Avian virulence and thermostable replication of the North American strain of West Nile virus

Richard M. Kinney,1 Claire Y.-H. Huang,1 Melissa C. Whiteman,1† Richard A. Bowen,2 Stanley A. Langevin,3 Barry R. Miller1 and Aaron C. Brault3

1Division of Vector-borne Infectious Diseases, National Center for Zoonotic, Vector-borne and Enteric Diseases, Coordinating Center for Infectious Diseases, Centers for Disease Control and Prevention, US Department of Health and Human Services, PO Box 2087, Fort Collins, CO 80522, USA
2Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80522, USA
3Center for Vector-borne Diseases and Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA

The NY99 genotype of West Nile virus (WNV) introduced into North America has demonstrated high virulence for American crows (AMCRs), whilst a closely related WNV strain (KEN-3829) from Kenya exhibits substantially reduced virulence in AMCRs [Brault, A. C., Langevin, S. A., Bowen, R. A., Panella, N. A., Biggerstaff, B. J., Miller, B. R. & Nicholas, K. (2004). Emerg Infect Dis 10, 2161–2168]. Viruses rescued from infectious cDNA clones of both the NY99 and KEN-3829 strains demonstrated virulence comparable to that of their parental strains in AMCRs. To begin to define parameters that might explain the different virulence phenotypes between these two viruses, temperature-sensitivity assays were performed for both viruses at the high temperatures experienced in viraemic AMCRs. Growth curves of the two WNV strains were performed in African green monkey kidney (Vero; 37–42 °C) and duck embryonic fibroblast (DEF; 37–45 °C) cells cultured at temperatures that were tolerated by the cell line. Unlike the NY99 virus, marked decreases in KEN-3829 viral titres were detected between 36 and 120 h post-infection (p.i.) at temperatures above 43 °C. Replication of KEN-3829 viral RNA was reduced 6500-fold at 72 h p.i. in DEF cells incubated at 44 °C relative to levels of intracellular virus-specific RNA measured at 37 °C. In contrast, replication of virus derived from the NY99 infectious cDNA at 44 °C demonstrated only a 17-fold reduction in RNA level. These results indicated that the ability of WNV NY99 to replicate at the high temperatures measured in infected AMCRs could be an important factor leading to the increased avian virulence and emergence of this strain of WNV.

INTRODUCTION

In North America, West Nile virus (WNV; family Flaviviridae, genus Flavivirus) has become the leading cause of arboviral encephalitis in humans and equines (O’Leary et al., 2004) and is associated with mortality in >200 avian species (Hayes et al., 2005). The North American WNV strain, NY99, is highly virulent for American crows (AMCRs; Corvus brachyrhynchos) (Komar et al., 2003; McLean et al., 2001). AMCRs inoculated with this virus develop peak viraemic titres in excess of 10 log10 p.f.u. ml−1 and suffer 100% mortality within 6 days post-infection (p.i.) (Brault et al., 2004; Komar et al., 2003).

Mortality in migratory storks and domesticated geese was identified in Israel between 1997 and 2000 (Bin et al., 2001; Malkinson et al., 2002), where a strain almost identical to the NY99 genotype has circulated (Lanciotti et al., 1999). Other studies have demonstrated that WNV strains from Africa and Australia cause reduced viraemia and mortality in AMCRs compared with the NY99 strain (Brault et al., 2004). These experimental avian infection data, coupled with the genetic relatedness of avian virulent strains, indicate that viral genetic determinants are responsible for the emergence of WNV-associated avian mortality.

The WNV genome is a single-stranded, positive-sense RNA of approximately 11 kb. The single open reading frame has 5′- and 3′-terminal non-coding regions (NCRs) and encodes a polyprotein, which is co- and post-translationally cleaved by viral and host proteases to yield three structural
proteins (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Lindenbach & Rice, 2003).

One of the 11 amino acid differences previously identified between the translated polyproteins of the AMCR-virulent NY99 strain and the relatively attenuated KEN-3829 strain of WNV is a Pro→Thr (NY99→KEN-3829) substitution at residue 249 of the NS3 protein (NS3–249) (Brault et al., 2000). An amino acid mutation at NS3–250 is a genetic determinant of temperature sensitivity of the candidate dengue serotype 2 (DEN-2) vaccine virus, strain PDK-53 (Butrapet et al., 2000). To investigate whether temperature sensitivity might contribute to the attenuated phenotype of WNV KEN-3829 in AMCRs, we measured the body temperatures of virus-infected AMCRs (40.5–44.5 °C) and determined the replication kinetics of the NY99 and KEN-3829 strains at different temperatures in mammalian and avian cell cultures. Additionally, we describe here in full the generation of a previously reported infectious clone of WNV NY99 (Beasley et al., 2005), as well as the construction of an infectious clone of the African KEN-3829 strain. We report phenotypic characterization of viruses generated from these constructs in temperature-sensitivity experiments in vitro and in the in vivo AMCR model.

