

WEST NILE FEVER

SUMMARY

Description and importance of the disease: West Nile fever is a mosquito-borne viral disease that can affect birds, humans and horses causing inapparent infection, mild febrile illness, meningitis, encephalitis, or death. West Nile virus (WNV) is a member of the genus *Flavivirus* in the family *Flaviviridae*. The arbovirus is maintained in nature by cycling through birds and mosquitoes; numerous avian and mosquito species support virus replication. For many avian species, WNV infection causes no overt signs while other birds, such as American crows (*Corvus brachyrhynchos*) and blue jays (*Cyanocitta cristata*), often succumb to fatal systemic illness. Among mammals, clinical disease is primarily exhibited in horses and humans.

Clinical signs of WNV infection in horses arise from viral-induced encephalitis or encephalomyelitis. Infections are dependent on mosquito transmission and are seasonal in temperate climates, peaking in the early autumn in the Northern Hemisphere. Affected horses frequently demonstrate mild to severe ataxia. Signs can range from slight incoordination to recumbency. Some horses exhibit weakness, muscle fasciculation, and cranial nerve deficits. Fever is not a consistently recognised feature of the disease in horses.

Identification of the agent: Bird tissues generally contain higher concentrations of virus than equine tissues. Brain and spinal cord are the preferred tissues for virus isolation from horses. In birds, kidney, heart, brain, liver or intestine can yield virus isolates. Virus can also be isolated from mosquitoes. Cell cultures are used most commonly for virus isolation. WNV is cytopathic in susceptible mammalian cell culture systems. Viral nucleic acid and viral antigens can be demonstrated in tissues of infected animals by reverse-transcriptase polymerase chain reaction (RT-PCR) and immuno-histochemistry, respectively.

Serological tests: Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA, plaque reduction neutralisation (PRN) or virus neutralisation (VN) tests. The ELISA, VN and PRN methods are most commonly used for identifying antibody against WNV in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus, Usutu virus, Japanese encephalitis virus, or tick-borne encephalitis (TBE) virus may be encountered.

Requirements for vaccines: A formalin-inactivated WNV vaccine derived from tissue culture, WNV live canarypoxvirus-vectored vaccine, a WNV DNA vaccine and chimeric vaccines are licensed for use in horses.

A. INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae* (Smithburn *et al.*, 1940). The genus *Flavivirus* also includes Japanese encephalitis virus (see Chapter 3.1.10 *Japanese encephalitis*), St Louis encephalitis virus, Murray Valley encephalitis virus, Usutu virus, and Kunjin virus, among others (Burke & Monath, 2001). WNV has a wide geographical range that includes parts of Europe, Asia, Africa, Australia (Kunjin virus) and in North, Central and South America. Migratory birds are thought to be primarily responsible for virus dispersal, including reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (Burke & Monath, 2001). WNV is maintained in a mosquito–bird–mosquito transmission cycle, whereas humans and horses are considered dead end hosts. Genetic analysis of WN isolates separates strains into multiple lineages (Mackenzie & Williams, 2009; Vazquez *et al.*, 2010). Lineage 1 isolates are found in northern and central Africa, the Middle East, Europe, Indian subcontinent, Australia (Kunjin virus) and

in North and Central America, and Colombia and Argentina in South America (Morales *et al.*, 2006). Lineage 2 strains are endemic in central and southern Africa and Madagascar, with co-circulation of both virus lineages in central Africa (Berthet *et al.*, 1997; Burt *et al.*, 2002). Circulation of WNV strains of lineage 2 have been reported in Austria (Wodak *et al.*, 2011), Greece (Danis *et al.*, 2011), Hungary (Bakonyi *et al.*, 2006), Italy (Savini *et al.*, 2012), Romania (Sirbu *et al.*, 2011) and Russia (Platonov *et al.*, 2011). Strains from either lineage 1 or lineage 2 viruses might be implicated in either human or animal disease.

WNV was recognised as a human pathogen in Africa during the first half of the 20th century. Although several WN fever epidemics were described, encephalitis as a consequence of human WN infection was rarely encountered prior to 1996; since then, outbreaks of human WN encephalitis have been reported from France, Greece, Israel, Italy, North America, Romania, Russia and Tunisia. During the 1960s, WN viral encephalitis of horses was reported from Egypt and France (Panthier *et al.*, 1966; Schmidt & El Mansoury, 1963). Since 1998, outbreaks of equine WNV encephalitis have been reported from Argentina, Canada, France, Israel, Italy, Morocco, Spain, and the United States of America. In 2011, an outbreak of equine encephalitis due to Kunjin virus, a lineage 1 subtype of WNV, was reported in Australia (Frost *et al.*, 2012; Hall *et al.*, 2001). There was no evidence of disease in humans or birds caused by this virus.

The occurrence of disease in humans and animals along with bird and mosquito surveillance for WNV activity demonstrate that the virus range has dramatically expanded including North, Central and South America as well as Europe and countries facing the Mediterranean Basin.

