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ORIGINAL ARTICLE

Setting up a SPF Chicken Model for the Pathotyping of West Nile Virus (WNV) Strains

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West Nile virus; SPF chickens; experimental infection; mortality rate; viremia; viral RNA load

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Summary

Birds play a central role in WNV epidemiology by spreading and amplifying the virus. Increasing numbers of WNV isolates are detected in Europe, and the virulence of these genetically variable isolates is not well characterized for birds. Therefore, we investigated whether SPF chickens could be a valuable avian model for the pathotyping of WNV strains. One-day-old SPF chickens were inoculated subcutaneously (SC) or intracerebrally (IC) with four lineage 1 WNV strains (Is98, It2008, Fr2000 or Kunjin) and were daily clinically monitored for 2 weeks after infection. Additionally, one-day-old SPF chickens were SC inoculated, and one-week-old SPF chickens were SC or IC inoculated with two Euro-Mediterranean isolates, Is98 and Fr2000, to sample blood and feathers at regular time points. These samples were analysed by WN NS2a-specific rRT-PCR and WN NS1 antigen-capture ELISA that were developed for the purpose of this study. Differences in strain virulence were evidenced after IC inoculation of one-day-old SPF chickens, with Is98 eliciting the highest mortality rates and Kunjin the lowest ones, while lethality of Fr2000 and It2008 was intermediate. Neither viral load in sera and feathers nor NS1 antigen in the serum correlated with the differential pathogenicity of Is98 and Fr2000. However, irrespective of the inoculated strain, younger chickens showed higher and longer-lasting viremias than older chickens. In all experimental groups, the detection window for viral RNA in feathers lasted up to 14 dpi. Altogether, the data presented in this study show that WNV strain virulence can be discriminated in a one-day-old SPF chicken model on the basis of mortality rates, while viremia and viral load in feathers appear to be age dependent rather than strain dependent.

Introduction

West Nile virus (WNV) is a mosquito-borne virus belonging to the *Flavivirus* genus (*Flaviviridae* family) (Murray et al., 2010). During the mid-20th century, sporadic cases and outbreaks of human WNV infections have been recorded in Africa, the Near and Middle East, Asia and Europe (Nir et al., 1972; George et al., 1984; Mathiot et al., 1984, 1988; Hubalek and Halouzka, 1999; Jupp, 2001), while other areas such as Egypt and Australasia became

endemic (Hurlbut et al., 1956; Smith et al., 2011). From the 1990s onward, WNV seemed to re-emerge in Europe as the frequency and severity of outbreaks increased for humans and horses, especially in Romania (Tsai et al., 1998), Russia (Malkinson et al., 2002), France (Murgue et al., 2001), Italy (Autorino et al., 2002; Rizzo et al., 2012) and Greece (Danis et al., 2011). Importantly, in 1998, high mortality rates were recorded for the first time among birds, a class so far considered as resistant, which indicated a shift in WNV pathogenicity (Malkinson et al., 2002; Ba-

net-Noach et al., 2003). Finally, in 1999, the introduction and rapid spread of WNV in North America with high rates of mortality and morbidity in humans as well as in horses and passerines established WNV as a global sanitary concern (van der Meulen et al., 2005; Murray et al., 2010).

In the United States, WNV has been detected in at least 326 species of wild and domestic birds showing variable susceptibilities (De Filette et al., 2012). American Passeriformes, especially the family *Corvidae*, appear as the most susceptible species as they develop the highest viremia levels, shed the largest quantity of virus in oral and cloacal fluids and show severe neurological signs and high mortality rates (Komar et al., 2003; van der Meulen et al., 2005). So far, psittacine and gallinaceous birds are considered the least susceptible species as viremia and virus shedding are generally more limited, and disease or death is rarely observed (van der Meulen et al., 2005). Nevertheless, viremia titres higher than $10^{6.3}$ PFU/ml of blood were described for WNV-infected 2- to 3-day-old chickens (Komar et al., 2003).

Unlike the sudden avian mass mortalities observed in the United States, WNV caused only limited to no disease in European birds (Dauphin et al., 2004). Only domestic geese (*Anser anser*, order anseriformes) have been reported to succumb massively to a natural infection with local WNV strains, and unusual deaths have been recorded in a few migrating white storks (*Ciconia ciconia*, order ciconiiformes), corvids (*Pica pica* and *Corvus corone*), rock pigeons (*Columba livia*), cormorants (*Phalacrocorax carbo*), raptors (*Accipiter gentilis*, *Falco rusticolus*) and gulls (*Larus michahellis*) (Banet-Noach et al., 2003; Dauphin et al., 2004; Zeller and Schuffenecker, 2004; Glavits et al., 2005; Calistri et al., 2010b; Wodak et al., 2011). Little is known about the susceptibility of European birds to European WNV isolates as experimental studies are still scarce (Sotelo et al., 2011; Ziegler et al., 2012).

Phylogenetic studies segregate WNV isolates in two main lineages: lineages 1 and 2, in addition to 5 other topotype lineages (Zeller and Schuffenecker, 2004; Bakonyi et al., 2005; Bondre et al., 2007; Calistri et al., 2010a,b; De Filette et al., 2012). So far, the majority of the strains responsible for the European and Mediterranean basin outbreaks belong to lineage 1 clade a (Zeller and Schuffenecker, 2004; Calistri et al., 2010a; Smith et al., 2011). Additionally, a WNV strain closely related to Central African lineage 2 viruses has been isolated in Hungary in 2004 (Bakonyi et al., 2006) before spreading and causing dramatic human outbreaks in Greece, especially in 2010 (Papa et al., 2011). As increasing strains and lineages are becoming involved in disease outbreaks in Europe, the European WNV issue appears even more complex than the American one where NY99 was the only viral strain associated with the North American outbreaks (Lanciotti et al., 1999), before being

displaced by a second U.S. genotype – WN02 – that originated from NY99 and emerged in 2002 (Ebel et al., 2004; Davis et al., 2005).