METHODS

Cells and viruses. Duck embryonic fibroblast (DEF; ATCC CCL-141) and African green monkey kidney (Vero) cells were utilized for temperature-sensitivity studies. Vero cells were maintained in Iscove’s modified Dulbecco’s modified Eagle’s medium (HyClone) containing 5% FBS, penicillin (100 U ml⁻¹) and streptomycin (100 µg ml⁻¹). DEF cells were maintained in Eagle’s minimal essential medium (MEM) with Hank’s salts (Invitrogen) containing 5% FBS and antibiotics. Incubator temperatures were calibrated with NIST-traceable, factory-calibrated thermometers (−1 to 51 °C; ERTC0).

Infectious cDNAs were generated from low-passage viruses. The NY99 virus (strain 382-99) was isolated from the brain of a Chilean flamingo (Phoenicopterus chilensis) and used at passage CE-1/LLC-MK₂(-1 (CE, chicken embryo fibroblast cells; LLC-MK₂, rhesus monkey kidney cells). The KEN-3829 virus was isolated from a pool of male mosquitoes (Culex univittatus; Miller et al., 2000) and used at passage Vero-1/BHK-21-1 (BHK, baby hamster kidney cells).

Crows. Hatch-year AMCRs were netted, banded, bled to determine pre-existing neutralizing antibodies against WNV and St Louis encephalitis virus, transported to Fort Collins, CO, USA, and housed at the USDA Wildlife Diseases Laboratory and the Colorado State University Animal Disease laboratory in groups of two in 1 m³ cages. Crows were fed a combination of cat and dog food ad libitum, as described previously (Braught et al., 2004).

Construction of a two-plasmid, infectious cDNA clone of WNV. We previously utilized the NY99 infectious clone (Beasley et al., 2005). Here, we describe in detail the construction of the pWN-AB and pWN-CG plasmids and recovery of the clone-derived WN/IC-P991 virus. Seven cDNA fragments (A–G) were amplified from WNV NY99 by RT–PCR (Titan One Tube RT–PCR kit; Roche) to permit splicing at restriction sites XhoI–453 (number indicates genomic nucleotide position), NgOMIV–2495, SplI–3623, KpnII–5336, StuI–7024 and XhoI–8870. The 5′-terminal primer included the sequence 5′-GAGCTC/ACGGCGT/AATAATTAACGACTCAGTCATA/ AGTATTGCGTGTCGAGTCTGCAAAAAC-3′ (delimiters indicate the SacI/MluI/T7 promoter and the 5′-terminal 28 nt of the WNV genome, respectively. The Nhel site was created and then changed to wild-type sequence to correct a clone error. A unique XbaI site was engineered at the 3′-terminus of the genomic cDNA. A natural NgOMIV–2076 site was removed by engineering a silent G7029T mutation, creating a SacI–7024 site and making the natural upstream NgOMIV–2495 site unique. Plasmids were replicated in Escherichia coli XL-1 Blue cells (Kinney et al., 1997). Although we derived infectious NY99 virus from a single, full-genome-length infectious clone (pWN/IC-24), we determined that such single clones were unstable during propagation in bacteria. Therefore, we employed a two-plasmid strategy, as described previously for our chimeric DEN-2/DEN-3 virus (Huang et al., 2003). Plasmid pWN-CG contained WN/Virulent NY99 nt 1–2495 cloned into the SacI/NgOMIV sites of the previously described intermediate plasmid, pD2U/D3 (Huang et al., 2003). Some residual DEN-2 cDNA remained in pWN-AB (Fig. 1). Plasmid pWN-CG, containing from WN/Virulent NY99 nt 2495 to the 3′ genomic terminus, was derived from subclones C, D, E, F and G. Plasmids pWN-AB and pWN-CG were digested with AseI and MluI, respectively, treated with calf intestinal alkaline phosphatase (CIP; New England Biolabs), purified over spin columns (QIAquick PCR Purification kit; Qiagen) and then digested with NgOMIV (Fig. 1). The small, excised AseI–NgOMIV and MluI–NgOMIV fragments were removed by eluting the larger DNA fragments from a second spin column. The cut pWN-AB and pWN-CG DNAs were ligated at the NgOMIV–2495 site, cut with XbaI, treated with protease K (Invitrogen), extracted with phenol: chloroform: isoamyl alcohol (25:24:1; Amresco) and chloroform, and ethanol precipitated (Fig. 1).

KEN-3829 virus-specific cDNA was spliced into the NY99-specific pWN-AB and pWN-CG clones to construct the pWN/IC-KEN infectious clone. Due to extensive sequence identity between the viruses, we constructed pWN/IC-KEN by retaining NY99-specific nucleotides between restriction sites KsnI–1481 and NgOMIV–2495 (26 silent nucleotide substitutions) in the pWN/AB plasmid and between BstBI–5146 and KpnI–5336 (eight silent substitutions) and KpnI–7757 and AatII–10198 (75 silent substitutions) in the pWN/CG plasmid. The remaining cDNA was contributed by the KEN-3829 virus. A capsid A60P amino acid substitution, which resulted from a G274C nucleotide mutation in a KEN-3829 virus-specific cDNA clone, was retained in pWN/IC-KEN.

