Comparison of assays for the detection of West Nile virus antibodies in equine serum after natural infection or vaccination

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West Nile virus (WNV) mainly infects birds, horses and humans. Outcomes of the infection range from light uncharacteristic signs to fatal neurologic disease. The main objectives of the present study were to measure serum IgG and IgM antibodies in naturally exposed and vaccinated horses and to compare results of hemagglutination-inhibition test (HIT), enzyme-linked immunosorbent assay (ELISA) and plaque reduction neutralization test (PRNT).

Altogether 224 animals were tested with HIT for WNV antibodies and 41 horses were simultaneously examined with ELISA and PRNT. After primary screening for WNV antibodies, horses were vaccinated. Samples were taken immediately before and 3-5 weeks after each vaccination. McNemar chi-squared and percent agreement tests were used to detect concordance between HIT, ELISA and PRNT.

Analyses by HIT confirmed the presence of WNV antibodies in 27/105 (25.71 %) from naturally exposed horses. Sera from 57/66 (86.36%) vaccinated animals were positive before the first booster and from 11/11 (100%) before the second booster. HIT was less sensitive when detecting IgG antibodies. We could detect post vaccination IgM in 13 cases with MAC-ELISA and in 7 cases with HIT.

WNV is endemic in Hungary causing regular natural infections. Protective antibodies could not be measured in each individual case 12 months after primary injections; protection is more reliable after the first yearly booster. Based on our findings it was not be possible to differentiate infected horses from recently vaccinated horses using IgM antibody capture ELISA (MAC-ELISA). HIT does not substitute ELISA or PRNT when detecting IgG, but was useful tool in this study to gain statistical information about the tendencies within a fixed population of horses.

Keywords: West Nile virus, hemagglutination-inhibition test, enzyme-linked immunosorbent assay, plaque reduction neutralization test, vaccination, natural infection

Abbreviations

Enzyme-linked immunosorbent assay (ELISA)

Hemagglutination (HA)

Hemagglutination-inhibition test (HIT)

IgM antibody capture ELISA (MAC-ELISA)

Plaque reduction neutralization test (PRNT)

West Nile virus (WNV)

1. Introduction

West Nile virus (WNV) is a mosquito-borne zoonotic arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (Smithburn et al., 1940). It is transmitted in natural cycles between mosquitoes, (mainly the genus Culex), and wild birds (Bakonyi et al., 2013, Szentpáli-Gavallér et al., 2014, Pradier and Parker, 2014). Horses and humans are incidental and dead-end hosts; however they can develop severe neurological disorders (Kutasi et al., 2011, Lim et al., 2011). Phylogenetic studies have identified 2 main lineages of WNV strains. Until the early 2000s strains from lineage 1 were present in Africa, India, and Australia and were responsible for outbreaks in Europe, in the Mediterranean Basin, and in North America, whereas lineage 2 strains had been reported only in sub-Saharan Africa and Madagascar. Circulation of WNV strains of lineage 2 have recently been reported in Hungary (Bakonyi et al., 2006), Austria (Wodak et al., 2011), Russia (Platonov et al., 2011), Romania (Sirbu et al., 2011), Greece (Danis et al., 2011) and Italy (Savini et al., 2012). The Hungarian equine WNV outbreak - reported in 2008 - was the first to be caused by a lineage 2 sub-Saharan strain in Europe. The pathogenicity of this lineage 2 strain resembled that of lineage 1 strains, and its sudden spread was unpredictable (Kutasi et al., 2011, Lim et al., 2011).