Further epidemiological, virological and entomological investigations are crucial for a better understanding of the factors triggering the spread of WNV. Given the central role of birds in spreading and amplifying WNV, *in vivo* bird models are needed for the assessment of WNV evolution dynamics and for rapid evaluation of new isolates. The potential risk should be monitored in a standardized experimental setting allowing a representative characterization of the interaction between avian hosts and WNV strains. As a consequence, developing a sensitive and robust *in vivo* avian model of WNV infection for diagnostic and research purposes appears as a relevant complementary tool for the pathotypic characterization of WNV isolates. Young SPF chickens are routinely used and officially recommended in the international legislation for the determination of the virulence of poultry viruses such as Newcastle Disease Virus or Avian Influenza (Alexander, 2000; Alexander et al., 2012). The aim of this study was to investigate whether a similar system could be valuable for WNV.

Materials and Methods

Viruses and virus preparations

WNV strains Kunjin (Kunjin 35911, GenBank accession no: JX014270), Fr2000 (PaAn001, GenBank accession no: AY268132.1), It2008 (15803, GenBank accession no: FJ483549) and Is98 (IS-98 STD1, GenBank accession no: AF481864) were used in this study. Kunjin belongs to lineage 1 clade b and the three other strains to lineage 1 clade a. The inocula were prepared by amplification in Vero cells (African green monkey kidney-derived cells provided by P. Desprès from the Pasteur Institute, Paris), for 1 passage for Kunjin and It2008, 5 passages for Is98 and for 2 to 5 passages for Fr2000. Infected cells were lysed by a freeze-thawed cycle after each passage, and both virus-containing cell lysates and supernatants were frozen in aliquots at -80°C .

Virus titration by plaque-forming unit (PFU) and tissue culture-infectious dose 50 (TCID₅₀) assays

The titre of the Is98 strain was estimated using a PFU assay as previously described (Bunning et al., 2002; Weingartl et al., 2004; Jerzak et al., 2007; Shirafuji et al., 2008; Bingham et al., 2010; Deardorff et al., 2011). Briefly, 100 μl of 1/10 serial viral dilutions ranging from 10^{-1} to 10^{-8} was added in serum-free medium to confluent Vero cells plated in 6-well plates. Cells and viruses were incubated at 37°C , 5% CO_2 for 1 h before addition of a DMEM high-glucose pyruvate solution (Life Technologies Europe

B.V., Belgium) containing 0.5% SeaPlaque agarose (cat no. 50101, Lonza, the Netherlands) and 2% foetal bovine serum (cat. no. A11-01, PAA Laboratory, Pasching, Austria). After incubation for 48 h, viable cells were stained with the overlay solution supplemented with 0.004% neutral red solution (cat no. 50101, Sigma-Aldrich, Bornem, Belgium). All samples were tested in duplicate, in three separate assays, and the plaques were manually counted.

The TCID₅₀ titration method was also implemented using 100 µl of the 1/10 dilutions used above to inoculate 8 wells of a 96-well plate. Freshly passaged Vero cells were subsequently added to each well (total volume: 200 µl/well, cells dilution: 1/8). After 3 days of incubation at 37°C and 5% CO₂, the cytopathic effect (CPE) was manually recorded. TCID₅₀/ml values were calculated according to the Reed and Muench method (Reed and Muench, 1938), where the tissue culture-infectious dose is the virus dilution where 50% of the wells are infected. The results are represented as log of the mean value obtained from three replicates.

Virus titration by NS2a-specific real-time RT-PCR

RNA was extracted from the above prepared serial 1/10 dilutions in addition to intermediate 1/10^{0.5} dilutions using a QIAamp Viral RNA Mini Kit (Qiagen Benelux B.V., Venlo, The Netherlands) according to the manufacturer's instructions. RNA was recovered in elution buffer (provided by the manufacturer) and stored at -80°C. Two µl (3.33% of total eluate) of each extract, in 25 µl final volume, was subjected to rRT-PCR amplification using the QuantiTect Probe RT-PCR Kit (Qiagen GmBh, Hilden, Germany) following manufacturer's instructions. The mix included primers (WNNS2-F: 5'-CCTTTTCAGTTGGGCCTTCTG-3' and WNNS2a-R: 5'-GATCTTGGCTGTCCA CCTCTTG-3') at 0.15 µM final concentration, and a FAM-labelled TAMRA probe (WNNS2A-6-FAM TAMRA: 5'-TTCTTGGCCACCCAGGAGGTC-3'-TAMRA) at 0.2 µM final concentration. Amplification conditions consisted of a first reverse-transcription step at 50°C for 30 min, followed by 10 min at 95°C and 50 cycles of 15 sec at 95°C, 34 sec at 54°C and 10 sec at 72°C. Samples for each experimental set and each dilution were run in triplicate, and results are presented as the mean.

The serum matrix effect on NS2a-specific rRT-PCR was evaluated for molecular viral detection in the serum of SPF chickens, our matrix of interest. A correction factor for negative serum matrix effect was calculated with 3 WNV-negative serum pools of 20-day-old SPF chickens. Each of these pools was spiked with an independent 1/10^{0.5} serial dilution series of an initial 1/20 dilution of an Is98 viral strain suspension aliquot (titre of the viral stock: 10^{5.67} TCID₅₀/ml). RNA was extracted from each dilution and

subjected in triplicate to the NS2a-specific rRT-PCR as described above.

Experimental inoculations of SPF chickens

Chickens

After hatching, SPF white Leghorn chickens provided by Lohmann Valo (Cuxhaven, Germany) were kept in biosecurity level 3 (BSL-3) isolators, and animal experiments were conducted under the authorization and supervision of the Biosafety and Bioethics Committees at the Veterinary and Agrochemical Research Center, following National and European regulations (procedure agreement no. 111202-01). The birds were provided with a commercial diet for poultry and water *ad libitum* throughout the experiments.

