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Extinction of West Nile Virus by Favipiravir through Lethal Mutagenesis

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ABSTRACT Favipiravir is an antiviral agent effective against several RNA viruses. The drug has been shown to protect mice against experimental infection with a lethal dose of West Nile virus (WNV), a mosquito-borne flavivirus responsible for outbreaks of meningitis and encephalitis for which no antiviral therapy has been licensed; however, the mechanism of action of the drug is still not well understood. Here, we describe the potent *in vitro* antiviral activity of favipiravir against WNV, showing that it decreases virus-specific infectivity and drives the virus to extinction. Two passages of WNV in the presence of 1 mM favipiravir—a concentration that is more than 10-fold lower than its 50% cytotoxic concentration (CC_{50})—resulted in a significant increase in mutation frequency in the mutant spectrum and in a bias toward A→G and G→A transitions relative to the population passaged in the absence of the drug. These data, together with the fact that the drug is already licensed in Japan against influenza virus and in a clinical trial against Ebola virus, point to favipiravir as a promising antiviral agent to fight medically relevant flaviviral infections, such as that caused by WNV.

KEYWORDS favipiravir, lethal mutagenesis, West Nile virus, antiviral agents, flavivirus

West Nile virus (WNV) is an enveloped plus-strand RNA neurotropic flavivirus transmitted by mosquitoes that constitutes a worrisome threat to global human and animal health (1). About 80% of human infections are asymptomatic, and of the 20% that display disease symptoms, 1% progress to severe neurological disease, including meningitis, encephalitis, and flaccid paralysis, which can be fatal (2). Nowadays, no specific drug is licensed for the control of any flavivirus, including WNV, and current treatment is only palliative (3).

RNA viruses encode an RNA-dependent RNA polymerase (RdRp) that lacks proofreading activity (4). The intrinsic error rates of flaviviral RNA polymerases have been established as ~1 mutation per genome and round of copying (5), which is the range estimated for other RNA viruses and seems adequate to maintain viral fitness. Variation of template-copying fidelity often entails a fitness cost for RNA viruses (6). An increase in replication fidelity (i.e., decreasing mutation rates of RdRp) restricts genetic diversity and limits phenotypic plasticity, which is particularly disadvantageous for arthropod-borne viruses, as they require successful infection, replication, and transmission in taxonomically divergent vertebrate and invertebrate hosts. Conversely, an induced decrease in replication fidelity lowers population fitness through the irreversible accumulation of deleterious and lethal mutations (7). Virus extinction through increased mutation rates is known as lethal mutagenesis. This antiviral strategy has been studied recently in the search of antiviral compounds that exploit the high error rates of RNA viruses, pushing them above an error threshold incompatible with viability (8). The purine analogue ribavirin reduced WNV infectivity and viral RNA synthesis, with a
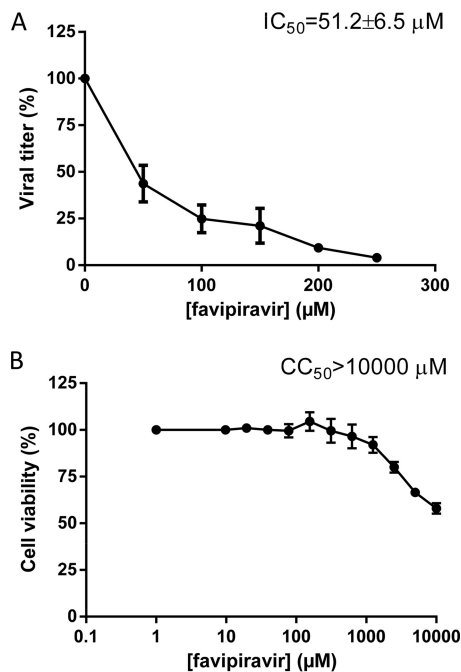
FIG 1 Reduction of WNV yield and cytotoxicity of favipiravir. (A) Percentage of WNV viral titer upon favipiravir treatment. Vero cells were pretreated for 16 h with the indicated favipiravir concentration and then infected with WNV (MOI, 0.1 PFU/cell) in the presence of the same drug concentration; at 48 h p.i., the cell culture supernatants were titrated by plaque assay, and the 50% inhibitory concentration (IC_{50}) was determined. (B) Percentage of viable Vero cells after favipiravir treatment. The 50% cytotoxic concentration was determined by measuring the cellular ATP content at 48 h posttreatment. Data are presented as the mean $\pm$ standard deviation (SD) from three independent determinations of both IC_{50} and CC_{50}.

