

# Pure

Scotland's Rural College

## Evaluation of cross-protection of a lineage 1 West Nile virus inactivated vaccine against natural infections from a virulent lineage 2 strain in horses, under field conditions

Chaintoutis, SC; Diakakis, N; Papanastassopoulou, M; Banos, G; Dovas, CI

*Published in:*

Clinical and Vaccine Immunology

*DOI:*

[10.1128/CVI.00302-15](https://doi.org/10.1128/CVI.00302-15)

First published: 15/07/2015

*Document Version*

Peer reviewed version

[Link to publication](#)

*Citation for published version (APA):*

Chaintoutis, SC., Diakakis, N., Papanastassopoulou, M., Banos, G., & Dovas, CI. (2015). Evaluation of cross-protection of a lineage 1 West Nile virus inactivated vaccine against natural infections from a virulent lineage 2 strain in horses, under field conditions. *Clinical and Vaccine Immunology*, 22(9), 1040 - 1049. <https://doi.org/10.1128/CVI.00302-15>

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal ?

### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

1 **Title**

2 Cross-protection evaluation of a lineage 1 West Nile virus inactivated vaccine against  
3 natural infections from a virulent lineage 2 strain in horses, under field conditions

4

5 **Authors**

6 Serafeim C. Chaintoutis<sup>a</sup>, Nikolaos Diakakis<sup>b</sup>, Maria Papanastassopoulou<sup>c</sup>, Georgios  
7 Banos<sup>d,e,f</sup>, Chrysostomos I. Dovas<sup>a\*</sup>

8

9 **Affiliations**

10 <sup>a</sup>Diagnostic Laboratory, Department of Clinical Sciences, School of Veterinary  
11 Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 11 Stavrou  
12 Voutyra str., 54627 Thessaloniki, Greece

13 <sup>b</sup>Equine Unit, Companion Animal Clinic, Department of Clinical Sciences, School of  
14 Veterinary Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki, 11  
15 Stavrou Voutyra str., 54627 Thessaloniki, Greece

16 <sup>c</sup>Laboratory of Microbiology and Infectious Diseases, School of Veterinary Medicine,  
17 Faculty of Health Sciences, Aristotle University of Thessaloniki, University Campus,  
18 54124 Thessaloniki, Greece

19 <sup>d</sup>Department of Animal Production, School of Veterinary Medicine, Faculty of Health  
20 Sciences, Aristotle University of Thessaloniki, University Campus, 54124 Thessaloniki,  
21 Greece

22 <sup>e</sup>SRUC, Easter Bush, Midlothian EH25 9RG, Scotland, United Kingdom

23 <sup>f</sup>The Roslin Institute, Royal (Dick) School of Veterinary Science, Easter Bush,  
24 Midlothian EH25 9RG, Scotland, United Kingdom

25

26 \*Corresponding author at: Diagnostic Laboratory, School of Veterinary Medicine,  
27 Faculty of Health Sciences, Aristotle University of Thessaloniki, 11 Stavrou Voutyra str.,  
28 54627 Thessaloniki, Greece. Tel.: +30 2310 999870, Fax: +30 2310 994487 (C.I. Dovas)

29

30 E-mail addresses: schainto@vet.auth.gr (S.C. Chaintoutis), diakakis@vet.auth.gr (N.  
31 Diakakis), mpapanas@vet.auth.gr (M. Papanastassopoulou), banos@vet.auth.gr (G.  
32 Banos), dovas@vet.auth.gr (C.I. Dovas)

33

#### 34 **Running title**

35 Horse WNV vaccine cross-protection field evaluation

36

#### 37 **Abstract**

38 Although experimental data regarding cross-protection of horse WNV vaccines against  
39 lineage 2 infections exist, their cross-protective efficacy under field conditions has not  
40 been demonstrated. This study was conducted to evaluate the capability of an inactivated  
41 lineage 1 vaccine (Equip<sup>®</sup> WNV) to protect against natural infections from the Nea  
42 Santa-Greece-2010 lineage 2 strain. In total 185 WNV-seronegative horses in  
43 Thessaloniki-Greece, were selected during two consecutive years (2011-2012). One  
44 hundred and forty were immunized and 45 were used as controls. Horses were examined  
45 for signs compatible with WNV-infection. Neutralizing antibody titers against the Greek  
46 strain and the PaAn001/France lineage 1 strain were determined in immunized horses.  
47 WNV circulation was detected during both years in the study area. It was estimated that  
48 37% and 27% of the horses were infected during 2011 and 2012, respectively. Three  
49 control animals developed clinical signs and WNV-diagnosis was confirmed. Signs

50 related to WNV infection were not observed in vaccinated animals. Non-vaccinated  
51 animals were associated with a  $7.58 \pm 1.82\%$  higher chance of exhibiting signs compared  
52 to immunized ( $P < 0.05$ ). Neutralizing antibodies raised against both strains in all  
53 immunized horses were detectable one month after the initial vaccination course. The  
54 cross-protective capacity of the lowest titer (1:40) was evident in 19 animals which were  
55 subsequently infected, and did not exhibit signs. Neutralizing antibodies were detectable  
56 until the annual booster, where strong anamnestic responses were observed  
57 ( $GMTR_{lin.1} = 30.2$ ,  $GMTR_{lin.2} = 27.5$ ). Results indicate that Equip<sup>®</sup> WNV is capable of  
58 inducing cross-protection in horses, against natural infections from a virulent lineage 2  
59 WNV strain.

60

61 **Keywords:**

62 West Nile virus, Lineage 1, Lineage 2, Nea Santa-Greece-2010, Horse, Inactivated  
63 vaccine, Cross-protective immunity, Neutralizing antibody titer, Natural infections, Field  
64 evaluation

65

66 **Introduction**

67 West Nile virus (WNV) is a single-stranded RNA virus within the Japanese  
68 encephalitis virus serocomplex, which belongs to the genus *Flavivirus* (family  
69 *Flaviviridae*) (1). WNV is maintained in nature by enzootic transmission cycles between  
70 certain bird species and ornithophilic mosquitoes (2). Mosquitoes mainly belonging to the  
71 genus *Culex* can also act as bridge vectors, transmitting the virus to other animal species,  
72 including incidental hosts (3-6). Humans and horses are regarded as incidental (dead-end)  
73 hosts, as the virus titer developed in their blood is generally too low to infect mosquitoes  
74 (7). Nevertheless, WNV infection in susceptible hosts may eventually cause neurological  
75 disease (8). Regarding horses, the reported clinical signs may vary and these include  
76 fever, paraparesis or tetraparesis and ataxia, recumbency and behavioral changes, while

77 in many clinically affected horses muscle fasciculation and tremors are also present. It is  
78 expected that deaths will occur in a small percent of the affected animals (9-13).

79 Phylogenetic analyses of WNV strains isolated worldwide have resulted in the  
80 identification of 8 genetic lineages of the virus so far (14). Until 2004, only viral strains  
81 belonging to lineages 1 and 3 had been found in Europe. The majority of the strains  
82 isolated from European outbreaks belong to lineage 1 (15, 16). Lineage 2 includes strains  
83 from sub-Saharan Africa and Madagascar, and these had been so far considered of low-  
84 virulence (17). Such strains belonging to lineage 2 were isolated in Hungary (2004), in  
85 Austria (2008), as well as in Italy (2008) (16, 18). However, a virulent lineage 2 strain  
86 (Nea Santa-Greece-2010) was found to be responsible for the occurrence of 4 consecutive  
87 epidemic periods (2010-2013) in Greece, with neuroinvasive disease (WNND) cases in  
88 humans and horses during all these years (19-20). An amino acid substitution (H<sub>249</sub>P) in  
89 the NS3 protein, absent from other closely related European strains, is suspected to be  
90 associated with the high virulence and neuroinvasiveness of the Greek strain (19).  
91 Enzootic transmission of the virus was detected once again in Central Macedonia, the  
92 epicenter of 2010 epidemic, during June 2014, using backyard chickens (21).

