Serum neutralising antibody response of seronegative horses against lineage 1 and lineage 2 West Nile virus following vaccination with an inactivated lineage 1 West Nile virus vaccine

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Lineage 2 West Nile virus (WNV) strains are endemic in South Africa and cause severe neurological disease in horses. An inactivated lineage 1 vaccine, Duvaxyn WNV, protects mice against challenge with a neuroinvasive South African lineage 2 strain of WNV. To evaluate the potential of Duvaxyn WNV to protect horses against lineage 2 strains of WNV, serum neutralising antibody responses of horses against lineage 1 WNV strain NY385/99 and lineage 2 WNV strain SPU93/01, isolated from a human with meningo-encephalitis in South Africa, were compared following vaccination with two doses of Duvaxyn WNV, 28 days apart, and a third dose one year later. Twenty-two seronegative horses were randomly assigned to two treatment groups: 16 to a vaccinated group and six retained as unvaccinated controls. Blood samples were taken from all horses on study days 0, 28, 35, 42, 49, 91, 141, 182, 231, 274, 322, 364 and 413. Primovaccination with Duvaxyn WNV resulted in high titres of serum neutralising antibodies against both strains. Following a single dose of Duvaxyn WNV on day 399, one year after primovaccination, there was a strong anamnestic response with a $\log_2$5-fold rise in the titres of neutralising antibodies against strains NY385/99 and SPU93/01. These results provide further evidence that Duvaxyn WNV is likely to protect horses against infection with lineage 2 strains of WNV and that a single annual booster may be sufficient to maintain immunity against lineage 2 WNV infection in horses.

Introduction

There are two major genetic lineages of West Nile virus (WNV): lineage 1, which is found in North America, North Africa, Europe and Australia, and lineage 2, which is endemic in central and southern Africa (Burt *et al*. 2002). Several minor lineages, including lineages 3–5 and possibly 6, occur in India, Eastern Europe and Spain (Bakonyi *et al.* 2005; Bondre *et al.* 2007; Vázquez *et al.* 2010). Initially, WNV was not thought to be a significant pathogen of horses in South Africa as few clinical cases were reported (Guthrie *et al.* 2003; Lanciotti *et al.* 2002). However, lineage 2 WNV infections have increasingly been associated with encephalitis in horses in South Africa (Venter *et al.* 2009). An 18-month pilot study of neurological disease in horses identified WNV infection in 19% of cases; all cases presented with severe neurological disease, of which 85% had to be euthanased or died with signs of encephalitis (Venter *et al.* 2009). All cases with a positive reverse transcriptase polymerase chain reaction were shown to be infected with lineage 2 WNV by DNA sequencing and phylogenetic analysis (Venter *et al.* 2009); clinical signs resembled those of lineage 1 WNV infections in the USA, such as ataxia, weakness, recumbency, muscle fasciculation and high case fatality rates (Dauphin *et al.* 2004; Durand, Simon & Tolou 2004; Schuler *et al.* 2004; Venter *et al.* 2009; Ward *et al.* 2004).

Vaccines to protect horses against WNV infection include a canarypox–WNV recombinant vaccine expressing prM and E genes from lineage 1 WNV strain NY385/99 (Minke *et al.* 2011), an inactivated lineage 1 WNV vaccine and a DNA vaccine (Dauphin & Zientara 2007). There are very limited data regarding the efficacy of these vaccines against lineage 2 WNV infection in horses. A recent study of a canarypox–WNV recombinant vaccine demonstrated efficacy against a European lineage 2 strain of WNV (Minke *et al.* 2011), but licensing of vaccines containing

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genetically modified organisms is problematic. An inactivated lineage 1 WNV vaccine, Duvaxyn WNV, has been shown to effectively protect mice against infection with a South African neuroinvasive strain of lineage 2 WNV (Venter *et al.* 2013). The present study examined the serum neutralising antibody response of seronegative horses against lineage 1 and lineage 2 WNV following vaccination with Duvaxyn WNV.

Materials and methods

Animals and study design

All 22 horses enrolled in the study were screened for the presence of IgG antibodies against WNV using a commercial enzyme-linked immunosorbent assay (ELISA) kit, ID Screen West Nile Competition ELISA (ID VET Innovative Diagnostics, Montpellier, France), 16 and 10 days before the day the study commenced (day 0). Of the 22 seronegative horses enrolled, 16 were assigned to the vaccinated group (T02) and six were retained as unvaccinated controls (T01). Horses in group T02 were vaccinated with Duvaxyn WNV on days 0, 28 and 399. Duvaxyn WNV was administered by deep intramuscular injection. One horse may have received an incomplete dose on day 399. Blood samples were taken from all horses on days 0, 28, 35, 42, 49, 91, 141, 182, 231, 274, 322, 364 and 413 and tested for the presence of serum neutralising antibodies against lineage 1 WNV strain NY385/99, which had been isolated from a dead bird in the USA in 1999 (Venter *et al.* 2005), and lineage 2 WNV strain SPU93/01, which had been isolated from a human patient with encephalitis in South Africa in 2001 (Venter *et al.* 2009).