Inoculations for clinical follow-up

In separate experiments, one-day-old SPF chickens were inoculated by the subcutaneous (SC) route with 100 µl inoculum/chicken or by the intracerebral (IC) route with 50 µl inoculum/chicken of Kunjin, Fr2000, It2008 or Is98 diluted in sterile PBS to 10³, 10² or 10¹ TCID₅₀. Experimental infections were repeated two to three times for each viral strain, and each experimental group included ten to fifteen chickens. Disease symptoms were observed daily for up to 14 days post-inoculation (dpi). Mortalities were recorded and cadavers removed daily from the isolators.

Inoculations for sample collection

In independent experiments, SPF chickens were inoculated subcutaneously (100 µl inoculum/chicken) at the age of one day or one week with a dose of, respectively, 10³ or 10⁴ TCID₅₀ Is98 or Fr2000 WNV diluted in sterile PBS (Table 1). For animal welfare reasons and considering that the mortality rate in one-day-old chickens inoculated intracerebrally with Is98 was very high and that the difference in survival rates between Is98 and Fr2000 at a dose of 10³ TCID₅₀ was still significant when the virus was inoculated by the SC route, we chose to skip the experimental condition 'IC inoculation of one-day-old birds' for this part of the study and to keep only the experimental condition 'SC

Table 1. Number of 1-day-old and 1-week-old SPF chickens involved in the viremia and virus detection experiments. Birds were inoculated subcutaneously (SC) or intracerebrally (IC) with WNV strains Is98 or Fr2000 at the age of 1 day or 1 week

Age at inoculation	Inoculation route	Dose	WNV strain	
			Is98	Fr2000
1 day	SC	10 ³ TCID ₅₀	N = 55	N = 27
1 week	SC	10 ⁴ TCID ₅₀	N = 30	N = 30
	IC	10 ² TCID ₅₀	N = 30	N = 40

inoculation of one-day-old birds', in order to limit as much as possible the number of inoculated birds. Thus, SPF chickens were inoculated intracerebrally (50 µl inoculum/chicken) at the age of one week with 10² TCID₅₀ of Is98 or Fr2000 WNV strains. A higher dose of 10⁴ TCID₅₀ was chosen for SC inoculation of chickens aged 1 week because preliminary studies have shown that one-week-old chickens are more resistant to WNV infection. On 2, 5, 7, 9, 12 and 14 dpi, 5 chickens per subgroup – or less if the number of surviving birds was limited – were sacrificed. Blood was collected and allowed to clot for 6 h at room temperature (RT°). After centrifugation at 8000 rpm for 5 min, serum was collected and aliquots stored at –20 or –80°C for further use in ELISA and RNA extraction, respectively. Two to three wing primary feathers were sampled from each sacrificed bird and stored in 600 µl of RNA later (RNA Stabilization Reagent, Qiagen Benelux B.V., The Netherlands) at –80°C for further RNA extraction.

Viremia monitoring

Viral genome load was measured in serum by NS2a-specific rRT-PCR. Quantification of WNV was realized using standard curves ($R^2 > 0.99$) generated with previously titrated Is98 WNV seed after correction of crossing points (Cp) for serum matrix effect.

Viral genome detection in feathers

Feathers were prepared by homogenization for 6 min at 30 cycles/s in 600 µl of Ambion® MagMAX™ Lysis/Binding Solution Concentrate (Life Technologies Europe B.V., Gent, Belgium) supplemented with 0.04 M DTT using a TissueLyser™ homogenizer in the presence of a 5-mm Ø stainless steel bead. After clarification at 8000 rpm for 10 min, RNA was extracted using a MagMAX™-96 Total RNA Isolation Kit (Life Technologies Europe B.V., Gent, Belgium) according to the manufacturer's instructions. RNA was recovered in 50 µl of elution buffer (provided by the manufacturer) and stored at –80°C. Two µl (4% of total eluate) of each extract was subjected to the NS2a-specific rRT-PCR in a final volume of 25 µl.

Secreted NS1-capture ELISA

Two NS1 specific monoclonal antibodies were produced to develop an in-house NS1-capture ELISA. Briefly, six-week-old female mice (BALB/c strain, Charles River, Lyon, France) were gene gun-immunized every 4 weeks during 3 months with gold beads coated with a pCI-NS1 plasmid. The immunized mice were boosted 2 weeks after the last DNA immunization with an intrasplenic inoculation of baculovirus-expressed NS1. Splenocytes were fused with SP2/0-Ag14 myeloma cells (CRL-1581, ATCC) using ClonaCell-Hy hybridoma kit (StemCell Technologies, Grenoble, France). After screening on WNV-infected Vero cells, two

specific clones were selected (3B8 and 5F7) to detect the secreted NS1 antigen (Ag) by Ag capture ELISA in the sera of infected birds.

Briefly, 96-microwell plates (F96 MaxiSorp Nunc-Immuno Thermo fisher) were coated for 1 h at 37°C with the 3B8 monoclonal antibody (mAb) diluted in PBS at 2 µg/ml. Plates were then washed three times with 300 µl of PBS supplemented with 0.1% Tween 80 and blocked for 30 min at 37°C with PBS containing 2.5% casein (cat. no C8654, Sigma-Aldrich, Steinheim, Germany). After washing, plates were incubated overnight at 4°C with serum samples and negative and positive controls (sera of 1-week-old naive and WNV-infected SPF chickens, respectively) diluted ½ in PBS. Biotin-labelled 5F7 mAb was then added for 1 h at RT°. The plates were then incubated with streptavidin-conjugated horseradish peroxidase (cat. no SNN2004, Invitrogen, Gent, Belgium) for 1 h. After six washes, enzyme activity was detected by adding 100 µl of tetra-methyl benzidine (TMB) peroxidase substrate solution (KPL, Gaithersburg, USA) for 15 min in the dark before stopping the reaction with 1 M H₃PO₄ buffer. Optical densities (O.D.) were determined at 450–620 nm with an ELISA plate reader (Original Multiskan Ex plate reader, Labsystems, Virginia, USA). The detection threshold had been preliminarily calculated as the mean O.D. (450 nm)-O.D. (620 nm) ± 3 S.D. for 50 random sera samples from 1-week-old naive SPF chickens. Samples with an O.D. > 0.115 are considered positive.