93 Experimental vaccinations in birds have been applied outside Europe (although  
94 bird vaccines against WNV are not commercially available) to a limited extent, especially  
95 in endangered bird species (e.g. in California condors) to protect them from fatal WNV  
96 infection, or in bird reservoir hosts (e.g. American crows and robins), aiming to reduce  
97 WNV viremia in them and prevent subsequent transmission of the virus to competent  
98 vectors (22-26). Regarding dead-end hosts, for humans only passive immunization  
99 (intravenous immunoglobulin or hyperimmune gammaglobulin administration) has been  
100 used to a limited extent for treatment of patients with WNND (27). No human vaccines  
101 against WNV are commercially available at this time, and as a result, active  
102 immunization of humans is not possible (28). In contrast, several inactivated and  
103 recombinant WNV vaccines for horses have been produced, evaluated and licensed in the  
104 USA. Specifically, two inactivated vaccines have been licensed and are being used at this  
105 time point in the USA; West Nile-Innovator<sup>®</sup> (Fort Dodge, IA, USA), and Vetera<sup>®</sup> WNV

106 (Boehringer Ingelheim Vetmedica, MO, USA). A recombinant vaccine with a Canarypox  
107 virus vector (Recombitek<sup>®</sup> Equine West Nile virus, Merial, GA, USA) is also marketed  
108 (19, 26, 29). It has been shown that all these immunologicals induce the production of  
109 WNV-specific neutralizing antibodies (NAbs) (30) and have proven to be very effective  
110 in protecting horses from meningoencephalitis in North America (31). Additionally, a  
111 DNA vaccine (West Nile-Innovator DNA<sup>®</sup>, Fort Dodge, IA, USA) was approved by the  
112 USDA in 2005. Finally, a chimeric vaccine (PreveNile<sup>™</sup>, Intervet, KS, USA) containing a  
113 strain of Yellow Fever virus (YFV-17D) was approved for marketing in 2006 by USDA,  
114 and was later remarketed as killed vaccine, under the name “EquiNile<sup>™</sup>” (19, 26, 32). All  
115 these immunologicals have been developed using lineage 1 WNV strains.

116         Along with the emergence of virulent lineage 2 WNV strains in Europe, two of  
117 the aforementioned vaccines, West Nile-Innovator [under the name Equip<sup>®</sup> WNV  
118 (Zoetis)] as well as Recombitek Equine West Nile virus [under the name Proteq West  
119 Nile<sup>™</sup>, (Merial)] were authorized in 2011 and are being commercialized in European  
120 countries (33, 34). Concomitantly, questions arose whether these commercially available  
121 WNV vaccines for horses are effective in protecting them against virulent strains  
122 belonging to lineage 2, since both of them contain lineage 1 antigens, and their protection  
123 had not been extensively evaluated for other lineages. Previous experimental studies  
124 indicated that both of these vaccines can lead to the development of cross-protective  
125 immunity. Specifically, the recombinant vaccine “ALVAC<sup>®</sup>-WNV” (Merial) is capable  
126 of immunity induction in horses challenged with the goshawk-Hungary/04 lineage 2  
127 strain (35). Another study which was conducted in mice immunized with the inactivated  
128 vaccine “Duvaxyn/Equip<sup>®</sup> WNV” has shown that it provided complete protection against  
129 challenge with the SPU93/01 lineage 2 strain (36). A more recent study was conducted in  
130 horses, showing that immunization with the Equip<sup>®</sup> WNV vaccine resulted in reduction  
131 of the number of viremic animals, the duration and severity of clinical signs of disease  
132 and mortality, following experimental infection with the virulent Nea Santa-Greece-2010  
133 lineage 2 WNV strain (37). As a result, Equip<sup>®</sup> WNV was recently authorized also for  
134 lineage 2, although the duration of immunity has not been established for these strains

135 (33). Nevertheless, results regarding the evaluation of the cross-protection of these  
136 vaccines in field conditions are lacking.

137           It has been evidenced that, under experimental conditions, the effects of needle  
138 WNV inoculation in chickens might differ significantly from those of mosquito-borne  
139 natural infections (38). It has also been demonstrated that experimental WNV challenge  
140 in horses via needle inoculation, or mosquito feeding were not able to induce significant  
141 clinical signs (30). In addition, under experimental conditions, cell culture-adapted and  
142 passaged viruses are used as challenge strains. All these cultivation procedures might  
143 have consequent effects on the virulence of the viral strains. Consequently, field  
144 evaluation of viral vaccines is of utmost importance, in order to truly estimate the degree  
145 of cross-protection among different strains. In the present study we evaluated the capacity  
146 of the inactivated Equip<sup>®</sup> WNV vaccine to offer cross-protective immunity in horses  
147 against natural infections from the highly virulent Nea Santa-Greece-2010 lineage 2  
148 WNV strain in field conditions.

149

## 150 **Materials and methods**

### 151 **Animals**

152           In total 185 mix-bred horses aged 5-18 years old were included in this 2-year  
153 study, which took place during the 2011 and 2012 epidemic periods in Greece. The  
154 horses belonged in 6 horse riding clubs in Central Macedonia the epicenter of the 2010  
155 Greek epidemic. None of the horses had been previously exposed to WNV, as indicated  
156 by serological testing with competitive enzyme-linked immunosorbent assay (cELISA)  
157 and serum neutralization test (SNT) as described below. Specifically, serological testing  
158 was conducted twice; i) one week prior to the initiation of the immunizations in both  
159 years, and ii) at the time the first dose of the primary vaccination was conducted, for both  
160 years. The health status of each horse was determined prior to its incorporation in the  
161 study. Immunizations, blood samplings and clinical examinations of the animals were

162 performed by experienced veterinarians. A mixture comprised of oats, muesli and  
163 hay/alfalfa hay, was being administrated to the horses, and water was available ad  
164 libitum. Trained technicians were responsible for animal husbandry procedures.

165

#### 166 Vaccine and immunization plan

167 The commercially available ready-to-use vaccine Equip<sup>®</sup> WNV (Zoetis, Louvain-  
168 la-Neuve, Belgium) was used in this 2-year study. This vaccine contains the inactivated  
169 lineage 1 WNV strain New York 1999/VM-2 (isolated from the brain of an infected horse  
170 during the 1999 epidemic period in New York, USA) formulated in MetaStim<sup>™</sup> oil  
171 emulsion adjuvant, consisting of Squalene, Poloxamer 401 (Pluronic<sup>®</sup> L121) and  
172 Polysorbate 80 (33). Vaccine lots 387BYC01L and 387BYA08A were used in 2011 and  
173 2012, respectively. Each dose was administrated via a single intramuscular injection in  
174 the neck of the animals.

175 During June-July 2011 an initial double primary vaccination (two doses  
176 administrated 3 weeks apart) of 85 horses was performed (Fig. 1), while 33 horses were  
177 used so as to form the control group (Table 1). During May-June 2012, 79 of the  
178 aforementioned vaccinated animals received an annual booster immunization dose of the  
179 vaccine. Six of the original 85 horses were excluded during the second year for various  
180 reasons, e.g. they were moved out of the study area, or were euthanized due to causes  
181 irrelevant to WNV-infection. In addition, in May-June 2012, another 55 horses which  
182 were seronegative to WNV received a double immunization with the vaccine (Fig. 1).  
183 During this period, 21 of the 2011's control animals which were determined as  
184 seronegative were kept, and along with 12 additional seronegative horses, were used as  
185 naïve controls for the 2012 epidemic period (Table 1). The total number of control horses  
186 ( $n = 45$ ) was intentionally limited to approximately 33% of the total number of horses  
187 used in the study for humane reasons. In each participating horse riding club the  
188 vaccinated and control animals were co-mingled and managed similarly.



189

## 190 Clinical examination and blood samplings

191 Physical and special neurological examination were being performed on each of  
192 the participating horses, at least one week prior to the initiation of immunizations, and  
193 until the end of the respective epidemic period. Monitoring was being performed  
194 regularly (every 5-6 days) for signs compatible with WNV infection, (e.g. anxiety,  
195 muscle fasciculation, head tremor, lip twitching, teeth grinding, ataxia, paresis, head  
196 shaking, etc.), along with any other abnormal conditions. Besides the evaluation for the  
197 presence of clinical signs due to WNV infection, horses were also being evaluated for  
198 local and systemic adverse reactions due to the vaccination. Clinical evaluations were  
199 done independent of knowledge of immunization status.

200 Blood was collected from all horses in 10 ml plain vacuum tubes at specific time  
201 points. For the animals participating from 2011, these time points were W (week) 0, W3,  
202 W7, W21, W34, W48, W52, W66, and W72 (Fig. 1). For the animals that participated in  
203 the study only during 2012, the respective time points for blood collection were W45(0),  
204 W48(3), W52(7), and W72(27) (Fig. 1). Numbers in parentheses indicate the exact week  
205 number in which samplings were being conducted from the horses that participated only  
206 in 2012, beginning from the week that these animals received the first dose of the vaccine  
207 (0). Numbers outside parentheses indicate the corresponding week number from the  
208 beginning of the study (2011). For example, W45(0) indicates week 0 for the horses  
209 participating in 2012 (conduction of the first immunization). Concomitantly, this is also  
210 week 45, counting from the day in which the first vaccine dose was administrated during  
211 2011 (W0).

