LD_{50}, lethal dose 50; ID50, infectious dose 50.

from Senegal [13, 14] and a new lineage identified (but not isolated) in mosquitoes in Spain [8]. Recently, a putative L9 was proposed for a WNV strain detected in *Uranotaenia* spp. mosquitoes in Austria [15]. However, the lineage classification of existing WNV strains is still a matter of controversy. Some authors have proposed alternative designations of new WNV lineages (from 6 to 9) and, therefore, there is an urgent need for the International Committee on Taxonomy of Viruses to establish clear rules on defining virus lineages and to harmonize WNV lineage numbering [15, 16].

Except for the Indian variant (L5), which has been involved in outbreaks of encephalitis and retinitis in humans [17], and the KOU strain, which caused clinical disease in a laboratory worker [18], the potential impact of the other lineages (apart from 1 and 2) in human and animal health is unknown and warrants further investigation.

In the past, WNV was considered a pathogen of lesser importance, causing only mild disease in humans, during sporadic, small, self-limiting outbreaks. This notion has changed, since over the last 18 years WNV has been responsible for major, persistent epidemics in humans and animals, mainly in North America (since its first occurrence there in 1999), but also in Europe, where its recent re-emergence has led to increasing outbreak frequency, human cases and virulence for certain wild birds [1, 19]. Likewise, in Australia, the first virulent strain of KUN WNV emerged recently, leading to an unprecedented severe outbreak of encephalitis in horses in 2011, which contrasts with the mild disease historically caused by native KUN virus [20, 21].

These cases, as well as other new incursions of enhanced virulence strains in distant areas, i.e. L2 WNV in Central Europe [5] or South Africa [22], demonstrates the continuous evolution of WNV strains and the permanent global risk of the emergence of highly pathogenic WNV variants.

The development and refinement of animal models that allow in-depth characterization of WNV strains is of paramount importance, particularly considering the current epidemiological situation, with an increasing number of different WNV lineages/strains/variants continuously emerging and co-circulating all over the world. Although hamsters and non-human primates have been used as models of human WNV infection, mice are by far the most commonly used models to examine particular aspects of WNV virulence (such as neuropathogenicity and neuroinvasiveness), to study host immune response after infection, to evaluate immunotherapy and vaccine efficacy [23] and to discover host–pathogen interactions that influence disease outcome in humans and other mammals [24].

The majority of the experimental research carried out in mice with WNV has focused on L1 and L2. Among L1 strains, abundant information exists regarding the North American strain that caused the devastating outbreak in New York in 1999, and the Australian KUN variants belonging to clade 1b [20, 21, 25–30]. As regards L2, most of the available information corresponds to old African isolates [25–27], while studies addressing the pathogenic potential of the new emerging L2 strains of Europe and South Africa are scarce [31]. Overall, these studies have provided valuable information about the usefulness of the mouse model for studies of the disease process and for pathogenicity determination of WNV variants. Also, they have indicated that virulence is not dependent upon the virus lineage or the geographical origin but is strain-specific [26].

Despite the intense activity of WNV in Europe in recent years, pathogenicity assessment of the new European isolates in the murine model is limited [31]. Given that WNV strains exhibit considerable genetic variation, one could predict that diverse WNV strains should demonstrate differential pathogenicity. Therefore, we considered that an in-depth evaluation of the ability of these emerging strains to cause disease might help us to better understand the particular eco-epidemiology of WNV in Europe and the Mediterranean area.

Likewise, little is known about the pathogenic force of other WNV isolates branching outside L1 and L2. Only a couple of recent studies [25, 32] have addressed this gap, and the authors found striking variations in virulence among these strains as compared to those belonging to L1 and L2.

The aim of this study was to characterize the pathogenicity of a wide variety of old (before 1985) and recent (from 1998 to 2008) WNV strains (n=12) belonging to four different lineages representative of five continents. Results obtained in this research will help to validate the model for human and horse infection, to better understand factors determining the differences in pathogenicity among WNV strains and to establish a protocol in a young mouse model that allows for comparisons in order to better discern between strains of WNV differing in virulence levels.