The protective immune response to WNV requires both innate and adaptive immunity (Da Filette, 2012). The importance of adaptive immunity has been demonstrated as passive transfer of immune monoclonal and polyclonal antibodies protected mice from lethal WNV infection (Diamond et al., 2012). IgM is critically important for the control of early WNV infection and is detectable approximately 4 to 7 days after infection. After four to five days of illness, IgG antibodies are measurable in patients presumably conferring long-term protection against WNV re-infection (Tardei et al., 2000). Cellular immune responses also control WNV infections. Cytolytic T cells clear WNV infection by lysing infected cells. (Da Filette et al., 2012)

Antibodies can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition test (HIT), IgG ELISA, plaque reduction neutralisation test (PRNT), and microtitre virus neutralisation (VN) (Beaty et al., 1989; Hayes, 1989). Virus-neutralizing antibody responses persist for longer than WNV-specific IgM levels in serum. The duration of IgM appears to be short-lived in horses; hence it is useful for identifying and differentiating recent infections from previously exposed animals. Horses infected with either WNV lineage 1 or lineage 2 elicit a similar antibody profile in serum samples and there is not any notable differences in the antibody profile (Castillo-Olivares et al., 2011).

The emergence of lineage 2 strains of WNV in Europe as a cause of clinical disease and mortality in horses raised the question whether the existing WNV vaccines - which are all based on lineage 1 strains - protect against circulating lineage 2 strains of WNV. Up until now the lineage 1 vaccines turned out to be effective in protecting against lineage 2 strains of the disease as well (Minke et al., 2011).

The main objectives of the present study were to measure serum IgG and IgM antibodies in naturally exposed and vaccinated horses and to compare results of HIT, competitive ELISA and PRNT.

2. Materials and methods

Altogether we examined 224 horses for the presence of WNV antibodies. Two different examinations were performed (Table 1.). In the first examination after collecting a primary sera sample for WNV antibodies 182 animals were vaccinated with an inactivated virus vaccine according to the user's manual of the product (Duvaxyn® WNV, Pfizer, in the EU now Equip® WNV, Zoetis; in the US WEST NILE-INNOVATOR®, Zoetis). Out of th182 horses 105 animals were naturally exposed and 77 had been vaccinated before. Naturally exposed horses without showing any clinical signs were randomly selected in different parts of the country. Samples were collected from diverse parts of the country, but the majority of samples originated from the central region of Hungary. Of the 77 immunized animals 66 were given the two primary vaccinations approximately 12 months ago and 11 were already vaccinated for 2 subsequent years. Based on the results of the primary samples (prior to vaccination), horses were divided into four groups: group A contained horses that were previously seronegative and the horses in group B were seroconverted secondary to natural infection. Group C incorporated horses that were previously vaccinated with the available West Nile inactivated virus vaccine for one year (n=66) with a double primary vaccination and horses in group D (n=11) had been vaccinated for two years with a plus booster in the second year. Sera samples were collected three times from Group A and B, the first ones immediately prior to the primary vaccination, the second ones were collected 3 weeks later – immediately prior to the second dosage – and the final samples 3-5 weeks after the second dosage. Sera samples were collected two times from horses in Group C and D, first immediately prior to the booster and the second 3-5 weeks after the booster. All sampling and vaccination took place in March and April to avoid recent seroconversion from natural infection and to finish vaccination protocol in time before the WNV season. Based on other papers clinical cases in Europe occur between August and November (Kutasi et al., Monaco

et al., 2010). Horses should be immunised prior to the mosquito season, in order to achieve a sufficient protection with the inactivated WNV vaccine.

At the time of the sera sample collection there were no ELISA or capture ELISA kits available in Hungary, so on the first occasion our samples were tested by the National Centre for Epidemiology, where IgG levels were measured with HIT validated for human screening. The HIT was performed as described by Clarke and Casals, with a microtitre-plate format. We briefly summarize the key steps of the method. Nonspecific inhibitors and natural hemagglutinins were removed by kaolin adsorption. Dilutions of kaolin-treated equine serum were mixed with 8 hemagglutination (HA) units of suckling mouse WNV antigen and incubated at 4°C overnight. Goose erythrocytes were then added to the mixture, and the solution was incubated for an additional hour at room temperature (Clarke and Casals, 1958). The HIT titre was determined as the highest dilution of equine serum that caused complete inhibition of erythrocyte agglutination by 8 HA units of viral antigen. IgG test was considered positive when titre was equal or higher than 1:40.