212 Additionally, for the confirmation of diagnosis of WNV infection in horses with  
213 neurological signs, blood samples were drawn shortly after clinical signs were being  
214 noticed. Blood was allowed to clot and tubes were centrifuged ( $3,000 \times g$ , 10 min,  $4^\circ\text{C}$ ).  
215 Sera were transferred to clear 2 ml microcentrifuge tubes and stored at  $-80^\circ\text{C}$  until they  
216 were assayed.

217

## 218 Serological and virological testing

219 Sera obtained from all control horses after the end of both 2011 and 2012  
220 epidemic periods (W21 and W72(27) of the study, respectively) were tested for WNV-  
221 specific antibodies (indication of seroconversion), using a commercially available  
222 cELISA kit (ID Screen<sup>®</sup> West Nile Competition, ID.vet, Montpellier, France). This  
223 analysis was performed in order to confirm that the virus was circulating in the  
224 participating horse riding clubs, as well as to estimate the percentage of animals which  
225 were exposed to the virus during each epidemic period.

226 In order to confirm that the vaccine induced the development of cross-protective  
227 immunity, sera obtained one month after the completion of the double primary  
228 vaccination course from all vaccinated horses of both years (W7 and W52(7),  
229 respectively) were tested for the presence of NABs specifically directed against the Nea  
230 Santa-Greece-2010 lineage 2 strain, following an existing SNT protocol (39) with slight  
231 modifications. Briefly, after heat inactivation at 56 °C for 30 min, sera were two-fold  
232 serially diluted (1:5 to 1:2560, in duplicates in 96-well cell culture plates) in Dulbecco's  
233 Modified Eagle's Medium (DMEM; Invitrogen-Gibco, Groningen, The Netherlands), and  
234 50 µl of DMEM containing 100 TCID<sub>50</sub> (50% tissue culture infectious doses) of the Nea  
235 Santa-Greece-2010 strain were added. Controls, reference sera and back titration of the  
236 antigen were also included. After incubation of the plates at 37 °C for 1.5 h, 2 × 10<sup>4</sup> Vero  
237 cells in 100 µl of DMEM with 2% penicillin (100 IU/ml) and streptomycin (100 µg/ml),  
238 2% sodium pyruvate and 10% fetal bovine serum (Invitrogen-Gibco, Groningen, The  
239 Netherlands) were added to every well. Plates were incubated at 37 °C for 5 days and  
240 wells were examined under an inverted light microscope for evidence of viral cytopathic  
241 effects. The NAb titer of each serum was calculated as the highest serum dilution in  
242 which protection of the cell monolayer was observed. Sera were being considered  
243 positive if cells were protected at a dilution  $\geq 1:10$ .

244 Moreover, sera obtained from all primo-vaccinated horses (naïve horses which  
245 received the initial two-dose vaccination course) after the end of each epidemic period  
246 (November, W21 and W72(27) of the study) were also tested with the aforementioned  
247 SNT protocol and NAb titers were compared to the respective ones developed 1 month  
248 after the double primary vaccination (W7 and W52(7), respectively), so as to detect the  
249 occurrence of anamnestic humoral immune responses, indicative of natural infections.

250 In order to evaluate the levels and the duration of the produced NABs, 23 primo-  
251 vaccinated animals of the first year (2011) which were not exposed to the virus as  
252 indicated by the results of the aforementioned analysis (~40% of the total number of  
253 vaccinated animals which were determined not to be exposed to WNV during that year),  
254 were tested at samples obtained from the day of the first immunization (W0), until one  
255 month after the annual booster (W52). SNTs were used to determine the NAb titers  
256 against two WNV strains; the Nea Santa-Greece-2010 lineage 2 strain, as well as the  
257 PaAn001/France lineage 1 strain (kindly provided by Dr. Sylvie Lecollinet, UMR 1161  
258 Virology, INRA-ANSES-ENVA, France).

259 For the confirmation of the diagnosis in control horses with clinical signs, the  
260 collected serum samples were tested for the presence of WNV-specific IgM antibodies,  
261 using a commercially available IgM antibody capture enzyme-linked immunosorbent  
262 assay (MAC-ELISA; IgM WNV Ab Test, IDEXX-Istitut Pourquier, Montpellier,  
263 France), following the manufacturer's instructions. Furthermore, RNA was extracted  
264 from the sera obtained of the horses with neurological signs using the NucleoSpin<sup>®</sup> RNA  
265 virus kit (Macherey-Nagel, Düren, Germany. Extracts were examined using a WNV-  
266 specific, one tube real-time RT-PCR, using the primer pair WNPoIUp (5'-  
267 TTTTGGGAGATGGTGGATGARGA-3') and WNPoIDo2 (5'-  
268 CCACATGAACCAWATGGCTCTGC-3'), at final concentration of 0.6 µM each, and  
269 the TaqMan probe WNPoIProb2 (5'-FAM-TCTCTCTCTTTCCCATCATGTTGT-  
270 ZNA5-BHQ1-3' at a final concentration of 0.2 µM) targeting a 144 bp part of the  
271 nonstructural protein 5 (NS5) genomic region of WNV. The limit of detection was  
272 previously determined to be 1 TCID<sub>50</sub>/ml (40). Amplification reactions were run in a

273 total volume of 25 µl using 5µl of RNA extract and 20µl of reaction buffer of a  
 274 commercial RT-PCR kit (One step RT-PCR Qiagen, Hilden, Germany). The thermal  
 275 cycling conditions were as follows; 50 °C for 30 min, followed by 95 °C for 15 min and  
 276 50 cycles in 2 steps: a) 95 °C for 30 sec (denaturation), and b) 60 °C for 40 sec  
 277 (annealing and extension). The fluorescence levels were measured at the end of each  
 278 cycle. The assay was performed using the CFX96 Touch™ Real-Time PCR detection  
 279 system (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of data was conducted  
 280 using the CFX™ Software (Version 3.0, Bio-Rad Laboratories, Hercules, CA, USA).

281

## 282 Statistical analysis

283 The effect of immunization on the presence of clinical signs was assessed with an  
 284 odds ratio and a mixed model analysis.

285 The odds ratio analysis considered non-vaccinated animals as the control and  
 286 vaccinated animals as the intervention (case) group. The odds ratio was calculated as  
 287 follows (41):

$$288 \quad OR = \frac{a}{b} / \frac{c}{d} \quad [1]$$

289 where  $OR$  = odds ratio,  $a$  = number of vaccinated horses with clinical signs,  $b$  = number  
 290 of vaccinated horses without clinical signs,  $c$  = number of non-vaccinated horses with  
 291 clinical signs and  $d$  = number of non-vaccinated horses without clinical signs.

292 The significance of the odd ratio was assessed by the confidence interval which was  
 293 calculated as follows:

$$294 \quad 95\% CI = e^{(\ln(OR) \pm 1.96 \cdot SE\{\ln(OR)\})} \quad [2]$$

295 where  $95\%CI$  = 95% confidence interval,  $\ln$  = natural logarithm,  $SE$  = standard error and  
 296  $OR$  = odds ratio as in equation 1; the standard error was calculated as follows:

297 
$$SE\{\ln(OR)\} = \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}} \quad [3]$$

298 where  $SE$  = as in equation 2 and  $a, b, c, d$  = as in equation 1.

299 In order to accommodate possible values equal to zero in the calculation of the  
300 odds ratio or its standard error, 0.5 may be added to all cells (42-43). An odds ratio  
301 significantly smaller than unity would suggest that intervention (i.e., immunization), is  
302 better than the control.

303 The mixed model analysis was based on the following model:

304 
$$Y_{ijklm} = \mu + CY_i + RC_j + VS_k + A_l + e_{ijklm} \quad [4]$$

305 where  $Y$  = presence or absence of clinical signs for the  $l^{\text{th}}$  animal,  $\mu$  = overall mean,  $CY$  =  
306 fixed effect of calendar year  $i$  ( $i = 2011-2012$ ),  $RC$  = fixed effect of riding club  $j$  ( $j = 1-6$ ),  
307  $VS$  = vaccination status  $k$  ( $k = 0$  for non-vaccinated and 1 for vaccinated animals),  $A$  =  
308 random animal effect reflecting the individual response of each horse and  $e$  = random  
309 residual.

310 Model 4 fitted a logit function to account for the binary nature of the trait  
311 (presence or absence of clinical signs). The outcomes of this model served as  
312 confirmation of the odds ratio analysis with the additional benefit of the quantification of  
313 the vaccination effect on presence of clinical signs, adjusted for all other factors included  
314 in model 4. The mixed model analysis was conducted with the ASReml software (44).

315 The two analyses, odds ratio and mixed model, were run once considering all  
316 animals and then a second time including only the infected horses.

317 NAb titers were used to calculate the geometrical mean titer (GMT) for each  
318 sampling time point and against each viral strain. The comparison of the GMTs against  
319 the two viral lineage antigens was performed at all sampling time points, using a paired  
320 two-tailed Student's  $t$ -test. A value of  $P < 0.05$  was considered to be statistically  
321 significant. In order to estimate the strength of the anamnestic immune responses (e.g.