The incubation period for equine WN encephalitis following mosquito transmission is estimated to be 3–15 days. A low titre level viraemia may precede clinical onset (Bunning *et al.*, 2002; Schmidt & El Mansoury, 1963). WN viral encephalitis occurs in only a small percentage of infected horses; the majority of infected horses do not display clinical signs (Ostlund *et al.*, 2000). The disease in horses is frequently characterised by mild to severe ataxia. Additionally, horses may exhibit weakness, muscle fasciculation and cranial nerve deficits (Cantile *et al.*, 2000; Ostlund *et al.*, 2000; 2001; Snook *et al.*, 2001). Fever is an inconsistently recognised feature. Treatment is supportive and signs may resolve or progress to terminal recumbency. The mortality rate is approximately one in three clinically affected unvaccinated horses. Differential diagnoses in horses include other arboviral encephalidites (e.g. eastern, western or Venezuelan equine encephalomyelitis, Japanese encephalitis), equine protozoal myelitis (*Sarcocystis neurona*), equine herpesvirus-1, Borna disease and rabies.

Most species of birds can become infected with WNV; the clinical outcome of infection is variable. Some species appear resistant while others suffer fatal neurologic disease. Neurological disease and death have been documented in domestic geese in Israel and Canada, and in many native and exotic zoo birds in the USA during the emergence of WNV (Austin *et al.*, 2004; Steele *et al.*, 2000). In Europe fatal neurological disease has been reported in wild birds (Zeller & Schuffenecker, 2004). WNV has been associated with sporadic disease in small numbers of other species, including squirrels, chipmunks, bats, dogs, cats, white-tailed deer, reindeer, sheep, alpacas, alligators and harbour seals during intense periods of local viral activity. WNV was isolated from a dromedary camel indicating this species as a possible source for the viral infection (Joseph *et al.*, 2016).

There has been confirmed transmission of WNV in humans by blood transfusion, organ transplantation and breast milk, but most human infections occur by natural transmission from mosquitoes. Laboratory acquired infections have also been reported (Campbell *et al.*, 2002).

Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). No vaccines are presently available for use in humans, however there is currently a WNV vaccine being used in human clinical trials. (NIH, 2015).

Due to the occurrence of inapparent WNV infections, diagnostic criteria must include a combination of clinical assessment and laboratory tests.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of West Nile fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement*	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification¹						
Nested RT-PCR	–	++	–	+++§	–	–
Real time RT-PCR	–	++	–	+++§	–	–
Immunohistochemistry	–	–	–	+	–	–
Isolation in tissue culture	–	++	–	++	–	–
Detection of immune response						
IgM capture ELISA	–	–	–	++	–	–
IgG indirect and competitive ELISAs ²	++	–	+	–	++	++
PRN	++	–	+	+	++	++
VN	++	–	+	+	++	++

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = purpose not applicable.

RT-PCR = reverse-transcriptase polymerase chain reaction; IgM = immunoglobulin M; ELISA = enzyme-linked immunosorbent assay; PRN = plaque reduction neutralisation; VN = virus neutralisation.

*RT-PCR methods may be used to declare domestic birds free from infection.

Restrictions to movements do not include dead-end hosts such as horses.

§RT-PCR positive results from horses are a rare event, thus to confirm suspect cases, serological tests such as IgM capture ELISA and seroconversion assessed by PRN or VN are recommended.

1. Identification of the agent

1.1. *In-vitro* and *in-vivo* culture

Attempts to detect virus from live, clinically ill horses are not usually successful due to the fleeting viraemia. Specimens for virus isolation include brain (particularly hindbrain and medulla) and spinal cord from deceased encephalitic horses (Ostlund *et al.*, 2000; 2001); a variety of bird tissues including brain, heart or liver may be used with success (Steele *et al.*, 2000). WNV can also be isolated from mosquitoes. In general, virus isolates are obtained more easily from avian specimens and to a lesser extent from mosquitoes and horses.

1 A combination of agent identification methods applied on the same clinical sample is recommended.

2 Both ELISA techniques lack specificity as they cross-react with antibodies directed to other flaviviruses, thus positive samples should be confirmed by a more specific test such as PRN or VN.

Virus may be propagated in susceptible cell cultures, such as rabbit kidney (RK-13), African green monkey kidney (Vero), baby hamster kidney (BHK-21), or pig kidney cells. Primary isolation in embryonated chicken eggs or *Aedes albopictus* (C6/36) cell lines followed by passages in mammalian cells can also be used. More than one cell culture passage may be required to observe cytopathic effect (CPE). Confirmation of WNV isolates is achieved by indirect fluorescent antibody staining of infected cultures or nucleic acid detection methods (see below).