Statistical analysis

Statistical analyses were performed using Minitab 13 and STATA 10 software (statistical programmes for Windows 2000), and differences were considered significant at $P < 0.05$.

Viremia values after inoculation with Is98 and Fr2000 were compared for each route of inoculation and for each dose. After checking the hypotheses' validity (normality of the criterion distribution for each group by the Ryan–Joiner test and homogeneity of the within-groups variances by the Levene test), the Student's t-test was carried out on this strain criterion to compare the two inoculated groups. When normality or homogeneity of variance tests failed, the nonparametric Mann–Whitney test was used. Similar statistical analyses were performed on the data from the detection of secreted NS1 Ag in serum and NS2a RNA in feathers to compare the two inoculated groups at different dpi and on the WNV titration data to compare the effect of serum matrix on the detection limit of the NS2a-specific rRT-PCR. Survival rates after inoculation with Is98, Fr2000, It2008 and Kunjin were compared for each route of inoculation in chickens aged 1 day at the time of infection. After checking the hypotheses' validity, the ANOVA

and Tukey's pairwise comparison tests were carried out on the strain criterion to compare the four inoculated groups. When normality or homogeneity of variance tests failed, the nonparametric Kruskal–Wallis test was used.

Results

Optimization of WNV molecular titration

To validate the NS2a-specific rRT-PCR technique as a titration method, the titres of three aliquots of Is98 virus stock were estimated using TCID₅₀, PFU- and NS2a-specific rRT-PCR assays (Fig. 1a). A linear correlation ($R^2 = 0.9918$) was observed between PFU or TCID₅₀ titres and values expressed in rRT-PCR Cp measurement, within a titre range of 10^3 to 10^5 TCID₅₀/ml of Is98 virus.

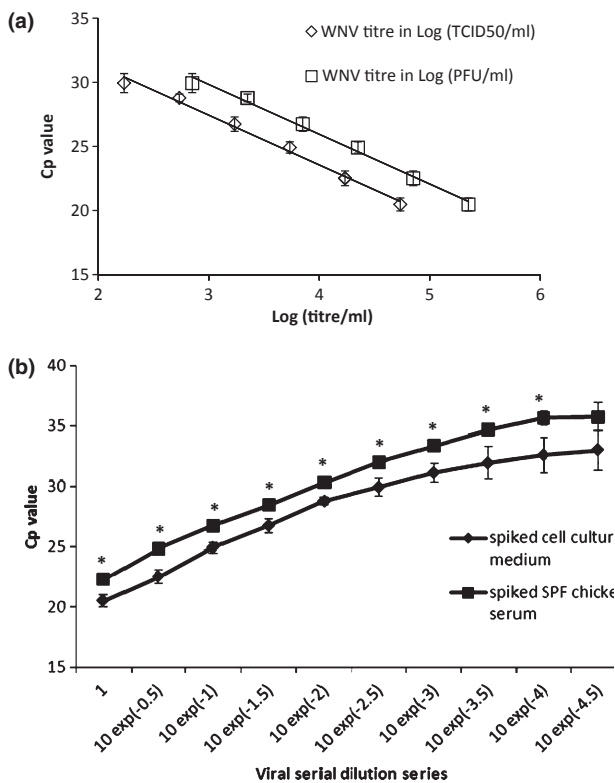


Fig. 1. rRT-PCR-mediated virus titration. (a) Correlation between the two virus isolation methods and the molecular technique used to determine the viral titre of West Nile virus (WNV) strain Is98. Cp values are plotted against log (TCID₅₀/ml) (open diamonds) and log (PFU/ml) (open squares). TCID₅₀/ml and PFU/ml are presented as $10^{-1/2}$ multiples of a viral stock titre. (b) Effect of SPF chicken serum matrix on the detection limit of the rRT-PCR NS2a. The results obtained with spiked cell culture medium are represented by filled diamonds; those for spiked serum samples are given by filled squares. * $P < 0.05$ for cell culture medium versus SPF chicken serum matrix. For both graphs, Cp values are presented as the mean of nine replicates \pm SD.

The influence of serum – as a matrix for WNV viremia monitoring – on molecular detection was investigated. The Cp values obtained for Is98 diluted in SPF chicken serum as a matrix were significantly higher ($P < 0.05$) when compared to virus-spiked culture medium, which indicates an inhibitory effect of serum on NS2a-specific rRT-PCR (Fig. 1b). A Cusum analysis (Van Dobben de Bruyn, 1968) showed that both curves do not significantly deviate from linearity ($P = 0.49$). This matrix effect could therefore be quantified by generating the following corrective Passing and Bablok (Passing and Bablok, 1983) regression equation: $y = -2,744046 + 1,035194 x$, where x represents the Cp measured in sera, and y represents the corrected Cp as it would be measured in the absence of matrix inhibitory effect. This equation was used in the rest of this study to correct NS2a Cp measured in test sera of SPF chickens. Corrected Cps were subsequently converted into TCID₅₀/ml equivalents by including on every rRT-PCR plate a standard curve generated with serial dilutions of a previously titrated stock of Is98 virus.

