Horizontal and Vertical Transmission of West Nile Virus Genotype NY99 by *Culex salinarius* and Genotypes NY99 and WN02 by *Culex tarsalis*

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Abstract. *Culex tarsalis* is a superior horizontal and vertical vector of West Nile virus (WNV) compared with *Culex salinarius*. *Culex salinarius* transmitted WNV genotype NY99 (CT 2741-99 strain) horizontally to suckling mice at significantly lower rates than *Cx. tarsalis* on Days 8, 9, 10, and 12 post-infection, and *Cx. salinarius* transmitted WNV genotype NY99 to offspring at a lower vertical transmission infection rate than *Cx. tarsalis*. *Culex tarsalis* transmitted WNV genotypes NY99 and WN02 (CT S0084-08 strain) with equal efficiency. Daily percent horizontal transmission of genotype NY99 by *Cx. tarsalis*-infected *per os* and by intra-thoracic infection was not significantly different from daily transmission of genotype WN02 from Days 5–23 and Days 2–9 post-infection, respectively. Our findings do not support the previously published hypothesis that genotype NY99 was replaced in the New World by WN02 because of a shorter extrinsic incubation of WN02.

INTRODUCTION

West Nile virus (family *Flaviviridae*, genus *Flavivirus*, WNV) in the northeastern United States amplifies during the summer months in an enzootic cycle by horizontal transmission and survives the winter months in non-blood-fed females infected by vertical transmission.^{1–3} *Culex pipiens pipiens* L. is the primary vector, but numerous isolations of WNV also have been made from other species.⁴ *Culex salinarius* Coquillett, which feeds on mammals and birds from New England to New Mexico and is frequently infected with WNV,^{4–10} is a likely bridge vector for transferring WNV from birds to humans.^{4,11} In the western United States, *Culex tarsalis* Coquillett is the likely primary vector in rural agricultural environments,¹² and species of the *Cx. pipiens* complex and *Cx. salinarius* may be the important vectors in urban ecosystems.^{6,7,12}

Culex tarsalis has been reported to have a relatively short WNV extrinsic incubation period,¹² to vertically transmit WNV,^{13,14} and, along with *Cx. pipiens*,¹⁵ to transmit the relatively newly recognized WN02 genotype more quickly than the original NY99 genotype.¹⁶ The extrinsic incubation period of the WN02 genotype was up to 2 days shorter in *Cx. pipiens* and 4 days shorter in *Cx. tarsalis* than that of the NY99 genotype and led the authors to suggest that genotype WN02 displaced genotype NY99 in North America by leading to higher infection rates of genotype WN02 in avian hosts. We examined this hypothesis by comparing the horizontal post-infection (pi) transmission rates at daily time points for WNV strains of genotype NY99 and genotype WN02 from Connecticut in *Cx. tarsalis* that probed or fed on suckling mice. We also compared the pi daily transmission rate for WNV strain genotype NY99 in *Cx. salinarius*, which had not previously been extensively studied as a vector of WNV and which we thought would have a contrasting extrinsic incubation period to *Cx. tarsalis*.¹¹ Additionally, we report the vertical transmission of WNV genotype NY99 by *Cx. salinarius* and *Cx. tarsalis* and the vertical transmission of WNV genotype WN02 by *Cx. tarsalis*.

MATERIALS AND METHODS

Mosquito colonies. The colony of *Cx. salinarius* was established in 2003 from specimens collected in Stratford, CT (41°10'41"N, 73°07'34"W) by procedures published previously.¹⁷ The *Cx. tarsalis* colony was established by William K. Reisen, University of California, Davis, from specimens collected from Coachella Valley, CA in 2005. *Culex tarsalis* collected from Coachella Valley in 2001 were reported to be most efficient at transmitting WNV after a 14-day incubation period.¹² This *Cx. tarsalis* colony was obtained from the U.S. Department of Agriculture/Agricultural Research Service (USDA/ARS) Laboratory, Gainesville, FL. Colonies of both species were kept at 25°C and a photoperiod of 16:8 (L:D) hr.

