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Susceptibility of European jackdaws (*Corvus monedula*) to experimental infection with lineage 1 and 2 West Nile viruses

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Abstract

Mass bird mortality has been observed in North America after the introduction of *West Nile virus* (WNV), most notably massive die-offs of American crows (*Corvus brachyrhynchos*). In contrast, WNV epidemic activity in Europe has been characterized by very low incidences of bird mortality. As the general susceptibility of European corvids to strains of WNV remains in question, European jackdaws (*Corvus monedula*) were inoculated with WNV strains circulating currently in Greece (Greece-10), Italy (FIN and Ita09) and Hungary (578/10), as well as a North American (NY99) genotype with a demonstrated corvid virulence phenotype. Infection with all strains except WNV-FIN resulted in mortality. Viraemia was observed for birds inoculated with all strains and virus was detected in a series of organs upon necropsy. These results suggested that jackdaws could potentially function as a sentinel for following WNV transmission in Europe; however, elicited viraemia levels might be too low to allow for efficient transmission of virus to mosquitoes.

INTRODUCTION

West Nile virus (WNV; genus *Flavivirus*; family: *Flaviviridae*) is maintained in an enzootic cycle between mosquitoes and birds, but may also infect humans and horses, which serve as incidental dead-end hosts. WNV is endemic in many parts of Africa, Australia, the Middle East and Asia, and more recently emerged in North America in 1999, and has since rapidly spread across North America, Mexico, South America and the Caribbean. Since 2008, WNV has emerged as a serious veterinary and public health problem in central and south-eastern Europe, affecting countries such as Greece (Barzon *et al.*, 2013b; Danis *et al.*, 2011; Papa *et al.*, 2010), Italy (Barzon *et al.*, 2009, 2011, 2012, 2013a), Hungary, Austria (Bakonyi *et al.*, 2013) and Romania (Sirbu *et al.*, 2011).

Birds were considered to be less susceptible to West Nile disease until high mortality rates were recorded in flocks of young domestic geese in Israel in 1998 (Banet-Noach *et al.*, 2003; Malkinson *et al.*, 2002), after which WNV has been implicated in deaths of members of 326 species of birds in North America (De Filette *et al.*, 2012). Among the passeriform

birds, the family *Corvidae* is ranked as the most highly susceptible species to WNV (Wheeler *et al.*, 2009) and, in particular, deaths among the American crow (*Corvus brachyrhynchos*) have been used to track the spread of the virus in North America (Eidson *et al.*, 2005; Julian *et al.*, 2002).

In contrast to the mass bird mortality observed in North America, sporadic isolated death events and low mortality rates have been reported among European birds, even during severe human and equine West Nile outbreaks (Calistri *et al.*, 2010; Dauphin *et al.*, 2004; Jourdain *et al.*, 2007; Valiakos *et al.*, 2011). Possible explanations for the lack of reporting of bird mortality could be that European birds are less susceptible to WNV-induced disease or that the North American strain of WNV exhibits an increased capacity for eliciting avian virulence. Other theories include poor detection of bird carcasses (due to their small size or scavenging) (Wobeser & Wobeser, 1992), limited monitoring of wild bird mortality or development of herd immunity due to local transmission spurred by avian migration through WNV endemic areas of the Middle East and Africa (Hubálek & Halouzka, 1999).

To date, the susceptibility of European jackdaws to WNV isolates circulating in Europe has not yet been addressed. In order to examine the role of a bird that is found ubiquitously across Europe as a natural reservoir for the transmission of WNV disease or as a potential sentinel for WNV activity, European jackdaws were inoculated with four different WNV isolates clinically relevant to Europe, including lineage 1 isolates Ita09 and FIN from Italy, as well as lineage 2 isolates from Greece and Hungary. The NY99 strain was used to assess and compare the susceptibility of the European birds to a strain known to be highly virulent in North American corvids, especially in American crows. Susceptibility was assessed in terms of mortality, median survival time, duration and magnitude of viraemia, and dissemination of virus to the different organs.

RESULTS

Morbidity and mortality

During a 9 day period, lethargy, low activity, anorexia and ruffled feathers were observed among some of the jackdaws inoculated with NY99, Greece-10, Ita09 and 578/10. Birds died within 24–48 h after onset of clinical symptoms. Among the five jackdaws that were inoculated with NY99 and Ita09 and followed for survival, three (60 %) died; among the five jackdaws inoculated with 578/10, two (40 %) died; and among the six jackdaws inoculated with Greece-10, three died (50 %). In contrast, all five birds inoculated with FIN survived the infection (Table 1). Comparison of the survival curves of the birds inoculated with the different virus strains revealed a significant difference between the survival curves of jackdaws infected with FIN compared with Ita09 (Fig. 1; $P=0.05$). Median day of death was 7 days post-infection (p.i.) for birds that succumbed due to infection with NY99, Ita09 and 578/10, and 8 days p.i. for birds that died due to infection with Greece-10 (Table 1).