RESULTS

Clinical signs, mortality rates and median survival times

A variable proportion of the mice, depending on the strain and dose inoculated, developed clinical disease. First clinical signs appeared at 6–7 days post infection (d.p.i.) and included ruffled fur, reduced activity and reluctance to move. Between 8 and 10 d.p.i., depending also upon strain, more severe symptoms were registered: hind leg paralysis, twitching and tremors, ataxia, hunchbacked posture, shuffling gait, distended abdomen and partially closed eyes due to swelling of the eyelids. Mice displaying immobility or severe leg paralysis were humanely euthanized for welfare reasons from 6 to 10 d.p.i.

All the mice that displayed clinical signs at some point after infection finally died or were euthanized (there were no recovery cases in any of the tested strains). PBS-inoculated controls remained healthy throughout the experiment.

The KOU strain exhibited the most virulent phenotype, with the shortest survival time and causing the death of

100 % of inoculated mice in all the assayed doses, except for the 1 p.f.u. group with a mortality rate of 33.3 % (Table 1). In most cases, KOU-inoculated mice died without showing previous clinical signs. In contrast, mice inoculated with other pathogenic strains usually showed overt signs of disease for 1 or 2 days before dying or being euthanized.

Mice infected with 1000 p.f.u. of all the strains showed clinical evidence of infection and finally died (Fig. 1, Table 1), except for B956, Kunjin (KUN) and Malaysian (MAL) that displayed an attenuated phenotype. In particular, the KUN and MAL strains were of low virulence for mice and caused only sporadic deaths that were not related to the inoculated dose.

MSTs were related to the inoculated dose in most strains, with increasing times as inoculated dose decreased.

Lethal dose 50 (LD_{50}) values allowed precise differentiation among strains, dividing them into three categories of virulence: high (HV), moderate (MV) and low (LV) (Table 1, Fig. 1). L1, recent L2 and KOU strains were HV (LD_{50} values ranging between 1.78 and 10). In contrast, MAL and KUN strains caused few or no deaths in inoculated mice, with LD_{50} values three (for KUN) to four (for MAL) orders of magnitude higher than the HV group, thus being classified as LV. As shown in Table 1, B956 strain (old L2), exhibited an LD_{50} value of $\geq$356 p.f.u. and was thus classified as MV, intermediate between HV and LV.

This classification also agrees with the data obtained when comparing survival curves among all the strains at a dose of 1000 p.f.u. using Kaplan–Meier analysis (Table 2). This analysis confirms that: (1) survival curves of NY, ISR, MO, SP, IT, FR, RSA and AUS are not statistically different and can therefore be grouped together (HV strains); (2) no difference exists between the KUN and MAL survival curves but both are significantly different (P<0.01) from all the strains of the first group, thus representing a second group (LV strains); (3) the KOU survival curve is statistically different from all other HV strains (P<0.05), showing it to be the most lethal isolate (LD_{50} 1.78) with the lowest median survival time (6 days for an inoculation dose of 1000 p.f.u.); and (4) the B956 strain again displays intermediate results (MV), with a survival curve significantly different from all the other (HV and LV) strains (with the exception of SP).

The majority of clinically affected mice did not have any visible abnormalities on necropsy. Only unspecific findings were observed in a small proportion of diseased mice, including ascites, swollen stomach and small intestine distended with liquid ingesta and gas.

Viral burden in tissues

High levels of viral RNA load were detected in the brains of all mice analysed, including those infected with the LV group of strains (Table 3). However, important variations between strains were evidenced as regards the extraneural replication of the virus. Viral RNA was detected in the heart, kidney, liver and spleen of all mice infected with the HV strains, whereas for the MV and LV strains the virus was completely absent (for B956 and MAL) or detected only in some organs at low concentration (KUN).

As shown in Table 3, differences exist in relation to viral RNA load in the organs of fatally infected mice. Overall, the highest viral loads were consistently found in the brain, which demonstrates the neuroinvasive capacity of all the tested strains, including MV and LV phenotypes.