significant increase of transition mutations, suggesting error catastrophe as one of the modes of action of ribavirin against WNV (9).

Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a broad-spectrum nucleoside analogue (10) that shows potent antiviral activity against several RNA viruses (11–28). It is known that intracellular host enzymes act upon favipiravir, converting it to its active form (favipiravir-4-ribofuranosyl-5′-triphosphate), which functions as a purine nucleotide analog (29). However, its antiviral mechanisms of action are still controversial, with some reports supporting that it directly inhibits the RdRp (11, 15, 29–32) and others favoring that it acts through a lethal mutagenesis mechanism (33–37). Here we quantify the antiviral activity of favipiravir on WNV infection in cell culture and provide for the first time evidence supporting lethal mutagenesis as at least part of its mode of action. The results open the possibility to apply lethal mutagenesis to combat WNV infections.

RESULTS

Antiviral activity and cellular toxicity of favipiravir *in vitro*. To test the effect of favipiravir on WNV infectious progeny production, Vero cells were infected in the presence of different concentrations of the drug (50 to 250 μM) and the virus titer was determined by plaque assay at 48 h postinfection (p.i.). Favipiravir significantly reduced the virus yield in a dose-dependent manner (Fig. 1A), with a 50% inhibitory concentration (IC_{50}) of 51.2 $\pm$ 6.5 μM.

To exclude the possibility that the observed antiviral activity of favipiravir could be due to an effect on the cellular polymerase or a metabolic inhibition of Vero cells, the drug-induced cytotoxicity was assayed. No apparent effect was observed by light microscopy in infected cells treated with the highest drug concentration after 48 h (data not shown), and the 50% cytotoxic concentration (CC_{50}) was >10,000 μM
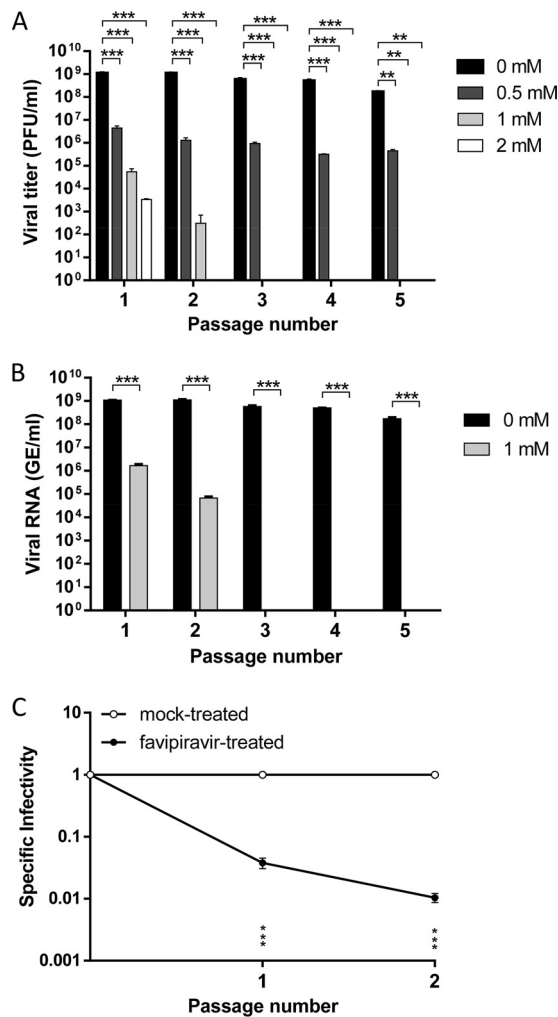
FIG 2 Antiviral activity of favipiravir against WNV in Vero cells. (A) Viral titers of WNV after each passage ($n = 5$) on Vero cells in the presence or absence of favipiravir. Virus yield in the supernatant of cells was titrated by plaque assay. (B) WNV RNA was extracted from the supernatants of untreated or 1 mM favipiravir-treated Vero cells after each passage ($n = 5$) and quantified in triplicate by qRT-PCR. (C) Specific infectivity of WNV in the presence of 1 mM favipiravir, calculated as the ratio between the number of infectious viruses and the total amount of viral RNA. Data are presented as the mean $\pm$ SD. Statistically significant differences are indicated: **, $P < 0.005$, and ***, $P < 0.0001$, by two-way ANOVA with Bonferroni's correction.