Recovery of clone-derived WNVs. Following XbaI linearization, virion genomic RNA was transcribed for 1–2 h at 37 °C (Ampliscribe T7 Transcription kit; Epicentre). Reactions included 6 mm mG (–5′)A cap analogue (New England Biolabs), 20 % of the manufacturer-recommended concentration of ATP and 0–2–0.5 µg pWN/IC-CG DNA or 0–5–2 µg in vitro-ligated pWN-AB/pWN-CG DNA. Reactions were treated with DNase I (Epicentre), extracted with phenol: chloroform: isoamyl alcohol and chloroform, and precipitated with ethanol. The RNA pellets were resuspended in 20 µl water and 2 µl aliquots were analysed by non-denaturing agarose gel electrophoresis to visualize genome-length RNA transcripts. Transcribed RNA was transfected by electroporation into BHK-21 cells (Kinney et al., 1997). Transfected cells were transferred to 75 cm² flasks. Viruses were harvested when cytopathic effects (CPE) were clearly evident, and titres were determined by plaque assay (Braught et al., 2004). Virus derived from pWN/IC-24 was designated WN/IC-24. The viruses WN/IC-P991 (parental NY99, clone 1) and WN/IC-KEN (KEN-3829) were recovered from the two-plasmid (pWN/IC-P991 and pWN/IC-KEN) strategy.

Nucleotide sequence analyses. Sequencing of WNV cDNA in plasmids and overlapping cDNA fragments amplified from viral RNA genomes by RT–PCR was performed (CEQ 8000 Genetic
Analysis System; Beckman Coulter). Primer sequences are available from the authors on request. The extreme 5'-terminal sequence of each WNV NY99 and KEN-3829 RNA genome was determined using 5'-rapid amplification of cDNA ends (RACE; Invitrogen) (Kinney et al., 1997). The extreme 3'-terminal sequence was determined by employing E. coli poly(A) polymerase to tail the RNA with poly(A), followed by RT-PCR using virus-specific and oligo(dT) primers, as described previously (Kinney et al., 1997).

Inoculation and serum sampling of AMCRs. AMCRs were injected subcutaneously in the breast with approximately 1500 p.f.u. per 0.1 ml of clone-derived WN/IC-P991 or WN/IC-KEN diluted in PBS. AMCRs were bled daily for 7 days after infection by jugular venipuncture and monitored daily for signs of disease for 14 days p.i. Each 0.2 ml blood sample was added to 0.8 ml BA-1 diluent [Hanks’ M-199 salts, 0.05 M Tris/HCl (pH 7.6), 1 % BSA, 0.35 g sodium bicarbonate ml⁻¹, 100 µg penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 1 µg Fungizone ml⁻¹]. Coagulation was allowed to take place at room temperature for 30 min. The samples were then placed on ice, centrifuged for 10 min at 4000 g and frozen at −80 °C until titrated for infectious units. Daily body temperatures were measured from the cloaca.

Temperature-sensitivity studies. Vero and DEF cultures grown in 75 cm² flasks were infected in triplicate at an m.o.i. of 0.01 with viruses NY99, WN/IC-24, WN/IC-P991, WN/IC-KEN or KEN-3829. Following adsorption for 1 h at 37 °C, the monolayers were washed with PBS, 20 ml of fresh medium was added and the cultures were incubated at various temperatures (Veros: 37, 40 and 42 °C; DEFs: 37, 43, 44-5 and 45 °C). Aliquots of culture medium were removed at 12–24 h intervals, diluted 1:20 in medium containing 20 % FBS and frozen at −80 °C.

Quantification of viral RNA. WN/IC-P991 and WN/IC-KEN were each inoculated at an m.o.i. of 0.01 on to six replicate DEF monolayers in 25 cm² flasks. Following a 1 h adsorption period, the cells were washed three times with PBS, supplemented with 6 ml medium and then incubated at 37 or 44 °C. At 24, 48 and 72 h p.i., the culture medium was removed from duplicate cell monolayers, each monolayer was washed three times with PBS and 250 µl Trizol (Invitrogen) was added to each flask. Each cell lysate was added to a QIAshredder spin-column (Qiagen) and total RNA was chloroform-extracted from each homogenate, ethanol precipitated and subjected to single-cycle reverse transcription using homologous positive-sense (5'-CACTCTTTTGAAGGAGAATGGTGTGG-3'; nt 2697–2722) and negative-sense (5'-GAGTTATCCAAAATCCAACTACTGAGG-3'; nt 4568–4594) WNV-specific primers. The resulting cDNAs were eluted from spin columns (Qiagen) in 50 µl nuclease-free water. A 10 µl aliquot of each sample was added to a master mix containing 250 nM probe (5'-6-FAM-AGAGCCATACACTTCCA-3'; nt 3955–3972), 700 nM of forward primer (5'-TGAATTCCTGGCG-GTACCTT-3'; nt 3923–3943) and 900 nM of reverse primer (5'-CGAAGACACCAACTGTTGATG-3'; nt 3977–3996) and WNV-specific
RNA was measured by quantitative PCR using homologous RNA standards on an ABI 7500 Real-time PCR System (Applied Biosystems).