For the eight strains displaying a HV phenotype, the virus was pantropic, infecting all harvested tissues. After the brain, the highest viral RNA burdens were observed in heart and kidney. The virus was also detected in spleen, although at lower concentrations. For all the strains with extraneural presence of the virus, the lowest viral RNA loads were consistently detected in the liver, failing to reach detectable levels in some cases, as for the B956 strain.

Antibodies

The presence of antibodies in sera from surviving mice was associated with the inoculated dose. Of those mice inoculated with the lowest dose (1 p.f.u.) of any strain, only one (inoculated with B956 strain) developed detectable antibodies. Higher viral doses increased the probability of developing antibodies, as shown in Table 4. A high proportion of surviving mice infected with $\geq$100 p.f.u. of LV or MV strains had seroconverted by day 21 post infection (100 % of mice for B956 and KUN and 88 % for MAL), with an infectious dose 50 (ID_{50}) much lower (between 3.9 and 40 p.f.u.) than their respective LD_{50}. Concerning HV strains, circulating antibodies were detected in few cases (only in 10 and 100 p.f.u. groups) and, consequently, ID_{50} values were identical (or very similar) to LD_{50} values in all HV strains (<10 p.f.u. in all cases). No mice survived in the 1000 p.f.u. group for those strains and therefore no serum was available for testing.

DISCUSSION

As WNV is relentlessly spreading across wide areas in Europe, North and South America, Africa and Australia, spillover events increase and new independently evolved strains arise worldwide, giving rise to a high genetic diversity with remarkable phenotypic variation, each with a particular ecological niche and epidemiological constrains. Full phenotypic characterization of emerging WNV strains, such as virulence, pathogenesis, neuroinvasiveness and neuropathogenicity, is needed to understand the variable epidemiology of this virus and to develop efficient vaccines and treatments adapted to locally circulating strains [25, 33]. In this study we used the Swiss mouse model to characterize the pathogenicity of 12 WNV strains representative of four different lineages, from five continents, spanning a time period of seven decades from the first (1937) to the last (2008) isolation.

IP inoculation of young Swiss (and to a lesser extent C57BL/6) mice has been widely used to determine the virulence and pathogenesis of different WNV variants. Extensive studies

Table 1. Mortality rates, median survival time (MST) and lethal dose 50 (LD_{50}) of 12 WNV strains following intraperitoneal (IP) inoculation in 4-week-old Swiss mice

Strain	Lineage	Viral dose (p.f.u.)	Mortality		MST (days)	LD_{50}
			dead/ total	%		
KOU	Putative 7	1	2/6	33.3	–	1.78
		10	6/6	100	7	
		100	6/6	100	7	
		10^3	6/6	100	6	
NY	1a	0.1	0/6	0	–	4.64
		1	3/6	50	14	
		10	3/6	50	11	
		100	5/6	83.3	9	
		10^3	6/6	100	8	
MO	1a	0.1	0/6	0	–	2.85
		1	3/6	50	9	
		10	4/6	66.6	9	
		100	5/6	83.3	9	
		10^3	6/6	100	7	
SP	1a	0.1	0/6	0	–	6.98
		1	2/6	33.3	–	
		10	3/6	50	10	
		100	5/6	83.3	9	
		10^3	6/6	100	9	
ISR	1a	0.1	0/6	0	–	10
		1	0/6	0	–	
		10	3/6	50	10	
		100	6/6	100	8	
		10^3	6/6	100	8	
IT	1a	0.1	0/6	0	–	3.16
		1	1/6	16.6	–	
		10	5/6	83.3	8	
		100	6/6	100	8	
		10^3	6/6	100	7	
FR	1a	0.1	1/6	16.6	–	3.36
		1	1/6	16.6	–	
		10	4/6	66.6	8	
		100	6/6	100	8	
		10^3	6/6	100	8	
AUS	2	0.1	0/6	0	–	10
		1	0/6	0	–	
		10	3/6	50	8	
		100	6/6	100	9	
		10^3	6/6	100	7	
RSA	2	0.1	0/6	0	–	2.51
		1	1/6	16.6	–	
		10	6/6	100	8	
		100	6/6	100	8	
		10^3	6/6	100	8	
B956	2	1	0/6	0	–	≥356
		10	2/6	33.3	–	
		100	2/6	33.3	–	
		10^3	3/6	50	11	
		10^4	5/6	83.3	10	
KUN	1b	1	0/6	0	–	$>10^4$
		10	1/6	16.6	–	
		100	1/6	16.6	–	
		10^3	0/6	0	–	
		10^4	3/6	50	12	
		10^5	0/6	0	–	
MAL	Putative 6	10	0/6	0	–	$>10^5$
		100	1/6	16.6	–	
		10^3	0/6	0	–	
		10^4	0/6	0	–	
		10^5	1/6	16.6	–	

