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West Nile Virus Vaccination in Horses – IgM and IgG responses after injection in different muscles

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Abstract

West Nile Virus (WNV) may cause significant morbidity and mortality in birds, humans and horses. WNV is not (yet) present in the Netherlands, but it is steadily approaching from south-eastern Europe. Recently, a WNV-vaccine (Duvaxyn®-WNV) became available in Europe. It is claimed that vaccination results not or only infrequently in an IgM response making it possible to differentiate acutely-infected horses from vaccinated horses by using an IgM-based ELISA. The aims of the study were to investigate the supposition that vaccination does not result in IgM production, to evaluate whether different intramuscular injection sites influence the immunological responses and whether any local or systemic adverse reactions would occur. Twenty horses and ponies, 3 to 21 years old, were divided into four groups and horses of each group were vaccinated twice (day 0 and 21) at different intramuscular sites (neck, pectoral muscles, rump and thigh). Weekly blood samples were collected over a period of 42 days and tested for Flavivirus IgM antibodies using a WNV-IgM ELISA and for Flavivirus Ig antibodies using a WNV blocking ELISA. None of the horses tested positive for WNV antibodies prior to vaccination. All horses showed a clear Ig (total antibody) response to the WNV vaccination, but in two horses this response was limited. Surprisingly, ten horses also gave a (limited) positive IgM response. This suggests that an IgM capture ELISA will not distinguish horses with an acute WNV infections from recently vaccinated horses with certainty. The location of the intramuscular injections had no significant effect on the immunogenic response. No systemic reactions were encountered nor were there local reactions at any of the injection sites.

Keywords: West Nile Virus, horses, vaccination response, ELISA

Das West Nile Virus(WNV) kann eine erhebliche Morbidität und Mortalität bei Vögeln, Menschen und Pferden verursachen. Bis jetzt existiert dieses Virus in den Niederlanden nicht, allerdings breitet es sich von Süd-Ost-Europa stetig aus. Seit kurzem ist in Europa eine WNV-Vakzine (Duvaxyn-WNV) erhältlich. Man geht davon aus, dass die Impfung nicht oder nur selten zu einer IgM-Antwort führt und somit eine Unterscheidung zwischen geimpften Pferden und akut infizierten Tieren durch einen die IgM-Konzentration erfassenden ELISA möglich ist. Die Ziele der Studie waren die Überprüfung der Hypothese, ob die Impfung keine Produktion von IgM hervorruft, die Beurteilung, ob unterschiedliche Injektionslokalisationen die immunologische Antwort beeinflussen und ob lokale oder systemische Nebenwirkungen auftreten. 20 Pferde und Ponies, in einem Alter von 3-21 Jahren wurden in vier Gruppen eingeteilt und die Pferde jeder Gruppe zweimalig im Abstand von 21 Tagen an unterschiedlichen Lokalisationen intramuskulär (Hals, Pectoralismuskel, Glutealmuskel, Oberschenkel) vakziniert. Über 42 Tage wurden wöchentlich Blutproben entnommen und zum einen mittels eines WNV-IgM-ELISA die Flavivirus-IgM-Antikörper bestimmt und zum anderen mittels einem das WNV-blockierenden ELISA die Flavivirus-Ig-Antikörper. Keines der Tiere wies vor der Vakzinierung WNV-Antikörper auf. Alle Pferde zeigten aufgrund der Impfung einen deutlichen Ig (Gesamt-Antikörper) Anstieg, allerdings war diese Reaktion bei zwei Pferden befristet. Überraschenderweise wiesen 10 Pferde eine positive IgM-Antwort auf. Daraus kann gefolgert werden, dass ein IgM erfassender ELISA nicht sicher zwischen Pferden mit einer akuten WNV-Infektion und geimpften Tieren differenzieren wird. Die Lokalisation der intramuskulären Injektion hatte keinen signifikanten Einfluss auf die immunologische Antwort. Es konnten weder systemische noch lokale Reaktionen unabhängig von der Lokalisation der Injektion erfasst werden.

Schlüsselwörter: West Nile Virus, Pferd, Immunantwort, ELISA, Impfreaktion

Introduction

West Nile Virus (WNV) is a positive stranded RNA virus belonging to the Flaviviridae. Arthropod-borne Flaviviruses are maintained in an enzootic cycle of birds and mammal hosts. The virus causes disease which is endemic in Africa, North America, South America and parts of Europe (*Castillo-Olivares* and *Wood* 2004). In USA the virus has been found in over 300 species of birds. Arthropods, such as mosquitos, act as vectors for WNV between avian and mammalian hosts. The Culex species are considered to be the most important group of vectors for WNV (*Shirafuji* et al. 2009), but numerous other mosquito species can also be infected. In the Netherlands at least four different mosquito species are potential vectors (*Reusken en Takken* 2006).