**In vitro transcription/quantification of RNA standards.** cDNA fragments (2-5 kb) of both pWN-CG-P991 and pWN-CG-KEN plasmids were subcloned into the pBluescript SKII (+) vector (Stratagene) using the PsiI sites at viral genomic positions 3405 and 5954. The recombinant plasmids were linearized with *SmaI*, treated with proteinase K, extracted with phenol:chloroform:isoamyl alcohol and precipitated with ethanol. RNA standards were treated with DNase I (Epicentre), purified using an RNA mini-elution kit (Qiagen) and eluted in 20 μl nuclease-free water. RNA was quantified by spectrophotometry (NanoDrop Technologies) and converted to RNA copy number ml⁻¹. A serial 10-fold dilution series, starting with 10⁹ copies, was made for each RNA standard, aliquoted into single-use tubes and stored at −80 °C until use.

**RESULTS**

**Generation of infectious viruses**

We recovered infectious virus from a single, full genome-length clone (pWN/IC-24) at day 7 post-transfection. Upon sequencing the pWN/IC-24 clone, we discovered a C3342A mutation, which encoded an in-frame NS1-291 TGC → TGA codon mutation (Cys to a stop codon). This substitution was not present in the pWN-DEFG subclone used in the construction of pWN/IC-24. Therefore, the mutation occurred during amplification of pWN/IC-24 in *E. coli*. WN/IC-24 virus contained an A3342T (plasmid-to-virus) mutation, probably a T7 polymerase transcription error at nt 3342. In three additional experiments, pWN/IC-24 transcription/transfection resulted in delayed recovery of virus containing C, mixed C/T or mixed T/C residues at the nt 3342 locus.

Infectious viruses were readily derived from the two-plasmid (pWN-AB and pWN-CG) infectious clone system within 4 days of electroporation, at which time the viruses were harvested. Viruses were amplified in LLC-MK₂ cells to produce the working seeds for phenotypic analyses.

**Sequence analyses of the viral genomes**

Sequence comparison of WNV NY99 and KEN-3829 has been reported previously (Charrel *et al.* 2003), with the strains differing at 22 nucleotide positions in the 3’ NCR and 11 amino acid residues in the translated polyprotein, including (changes indicated as NY99 → KEN-3829): C-L3N, C-V8A, E-I126T, E-V159I, N51-A70S, N52A-T52A, N52B-V103A, N53-P249T, N53-T356I, N54A-A85V and N54B-E249D. The KEN-3829 sequence (GenBank accession no. AY262283) lacked the 5’-terminal 36 nt and 3’-terminal 8 nt. In this study, we determined that the NY99 and KEN-3829 5’- and 3’-terminal sequences were identical (5’ terminus: 5’-AGTAGTTCGCTGTGAGCTGACAAACTTAGT-AGTGT TT-3’; 3’ terminus: 5’-CCTGGTGTGCGAGAACACAGAGATC-3’).

A comparison of the nucleotide sequences of WNV NY99 CE-1 and CE-1/LLC-MK₂-1 seeds and WN/IC-P991 virus with the published sequence of NY99 strain 382-99 (GenBank accession no. AF196835; Lanciotti *et al.*, 1999) is shown in Table 1. A comparison of AF196835 and WN/IC-P991 has been reported previously (Beasley *et al.*, 2005). WN/IC-P991 virus contained three silent nucleotide substitutions, a single amino acid substitution each in

<table>
<thead>
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<th>Genome nucleotide position</th>
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<th>WN NY99 viruses reported in this study†</th>
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<td></td>
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<td>CE-1</td>
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<td>T/A (Ile/Asn)§</td>
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<td>G (3’ NCR)</td>
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*GenBank accession number AF196835 (Lanciotti *et al.*, 1999).†Virus seeds at cell culture passage levels CE-1 and CE-1/LLC-MK₂-1 and after recovery of clone-derived, infectious WN/IC-P991 virus (passage BHK-21-0/LLC-MK₂-1).§The nucleotide is shown, with the encoded amino acid in parentheses.¶Mixed locus in the viral population.¶¶Silent cDNA error retained as a clone marker.¶¶¶Engineered silent mutation in the pWN/IC-P991 infectious cDNA clone to remove the natural NgoMIV-7026 restriction enzyme site.
NS2A and NS3 and a nucleotide substitution in the 3’ NCR relative to the previously published sequence.