are available regarding the NY99 strain as well as other old isolates from Africa and Australia. However, and despite the growing number of WNV outbreaks caused by L1 and L2 in the last decade in Europe [34, 35] and South Africa [22], only a few recent isolates have been characterized in mice to date [28, 31]. The lack of pathogenicity assessments is even more evident for WNV lineages other than L1 and L2 [25, 32]. In this study we investigated the pathogenicity of 10 L1 and L2 strains, as well as one strain belonging to L6 (MAL) and another proposed as L7 (KOU). Of those, four recent L1 and L2 strains had never before been fully characterized using a mouse model (IT, FR, AUS and RSA). For other strains (SP, MO, KOU, MAL) only limited information is available [25, 28, 32].

The inoculation of 10-fold dilutions of each strain in young Swiss mice allowed us to accurately determine LD_{50} values and classify the assayed isolates into three groups based on these results: HV, with LD_{50} <20 p.f.u. (KOU, NY, SP, MO, AUS, ISR, FR, IT, RSA); MV, with LD_{50} ≥356 p.f.u. (B956); and LV, with LD_{50} $>10^4$ p.f.u. (MAL and KUN). LD_{50} data obtained for the strains classified within the highly

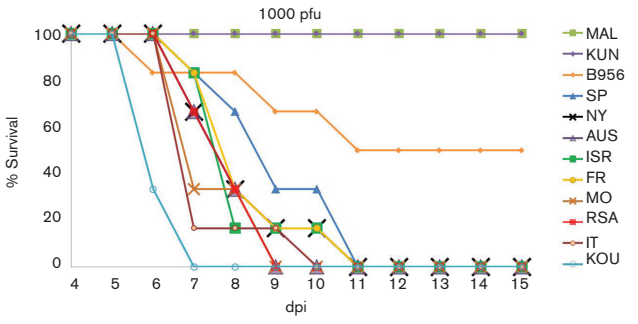

Fig. 1. Survival curves for all the strains after IP inoculation of 1000 p.f.u. in 4-week-old Swiss mice (six mice per strain).

Table 2. Results (*P* values) of the Kaplan–Meier analysis (log-rank test) for comparisons among all tested strains at a dose of 1000 p.f.u.

	KOU	NY	MO	SP	ISR	IT	FR	AUS	RSA	B956	MAL
NY	≤0.01										
MO	≤0.01	NS									
SP	≤0.01	NS	NS								
ISR	≤0.01	NS	NS	NS							
IT	≤0.05	NS	NS	NS	NS						
FR	≤0.01	NS	NS	NS	NS	NS					
AUS	≤0.01	NS	NS	NS	NS	NS	NS				
RSA	≤0.01	NS	NS	NS	NS	NS	NS	NS			
B956	≤0.01	≤0.05	≤0.05	NS	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05		
MAL	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	
KUN	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	≤0.01	NS

NS, non significant.

pathogenic group were consistent with the range of values (≤20 p.f.u.) previously observed for NY [20, 25, 28], ISR [29], MO and SP [28]. In the case of the Italian strain analysed in this study, the LD_{50} value was also similar to that described for another L1 strain isolated in Italy the following year (2009) [31]. Isolates from AUS (L2), RSA (L2) and FR (L1) were characterized in the mouse model for the first time and they all displayed LD_{50} values <20 p.f.u. MAL and KUN strains were classified as LV with very high LD_{50} values (above 10^4 p.f.u.). Although it could be related to the unknown (probably high) number of cell passages of the strains, this result is in accordance with previous studies where old KUN strains have repeatedly been associated with a LV phenotype both *in vivo* and *in vitro* [20, 25–27, 29], and two previous studies where the MAL strain exhibited a LV phenotype in the mouse model [25, 32].