West Nile virus. The CT 2741-99 strain of WNV from *Cx. p. pipiens* is a NY99 genotype.¹⁸ Strain CT S0084-08 was from *Cx. p. pipiens* collected September 16, 2008 in Stratford, CT and is a WN02 genotype. The two strains were sequenced at the DNA Analysis Facility on Science Hill at Yale University. Primers used were WN1101-GATGAATATGG AGGCGGCCA and WN1816A-CCGACGTCAACTTGA CAGTGTG.¹⁹ Genotype NY99 strain CT 2741-99 (GenBank accession JN182684) differed from genotype WN02 strain CT S0084-08 (GenBank accession JN182683) by a U to C substitution at position 1442, resulting in a Val to Ala amino acid substitution at position 159 of the E protein, and by silent changes A to G at position 1482 and C to U at position 1629. The WNV CT 2741-99 strain was passed three times and the CT S0084-08 strain two times through Vero cells and either mixed 1:1 with fresh heparinized hamster blood as described previously,²⁰ or frozen virus of each strain was added to Vero cell growth medium and mixed 1:1 with fresh mouse blood to produce approximately equal titers. Complete genomes of WNV strains CT 2741-99 (GenBank accession no. AF206518) and CT S0084-08 (GenBank accession no. HM488170) have been published.²¹

Per os infection of *Cx. salinarius* and *Cx. tarsalis*. Seven- to 10-day-old females of each species were deprived of raisins for 1 d before being allowed to feed on an infectious mixture of hamster or mouse blood and WNV through a membrane feeder (Hemotek 5W1, Discovery Workshops, Accrington, UK) placed

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on top of each cage. Each engorged female was transferred into a 296-mL numbered clear plastic cup covered with netting and kept at 26°C and a photoperiod of 16:8 (L:D) hr. Approximately 25 mL of hay infusion was added to each cup for deposition of eggs. A raisin was provided to each female before and after oviposition. Each female that had fed on infectious blood was examined daily, and when it died or was killed, it was frozen at -80°C and later tested for virus in Vero cells.

Laboratory animals. Suckling mice from pregnant CD-1 mice were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Suckling mice were tattooed and exposed to individual female mosquitoes as described previously.²⁰ A tattooed suckling mouse, 2–4 d old, was securely placed over the netting of a cup containing a single mosquito. The suckling mouse was later returned to its mother. A non-tattooed mouse that was not exposed to a mosquito served as a negative control for each litter. Suckling mice were examined daily for signs of illness. Sick mice were euthanized with CO₂ and frozen at -80°C. The missing suckling mice, presumably eaten by their mothers, were not included in the analysis of extrinsic incubation. All live mice on Day ≥ 4 pi exposed to *Cx. salinarius* were euthanized with CO₂ and frozen at -80°C. Suckling mice exposed to *Cx. tarsalis* were euthanized on Days 3 or 4 pi. Brain tissue was removed from each suckling mouse and triturated in phosphate-buffered saline (PBS) containing gelatin (0.5% gelatin, 30% rabbit serum, antibiotic, and antimycotic). This mixture was centrifuged at 5°C, and 100 µL of the supernatant was transferred onto a confluent layer of 1- and 2-day-old Vero cells for attempted isolation of virus. Reverse transcription-polymerase chain reaction (RT-PCR) was used to confirm all isolates.²² Isolation of WNV from suckling mice was evidence of transmission. Groups of mice with an infected untreated control suckling mouse were not included in the analysis.

The Connecticut Agricultural Experiment Station's Animal Care and Use Committee approved the procedures for handling animals.

Comparison of horizontal transmission of West Nile virus genotype NY99 by *Cx. salinarius* and *Cx. tarsalis* at 26°C. Females of each species were exposed daily to suckling mice on Days 6 through 30 pi. Infectious hamster blood fed to *Cx. salinarius* contained 5.9–28.0 × 10⁵ plaque-forming units (PFU)/mL, and infectious hamster blood fed to *Cx. tarsalis* contained 1.7–3.2 × 10⁸ PFU/mL.²³ These quantities of WNV were within the range of PFU reported in experimentally infected birds.²⁴ Suckling mice and mosquitoes were tested for WNV as described previously.