322 due to the booster) between two sampling points ( $w$ ,  $z$ ) and against the same lineage ( $x$ ),  
323 geometrical mean titer ratio (GMTR) was calculated as follows:  $GMTR_x = GMT_z /$   
324  $GMT_w$ .

325

326 Investigation of the immunological similarity between the vaccine and the circulating  
327 viral strains

328 In an effort to interpret the immunological cross-reactivity between the NABs  
329 produced against the vaccine strain (New York 1999/VM-2, GenBank Acc. No.  
330 AF260967) and the lineage 2 strain circulating in the study area (Nea Santa-Greece-2010,  
331 GenBank Acc. No. HQ537483), we compared the identity of the Envelope (E) protein  
332 peptide sequences of the two strains. Furthermore, the respective peptide sequence of a  
333 lineage 2 strain isolated in South Africa (SA93/01, Gen.Bank. Acc. No. EF429198) was  
334 included in these comparisons. Multiple alignments of E protein sequences were  
335 conducted using the MEGA v.6.06 software (45), and amino acid substitutions were  
336 visualized using BioEdit v.7.2.5 software (46).

337

338 Animal ethics

339 Animal studies have been performed in accordance to the International Guiding  
340 Principles for Biomedical Research involving animals, as issued by the Council for  
341 International Organizations of Medical Sciences. All horse owners gave their consent for  
342 the immunizations, blood sampling and serological testing prior to the commencement of  
343 the study. This study was performed in compliance with national guidelines and EU  
344 regulations, as well as by local Ethics Committees of the School of Veterinary Medicine,  
345 Aristotle University of Thessaloniki.

346

347 **Results**

348 WNV circulation in the study area

349           The presence and circulation of the virus in the study area was confirmed for both  
350 2011 and 2012, by studies conducted in captive sentinel chickens and mosquitoes, as  
351 already described (40, 47-48). Specifically, chickens were placed in cages in close  
352 proximity to the participating horse riding clubs, and exposed to mosquitoes throughout  
353 both epidemic periods, followed by serological and virological testing. Mosquitoes which  
354 were being collected throughout May-October of both years were also tested. Molecular  
355 characterization of the circulating viral strain during both 2011 and 2012 in chickens and  
356 mosquitoes confirmed that the virulent Nea Santa-Greece-2010 was the only strain  
357 detected in Central Macedonia (40, 47-48).

358

359 WNV natural infections in control and immunized horses

360           Serological testing of control horses during November of each year [W21,  
361 W72(27)] indicated that WNV circulated in all the participating horse riding clubs,  
362 during both 2011 and 2012 epidemic seasons. Specifically, 12 out of 33 control animals  
363 of 2011 (36%) and 9 out of 33 control animals of the 2012 period (27%) seroconverted to  
364 WNV as evidenced by cELISA testing (Table 1).

365           Comparative evaluation of NAb titers of sera obtained from all primo-vaccinated  
366 animals one month after the double vaccination and after the end of each epidemic period  
367 (November), indicated that anamnestic humoral responses (WNV infections) were  
368 evident in 32 of 85 primo-vaccinated horses of 2011 (38% of the immunized horses) and  
369 in 14 of 55 primo-vaccinated horses of 2012 (26% of the horses which received a primary  
370 immunization during the later year). Specifically, GMT increased from 1:67 to 1:1083  
371 (GMTR = 16.2,  $\sim\log_2$ 4-fold increase). Natural infections, during 2012, of those horses  
372 that were exposed for a second consecutive year could not be determined directly by  
373 SNTs (due to the booster), but were calculated indirectly based on the respective  
374 percentages of the seroconverted control horses, as well as on the percentages of primo-

375 vaccinated horses in which anamnestic humoral responses due to infections were  
376 detected, for the two years. As a result, it is estimated that from the 52 horses which  
377 remained uninfected during 2011 and received an annual booster in 2012, 15 animals  
378 were subsequently infected from WNV during the second epidemic season (Table 1).

379           Combinatory analysis of all these results obtained from serological testing applied  
380 in control and vaccinated horses indicate in total 44 out of 118 horses (37%) were  
381 infected during 2011. The respective infection rate for 2012 was estimated to be 27%  
382 (Table 1). Infection rates between the horse riding clubs ranged between 18 and 60% for  
383 2011 and between 18 and 47% for 2012.

384

#### 385 Vaccine safety

386           Regarding the adverse reactions of the applied vaccine, only one out of 140  
387 immunized animals (0.7%) developed a local reaction, on the site of the injection. This  
388 was a mild swelling which was developed after the second injection of the first year, and  
389 it was observed again in the same animal after the annual booster. During the  
390 aforementioned occurrences, resolution of the lesion was observed within a few days,  
391 without any interventions, and without other effects on the health of the animal.

392

#### 393 Neutralizing antibody responses in immunized horses

394           One month after the initial double vaccination, NABs against the Greek lineage 2  
395 strain were induced in all vaccinated animals with a GMT of 1:102 (titer range: 1:40 to  
396 1:320). Briefly, in 67 out of the 140 vaccinated animals (47.9%; 95% CI: 39-56%), an  
397 intermediate neutralizing activity was observed (titers 1:40-1:80). Twenty-six of these  
398 animals developed a NAb titer of 1:40 and 41 animals were presented with a titer of 1:80.  
399 Higher neutralizing responses were observed in sera from the remaining 73 vaccinated  
400 animals (52.1%; 95% CI: 44-61%). Nineteen out of the 46 primo-vaccinated horses of



401 2011 and 2012 (41%) which were subsequently infected (32 and 14, respectively) as  
402 determined by SNT (Table 1), had a NAb titer of 1:40 against the Nea Santa-Greece-  
403 2010 strain, one month after the primary immunization course.

404           Moreover, application of SNT in 23 of the 53 primo-vaccinated horses of 2011  
405 which were revealed to not be naturally infected indicated that GMTs against the lineage  
406 1 strain were higher than the respective titer against the lineage 2 strain, at all sampling  
407 points. NABs in the sera of these animals were being consistently detected against the two  
408 viral lineage antigens, at all sampling points, and until the annual booster immunization  
409 (Fig. 2). However, paired *t*-test analysis revealed no significant differences in the GMTs  
410 against the two WNV strains in the sera of the 23 vaccinated horses, at all sampling  
411 points ( $P > 0.28$ ). Specifically, analysis of the NAb titers raised against the two viral  
412 lineage antigens one month after the completion of the double primary vaccination course  
413 (i.e. on W7) indicated that the GMT for lineage 1 was 1:175 (titer range: 1:80 to 1:320),  
414 while the respective value for lineage 2 was 1:112 (titer range: 1:40 to 1:160). Individual  
415 NAb titers indicated that in 9 out of 23 animals (39.1; 95% CI: 20-61%) NAb titers were  
416 the same against both antigens. In 13 out of 23 animals (56.5; 95% CI: 35-76%) titers  
417 against the lineage 1 antigen were higher than the respective titer raised against lineage 2  
418 by one serial dilution, while in one serum (4.4; 95% CI: 0.1-24%) a titer difference of 2  
419 dilutions between the two viral lineage antigens was observed. Further comparison of  
420 NAb titers throughout the year indicate that those for lineage 1 were consistently higher  
421 than the respective values determined for lineage 2.

422           One month after the annual booster a strong titer increase was observed against  
423 both strains (Fig. 2). Achieved NAb titers were well above their initial peak (one month  
424 after the initial double primary vaccination). Specifically,  $\geq \log_2 4$ -fold titer increase was  
425 observed in all cases, regarding the NAb titers against both PaAn001/France lineage 1  
426 ( $\text{GMTR}_{lin,1} = 30.2$ ) and Nea Santa-Greece-2010 lineage 2 ( $\text{GMTR}_{lin,2} = 27.5$ ) strains. A  
427 similar degree of immunoreactivity (titer increase  $\geq 4$  two-fold serial dilutions) was also  
428 observed in the vaccinated animals which were naturally infected. In all these anamnestic  
429 immune responses NAb titers were determined to be  $\geq 1:320$ . The GMT for lineage 1 one

430 month after the annual booster (i.e. on W52) was determined to be 1:1894 (titer range:  
431 1:640 to  $\geq$ 1:2560), whereas the respective value for the lineage 2 antigen was 1:722 (titer  
432 range: 1:320 to 1:1280). In terms of NAb titer differences between the two viral lineage  
433 antigens, it was indicated that in 15 out of 23 animals (65.2; 95% CI: 43-83%) NAb titers  
434 against the lineage 1 antigen were higher than the respective titer raised against lineage 2  
435 by one serial dilution. In 7 out of 23 animals (30.4; 95% CI: 14-53%) titers differed by  
436 two serial dilutions, and in one serum (4.4; 95% CI: 0.1-24%) a titer difference of 3  
437 dilutions was observed.