Clinical characterization in SPF chickens of four WNV strains presenting different epidemiological profiles

During the clinical follow-up experiments, Is98, Fr2000, It2008 and, to a lesser extent, Kunjin strains were all pathogenic for SPF chickens inoculated at the age of 1 day with a dose of 10^3 TCID₅₀. Whatever the inoculated WNV strain, most of the birds that succumbed had developed signs of the disease, such as prostration, lethargy, immobility and unresponsiveness. The onset of disease was observed at variable time points within different experimental groups and did not show any significant trend. The majority of birds that developed clinical signs succumbed within 24 to 48 h after the onset of symptoms. Time to death ranged from 2 to 12 dpi, but no correlation could be drawn with the inoculated strain or the inoculation route. Survival rates were not significantly different between the Fr2000- and the It2008-inoculated chickens, whatever the inoculation route (Figs 2 a,b). However, significant differences ($P < 0.05$) were observed between the survival rates of the Is98 and the Kunjin groups for both inoculation routes. Is98 appeared to be the most lethal in one-day-old chickens (50 to 80% mortality for the SC route and 90 to 100% for the IC route), followed by Fr2000 and It2008 (30 to 70% mortality for the SC route and 40 to 70% for the IC route). Kunjin was the less virulent WNV strain for chickens as it elicited no mortality using the SC route and 10 to 20% mortality using the IC route. As Is98 and Fr2000 were the most virulent strains in our model, they were selected for the dose effect study in one-day-old SPF chickens. Mortality rates obtained with doses of 10^2 and 10^1 TCID₅₀ were recorded and compared with the survival rates obtained with the 10^3

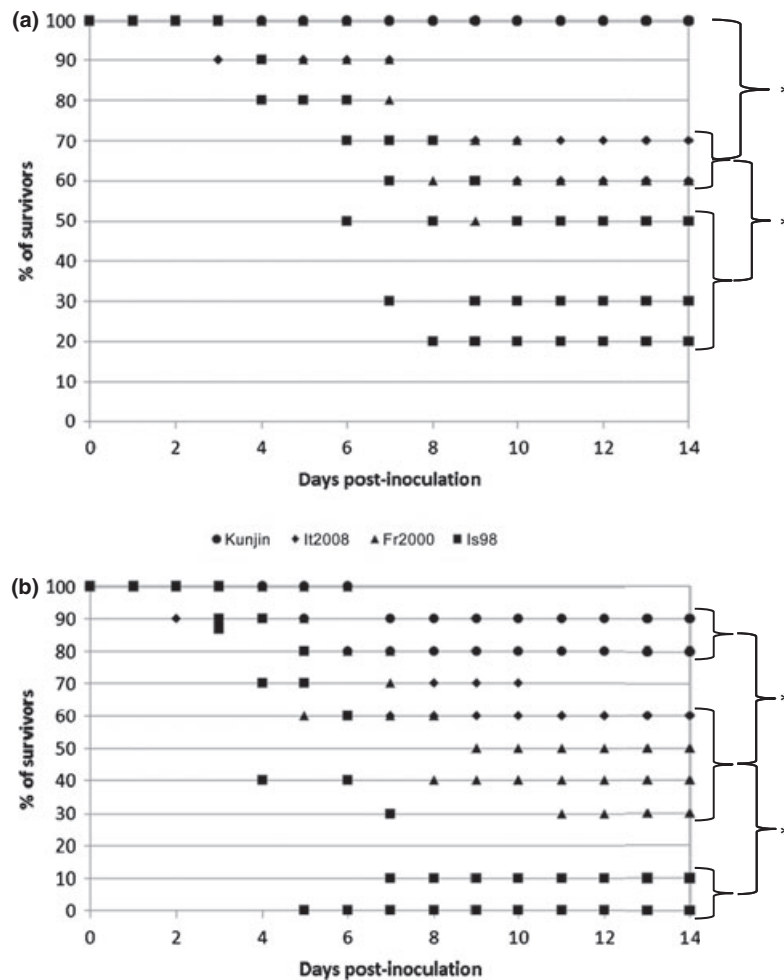


Fig. 2. Survival of SPF chickens as a function of infection by the subcutaneous (SC) (a) or the intracerebral (IC) (b) route with WNV isolates Kunjin, Fr2000, It2008 or Is98, at a dose of 10^3 TCID₅₀ at one day of age. For both graphs, discs, diamonds, triangles and squares represent SPF chickens inoculated with, respectively, Kunjin, It2008, Fr2000 or Is98. The percentages of surviving SPF chickens in each group are plotted against time after infection in days and represent data collected during two to three independent experiments. * $P < 0.05$ for Is98, Fr2000, It2008 and Kunjin versus each other over the whole infection course.

TCID₅₀ doses. For both viral strains, survival rates did not appear to be dose dependent. However, survival rates were lower for the Is98-inoculated chickens in comparison with the Fr2000 cohorts, whatever the inoculated dose. This difference was significant ($P < 0.05$) between the subgroups inoculated via the SC route with a dose of 10^3 TCID₅₀ (Fig. 3a) and the subgroups inoculated via the IC route with a dose of 10^2 TCID₅₀ or 10^1 TCID₅₀ (Fig. 3b). Survival rate differences were highly significant ($P < 0.01$) within the subgroups inoculated by the IC route with a dose of 10^3 TCID₅₀ (3% versus 40% survival for Is98- and Fr2000-inoculated chickens, respectively) (Fig. 3b).

In a latter set of experiments, SPF chickens inoculated at the age of 1 week with doses of 10^2 (IC inoculation) or 10^4 TCID₅₀ (SC inoculation) of the Is98 or Fr2000 strains did

not develop any sign of disease and survived the infection, whatever the inoculation route (data not shown).