Viraemia profiles

We determined WNV viraemia profiles for the jackdaws inoculated with the five different virus strains and found that all birds developed viraemia within 96 h p.i (Table 1; Fig. 2).

For NY99-infected birds, peak serum viraemia titres ranged from 4.1 to 9.6 log₁₀RNA copies ml⁻¹ (mean 6.2 log₁₀RNA copies ml⁻¹). Birds infected with the Greece-10 strain had peak serum viraemia titres ranging from 4.2 to 8.4 log₁₀RNA copies ml⁻¹ (mean 5.9 log₁₀RNA copies ml⁻¹). For FIN-infected birds, peak serum viraemia titres ranged from 3.3 to 5.1 log₁₀RNA copies ml⁻¹ (mean 4.0 log₁₀RNA copies ml⁻¹). Ita09-infected birds displayed peak serum viraemia titres ranging from 3.4 to 7.5 log₁₀RNA copies ml⁻¹ (mean 4.7 log₁₀RNA copies ml⁻¹). Among jackdaws infected with the 578/10 strain, peak serum viraemia titres ranged from 3.1 to 6.9 log₁₀RNA copies ml⁻¹ (mean 5.0 log₁₀RNA copies ml⁻¹).

These data demonstrate that birds inoculated with NY99 and Greece-10 had the highest mean peak viraemia titres, followed by 578/10 and Ita09, with the lowest viraemia peaks for FIN-infected birds. Mean peak viraemia titres were significantly different between Greece-10 and FIN ($P=0.02$) and NY99 and FIN ($P=0.03$). Onset of viraemia occurred significantly later for FIN-infected birds compared with Greece-10 ($P=0.02$), NY99 and 578/10 ($P=0.03$, for both) (Table 1). The peak of viraemia was reached the earliest by birds inoculated with Greece-10, followed by the other viral strains (Table 1), but differences were not statistically significant ($P=0.8$). In terms of duration, viraemia lasted the longest for FIN and 578/10, followed by Greece-10, NY99 and Ita09 (Table 1), but these differences were also not statistically significant ($P=0.4$).

Determination of the viraemia profiles in terms of infectious virus (TCID₅₀) titres was only successful for a limited set of birds. The infectious titres recorded successfully were for the birds with the highest peak viraemia titre (in terms of viral RNA) in each group. These titres were specifically 10^{6.3} TCID₅₀ ml⁻¹ (10^{9.6} RNA copies) for the NY99-infected bird with the highest peak viraemia, 10^{5.3} TCID₅₀ ml⁻¹ for the Greece-10- and Ita09-infected birds (10^{8.4} and 10^{7.5} RNA copies, respectively), and 10^{2.3} TCID₅₀ ml⁻¹ for the 578/10-infected bird (10^{6.9} RNA copies).

Tissue tropism

Viral loads were determined in the heart, liver, spleen, kidney, bone marrow and brain for all birds. In order to assess the spread of virus to the different organs at the approximate peak of viraemia, two birds per group were euthanized on day 4 p.i. Virus was detected in all organs of these birds (Fig. 3), with the highest mean tissue viral titres found in the spleen and bone marrow (7.1 and 6.9 log₁₀RNA copies g⁻¹, respectively), followed by the liver, kidney and heart (5.8, 5.7 and 5.5 log₁₀RNA copies g⁻¹, respectively). The lowest tissue viral titres were found in the brain (3.8 log₁₀RNA copies g⁻¹). Between the different virus strains, higher tissue viral RNA titres were found in the organs infected by Greece-10 (7.0 log₁₀RNA copies g⁻¹), followed by 578/10 (6.4 log₁₀RNA copies g⁻¹), NY99 and Ita09 (5.4 and 5.3 log₁₀RNA copies g⁻¹, respectively), whilst FIN-infected birds had the lowest tissue viral RNA burden (4.8 log₁₀RNA copies g⁻¹). Viral titres in the organs were significantly different for Greece-10 compared with NY99 ($P=0.05$), FIN ($P=0.004$) and Ita09 ($P=0.02$), and in the organs infected with 578/10 compared with FIN ($P=0.01$).

For birds euthanized due to morbidity, virus was also found in all the organs (Fig. 4), with the tendency for the spleen, kidney and heart to contain the highest mean tissue viral RNA

load (6.7, 6.6 and 6.2 log₁₀RNA copies g⁻¹, respectively), followed by the liver, brain and bone marrow (5.5, 5.4 and 5.2 log₁₀RNA copies g⁻¹, respectively). Viral RNA titres were highest in organs of birds infected by Greece-10 (6.2 log₁₀RNA copies g⁻¹), followed by 578/10 and NY99 (6.1 log₁₀RNA copies g⁻¹, for both), with the lowest titres exhibited by Ita09-infected birds (5.4 log₁₀RNA copies g⁻¹). However, these titres were not found to be significantly different ($P=0.4$).