438

439 Clinical signs, confirmation of the diagnosis and cross-protective efficacy of the vaccine

440         None of the 140 vaccinated horses (0%) showed any clinical signs related to  
441 WNV infection during both epidemic periods. In contrast, 3 out of 45 control animals  
442 (7%) showed clinical signs due to WNV infection. The odds ratio, considering all  
443 animals, was 0.0432 (95% CI: 0.0022-0.8534). The fact that the CI did not cross 1  
444 implies a statistically significant ( $P < 0.05$ ) difference between vaccinated and non-  
445 vaccinated horses with regards to presence of clinical signs. The same analysis based on  
446 the infected animals only (61 immunized and 21 controls) returned an odds ratio of  
447 0.0423 (95% CI: 0.0021-0.08562), implying that immunization was also beneficial for  
448 animals that were naturally infected.

449         More specifically, during the 2011 epidemic period, clinical signs were detected  
450 in one out of the 12 seroconverted control animals (August 13). During the 2012  
451 epidemic period, 2 out of the 9 seroconverted horses of the control group showed clinical  
452 signs (August 25 and September 8, respectively). These signs included fever, weakness  
453 of hind limbs, ataxia, muscle twitching and tremors in all 3 affected animals. Diagnosis  
454 was confirmed, as WNV-specific IgM antibodies were detected in all of them by MAC-  
455 ELISA testing. WNV RNA was not detected (no Ct values obtained). Consequently, it  
456 was not possible to detect the virus in the obtained blood sera, since they were drawn  
457 after the initiation of the clinical signs and probably past the viremia stage. The 3 horses

458 received supportive treatment (dexamethasone, vitamin B complex supplements and  
459 phenylbutazone) that led to the resolution of clinical signs within a few days.

460 The effect of vaccination on preventing clinical signs was confirmed and  
461 quantified with mixed model analysis. Vaccination status had a significant effect on  
462 clinical signs, with non-immunized animals being associated with a  $7.58 \pm 1.82\%$  ( $P <$   
463  $0.05$ ) higher chance of exhibiting signs compared to immunized animals. This value was  
464 derived from the analysis of all animals with mixed model 4. The value reflects the effect  
465 of immunization on the presence of clinical signs and describes the difference between  
466 the marginal means of vaccinated and non-vaccinated animals, adjusted for all other  
467 effects in model 4. The corresponding value from the analysis of infected animals only  
468 was  $14.22 \pm 1.43\%$  ( $P < 0.05$ ), suggesting that the vaccination effect was even stronger for  
469 naturally infected horses.

470

471 E protein amino acid sequence comparisons

472 For the interpretation of the reactivity between the NAbs raised against the  
473 vaccine strain and virus strains belonging to lineage 2, the immunological similarity of E  
474 protein peptide sequences was investigated. No differences were observed between the  
475 peptide sequences of the Greek and African lineage 2 strains. Sequence comparison of  
476 the lineage 1 vaccine strain with those of the lineage 2 strains, indicated 23 amino acid  
477 substitutions. Specifically, 3 of these substitutions were observed in structural domain I  
478 (DI; L<sub>131</sub>Q, V<sub>159</sub>I, and A<sub>172</sub>S), 15 substitutions were identified in DII (E<sub>55</sub>D, T<sub>64</sub>S, K<sub>71</sub>R,  
479 D<sub>83</sub>E, R<sub>93</sub>K, S<sub>122</sub>T, I<sub>126</sub>T, R<sub>128</sub>W, T<sub>129</sub>I, N<sub>199</sub>S, T<sub>205</sub>S, T<sub>208</sub>A, T<sub>210</sub>S, V<sub>232</sub>T, and I<sub>253</sub>V), 2  
480 substitutions were present in DIII (L<sub>312</sub>A, and A<sub>369</sub>S), and 3 more substitutions were  
481 found in the transmembrane domain/stem region (K<sub>413</sub>R, V<sub>442</sub>I, and L<sub>483</sub>M) (Fig. 3).

482

483 **Discussion**

484 Two doses of the vaccine, administrated 3 weeks apart in immunologically naïve  
485 horses resulted in the development of adequate cross-protective immunity against  
486 development of neurological signs due to natural infections from the Nea Santa-Greece-  
487 2010 lineage 2 strain during the following epidemic period, as indicated by the lack of  
488 occurrence of clinical signs in any of the immunized animals. Immunization using the  
489 aforementioned vaccine may not prevent horses from being infected from lineage 2  
490 strains, but can reduce the number of viremic horses, the viremia duration and titer in the  
491 infected animals, the duration and severity of clinical signs and the mortality, as it has  
492 already been described (34, 37). In our case, although detection of severe cases in horses  
493 was effective due to the experience of the involved veterinarians, it is possible that mild  
494 clinical occurrences could have not been not iced. However, since no supportive  
495 treatment was required, the impact of these cases was insignificant. Adverse reactions  
496 due to the vaccine were minimal. Our findings confirm that although the majority of  
497 infections in horses were subclinical, a high percentage (14%) of the seroconverted non  
498 vaccinated horses exhibited neurological signs. This is in agreement to a similar  
499 percentage (19%) of neurological manifestations-to-infections reported for this virus  
500 strain during the 2010 epidemic in Greece (49). Interestingly, slightly lower morbidity  
501 (10%) within infected horses has been reported for lineage 1 WNV strains (50-52).

502 Despite the use of adjuvants, long-term immunity is not a feature of inactivated  
503 vaccines. Although the duration of immunity for lineage 1 strains has been determined  
504 (12 months after the primary vaccination course) (33), relevant information for lineage 2  
505 is lacking. Previous studies with Equip<sup>®</sup> WNV have indicated that immunized horses  
506 maintained NAb titers  $\geq 1:100$  against lineage 1, for 5-7 months, as determined by plaque  
507 reduction neutralization test (PRNT) (53). In another study it was shown that neutralizing  
508 responses were maintained for 6 months after vaccination of immunologically naïve  
509 horses (54). In a more recent study, it has been demonstrated that NABs could be detected  
510 at samples obtained one year after the primary vaccination course of naïve horses,  
511 although a decline in neutralizing titers was observed (55). In our case, NABs against the  
512 lineage 2 strain were developed in all vaccinated animals (titer range: 1:40 to 1:320,

513 GMT = 1:102) one month after the initial double vaccination. Although a titer decline  
514 was observed through time, as evidenced by testing of vaccinated animals that were not  
515 infected (GMT < 1:100 on week 34), NABs were detectable until the annual booster. The  
516 lowest neutralizing response of 1:40 against the lineage 2 strain was observed in 41% of  
517 the primo-vaccinated horses of both years ( $n = 19$ ) which were subsequently infected.  
518 The fact that these 19 horses did not exhibit clinical signs due to WNV infection indicates  
519 that NAb titers as low as 1:40 one month after the primary immunization course can be  
520 protective against natural infections from the Nea Santa-Greece-2010. It can be  
521 hypothesized that humoral immunity against lineage 2 lasts at least until W21, based  
522 solely on the GMTs against the lineage 2 strain which were >1:100 for these sampling  
523 time points, although individual titers >1:100 were detected until W34. GMTs against the  
524 PaAn001/France lineage 1 were higher than the respective titers against lineage 2, which  
525 is in agreement with other studies (36, 55). In our case comparison of GMTs against the  
526 two lineages revealed no significant differences. The applied immunization scheme  
527 resulted in development of adequate B-cell memory, as indicated by the strong responses  
528 observed after annual boosters and natural infections. Results regarding these responses  
529 are supported by a previous study, in which significant NAb titer increase ( $\log_2 5$ -fold)  
530 against both lineages is described, and titers achieved were well above their peak  
531 observed after the initial vaccination (55). It has been previously demonstrated that  
532 horses immunized with this vaccine also developed antigen-specific cellular responses  
533 ( $CD4^+$  and  $CD8^+$  IFN- $\gamma$  expression, cellular proliferation and IL-4 expression in  $CD4^+$   
534 PBMCs) (54), indicating that, besides humoral immunity, the vaccine induces T-cell  
535 responses, which might have an additional contribution to the cross-protection of the  
536 naturally infected horses.