**Avian virulence**

Fig. 2(a) shows the mean body temperatures of AMCRs following inoculation with WN/IC-P991 or WN/IC-KEN. Although 16 AMCRs were inoculated with WN/IC-KEN, temperatures were measured only in a subset of eight individuals. Mean temperatures varied from about 41°C to 43.6°C. Temperatures at time 0 averaged somewhat higher than those at 1–3 days p.i. Stress induced by the first prolonged handling may have caused this apparent early temperature spike (Cabanac & Aizawa, 2000; Kendeigh, 1969). A febrile response was apparent by 4–5 days p.i. in both groups; however, there was considerable variation in the measured body temperatures and no uninfected control group was included for comparison. Temperatures of 43.0–44.0°C were measured in all eight AMCRs in the WN/IC-P991 group at 4 days p.i. and temperatures of 43.5–44.0°C were measured in five of the six AMCRs in this group that survived at 5 days p.i. The sixth day 5 survivor had a measured temperature of 42.5°C. Temperatures in the WN/IC-KEN group ranged from 43.0–44.5°C in seven, seven, five and six of the eight animals at 4–7 days p.i., respectively. The highest temperature measured was 44.5°C in one WN/IC-KEN-infected bird at 5 days p.i. Overall, no temperature differences were identified between the WN/IC-P991 and WN/IC-KEN groups.

Despite the high temperatures observed in virus-infected AMCRs (Fig. 2a), WN/IC-P991 replicated efficiently, reaching a mean peak viraemic titre of 9.4 log_{10} p.f.u. ml^{-1} by 4 days p.i. (Fig. 2b). The WN/IC-P991 viraemic profile was similar to that reported previously for AMCRs infected with WNV NY99 (Brault et al., 2004). WN/IC-P991 was also fatal for 100% of the infected birds. Mean survival times were similar following challenge with WNV NY99 (5.1 ± 0.6 days) (Brault et al., 2004) or WN/IC-P991 (5.8 ± 0.7 days) (current study).

The NY99 results contrasted with the poor in vivo replication profile of WN/IC-KEN in AMCRs (Fig. 2b). This virus replicated to a mean peak viraemic titre of 5.1 log_{10} p.f.u. ml^{-1} by 5 days p.i. Although the viraemic profile of WN/IC-KEN in this experiment (Fig. 2b) was somewhat higher in magnitude than that reported earlier for KEN-3829 WNV (4.0 log_{10} p.f.u. ml^{-1} mean peak titre; Brault et al., 2004), the difference was not statistically significant. Measurable viraemia did not develop until 2–3 days p.i. with either of these two viruses. AMCRs infected with WN/IC-KEN-3829 and WN/IC-KEN suffered only 25% (Brault et al., 2004) and 31–25% mortality (Fig. 2c), respectively, and the survival times of those birds that succumbed to infection were extended to 8 days p.i. or longer, compared with 4–6 days for crows infected with the NY99 virus-specific strains. The mean morbidity/mortality survival times of the birds infected with KEN-3829 (10.5 ± 2.1 days) (Brault et al., 2004) and WN/IC-KEN (9.6 ± 1.1 days) (Fig. 2c) were similar.

**Phenotypes of WNVs NY99 and KEN-3829 and their clone-derived viruses**

WNV NY99, WN/IC-P991, WN KEN-3829 and WN/IC-KEN exhibited almost identical plaque phenotypes at 4 days p.i. in Vero cells (Fig. 3a–d, respectively). Mean diameters of 12 plaques for each virus were 3.3 ± 0.3, 3.2 ± 0.3, 3.1 ± 0.3 and 3.3 ± 0.1 mm, respectively. WNV NY99 and WN/IC-P991 (Fig. 3e) exhibited essentially identical robust replication profiles at 37°C in Vero cells.
as did WNV KEN-3829 and WN/IC-KEN (Fig. 3f) in a separate experiment. These results agreed with previously determined replication profiles for WNV NY99 and its clone-derived NY99ic virus (WN/IC-P991 here) in Vero cells (Beasley et al., 2005). Similarly, WNV NY99 and WN/IC-P991 (Fig. 3g) showed similar replication profiles at 37°C in DEF cells, as did WNV KEN-3829 and WN/IC-KEN (Fig. 3h). WNV NY99 and its clone-derived virus have also been shown to exhibit almost identical neuroinvasive and neurovirulence phenotypes in mice (Beasley et al., 2005). These comparative phenotypic data provided confidence that WN/IC-P991 and WN/IC-KEN were faithful representations of their parental viruses.

**Temperature-sensitivity studies in Vero cells**

We tested the abilities of WNV KEN-3829 and NY99 and the two NY99 virus-specific clone-derived viruses WN/IC-P991 and WN/IC-24 to replicate in Vero cells at 37, 40 and 42°C (Fig. 4). CPE was evident in control Vero cultures within 36 h of being transferred from 37 to 43°C or higher, and virus replication was not supported (data not shown). No significant replication differences were observed among the NY99-specific viruses at any of the three temperatures tested. Although WNV KEN-3829 replicated similarly to the NY99 viruses from 12 to 48 h.p.i. in Vero cells at 42°C, it showed more rapidly declining titres between 60 and 120 h.p.i., with an approximately 10–20-fold reduction in titre at 72 h.p.i. relative to the NY99-specific viruses.

