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Running title

Horse WNV vaccine cross-protection field evaluation

Abstract

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

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

Keywords:

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

Introduction

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

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

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

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

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

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

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

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

Materials and methods

Animals

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

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

Vaccine and immunization plan

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

During June-July 2011 an initial double primary vaccination (two doses administrated 3 weeks apart) of 85 horses was performed (Fig. 1), while 33 horses were used so as to form the control group (Table 1). During May-June 2012, 79 of the aforementioned vaccinated animals received an annual booster immunization dose of the vaccine. Six of the original 85 horses were excluded during the second year for various reasons, e.g. they were moved out of the study area, or were euthanized due to causes irrelevant to WNV-infection. In addition, in May-June 2012, another 55 horses which were seronegative to WNV received a double immunization with the vaccine (Fig. 1). During this period, 21 of the 2011's control animals which were determined as seronegative were kept, and along with 12 additional seronegative horses, were used as naïve controls for the 2012 epidemic period (Table 1). The total number of control horses (n = 45) was intentionally limited to approximately 33% of the total number of horses used in the study for humane reasons. In each participating horse riding club the 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 × *g*, 10 min, 4 °C).
215 Sera were transferred to clear 2 ml microcentrifuge tubes and stored at -80 °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® 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 $TCID_{50}$ (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, 2×10^4 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 ≥1:10.

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

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

For the confirmation of the diagnosis in control horses with clinical signs, the collected serum samples were tested for the presence of WNV-specific IgM antibodies, using a commercially available IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA; IgM WNV Ab Test, IDEXX-Istitut Pourquier, Montpellier, France), following the manufacturer's instructions. Furthermore, RNA was extracted from the sera obtained of the horses with neurological signs using the NucleoSpin® RNA virus kit (Macherey-Nagel, Düren, Germany. Extracts were examined using a WNV-specific, one tube real-time RT-PCR, using the primer pair WNPolUp (5'-TTTTGGGAGATGGTGGATGARGA-3') and WNPolDo2 (5'-CCACATGAACCAWATGGCTCTGC-3'), at final concentration of 0.6 μM each, and the TaqMan probe WNPolProb2 (5'-FAM-TCTCTCTCTTTCCCATCATGTTGT-ZNA5-BHQ1-3' at a final concentration of 0.2 μM) targeting a 144 bp part of the nonstructural protein 5 (NS5) genomic region of WNV. The limit of detection was previously determined to be 1 $TCID_{50}$/ml (40). Amplification reactions were run in a

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

Statistical analysis

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

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

$$OR = \frac{a}{b} \Big/ \frac{c}{d} \qquad [1]$$

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

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

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

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

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

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

305 where *Y* = presence or absence of clinical signs for the l^{th} animal, μ = 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.

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

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

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

Animal ethics

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

Results

WNV circulation in the study area

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

WNV natural infections in control and immunized horses

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

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

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

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

Vaccine safety

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

Neutralizing antibody responses in immunized horses

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

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

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

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

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

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

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

More specifically, during the 2011 epidemic period, clinical signs were detected in one out of the 12 seroconverted control animals (August 13). During the 2012 epidemic period, 2 out of the 9 seroconverted horses of the control group showed clinical signs (August 25 and September 8, respectively). These signs included fever, weakness of hind limbs, ataxia, muscle twitching and tremors in all 3 affected animals. Diagnosis was confirmed, as WNV-specific IgM antibodies were detected in all of them by MAC-ELISA testing. WNV RNA was not detected (no Ct values obtained). Consequently, it was not possible to detect the virus in the obtained blood sera, since they were drawn 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±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±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; $L_{131}Q$, $V_{159}I$, and $A_{172}S$), 15 substitutions were identified in DII ($E_{55}D$, $T_{64}S$, $K_{71}R$,
479 $D_{83}E$, $R_{93}K$, $S_{122}T$, $I_{126}T$, $R_{128}W$, $T_{129}I$, $N_{199}S$, $T_{205}S$, $T_{208}A$, $T_{210}S$, $V_{232}T$, and $I_{253}V$), 2
480 substitutions were present in DIII ($L_{312}A$, and $A_{369}S$), and 3 more substitutions were
481 found in the transmembrane domain/stem region ($K_{413}R$, $V_{442}I$, and $L_{483}M$) (Fig. 3).