1.2. Molecular methods – detection of nucleic acid

Several polymerase chain reaction (PCR) methods have been described for the identification of WNV and some are available as commercial kits. Included here is a real-time reverse-transcriptase (RT)-PCR with the capacity to detect both lineage 1 and lineage 2 WNV (Eiden *et al.*, 2010). Additionally, a conventional, gel-based RT-PCR designed to detect lineage 1 North American strains is described (Johnson *et al.*, 2001). Both assays have been successfully employed with field-collected samples. Lineage 1 WNV from France, Egypt, Israel, Italy, Kenya, Mexico and Russia demonstrate a highly conserved nucleotide sequence in the target region, regardless of species of origin (Lanciotti *et al.*, 2000). While the laboratory practices required to avoid contamination in a nested method are stringent, there is higher sensitivity for detection of North American strains of WNV RNA with the conventional nested procedure, particularly in equine field samples. The efficiency of the conventional RT-PCR to detect other WNV lineages is not known. In view of the continued evolution and possible emergence of new WNV strains, it is important that the designs of PCR tests are constantly monitored and updated when necessary. Samples appropriate for WNV RT-PCR include mammalian brain, avian brain, kidney, heart, liver, spleen, intestine, and insect pools. In any PCR assay it is imperative to include positive and no-template controls. For the nested RT-PCR, measures must be employed to avoid cross-contamination with products of the primary RT-PCR during the transfer of the outer primer product to the nested PCR reaction tubes. For any PCR reaction to be valid, the control reactions must fall within the expected range.

1.2.1. Extraction of viral RNA

Several commercial kits are available for RNA extraction. Select a kit appropriate for the type of sample and follow the manufacturer's recommendations.

1.2.2. Real-time reverse-transcriptase PCR

The following method was developed by Eiden *et al.* (2010) for concurrent identification of lineage 1 and lineage 2 WNV. Strain identification may be achieved by sequencing of the resultant amplicon and alignment with WNV reference strains. The procedure has been slightly modified from the published method and included here are the primers and probe directed to the NS2A region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements and melting temperatures of primers and probe. Primer and probe concentrations may be adjusted to achieve optimal results. An internal control and appropriate control primers and probe may be included to confirm valid test conditions.

Primers/probe (NS2A region of genome):

Forward primer: GGG-CCT-TCT-GGT-CGT-GTT-C

Reverse primer: GAT-CTT-GGC-YGT-CCA-CCT-C

Probe: FAM-CCA-CCC-AGG-AGG-TCC-TTC-GCA-A-BHQ

Per sample, prepare 20 µl volume of the RT-PCR reagents (per kit instructions) containing a 0.9 µM concentration of each primer and a 0.25 µM probe concentration. Dispense 20 µl of the mixture into each sample PCR tube or well. Add 5.0 µl of the extracted RNA sample, seal the tube/plate, and place in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse transcription incubation, followed by 45 cycles of amplification. Ct values of 37 or less are considered positive for WNV. Ct values of 37.1 through 42 are considered suspect, and should be repeated. Values higher than 42 are negative. For the PCR to be valid, the Ct values of the positive controls should fall within the expected range. No-template controls must be negative.

1.2.3. Conventional reverse-transcriptase PCR

The following method was developed for detection of lineage 1 WNV (Johnson *et al.*, 2001). The procedure may be conducted as a one-step RT-PCR using the outer primers only, or as a nested assay. The nested assay is the most sensitive RT-PCR and is recommended for testing of mammalian brain tissues or other samples that may contain a low amount of virus. The target of this RT-PCR is the E region of the WNV genome. The assay may be performed with a commercial kit of choice that provides the expected amplification of the controls. The cycling parameters must be adjusted to conform to the kit requirements.

Outer primers:

1401F: ACC-AAC-TAC-TGT-GGA-GTC

1845R: TTC-CAT-CTT-CAC-TCT-ACA-CT

Nested primers:

1485F: GCC-TTC-ATA-CAC-ACT-AAA-G

1732R: CCA-ATG-CTA-TCA-CAG-ACT

Per sample, prepare 45 µl volume of the RT-PCR reagents containing a 0.6 µM concentration of each outer primer. Dispense 45 µl of the mixture into each sample PCR tube. Add 5.0 µl of the extracted RNA sample and place the tubes in the thermocycler. Run the samples under the conditions described for the kit in use, beginning with a reverse-transcription incubation, for 60 minutes or according to the kit's recommendation, at the temperature specified by the manufacturer for the enzyme provided, followed by sample denaturation and 35 cycles of amplification. For the nested reaction, prepare a similar reaction mixture (without reverse transcriptase) of 49 µl per sample containing the nested primers and dispense into PCR tubes. Transfer 1.0 µl of the outer primer amplification product to the nested tube and place in the thermocycler. Use caution when transferring the amplification product to avoid cross contamination of samples. Perform 35 amplification cycles per kit recommendations. Based on the melting temperatures of the primers, the annealing temperature for primary RT-PCR and nested PCR should be 46°C. Following amplification, mix 8–10 µl of each PCR product with a loading buffer containing a DNA stain. Load the mixture into a gel and analyse by agar gel electrophoresis and ultraviolet visualisation. WNV-positive samples will be identified by a 445 base-pair band (outer primer amplification only) and/or a 248 base pair band (nested amplification). For the PCR reaction to be valid, positive controls must have the bands of the expected size, and template controls should not have bands. PCR amplicons may be sequenced for identity confirmation.

