Immunological response in horses following West Nile virus vaccination with inactivated or recombinant vaccine

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Veterinaria Italiana 2019, **55** (1), 73-79. doi: 10.12834/VetIt.1820.9611.1
Accepted: 24.10.2018 | Available on line: 31.03.2019

Keywords
West Nile virus,
Vaccine,
Horses,
Immunological response.

Summary
To evaluate the immunological response following vaccination, 40 WNV serologically negative horses were selected and divided in two groups of 20 animals. One group was vaccinated (booster after 28 days) with a whole inactivated viral strain and the second group with a live recombinant canarypox virus expressing the genes coding for the WNV preM/E viral proteins. IgM, IgG and neutralizing antibodies were monitored by class specific ELISAs and serum neutralization assay for 360 days. In both groups, IgM antibodies were first detected 7 days post vaccination (dpv). However, in the group vaccinated with inactivated vaccine, IgM antibodies were detected until day 42 pv, whereas in the group vaccinated with the recombinant vaccine, they were detected up to day 52 pv. A similar ($P > 0.05$) proportion of horses showed IgM antibodies after vaccination with either recombinant [30%; 95% confidence interval (CI): 14.59%-52.18%] or inactivated (32%; 95% CI: 15.39-54.28%) vaccine. Both vaccines induced in vaccinated horses a detectable IgG antibody response starting from day 7 pv and lasting till the end of the trial. Analogously, both products elicited WNV specific neutralizing antibodies. The response induced by the live canarypox virus-vectored vaccine was higher (mean titres 1:298 vs 1:18.9) and lasted longer than did that induced by the killed-virus vaccines. In fact, one year after the vaccination, neutralizing antibodies were still detectable in the horses which received the canarypox virus-based vaccine but not in the group vaccinated with the killed product. The use of vaccines is a valuable tool to prevent WNV disease in horses and the availability of different products facilitates the control of the disease in endemic areas.

Risposta immunitaria in cavalli vaccinati per la febbre del Nilo occidentale con vaccino inattivato o ricombinante

Parole chiave
Cavalli,
Risposta immunologica,
Vaccino,
Virus della West Nile.

Riassunto
Per valutare la risposta immunitaria alla vaccinazione nei confronti della febbre del Nilo occidentale sono stati scelti quaranta cavalli sierologicamente negativi al virus della West Nile (WNV) e divisi in due gruppi da venti unità. Un gruppo è stato vaccinato (richiamo dopo 28 giorni) con un vaccino inattivato e il secondo gruppo con un vaccino vivo contenente il virus del vaiolo del canarino, ricombinante per WNV, che esprime i geni codificanti per le proteine virali preM/E del virus della West Nile. Per 360 giorni IgM, IgG e anticorpi neutralizzanti sono stati monitorati con ELISA specifiche per classe e test di sieroneutralizzazione. In entrambi i gruppi, gli anticorpi IgM sono stati rilevati per la prima volta 7 giorni dopo la vaccinazione (gdv). Nel gruppo vaccinato con il prodotto inattivato (K-WNV), le IgM sono state rilevate fino a 42 gdv mentre, in quello vaccinato con prodotto ricombinante (Rec-WNV), fino a 52 gdv. Un numero simile ($P > 0.05$) di cavalli di entrambi i gruppi sono risultati positivi all'ELISA IgM dopo vaccinazione. Entrambi i vaccini hanno indotto una risposta anticorpale IgG rilevabile a partire dal 7° gdv fino alla fine dello studio; entrambi i prodotti hanno stimolato la produzione di anticorpi neutralizzanti. Tuttavia, i titoli anticorpali rilevati nel gruppo vaccinato con Rec WNV (titoli medi 1: 298) sono stati più elevati e duraturi rispetto a quelli rilevati nel gruppo vaccinato con K WNV

(titoli medi 1: 18,9). A un anno dalla vaccinazione infatti, questi erano ancora rilevabili solo nei cavalli vaccinati con Rec WNV. I vaccini sono uno strumento prezioso per prevenire la malattia del WNV nei cavalli e la disponibilità di diversi prodotti facilita il controllo della malattia nelle aree endemiche.