All birds that had survived infection by day 14 were euthanized and necropsied to determine whether virus could still be found in any of the organs (Fig. 5). Viral RNA was detected in all organs of the three Greece-10-infected survivor birds except in the bone marrow of one bird, whilst NY99-infected birds had a minimum of three positive organs (out of six), with the liver being completely negative for both birds. FIN-infected survivor birds had a minimum of two organs (out of six) positive for viral RNA, whilst the bone marrow was consistently negative for all five birds. The two birds that survived infection with Ita09 were both positive for viral RNA in five out of six organs, with the liver negative in one bird and the bone marrow negative in the other bird. Viral RNA was detected in all organs of the two 578/10-survivor birds except for in the liver of one bird. Between the different virus strains, mean viral RNA titres in the organs were significantly higher in Greece-10-infected birds compared with birds infected with FIN ($P=0.03$).

Overall, mean tissue viral titres in the organs of the survivor birds were lower (2.7 log₁₀RNA copies g⁻¹) compared with the birds necropsied on day 4 (5.8 log₁₀RNA copies g⁻¹) or after illness/death (5.9 log₁₀RNA copies g⁻¹), with most virus persisting in the kidney and spleen (4.1 and 3.7 log₁₀RNA copies g⁻¹, respectively), followed by the brain and heart (3.0 and 2.3 log₁₀RNA copies g⁻¹, respectively), and the liver and bone marrow (1.7 and 1.6 log₁₀RNA copies g⁻¹, respectively).

Immunohistochemistry

Sections of organs of birds euthanized on day 4 p.i. were stained with polyclonal anti-WNV NS3 to determine replication of the virus in these tissues and rule out any positive quantitative real-time (qRT)-PCR detection as a result of spillover from blood (Table 2). Tissues most consistently positive for WNV antigen were the kidney (100 %), followed by the heart, liver, spleen (90 %) and bone marrow (80 %), with the brain only positive in 50 % of the cases. Antigen was most abundant in the spleen, followed by the kidney, bone marrow, heart and liver (Fig. S1, available in the online Supplementary Material). The abundance of viral antigen found in the positive brains was minimal. Overall, organs of birds most often positive, as well as most abundant for viral antigen, were those infected with Greece-10 and 578/10, followed by FIN and Ita09, with the lowest amount of antigen found in the organs of the two NY99-infected birds.

Viral antigen appeared to be slightly more abundant in birds euthanized due to morbidity (Table 3) as compared with the birds euthanized on day 4, with the spleen and kidney consistently positive for viral antigen (100 %), followed by the heart and brain (91 %), whilst the bone marrow (64 %) and liver (45 %) were the least often positive. In terms of antigen abundance, staining was most prominent in the heart, kidney and spleen, whilst lower amounts of antigen were present in the brain, bone marrow and liver (Fig. S1).

Between the different virus strains, the abundance of viral antigen in the organs was very similar in Greece-10, 578/10 and Ita09-infected birds, and was also higher than the amount of antigen found in the organs of NY99-infected birds.

DISCUSSION

In this study, European jackdaws appear to be susceptible to infection with European strains of WNV from both lineages 1 and 2, as well as the North American strain NY99. However, the same extent of susceptibility as shown by the American crow upon infection with NY99, with 100 % mortality rates and production of high viraemia titres reaching peaks $>10^7$ p.f.u. ml^{-1} (Brault *et al.*, 2004, 2007; Komar *et al.*, 2003; Nemeth *et al.*, 2011; Weingartl *et al.*, 2004), was not observed in this study. Herein, mortality rates ranged between 40 and 60 % for four out of five strains and the highest mean peak serum viraemia was $\sim 6.2 \log_{10}$ RNA copies ml^{-1} , reached by birds inoculated with NY99.

Viral RNA was detected in all organs on day 4 p.i. and at the time of euthanasia due to morbidity. Viral RNA titres were observed to be significantly higher on day 4 p.i. in the organs of birds inoculated with Greece-10 compared with NY99, FIN and Ita09, and in the organs of birds inoculated with 578/10 compared with FIN. Even though at this time point spillover of virus from blood most likely resulted in higher viral RNA titres in the organs, immunohistochemistry confirmed that at least the relative differences were the same, as the amount of viral antigen staining confirmed that the two Greece-10- and 578/10-inoculated birds did indeed have the most viral antigen present in their organs as a whole, suggesting that spillover of virus from the circulation did not generally influence these results. Interestingly, Greece-10 and 578/10 are both lineage 2 viruses with 99 % identity, possibly indicating a strain-related tropism. Nonetheless, by the time the birds were euthanized due to morbidity (between days 5 and 9), any virus-related significant differences were no longer observed in terms of viral RNA titres, although immunohistochemical staining suggested a tendency for viral antigen to be more abundant in organs of Greece-10, 578/10 and Ita09-infected birds.