1.3. Antigen detection – immunolabelling techniques

Immunohistochemical (IHC) staining of formalin-fixed avian tissues is a reliable method for identification of WNV infection in birds. Brain, heart, kidney, spleen, liver, intestine, and lung are often IHC-positive tissues in infected birds. The success rate of IHC detection in positive birds is enhanced by the examination of multiple tissues. The specificity of identification (e.g. flavivirus specific or WNV specific) depends on the selection of detector antibody. The brain and spinal cord tissues of horses with WN viral encephalitis are inconsistently positive in IHC tests; many equine encephalitis cases yield false-negative results. Failure to identify WNV antigen in equine central nervous system does not rule out infection. For further advice, consult OIE Reference Laboratories.

2. Serological tests

Antibody can be identified in equine serum by IgM capture enzyme-linked immunosorbent assay (IgM capture ELISA), haemagglutination inhibition (HI), IgG ELISA, plaque reduction neutralisation (PRN), and microtitre virus neutralisation (VN) (Beaty *et al.*, 1989; Hayes, 1989). The IgM capture ELISA described below is particularly useful for detecting equine antibodies resulting from recent natural exposure to WNV. Equine WNV-specific IgM antibodies are usually detectable from 7–10 days post-infection to 1–2 months post-infection. Most horses with WN encephalitis test positive in the IgM capture ELISA at the time that clinical signs are first observed. WNV neutralising antibodies are detectable in equine serum by 2 weeks post-infection and can persist for more than 1 year. The ELISA, HI, VN and PRN methods are most commonly used for identifying WNV antibody in avian serum. In some serological assays, antibody cross-reactions with related flaviviruses, such as St Louis encephalitis virus or Japanese encephalitis virus, will be encountered. The PRN test is the most specific among

WNV serological tests; when needed, serum antibody titres against related flaviviruses can be tested in parallel. Finally, WN vaccination history must be considered in interpretation of serology results, particularly in the PRN and VN tests and IgG ELISA. An IgM capture ELISA may be used to test avian or other species provided that species-specific capture antibody is available (e.g. anti-chicken IgM). The PRN test is applicable to any species, including birds.

2.1. ELISA and related methods

2.1.1. Equine IgM capture ELISA

Several kits for IgM detection from equine specimens are commercially available, alternatively WNV and negative control antigens for the IgM capture ELISA may be prepared from mouse brain (see Chapter 3.5.5 *Equine encephalomyelitis [Eastern and Western]*), tissue culture or recombinant cell lines (Davis *et al.*, 2001). Commercial sources of WNV testing reagents are available in North America. Characterised equine control serum, although not an international standard, can be obtained from the National Veterinary Services Laboratories, Ames, Iowa, USA. Virus and negative control antigens should be prepared in parallel for use in the ELISA. Antigen preparations must be titrated with control sera to optimise sensitivity and specificity of the assay. Equine serum samples are tested at a dilution of 1/400 and equine cerebrospinal fluid samples are tested at a dilution of 1/2 in the assay, or as specified by the kit manufacturer. To ensure specificity, each serum sample is tested for reactivity with both virus antigen and control antigen.

2.1.2. Example test procedure

- i) Coat flat-bottom 96-well ELISA plates with 100 µl/well anti-equine IgM diluted in 0.5 M carbonate buffer, pH 9.6, according to the manufacturer's suggested dilution for use as a capture antibody.
- ii) Incubate plates overnight at 4°C in a humid chamber. Coated plates may be stored for several weeks in a dry or desiccated chamber.
- iii) Prior to use, wash plates twice with 200–300 µl/well 0.01 M phosphate buffered saline, pH 7.2, containing 0.05% Tween 20 (PBST).
- iv) Block plates by adding 300 µl/well freshly prepared 5% nonfat dry milk in PBST (or as specified by the kit manufacturer) and incubate 60 minutes at room temperature. After incubation, remove blocking solution and wash plates three times with PBST.
- v) Test and control sera are diluted 1/400 (cerebrospinal fluid is diluted 1/2) in PBST and 50 µl/well of each sample is added to duplicate sets of wells (total of four wells per sample) on the plate. Include control positive and negative sera prepared in the same manner as samples.
- vi) Cover the plates and incubate 75 minutes at 37°C in a humid chamber.
- vii) Remove serum and wash plates three times in PBST.
- viii) Dilute virus and negative control antigens in PBST and add 50 µl of virus antigen to one set of wells per test and control sera and add 50 µl normal antigen to the second set of wells per test and control sera.
- ix) Cover the plates and incubate overnight at 4°C in a humid chamber.
- x) Remove antigens from the wells and wash the plates three times in PBST.
- xi) Dilute horseradish peroxidase conjugated anti-*Flavivirus* monoclonal antibody³ in PBST according to manufacturer's directions and add 50 µl per well.
- xii) Cover the plates and incubate at 37°C for 60 minutes.
- xiii) Remove conjugate and wash plates six times in PBST.
- xiv) Add 50 µl/well freshly prepared ABTS (2,2'-azino-di-[3-ethyl-benzthiazoline]-6-sulphonic acid) chromogen with hydrogen peroxide (0.1%) and incubate at room temperature for 30 minutes. Alternative chromogens may be used as indicated by the kit manufacturer.