**Temperature-sensitivity studies in DEF cells**

The replication profile of WNV KEN-3829 in Vero cells at 42°C indicated that this strain might exhibit a more significant temperature-sensitive (ts) phenotype at even higher temperatures. DEF cells were cultured successfully at temperatures of up to 45°C for up to 6 days. These cells were infected at 37°C with WN/IC-P991 or WN/IC-KEN and then cultured at 37, 43, 44, 44.5 and 45°C (Fig. 5). These temperatures encompassed the range of temperatures that was observed in virus-infected AMCRs (Fig. 2a). These two viruses demonstrated essentially identical replication profiles in DEF cells at 37°C. Although the replication efficiencies of both viruses progressively declined as the temperature was increased, WN/IC-KEN exhibited much greater reductions in titre after the first 36–48 h.p.i. (Fig. 5). At 44°C, a temperature that was comparable to the high temperatures that were frequently identified in

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**Fig. 3. In vitro replication phenotypes of parental and clone-derived WNVs. (a–d) Plaques at 4 days p.i. in Vero cells overlaid with agarose and stained with neutral red. Images were taken with a Bio-Rad VersaDoc Imaging System. (e, f) Replication profiles at 37°C in Vero cells. (g, h) Replication profiles at 37°C in DEF cells. Mean (±SD) titres are from triplicate cultures. The detection limit was 100 p.f.u. ml⁻¹. ■, WN/IC-P991; □, WNV NY99; ○, WNV KEN3829; ●, WN/IC-KEN.**
as observed in two independent experiments conducted at 84–96 h p.i. These were reproducible replication patterns, and complete CPE developed in the cultures at 37°C (a), 40°C (b) and 42°C (c). Mean (±SD) titres are from triplicate cultures. The detection limit was 100 p.f.u. ml⁻¹.

Fig. 4. Growth curves of NY99 ( ■ ), WN/IC-P991 ( ■ ), WN/IC-24 ( ▲ ) and KEN-3829 ( ○ ) in Vero cells incubated at 37°C (a), 40°C (b) and 42°C (c). Mean (±SD) titres are from triplicate cultures. The detection limit was 100 p.f.u. ml⁻¹.

WNV-infected AMCRs, WN/IC-KEN-infected cells developed CPE and the virus grew to titres that were comparable to those of WN/IC-P991 during the initial 36–48 h p.i. in DEF cells. However, following the initial 48 h p.i. period, the KEN-3829 virus-specific strain failed to generate any additional CPE and titres rapidly waned up to 120 h p.i. At 44°C, the WN/IC-P991 viral titre peaked at 60 h p.i. and complete CPE developed in the cultures at 84–96 h p.i. These were reproducible replication patterns, as observed in two independent experiments conducted at 44°C (Fig. 5c, d). At 45°C, WN/IC-P991 still replicated to a peak titre of nearly 5 log₁₀ p.f.u. ml⁻¹ by 96 h p.i. (Fig. 5f), whereas replication of WN/IC-KEN was undetectable (<100 p.f.u. ml⁻¹). Although temperature-related CPE became evident in uninfected control cultures at 45°C, CPE development was more rapid and extensive in the DEF flasks infected with WN/IC-P991.

WN/IC-KEN exhibited a consistent decrease in replicative ability at temperatures above 43°C in DEF cells relative to the NY99 strain (Fig. 5). We performed a third growth curve experiment at 44°C in DEF cells to compare the replication profiles of the parental WNV KEN-3829 and its clone-derived WN/IC-KEN virus, relative to replication of WN/IC-P991, at this high incubation temperature. WNV KEN-3829 and WN/IC-KEN exhibited almost identical replication profiles at 37°C (Fig. 6a) and 44°C (Fig. 6b), including the decreased replicative ability that was previously demonstrated (Fig. 5c, d) for WN/IC-KEN at 44°C at 36–48 h p.i. To ensure that we were measuring differences in virus replication rather than physical stability, WN/IC-P991 and WN/IC-KEN were diluted in MEM containing 5% FBS and incubated for 120 h in tubes at room temperature, 37 and 44°C. Both viruses exhibited less than a 10-fold decrease in titre over 120 h at room temperature (Fig. 6c, d). Rapid loss of infectivity occurred for both viruses at 37 and 44°C. At 44°C, both viruses lost ≥1·5 log₁₀ p.f.u. ml⁻¹ infectious titre per 24 h period during the first 48 h of incubation. These data supported the contention that the different replication profiles of these two viruses represented genuine differences in their replicative abilities at 44–44·5°C in DEF cultures (Fig. 5).

RNA quantification

We also measured intracellular levels of viral RNA in DEF cells incubated at 37 and 44°C (Fig. 7). RNA extracted from lysates of DEF cells infected with WN/IC-P991 and WN/IC-KEN demonstrated comparable levels of RNA at 24 h p.i. (Fig. 7). However, no significant increase in total WN/IC-KEN RNA was identified between 24 and 48 h p.i. at 44°C, despite the approximately 100-fold increase in WN/IC-KEN RNA during this period at 37°C. Additionally, WN/IC-P991 viral RNA replication was reduced by only 17-fold at 72 h p.i. at 44°C relative to the RNA level of this virus measured at 37°C. In contrast, WN/IC-KEN RNA levels were reduced in excess of 6500-fold at 44°C compared with at 37°C. This marked reduction in the intracellular level of WN/IC-KEN viral RNA correlated well with the reduction in progeny WN/IC-KEN (and WNV KEN-3829) released from virus-infected DEF cells incubated at 44°C and quantified by plaque assay of infectious cell-culture medium (Figs 5c, d, and 6b).