In a previous study (Lim *et al.*, 2013b), mice inoculated with WNV FIN displayed a slightly reduced mortality in comparison with the other WNV strains (also used here). In this study, however, the virulence phenotype of FIN has become much more pronounced, as birds inoculated with FIN suffered no mortality and had significantly lower peak viraemia titres as well as delayed onset of viraemia compared with the other groups. Even though viral virulence generally correlates well with high and prolonged viraemia (Kinney *et al.*, 2006), in this case, viraemia was not significantly shorter in FIN-infected birds compared with the other virus strains, although onset of viraemia did occur significantly later.

Interestingly, the two birds inoculated with FIN and euthanized on day 4 were both positive for virus in the majority of their organs (qRT-PCR and immunohisto-chemistry) despite the absence of mortality seen in this group. This, in combination with the presence of viraemia observed in all birds of this group, suggests that even in birds that are not susceptible to lethal WNV infection, the virus is able to elicit viraemia and disseminate to the organs, including the brain. A similar observation was demonstrated in chickens, which experienced

no mortality following infection with WNV, but exhibited titres as high as $5 \log_{10} \text{p.f.u. ml}^{-1}$ and virus could also be isolated from several organs, including the spleen and kidney (Senne *et al.*, 2000).

The introduction of a T249P amino acid substitution (present in North American WNV) in the NS3 helicase of a low virulence strain of WNV has been demonstrated to result in increased virulence in American crows (Brault *et al.*, 2007). The virus strains used in this study all contain a proline at this site, with the exception of FIN, which contains a threonine at this position (Lim *et al.*, 2013b) (Table S1). Ita09, which is 99.7 % identical to the nucleotide sequence of FIN, did not display the same attenuation in jackdaws as FIN. It is therefore very likely that the attenuated phenotype of FIN demonstrated in this study is the result of this P249T substitution. Studies are ongoing to test the relevance of the T249P substitution present in the Italian backbone of WNV in both European and American corvids.

It has been shown that a blood titre of $10^6 \text{ p.f.u. ml}^{-1}$ is required for transmission to feeding mosquitoes (van der Meulen *et al.*, 2005). Additionally, viraemic titres $>10^5 \text{ p.f.u. ml}^{-1}$ were considered infectious for *Culex pipiens* (Turell *et al.*, 2000) and *Culex quinquefasciatus* (Sardelis *et al.*, 2001). This suggests that only the NY99-, Greece-10- and Ita09-infected birds that reached peak viraemia RNA titres $>10^{7.5} \text{ RNA copies ml}^{-1}$ (which resulted in $10^{5.3} \text{ TCID}_{50} \text{ ml}^{-1}$ upon virus isolation) would be likely to infect feeding mosquitoes, as the lowest cut-off of $10^{5.0} \text{ p.f.u. ml}^{-1}$ will result in $\sim 10^{5.2} \text{ TCID}_{50} \text{ ml}^{-1}$, according to a conversion factor of 1 TCID_{50} to 0.7 p.f.u. (Saunders, 2007). It is possible that the infectious viraemia titres of the other samples were below the detection limit of the assay. As the majority of the birds did not attain viraemia titres $>10^{7.5} \text{ RNA copies ml}^{-1}$, it suggests that jackdaws would most likely not serve as efficient amplifying hosts in the transmission cycle of WNV in Europe. However, it is also possible that a higher peak of viraemia was missed due to sampling on intervening days. Similar results were observed in a study using a French strain (Fr2000) in carrion crows (Dridi *et al.*, 2013), where the highest viral RNA titre recorded was equivalent to only $10^4 \text{ TCID}_{50} \text{ ml}^{-1}$, which is also below the infectious viral titre required for transmission to feeding mosquitoes.

None of the isolates used in this study has either caused massive die-offs of birds in the field or has spread swiftly across Europe as observed during the North American invasion by WNV (Hofmeister, 2011). Thus far, experimental studies using European strains of WNV and European birds have shown fairly low mortality rates (Dridi *et al.*, 2013; Sotelo *et al.*, 2011; Ziegler *et al.*, 2013), with the highest mortality rate of 33 % observed following inoculation of carrion crows with the Fr2000 strain (Dridi *et al.*, 2013). The study presented herein suggests that the susceptibility of European birds could be related to the WNV strain, as much higher mortality was observed in this study with alternative strains compared with the French strain. At the same time, host factors present in the jackdaw or other European avian species could also play an important role in susceptibility, as the mortality induced by the North American strain NY99, known to be highly virulent in corvids, was similar to that exhibited by Ita09 (60 %) in jackdaws. This mortality rate, as well as the viraemia levels, was also not comparable with those usually seen upon infection of American crows with

NY99. This therefore suggests that host factors present in European jackdaws could potentially reduce their susceptibility to WNV.