However, variations in LD_{50} were observed for the moderately pathogenic strain (B956) with regard to previous studies. While we obtained an intermediate LD_{50} value of ≥356 p.f.u., the two previous studies in which the B956 strain had been characterized assigned different LD_{50} results: 39.2 p.f.u. in [28] and >10^4 p.f.u. for the same strain

Table 3. Viral burden in organs of inoculated mice (Ct values)

Organs were harvested from mice that died due to the infection or were euthanized for welfare reasons. For AUS, FR, ISR, IT, NY, RSA, MO, SP and KOU, organs were collected from mice infected with 10, 10^2 and 10^3 p.f.u. For the remaining strains, samples were obtained from mice inoculated with lethal doses (for B956, 10^3 and 10^4, for KUN, 10^4 and MAL, 10^3).

Strain	d.p.i.	Spleen	Heart	Liver	Kidney	Brain
KOU (*n*=4)	6 (*n*=1), 7 (*n*=3)	++	+++	+	++	++++
NY (*n*=5)	8 (*n*=4), 9 (*n*=1)	+	+++	+	++	++++
MO (*n*=6)	7 (*n*=5), 9 (*n*=1)	++	+++	+	+++	++++
SP (*n*=6)	7 (*n*=1), 8 (*n*=2), 9 (*n*=3)	+	++	+	++	++++
ISR (*n*=4)	7 (*n*=1), 9 (*n*=3)	++	++	+	++	++++
IT (*n*=3)	7 (*n*=1), 8 (*n*=2)	++	+++	++	+++	++++
FR (*n*=4)	8 (*n*=3), 9 (*n*=1)	++	++	+	++++	++++
AUS (*n*=4)	8 (*n*=3), 9 (*n*=1)	+	++	+	+++	++++
RSA (*n*=5)	8 (*n*=4), 9 (*n*=1)	+	++	−	++	++++
B956 (*n*=5)	6 (*n*=1), 9 (*n*=2), 10 (*n*=1), 12 (*n*=1)	−	−	−	−	++++
KUN (*n*=3)	9 (*n*=1), 10 (*n*=1), 11 (*n*=1)	−	++	−	+	+++
MAL (*n*=1)	13 (*n*=1)	−	−	−	−	+++

++++ mean Ct value: 15–25.

+++ mean Ct value: 25.1–30.

++ mean Ct value: 30.1–35.

+ mean Ct value: 35.1–39.9.

− negative.

n indicates the number of mice of each strain sampled for viral load determination.

Table 4. Antibody detection in surviving mice (at 21 d.p.i.) and infectious dose 50 (ID_{50}) of each strain

Antibodies were detected using a commercially available epitope-blocking ELISA (Ingezym West Nile Compac, INGENASA). Number of seropositive mice/number of surviving mice (% of seropositive).

Strain	Dose (p.f.u.)					ID_{50} (p.f.u.)
	1	10	100	1000	10 000	
KOU	0/4	–	–	–	NA	1.78
NY	0/3	0/3	0/1	–	NA	4.64
MO	0/3	0/2	1/1 (100 %)	–	NA	4.22
SP	0/4	1/3 (33 %)	0/1	–	NA	2.04
ISR	0/6	0/3	–	–	NA	10
IT	0/5	0/1	–	–	NA	3.16
FR	0/5	1/2 (50 %)	–	–	NA	2.51
AUS	0/6	0/3	–	–	NA	10
RSA	0/5	–	–	–	NA	2.51
B956	1/6 (16.6 %)	1/4 (25 %)	4/4 (100 %)	3/3 (100 %)	1/1 (100 %)	≤7
KUN	0/6	4/5 (80 %)	5/5 (100 %)	6/6 (100 %)	3/3 (100 %)	3.98
MAL	NA	1/6 (16.6 %)	4/5 (80 %)	5/6 (83.3 %)	6/6 (100 %)	≤39.81

NA, not applicable (dose not inoculated).