DISCUSSION

Dead bird densities, notably from AMCRs and other corvids, have served as a hallmark sentinel for tracking the spread of WNV in North America (Eidson et al., 2001a, b, c; Watson et al., 2004). WNV NY99 causes high-titre viraemia and mortality in naturally (Steele et al., 2000; Wunschmann et al., 2004; Yaremchuk et al., 2004) and experimentally (Komar et al., 2003; McLean et al., 2001; Weingartl et al., 2004) infected AMCRs. Experiments with WNV strains that are not associated with epizoonitcs have confirmed the viral genetic basis for the high virulence of WNV NY99 (Braught et al., 2004; Langevin et al., 2005). The clone-derived viruses WN/IC-P991 and WN/IC-KEN reported in the current study exhibited virulence phenotypes in AMCRs similar to those reported for the parental viruses. Viral virulence often correlates well with high and prolonged viraemia. Whilst extensive pathology has been demonstrated in AMCRs following infection with WNV NY99 (Anderson et al., 1999; Panella et al., 2001; Steele et al.,
Genetic determinants of viral virulence and attenuation can occur in both structural and non-structural genes. Chimeric flaviviruses expressing the prM/E gene region of WNV NY99 have exhibited attenuated phenotypes with respect to both WNV and the flaviviral vector, possibly indicating that one or more WNV virulence determinants resides outside of this structural gene region. Experimental infection of fish crows (Corvus ossifragus) with chimeric yellow fever virus (YFV)/WNV virus resulted in low viraemias and poor seroconversion rates (Langevin et al., 2003). However, the 40 °C or higher core body temperature of birds (Kendeigh, 1969; Page, 1965; Torre-Bueno, 1976; Verbeek & Caffrey, 2002) might interfere with YFV replication proteins. Chimeric DEN-2 (Huang et al., 2005) and DEN-4 (Pletnev et al., 2002) viruses expressing WNV prM/E were attenuated for newborn and 3-day-old mice, respectively, following intracranial challenge with 4 log_{10} p.f.u. of chimeric virus. Chimeric DEN-2/WNV was also avirulent for adult AG129 mice deficient in alpha/beta interferon (IFN-α/β) and IFN-γ responses (Calvert et al., 2006). Temperature sensitivity of a candidate DEN-2 vaccine virus, strain PDK-53, has been mapped to amino acid substitutions at NS1-53 and NS3-250 (Butrapet et al., 2000). Interestingly, WNV KEN-3829 has amino acid substitutions in both NS1 and NS3, relative to WNV NY99, and the sole non-conservative amino acid substitution between NY99 and KEN-3829 consists of a Pro→Thr substitution (NY99→KEN-3829) at NS3-249. We hypothesize that substitutions in non-structural genes might encode the in vitro ts and avian attenuation phenotypes of WNV KEN-3829 relative to WNV NY99.

Temperatures of 40·2 (±0·6 SD) to 41·4 °C (±0·8 SD) have been reported for AMCRs, although there is little information regarding temperature in these birds (Verbeek & Caffrey, 2002). Core body temperatures of passerine birds, such as the house sparrow (Passer domesticus), average approximately 40·5 °C, although their temperature may increase to 45 °C during periods of stress or activity.
Temperatures of 43–45°C represent high physiological temperatures for birds, as core body temperatures of 46–47°C are rapidly fatal for some bird species (Kendeigh, 1969; Torre-Bueno, 1976). The greater apparent temperature sensitivity of KEN-3829 at 42°C in Vero cells and at 43–45°C in DEF cells relative to the NY99 genotype was an interesting finding in light of the high temperatures that occurred in the WNV-infected AMCRs and the decreased replicative ability of KEN-3829-specific strains in these birds. Although WN/IC-P991 exhibited increasingly significant reductions in replicative capability in DEF cells as incubation temperature varied from 43–45°C, this NY99 strain nevertheless maintained a replicative advantage over WN/IC-KEN.

Although WN/IC-KEN replication was not detected in DEF cells incubated at 45°C, its replication profile at 43–44.5°C was similar to that of WN/IC-P991 during the initial 36–48 h p.i., after which WN/IC-KEN viral titres rapidly waned. The replication of KEN-3829 viral RNA was greatly reduced at 44°C in DEF cells, particularly after 48 h p.i., indicating that compromised replication of viral RNA was a major factor in the reduction of infectious progeny at higher temperatures.