Although HIT it is still used in laboratories, this test is gradually being replaced by other techniques. The disadvantage of HIT is the increasing tendency with time for sera to cross-react with other virus antigens within a given virus family (Specter et al., 2000). Therefore in our study 41 horses were simultaneously examined for both IgG and IgM with ELISA and also with PRNT. IgM antibody capture ELISA (MAC-ELISA), competitive ELISA tests and PRNT were used according to the OIE Terrestrial Manual 2013. Equine serum samples were tested at dilution of 1/400. All samples were examined for IgG but only post vaccination samples were tested for IgM.

HIT intervals were calculated with two sample z tests at 0.95 confidence levels. McNemar chi-squared and percent agreement tests were used to detect concordance between HIT, ELISA and PRNT. We calculated sensitivity and specificity values for both HIT and ELISA taking PRNT results as reference values.

During the second examination, 42 out of the 224 cases were evaluated for seroprevalence no further tests were carried out. The selection of this group of 42 horses was restricted to areas of the country where previously WNV disease was detected since our intention was to look for subclinical seroconversion rates by measuring the IgG titer with HIT.

Groups	Horses (n)
I. examination	
Group A no vaccination history , seronegative	78
Group B no vaccination history , seropositive	27
Group C vaccination history of 1 year	66
Group D vaccination history of 2 years	11
II. examination	
Not vaccinated horses	42
Altogether	**224**

Table 1: Summary of the examinations

3. Results

3.1. I examination

3.1.1. HIT

Before presenting our results, we call the attention of the reader to averages shown in our tables and figures were calculated on a logarithmic scale. The rationale behind this method was to avoid a few extremely high (1:5120 and higher) values heavily altering our results. Since one cannot assign any biological significance to these outstanding values (i.e., we cannot assume that a horse with 1:10240 IgG titer is better protected against the disease than one with 1:5120 IgG titer), reducing their effect on the averages by moving to the logarithmic scale seemed a more relevant approach. By the same argument one can conclude that the averages themselves, regardless of the method they were calculated with, contain little information about the studied groups. To remedy this we will provide some additional descriptive statistics.

Altogether we examined 105 horses with no vaccination history. According to the IgG titre of the primary serology test we divided these horses into groups A (n=78) and B (n=27). Based on these results 74.29 % of the naturally exposed horses were seronegative and 25.71 % were seropositive. According to statistical analysis (two sample z-test, 0.95 confidence level) the rate of seropositive horses is between 16-38%. Following the first vaccination 65 (83.33%) of 78 horses in Group A seroconverted and all horses of group B remained seropositive. Sequentially to the second vaccination in group A 76 (97%) seropositive and 2 seronegative horses were observed, and in group B all horses were seropositive (Tables 2. and 3.).

	1. sera sample (prior to vaccination)		**2. sera sample (after first vaccination)**		**3. sera sample (after second vaccination)**	
	positive	**negative**	**positive**	**negative**	**positive**	**negative**
Number of horses	0	78	65	13	76	2
Ratio	0%	100%	83.33%	16.67%%	97.44%	2.56%
Titre averages[a]	-	<1:10	1:160-1:320	<1:10	1:640-1:1280	1:10-1:20

Table 2: Summery of IgG levels from horses in Group A (n=78)

	1. sera sample (prior to vaccination)		**2. sera sample (after first vaccination)**		**3. sera sample (after second vaccination)**	
	positive	**negative**	**positive**	**negative**	**positive**	**negative**
Number of horses	27	0	27	0	27	0
Ratio	100%	0%	100%	0%	100%	0%
Titre averages[a]	1:40-1:80	-	1:640-1:1280	-	1:2560-1:5120	-

Table 3: Summery of the IgG levels from horses in Group B (n=27)

We also present a figure showing the ratio of horses with measured antibody levels 1:20 or lower, between 1:40 and 1:160, and 1:320 or higher (we refer to these three categories as negligible, satisfying and high respectively) (Figure 1.).