537 WNV E protein is a major determinant of tropism and the primary target of NABs.  
538 Neutralizing epitopes have been identified mainly on DIII of E protein, and specifically,  
539 on residues 306, 307, 330 and 332 (56-58). Additional neutralizing epitopes have been  
540 identified on several residues of DI and DII, although the observed neutralizing activity  
541 for these regions is weaker (59). In our case, no changes were observed at residues S<sub>306</sub>,

542 K<sub>307</sub>, T<sub>330</sub> and T<sub>332</sub>, which serve as major DIII neutralizing epitopes (56-58). However  
543 escape from neutralization has been associated with the L<sub>312</sub>A substitution, which was  
544 present in “Nea Santa-Greece-2010”, as it is in several WNV strains (60). No changes  
545 were observed in residues W<sub>101</sub>, G<sub>106</sub> and L<sub>107</sub>, antigenic sites of the fusion loop located  
546 within DII (DII-FL, residues 98-109), which act as target of cross-NAbs among different  
547 species of the genus *Flavivirus* (61-63). Despite the L<sub>312</sub>A substitution, the findings of  
548 the present study ultimately suggest that under field conditions, adequate cross-  
549 neutralization is capable of providing a high degree of protection.

550 Different WNV lineages, characterized by varying virulence and  
551 neuroinvasiveness co-circulate in Europe (63), and knowledge regarding the cross-  
552 protection is prerequisite. However, since outbreaks in horses were limited and  
553 unpredictable, immunizations have been performed extensively (26), regardless the  
554 degree of cross-protection between circulating and the vaccine strain. For the purpose of  
555 in-field evaluations of arbovirus vaccines, identification of the circulating strain  
556 comprises a necessity. In our case, it was not possible to detect the virus in the affected  
557 horses. This was anticipated, given that in horses WNV detection is hampered by the  
558 short viremia duration which precedes the onset of clinical signs (7, 49, 51). Therefore,  
559 WNV surveillance data from birds and mosquitoes, indicating that the only strain  
560 circulating during both years was the Nea Santa-Greece-2010 were utilized (46-48).  
561 Mixed model analysis seems to be a more accurate approach for in-field vaccine  
562 evaluations, as many factors are involved and should be taken into consideration. It was  
563 also possible to quantify the favorable effect of the immunization on the presence of  
564 clinical signs. Immunizations using inactivated lineage 1 vaccines can effectively protect  
565 horses from the development of neurological signs due to natural infections of virulent  
566 lineage 2 WNV strains. Since pathogenesis and antiviral immune responses against WNV  
567 in horses and humans are similar, our results could be of value in the future, for the  
568 possible evaluation of a candidate human vaccine.

569

570 **Conflict of interest**

571 Funding was provided by Zoetis Inc.

572

573 **Acknowledgements**

574 Eleni Pavlidou (Zoetis Inc., Athens, Greece) is acknowledged for her support.

575 Acknowledgements are expressed to all the horse owners who gave their consent for the  
576 immunizations, blood samplings and laboratory testing, as well as all horse riding club  
577 owners and the animal husbandry technical staff who allowed access to their premises  
578 and aided us with our study. Panagiota Tyrnenopoulou (Equine Unit, Companion Animal  
579 Clinic, School of Veterinary Medicine, Aristotle University of Thessaloniki) is thanked  
580 for her contribution in immunizations and blood samplings. The authors would also like  
581 to express their acknowledgments to Dr. Sylvie Lecollinet (UMR 1161 Virology, INRA-  
582 ANSES-ENVA, France) for providing the PaAn001/France lineage 1 WNV strain. Prof.  
583 Orestis Papadopoulos is also thanked for valuable discussions. Serafeim C. Chaintoutis  
584 was supported by the Alexander S. Onassis Public Benefit Foundation.

585

586 **References**

- 587 1. Zeller HG, Schuffenecker I. 2004. West Nile Virus: An Overview of Its Spread in  
588 Europe and the Mediterranean Basin in Contrast to its Spread in the Americas.  
589 *Eur. J. Clin. Microbiol. Infect. Dis.* 23:147–56.
- 590 2. Hayes EB, Komar N, Nasci RS, Montgomery SP, O’Leary DR, Campbell GL.  
591 2005. Epidemiology and transmission dynamics of West Nile virus disease.  
592 *Emerg. Infect. Dis.* 11:1167–73.
- 593 3. Rappole JH, Hubálek Z. 2003. Migratory birds and West Nile virus. *J. Appl.*  
594 *Microbiol.* 94:47–58.

- 595 4. Linke S, Niedrig M, Kaiser A, Ellerbrok H, Müller K, Müller T, Conraths FJ,  
596 Mühle RU, Schmidt D, Köppen U, Bairlein F, Berthold P, Pauli G. 2007.  
597 Serologic evidence of West Nile virus infections in wild birds captured in  
598 Germany. *Am. J. Trop. Med. Hyg.* 77: 358–64.
- 599 5. Kramer LD, Styer LM, Ebel GD. 2008. A global perspective on the epidemiology  
600 of West Nile virus. *Annu. Rev. Entomol.* 53:61–81.
- 601 6. Engler O, Savini G, Papa A, Figuerola J, Groschup MH, Kampen H, Medlock J,  
602 Vaux A, Wilson AJ, Werner D, Jöst H, Goffredo M, Capelli G, Federici V,  
603 Tonolla M, Patocchi N, Flacio E, Portmann J, Rossi-Pedruzzi A, Mourelatos S,  
604 Ruiz S, Vázquez A, Calzolari M, Bonilauri P, Dottori M, Schaffner F, Mathis A,  
605 Johnson N. 2013. European surveillance for West Nile virus in mosquito  
606 populations. *Int. J. Environ. Res. Public Health* 10:4869–95.
- 607 7. Dauphin G, Zientara S, Zeller H, Murgue B. 2004. West Nile: worldwide current  
608 situation in animals and humans. *Comp. Immunol. Microbiol. Infect. Dis.* 27:343–  
609 55.
- 610 8. Hayes EB, Sejvar JJ, Zaki SR, Lanciotti RS, Bode AV, Campbell GL. 2005.  
611 Virology, pathology, and clinical manifestations of West Nile virus disease.  
612 *Emerg. Infect. Dis.* 11:1174–9.
- 613 9. Cantile C, di Guardo G, Eleni C, Arispici M. 2000. Clinical and  
614 neuropathological features of West Nile virus equine encephalomyelitis in Italy.  
615 *Equine Vet. J.* 32:31–5.
- 616 10. Steinman A, Banet C, Sutton GA, Yadin H, Hadar S, Brill A. 2002. Clinical signs  
617 of West Nile virus encephalomyelitis in horses during the outbreak in Israel in  
618 2000. *Vet. Rec.* 151:47–9.



- 619 11. Weese JS, Baird JD, DeLay J, Kenney DG, Staempfli HR, Viel L, Parent J,  
620 Smith-Maxie L, Poma R. 2003. West Nile virus encephalomyelitis in horses in  
621 Ontario: 28 cases. *Can. Vet. J.* 44:469–73.
- 622 12. Venter M, Human S, Zaayman D, Gerdes GH, Williams J, Steyl J, Leman PA,  
623 Paweska JT, Setzkorn H, Rous G, Murray S, Parker R, Donnellan C, Swanepoel  
624 R. 2009. Lineage 2 West Nile virus as cause of fatal neurologic disease in horses,  
625 South Africa. *Emerg. Infect. Dis.* 15:877–84.
- 626 13. Kutasi O, Bakonyi T, Lecollinet S, Biksi I, Ferenczi E, Bahuon C, Sardi S,  
627 Zientara S, Szenci O. 2011. Equine encephalomyelitis outbreak caused by a  
628 genetic lineage 2 West Nile virus in Hungary. *J. Vet. Intern. Med.* 25:586–91.
- 629 14. Vazquez A, Sánchez-Seco MP, Ruiz S, Molero F, Hernández L, Moreno J,  
630 Magallanes A, Tejedor CG, Tenorio A. 2010. Putative new lineage of West Nile  
631 virus, Spain. *Emerg. Infect. Dis.* 16:549–52.
- 632 15. Bakonyi T, Ivanics E, Erdélyi K, Ursu K, Ferenczi E, Weissenböck H, Nowotny  
633 N. 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe.  
634 *Emerg. Infect. Dis.* 12:618–23.
- 635 16. Calistri P, Giovannini A, Hubalek Z, Ionescu A, Monaco F, Savini G, Lelli R.  
636 2010. Epidemiology of West Nile in Europe and in the Mediterranean basin. *Open  
637 Virol. J.* 4:29–37.
- 638 17. Murgue B, Zeller H, Deubel V. 2002. The ecology and epidemiology of West  
639 Nile virus in Africa, Europe and Asia. *Curr. Top. Microbiol. Immunol.* 267:195–  
640 221.
- 641 18. Savini G, Capelli G, Monaco F, Polci A, Russo F, Di Gennaro A, Marini V,  
642 Teodori L, Montarsi F, Pinoni C, Piscicella M, Terregino C, Marangon S, Capua I,  
643 Lelli R. 2012. Evidence of West Nile virus lineage 2 circulation in Northern Italy.  
644 *Vet. Microbiol.* 158:267–73.