–, No surviving mice.

in [25]. Discrepancies in the phenotypic expression of viral infection have also been evidenced in other studies [20, 29] and different factors such as age and strain of mice, inoculation site, genetic variation of the virus and history of the viral isolate have been outlined as potential causes of inter-assay variations [36]. In fact, a high number of cell passages can induce virus attenuation and therefore the passage history of each strain should be taken into account to accurately interpret the results of pathogenicity studies. Likewise, it is important to consider that differences in sample size (number of inoculated mice by dose) among studies can have a great impact on LD_{50} calculations (and to a lesser extent also in MSTs). This limitation must be taken into account when comparing and interpreting LD_{50} results from different experiments.

As previously observed by [25], KOU was the most virulent strain for all the assayed doses (LD_{50}=1.78) and displayed the lowest MST (6 days for an inoculation dose of 1000 p.f.u.). The murine origin of the virus (it has been isolated from different species of rodents in Africa [18]) could be related to the high virulence evidenced in our mouse model. Although only one human infection case caused by KOU has been confirmed (in a laboratory worker [18]), there has been at least one other suspicious outbreak of neurological disease in children in Sudan in 2002 in an area close to the region where KOU was first isolated. Unfortunately, the causative virus ('West Nile-like' virus) of this outbreak was not isolated, precluding definitive identification of the etiologic agent [37]. These epidemiological data, together with our findings of enhanced virulence and the extremely rapid capacity of the virus to cause systemic infection, should be considered to envisage future studies aiming to provide detailed information about virus and host pathogenicity determinants.

Very high levels of WNV RNA were consistently detected in the central nervous system of all lethally infected mice, regardless of the inoculated strain, proving the neuroinvasiveness capacity of all tested isolates. However, relevant differences exist in WNV detection in other organs. Overall, in mice inoculated with HV strains, the virus was also detected in liver (except for RSA) and spleen and particularly high viral loads were observed in heart and kidney. In contrast, in mice inoculated with MV and LV strains, the virus was absent from peripheral organs, with the exception of heart and kidney in KUN-infected mice. These results are consistent with previous studies which suggest that the main pathogenic difference between high- and low-virulent variants is the increased capacity of the former to replicate efficiently in extraneural organs to establish systemic infection [30].

Interestingly, the lowest viral loads were consistently found in the liver of infected mice. In some cases (KUN, MAL, B956 and RSA), the virus was completely absent from the liver in all lethally infected animals. Similar results have been obtained in previous studies [38] but it is still not clear why WNV strains do not replicate efficiently in the murine liver.

The strains MV (B956) and LV (KUN, MAL) were capable of infecting neural tissue, but seroconversion in surviving mice at the end of the trial (21 d.p.i.) demonstrates that these animals were able to overcome the infection. Similar results were also observed for old KUN isolates by other authors [30]. In general, antibody response to the infection increased with the inoculated dose, as shown in surviving mice inoculated with the MV and LV strains (B956, KUN, MAL). In fact, a very low proportion of mice seroconverted after inoculation of the lowest doses (1 and 10 p.f.u.), as

already described in previous studies where mice were inoculated with low doses of both LV and HV strains [28, 30]. For HV strains, ID_{50} and LD_{50} values were identical or very similar, indicating very low proportion of sublethal infections.