WNV replicates in avian species and insects, whereas mammals including horses and humans, are dead-end hosts (*Higgs* et al. 2004). In those species the multiplication of the virus is so low that arthropods are not infected when taking a blood meal (*Castillo-Olivares* and *Wood* 2004) and the transmission cycle ends.

The clinical symptoms in the dead-end hosts may, however, be severe. The clinical progression of WNV infection varies greatly, with symptoms varying from subclinical (asymptomatic), to a mild flu-like disease (West Nile Fever), to a severe form involving neurological symptoms with considerable mortality (*Weese* et al. 2003). The important complications of a WNV infection are meningo-encephalitis and other neurological symptoms such as acute paralysis or paresis (*Weese* et al. 2003, *Chu* et al. 2007). The severity of the disease is influenced by the age and physical condition of the host and by the specific virulence of the virus strain involved. In horses the asymptomatic state is the most common form. In humans fewer than 1% of the infected patients develop neurological symptoms (*Castillero-Olivares* and *Wood* 2004). However, over the last two decades there has been an increased incidence of the neurological form amongst both horses and

people (*Marfin* et al. 2001). In a virulent lineage 1 strain epidemic in the USA about a third of the affected horses developed the severe neurological form of the disease and either died or were euthanized. The common milder symptoms in horses include mild pyrexia, mild ataxia and lethargy. The symptoms associated with severe infections include acute or progressive ataxia, paralysis of the facial nerve, muscle fasciculation and seizures (*Castillero-Olivares* and *Wood* 2004).

West Nile Virus appeared in horses along the eastern seaboard of North America in 1999 and rapidly spread over the whole of the continent (*Malan* et al. 2004). More than 25,000 cases were reported with a case fatality rate of 30-40%. Nowadays, it is endemic in North America. Since 2008, cases of WNV infection have been reported in Europe: Italy, Rumania, Austria and Greece (ECDC website). It is possible that the disease will spread further over Europe in the (near) future (*Sloet* et al. 2009).

Several WNV vaccines for horses have been marketed in the USA. The first vaccine available in the USA in 2001-2002, was based on inactivated whole virus particles combined with a potent adjuvant. (West Nile Innovator® (Fort Dodge), In 2003 a vaccine based on recombinant DNA technology using canary-pox virus vector was marketed (Recombitek® West Nile, Merial). This was followed in 2006 by a live-chimeric vaccine (PriveNile®, Virbac) and in 2009 by another killed-virus vaccine (Vitera® West Nile - Boehringer Vetmedica). Most of these vaccines have provided good clinical protection against WNV infections without any significant adverse reactions being reported (*Seino* et al. 2007, *Long* et al 2007a, *Long* et al. 2007b). In November 2008, the first WNV vaccine (inactivated whole virus; Duvaxyn WNV®, Fort Dodge) was approved by the European Medicines Agency (EMEA) for European use. The product became available in the Netherlands during the summer of 2009.

The general assumption is that killed vaccines do not produce an IgM response that is typical of the acute field infection (CDC website). This is especially important when the vaccine is used in a WNV-free country as this assumption should make it possible using an IgM specific WNV ELISA to distinguish a horse with a WNV field infection from one that has been (recently) vaccinated. Further studies, like Long et al. (2006), also stated that detectable IgM rarely occurs following vaccination. On the other hand, *Porter* et al. (2004) found that 3 out of 20 horses that were vaccinated with WNV vaccine tested positive for WNV anti-IgM at 1:400 serum dilution. However, these authors stated that current anti-WNV vaccination protocols have minimal effect on testing serum when using MAC-ELISA (IgM Capture ELISA). *Davis* et al. (2008) noted seroconversion of IgG and IgM after a WNV vaccination protocol. In that study, serologic evidence of enhanced antigen-specific IgM expression was evident after completion of the vaccination protocol and was maintained for 14 weeks. From these conflicting results it is clear that it can not be assumed without further research that Duvaxyn WNV® does not produce IgM antibodies.

The main aim of the present study was to verify the assumption that the WNV vaccine will trigger a strong Ig response and no or a very poor IgM response. In addition the effect of different sites of injection on IgG and IgM antibody responses is examined, as well as whether vaccination induces any adverse reactions.