(Fig. 1B), indicating that favipiravir has low toxicity under the tested conditions. These data resulted in a selective index (SI [CC_{50}/IC_{50}]), of over 195.

Favipiravir reduces virus-specific infectivity *in vitro*. Based on the antiviral activity and cytotoxicity of favipiravir, serial passages of WNV in Vero cells were carried out in the presence of different drug concentrations (0.5 to 2 mM). All tested concentrations decreased viral infectivity, measured by plaque assay, although 0.5 mM favipiravir was unable to completely inhibit the infection, even when the drug was maintained for 5 consecutive passages (Fig. 2A). Then viral RNA present in the supernatants of cells infected in the absence or presence of 1 mM favipiravir was quantified by quantitative reverse transcription-PCR (qRT-PCR). Whereas the viral RNA load in the supernatant of cells infected in the absence of favipiravir remained almost invariable after 5 passages, viral RNA when favipiravir was present drastically decreased after just one passage and was undetectable beginning on the third passage (Fig. 2B). The specific infectivity, determined as the ratio between the number of infectious viruses and the total amount of viral RNA, was significantly reduced (Fig. 2C). No infectivity or
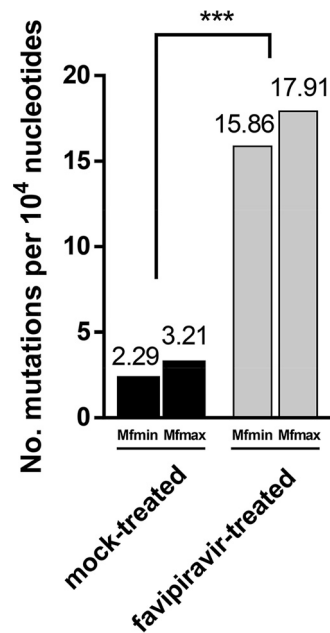
FIG 3 Increased WNV mutation frequency induced by favipiravir. The minimum (Mfmin) and maximum (Mfmax) mutation frequencies of a 909-bp fragment of the NS5 gene amplified by RT-PCR from WNV were determined from supernatants of infected Vero cells after two passages in the absence or presence of 1 mM favipiravir. The mean number of mutations per 10^4 nucleotides sequenced is displayed. The minimum mutation frequency is the number of different mutations found in the mutant spectrum relative to the consensus sequence of the corresponding population divided by the total number of nucleotides sequenced and normalized to 10^4 nucleotides. The maximum mutation frequency is the number of total mutations found in the mutant spectrum relative to the consensus sequence of the corresponding population divided by the total number of nucleotides sequenced and normalized to 10^4 nucleotides. Statistically significant difference is indicated: ***, $P < 0.0001$, by χ^2 test with Yates' correction.

viral RNA was rescued when the cell culture supernatant from the fifth passage in the presence of 1 mM favipiravir was subjected to five additional passages in the absence of the drug.