3 Available from the Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia, 30333, USA.

- xv) Measure absorbance at 405 nm. A test sample is considered to be positive if the absorbance of the test sample in wells containing virus antigen is at least twice the absorbance of negative control serum in wells containing virus antigen and at least twice the absorbance of the sample tested in parallel in wells containing control antigen.

2.1.3. Indirect and competitive ELISAs

Numerous indirect and competitive commercial and in-house ELISAs have been developed and are used to detect WNV antibodies. While competitive assays are applicable for sera of all species, indirect assays require enzyme-labelled species-specific secondary antibodies. Most ELISA techniques lack specificity as they cross-react with antibodies directed to other flaviviruses especially those of the Japanese encephalitis serogroup. They are very useful for epidemiological and surveillance purposes as well as a screening method. A positive ELISA result however should be confirmed by a more specific test such as VN or PRNT. Some indirect or competitive ELISAs can detect antibodies of any immunoglobulin class (IgM, IgG, etc.). Vaccinated horses often test positive on the indirect or competitive ELISAs.

2.2. Neutralisation

2.2.1. Plaque reduction neutralisation (applicable to serum from any species)

The PRN test is performed in Vero cell cultures in either 25 cm² flasks or 6-well plates. The sera can be screened at a 1/10 and 1/100 final dilution or may be titrated to establish an endpoint. A description of the test as performed in 25 cm² flasks using 100 plaque-forming units (PFU) of virus is as follows.

Prior to testing, serum is heat inactivated at 56°C for 30 minutes and diluted (e.g. 1/5 and 1/50) in cell culture medium. Virus (200 plaque-forming units per 0.1 ml) working dilution is prepared in media containing 10% guinea-pig complement. Equal volumes of virus and serum are mixed and incubated at 37°C for 75 minutes before inoculation of 0.1 ml onto confluent cell culture monolayers. The inoculum is adsorbed for 1 hour at 37°C, followed by the addition of 4.0 ml of primary overlay medium. The primary overlay medium consists of two solutions that are prepared separately. Solution I contains 2 × Earle's Basic Salts Solution without phenol red, 4% fetal bovine serum, 100 µg/ml gentamicin and 0.45% sodium bicarbonate. Solution II consists of 2% Noble agar that is sterilised and maintained at 47°C. Equal volumes of solutions I and II are adjusted to 47°C and mixed together just before use. The test is incubated for 72 hours at 37°C. A second 4.0 ml overlay prepared as above, but also containing 0.003% neutral red is applied to each flask. Following a further overnight incubation at 37°C, the number of virus plaques per flask is assessed. Endpoint titres are based on 90% reduction compared with the virus control flasks, which should have about 100 plaques.

2.2.2. Virus neutralisation – microtitre format

The microtitre VN assay is capable of identifying and quantifying antibodies against WNV present in test samples. Its performance is comparable to the PRN test (Di Gennaro *et al.*, 2014); however, it requires less sample volume than PRN and is more suitable when only small volumes of samples are available (Weingartl *et al.*, 2003). Appropriate precautions are necessary to prevent human exposure when using live WNV in unsealed microtitre plates.

2.2.3. Test procedure

The cell line commonly used is African green monkey kidney (Vero). The VN test requires 3–5 days to be completed.

- i) Twenty-five µl each of several dilutions of the test serum, starting from 1/5 to 1/640, are added to each test well of a sterile flat-bottomed microtitre plate and mixed with an equal volume of 100 TCID₅₀ of WNV reference virus. Plates are incubated at 37°C in 5% CO₂ in a humidified incubator.
- ii) After 1 hour of incubation, approximately 10⁴ Vero cells are added per well in a volume of 50 µl of Minimal Essential Medium containing antibiotics. Following incubation for 3–5 days, the test is read using an inverted microscope
- iii) Wells are scored for the degree of CPE observed. A sample is considered positive when it shows more than 90% of CPE neutralisation at the lowest dilution (1:10). The serum titre represents the highest serum dilution capable of neutralising more than 90% of the CPE in the tissue culture.

A VN titre greater or equal 1/10 is usually considered specific for WNV. However it should be noted that birds and mammals may show cross reactions at this level after infection with, or vaccination against, Japanese encephalitis or St Louis encephalitis viruses. Cross-reactivity also exists between WNV and the Usutu virus however, specific antibodies can be attributed to one or the other virus as there is a neutralising titre of fourfold or higher for one virus compared with the other when independently tested.