**Fig. 6.** (a, b) Growth curves of WN/IC-P991 (■), WNV KEN-3829 (○) and WN/IC-KEN (●) in DEF cells incubated at 37°C (a) or 44°C (b). Mean (±SD) titres are from triplicate cultures. The detection limit was 100 p.f.u. ml⁻¹. (c, d) WN/IC-P991 and WN/IC-KEN were incubated at room temperature (■), 37°C (○) or 44°C (●) in tubes containing cell culture medium. Mean (±SD) titres of duplicate samples are plotted.

**Fig. 7.** Quantitative RT-PCR of viral RNAs extracted from cellular lysates of virus-infected DEF cultures incubated at 37 and 44°C. Mean (±SD) values of duplicate cultures are plotted. The detection limit was 800 RNA copies per flask. □, WN/IC-P991 (37°C); ■, WN/IC-P991 (44°C); ○, WN/IC-KEN (37°C); ●, WN/IC-KEN (44°C).
temperatures. These data suggested that WN/IC-P991 and WN/IC-KEN replicated to a similar extent early after infection, but that thereafter replication of WN/IC-KEN was suppressed, perhaps by a delayed, inducible host-cell response.

Upon viral infection of a host cell, latent intracellular transcription factors are induced, resulting in the mounting of an innate antiviral defence (Sen, 2001). Type I IFN-α/β and the IFN-stimulated genes (ISGs) constitute a major component of this innate antiviral response. WNV NY99 has been shown to delay the phosphorylation and activation of the transcription factor IFN regulatory factor 3, resulting in the delayed transcription of a number of ISGs until 24–36 h p.i. in mammalian cell cultures (Fredericksen & Gale, 2006; Fredericksen et al., 2004). Similar results were demonstrated following infection of avian cells with a negative-strand RNA virus, Newcastle disease virus (Munir et al., 2005). The latter investigators demonstrated delayed induction of five out of seven ISGs until 36 h p.i. following infection of primary chick embryo cells with this paramyxovirus. These reported delays in the induction of critical antiviral response genes are very similar to the 36–48 h window of equivalent replication that we observed for WN/IC-P991 and WN/IC-KEN immediately after infection of cells. We speculate that both WN/IC-P991 and WN/IC-KEN suppress intracellular antiviral response factors such as IFN production and transcription of ISGs early during the infection cycle. The more significant ts phenotype exhibited by WN/IC-KEN at 36–48 h p.i. may have been due to a greater sensitivity to delayed antiviral host factors. To date, the flaviviral non-structural proteins NS2A, NS2B, NS3, NS4A, NS4B of Kunjin virus (KUNV) (Liu et al., 2004, 2005) and NS2A, NS4A and NS4B of DEN-2 (Muñoz-Jordán et al., 2003) have been shown to inhibit IFN signalling in mammalian cell cultures. Furthermore, an A30P amino acid substitution in NS2A of KUNV disabled the ability of this virus to block induction of IFN-α/β in cell culture and attenuated viral virulence in mice (Liu et al., 2006). WNV KEN-3829 has amino acid substitutions in the C, E, NS1, NS2A, NS2B, NS3, NS4A and NS4B proteins relative to the AMCR-virulent WNV NY99 (Brault et al., 2004; Charrel et al., 2003). We speculate that one or more of the WNV KEN-3829 virus-specific amino acid substitutions generated the ts phenotype and compromised the ability of this virus to fully inhibit, to an NY99-specific level, one or more components of the innate antiviral response in virus-infected cells. A possible temperature-dependent increased sensitivity to effectors of innate immunity, such as IFN and ISGs, might contribute to decreased KEN-3829 virus replication and spread, and result in the relatively attenuated phenotype of this virus observed in AMCRs. Other mechanisms may be involved in the relative ts phenotype of KEN-3829. At the high incubation temperatures employed in this study, less than optimal conformations of WNV KEN-3829 viral structural or non-structural proteins resulting from amino acid substitutions, relative to the NY99 strain, might lead to a variety of impaired interactions between viral and host proteins or between proteins and RNA, as well as depressed enzymic activities, affecting the intracellular replication of this virus.

The development of infectious cDNA clones of WNV, including the NY99 strain, has been reported previously (Beasley et al., 2005; Li et al., 2005; Shi et al., 2002; Yamshchikov et al., 2001). In particular, the two-plasmid pWN-AB/pWN-CG system described in further detail here has been employed previously to investigate protein glycosylation status in WNV strains (Beasley et al., 2005). The clone-derived WN/IC-P991 and WN/IC-KEN were extensively characterized in this study to: (a) establish their authentic phenotypic characteristics relative to the parental viruses, and (b) establish the recombinant pWN-AB and pWN-CG plasmids as suitable cDNA target templates for site-directed mutagenesis and gene recombination for further investigation of WNV genetic determinants of avian and mammalian virulence and attenuation, including refinement of the genetic basis for the apparent ts phenotype of WNV KEN-3829. Data presented in this report indicate the potential importance of a natural temperature-resistant phenotype of WNV introduced into New York in 1999 that could be a major factor in the enhanced replication of WNV in birds, and AMCRs in particular, and might be associated with the rapid spread of the virus across North America. Our results reveal the possibility of using a temperature-sensitive model in DEF cells for predicting avian virulence of WNV strains.

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