Mutagenic effect of favipiravir on WNV. Since a reduction in specific infectivity is a hallmark of lethal mutagenesis, the mechanism behind the observed viral extinction induced by favipiravir was investigated at the level of nucleotide sequences of viral RNA. For this purpose, a fragment (909 bp) from the relatively conserved NS5 viral genomic region of WNV (38), which encodes the viral RdRp, was amplified by RT-PCR from the supernatant of infected cells at the second viral passage in absence or presence of 1 mM favipiravir, cloned, and bidirectionally sequenced. A total of 43 and 48 clones were sequenced from the populations passaged in presence and absence of favipiravir, respectively, representing a total of 39,087 and 43,632 nucleotides sequenced. Favipiravir induced a statistically significant 5.6-fold increase of mutation frequency in the mutant spectrum (Fig. 3). A significant difference was also found in the proportion of mutated clones: 0.74 (32/43) with favipiravir and 0.20 (10/48) without it. No modification of the consensus sequence of the populations passaged in the presence or absence of favipiravir was noted.

In addition to the difference in point mutations, one deletion (G at position 8294) was detected in the genomic region analyzed of the population passaged in the presence of favipiravir. In line with the ambiguous purine-pseudobase feature of favipiravir (39), a significant increase of G→A, A→G, and, to a lesser extent, U→C mutations was observed (Table 1). No differences in mutation hot spots, ratios of synonymous to nonsynonymous mutations, or nucleotide types present at the 3′ or 5′ side of the mutated sites were noted (Fig. 4), suggesting that the observed mutagenesis was basically random. The results demonstrate a mutagenic activity of favipiravir for

TABLE 1 Type of mutations found in WNV populations untreated or treated with 1 mM favipiravir[a]

WNV population	No. of mutated clones/total	No. of deletions	No. of mutations[b]				Total nucleotides sequenced
			A→G	U→C	G→A	C→U	
Untreated	10/48	0	4 (0.01)	2 (0.01)	0 (0.00)	8 (0.04)	43,632
Treated	32/43**	1	26** (0.1)*	9* (0.05)	21** (0.08)*	13 (0.06)	39,087

[a]Shown are the number of mutated clones and type of mutated nucleotides observed in the untreated or favipiravir-treated WNV population relative to the consensus sequence. The proportion of each type of mutation normalized to the number of each nucleotide type in the 909-base fragment sequenced is given in parentheses.

[b]Statistically significant differences compared to untreated samples are indicated: *, $P = 0.05$, and **, $P < 0.0001$, by Fisher's exact test and χ^2 test with Yates' correction, respectively.

WNV and suggest that the observed viral extinction was associated with an accumulation of mutations that led to an unviable viral progeny. We conclude that the broad-spectrum antiviral agent favipiravir can act as a lethal mutagen for WNV.