C. REQUIREMENTS FOR VACCINES

1. Background

In 2003, the United States Department of Agriculture (USDA) issued a license for a formalin-inactivated WNV vaccine derived from tissue culture for use in horses. The European Committee for Medicinal Products for Veterinary Use (CVMP) approved this product in 2008. In 2011, the product was conditionally licensed by USDA for use in alligators. In 2004, an inactivated human cell line-derived WNV vaccine obtained a market authorisation in Israel as a veterinary vaccine for geese. Information on Biotechnology-derived vaccines has also been licensed, as detailed in section C.3 below. These vaccines have demonstrated sufficient efficacy and safety in adequately vaccinated horses. Vaccination may be helpful in preventing neurological signs associated with WNV infection.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given here and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See chapter 1.1.8 for general requirements for master seeds and allowable passages for vaccine production.

2.1.1. Biological characteristics of the master seed (MS)

The isolate of WNV used as the master seed virus (MSV) for vaccine production must be accompanied by documentation describing its origin and passage history.

i) Inactivated vaccines

Virulent virus potentially may be used in inactivated vaccines, provided that manufacturing methods ensure complete inactivation of the MSV. The completed vaccine must be safe in host animals at the intended age of vaccination and provide protection after challenge.

ii) Live vaccines

The MSV must be safe in host animals at the most susceptible age for infection. It should not increase in virulence or undergo detectable genetic changes with repeated passage through host animals. Ideally, the MSV should not be shed from vaccinated animals into the environment; otherwise, shedding should be minimal and transient. The MSV should not adversely impact non-target species with which a vaccinated animal may have contact. The completed vaccine must provide protection after challenge.

iii) Recombinant vaccines

Recombinant vaccines may be live or inactivated, and recombinant MSVs are subject to the same guidelines as conventional MSVs. In addition, recombinant MSVs expressing foreign genes should stably produce the foreign antigens.

iv) DNA vaccines

The MS is the host organism (e.g. *Escherichia coli*) that expresses the plasmid used in the vaccine. The completed DNA vaccine is subject to the same requirements as listed above.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

The MSV must be tested for purity, identity, and freedom from extraneous agents at the time before it is used in the manufacture of vaccine. The MSV must be free from bacteria, fungi and mycoplasma. It also must be free of extraneous viruses, including equine herpesvirus, equine adenovirus, equine viral arteritis virus, bovine viral diarrhoea virus, reovirus, and rabies virus by the fluorescent antibody technique. The MSV must be free from extraneous virus by CPE and haemadsorption on the Vero cell line and an embryonic equine cell type.

2.1.3. Validation as a vaccine strain

In an immunogenicity trial, the MSV at the highest passage level intended for production must protect susceptible horses against a virulent challenge isolate found in the geographical area of intended vaccine use. Ideally the challenge isolate and MSV should be heterologous. MSVs intended for use in live vaccines also must be tested for innocuity in susceptible non-target species, such as birds.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

In the event that an emerging strain or variant of WNV results in an emergency epizootic situation that cannot be controlled by currently available vaccines, provisional acceptance of a new MSV should be considered, ideally for use in an inactivated vaccine. Such acceptance should be based on a risk analysis of potential contamination of the new MSV with extraneous agents. This risk assessment should consider the source and passage history of the MSV and characteristics of the vaccine manufacturing process, including the nature and concentration of the virus inactivant.

2.2. Method of manufacture

2.2.1. Procedure

The MSV should be propagated in cell lines known to support the growth of WNV. See chapter 1.1.8 for additional guidance on the preparation and testing of master cell stocks. Cell lines should be free from extraneous viruses, bacteria, fungi, and mycoplasma. Viral propagation should not exceed five passages from the MSV, unless further passages prove to provide protection in the host animal.

The susceptible cell line is seeded into suitable vessels. Minimal essential medium, supplemented with fetal bovine serum (FBS), may be used as the medium for production. Incubation is at 37°C.

Cell cultures are inoculated directly with WNV working virus stock, which is generally 1 to 4 passages from the MSV. Inoculated cultures are incubated for 1–8 days before harvesting the culture medium. During incubation, the cultures are observed daily for CPE and bacterial contamination.

Inactivated vaccines may be chemically inactivated with either formalin or binary ethylenimine and mixed with a suitable adjuvant. The duration of the inactivation period is based on demonstrated inactivation kinetics.

DNA vaccine expression cassettes are amplified in *E. coli* (or other suitable vector). Purified plasmids are formulated into a vaccine.

2.2.2. Requirements for ingredients

All ingredients used in the manufacture of WNV vaccine should be defined in approved manufacturing protocols and consistent from batch to batch. See chapter 1.18 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies (TSEs).

2.2.3. In-process controls

Production lots of WNV must be titrated in tissue culture before inactivation to standardise the product. Low-titred lots may be concentrated or blended with higher-titred lots to achieve the correct titre.

Inactivated WNV lots must be tested for completeness of inactivation. Ideal protocols incorporate concentration and amplification steps, to enhance detection of residual live virus.