Introduction

West Nile virus (WNV) is a member of the *Flavivirus* genus family *Flaviviridae*. Included in the Japanese encephalitis group, it is one of the most widespread arbovirus in the world. In Europe, WNV has been reporting since the mid 1960s (Filipe *et al.* 1969, Joubert *et al.* 1970) but the virus circulation has increased dramatically in the last decades (Calistri *et al.* 2010, Savini *et al.* 2018). In Italy, WNV epidemics caused by genetically divergent isolates have been recorded since 2008 (Savini *et al.* 2008, Monaco *et al.* 2010, Savini *et al.* 2012) and most of the territory is nowadays endemic.

The infection is maintained in nature in a mosquito-bird cycle involving *Culex* species of ornitophilic mosquitoes as main vectors and an extensive variety of birds as reservoir hosts (Komar *et al.* 2001, Mancini *et al.* 2017). Mammals including humans and equidae are susceptible to the infection and can show clinical symptoms ranging from a flu-like syndrome to a fatal meningoencephalitis (Komar 2000, Debiasi and Tyler 2006).

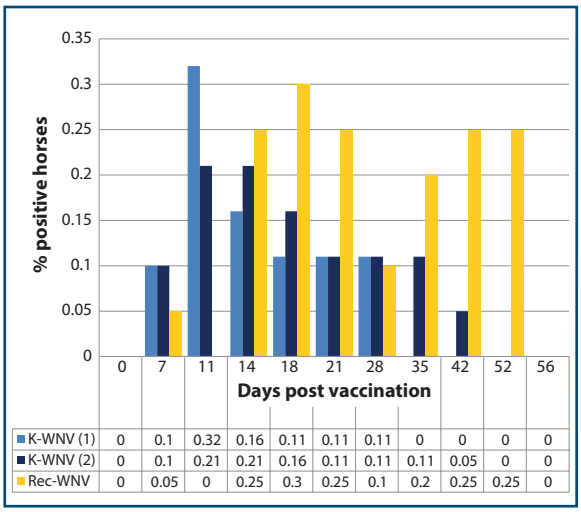
Because of the low grade of viraemia and the lack of viral shedding, the virus cannot be transferred further and an infected horse acts as a dead-end host (Bunning *et al.* 2002). Nevertheless, the development of the severe clinical symptoms might raise devastating emotional effects and significant financial burden to owners. There is no specific anti-viral treatment for the disease and prevention can be achieved by minimizing the exposure to the vector or, in equines, through vaccination (Amanna and Slifka 2014).

To date, three vaccines have obtained the marketing authorization in European Union (EU) member countries: the inactivated vaccine, produced from the VM-2 strain (Equip® WNV, Zoetis, Belgium previously Duvaxyn® WNV, Pfizer, US) (EMEA 2008), the recombinant canarypox virus vCP2017 strain, that expresses the WNV prM/prE genes (Recombitek equine WNV vaccine, Merial) (EMEA 2011) and the inactivated chimaeric flavivirus strain of Yellow fever virus presenting the genes for the structural proteins E and prM of WNV (Equilis® West Nile, Intervet International BV, Netherlands) (EMEA 2013). These products are capable of protecting horses against possible development of clinical manifestations of the infection which can lead to a severe and long lasting neurological impairment of the animal. Aim of this study was to look into the dynamic of the serological response in horses vaccinated with two different products widely used in EU member countries to protect horse population: the inactivated vaccine (Equip® WNV) and the recombinant canarypox virus expressing the WNV prM/prE genes (Recombitek equine WNV).

Materials and methods

Forty healthy horses, serologically negative to WNV, were randomly selected and divided into two groups of twenty animals. One group was vaccinated with the inactivate WNV strain, the other with the recombinant canarypox expressing the WNV prM/prE genes. Both groups received a booster shot 28 days after the first dose.