Materials and Methods

Horses

Twenty horses and ponies aged between 3 and 21 years old were used in the study, for breed and gender see Table 1. The

Table 1 Breed, age and gender of horses involved in the WNV vaccination study and the location of the intramuscular injection. (KWPN is a warm-blood horse of the Royal Studbook of the Netherlands)

nr	breed	sex	age	location of injection
1	Trakhener	gelding	3	rump
2	Arab	stallion	20	pectoral muscles
3	Welsh	mare	17	thigh
4	KWPN	mare	11	pectoral muscles
5	Thoroughbred	mare	4	pectoral muscles
6	KWPN	mare	16	pectoral muscles
7	Frysian	gelding	21	rump
8	Thoroughbred	mare	4	neck
9	KWPN	mare	8	neck
10	Friesian	gelding	9	thigh
11	KWPN	mare	6	neck
12	KWPN	mare	9	neck
13	unknown	mare	8	neck
14	Shetland pony	gelding	9	rump
15	Shetland pony	gelding	10	rump
16	Shetland pony	gelding	10	rump
17	Shetland pony	gelding	7	thigh
18	Shetland pony	gelding	12	thigh
19	Thoroughbred	gelding	18	pectoral muscles
20	KWPN	gelding	16	thigh

study was approved by the Ethical Commission for Animals (DEC) of Utrecht University.

Vaccination

On days 0 and 21 an intramuscular injection was administered with the licensed European vaccine (Duvaxyn WNV®, Fort Dodge) using a single dose of 1 mL per animal. The horses were at randomly divided in four groups and for each group a different injection site was used (neck, pectoral muscles, rump and thigh). On days 1, 2, 22, and 23, all horses were checked for local and/or systemic reactions such as appetite and behaviour changes, and the injection sites were inspected and palpated for evidence of oedema, inflammation and/or a pain reaction.

Blood sampling

For serological examinations twenty millilitres of blood was taken from the left or right jugular vein using a vacuum system. In total 6 sequential blood samples were collected, starting on day 0 just before the first vaccination, on days 7, 14, 21 (just before the second vaccination), and on days 28 and 42. Serum was collected by centrifugation (10 minutes at 6,000 rpm) and stored at -18°C until tested.

WNV ELISAs

All the samples were semi-quantitatively tested for antibodies against WNV using either a commercial competition/blocking ELISA detecting total Ig (ID-Vet, Montpellier, France) or an IgM-capture ELISA based on commercially available reagents (Hennessy Research, Shawnee, USA). The WNV IgM capture ELISA was performed according to the protocol provided with the reagents (Hennessy Research, Shawnee, USA). Shortly, ELISA plates were coated with a rabbit polyclonal antibody directed against horse IgM (Bethyl Laboratories Inc., Montgomery, USA). Subsequently, unbound coating sites were blocked using Bovine Serum Albumin. After washing, serum samples diluted 1:400 were added to duplicate wells and incubated. After further washing either recombinant WNV-antigen or control antigen was added and incubated. After another washing conjugate, which consisted of a monoclonal antibody directed against WNV, labelled with an enzyme, was added. After a final washing, a chromogenic substrate solution was added and incubated. The enzyme reaction was stopped and the optical densities (OD's) were measured using an ELISA reader. The difference between the OD of the WNV antigen well and the control antigen well was compared to a positive control sample. The positive to negative (P/N) ratio is proportional to the concentration of Flavivirus specific IgM antibodies present in the sample. For qualitative interpretations, a P/N-ratio higher than 2.0 was considered positive, while a P/N-ratio of 2.0 or lower was considered negative.

The WNV competition/blocking ELISA was performed according to the manufacturer's instructions (ID-VET, Montpellier, France). Shortly, samples were diluted 1:2 and added to the wells of ELISA plates coated with a recombinant WNV antigen. After incubation, plates were washed and a conjugate, consisting of a monoclonal antibody directed against WNV labelled with an enzyme was added and incubated. Then the plates were washed again and a chromogenic substrate solution was added. The enzyme reaction was stopped and the optical densities (OD's) were measured using an ELISA reader. The OD's were related to a negative control sample. The resulting sample to negative (S/N) percentage (S/N%) is inverse proportional to the concentration of Flavivirus specific Ig antibodies present in the sample. For qualitative interpretations, a sample with a S/N% less than 40% is considered positive, a sample with a S/N% ≥ 40 and <50% as doubtful, and a sample with a S/N% ≥ 50% as negative.

Data analysis

The results of the four different locations of intramuscular injection were analysed using an Anova method with the statistical program SPSS 17.0 (IBM).

Results

Before the first vaccination, none of the horses had any antibodies against WNV.