However, a substantial proportion of the jackdaws did die as a result of infection, which therefore suggests that perhaps some birds in Europe are indeed succumbing to WNV infection in the field, but that the mortality rates of these birds are simply too low to be detected by the current monitoring systems present in Europe. It is also possible that natural infection via mosquito feeding could result in higher serum viraemia titres in jackdaws or that other ecological factors such as infection due to carcass scavenging by more susceptible birds could play a more important role in the transmission and maintenance of WNV in Europe. Nevertheless, this study shows that jackdaw mortality could be potentially useful for tracking WNV transmission in Europe.

METHODS

Source of virus and birds

Five different isolates of WNV were utilized (Table 4). The NY99-4132 strain (NY99; lineage 1a) (Brault *et al.*, 2004) was isolated originally from the brain of an American crow and was passed two times in Vero cells before being used in this study. The lineage 2 Greek strain Nea Santa-Greece-2010 (Greece-10; GenBank accession number HQ537483.1) was isolated from a pool of *C. pipiens* mosquitoes (Papa *et al.*, 2011) and passaged once in Vero cells. Two lineage 1a Italian strains, FIN (provided by Dr Vittorio Sambri, University of Bologna, Italy; two Vero E6 passages; GenBank accession number KF234080) and Ita09 (provided by Dr Luisa Barzon, University of Padova, Italy, one Vero E6 passage; GenBank accession number GU011992.2) were both isolated from a patient with neuroinvasive disease (Barzon *et al.*, 2009). The Hungarian lineage 2 strain 578/10 (provided by Dr Tamás Bakonyi, Szent István University, Hungary, two passages on Vero E6 cells; GenBank accession number KC496015) was isolated from the brain of a horse that died of WNV neuroinvasive disease. Virus stocks used in this study (not including NY99 and Greece-10) were prepared by growing the viruses once in C6/36 insect cells (Table 1).

Jackdaws were captured using walk-in traps in the municipality of Rotterdam, The Netherlands. They were transported to indoor housing where they were kept in groups of seven or eight in isolators under negative pressure. Only seronegative birds were used in this study. All birds were cared for in animal holding facilities at the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands.

Detection of pre-existing WNV antibodies

To confirm that jackdaws had not been exposed previously to WNV, the birds were bled before experimental infection and serum was tested for neutralizing antibodies using TCID₅₀ neutralization assays. Serum was heat-inactivated at 56 °C for 30 min, serially diluted twofold and incubated with an equal volume of virus (strain NY99, originally isolated from a dead Chilean flamingo at the Bronx Zoo in New York, obtained from the Health Protection Agency, Porton Down, UK; P5 on Vero E6 cells; GenBank accession number AF196835.2) to a final concentration of 100 TCID₅₀ per 0.1 ml. Samples were

incubated at 37 °C for 1 h and subsequently added to an 80 % confluent monolayer of Vero E6 cells in CELLSTAR 96-well plates (Greiner Bio-One). Plates were incubated at 37 °C for 5 days. Samples were read and a 100 % reduction in cytopathic effect (CPE) as compared with the serum-negative control was used for the determination of neutralization. Detection of any neutralizing activity to WNV in the serum of any bird precluded its use for experimental inoculation.

Experimental infection and sampling protocol

Jackdaws were subcutaneously inoculated in the thigh region with 2000 TCID₅₀ virus in 0.1 ml Dulbecco's modified Eagle's medium (DMEM) containing no FBS. Jackdaws were injected with NY99 (*n*=7), Greece-10 (*n*=8), FIN (*n*=7), Ita09 (*n*=7) and 578/10 (*n*=7). Blood was collected from all birds (~0.1 ml) at 2 day intervals for a period of 8 days p.i. Coagulated blood was centrifuged at 1300 *g* for 5 min in MiniCollect vials (Greiner Bio-One) in order to separate serum, which was stored subsequently at -80 °C. All jackdaws were examined for signs of disease twice daily for 14 days following inoculation and euthanized under isoflurane anaesthesia upon display of clinical symptoms. Additionally, two birds per group were euthanized at day 4 p.i. for monitoring the dissemination of virus to the organs at the approximate time of peak viraemia, as well as all surviving birds at day 14 p.i.

Necropsies were performed on all euthanized birds and the following tissues were collected: heart, liver, spleen, kidney, bone marrow and brain. A small section of each tissue was collected, and subsequently weighed and homogenized using a metal bead in 1 ml DMEM containing antibiotics (100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹). The remaining portion of the tissues was collected in formalin for use in immunohistochemical staining.

Determination of viral loads

To determine viral loads in the serum samples and tissue homogenates, we used qRT-PCR to measure viral RNA titres (serum and tissue) and TCID₅₀ titration for the calculation of infectious virus titres (serum only). Briefly, RNA was isolated from 50 µl serum or 100 µl homogenized tissue using the MagNA Pure LC Total Nucleic Acid Isolation kit (Roche) and an automated nucleic acid robotic workstation (Roche) according to the manufacturer's instructions. RNA was eluted in 100 µl elution buffer (Roche) and stored at -80 °C until assayed. RNA copy numbers were quantified using unmodified primers as described previously (Lim *et al.*, 2013a). The limit of detection of the assay was 0.95 log₁₀RNA copies.