Production lots of DNA are quantified by analytical methods and characterised before standardisation and blending at the correct DNA content. Production lots must not exceed the highest acceptable level of lipopolysaccharide (LPS) content, based on safety testing.

2.2.4. Final product batch tests

i) Sterility

Inactivated and live vaccine samples are examined for bacterial and fungal contamination. The volume of medium used in these tests should be enough to nullify any bacteriostatic or fungistatic effects of the preservatives in the product. To test for bacteria, ten vessels, each containing a minimum of 120 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The ten vessels are incubated at 30–35°C from 10 days and observed for bacterial growth. To test for fungi, ten vessels, each containing a minimum of 40 ml of soybean casein digest medium, are inoculated with 0.2 ml from ten final-container samples. The vessels are incubated at 20–25°C from ten days and observed for fungal growth. Individual countries may have other requirements.

ii) Identity

Separate batch tests for identity should be conducted if the batch potency test, such as tissue-culture titrations of live virus vaccines, does not sufficiently verify the identity of the agent in the vaccine. Identity tests may include fluorescent antibody or serum neutralisation assays.

Additionally, if all-in, all-out manufacturing processes and sanitation are not used and more than one agent is propagated in a laboratory, identity tests shall demonstrate that no other vaccine strain is present.

iii) Safety

Safety tests in target animals are not required in many regions for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

Batch safety tests may be conducted in guinea pigs, mice, or host animals. The product should be administered according to label recommendations in host animals. Individual countries may require dose overage (e.g. 2x–10x). No systemic or local adverse reactions should occur after vaccination.

The requirement for an *in-vivo* batch safety test may be exempted if the safety of the product is demonstrated and approved in the registration dossier and production is consistent with that described in chapter 1.1.8 and Chapter 2.3.3 *Minimum requirements for the organisation and management of a vaccine manufacturing facility*).

iv) Batch potency

Killed virus vaccines may use host animal or laboratory animal vaccination/serology tests or vaccination/challenge tests to determine potency of the final product. In vitro techniques to compare a standard with the final product are also acceptable in determining the relative potency of a product (USDA, 2011). The standard should be shown to be protective in the host animal.

Live viral products are titred in cell cultures to determine the potency of the final product. Compared to the minimum protective dose established in the immunogenicity trial, the batch release potency titre should include overages for batch test variability and loss of potency over product dating. In the absence of specific data to support an alternative, overages of 0.7 log₁₀ and 0.5 log₁₀, respectively, are recommended.

DNA vaccines may be tested for bioactivity and DNA content using parallel-line direct quantification methods that compare a standard preparation to the final product.

2.3. Requirements for authorisation/registration/licensing

2.3.1. Manufacturing process

For registration of WNV vaccine, all relevant details concerning manufacture of the vaccine, in-process controls, and quality control testing (see Sections C.2.a and C.2.b) should be submitted to the authorities. Test results shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume

2.3.2. Safety requirements

The inherent safety of the vaccine strain, if it will be used in the manufacture of a live vaccine, is tested at the Master Seed level.

i) Target and non-target animal safety

The Seed should be tested in host animals of the most susceptible age. The animals should be monitored for clinical disease and virus shed/spread. The organism dose should be no less than that expected in completed vaccine. Individual countries may have overdose requirements. If the vaccine will be intended for use in specific subpopulations (e.g. pregnant animals), they also should be included in target animal safety studies. Additionally, similar studies should be conducted in susceptible non-target species (e.g. birds).

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Demonstrate that repeated *in-vivo* passage of the MSV does not increase virulence. (For additional guidance see Section: *Increase in Virulence Tests* in chapter 1.1.8.)

The final vaccine formulation (inactivated or live) should be tested in a limited number of target animals prior to a larger-scale field study. The final vaccine formulation should not cause adverse reactions.

Field safety studies should be conducted before any vaccine receives final approval. Generally, two serials should be used, in three different geographical locations under typical animal husbandry conditions, and in a minimum of 600 animals. The vaccine should be administered according to label recommendations (including booster doses) and should contain the maximum permissible amount of WNV antigen. (If no maximum antigen content is specified, serials should be of anticipated typical post-marketing potency.) About one-third of the animals should be at the minimum age recommended for vaccination.

iii) Precautions (hazards)

Vaccine should be identified as harmless or pathogenic for vaccinators. Manufacturers should provide adequate warnings that medical advice should be sought in the case of self-injection. Warnings should be included on the product label/leaflet so that the vaccinator is aware of any danger.

2.3.3. Efficacy requirements

To register a WNV vaccine, a batch produced according to the standard method and containing the minimum amount of antigen or potency value must provide protection against virulent challenge in the minimum-aged animal recommended for vaccination. Each future commercial batch shall be tested before release to ensure it has at least the same potency demonstrated by the batch used for the efficacy study.