Viral load in sera and feathers

Viremia was detectable from 2 to 7 dpi after SC inoculation of one-day-old chickens with both Is98 and Fr2000 strains at a dose of 10^3 TCID₅₀ (Fig. 4a). Interestingly, after subcutaneous inoculation at the age of 1 week at a dose of 10^4 TCID₅₀, the Is98 strain was detected in the serum of SPF chickens at only 2 dpi, while the Fr2000 strain induced a viremia up to 7 dpi. When both WNV strains were inoculated by the IC route at a dose of 10^2 TCID₅₀ to one-week-old chickens, viremia was only observed at 2 dpi. From a quantitative point of view, the viral load was significantly

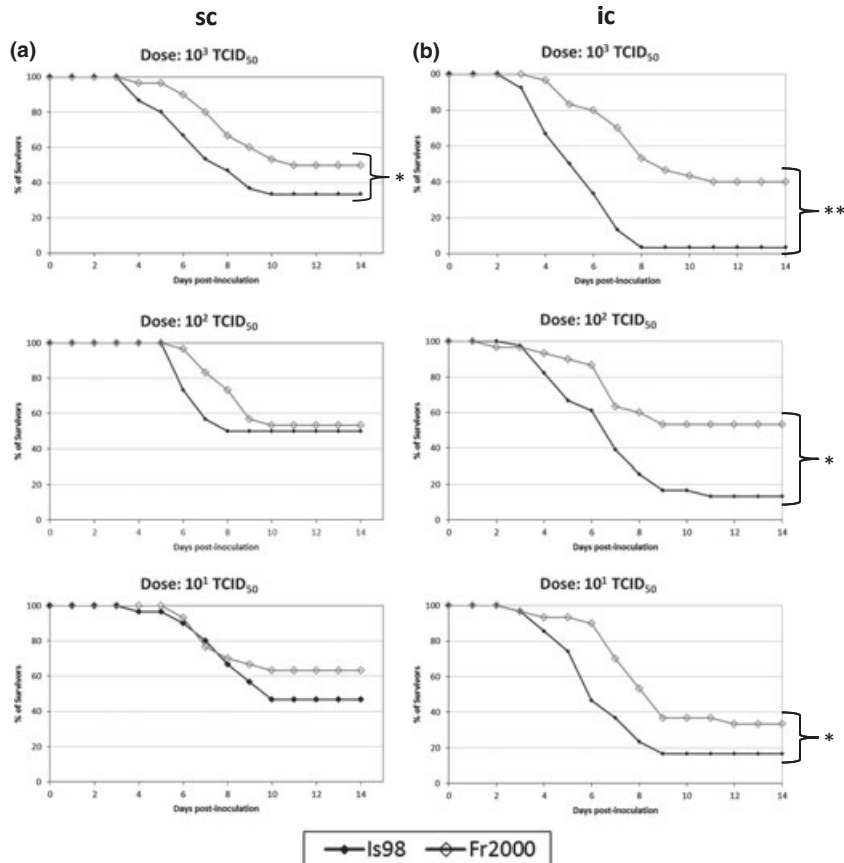


Fig. 3. Survival of one-day-old SPF chickens as a function of infection by the subcutaneous (SC) (a) or the intracerebral (IC) (b) route with WNV isolates Is98 or Fr2000 at different doses. For all graphs, filled diamonds represent SPF chickens inoculated with Is98, and open diamonds represent SPF chickens inoculated with Fr2000 at a dose of 10^3 , 10^2 or 10^1 TCID₅₀ (upper, middle and lower panels, respectively). The percentage of surviving SPF chickens in each group is plotted against time after infection in days and represents the average of three independent experiments. * $P < 0.05$ for Is98 versus Fr2000 over the whole infection course. ** $P < 0.01$ for Is98 versus Fr2000 over the whole infection course.

higher ($P < 0.05$) when WNV was inoculated subcutaneously in one-day-old chickens in comparison with one-week-old chickens, irrespective of the strain and the route of inoculation used. Viremia – measured as titre equivalents – peaked at 2 dpi whatever the viral strains, routes and ages at inoculation and was at that time point significantly higher ($P < 0.05$) in chickens inoculated at one week of age by the SC route with the Fr2000 WNV strain in comparison with the Is98 WNV strain ($10^{3.9}$ and $10^{3.3}$ TCID₅₀/ml equivalents, respectively).

In feathers, viral genome was detectable up to 14 dpi for both viral strains (Fig. 4b). In chickens inoculated at the age of one day, detection in feathers reached a high plateau at 5 dpi, whereas in one-week-old chickens inoculated by either route, viral load was moderate all over the infection period. At 7 dpi, viral load was significantly higher ($P < 0.05$) in chickens inoculated subcutaneously at the age of 1 day with Is98 as compared to the Fr2000 group. Whatever the age and inoculation route, the two viral

strains elicited similar viral loads in feathers all over the infection period.

Measurement of secreted NS1 in serum

Significant levels of secreted NS1 were detected from 5 to 9 dpi in chickens inoculated subcutaneously with the Is98 strain at the age of 1 day, whereas O.D. values remained below detection limit in chickens inoculated at the age of 1 week by either route (Table 2). Conversely, no secreted NS1 could be detected in chickens inoculated subcutaneously with the Fr2000 strain at the age of 1 day, while surprisingly chickens inoculated at the age of 1 week were positive from 5 to 9 dpi and at 14 dpi for the SC group and at 9 dpi for the IC group. NS1 secretion levels were significantly higher at 5 dpi after SC inoculation of day-old chickens with the Is98 strain ($P < 0.05$) and at 9 dpi after SC inoculation of one-week-old chickens with the Fr2000 strain.

