Experimental infection of house sparrows (*Passer domesticus*) with West Nile virus strains of lineages 1 and 2.

Javier Del Amo^a, Francisco Llorente^a, Elisa Pérez-Ramírez^a, Ramón C. Soriguer^b, Jordi Figuerola^b, Norbert Nowotny^{c,d}, Miguel Angel Jiménez-Clavero^{a,*}

^aCentro de Investigación en Sanidad Animal (CISA)-INIA, Ctra. Algete-El Casar s/n, 28130 Valdeolmos, Spain,

^bEstación Biológica de Doñana-CSIC, Seville, Spain.

^cInstitute of Virology, Viral Zoonoses, Emerging and Vector-Borne Infections Group, University of Veterinary Medicine, Vienna, Austria.

^dDepartment of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat, Oman.

*Corresponding autor.

Address:

Centro de Investigación en Sanidad Animal (CISA)-INIA

Ctra. Algete-El Casar s/n, 28130 Valdeolmos, Spain

Telephone: +34 91 6202300

Fax No. +34 91 6202247

e-mail: majimenez@inia.es

Abstract

West Nile virus (WNV) is a zoonotic pathogen which is maintained in an enzootic cycle between mosquitoes and birds; humans, equines, other mammals and some bird species are dead-end hosts. Lineage 1 WNV strains have predominated in Europe since the 1960's. However, in 2004 lineage 2 strains emerged in Hungary and Russia, respectively, spreading since then to a number of neighbouring countries (e.g., Austria, Greece, Italy, Serbia and Romania). Wild bird mortality is a hallmark of North American WNV outbreaks, a feature uncommon in Europe. This study aimed to compare the course of infection of lineage 1 (NY99) and lineage 2 (Austria/2008) WNV strains in the house sparrow, a bird species common in Europe and North America. House sparrows were inoculated with either NY99 or Austria/2008 WNV strains, or sham-inoculated, and clinical and analytic parameters (viraemia, viral load, antibodies) were examined until 14 days after inoculation. Although all inoculated sparrows became infected, no mortality or clinical signs were observed due to the infection. However, the magnitude and duration of viraemia were higher for NY99- than for Austria/2008- infected birds. The house sparrow proved to be a competent host for both strains, although the competence index calculated for NY99 was higher than for Austria/2008. Viral load in tissues and swabs was also higher in NY99-inoculated sparrows. In conclusion, the house sparrow is a convenient avian model for studying host competence of WNV strains. The observed differences between NY99 and Austria/2008 strains might have important epidemiological consequences for disease incidence and dispersal capacity.

Keywords

West Nile virus; Experimental infection; house sparrow; Host competence; WNV lineage 1 and 2

1. Introduction

West Nile virus (WNV, family *Flaviviridae*, genus *Flavivirus*) is an neurotropic arbovirus affecting man, horses and birds, that is maintained in an enzootic cycle between mainly *Culex* mosquito vectors and birds acting as primary vertebrate hosts (Work et al., 1955). Two major genetic lineages, 1 and 2 (hereafter L1 and L2, respectively) are phylogenetically distinguishable. In North America, all circulating strains have been derived from a single introduction of L1, first detected in 1999 (type strain: NY99)(Lanciotti et al., 1999). North American outbreaks are characterized by wild bird mortality and high pathogenicity for humans and horses (Komar et al., 2003, 2001). In Europe, where WNV is known since the 1960s (Calistri et al., 2010), this virus was absent for nearly three decades and reemerged in the 1990s. Since then, several outbreaks of different magnitudes have occurred, mostly caused by strains belonging to L1 (Calistri et al., 2010). The first European L2 strains of WNV were reported in 2004 in Hungary (Bakonyi et al., 2006) and in Southern Russia (Platonov et al., 2008). Subsequently, L2 strains were detected in Austria (Bakonyi et al., 2013; Wodak et al., 2011), Greece (Papa et al., 2011), Italy (Savini et al., 2012), Serbia (Petrović et al., 2013), and Romania (Sirbu et al., 2011). The pathogenesis of L1 and L2 WNV strains has been compared experimentally only in gyrfalcons (Ziegler et al., 2013), but not in passerines, regarded as main amplifying hosts for WNV (Komar et al., 2003). The house sparrow (*Passer domesticus*) is an ubiquitous bird species, abundant in urban and peri-urban areas, being a competent host for WNV L1 (Komar et al., 2003). To date, no experimental study has been attempted to assess the infection potential and host capacity of house sparrows by WNV L2 strains, but recently it has been reported that house sparrows have been infected naturally with lineage 2 WNV in Hungary in 2009 (Bakonyi et al, 2013). This passerine species has become a model for the study of WNV infection in