DISCUSSION

The mechanism of favipiravir activity as a broad-spectrum antiviral nucleoside analogue is still controversial, as both direct inhibition of the viral RdRp (11, 15, 29–32) and a lethal mutagenesis activity that increases the virus mutation frequency (33–37) have been proposed. It is difficult to discern whether an inhibitory or mutagenic activity prevails in the antiviral activity of a nucleoside analogue: there are several reasons for the difficulty, including that a mutagenic activity *per se* often entails inhibition of viral progeny production. It is possible that, depending on the drug concentration and on the RdRp targeted by favipiravir-4-ribofuranosyl-5′-triphosphate, an inhibitory or mutagenic activity may predominate. In fact, even with picornaviral RdRps that show remarkable amino acid sequence identity, the same amino acid substitution can have very different effects on the recognition of mutagenic nucleotide analogues (compare
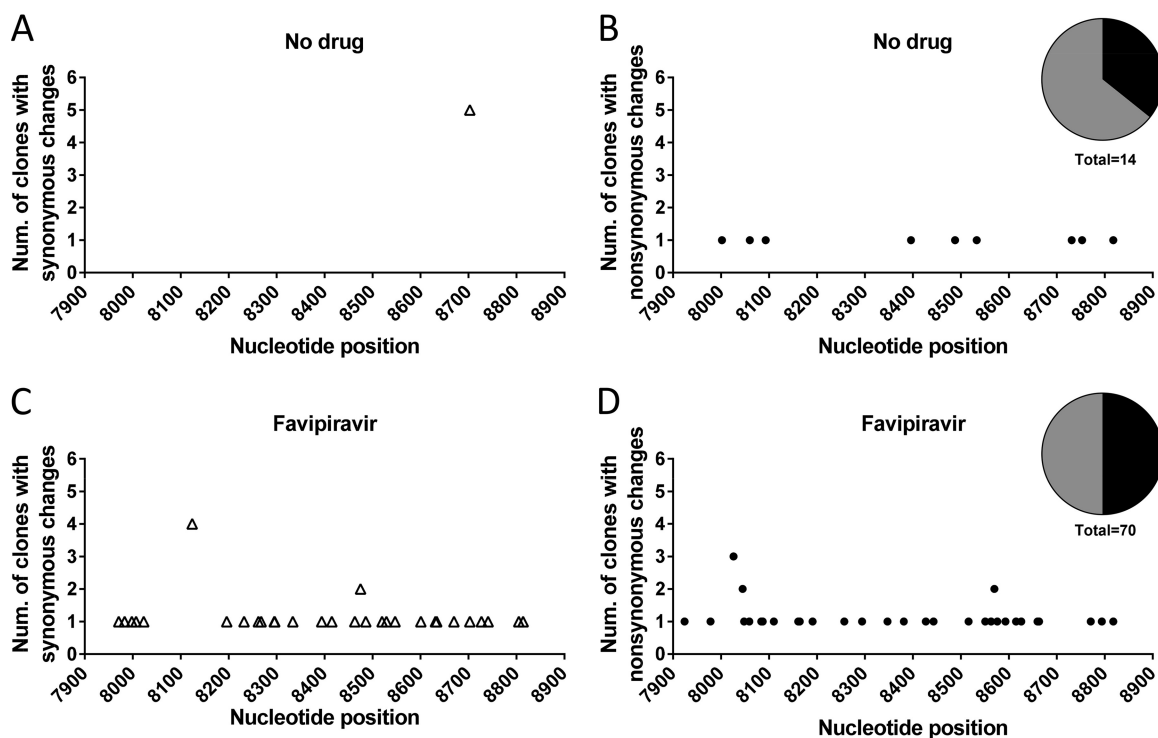

FIG 4 Distribution of mutations induced by favipiravir. Schematic representation of the number of clones with point mutations found in the mutant spectrum of the WNV NS5 gene fragment analyzed in the absence (A and B) or presence (C and D) of 1 mM favipiravir. Nucleotide positions of synonymous (open triangles in panels A and C) and nonsynonymous (solid circles in panels B and D) mutations are displayed. Pie charts representing the percentages of synonymous (black) and nonsynonymous (gray) mutations are included.

references 40 and 41). Studies of favipiravir-induced mutagenesis with purified RdRps may help in settling this issue.

Favipiravir administered orally (200 mg/kg of body weight) twice a day protected mice against experimental infection with WNV and reduced WNV RNA loads in animal tissues (42). Similar results were observed in a hamster treated with the same doses of favipiravir and infected with yellow fever virus (YFV) (43), a flavivirus also transmitted by mosquitoes, but the underlying mechanism was not addressed in any of these reports. Here, the mechanism of favipiravir inhibition of WNV multiplication *in vitro* has been analyzed. First, once it was established that favipiravir presented a low toxicity in Vero cells, its anti-WNV capacity was assayed using a wide range of drug concentrations. The CC_{50}, IC_{50}, and SI values suggest that favipiravir can be a good anti-WNV candidate, in agreement with its *in vivo* effect (42). These results are in line with those first reported for influenza virus (10, 29) and then for other RNA viruses, such as filoviruses (44), bunyaviruses (45), and hantaviruses (46).