Following the vaccination, all horses were regularly bled up to 1 year to monitor the immune response. To this purpose, blood samples were collected by



	0	7	11	14	18	21	28	35	42	52	56
K-WNV (1)	0	0.1	0.32	0.16	0.11	0.11	0.11	0	0	0	0
K-WNV (2)	0	0.1	0.21	0.21	0.16	0.11	0.11	0.11	0.05	0	0
Rec-WNV	0	0.05	0	0.25	0.3	0.25	0.1	0.2	0.25	0.25	0

Figure 1. *Detection of IgM antibodies in 2 groups of horses (n = 20) vaccinated either with an inactivated West Nile virus vaccine (K-WNV) or with a recombinant canarypox WNV vaccine (Rec-WNV).*
K-WNV (1) and Rec-WNV = IgM response detected by 'West Nile Virus IgM Antibody' Test (IDEXX Laboratories, Inc., Maine, US).
K-WNV (2) = IgM response detected by ID Screen West Nile IgM Capture (IDvet, Grabels, France).

jugular venipuncture in dry tubes at the indicated time points (Figure 1). Sera were separated and stored at -20 °C until analysis. A commercial ELISA kit (ID Screen West Nile Competition Multi-species - ID-Vet, Montpellier, France) was used to detect IgG immunoglobulins while the West Nile Virus IgM Antibody ELISA kit (IDEXX Laboratories, Inc., Maine, US) to evaluate the IgM response. IgM response in the group of animals receiving the recombinant product was also assessed by the ID Screen West Nile IgM Capture (IDvet, Grabels, France). The tests were performed according to the manufacturer's instructions. To confirm the presence of WNV antibodies, to define their titers and to exclude any cross reaction with other co-circulating related flaviviruses such as Usutu (Savini *et al.* 2011), serum samples were also examined by serum neutralization (SN) assay in microtitre format (Di Gennaro *et al.* 2014). For each proportion, 95% confidence intervals (CI) were calculated through the Bayesian approach using the Beta (s+1, n-s+1) distribution where s is the total number of positives and n is the total number of tested animals.

Any WNV circulation in the area where the animals were kept was investigated according to the procedure defined in the WNV National Surveillance Plan (Italian Ministry of Health, 2016[1]).

Results

No adverse effects were observed in the animals following vaccination. IgM antibodies were detected in both groups of vaccinated animals (Figure 1). A similar ($P > 0.05$) proportion of horses developed IgM antibodies after vaccination with either the recombinant vaccine (Rec-WNV) [30%; 95% Confidence interval (CI): 14.59%-52.18%] or the inactivated vaccine (K-WNV) (32%; 95% CI: 15.39-54.28%). In both groups, IgM antibodies were first detected after 7 days. However, in the group vaccinated with the inactivated product, the IgM antibodies were detected up to 42 dpv (in 1 animal) while in the group immunized with the recombinant product IgM antibodies were detected up to 52 dpv (in 5 animals) (Figure 1). Although in the K-WNV group the IDEXX ELISA was capable of detecting a higher percentage of positive animals (32%; 95% CI: 15.39-54.28%) than the ID-VET kit (21%; 95% CI: 8.66-43.66%), this difference was not statistically significant ($P > 0.05$) (Figure 1).

Both vaccines induced a detectable IgG response as early as 7 days following the vaccinations whatever the vaccine used. In the K-WNV group, all animals seroconverted on day 18 pv while in the Rec-WNV group, all the animals became IgG positive following the booster injection (on day 35 pv). IgG antibodies were detected in the animals of both groups until the end of the trial (one year after vaccination).

In the group vaccinated with the modified canarypox strain, neutralizing antibodies were first detected on day 18 pv in 1 horse. From day 42 pv till the end of the trial, neutralizing titers were detected in all vaccinated animals. The peak was observed on day 56 pv (mean antibody titer = 1:298) (Figure 2). In contrast, not all the animals vaccinated with the killed WNV strain developed a neutralizing response following vaccination. In fact, neutralizing titers were first detected on day 21 pv and, 3 weeks later (day 42 pv), all except one animal seroconverted. After peaking on day 42 (mean antibody titer = 1:18.9), the neutralizing titers decreased and, at the end of the trial, only 4 animals showed detectable titers (1:5) (Figure 2).