Serum neutralisation tests

Testing for serum neutralising antibodies against lineages 1 and 2 WNV was conducted at the National Institute for Communicable Diseases (National Health Laboratory Service, South Africa) in accordance with a modification of the OIE manual using $TCID_{50}$ serum neutralisation assays. Duplicates of serial two-fold dilutions of sera inactivated at 56 °C for 30 min were tested using a microneutralisation procedure (Swanepoel *et al.* 1986), with the slight modification of using WNV isolate NY385/99 representing lineage 1 and WNV isolate SPU93/01 representing lineage 2 to assess neutralising antibodies against the two lineages. Vero cells were cultivated in Eagles Minimal Essential Medium (BioWhitaker, MD, USA) containing L-glutamine, non-essential amino acids, antibiotics (100 IU penicillin, 100 µg streptomycin and 0.25 µg amphotericin B, per mL) and 10% foetal bovine serum (Gibco), and incubated at 37 °C in 5% CO_2. Virus stocks comprised supernatant fluids from Vero cell cultures infected with the aforementioned WNV isolates. Inactivated sera were serially diluted two-fold from a 1:10 dilution in 50 µL medium in 96-well flat-bottom cell culture microplates. Equal volumes of virus suspension, containing a calculated 100 $TCID_{50}$ of virus, were added to each well and incubated for 60 min at 37 °C in 5% CO_2 before plates were seeded with 2×10^4 cells per well in 100 µL medium. The plates, with loose lids, were incubated at 37 °C in 5% CO_2 and the results were read after 10 days. Titres were expressed as the reciprocal of the serum dilution that inhibited ≥ 75% of viral cytopathic effect. A serum sample was considered positive when it had a titre ≥ $\log_{10}$ 1.0, equivalent to a serum dilution ≥ 1:10.

Statistics

Data were analysed using SAS v. 9.2.3 (SAS Institute, Cary, North Carolina). Serum neutralisation titres were analysed using a linear mixed model with repeated measures. Titres reported as < 10 were adjusted to five for analysis. Titres were natural log transformed before analysis. Test virus lineage, treatment (i.e. vaccination or control), day, lineage × treatment interaction, lineage × day interaction, treatment × day interaction and lineage × treatment × day 3-way interaction were entered as fixed effects. Animal and residual were entered as random effects. Since lineage, treatment, lineage × treatment interaction and treatment × day interaction were all significant at the 0.05 level, treatment comparisons were conducted within each lineage and day. Similarly, lineage comparisons were conducted within each treatment and day.

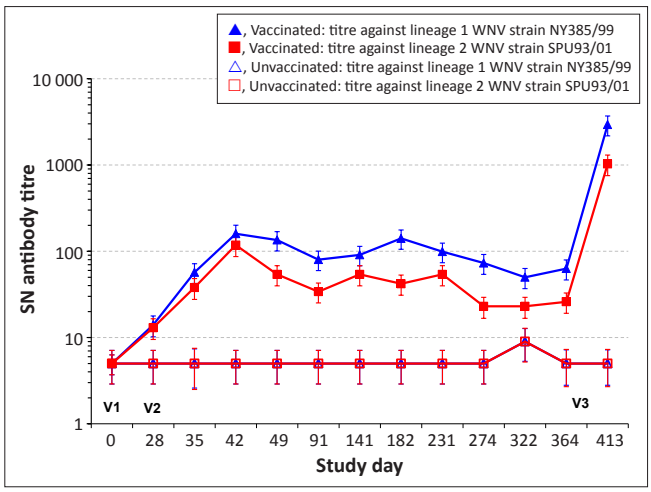
Ethical considerations

This study was approved by the National Health Laboratory Service Animal Ethics Committee and the Pfizer Animal Health Ethical Review Assessment Committee.

Results

One horse in the vaccinated group was sold and withdrawn from the study on day 280 and one unvaccinated horse was sold and withdrawn from the study on day 341.