Altogether 77 horses had a history of vaccination, of these 66 had received primary vaccination in the previous year (group C) and 11 were vaccinated twice during the previous two years (group D). Group C consisted of 9 (13.64%) seronegative and 57 (86.36%) seropositive horses. In group D all horses were seropositive.

Following the first vaccination in group C 65 (98.48%) seropositive and 1 (1.52%) seronegative horses were found. All of the IgG levels of seropositive horses except for one were higher than 1:320. In group D all horses remained seropositive, with 1:640 or higher IgG titres (Tables 4. and 5.). For easier comparison we summarized the results of each group in Figure 2.

	1. sera sample (prior to vaccination)		**2. sera sample (after the vaccination)**	
	positive	**negative**	**positive**	**negative**
Number of horses	57	9	65	1
Ratio	86.36%	13.64%	98.48%	1.52%
Titre averages[a]	1:320-1:640	<1:10	1:2560-1:5120	1:10-1:20

Table 4: Summary of group C IgG levels

	1. sera sample (prior to vaccination)		**2. sera sample (after first vaccination)**	
	positive	**negative**	**positive**	**negative**
Number of horses	11	0	11	0

Ratio	100%	0%	100%	0%	
Titre averages[a]	1:320-1:640	-	1:2560-1:5120	-	

Table 5: Summary of group D IgG levels

3.1.2. ELISA/PRNT/ HIT

Altogether 90 sera samples were examined for IgG levels, which we obtained from the 41 horses as shown in Table 6.

Groups	**Number of horses**	**Sera samples per horses**	**Sera samples per groups**
Group A	7	3	21
Group B	7	3	21
Group C seronegative prior to buster vaccination	7	2	14
Group C seropositive prior to buster vaccination	7	2	14
Group D	7	2	14
Horses from II. examinations	6	1	6
Altogether	7*3+7*3+7*2+7*2+7*2+7*2+6*1		**90**

Table 6: Sera samples used for the comparison of HIT/ELISA/PRNT

The table below compares the IgG results from ELISA/PRNT/HIT (Table 7). Overall the 3 tests yielded the same result in 74 of the 90 cases (82%).

	HIT+ PRNT+	**HIT+ PRNT –**	**HIT – PRNT+**	**HIT – PRNT –**
ELISA+	63	0	10	1

ELISA –	0	1	4	11

Table 7: IgG results from ELISA/PRNT/HIT

(Green numbers show the values where all three results agree and red numbers indicate the cases when one test gave a different value from the others)

We provide contingency tables for pairwise comparison of the three methods, below each table we indicate overall percent agreement (Table 8./ A, B, C).

	HIT+	**HIT-**
ELISA+	63	11
ELISA –	1	15

Table 8. /A: Contingency table of ELISA/HIT

(63+15)/90 : 87%

	PRNT+	**PRNT –**
ELISA+	73	1
ELIS –	4	12

Table 8. / B: Contingency table of ELISA/PRNT

85/90 : 94%

	PRNT+	**PRNT –**
HIT+	63	1
HIT –	14	12

Table 8/ C: Contingency table of HIT/PRNT

75/90 : 83%

We used McNemar's test (with continuity correction) to verify that the difference shown in the contingency tables is significant. The result of the test is that both ELISA and HIT ($p = 0.0094$, McNemar's chi-squared = 6.750, df = 1) and HIT and PRNT ($p = 0.0019$, McNemar's chi-squared = 9.6, df = 1) differ (very) significantly, while the test does not show a significant difference between ELISA and PRNT results ($p = 0.3711$, McNemar's chi-squared = 0.8, df = 1).

We calculated sensitivity and specificity values for both HIT and ELISA taking PRNT results as reference values (These are just the positive/negative percent agreements from the above contingency tables) Sensitivity was 82% and specificity was 92% for HIT, while sensitivity was 95% and specificity was 92% for ELISA (Table 9).