WNV Competition ELISA

In the competition ELISA, the average S/N% of the groups of horses vaccinated against WNV in different muscles sharply declined with time following vaccination on day 0 (Figure 1). At day 14 all group averages were below the cut-off level of the test; i.e. most samples were already positive. The horses that had received a croup injection had slightly higher average S/N% in comparison with the horses injected in other muscles on day 14 and 21. From day 28 onwards all samples were strongly positive. Individual S/N% showed that two horses (7 and 13) had a delayed response, while the response of horse 9 remained rather low. Upon retesting, these results proved to be reproducible (data not shown).

WNV IgM Capture ELISA

The average P/N-ratio of the horses showed an increase between day 0 (vaccination) and day 14 (Figure 2). The average P/N-ratio exceeded the cut-off level at day 7 and 14 (i.e. positive results). At the second vaccination (day 21) the average P/N-ratio had declined below the cut-off level, but this was followed by another increase in average P/N-ratio, which was again above the cut-off level at day 28, after which the mean antibody levels declined again. At day 42 the average P/N-ratio was below the cut-off level, but still higher than at day 0.

The sequential P/N-ratios of the 10 individual horses which had one or more positive results in the WNV IgM Capture ELISA are shown in Figure 3. In seven animals the P/N-ratios were temporarily weak-positive (just above the cut-off level). However, in three horses (numbers 2, 6, and 16) relatively high P/N-ratio's were found. The results were confirmed when the samples were retested (data not shown). In most of these 10 animals the P/N-ratio's increased on day 0 and day 14 after the first vaccination, followed by a decrease. A slightly lower increase and decrease was identified in some horses following the second vaccination at day 21. The exception was horse 2 that hardly responded to the first vaccination, but showed the highest P/N-ratios following the second vaccination.

Effect of the vaccination site on IgG and IgM antibody responses

The location of the vaccination site (neck, pectoral muscles, rump and thigh) had no significant effect on the antibody response to the vaccine.

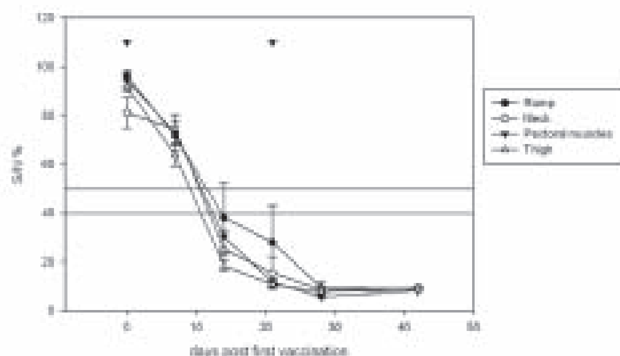

Fig. 1 Sequential results of the WNV competition ELISA per location of the injection sites of horses vaccinated against WNV on day 0 and 21. Means and standard errors of the mean (S.E.M.) are indicated. The horizontal lines indicate the test cut-off (<40% positive, 40-50% doubtful, >50% negative). The black triangles on day 0 and 21 mark the vaccination times.

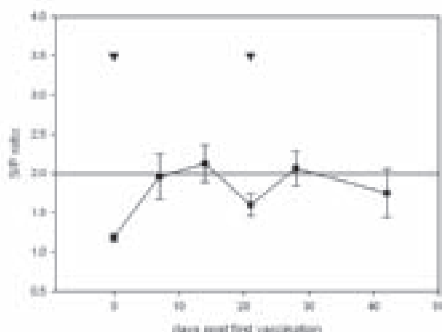

Fig. 2 Sequential average results of the WNV IgM capture ELISA of horses vaccinated against WNV on day 0 and 21. The standard error of the mean (S.E.M.) is indicated. The horizontal line indicates the test cut-off (<2.0 negative, >2.0 positive). The black triangles on day 0 and 21 mark the vaccination times.

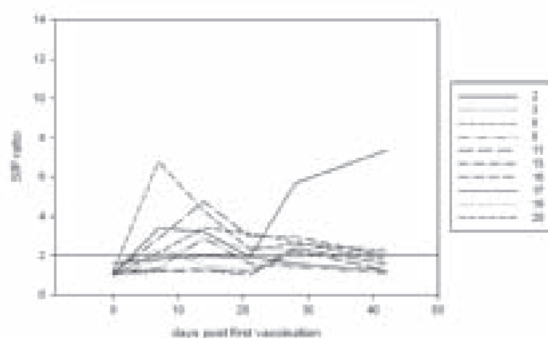

Fig. 3 Sequential individual results of the 10 horses vaccinated against WNV on day 0 and 21 that scored positive in the WNV IgM capture ELISA at some point in time. The horizontal line indicates the test cut-off (<2.0 negative, >2.0 positive).