482

483 **Discussion**

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

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

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

WNV E protein is a major determinant of tropism and the primary target of NAbs. Neutralizing epitopes have been identified mainly on DIII of E protein, and specifically, on residues 306, 307, 330 and 332 (56-58). Additional neutralizing epitopes have been identified on several residues of DI and DII, although the observed neutralizing activity for these regions is weaker (59). In our case, no changes were observed at residues S_{306},

K_{307}, T_{330} and T_{332}, which serve as major DIII neutralizing epitopes (56-58). However escape from neutralization has been associated with the $L_{312}A$ substitution, which was present in "Nea Santa-Greece-2010", as it is in several WNV strains (60). No changes were observed in residues W_{101}, G_{106} and L_{107}, antigenic sites of the fusion loop located within DII (DII-FL, residues 98-109), which act as target of cross-NAbs among different species of the genus *Flavivirus* (61-63). Despite the $L_{312}A$ substitution, the findings of the present study ultimately suggest that under field conditions, adequate cross-neutralization is capable of providing a high degree of protection.

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

Conflict of interest

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References

1. Zeller HG, Schuffenecker I. 2004. West Nile Virus: An Overview of Its Spread in Europe and the Mediterranean Basin in Contrast to its Spread in the Americas. Eur. J. Clin. Microbiol. Infect. Dis. 23:147–56.

2. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. 2005. Epidemiology and transmission dynamics of West Nile virus disease. Emerg. Infect. Dis. 11:1167–73.

3. Rappole JH, Hubálek Z. 2003. Migratory birds and West Nile virus. J. Appl. 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.

11. Weese JS, Baird JD, DeLay J, Kenney DG, Staempfli HR, Viel L, Parent J, Smith-Maxie L, Poma R. 2003. West Nile virus encephalomyelitis in horses in Ontario: 28 cases. Can. Vet. J. 44:469–73.

12. Venter M, Human S, Zaayman D, Gerdes GH, Williams J, Steyl J, Leman PA, Paweska JT, Setzkorn H, Rous G, Murray S, Parker R, Donnellan C, Swanepoel R. 2009. Lineage 2 West Nile virus as cause of fatal neurologic disease in horses, South Africa. Emerg. Infect. Dis. 15:877–84.

13. Kutasi O, Bakonyi T, Lecollinet S, Biksi I, Ferenczi E, Bahuon C, Sardi S, Zientara S, Szenci O. 2011. Equine encephalomyelitis outbreak caused by a genetic lineage 2 West Nile virus in Hungary. J. Vet. Intern. Med. 25:586–91.

14. Vazquez A, Sánchez-Seco MP, Ruiz S, Molero F, Hernández L, Moreno J, Magallanes A, Tejedor CG, Tenorio A. 2010. Putative new lineage of West Nile virus, Spain. Emerg. Infect. Dis. 16:549–52.

15. Bakonyi T, Ivanics E, Erdélyi K, Ursu K, Ferenczi E, Weissenböck H, Nowotny N. 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg. Infect. Dis. 12:618–23.

16. Calistri P, Giovannini A, Hubalek Z, Ionescu A, Monaco F, Savini G, Lelli R. 2010. Epidemiology of West Nile in Europe and in the Mediterranean basin. Open Virol. J. 4:29–37.

17. Murgue B, Zeller H, Deubel V. 2002. The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. Curr. Top. Microbiol. Immunol. 267:195–221.

18. Savini G, Capelli G, Monaco F, Polci A, Russo F, Di Gennaro A, Marini V, Teodori L, Montarsi F, Pinoni C, Pisciella M, Terregino C, Marangon S, Capua I, Lelli R. 2012. Evidence of West Nile virus lineage 2 circulation in Northern Italy. Vet. Microbiol. 158:267–73.