Infectious titres in the serum were determined by log₁₀ titration of the serum samples on Vero E6 cells and calculating the TCID₅₀ using the Spearman-Kärber method (Kärber, 1931; Spearman, 1908) after the determination of CPE 5 days p.i. Initial 1 : 10 dilution of serum resulted in a limit of detection of 10^{1.75} TCID₅₀ ml⁻¹.

Immunohistochemistry

Sagittal organ paraffin sections (4 µm thick) were processed for peroxidase immunohistochemistry of virus non-structural protein markers. Sections were deparaffinized

in xylene, rehydrated in descending concentrations of ethanol and incubated for 10 min in 3 % H₂O₂ diluted in PBS in order to block endogenous peroxidase activity. Antigen exposure was performed by 15 min incubation at 121 °C in citrate buffer (0.01 M, pH 6.0). Sections were incubated overnight at 4 °C with primary goat anti-WNV NS3 (1 : 100; R&D Systems) or goat serum (1 : 100; Dako) for isotype controls, and detected with secondary rabbit anti-goat IgGPO (Dako) antibody. Sections were counterstained with Mayer's haematoxylin, mounted with Kaiser's glycerol gelatin and analysed using a light microscope.

Statistical analyses

Survival curves were analysed using the log-rank (Mantel–Cox) test. All other statistical analyses were performed by Kruskal–Wallis ANOVA and any significant difference found was more closely analysed between the groups using the Mann–Whitney *U* test.

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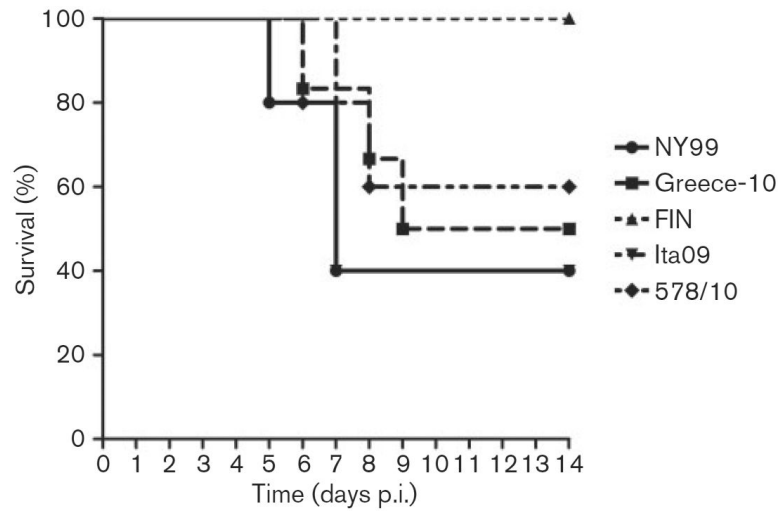


Fig. 1. Survival of five or six European jackdaws, each inoculated with 2000 TCID₅₀ WNV strains NY99, Greece-10, FIN, Ita09 or 578/10. Jackdaws were monitored daily for signs of disease up to 14 days p.i.

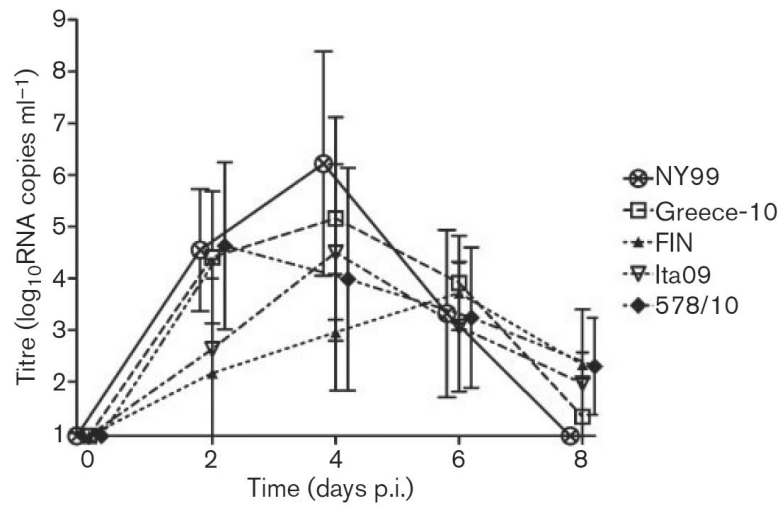


Fig. 2. Serum viraemia profiles for WNV-infected European jackdaws after inoculation with 2000 TCID₅₀ NY99 ($n=5$), Greece-10 ($n=6$), FIN ($n=5$), Ita09 ($n=5$) or 578/10 ($n=5$). Viral titres were determined by RNA copy numbers and are represented as geometric mean \pm SD. A detection limit of 0.95 log₁₀RNA copies ml⁻¹ was determined.