susceptible avian hosts, which has been used to compare the course of infection of different WNV strains, including North American, Australian, African (Langevin et al., 2005) and European (Del Amo et al., 2014) isolates, all belonging to L1. The present study aims to compare the course of infection of house sparrows experimentally infected with two WNV strains belonging to two different lineages, L1 (NY99, American origin) and L2 (Austria/2008, European origin).

2. Materials and Methods

2.1. Viruses and virus preparations

WNV strains used in this work were: NY99-crow-V76/1 (obtained through the National Veterinary Service Laboratories, USDA, lot n° 034EDV0601), a North American WNV strain, isolated from a diseased crow during the WNV outbreak in New York in 1999 (GenBank accession n° FJ151394; hereafter NY99) and Austria/2008_gh, isolated from the brain of a goshawk found dead in Vienna, Austria in 2008 (Bakonyi et al., 2013)(GenBank accession n° KF179640; hereafter Austria/2008). All the above virus strains were propagated and titrated by plaque assays in Vero cells (ATCC CCL-81).

2.2. Birds and animal care

Free-living house sparrows were captured using mist nets and banded in the province of Sevilla (Spain) in December, 2011. After a two months' period of habituation to captivity in the Cañada de los Pájaros facilities (Sevilla), they were transported to the BSL-3 biocontainment facility at CISA (Valdeolmos, Spain) where they were bled to determine pre-existing immunity to WNV and housed in individual cages. The house sparrows (n=24) were randomly distributed in 3 groups of 8 individuals, composed (whenever possible) of 50% males and 50% females. Mixed bird-seed and water were supplied *ad libitum*. All the animal care, handling and experimental procedures

performed in this work were supervised and approved by the National Committee for Ethics and Welfare in Animal Experimentation in Spain according to European legislation (Council Directive 86/609/EEC).

2.3. Experimental inoculation

The inoculation was carried out essentially as described previously (Del Amo et al., 2014). Briefly, birds were inoculated subcutaneously in the neck with 0.1 ml of viral suspension containing approximately 10,000 plaque-forming units (pfu)/bird of NY99 WNV strain (n=8) or Austria/2008 WNV strain (n=8). The control group (n=8), was sham-inoculated with an equivalent volume of diluent (DMEM plus antibiotics) and manipulated in the same way as the virus-inoculated birds.

2.4. Clinical follow-up and collection of samples

The experimental procedure was based on Sotelo *et al.* (Sotelo et al., 2011a), adapted to house sparrows as described (Del Amo et al., 2014), with some modifications, as follows: Clinical signs were monitored daily. Blood samples (0.1 ml obtained from the jugular vein) were collected at 1, 3, 5, 7 and 9 days post-infection (dpi) from all birds to determine viraemia and viral loads. Similarly, oropharyngeal and cloacal swabs were collected from all birds one day before inoculation, and at 1, 3, 5, 7 and 9 dpi. Blood samples were collected in sterile polypropylene tubes filled with 0.9 ml BA-1 diluent as previously described (Sotelo et al., 2011a), mixed and stored at -70°C until analysis. Swab samples were placed in sterile polypropylene tubes containing 1 ml PBS and immediately stored at -70°C until analysis. Two birds per group were euthanized at 7 and 10 dpi. The remaining birds were euthanized at the end of the experiment (14 dpi) except one, from the Austria/2008-inoculated group, which died at 7 dpi, likely as a consequence of manipulation during bleeding. Detailed necropsies were performed and tissue samples from brain, kidney, liver, heart and spleen were obtained from each individual and homogenized in PBS as described (Sotelo et al., 2011a).