Comparison	Sensitivity	Specificity
PRNT / HIT	63/(63+14) = **82%**	12/(12+1) = **92%**
PRNT / ELISA	73/(73+4) = **95%**	12/(12+1) = **92%**

Table 9: Sensitivity and specificity values

Only the post vaccination samples were tested for IgM with Capture ELISA. We detected 13 IgM positive samples.

3.2. II examination

42 of the horses were not participating in the vaccination program, in these cases we investigated if the horses had seroconversion due to natural infection.

40 of the horses examined previously shared a stall with confirmed cases of neuroinvasive WNV infection. 27 horses out of the 40 were seronegative and 13 were seronegative.

Two of the 42 horses were survivors of neuroinvasive WNV, both confirmed by high IgG and IgM levels at the acute phase of the disease. One of these two cases was shown no humoral protection against WNV 3 years after infection; the other horse had sufficient level of IgG (1:160), 1 year after infection, to be able to prevent a re-infection.

4. Discussion

The sera sampling prior to vaccination was performed in March, when there was a negligible chance of a recent infection, due to the seasonal nature of the disease (Smithburn et al., 1940). Therefore the 25.71% of IgG positive samples, from the horses with no vaccination history, were due to infections from previous year(s).

Although HIT was the least sensitive (82 %) serological test we performed to measure IgG levels and the sample of 105 horses (Group A and B) is not suitable to give an accurate account of the rate of seropositive horses in the whole Hungarian horse population, we can still conclude that there is a high seroconversion rate in the country. According to statistical analysis (two sample z-test, 0.95 confidence level) the rate of seropositive horses is between

16-38%. While this is clearly not a very accurate estimate, even the lower end of the estimated interval (16%) would indicate a high seroconversion rate in the country.

According to the vaccine description only 1 booster is needed per year following the primary vaccination course (2 subsequent vaccinations in 3 weeks intervals) in order to maintain a sufficient immunity (EMA/510730/2008). Also it was reported that the immune response after the booster is stronger compared to the primary vaccination (EMEA, 2008). We found that all horses from group D had 1:80 or higher IgG levels prior to the booster and 9 out of 11 horses had 1:320 or higher, so we concluded that all horses could indeed maintain their immunity for one year in this group. On the contrary, not all horses show humoral protection prior to booster vaccination from group C. That is, horses in this group did not always maintain their immunity for 12 month. With the possibility of climate change affecting the seasonal nature of the disease this result could become significant for veterinary practice in the future. Due to climate change WNV might appear in other parts of the year than early autumn. Consequently, vaccination which induces a protection during the whole year would be beneficial. Since it was verified by all three serological methods in this study that some horses lose protective level of antibody in less than 12 months after the primary vaccination, we suggest that the first booster should be applied sooner.

Comparing the post vaccination titres we found that the IgG average levels of groups A and B and groups C and D did not significantly diverge. Taking a closer look at the actual distribution of the values within the groups, we found that data from groups C and D showed a more homogeneous profile; hence the post-vaccination IgG levels in these groups were more reliably in the high domain than those of the horses vaccinated for the first time in the year of the study.

We do not have accurate knowledge of how the IgG levels predict the length of the immunity; however our results suggest a hypothesis, that one should expect the presence of antibodies to persist if the initial IgG level is sufficiently high. Naturally to make such a hypothesis quantitative and to confirm it one would need to conduct a longitudinal study.

Hemagglutination is a general property of the arbovirus group where WNV also belongs. (Sabin, 1951) The descriptive statistics indicate that the HIT is less sensitive than the ELISA and PRNT. However it is worth noting that out of the 14 cases where the PRNT result was positive and the HIT result was negative, 11 cases gave border PRNT results. According to

these results HIT is not a sufficient method to detect slightly elevated, border positive antibody titres.

Some strains of a given virus may show greater capacity than others to yield an HA antigen (Clarke and Casals, 1958). Based on this, sensitivity of the HIT does not only depend on the test itself but also on the WNV strain.