None of the animals in the trial developed neutralizing antibodies against Usutu virus nor WNV circulation was detected in the area during the study period.

Discussion

In accordance with the Directive 2003/99/EC on monitoring of zoonoses and zoonotic agents, European Member States collect data in order to define hazards, to evaluate exposures and to assess risks related to zoonoses and zoonotic agents. As a consequence, Member States have implemented surveillance programs and, since 2012, they have been collecting data and reporting cases of West Nile disease (WND). In Austria, France, Greece, Italy, and United Kingdom, an integrated

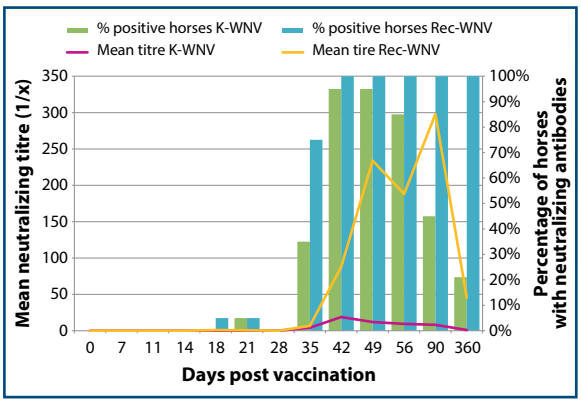


Figure 2. *Mean titres and percentage of animals with detectable neutralizing antibodies in two groups of horses following vaccination against the West Nile virus (WNV) with an inactivated (K-WNV) or a recombinant canaripox WNV vaccine (Rec-WNV).*

[1] Italian Ministry of Health. 2016. Piano Nazionale integrato di sorveglianza e risposta al virus della West Nile. Circolare 10/08/2016, n. 23689.

animal-human-vector approach is already in place (Gossner *et al.* 2017). Veterinary surveillance usually relies on the combination of passive measures, based on reporting WND clinical cases in horses, and active surveillance, based on detecting seroconversion in sentinel horses (Humblet *et al.* 2016). The serological tests most commonly used are ELISAs (for detection of IgG and IgM antibodies) as screening test and VNT as confirmatory test. The humoral response following WNV infection includes the production of IgM antibodies, which are detectable 4-7 days after infection, and of IgG, detectable 5 to 7 days after infection (Bunning *et al.* 2002). As the IgM lifespan is considered to be less than three months in horses, the presence of IgM antibodies in this species is regarded as a valuable indicator of recent infections (Castillo-Olivares and Wood 2004) and, as a consequence, the WNV IgM ELISA is considered the test of choice for diagnosis of recent infection.

It is also claimed that vaccination only occasionally elicits an IgM response making it possible to differentiate acutely-infected from recently vaccinated horses by using an IgM-based ELISA (EMEA 2008). The possibility to differentiate a recently infected horse from one that has been (recently) vaccinated is particularly useful to early identify viral circulation either in WNV-free or endemic areas. In this study, WNV recombinant and killed vaccines were examined and even if the IgM response of the inactivated vaccine was lower, shorter and involving fewer animals, both products were capable of stimulating the production of IgM antibodies in vaccinated horses. These results were in line with what found in similar trials by other authors (Porter *et al.* 2004, Jonquiere *et al.* 2011, Joò *et al.* 2017). Surprisingly, in few animals vaccinated with the recombinant vaccine, the IgM antibodies persisted for 52 days after vaccination showing a kinetic similar to that observed in naturally infected horses in which IgM antibodies can be detected 3 months after infection (Ostlund *et al.* 2000). This study confirmed, once more, how difficult is to predict the IgM kinetic following vaccination suggesting that, in endemic areas or within a WNV surveillance plans, it is not possible to differentiate infected horses from recently vaccinated horses based on the presence/absence of IgM antibodies. As a consequence, positive IgM results should be carefully interpreted by verifying the vaccination history of the horses especially when vaccination against WNV is commonly practiced. When dealing with WNV IgM antibodies, particular attention should also be paid to the IgM ELISAs used since variations may exist in the IgM ELISA performances which often demonstrate different diagnostic sensitivity and potential for false positive results (Davidson *et al.* 2005, Beck *et al.* 2017). In this study, although the number of positive animals detected by the two ELISAs after the vaccination with the inactivated product was different, this difference was not statistically significant.