Considering results from survival curves for each WNV strain and inoculation dose, we selected the dose 1000 p.f.u. as the most useful to clearly discriminate pathotypes in this mouse model. Comparison of survival curves at this dose allowed statistically significant differentiation of strains as HV, MV and LV. Moreover, within the HV group, two subgroups could be identified: KOU (highest virulence, HV+) and the remaining HV strains (NY, ISR, MO, SP, IT, FR, AUS, RSA). MSTs were in agreement with this classification, being 6 days for KOU, 7–9 days for the remaining HV strains and 11 days for the MV group. MSTs could not be calculated for KUN and MAL due to insufficient lethal events. Likewise, a strong consistency was evident between this classification and LD_{50} values obtained for each strain. Therefore, the inoculation of a single dose (1000 p.f.u.) of each strain in 4-week-old Swiss mice would be sufficient to classify WNV strains by pathotype. This approach would considerably reduce the number of mice necessary for this type of *in vivo* trial, which is an important animal welfare improvement in order to fulfil one of the main guideline criteria in animal experimentation. Also, the overall reduction in the total number of mice needed for each experiment would enable an increase in the sample size for the selected dose (1000 p.f.u.) to levels that would allow for a substantial increase in statistical significance when comparing survival curves.

Overall, our experimental results, in agreement with previous studies [26, 31], indicate that differences in pathogenicity among WNV strains do not correlate with the phylogenetic lineage, geographic origin or year of isolation. Rather, virulence appears to be an evolving phenotype acquired independently of the genetic background during virus adaptation to varying ecological niches. We confirmed the enhanced virulence of the putative L7 strain (KOU), with prominent neuroinvasive and neurovirulent features and high capacity to cause rapid systemic infection. We also corroborated that recent European WNV strains belonging to both L1 and L2 do not differ in their virulence profile compared to the prototype NY99 strain. Hence, the virulence found recently in Europe for humans in certain instances [34, 35] is compatible with the high virulence observed here in mice for the recent European strains of L1 and L2. Differences in WNV eco-epidemiology between Europe and North America, therfore, must be explained not based on strain virulence for humans (or mammals) but taking into consideration other phenotypic traits that may affect viral transmission such as host and vector competence, past exposure to other flaviviruses, co-circulation of a wide diversity of WNV strains or other ecological features such as biodiversity or species richness.

METHODS

Viruses and virus preparations

All viral strains and isolates used in this study, along with their origins and sources and the number of cell passages, are listed in Table 5. All the strains were titrated by plaque assay in Vero cells and virus titres are given in p.f.u.

Mouse infection and survival studies

Three-week-old female Swiss HSD ICR (CD1) outbred mice were purchased from Harlan (Switzerland) and maintained in a BSL3 facility at CISA. After 1 week of acclimatization (that is, at four weeks of age) groups of six mice were inoculated intraperitoneally with 100 µl of virus at doses ranging from 0.1 to 10^3 p.f.u. diluted in cell culture medium + 0.2 % endotoxin-free bovine serum albumin (BSA). One additional dose (10^4 p.f.u.) was also assayed for the B956 strain and two higher doses (10^4 and 10^5 p.f.u.) were included for the MAL and KUN strains, instead of the lowest 0.1 and 1 p.f.u. doses.

A group of six mice inoculated in parallel with an equivalent volume (100 µl) of cell culture medium + 0.2 % BSA was maintained as a control.

The mice were monitored daily for symptoms and death, up to 3 weeks after inoculation. The mice were euthanized either 21 days after infection when healthy or at a pre-mortem stage when severely diseased. The mortality rate of each group was used to calculate the LD_{50} for each strain using the Reed–Muench method [39].

Viral burden determination in brain and extraneural tissues

Mice that succumbed to the infection were subjected to necropsy and samples of brain, liver, kidney, spleen and heart were collected for PCR analysis. During necropsy, care was taken to avoid cross-contamination of tissues by using single-use scalpels.

Tissues were homogenized for 2 min at 30 cycles/s in PBS using a Tissuelyser homogenizer (QIAGEN), followed by clarification for 10 min at 850×g.

RNA was extracted using Biosprint-15 automated extraction (QIAGEN, Valencia, CA, USA), and 4 µl (4 % of total eluate) of each extract, in 25 µl final volume, was subjected to real-time RT-PCR amplification for detection of L1 and L2 using the QuantiTect Probe RT-PCR kit (QIAGEN) as described previously [40]. Samples of mice infected with WNV strains other than L1 and L2 (MAL and KOU) were analysed using a recently developed duplex real-time RT-PCR for simultaneous detection of Japanese encephalitis and Ntaya serogroups within the flavivirus genus (Elizalde *et al.*, unpublished results). Samples were considered positive in these tests at Ct $\leq$40.