Vaccination reactions

None of the vaccinated horses showed any detectable systemic reaction and neither was there any significant local reaction at the injection sites.

Discussion

As expected in a WNV free country, all horses were negative for antibodies against WNV before the first vaccination confirming that none had recent contact with WNV. None of the animals showed any adverse effects to the vaccinations. This corresponds with the findings in the USA where thousands of horses are vaccinated every year with comparable vaccines without any reported problems. *Porter* et al. (2004) stated that diagnosis of WNV infection in horses can be confirmed by an IgM titre of 1:400 or higher and these authors also confirmed that the IgM Capture ELISA is a highly sensitive, specific and accurate diagnostic test for WNV infected horses.

A Flavivirus blocking ELISA is suitable for detection of both IgG and IgM antibodies against Flaviviridae including WNV, and can also be used for both horses and chickens. In a WNV free area it is well-suited as a monitoring tool. In a validation study the blocking-ELISA had a specificity of 1.00 and a sensitivity of 0.98 (*Van Maanen* et al, 2010). An IgM capture ELISA is suited for detection of recent infections and can also be applied in endemic areas. This ELISA had a specificity of 1.00 and a sensitivity of 0.96 (*Van Maanen* et al. 2010). *Long* et al. (2006) confirmed that an IgM capture ELISA was a useful tool in regards to sensitivity and specificity for diagnosis of recent WNV exposed horses.

After the first and second vaccinations all the horses seroconverted in the WNV competition ELISA, indicating that they developed a humoral antibody response to WNV. This corresponds with the claims of the manufacturer of the vaccine.

The most important finding of the present study was the fact that three horses also showed a definite IgM response to the vaccination, and another seven horses showed a short and weak response (just above the cut-off level). Samples with a relatively high P/N-ratio were retested resulting in almost identical results. This suggests that during a WNV outbreak or WNV surveillance it may not be possible to differentiate infected horses from recently vaccinated horses using the IgM capture ELISA. This will have implications for the interpretation of positive IgM results in monitoring programmes such as have been implemented by the Animal Health Service at Deventer (GD), especially when vaccination against WNV becomes common practice. Therefore, for a correct interpretation of IgM capture ELISA results, the vaccination history of the horses should be known.

In a serologic surveillance programme in a WNV free area, positive ELISA results should always be confirmed by using plaque reduction neutralization tests (PRNT) which allows to identify the flavivirus causing the seroconversion (*El Garch* et al. 2008, *Davis* et al. 2008) The PRNT is considered the most specific test for the arthropod-borne flaviviruses and can be used to help distinguish false-positive results in an IgM antibody-capture enzyme-linked immunosorbent assay or other assays (for example, indirect immunofluorescence and

hemagglutination inhibition tests). The plaque-reduction neutralization test may also help distinguish serologic cross-reactions among the flaviviruses (CDC website).

In case of a positive PRNT, further steps can be taken to detect the virus, e.g. by using a real-time WNV PCR on serum samples and CSF samples of suspected animals. However, in vaccinated animals the PRNT is probably also not a good diagnostic test for WNV infections in horses, as it is very likely that antibodies resulting from WNV vaccination will also test positive in a WNV PRNT. In the event of a suspected WNV case it is also important to consider the use of other diagnostic tests besides serological tests . Real-time PCR is a highly sensitive and specific diagnostic tool that demonstrates the presence of viral DNA or RNA. However, its use as a diagnostic tool can be limited because of transient and low viraemia. Also, cytology of cerebrospinal fluid can be helpful as a diagnostic aid, because WNV infection in horses may cause mononuclear pleocytosis (*Porter* et al. 2004). Porter et al. also stated that immunohistochemistry of tissue of the central nervous system and spinal cord is a reliable test for WNV infection. However, this can only be performed after the patient has deceased.

Conclusions

None of the horses in this group tested positive for WNV antibodies on Day 0. All horses demonstrated a clear Ig (total antibody) response to the WNV vaccinations, but in two horses this response was less pronounced. Ten out of the 20 horses demonstrated a positive IgM response at some point after first and/or second vaccination, indicating that the IgM capture ELISA used will not distinguish with certainty horses with an acute WNV infection from recently vaccinated horses. No statistically significant association was found between the specific location of the intramuscular injection and the WNV IgM and IgG responses. No side effects were found following the first WNV vaccination nor the booster.

Conflict of interest statement

The authors have declared no potential conflicts.

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