Vertical transmission relative to days pi. Eggs were collected on days pi from female *Cx. salinarius* infected with genotype NY99 and from *Cx. tarsalis* infected with genotypes NY99 and WN02. Egg rafts were from females that had fed relatively recently on the membrane and from females that had fed on the membrane and later on suckling mice. Egg rafts were each transferred into a 16-mL glass vial (Wheaton, Science Products, Millville, NJ) containing 10-mL distilled water and 0.02 g of rodent chow. Upon hatching, F₁ larvae were reared at 26°C and a photoperiod of 16:8 (L:D) hr in clear plastic, disposable 540-mL containers and fed rodent chow as needed. Larvae were reared in groups of 40–50 per container. Adults were separated according to sex, frozen at -80°C, and processed for virus isolation in Vero cells in groups of 25 to determine if vertical transmission had occurred.

Comparison of transmission of West Nile virus genotypes NY99 and WN02 by *Cx. tarsalis* at 26°C. Females, which were infected with WNV genotypes NY99 or WN02, were exposed daily to suckling mice from Day 5 pi until Day 23 pi. An observer carefully monitored each mosquito during a 2-hr exposure period and recorded the mosquito as probed, fed, or not probed. *Culex* females transmit WNV after probing without ingesting blood.²⁵ Suckling mice that were not probed or fed upon by a mosquito were not included in the analysis. The titers of infectious mouse blood were 2.25–23.0 × 10⁵ PFU/mL for genotype NY99 and 2.0–22.0 × 10⁵ PFU/mL for genotype WN02. Suckling mice and mosquitoes were tested for WNV as described previously. Mosquitoes that tested negative were not included in the analysis.

Comparison of transmission of West Nile virus genotypes NY99 and WN02 by *Cx. tarsalis* at 26°C after thoracic microinjection of virus. The stains of genotype NY99 and genotype WN02 were titrated in *Cx. tarsalis* females by injection of virus into the thorax.²⁶

The dose of each strain was determined by 10-fold serial dilutions (i.e., 10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷) in PBS. The mosquitoes (10 in each group) were inoculated in the thorax by microinjection with 300 nL of diluted virus. On Day 6 pi, the mosquitoes were killed in dry ice, total RNA was isolated, and the viral load was determined by quantitative polymerase chain reaction (qPCR). Infected mosquitoes were homogenized in 350-µL RLT Buffer (RNeasy Mini kit, Qiagen) with a Pestle Grinder System (Fisher Scientific, Pittsburgh, PA). Total RNA was isolated, and cDNA was transcribed for qPCR detection. The specific RNA of the WNV-E gene was quantified by Taqman qPCR. The primers and probe were: WNV-F: TTCTCGAAGGCGACAGC TG; WNV-R: CCGCCTCCATATTCATCATC; WNV-Probe: FAM6-ATGTCTAAGGACAAGCCTACCATC-TRAMA. The *C. quinquefasciatus* actin (CPIJ012570) gene was used as an internal control in qPCR detection: Actin-F: CCC CTGAGCGCAAGTACT; Actin-R: CGTCGTATTCTG CTTGGAGAT; Actin-Probe: FAM6-TGGCCTCGCTGTCC ACCTTCCA-TRAMA. The qPCR was performed in a Bio-Rad iQ5 Cyclor with a Taqman Universal PCR Master Mix system (Applied Biosystems, Foster City, CA). The PCR mixtures were preincubated at 50°C for 2 min in the thermal cycle, denatured at 95°C for 10 min, and cycled 40 times at 95°C for 30 sec and 60°C for 1 min. There were no nucleotide differences in the PCR amplified gene of genotypes NY99 (1019–1099 bp, GenBank accession no. AF206518) and WN02 (1037–1117 bp, GenBank accession no. HM488170).²¹ These data indicate that the PCR efficiency for the two genotypes should be the same. The standard curves were measured by 10-fold dilutions of WNV E gene (PCR amplified fragments) and the *C. quinquefasciatus* actin gene.

The 50% mosquito infective dose (MID₅₀) was estimated by Reed-Muench.²⁷ Equivalent quantities of virus of the two strains were then made, and 300 nL of each strain containing 1,000 MID₅₀ virus was injected into each female mosquito. Female mosquitoes injected with genotype NY99 or genotype WN02 were allowed to feed or probe on suckling mice as described previously on Days 2–9 after injection with WNV to determine if there were differences in extrinsic incubation between the two strains. Suckling mice were returned to their mothers after exposure to female mosquitoes, and each was euthanized on Day 3 after exposure to a female mosquito. Female mosquitoes and suckling mice were tested for WNV as described previously.