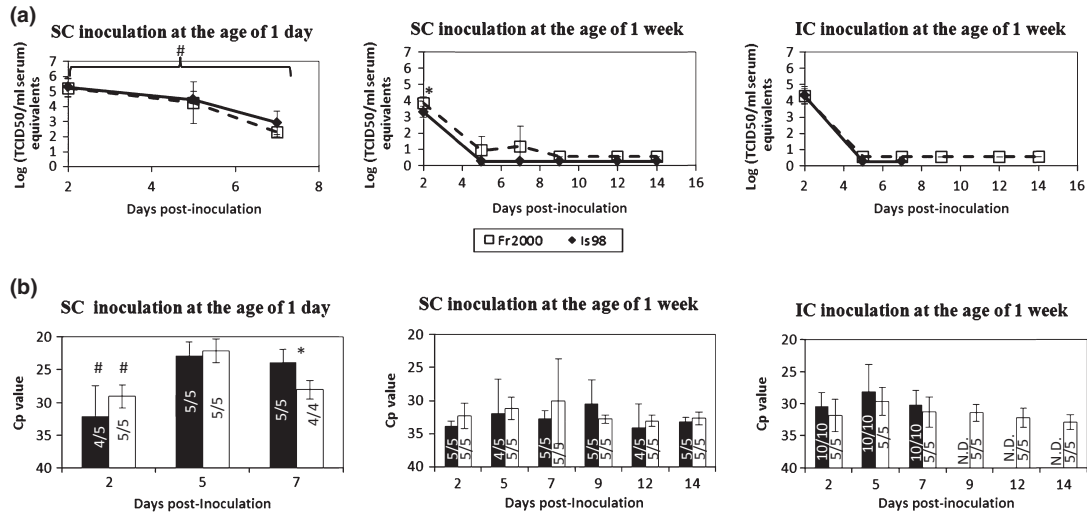


Fig. 4. Mean viremia titres (a) and viral load in feathers (b) as estimated by NS2a-specific rRT-PCR in SPF chickens inoculated subcutaneously (SC) at the age of 1 day with a dose of 10^3 TCID₅₀ (left panels) or 1 week with a dose of 10^4 TCID₅₀ (middle panels) or intracerebrally (IC) (right panels) at the age of 1 week with a dose of 10^2 TCID₅₀ of Is98 (filled diamonds/black bars) or Fr2000 (open squares/white bars). Error bars represent the standard error of the represented means. Within the bars, numbers of positives/examined feather samples are indicated. N.D.: not determined. **P* < 0.05 for Is98 versus Fr2000. #*P* < 0.05 for 2 dpi versus 5 and 7 dpi and for SC inoculation at the age of 1 day versus SC and IC inoculation at the age of 1 week.

Table 2. NS1 antigen secretion in serum measured by capture ELISA and expressed as optical densities (O.D.) and numbers of positives/examined serum samples. Underlined values are above the detection threshold

Age at Inoculation	Inoculation route	Dose	Days post-infection	Is98		Fr2000	
				Average O.D. ± SD	Positives ratio	Average O.D. ± SD	Positives ratio
1 day	SC	10^3 TCID ₅₀	2	0.055 ± 0.004	0/5	0.037 ± 0.008	0/5
			5	<u>0.377</u> *# ± 0.051	3/3	0.105* ± 0.046	1/5
			7	<u>0.156</u> ± 0.025	2/2	0.086 ± 0.021	0/4
			9	<u>0.130</u> ± 0.046	2/3	N.D.	N.D.
			12	0.049 ± 0.034	0/2	N.D.	N.D.
			14	0.058 ± 0.012	0/2	N.D.	N.D.
1 week	SC	10^4 TCID ₅₀	2	0.033 ± 0.010	0/5	0.029 ± 0.006	0/5
			5	0.033 ± 0.006	0/5	<u>0.143</u> ± 0.152	2/5
			7	0.029 ± 0.005	0/5	<u>0.362</u> ± 0.341	3/5
			9	0.057*±0.076	1/4	<u>0.216</u> * ± 0.115	4/5
			12	0.034 ± 0.013	0/5	0.114 ± 0.102	1/5
			14	0.043 ± 0.027	0/5	<u>0.181</u> ± 0.170	2/5
			IC	10^2 TCID ₅₀	2	0.046 ± 0.013	0/10
	5	0.064 ± 0.056			1/9	0.098 ± 0.118	1/5
	7	0.062 ± 0.032			1/10	0.043 ± 0.009	0/5
	9	N.D.			N.D.	<u>0.191</u> ± 0.301	1/5
	12	N.D.			N.D.	<u>0.047</u> ± 0.013	0/5
	14	N.D.			N.D.	0.057 ± 0.017	0/5

S.D., standard deviation; N.D., not determined.

**P* < 0.05 for Is98 versus Fr2000.

#*P* < 0.05 for O.D. at 5 dpi versus O.D. at another dpi within the same group.

Discussion

To set up a model for the pathotyping of WNV strains, SPF chickens aged 1 day or 1 week were inoculated with four selected WNV strains that were suspected to have different pathogenicities in birds. Kunjin viruses, among which a 1984 isolate was used in this study, are endemic in Australia since the 1960s, where they affect humans, horses and birds. In the field, they are characterized by a weak pathogenicity as they usually cause no deaths in birds or horses, and the disease in humans is benign (Smith et al., 2011). Nevertheless, a WNV isolate that is antigenically and genetically closely related to Australian Kunjin viruses caused a case-fatality rate of 10–15% in up to 1000 horses showing neurological disease in south-eastern Australia in 2011 (Frost et al., 2012). Fr2000 was isolated in the early 2000s from a horse in France during an epidemic affecting horses, but not birds (Murgue et al., 2001). It2008 was isolated in 2008 from the brain of a magpie in Italy, during an outbreak affecting mainly humans and horses, but was also detected in the carcasses of corvids and rock pigeons (Calistri et al., 2010b). The Is98 strain was isolated from a stork in Israel where it caused high rates of avian deaths in 1998 (Malkinson et al., 2002; Banet-Noach et al., 2003; Dauphin et al., 2004).

Our experimental infections demonstrated that WNV pathotypes can be discriminated in a SPF chicken model by comparing survival rates of experimentally infected one-day-old chickens. In our hands, the sharpest discrimination between Is98, Fr2000, It2008 and Kunjin WNV strains was achieved in the IC inoculated cohorts. Using the SC route of inoculation, Murata and colleagues have tested two New York (NY) strains of WNV that differ in the glycosylation status of the E protein – a polymorphism pointed out as a determinant of virulence – and have also found a correlation between survival rates in 2-day-old Boris Brown chickens and virulence of these variants (Murata et al., 2010). Moreover, in our experiments, deaths did not occur markedly earlier in the groups inoculated by the IC route as compared to the SC route, suggesting that neuroinvasion did not speed up fatal outcome.