- 645 19. Marka A, Diamantidis A, Papa A, Valiakos G, Chaintoutis SC, Doukas D,  
646 Tserkezou P, Giannakopoulos A, Papaspyropoulos K, Patsoula E, Badieritakis E,  
647 Baka A, Tseroni M, Pervanidou D, Papadopoulos NT, Koliopoulos G, Tontis D,  
648 Dovas CI, Billinis C, Tsakris A, Kremastinou J, Hadjichristodoulou C. 2013.  
649 West Nile Virus State of the Art Report of MALWEST Project. *Int. J. Environ.*  
650 *Res. Public Health* 10:6534–610.
- 651 20. Hellenic Center for Disease Control and Prevention (HCDCP). Report on West  
652 Nile Virus Epidemic; 2013. <http://goo.gl/lsJaeC> [cited 2015 March 15].
- 653 21. Chaintoutis SC, Gewehr S, Danis K, Kalaitzopoulou S, Antalis V,  
654 Papanastassopoulou M, Mourelatos S, Panagiotopoulos T, Hadjichristodoulou C,  
655 Dovas CI. 2014. Surveillance and early warning of West Nile virus lineage 2  
656 using domestic pigeons and backyard chickens. Proceedings of the 19<sup>th</sup> Congress  
657 of the European Society for Vector Ecology.
- 658 22. Turell MJ, Bunning M, Ludwig GV, Ortman B, Chang J, Speaker T, Spielman A,  
659 McLean R, Komar N, Gates R, McNamara T, Creekmore T, Farley L, Mitchell  
660 CJ. 2003. DNA vaccine for West Nile virus infection in fish crows (*Corvus*  
661 *ossifragus*). *Emerg. Infect. Dis.* 9:1077–81.
- 662 23. Bunning ML, Fox PE, Bowen RA, Komar N, Chang G-JJ, Speaker TJ, Stephens  
663 MR, Nemeth N, Panella NA, Langevin SA, Gordy P, Teehee M, Bright PR, Turell  
664 MJ. 2007. DNA vaccination of the American crow (*Corvus brachyrhynchos*)  
665 provides partial protection against lethal challenge with West Nile virus. *Avian*  
666 *Dis.* 51:573–7.
- 667 24. Chang G-JJ, Davis BS, Stringfield C, Lutz C. 2007. Prospective immunization of  
668 the endangered California condors (*Gymnogyps californianus*) protects this  
669 species from lethal West Nile virus infection. *Vaccine* 25:2325–30.

- 670 25. Kilpatrick AM, Dupuis AP, Chang G-JJ, Kramer LD. 2010. DNA vaccination of  
671 American robins (*Turdus migratorius*) against West Nile virus. *Vector Borne*  
672 *Zoonotic Dis.* 10:377–80.
- 673 26. Beck C, Jimenez-Clavero MA, Leblond A, Durand B, Nowotny N, Leparç-  
674 Goffart I, Zientara S, Jourdain E, Lecollinet S. 2013. Flaviviruses in Europe:  
675 Complex Circulation Patterns and Their Consequences for the Diagnosis and  
676 Control of West Nile Disease. *Intern. J. Environ. Res. Public Health* 10:6049–83.
- 677 27. Agrawal A-G, Petersen LR. 2003. Human Immunoglobulin as a Treatment for  
678 West Nile Virus Infection. *J. Infect. Dis.* 188:1–4.
- 679 28. Brandler S, Tangy F. 2013. Vaccines in development against West Nile virus.  
680 *Viruses* 5:2384–409.
- 681 29. Ng T, Hataway D, Jennings N, Champ D, Chiang YW, Chu HJ, 2003. Equine  
682 vaccine for West Nile virus. *Dev. Biol.* 114:221–7.
- 683 30. Seino KK, Long MT, Gibbs EP, Bowen RA, Beachboard SE, Humphrey PP,  
684 Dixon MA, Bourgeois MA. 2007. Comparative efficacies of three commercially  
685 available vaccines against West Nile virus (WNV) in a short-duration challenge  
686 trial involving an equine WNV encephalitis model. *Clin. Vaccine Immunol.*  
687 14:1465–71.
- 688 31. Epp T, Waldner C, Townsend HGG. 2007. A case-control study of factors  
689 associated with development of clinical disease due to West Nile virus,  
690 Saskatchewan 2003. *Equine Vet. J.* 39:498–503.
- 691 32. EquiNile with Havlogen, Material Safety Data Sheet. Intervet Shering-Plough  
692 Animal Health Nr. SP002579. Retrieved from  
693 <http://merckusa.compassites.com/product/view/6324025> [cited 2015 March 15]
- 694 33. European Medicines Agency (EMA) EPAR Product information Equip WNV  
695 [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/EPAR\\_-](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-)

- 696 [Product Information/veterinary/000137/WC500063683.pdf](#) [cited 2015 March  
697 15].
- 698 34. European Medicines Agency (EMA). EPAR Summary for the public Proteq West  
699 Nile [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/EPAR -  
700 \\_Summary\\_for\\_the\\_public/veterinary/002005/WC500110366.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/veterinary/002005/WC500110366.pdf) [cited 2015  
701 March 15].
- 702 35. Minke JM, Siger L, Cupillard L, Powers B, Bakonyi T, Boyum S, Nowotny N,  
703 Bowen R. 2011. Protection provided by a recombinant ALVAC<sup>®</sup>-WNV vaccine  
704 expressing the prM/E genes of a lineage 1 strain of WNV against a virulent  
705 challenge with a lineage 2 strain. *Vaccine* 29:4608–12.
- 706 36. Venter M, van Vuren PJ, Mentoer J, Paweska J, Williams J. 2013. Inactivated  
707 West Nile Virus (WNV) vaccine, Duvaxyn WNV, protects against a highly  
708 neuroinvasive lineage 2 WNV strain in mice. *Vaccine* 31:3856–62.
- 709 37. Bowen RA, Bosco-Lauth A, Syvrud K, Thomas A, Meinert TR, Ludlow DR,  
710 Cook C, Salt J, Ons E. 2014. Protection of horses from West Nile virus Lineage 2  
711 challenge following immunization with a whole, inactivated WNV lineage 1  
712 vaccine. *Vaccine* 32:5455–9.
- 713 38. Styer LM, Bernard KA, Kramer LD. 2006. Enhanced early West Nile virus  
714 infection in young chickens infected by mosquito bite: effect of viral dose. *Am J  
715 Trop Med Hyg.* 75:337-45.
- 716 39. Chaintoutis SC, Dovas CI, Papanastassopoulou M, Gewehr S, Danis K, Beck C,  
717 Lecollinet S, Antalis V, Kalaitzopoulou S, Panagiotopoulos T, Mourelatos S,  
718 Zientara S, Papadopoulos O. 2014. Evaluation of a West Nile virus surveillance  
719 and early warning system in Greece, based on domestic pigeons. *Comp. Immunol.  
720 Microbiol. Infect. Dis.* 37:131–41.

- 721 40. Chaskopoulou A, Dovas CI, Chaintoutis SC, Kashefi J, Koehler P,  
722 Papanastassopoulou M. 2013. Detection and early warning of West Nile virus  
723 circulation in Central Macedonia, Greece, using sentinel chickens and  
724 mosquitoes. *Vector Borne Zoonotic Dis.* 13:723–32.
- 725 41. Altman DG. 2006. *Practical Statistics for Medical Research*, 2nd ed. Chapman &  
726 Hall/CRC Tests in Statistical Science, London, UK
- 727 42. Pagano M, Gauvreau K. 2000. *Principles of biostatistics*. 2nd ed. Belmont, CA:  
728 Brooks/Cole.
- 729 43. Deeks JJ, Higgins JPT. 2010. Statistical algorithms in Review Manager 5.  
730 Retrieved from [http://ims.cochrane.org/revman/documentation/Statistical-](http://ims.cochrane.org/revman/documentation/Statistical-methods-in-RevMan-5.pdf)  
731 [methods-in-RevMan-5.pdf](http://ims.cochrane.org/revman/documentation/Statistical-methods-in-RevMan-5.pdf)
- 732 44. Gilmour AR, Gogel BJ, Cullis BR, Thompson R. 2009. *ASReml User Guide*  
733 Release 3.0 VSN International Ltd, Hemel Hempstead, HP1 1ES, UK
- 734 45. Tamura K, Stecher G, Peterson D, Filipksi A, and Kumar S. 2013. MEGA6:  
735 Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30:2725-  
736 9.
- 737 46. Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and  
738 analysis program for Windows 95/98/NT. *Nucl. Acids Symp. Ser.* 41:95-8.
- 739 47. Chaskopoulou A, Dovas CI, Chaintoutis SC, Bouzalas I, Ara G,  
740 Papanastassopoulou M. 2011. Evidence of enzootic circulation of West Nile virus  
741 (Nea Santa-Greece-2010, lineage 2), Greece, May to July 2011. *Euro Surveill.*  
742 16:pii=19933.
- 743 48. Chaintoutis SC, Chaskopoulou A, Chassalevris T, Koehler PG,  
744 Papanastassopoulou M, Dovas CI. 2013. West Nile virus lineage 2 strain in  
745 Greece, 2012. *Emerg. Infect. Dis.* 19:827-9.