Both vaccines were capable of evoking an IgG response in all vaccinated animals even if differences between groups were observed in the timeframe elapsed to stimulate the complete seroconversion. All horses immunized with the inactivated product developed a detectable IgG response starting from the 18th dpv while 35 days were necessary to achieve the complete seroconversion in the group which received the recombinant vaccine. Such difference may rely on the different nature of the two vaccines. The viral antigens are *'*ready to be used*'* in the inactivated vaccines, while, with the recombinant product, the same antigens need to be expressed by the vaccinated host. Differences were also noted in the duration and intensity of the neutralizing antibody response elicited by the two vaccines. Both vaccines induced the rise in neutralizing antibodies as observed by other authors (Davies *et al.* 2008, Joò *et al.* 2017, Seino *et al.* 2007) even though neutralizing antibody responses induced by the live canarypox virus-vectored vaccine were higher and lasted longer than did those induced by the killed-virus vaccines. One year after the vaccination, neutralizing antibodies were still detectable in the horses which received the canarypox virus-based vaccine but not in the group vaccinated with the killed product. Long-term immunity is not a characteristic of inactivated vaccines, and field studies demonstrated the drop of neutralizing titers 5 to 7 months after vaccination (Davidson *et al.* 2005). In our K-WNV group of horses, the presence of neutralizing antibodies was detected only in four animals with titers below the threshold (< 1:10).

It is known that the protective immune response to WNV requires both innate and adaptive immunity (De Filette *et al.* 2012) and there is strong evidence that neutralizing antibodies provide long-term protection from clinical signs of the disease (Pierson and Diamond 2015). Different neutralizing responses however do not necessarily reflect dissimilarities in the protective capacity induced by the two vaccines. Many trials demonstrated that the level of neutralizing antibody titer is not predictive of protective immunity in horses or hamsters (Tesh *et al.* 2002, Seino *et al.* 2007) since protection may occur in the absence of detectable antibodies. The requirements of significant levels of neutralizing antibodies at the time of exposure may not be critical as long as vaccination properly primes the immune system and response is rapid (Minke *et al.* 2004).

The absence or scarce level of humoral response

does not preclude the efficacy of the cellular response. In fact, the role of cell mediated immunity in protecting against WNV and other related flaviviruses has been demonstrated in experimental murine studies (Diamond *et al.* 2003, Shrestha and Diamond 2004). The capacity of flavivirus infection to induce both innate and adaptive immune response is crucial to prevent the dissemination of these viruses in the organism (Diamond 2003). Effective vaccine-based protection requires both responses and if the humoral has been frequently reported, the cell-mediated response is still poorly explored (Nelson *et al.* 2010).

The use of vaccines has been demonstrated as a valuable preventative strategy against WND in horses (Grosenbaugh *et al.* 2004, Siger *et al.* 2004, Joò *et al.* 2017). The 2018 has been regarded as an exceptional year referring to WNV circulation in EU countries (Haussig *et al.* 2018, Riccardo *et al.* 2018) with the infection of a high number of horses even in endemic areas (https://westnile.izs.it/j6_wnd/docBolletMeditPeriodico?annoDocumento=2018). Thus, the availability of different products provides a valuable tool to reduce the impact of the severe clinical symptoms in this species.

Funding

This work was supported by the Italian Ministry of Health (MSRCSI 01, 2012). Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the OIE Reference Laboratory for West Nile Fever of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Teramo, Italy.

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