To assess whether the anti-WNV activity of favipiravir could be associated with a mutagenic activity, the virus was serially propagated in Vero cells in the presence of different concentrations of the drug. At 2 mM, the virus was no longer detected after just one passage, whereas at 1 mM, it was undetectable by the third passage—results that are similar to those reported for influenza virus (34). Likewise, no virus was recovered after 10 serial passages in which the drug was present only during the first five passages. In addition, the specific infectivity, measured as the ratio of viral yield to genomic RNA, was reduced 26- and 95-fold after the first and second passages, respectively, in the presence of 1 mM favipiravir. The consensus sequence remained invariant. All these data, together with the low toxicity observed, point toward WNV extinction being associated with lethal mutagenesis.

Viral RNA from cell culture supernatants after two passages in the absence or presence of 1 mM favipiravir was amplified by RT-PCR, cloned, and sequenced to analyze the mutant spectra of the viral populations. Sequence analysis showed a 5.6-fold increase in the mutation frequency of the mutant spectrum associated with replication in the presence of favipiravir. Consistent with the structure of favipiravir, which renders it a substrate alternative to GTP and ATP (31, 39), a significant increase in G→A (21-fold) and A→G (6.5-fold) frequencies was associated with the presence of favipiravir. Different mutation patterns upon replication in the presence of favipiravir have been reported for other viruses: an enrichment in G→A and C→U mutations was observed in the case of influenza virus (32, 34) and hepatitis C virus (35), a slight increase of A→G and U→C mutations was found for murine norovirus (33), and no obvious outstanding mutation type was found in the case of foot-and-mouth disease virus (37). These differences suggest that the RdRp recognition of favipiravir varies among RNA viruses, again rendering plausible that depending on the virus-host system, an inhibitory or mutagenic activity by favipiravir may prevail. To have elucidated that mutagenesis is at least part of the mechanism of favipiravir against WNV is relevant to the design of effective antiviral strategies against this important pathogen. It is essential to use two or more antiviral agents to delay or prevent selection of RNA virus mutants that escape antiviral interventions. In fact, when one of the agents has a mutagenic activity, maximum efficacy is predicted with the sequential administration of a nonmutagenic inhibitor first, followed by the mutagen, but not the converse (47, 48). Therefore, our study positions favipiravir as an adequate antiviral agent to reach its maximum efficacy not in combination with nonmutagenic inhibitors but as the second drug in a sequential administration protocol.

Overall, our results demonstrate, for the first time, a mutagenic activity as part of the antiviral action of favipiravir on WNV infection in cell culture. This, together with the clinical safety record of favipiravir, which is already licensed in Japan against influenza virus (Avigan; Toyama Chemical Co., Ltd.) and in a clinical trial against Ebola virus (U.S. National Institutes of Health identifier NCT02662855; https://clinicaltrials.gov/ct2/show/NCT02662855) point to favipiravir as an interesting antiviral candidate against WNV and other medically relevant flaviviruses.

MATERIALS AND METHODS

Cells, virus infections, and virus titrations. Vero CCL-81 cells (ATCC, Manassas, VA) were grown in Eagle's minimal essential medium (EMEM [Lonza, Verviers, Belgium]) supplemented with 5% fetal bovine serum (HyClone; GE Healthcare, United Kingdom), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Lonza). Cells were maintained at 37°C and 5% CO_2 in a humidified incubator and used at low passage. The origin and passage history of the WNV strain NY99 (GenBank accession no. KC407666) have been described previously (49). For infections in liquid medium, WNV was incubated with Vero CCL-81 cell monolayers for 1 h at 37°C, and then the inoculum was removed and fresh medium containing 2.5% fetal bovine serum was added. The viral titer was determined 48 h postinfection by plaque assay in semisolid agarose medium (50). All infectious virus manipulations were performed in biosafety level 3 (BSL3) facilities.