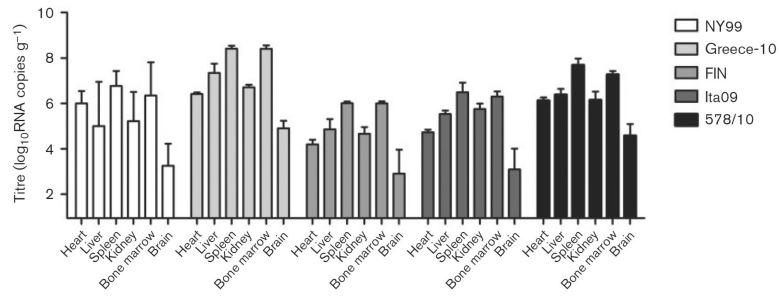


Fig. 3. Tissue viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different WNV strains and euthanized on day 4 p.i. (two per group). Viral titres are represented as geometric mean±SD. A detection limit of 0.95 log₁₀RNA copies g⁻¹ was determined.

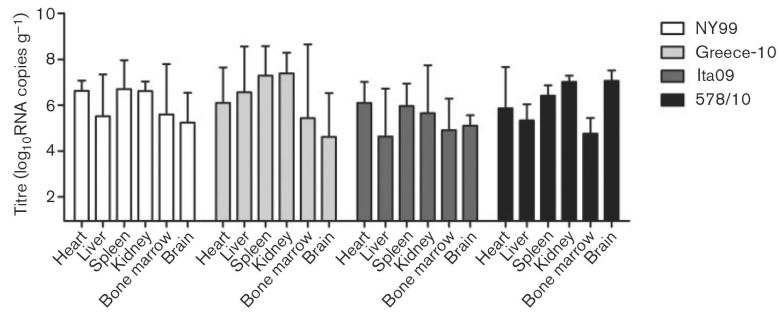


Fig. 4. Tissue viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different WNV strains and euthanized due to morbidity. NY99 ($n=3$): one bird euthanized on day 5 p.i., two birds on day 7 p.i.; Greece-10 ($n=3$): one bird euthanized on day 6 p.i., one bird on day 8 p.i. and one bird on day 9 p.i.; Ita09 ($n=3$): three birds euthanized on day 7 p.i.; and 578/10 ($n=2$): one bird euthanized on day 6 p.i. and one bird on day 8 p.i. Viral titres are represented as geometric mean \pm SD. A detection limit of 0.95 log₁₀RNA copies g⁻¹ was determined.

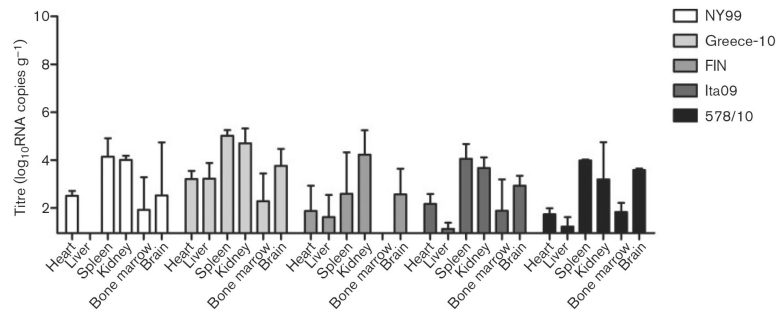


Fig. 5. Tissue viral load, as determined by RNA copy numbers, in organs harvested from birds experimentally infected with different WNV strains and euthanized on day 14 p.i. NY99, two birds euthanized; Greece-10, three birds; FIN, five birds; Ita09, two birds; and 578/10, three birds. Viral titres are represented as geometric mean \pm SD. A detection limit of 0.95 log₁₀RNA copies g⁻¹ was determined.

Clinical profile of five or six European jackdaws infected with WNV strains NY99, Greece-10, FIN, Ita09 and 578/10

Table 1

| Virus group | Mortality [n/N (%)] | Median day of death | Mean \pm sd day of onset of viraemia | Mean \pm sd peak serum viraemia (log ₁₀ RNA copy number ml ⁻¹) | Mean \pm sd day of peak viraemia | Mean \pm sd duration of viraemia (days) |
|-------------|---------------------|---------------------|--|---|------------------------------------|---|
| NY99 | 3/5 (60) | 7 | 2.0 \pm 0 | 6.2 \pm 1.9 | 4.0 \pm 0 | 5.2 \pm 1.0 |
| Greece-10 | 3/6 (50) | 8 | 2.0 \pm 0 | 5.9 \pm 1.3 | 3.3 \pm 1.0 | 6.0 \pm 1.2 |
| FIN | 0/5 (0) | NA | 3.2 \pm 1.0 | 4.0 \pm 0.7 | 4.0 \pm 1.3 | 6.4 \pm 0.8 |
| Ita09 | 3/5 (60) | 7 | 2.8 \pm 1.0 | 4.7 \pm 1.5 | 4.0 \pm 1.3 | 5.2 \pm 1.6 |
| 578/10 | 2/5 (40) | 7 | 2.0 \pm 0 | 5.0 \pm 1.3 | 4.0 \pm 2.2 | 6.4 \pm 1.5 |

NA, not applicable.