Statistics. The vertical transmission rate and the vertical minimal infection rate were determined using previously described methods.²⁸ The vertical minimal infection rate was calculated using the formula for bias corrected maximum likelihood estimate.²⁹ Fisher's exact test was used to compare infection and daily horizontal transmission rates of *Cx. salinarius* and *Cx. tarsalis* and the infection and daily horizontal transmission rates of the two WNV genotypes by *Cx. tarsalis*. Only females that tested positive for WNV were included in the analyses.

RESULTS

Comparison of horizontal transmission of WNV genotype NY99 by *Cx. salinarius* and *Cx. tarsalis* relative to days pi.

Horizontal transmission of WNV genotype NY99 by *Cx. salinarius* to suckling mice was recorded in <2% of the females that fed on Days 6 and 7 pi (Figure 1). Percent infection increased to 17–47% on Days 10–12 pi and then to 69–80% on Days 13–17 pi. Thereafter, until Day 30 pi, percent transmission ranged from 80% to 100%. Females that were 42, 43, 47, and 48 days pi also transmitted virus to suckling mice (data not shown in Figure 1). In contrast, transmission of WNV genotype NY99 by *Cx. tarsalis* increased from 5% on Day 6 pi to 67% on Day 9 pi to 77–100% on Days 11–30 pi with the exception of 63% transmitting on Day 14 pi. A significantly higher percentage of *Cx. tarsalis* kept at 26°C transmitted WNV genotype NY99 to suckling mice than did *Cx. salinarius* during Days pi 8, 9, 10, and 12. Seventy-eight percent ($N = 1,526$ fed females) and 80% ($N = 594$ fed females) of *Cx. salinarius* and *Cx. tarsalis* that fed on WNV genotype NY99 were infected, respectively. Infection rates were not significantly different (Fisher exact test, degrees-of-freedom [df] = 1, $P > 0.05$).

Vertical transmission by *Cx. salinarius* and *Cx. tarsalis*. One of 222-infected *Cx. salinarius* females that laid eggs vertically transmitted WNV genotype NY99 for a vertical transmission rate of 0.45%. Virus was isolated from one pool of 25 F_1 females from an egg raft that was laid 43 days pi. The female that vertically transmitted WNV had previously horizontally transmitted WNV to suckling mice on three occasions. In

all, 48,101 F_1 adults (24,308 males and 23,793 females) from infected females were tested. The vertical minimal infection rate was 0.04 WNV-infected mosquitoes/1,000 mosquito specimens tested.

Fifteen of 379-infected *Cx. tarsalis* females vertically transmitted WNV genotype NY99 and one of 57 infected females vertically transmitted WNV genotype WN02 to F_1 adults for vertical transmission rates of 3.69% and 1.75%, respectively. Seven, 7, and 1 females transmitted WNV genotype NY99 to progeny in egg rafts 1, 2, and 3, respectively. Two, 8, and 5 females vertically transmitted on Days 5 and 8, 10–16, and 24–30 pi, respectively. The female infected with WNV genotype WN02 transmitted virus to her second egg raft on Day 22 pi. One pool of 25 males and one pool of 25 females were infected.

A total of 28,372 and 5,450 F_1 adults from *Cx. tarsalis* females infected with genotypes NY99 and WN02, respectively, were tested for WNV. The vertical minimal infection rates were 0.96 WNV-infected genotype NY99 mosquitoes per 1,000 mosquito specimens tested and 0.37 WNV-infected genotype WN02 mosquitoes per 1,000 mosquito specimens tested.

Comparison of horizontal transmission of WNV genotype NY99 and genotype WN02 by *Cx. tarsalis* relative to days pi.

The percent of females that became infected with WNV after feeding on infectious blood containing genotypes NY99 and WN02 were 56% ($N = 189$ fed females) and 59% ($N = 209$ fed females), respectively. Infection rates were not significantly different (Fisher exact test, df = 1, $P > 0.05$). Horizontal transmission of genotype NY99 increased from 15% on Day 6 pi to 54% on Day 9 pi to 70–100% on Days 12–23 pi (Figure 2). Similar horizontal transmission results were obtained with genotype WN02. Significant differences in transmission were not noted in the two strains on any of the days from 5 to 23 pi (Fisher exact test, df = 1, $P > 0.05$).