- 746 49. Bouzalas IG, Diakakis N, Chaintoutis SC, Brellou GD, Papanastassopoulou M,  
747 Danis K, Vlemmas I, Seuberlich T, Dovas CI. 2015. Emergence of equine West  
748 Nile encephalitis in Central Macedonia, Greece, 2010. *Transbound. Emerg. Dis.*  
749 doi:10.1111/tbed.12334
- 750 50. Long MT, Porter MB, Hernandez J, Giguere S, Fontaine GL, Jodoin EA, Gillis  
751 KD. 2003. Preliminary data regarding the subclinical exposure rate of horses to  
752 West Nile virus during the 2001 Florida enzootic. *Proc. Am. Assoc. Equine.*  
753 *Pract.* 49:397–8.
- 754 51. Castillo-Olivares J, Wood J. 2004. West Nile virus infection of horses. *Vet. Res.*  
755 35:467-83.
- 756 52. Gardner IA, Wong SJ, Ferraro GL, Balasuriya UB, Hullinger PJ, Wilson D, Shi  
757 PY, MacLachlan NJ. 2007. Incidence and effects of West Nile virus infection in  
758 vaccinated and unvaccinated horses in California. *Vet. Res.* 38:109–16.
- 759 53. Davidson AJ, Traub-Dargatz JL, Rodeheaver RM, Ostlund EN, Pedersen DD,  
760 Moorhead RG, Stricklin JB, Dewell RD, Roach SD, Long RE, Albers SJ, Callan  
761 RJ, Salman MD. 2005. Immunologic responses to West Nile virus in vaccinated  
762 and clinically affected horses. *J. Am. Vet. Med. Assoc.* 226:240–5.
- 763 54. Davis EG, Zhang Y, Tuttle J, Hankins K, Wilkerson M. 2008. Investigation of  
764 antigen specific lymphocyte responses in healthy horses vaccinated with an  
765 inactivated West Nile virus vaccine. *Vet. Immunol. Immunopathol.* 126:293–301.
- 766 55. Pearce MC, Venter M, Schouwstra T, Van Eeden C, Jansen van Vuren P,  
767 Paweska J, Liu B, du Plessis A. 2013. Serum neutralising antibody response of  
768 seronegative horses against lineage 1 and lineage 2 West Nile virus following  
769 vaccination with an inactivated lineage 1 West Nile virus vaccine. *J. S. Afr. Vet.*  
770 *Assoc.* doi:10.4102/jsava.v84i1.1052.

- 771 56. Beasley DWC, Barrett ADT. 2002. Identification of Neutralizing Epitopes within  
772 Structural Domain III of the West Nile Virus Envelope Protein. *J. Virol.*  
773 76:13091–100.
- 774 57. Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S,  
775 Mehlhop E, Marri A, Chung KM, Ebel GD, Kramer LD, Fremont DH, Diamond  
776 MS. 2005. Development of a humanized monoclonal antibody with therapeutic  
777 potential against West Nile virus. *Nat. Med.* 11:522–30.
- 778 58. Sánchez MD, Pierson TC, McAllister D, Hanna SL, Puffer BA, Valentine LE,  
779 Murtadha MM, Hoxie JA, Doms RW. 2005. Characterization of neutralizing  
780 antibodies to West Nile virus. *Virology* 336:70–82.
- 781 59. Oliphant T, Nybakken GE, Engle M, Xu Q, Nelson CA, Sukupolvi-Petty S, Marri  
782 A, Lachmi BE, Olshevsky U, Fremont DH, Pierson TC, Diamond MS. 2006.  
783 Antibody Recognition and Neutralization Determinants on Domains I and II of  
784 West Nile Virus Envelope Protein. *J. Virol.* 80:12149–59.
- 785 60. Alan LL, Barrett DT, Beasley DWC. 2005. Differential expression of domain III  
786 neutralizing epitopes on the envelope proteins of West Nile virus strains. *Virology*  
787 335:99–105.
- 788 61. Crill WD, Chang GJ. 2004. Localization and characterization of flavivirus  
789 envelope glycoprotein cross-reactive epitopes. *J. Virol.* 78:13975–86
- 790 62. Stiasny K, Kiermayr S, Holzmann H, Heinz FX. 2006. Cryptic properties of a  
791 cluster of dominant flavivirus cross-reactive antigenic sites. *J. Virol.* 80:9557–68.
- 792 63. Donadieu E, Bahuon C, Lowenski S, Zientara S, Couplier M, Lecollinet S. 2013.  
793 Differential virulence and pathogenesis of West Nile viruses. *Viruses* 5:2856–80.
- 794

795 **Figure captions**

796 **Fig. 1.** Timeline of the immunizations and blood serum samplings performed in horses,  
797 for the evaluation of the cross-protective immunity offered from the inactivated vaccine.  
798 Black syringes indicate the double primary vaccinations; white syringe indicates annual  
799 booster vaccination. Arrows depict the time points of blood serum samplings. Weeks in  
800 which these samplings were conducted are displayed above the arrows. The two black  
801 arrows marked with asterisks (\*) depict samplings performed one week prior to the  
802 initiation of the primary vaccinations of 2011 and 2012, respectively, in order to detect  
803 and select WNV-seronegative horses.

804 **Fig. 2.** Neutralizing antibody (NAb) geometrical mean titers (GMTs) of 23 immunized  
805 horses which were not infected, against the Nea Santa-Greece-2010 lineage 2 strain (grey  
806 curve, ◆) as well as the PaAn001/France lineage 1 strain (black curve, ▲). SNTs were  
807 performed in sera collected from W0 (time of the first dose of the double primary  
808 vaccination in 2011) and until W52 (i.e., one month after the annual booster  
809 immunization). Paired *t*-test analysis revealed no significant differences in the NAb  
810 GMTs against the two antigens, at all sampling points ( $P > 0.28$ ). Range error bars  
811 encompass the range of the individual NAb titers against each antigen, and for every  
812 sampling time point. Geometrical mean titer ratio (GMTR) calculated for the annual  
813 booster against each strain is also presented.

814 **Fig. 3.** Alignment of the E protein amino acid sequences from the vaccine lineage 1 strain  
815 “New York 1999/VM2” (GenBank Acc. No. AF260967), the circulating lineage 2 strain  
816 “Nea Santa-Greece-2010” (GenBank Acc. No. HQ537483), and the South African  
817 lineage 2 strain “SA93/01” (GenBank Acc. No. EF429198) of West Nile virus. Dots  
818 indicate amino acid identities. The domains are indicated by bars, as explained at the  
819 figure legend. Investigation of the immunological similarity between the three peptides  
820 revealed no differences between the two lineage 2 strains. Comparison of the lineage 1  
821 (vaccine) and the lineage 2 peptide sequences of the E protein revealed 23 amino acid  
822 substitutions.



823 **Table 1.** Numbers of immunized and control horses which were included in the efficacy  
 824 study during 2011 and 2012. Numbers and infection rates of horses per year, as well as  
 825 numbers of horses which exhibited neurological signs due to WNV infection are also  
 826 included in the table.

Horse group	2011		2012		
	IH (WNV-seronegative, primo-vaccinated)	CH (WNV-seronegative)	IH (WNV-seronegative, primo-vaccinated)	IH (primo-vaccinated in 2011 but not infected, received annual booster vaccine dose in 2012)	CH (WNV-seronegative of 2011 but not infected + WNV-seronegative, selected in 2012)
No. of horses per group	85	33	55	52	33 (21+12)
No. (%) of WNV naturally infected horses determined by cELISA and/or SNT	32/85 (38%)	12/33 (36%)	14/55 (26%)	ID: 15/52 (29%)	9/33 (27%)
No. (%) of WNV naturally infected horses with clinical signs, confirmed by MAC-ELISA	0/32 (0%)	1/12 (8%)	0/14 (0%)	0/15 (0%)	2/9 (22%)
Total No. (%) of infected horses per year	44/118 (37%)		38/140 (27%)		

827 IH, immunized horses; CH, control horses; cELISA, competitive enzyme-linked  
 828 immunosorbent assay; SNT, serum neutralization test; ; MAC-ELISA, IgM antibody  
 829 capture enzyme-linked immunosorbent assay; ID, indirect determination

830