Immunohistochemical analysis of WNV antigen distribution in European jackdaws euthanized on day 4 p.i.

Table 2

| Virus strain | Bird no. | Heart | Liver | Spleen | Kidney | Bone marrow | Brain | Total score per bird | Mean score per virus strain | No. of positive organs/bird |
|------------------------------|----------|-------|-------|--------|--------|-------------|-------|----------------------|-----------------------------|-----------------------------|
| NY99 | 6 | ± | ± | ± | ± | + | ± | 7 | 6.5 | 6/6 |
| | 7 | + | ± | - | + | - | ± | 6 | | 4/6 |
| Greece-10 | 1 | ++ | - | ++ | + | ++ | ± | 12 | 12.5 | 5/6 |
| | 4 | + | + | ++ | + | ++ | ± | 13 | | 6/6 |
| FIN | 6 | ± | ± | + | + | ± | ± | 8 | 7 | 6/6 |
| | 7 | - | ± | + | + | ± | - | 6 | | 4/6 |
| Ita09 | 1 | ± | + | + | + | - | - | 7 | 8.5 | 4/6 |
| | 2 | + | + | + | + | + | - | 10 | | 5/6 |
| 578/10 | 6 | + | + | ++ | + | + | - | 11 | 10.5 | 5/6 |
| | 7 | ± | + | ++ | + | + | - | 10 | | 5/6 |
| Score per organ | | 15 | 14 | 21 | 19 | 16 | 5 | | | |
| No. of positive birds/organs | | 9/10 | 9/10 | 9/10 | 10/10 | 8/10 | 5/10 | | | |

Subjective determinations of the amount of antigen in each organ were made: -, negative; ±, minimal; +, moderate; ++, abundant. Each determination was given a score from 0 to 3; 0, negative; 1, minimal; 2, moderate; 3, abundant.

Table 3
 Immunohistochemical analysis of WNV antigen distribution in European jackdaws euthanized due to morbidity (days 5–9 p.i.)

| Virus strain | Bird no. | Heart | Liver | Spleen | Kidney | Bone marrow | Brain | Total score per bird | Mean score per virus strain | No. of positive organs/bird |
|------------------------------|----------|-------|-------|--------|--------|-------------|-------|----------------------|-----------------------------|-----------------------------|
| NY99 | 1 | - | ± | + | ± | + | - | 6 | 7.7 | 4/6 |
| | 3 | + | - | + | + | - | + | 8 | | 4/6 |
| | 5 | ++ | - | + | + | - | + | 9 | | 4/6 |
| Greece-10 | 5 | + | - | ± | + | - | + | 7 | 10.7 | 4/6 |
| | 6 | + | ++ | ++ | ++ | ++ | ± | 15 | | 6/6 |
| Ita09 | 8 | ++ | - | + | + | ± | + | 10 | 10 | 5/6 |
| | 3 | + | + | + | ++ | + | ++ | 14 | | 6/6 |
| | 4 | + | ± | ± | + | ± | ± | 8 | | 6/6 |
| 578/10 | 5 | + | - | + | ++ | - | ± | 8 | 10.5 | 4/6 |
| | 4 | + | ± | + | + | ± | ++ | 11 | | 6/6 |
| | 5 | ++ | - | + | + | ± | + | 10 | | 5/6 |
| Score per organ | | 13 | 4.5 | 11 | 13.5 | 6 | 10 | | | |
| No. of positive birds/organs | | 10/11 | 5/11 | 11/11 | 11/11 | 7/11 | 10/11 | | | |

Subjective determinations of the amount of antigen in each organ were made: -, negative; ±, minimal; +, moderate; ++, abundant. Each determination was given a score from 0 to 3: 0, negative; 1, minimal; 2, moderate; 3, abundant.

Table 4

WNV strains used for susceptibility studies in European jackdaws

| Virus | Strain | Source | Passage history* | Location | Genetic lineage |
|--------------|-----------------------|------------------------------------|-------------------------|-----------------|------------------------|
| NY99 | NY99-4132 | American crow (brain) | V2 | USA | 1a |
| Greece-10 | Nea Santa-Greece-2010 | <i>Culex pipiens</i> | V1 | Greece | 2 |
| FIN | FIN | Patient with neuroinvasive disease | V2, C1 | Italy | 1a |
| Ita09 | Ita09 | Patient with neuroinvasive disease | V1, C1 | Italy | 1a |
| Hungary | 578/10 | Horse (brain) | V2, C1 | Hungary | 2 |

* Viruses were propagated in Vero (V) or C6/36 insect cells (C). Numbers following passage source represent the number of viral passages.