Horizontal transmission of WNV genotypes NY99 and WN02 by *Cx. tarsalis* injected with virus was similar (Table 1). Significant differences in transmission were not observed in the two strains on any of the days from 2 to 9 pi (Fisher exact test, df = 1, $P > 0.05$).

FIGURE 1. Percent *Cx. salinarius* and *Cx. tarsalis* horizontally transmitting WNV genotype NY99 at 26°C to suckling mice on days 6–30 pi. The number of infected mosquitoes that fed each day is shown above or below each data point. *Differences in transmission between the two species at specific time-points pi significant at $P < 0.05$, df = 1, Fisher exact test.

FIGURE 2. Percent *Cx. tarsalis* horizontally transmitting WNV genotypes NY99 and WN02 to suckling mice on days 5–23 pi at 26°C. The number of infected mosquitoes that probed or fed each day is shown above or below each data point. There were no significant differences in transmission between the two strains at any of the time points pi at $P > 0.05$, $df = 1$, Fisher exact test.

DISCUSSION

Culex salinarius is a relatively abundant species in the northeastern United States. It feeds readily on mammals, including humans and on birds,⁵ and is naturally infected with WNV.^{4,30} Although Sardelis and others¹¹ reported an estimated 34% transmission rate of WNV by *Cx. salinarius* on Day 14 pi at 26°C, our transmission values were higher and similar to those reported for *Cx. pipiens*.^{20,31} The extrinsic incubation periods for transmission of WNV for both *Cx. salinarius* and *Cx. pipiens* were longer than what we and others reported for *Cx. tarsalis*.^{12,32,33} Although the concentration of virus ingested by *Cx. tarsalis* was greater than by *Cx. salinarius* in our experiments, the infection rates in both species were relatively high and not significantly different from one another, indicating that the viral meals for both species were well above the threshold levels of infection. On the basis of our results, *Cx. salinarius*, although not as efficient as *Cx. tarsalis*, is as efficient as *Cx. p. pipiens* in transmitting WNV in the northeastern United States, and although minimum field infection rates of WNV have been reported to be lower for *Cx. salinarius* than for

Cx. pipiens,^{4,30} the opportunistic feeding of *Cx. salinarius* on mammals, compared with *Cx. pipiens*,^{5,10} likely results in a greater percentage of females feeding on humans. It is noteworthy that in urban areas of New Mexico in the southwestern United States, WNV infection rates were relatively high in *Cx. salinarius* and in *Cx. pipiens quinquefasciatus* Say compared with the rates reported for *Cx. tarsalis*.⁷

Vertical transmission of WNV has been reported in eight species.^{2,3,13,14,34–38} We now document *Cx. salinarius* to also vertically transmit WNV. The 0.45% vertical transmission rate and the vertical minimal infection rate of 0.04/1,000 mosquitoes tested, although lower than those reported for *Cx. p. pipiens*, suggest to us that WNV could survive winter in non-blood-fed, vertically infected females of *Cx. salinarius* as previously reported for *Cx. p. pipiens* and possibly contribute to the field infection rate during the summer.^{2,3} This ability to vertically transmit and to horizontally transmit WNV similarly to *Cx. p. pipiens* suggests to us that *Cx. salinarius* may have a role in maintaining this virus in the northeastern United States.

The minimum incubation for vertical transmission of strain WNV genotype NY99 is shorter in *Cx. tarsalis* than in *Cx. p. pipiens* at 26°C. Transmission occurred in *Cx. tarsalis* females as early as 5 and 8 days pi during deposition of the first egg raft, whereas vertical transmission in *Cx. p. pipiens* did not occur until the second egg raft was laid 13 or more days pi.²⁰ Higher rates of vertical transmission by *Cx. tarsalis* occurred 10 days or later pi. Vertical transmission likely enables WNV to survive in *Cx. tarsalis*, as it does in *Cx. p. pipiens*, during winter in the northern part of its geographical range and likely enhances the field infection rates during the warm months of the year.