Additional blood samples (0.1-0.2 ml/individual) were collected prior to inoculation and just before euthanasia, in order to obtain serum for antibody detection.

2.5. Viraemia and viral load assays

Viraemia and viral load were measured as previously described (Del Amo et al., 2014). Briefly, viraemia was measured by a standard plaque-formation assay in Vero cells, and viral load in blood, tissue samples and oropharyngeal and cloacal swabs was measured using a semi-quantitative real-time RT-PCR method for the detection of L1 and L2 WNV genomes (Del Amo et al., 2013). Samples were considered positive in this test at Ct<40.

2.6. Antibody detection assays

Antibodies to WNV were detected in serum by a commercially available epitope-blocking ELISA (Ingezym West Nile Compac, INGENASA, Madrid, Spain) suitable for the detection of WNV antibodies in wild birds with low requirement of sample volume (Sotelo et al., 2011b). Neutralizing antibody titres were assessed by a standard virus-neutralization test (Sotelo et al, 2011b), using homologous virus as antigen.

2.7. Calculation of host competence index (Ci) values

The calculation of competence index values for the inoculated house sparrows was based on the formula described elsewhere (Komar et al., 1999), considering viraemia values above $10^{5.0}$ pfu/mL as infectious for *Culex pipiens* mosquitoes (Turell et al., 2000). The duration of infectious viraemia (d) was calculated by interpolation of the points crossing the value $y=10^{5.0}$ pfu/mL in the viraemic curve.

3. Results

Neither mortality nor clinical signs were observed as a consequence of the infection of house sparrows with NY99 (L1) or Austria /2008 (L2) WNV. The mean peak viraemia was reached at 3

139 dpi in both inoculated groups, but house sparrows inoculated with NY99 strain developed higher
140 virus titre and longer duration of the viraemic period than those inoculated with Austria/2008 (9.62
141 $\log_{10}$ vs. $6.72 \log_{10}$ and 8 vs. 6 days, respectively, Figure 1). The magnitude and duration of
142 viraemia indicated that the house sparrow is a competent host for transmission of both NY99 and
143 Austria/2008 WNV strains. However, the competence index (Ci) calculated for NY99 strain was
144 2.09 whereas Ci was 0.60 for Austria/2008 strain (Table 1). All WNV-inoculated individuals
145 seroconverted, as measured by ELISA, including those examined at 7 and 10 dpi. Neutralizing
146 antibodies were detected in all NY99-inoculated individuals, with titres ranging from 1:20 to 1:80,
147 and in birds inoculated with L2 Austria/2008 strain, with titres between 1:10 and 1:40. The sham-
148 inoculated (control) group, subjected to the same handling and sampling procedures as the
149 inoculated groups, remained asymptomatic, seronegative and aviraemic during the whole
150 experiment.

151 In order to assess systemic infection and the extent of virus distribution in organs at different time
152 points, two individuals from each group were euthanized at 7 and 10 dpi and the rest at 14 dpi. At 7
153 dpi, one of the Austria/2008-inoculated birds (#22) died during manipulation and was also subjected
154 to necropsy. This particular bird developed lower viraemia and the lowest viral loads in tissues, as
155 shown in Table 2, suggesting altogether that it died as a consequence of the manipulation and not
156 due to the infection. Viral load was determined in heart, liver, brain, kidney and spleen samples
157 (Table 2). Overall, WNV infection was widely disseminated throughout the body, but birds
158 inoculated with the NY99 strain reached higher viral loads (lower Ct) than those inoculated with the
159 Austria/2008 strain, in all organs analyzed. Also, WNV RNA was more consistently detected in
160 organ samples from individuals inoculated with NY99 (38 positives out of 40 tissue samples
161 analyzed) than in those with Austria/2008 (22 positives out of 40 analyzed). In general, viral

genome load in tissues declined over time, being higher at 7 dpi and then fading progressively at 10 and 14 dpi. However, a significant variation between individuals was observed in this regard. For instance, in the group inoculated with Austria/2008, at 14 dpi, one individual (#20) still showed remarkable viral loads in all tissues, while others had almost (#19) or completely (#24) cleared the virus from the tissues examined. In kidney, the viral genome load remained slightly higher at the end of the experiment (14 dpi) than in the other organs, both in Austria/2008 and in NY99-inoculated birds, though, again, individual variations were observed (Table 2).