19. Marka A, Diamantidis A, Papa A, Valiakos G, Chaintoutis SC, Doukas D, Tserkezou P, Giannakopoulos A, Papaspyropoulos K, Patsoula E, Badieritakis E, Baka A, Tseroni M, Pervanidou D, Papadopoulos NT, Koliopoulos G, Tontis D, Dovas CI, Billinis C, Tsakris A, Kremastinou J, Hadjichristodoulou C. 2013. West Nile Virus State of the Art Report of MALWEST Project. Int. J. Environ. Res. Public Health 10:6534–610.

20. Hellenic Center for Disease Control and Prevention (HCDCP). Report on West Nile Virus Epidemic; 2013. http://goo.gl/lsJaeC [cited 2015 March 15].

21. Chaintoutis SC, Gewehr S, Danis K, Kalaitzopoulou S, Antalis V, Papanastassopoulou M, Mourelatos S, Panagiotopoulos T, Hadjichristodoulou C, Dovas CI. 2014. Surveillance and early warning of West Nile virus lineage 2 using domestic pigeons and backyard chickens. Proceedings of the 19th Congress of the European Society for Vector Ecology.

22. Turell MJ, Bunning M, Ludwig GV, Ortman B, Chang J, Speaker T, Spielman A, McLean R, Komar N, Gates R, McNamara T, Creekmore T, Farley L, Mitchell CJ. 2003. DNA vaccine for West Nile virus infection in fish crows (*Corvus ossifragus*). Emerg. Infect. Dis. 9:1077–81.

23. Bunning ML, Fox PE, Bowen RA, Komar N, Chang G-JJ, Speaker TJ, Stephens MR, Nemeth N, Panella NA, Langevin SA, Gordy P, Teehee M, Bright PR, Turell MJ. 2007. DNA vaccination of the American crow (*Corvus brachyrhynchos*) provides partial protection against lethal challenge with West Nile virus. Avian Dis. 51:573–7.

24. Chang G-JJ, Davis BS, Stringfield C, Lutz C. 2007. Prospective immunization of the endangered California condors (*Gymnogyps californianus*) protects this 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, Leparc-
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_-

_Product_Information/veterinary/000137/WC500063683.pdf [cited 2015 March 15].

34. European Medicines Agency (EMA). EPAR Summary for the public Proteq West Nile http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/veterinary/002005/WC500110366.pdf [cited 2015 March 15].

35. Minke JM, Siger L, Cupillard L, Powers B, Bakonyi T, Boyum S, Nowotny N, Bowen R. 2011. Protection provided by a recombinant ALVAC®-WNV vaccine expressing the prM/E genes of a lineage 1 strain of WNV against a virulent challenge with a lineage 2 strain. Vaccine 29:4608–12.

36. Venter M, van Vuren PJ, Mentoor J, Paweska J, Williams J. 2013. Inactivated West Nile Virus (WNV) vaccine, Duvaxyn WNV, protects against a highly neuroinvasive lineage 2 WNV strain in mice. Vaccine 31:3856–62.

37. Bowen RA, Bosco-Lauth A, Syvrud K, Thomas A, Meinert TR, Ludlow DR, Cook C, Salt J, Ons E. 2014. Protection of horses from West Nile virus Lineage 2 challenge following immunization with a whole, inactivated WNV lineage 1 vaccine. Vaccine 32:5455–9.

38. Styer LM, Bernard KA, Kramer LD. 2006. Enhanced early West Nile virus infection in young chickens infected by mosquito bite: effect of viral dose. Am J Trop Med Hyg. 75:337-45.

39. Chaintoutis SC, Dovas CI, Papanastassopoulou M, Gewehr S, Danis K, Beck C, Lecollinet S, Antalis V, Kalaitzopoulou S, Panagiotopoulos T, Mourelatos S, Zientara S, Papadopoulos O. 2014. Evaluation of a West Nile virus surveillance and early warning system in Greece, based on domestic pigeons. Comp. Immunol. 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-
731 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, Filipski 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 Porotein. 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, Coulpier 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.

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

	2011		2012		
Horse group	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 cinical 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%)		

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