Favipiravir treatment. Favipiravir (Atomax Chemicals Co., Ltd., Shenzhen, China) was diluted in distilled H_2O at a concentration of 20 mM, sterilized by filtration, and stored at −80°C until use. Vero cells were infected with WNV NY99 as described above in the presence of different concentrations of favipiravir. The 50% inhibitory concentration (IC_{50}) was determined as the concentration required to reduce the number of plaques to 50% relative to the untreated controls. WNV was subjected to serial passages in the presence of different concentrations of favipiravir in Vero cells seeded in 6-well plates (2×10^5 cells per well). In the first passage, cell monolayers were pretreated with the drug (or EMEM in untreated wells) for 16 h prior to infection. Viral adsorption (multiplicity of infection [MOI], 0.01 PFU/cell) was carried out in fresh medium containing the drug for 1 h at 37°C. Particles not attached to the cell were discarded by washing three times with EMEM, and infected cells were left in fresh medium in the presence or absence of favipiravir for 48 h, a time point at which 1/4 of the supernatant was used to infect fresh new monolayers of Vero cells in the presence of the same concentration of the drug. For serial blind passages ($n = 10$), the same protocol was used, except that no drug was present during the last five passages. Plaque assays were performed as previously described (50).

Selective index determination. Drug toxicity was examined by measuring the cellular ATP content with the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). The 50% cytotoxic concentration (CC_{50} [i.e., the concentration required to kill 50% of the cell population]) was determined after culturing confluent Vero cell monolayers with a wide range of drug concentrations (from 0 to 10 mM) in triplicate for 48 h. Then the selective index (SI) was calculated as the CC_{50}/IC_{50} ratio.

RNA extraction and qRT-PCR. Viral RNA was extracted from clarified supernatants of infected cells using a commercial kit (Speedtools RNA virus extraction kit, Biotools B&M Labs S.A, Madrid, Spain). Quantitative reverse transcription-PCR (qRT-PCR) was performed on a 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA) using a High Scriptools-Quantimix Easy Probes kit (Biotools) and previously described (51) primers and probe matching the 3' noncoding (NC) region to ensure detection of full-length RNA genomes. Viral RNA was quantified as genomic equivalents (GE) to PFU by comparison with RNA extracted from previously titrated samples (52, 53).

Viral mutation analysis. RNA extracted from supernatants of infected cells was amplified by RT-PCR (SuperScript One Step RT-PCR system, Invitrogen, Carlsbad, CA, USA), using specific primers (forward, 5'-CGAACCGGTCGGAAAAGTGA-3'; reverse, 5'-CAAAAACGCCCACAACCAGT-3'; enclosing nucleotides 7893 to 8841 of the WNV strain [GenBank accession no. KC407666]) (49), to yield a 949-bp cDNA fragment of the virus NS5 gene. Amplified fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI), and over 40 positive colonies from each sample were randomly chosen for bidirectional sequencing (Macrogen, The Netherlands). To avoid complexity biases due to redundant amplifications of the same initial RNA templates (54), amplifications were carried out with the undiluted samples only if a 1:1,000 dilution of the sample yielded an amplification band that was visible in stained agarose gels. The sequences were aligned and compared (SeqMan II software in DNAStar Package version 5.01) with the parental virus sequence as a reference, and each point mutation was recorded.

Statistical analyses. Statistical analysis was performed using Graph Pad Prism for Windows, version 6 (Graph Pad Software, Inc., San Diego, CA, 2005). Two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple comparisons and the chi-square test were used. Statistically significant differences are indicated by asterisks.

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