All members of the genus *Flavivirus* share antigenic epitopes, as revealed by cross-reactivity in the HIT (Casals et al., 1954). Therefore, commercially available plates, not necessarily coated with homologous antigen, can be used for antibody detection in serum by ELISA. Both HIT and ELISA should be followed by a neutralization assay to complete identification within the serologic group (Hann et al., 2003). However, PRNT is a laborious test and must be carried out in a biosafety level 3 (BSL-3) facility as viable WNV are used in this assay (Da Filette et al., 2012). ELISA has the advantage of being rapid, reproducible and less expensive than other methods.

In two cases from group A even the second vaccination and in one case from group C even the booster vaccination did not induce humoral immunity, according to the HIT results. Besides the lower sensitivity rate of HIT compared to ELISA or PRNT, this phenomenon could also be explained by a possible immune tolerance or immunosuppression of these horses, which can lead to a low- or non-reactivity of the immune system. While immune tolerance describes a state of unresponsiveness of the immune system to particular agents (Garnett et al., 2011) immunosuppression generally reduces or eliminates an immune response to all antigen stimuli. The most common iatrogenic cause of corticosteroid treatment is immunosuppression, which can bias adaptive immune response to vaccination in horses (Slack et al., 2000).

IgM can be detected after a recent infection, approximately for 4 to 7 days (Tardei et al., 2000). All of our samples were collected between March and April when there is a negligible chance of a recent infection, therefore the 13 post vaccination IgM positive results detected with capture ELISA, could only develop secondary to the vaccination. So we can conclude that the presence of IgM is not indicating natural WNV infections, as it has been already reported in a previous study (Jonquiere, 2010).

In the second examination we tested for seroconversion due to natural infection, on 40 cases, which previously shared a stall with confirmed cases of neuroinvasive WNV infection. 32.5 % of the studied cases were seropositive. This is a higher value compared to our findings in

the first examination's naturally exposed cases (25.71%), but according to statistical analysis (two sample z-test, 0.9 confidence level) the difference between the two measured value is not significant. Based on this it is not possible to rule out that horses coming from environments where infected horses were detected are not more exposed to the disease, but to confirm or reject this statement a larger sample would be needed.

Among flaviviruses, Yellow fever virus results in a lifelong natural immunity. In contrast protection against Japan encephalitis virus (JEV) or WNV may lessen later in lifetime (Parker et al., 2003).

Our result was in accordance with this, since from the two horses which survived from neuroinvasive WNV infection only one had a protective level of IgG, which was latter infected.

Lack of or lower humoral immune protection following a natural infection does not imply that the horse does not have a cellular immunity. A possible way of evaluating cellular immunity in these cases could be an investigation of the cytotoxic, necrotising effect of the T-killer cells.

Effective vaccine-mediated protection against WNV disease will most likely require both virus-specific antibody and cell-mediated immune responses. Many groups have reported flavivirus vaccine candidates that can efficiently induce virus-specific antibody responses, but the induction of appropriate T cell responses have been less frequently reported. In this regard, live attenuated vaccines have been shown to be very effective for inducing high magnitude, virus-specific T cell responses (Nelson 2010).

5. Conclusions

The ratio of seropositive horses is high in Hungary. There were cases where protective antibodies could not be measured in 12 months after primary vaccination; therefore we suggest that the first booster should be repeated earlier than a year after. Flavivirus infected sera show cross-reactions in serodiagnosis with heterologous flavivirus infections. Therefore PRNT is still used as the reference assay for specific diagnosis of WNV infection. ELISA has the advantage of being a faster and less expensive alternative which is more accessible for everyday use. HIT is not suitable for diagnostics; however it was a useful tool in this study to gain statistical information about the tendencies within a fixed population of horses. Based on our findings it was not possible to differentiate infected horses from recently vaccinated

horses using the MAC-ELISA. Following neuroinvasive infections there is no available data about the duration of protective humural or cellular immunity.

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[a] As it has been explained at the start of this section, averages have been calculated on a logarithmic scale. Due to the size of the error we indicate the magnitude of the averages in each table rather than the actual values.