The lethality observed for Is98 and Fr2000 WNV strains was dose independent as reported previously for the NY WNV variants (Murata et al., 2010). This result might reflect the high virulence of several WNV subclones that are rapidly selected from the quasispecies population (Jerzak et al., 2005) and/or heterogeneity in the genotype of the host chickens. It was indeed shown for humans that single nucleotide polymorphisms (SNPs) affecting, for example, *interferon regulatory factor 3* (*IRF3*, alias of *IRF7* in *Gallus gallus*) or *myxovirus resistance 1* (*MX1*) genes are associated with symptomatic WN disease (as opposed to asymptomatic infection) and progression towards

encephalitis and/or paralysis, respectively (Bigam et al., 2011). Moreover, in Marek's disease, mortality is determined by the interaction between the susceptible host chicken genotype and the virus quasispecies population (Smith and Calnek, 1974).

In this study, no clinical symptoms were observed in SPF chickens infected at the age of 1 week with Is98 and Fr2000 WNV strains. A similar low susceptibility to WNV was reported in older chickens aged 3 to 20 weeks (Senne et al., 2000; Buckley et al., 2006; Phipps et al., 2007; Totani et al., 2011). Decreasing susceptibility to viral infection as birds grow was also described for Aigamo ducks inoculated with WNV (Shirafuji et al., 2009). Data from the present study consolidate this observation as, regardless of the inoculated strain, viremia was higher and longer-lasting in SPF chickens inoculated at the age of 1 day compared with those which were inoculated at the age of 1 week by either route. The failure of young SPF chickens to clear WNV particles and limit their replication can be explained by the immaturity of their immune system (Lowenthal et al., 1994) and particularly of their innate immune response.

For the purpose of this study, a one-step rRT-PCR assay using a FAM-labelled TAMRA probe targeting the NS2a viral gene, that is quite conserved within the group of mosquito-borne viruses causing encephalitic diseases such as WNV (Danecek and Schein, 2010), was developed. Previously, Eiden and co-workers had designed NS2a primers on the basis of 95 WNV isolates that were able to detect WNV strains of lineages 1 and 2 with an extremely high sensitivity and an acceptable specificity (Eiden et al., 2010). In the present study, the results from the NS2a-specific rRT-PCR assay show a strong linear correlation between viral titres (PFU and TCID₅₀) and recorded Cp values. Subsequently, we estimated the serum matrix effect on the sensitivity of the NS2a-specific rRT-PCR. To our knowledge, our approach for matrix effect quantification by a Passing and Bablok regression equation is unprecedented.

Viremia, as measured by the NS2a rRT-PCR in infected one-day-old SPF chickens, did not allow significant discrimination between the Is98- and Fr2000-infected groups. Consistent with our observation, Sotelo and colleagues, who inoculated red-legged partridges – a gallinaceous bird – with two Mediterranean WNV strains, also recorded a significant difference of pathogenicity in the survival rates, but neither in the level of viremia nor in the characteristics of the internal lesions (Sotelo et al., 2011). However, in the one-week-old cohorts, viremia levels tended to be inversely correlated with the hypothetical virulence of the inoculated viral strains as the group inoculated with Fr2000 showed the highest TCID₅₀/ml values compared with the Is98 group, with a significant difference at 2 dpi ($P < 0.05$). Such an antagonistic result might be explained by differences in the tropism and multiplication sites of Fr2000 and

Is98 and/or a different susceptibility of the two WNV strains to mature immune responses.

WNV was previously detected by others in the feather pulp of carcasses of naturally infected corvids (Docherty et al., 2004; Godhardt et al., 2006), moribund white pelicans (Johnson et al., 2010) as well as experimentally infected jungle crows and Aigamo ducks (Shirafuji et al., 2008, 2009). Nemeth and colleagues, who used whole feathers for molecular WNV detection in experimentally infected eastern screech owls (*Megascops asio*), found that viral detection was limited within the course of WNV infection and did not extend beyond the period where the birds were viremic (Nemeth et al., 2006). Interestingly, in our hands, WNV RNA was detected in the feathers of all experimental groups infected with both Is98 and Fr2000 during the whole infection period, which suggests that the clearance in chicken feathers is slower than in serum. Similarly to what was observed for the serum matrix, age at inoculation influenced NS2a expression levels in feathers as those were higher in one-day-old SPF chickens.

The secretion pattern of NS1 antigen in serum seemed to parallel the profile of pathogenicity in the one-day-old cohorts. Indeed, the levels of NS1 were higher in the Is98 group with a significant difference at 5 dpi ($P < 0.05$). However, in the 1-week-old cohorts inoculated by the SC route, the secreted NS1 levels were higher in the Fr2000 group than in the Is98 group, consistent with the shift observed in serum by the NS2a-specific rRT-PCR.

In summary, the present study shows that WNV strains differing in pathogenicity – clade 1a Is98, Fr2000 and It2008 and clade 1b Kunjin – could be discriminated in a SPF chicken model of 1 day of age on the basis of survival rates. Therefore, the development of a standardized intracerebral pathogenicity index test in one-day-old chickens for pathotyping WNV isolates, as described for Newcastle disease virus (OIE, 2012), another neurotropic virus for birds, could be considered. The other parameters, namely viremia and viral load in feathers, appeared to be rather host age dependent than strain dependent. The outcome of experimental infections carried out recently in wild-caught Carrion crows (wild European corvids) with Is98 and Fr2000 WNV strains corroborates the conclusions obtained from the SPF chicken model described herein (Drudi et al., 2013).

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Conflicts of interest

The authors disclose any financial and personal relationship with other people or organizations that could inappropriately influence their work. All authors declare they have no competing interests.

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