The original NY99 genotype of WNV was replaced by genotype WN02 throughout the United States in the early 2000s.^{15,39,40} The WN02 strains consistently differed by a Val to Ala amino acid substitution in the E protein at position 159. The WNV strains of genotype WN02 were reported to infect a larger proportion of *Culex* and to have shorter extrinsic incubation periods than did females infected with genotype strains

TABLE 1

Horizontal extrinsic incubation at 26°C in *Culex tarsalis* injected with 300 nL of WNV genotype NY99 and WNV genotype WN02 containing 1,000 mosquito infective dose₅₀*

Days pi	Viral strain injected	
	NY99	WN02
	No. infected mice/no. mice probed or fed upon (%)	No. infected mice/no. mice probed or fed upon (%)
2	1/8 (12.5)	0/4 (0)
3	7/12 (58.3)	3/10 (30)
4	13/14 (92.9)	9/10 (90)
5	16/16 (100)	16/18 (88.9)
6	11/12 (91.7)	8/11 (72.7)
7	2/2 (100)	1/1 (100)
8	5/5 (100)	2/2 (100)
9	7/7 (100)	1/1 (100)

*There were no significant differences in transmission between the two stains at any of the days pi ($P > 0.05$, $df = 1$, Fisher exact test).

of NY99.^{15,16} A more recent study reported transmission of the WN02 genotype to be advantageously increased in *Cx. pipiens* with increasing temperature and time compared with the NY99 genotype.⁴¹ The authors of these studies suggested that the increased transmission efficiency, resulting from the decreased extrinsic incubation period in *Culex* infected with genotype WN02 strains compared with those infected with NY99 genotype strains, was the likely reason for the displacement of the original genotype.

The initial descriptions of the WN02 genotype were made from birds and mosquitoes collected in June and July 2002 in Houston, TX where *Cx. p. quinquefasciatus* is relatively abundant,¹⁹ and in 2002 and 2003 from mosquitoes, birds, and mammals in New York State where *Cx. p. pipiens* is relatively abundant, and *Cx. tarsalis* is essentially absent.¹⁵ We examined the hypothesis that genotype WN02 has a shorter incubation period in *Culex* compared with NY99 by exposing the western United States mosquito vector of WNV *Cx. tarsalis* infected with Connecticut genotypes NY99 or WN02 to suckling mice at specific time points *pi*. Isolation was attempted from each mouse that had been probed or fed upon by infected *Cx. tarsalis*. Even though the initial reports of genotype WN02 were made in species other than *Cx. tarsalis*, we selected *Cx. tarsalis* as the model in our studies because 1) our initial studies confirmed that *Cx. tarsalis* is a superior vector of WNV,^{12,33} 2) we thought *Cx. tarsalis* would more readily probe and feed on suckling mice during the day than *Cx. p. pipiens*, and 3) the extrinsic incubation period of genotype WN02 was reported to be up to 4 days shorter than genotype NY99 in *Cx. tarsalis*.¹⁶

Our studies, which used a different strain of *Cx. tarsalis* and different genotype NY99 and WN02 strains than reported by Moudy and others,¹⁶ did not confirm a shorter extrinsic incubation period of genotype WN02 compared with genotype NY99 in *Cx. tarsalis*. We detected no significant differences at daily time points in transmission between the two genotypes from Days 5 through 23 *pi* when infected *per os* or from Days 2–9 when infected by intra-thoracic injection. Our percentages of transmission following *per os* infection, based upon natural transmission of WNV from *Cx. tarsalis* to suckling mice and subsequent isolation of virus, were considerably higher than those reported by Moudy and others.¹⁶ Their transmission rates at 27°C for genotype NY99 on Days 7, 9, and 14 *pi* were 0%, 2%, and 12%, respectively, in contrast to our transmission rates at 26°C on Days 7, 9, and 14 *pi* of 26%, 54%, and 75%, respectively. Others also have reported relatively high rates of transmission of genotype NY99 by *Cx. tarsalis* at 14 days *pi* or less.^{12,32} Although our transmission data following intra-thoracic inoculation were consistent with Moudy and others,¹⁶ our data from *per os* infections suggest that the replacement of genotype NY99 by WN02 was the result of factors other than increased transmission efficiency of WN02 by overcoming the midgut barrier. Representative mutations have yet to be identified that might explain the selective or adaptive processes for replacement of the original NY99 genotype by the WN02 genotype in North America.

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