Back-transformed least-squares geometric mean (BT-LSGM) titres of serum neutralising antibodies against lineage 1 and lineage 2 WNV are shown in Figure 1. The effect of WNV test strain was significant ($p = 0.0112$) and there were significant effects of treatment ($p < 0.0001$), time ($p < 0.0001$), treatment ×



Note: Bars represent standard errors.
SN, serum neutralising; V1, vaccination 1 on day 0; V2, vaccination 2 on day 28; V3, vaccination 3 on day 399

FIGURE 1: Back-transformed least-squares geometric mean titres of serum neutralising antibody against West Nile virus strains NY385/99 (lineage 1) and SPU93/01 (lineage 2), by treatment group and virus strain.

time interaction ($p < 0.0001$) and WNV test strain × treatment interaction ($p = 0.0112$) on titres of serum neutralising antibodies.

There were significant pairwise differences in BT-LSGM serum neutralising antibody titres against lineage 1 WNV strain NY385/99 between unvaccinated (T01) and vaccinated (T02) horses at all sampling points from day 28 to day 413 ($p < 0.0307$ in all cases). After day 0, BT-LSGM titres of serum neutralising antibodies against strain NY385/99 were consistently higher in vaccinated horses than in unvaccinated controls at all sampling points. There were significant pairwise differences in BT-LSGM serum neutralising antibody titres against lineage 2 WNV strain SPU93/01 between unvaccinated (T01) and vaccinated (T02) horses at all sampling points from day 35 to day 413 ($p < 0.0028$ in all cases), except on day 322. BT-LSGM titres of serum neutralising antibodies against strain SPU93/01 were consistently higher in vaccinated horses than in unvaccinated controls at all sampling points after day 35.

With the exception of day 322, serum neutralising antibody titres against both strain NY385/99 and strain SPU93/01 were < 10 in all unvaccinated horses. On day 322, the test result for one unvaccinated horse indicated a serum neutralising antibody titre of 160 against both strain NY385/99 and strain SPU93/01. However, at all other samplings this horse had a titre < 10 against both strains.

In vaccinated horses, there were significant pairwise differences in BT-LSGM serum neutralising antibody titres against strain NY385/99 compared with strain SPU93/01 at all sampling points from day 49 to day 413 ($p < 0.0163$ in all cases). After day 49, BT-LSGM titres of serum neutralising antibodies against strain NY385/99 were consistently higher than against strain SPU93/01 at all tested time points.

From day 49 to day 364, BT-LSGM neutralising titres against strain NY385/99 were two to three times higher than those against strain SPU93/01. On day 413, compared with day 364, the BT-LSGM neutralising titre against strain NY385/99 was 47 times higher, and against strain SPU93/01 it was 40 times higher.

Discussion

These results demonstrate that vaccination of horses with commercial Duvaxyn WNV stimulates the production of high titres of serum neutralising antibodies against a neuroinvasive South African lineage 2 WNV strain. Following primovaccination with two doses of Duvaxyn WNV four weeks apart, the peak and subsequent titres of serum neutralising antibodies against WNV strain SPU93/01 were lower than for lineage 1 WNV strain NY385/99, but the response following the second administration of Duvaxyn WNV and the subsequent decline in serum neutralising antibodies against lineage 2 strain SPU93/01 were comparable to the pattern seen for serum neutralising antibodies against lineage 1 WNV strain NY385/99.

Following a single dose of Duvaxyn WNV on day 399, one year after primovaccination, there was a strong anamnestic response with a large rise in the titres of neutralising antibodies against both lineage 1 WNV strain NY385/99 and lineage 2 WNV strain SPU93/01 to values well above their peak following primovaccination.

Duvaxyn WNV was shown to protect mice against challenge with a neuroinvasive South African lineage 2 strain of WNV (Venter *et al.* 2013). These results provide further evidence that Duvaxyn WNV is likely to protect horses against infection with lineage 2 strains of WNV and that a single annual booster may be sufficient to maintain immunity against lineage 2 WNV infection in horses.

Acknowledgements

Competing interests

This work was funded by Pfizer Animal Health.

Authors' contributions

M.C.P. (Veterinary Medicine Research and Development, Zoetis, Belgium) was the project leader, contributed to the study design and drafted the manuscript. M.V. (University of Pretoria as well as the Institute for Communicable Diseases of the National Health Laboratory Service) contributed to the study design, was responsible for laboratory analysis and reviewed the manuscript. T.S. (Aran Veterinary Clinic, Rustenburg) contributed to the study design, conducted the field component of the study and reviewed the manuscript. C.v.E. (University of Pretoria) and P.J.v.V. (National Institute for Communicable Diseases of the National Health Laboratory Service) conducted the serological assays and reviewed the manuscript. J.P. (National Institute for Communicable Diseases of the National Health Laboratory Service) supervised the neutralisation assays and reviewed the manuscript. B.L. (Biometrics, Zoetis, united States of America) conducted all statistical analyses. A.d.P. (Zoetis, South Africa) contributed to the study design, monitored the study and reviewed the manuscript.

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