Assessment of viral genome shedding through the faecal and oral routes, yielded results consistent with those observed in blood and tissues: viral genome loads were higher, more persistent and more consistently detected in oropharyngeal and cloacal swabs from the group inoculated with the NY99 strain than from the group inoculated with the Austria/2008 strain (Figure 2). Viral genome shedding in oral and cloacal swabs was detectable at 3 dpi in both groups. However, NY99-inoculated birds were still shedding virus genome through both routes at 9 dpi, while virus genome shedding ceased at 5 dpi in the Austria/2008-inoculated birds (with the exception of one bird found positive orally at 7 dpi).

4. Discussion

The house sparrow is an abundant passerine species often sharing habitat with humans in urban and peri-urban locations. This species is distributed world-wide although its native range is restricted to the Palearctic. The house sparrow is susceptible to WNV infection and disease, and a competent host for virus transmission to blood-sucking mosquitoes (Komar et al., 2003; Nemeth et al., 2009, 2008). Some important phenotypic traits of the virus, such as virulence, intensity of virus amplification and/or competence for virus transmission in this host can vary between different WNV strains (Del Amo et al., 2014; Langevin et al., 2005). In this context, it is worth mentioning

that up to now, approximately two-thirds of the experimental studies made on WNV infection in wild birds used the North American L1 type strain (NY99) (Pérez-Ramírez et al., 2014), and only few studies have focused on more recent Euro-Mediterranean WNV strains. To date, only two experimental studies have specifically addressed the course of infection of European L2 strains in wild birds, one using gyrfalcons (Ziegler et al., 2013) and another one using jackdaws (Lim et al, 2014). Hence, the present paper is the first one reporting the outcomes of an experimental infection of house sparrows with a L2 WNV strain, and comparing the effects of infection by L1 and L2 virus strains in a passerine species.

In the experimental design of the study it was considered important to include a reference strain to enable a comparison with other preceding studies. Strain NY99 was selected because it is the most commonly used strain in experimental studies in wild birds, including house sparrows. In these previous investigations, NY99 was found to be lethal for house sparrows in a range from 0% to 50% (0 to 38% in case of needle-inoculation) (Komar et al., 2003; Langevin et al., 2005; Lapointe et al., 2009; Nemeth et al., 2009). In the present experiment NY99 did not cause any mortality, but in a recent study using identical experimental procedures, the same virus batch and house sparrows captured at the same location (Sevilla, Spain), 25% mortality was observed after infection with NY99 (Del Amo et al., 2014). Mortality rates in both experiments fall in the range already described but the differences observed deserve further discussion. First, they may just reflect random variation because these differences in mortality rate with respect to Del Amo et al. (2014) were not statistically significant (F-Fisher exact test, $p=0.15$), possibly due to the small sample size. Second, the birds used in our experiment were captured 5 months later than in (Del Amo et al., 2014), which might have caused a bias in the age of the birds employed. Birds' age is known to be an important factor associated to host susceptibility (Pérez-Ramírez et al., 2014). Examination of plumage

characteristics additionally indicated that in the previous experiment the proportion of young birds was higher than in the present experiment. The relevance of age as a determinant factor in the susceptibility of the house sparrow to WNV disease has been proven in the study by Nemeth et al (2009), where differences in mortality were observed between adults (10%) and juveniles (23%) after inoculation with NY99 strain.