Antibody detection assays

Surviving mice (except those inoculated with 0.1 p.f.u.) were bled by puncture of the submandibular vein at the end of

Table 5. Viral strains used in this study, along with their origins and sources and the number of passages in cells

	Lineage	Geographic origin	Species	Year of isolation	Cell passage number	GenBank accession number	Source of the strain/isolate
SP (GE-1b/B-2007)	1a	Spain	Golden eagle	2007	BSR-3p	FJ766331	Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, Spain
MO (MO20034-04.05)	1a	Morocco	Horse	2003	VR-7p	AY701413	Spanish National Reference Laboratory for viral zoonoses, Algete, Spain
IT (Italy 15 803/08)	1a	Italy	Magpie	2008	VR-3p	FJ483549	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER), Brescia, Italy
ISR (IS-98 STD1)	1a	Israel	White stork	1998	AP61-3p/ VR-2p	AF481864	Institut Pasteur Paris, France
FR (PaAn001)	1a	France	Horse	2000	C6/36-1p/ VR-3p	AY268132	Agence nationale de sécurité sanitaire, de l'alimentation, de l'environnement et du travail (ANSES), Maisons-Alfort, France
NY (NY99-crow-V76/1)	1a	NY (USA)	American crow	1999	VR-6p	FJ151394	Diagnostic Virology Laboratory Dept. of Agriculture (USDA), Ames, IA, USA
KUN (KUN-KJ359)	1b	Australia	Horse	1984	Unknown	GU047874 HQ840762	Instituto de Salud Carlos III (ISCIII), Madrid, Spain
B956	2	Uganda	Human	1937	Mouse-31p/ VR-10p	AY532665	American Type Culture Collection (ATCC), Manassas, VA, USA
AUS (Austria/2008_gh)	2	Austria	Goshawk	2008	VR-2p	KF179640	Institute of Virology University of Veterinary Medicine, Vienna, Austria
RSA (HS101/08)	2	South Africa	Horse	2008	Mouse-2p/ VR-4p	FJ464378 FJ464381	Agricultural Research Council – Onderstepoort Veterinary Institute, South Africa
MAL (MP502-66)	Putative 6	Sarawak, Malaysia	*Culex pseudovishnui*	1966	Unknown	GU047874	Instituto de Salud Carlos III (ISCIII), Madrid, Spain
KOU (DakArD96655)	Putative 7	Senegal	*Rhipicephalus guihoni*	1993	AP61-8p/ VR-1p	KJ131501	CRORA- Institut Pasteur de Dakar, Senegal

BSR, cell line derived from BHK-21 baby hamster kidney cells; VR, Vero cells; AP61, *Aedes pseudoscutellaris* cells; C6/36, *Aedes albopictus* cells.

the experiment (day 21). Blood was collected in dry tubes and allowed to clot at 37 °C for 1 h, followed by incubation at 4 °C overnight. After centrifugation, sera were separated and frozen at −20 °C until analysis.

Serum antibodies against WNV were detected by a commercially available epitope-blocking ELISA (Ingezym West Nile Compac, INGENASA, Madrid, Spain) as described previously [41].

The ID_{50} value of each strain was calculated using the Reed–Muench method [39], considering as positives all mice with demonstrable infection (defined as either mortality or survival with seroconversion).

Data analysis

Differences in survival curves between strains were calculated by Kaplan–Meier analysis and analysed by log-rank test, using SPSS 15.0. *P*-values equal to or less than 0.05 were considered to be statistically significant. MSTs were calculated using SPSS 15.0.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All the animal care, handling and experimental procedures performed in this work were supervised and approved by the Ethics and Animal Welfare Committee of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) according to European and Spanish laws on the protection of animals for experimental and other scientific purposes (Spanish Royal Decree 53/2013 and Council Directive 2010/63/EU).

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