Similarly to the NY99 L1 strain, the L2 strain Austria/2008 resulted in an asymptomatic infection in the house sparrow. Despite this, the experiment highlights several interesting differences between both strains: NY99 produced an enhanced virus amplification which yielded higher and longer viraemias, generated higher and more persistent viral loads in organs, and more intense, consistent and persistent virus genome shedding through the oral and cloacal routes, compared to the strain Austria/2008. Accordingly, the host competence index calculated for NY99 was also higher than for Austria/2008, which suggests that mosquito vectors feeding on NY99-infected house sparrows may acquire the infection more readily than if feeding on Austria/2008-infected ones. In other words, house sparrows infected with NY99 would transmit the virus much more efficiently and for longer periods than those infected with the Austria/2008 strain, not only through mosquito bites, but possibly also when preyed or scavenged by other animals, like raptors (Nemeth et al., 2009), since the virus lasted for longer periods also in their organs. Despite seroconversion was observed in all house sparrows inoculated with either of the strains tested, a slightly lower (1:10-1:40 titre) neutralizing antibody response was detected in house sparrows of the group inoculated with the L2 strain Austria/2008, with respect to the neutralizing antibody response observed in those inoculated with L1 (NY99), which was present in all individuals of this group, with titres ranging from 1:20 to 1:80. This slight difference in immunogenicity could be consistent with a less intense infection by WNV Austria/2008, compared with WNV NY99, as the other data from this paper already indicate.

In this study, the strain NY99 exceeded the amplification capacity of the L2 strain Austria/2008 in the house sparrow. This has also been observed for other L1 strains recently isolated in Southern Europe (Del Amo et al., 2014). Overall, it appears that “NY99-like” WNV strains would be transmitted more efficiently in a mosquito-bird cycle than the strains circulating in Europe in recent years. This may provide a suitable explanation for the epidemic spread and extensive wild bird mortality observed in North America, in comparison to Europe. Even though North American WNV isolates do not appear to be intrinsically more virulent than the European strains, at least for the house sparrow, they are likely more transmissible, leading to a more exacerbated cycle, a greater basic reproductive ratio (R_0) and more infection events. Although an increased transmissibility does not necessarily mean a higher mortality, for WNV strains similar to NY99, which cause certain levels of mortality in house sparrows and other birds, it is likely, as the number of infections grows, the observed number of birds dying as a consequence of WNV disease would also increase.

In this study, the house sparrow has been confirmed to be a suitable model for estimating key parameters of WNV infection in the avian host, and how they differ between different virus strains. It would be interesting to apply this model to the study of newly emerging WNV variants, such as those involved in the recent outbreaks of human West Nile neuroinvasive disease in Europe.

Acknowledgements:

We would like to thank the personnel at the Doñana Biological Station and Cañada de los Pájaros, involved in the capture and acclimatization of the house sparrows used in the experiments, Consejería de Medio Ambiente, Junta de Andalucía, for permissions to capture the house sparrows, and staff at the CISA-INIA BSL-3 animal facilities, where the experiments were carried out. This study was funded by grants from INIA (FAU2008-00002-00-00), European Commission

(HEALTH.2010.2.3-3-3 261391 EuroWestNile project) and Junta de Andalucía (Proyecto de Excelencia RNM-7038).

Conflict of interest:

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Table legends

Table 1. Host competence index for each group of house sparrows. Competence index is determined by susceptibility (s), infectiousness (proportion of vectors that could become infected per day) (i) and duration of transmissible viraemia (d). The equation to calculate competence index is $C_i = s \times i \times d$. Host competence index (C_i) is a value that reflects the relative number of infectious mosquitoes that would be derived from feeding these hosts.

Table 2. Viral genome load in house sparrow tissues at different days post inoculation. Each tissue was analyzed by semi-quantitative real time RT-PCR and the viral load is shown as the C_t value. Except bird #22 that died during manipulation (bleeding), all others were subjected to programmed necropsies.

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Figure legends

Figure 1. Mean daily blood viral genome (left panel) and viraemia titers (right panel), plotted for two groups of eight house sparrow either inoculated with WNV strain NY99 (open squares) or WNV strain Austria/2008 (closed circles). Error bars represent the standard error of the data. The efficiency of the semi-quantitative real-time RT-PCR for viral genome load determinations was $E = 0.908$.

Figure 2. Viral genome shedding through the oral (upper panel) and faecal (lower panel) routes as estimated by real-time RT-PCR analysis of the oropharyngeal and cloacal swabs, respectively, taken at different days post-inoculation of: WNV NY99 strain (white bars) and WNV strain Austria/2008 (black bars). Error bars represent standard error of the data. Over each bar, n° of positive/examined swab samples are indicated.