WNV vaccine efficacy is often estimated in vaccinated horses by evaluating post-challenge viraemia and/or neurological signs (e.g. muscle fasciculation, ataxia, seizures). Efficacy is estimated in alligators by evaluating post-challenge viraemia and/or lymphohistiocytic skin (pix) lesions. Twenty-five vaccinates and ten placebo-vaccinated controls are recommended.

Horses may be challenged intrathecally or via controlled exposure to infected mosquitoes. Alligators may be challenged subcutaneously. All animals should be monitored for 14–21 days after challenge. Viraemia should be evaluated qualitatively (i.e., detectable presence or absence) by validated assays. The vaccination effect may be assessed by calculating prevented fraction with a 95% confidence interval. Individual countries may have minimum efficacy requirements, but in no case should the lower confidence interval include zero.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

Vaccination is only recommended for horses and alligators in WN-positive areas. Vaccinated horses may develop a serological titre that may interfere with the ability to export the horse.

2.3.5. Duration of immunity

Studies to determine a minimum duration of immunity should be conducted before the vaccine receives final approval. Duration of immunity should be demonstrated in a manner similar to the original efficacy study, challenging animals at the end of the claimed period of protection. At a minimum, the duration should be for the length of the mosquito season in seasonally infected areas. It may be desirable to demonstrate longer immunity for animals at higher risk and in infected areas with year-round mosquito activity.

2.3.6. Stability

Live and inactivated vaccines are typically assigned an initial expiry date of 18 or 24 months, respectively. Real-time stability studies should be conducted to confirm the appropriateness of all expiration dating. Product labelling should specify proper storage conditions.

3. Vaccines based on biotechnology

In 2003, the USDA licensed a live canarypoxvirus-vectorized WNV vaccine for use in horses. In 2005, the USDA issued the first fully licensed WNV DNA vaccine for animals in the USA. The vaccine contains genes for two WNV proteins, and therefore, does not contain any whole WNV, live or killed. In late 2006, a chimeric vaccine, based on a yellow fever (YF) virus vector, was licensed by USDA for use in horses. The CVMP approved a recombinant canarypox virus WNV product in 2011 and an inactivated chimeric flavivirus strain YF-WNV product in 2013. In addition to meeting requirements for efficacy, potency, purity and safety, recombinant seeds must undergo a risk analysis. The conclusion of the risk analysis must be that the vaccine does not have significant environmental impact.

REFERENCES

- AUSTIN R.J., WHITING T.L., ANDERSON R.A. & DREBOT M.A. (2004). An outbreak of West Nile virus-associated disease in domestic geese (*Anser anser domesticus*) upon initial introduction to a geographic region, with evidence of bird to bird transmission. *Can. Vet. J.*, **45**, 117–123.
- BAKONYI T., IVANICS E., ERDELYI K., URSU K., FERENCZI E., WEISSENBOCK H. & NOWOTNY N. (2006). Lineage 1 and 2 strains of encephalitic West Nile Virus, central Europe. *Emerging Infect. Dis.*, **12**, 618–623.
- BEATY B.J., CALISHER C.H. & SHOPE R.E. (1989). Arboviruses. *In: Diagnostic Procedures for Viral Rickettsial and Chlamydial infections*, Sixth Edition, Schmidt N.H. & Emmons R.W., eds. American Public Health Association, Washington DC, USA, 797–856.
- BERTHET F.-X., ZELLER H.G., DROUET M.-T., RAUZIER J., DIGOUTTE J.-P. & DEUBEL V. (1997). Extensive nucleotide changes and deletions within the envelope glycoprotein gene of Euro-African West Nile viruses. *J. Gen. Virol.*, **78**, 2293–2297.
- BUNNING M.L., BOWEN R.A., CROPP B.C., SULLIVAN K.G., DAVIS B.S., KOMAR N., GODSEY M., BAKER D., HETTLER D.L., HOLMES D.A., BIGGERSTAFF B.J. & MITCHELL C.J. (2002). Experimental infection of horses with West Nile virus. *Emerg. Infect. Dis.*, **8**, 380–386.
- BURKE D.S. & MONATH T.P. (2001). Flaviviruses. *In: Fields Virology*, Fourth Edition, Knipe D.M. & Howley P.M., eds. Lippincott Williams & Wilkins, Philadelphia, Pennsylvania, USA, 1043–1125.
- BURT F.J., GROBBELAAR A.A., LEMAN P.A., ANTHONY F.S., GIBSON G.V.F. & SWANEPOEL R. (2002). Phylogenetic relationships of Southern African West Nile virus isolates. *Emerg. Infect. Dis.*, **8**, 820–826.
- CAMPBELL G., LANCIOTTI R., BERNARD B. & LU H. (2002). Laboratory-acquired West Nile virus infections – United States, 2002. *Morbidity and Mortality Weekly Report (MMWR)*, **51**, 1133–1135. <http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5150a2.htm>
- CANTILE C., DI GUARDO G., ELENI C. & ARISPICI M. (2000). Clinical and neuropathological features of West Nile virus equine encephalomyelitis in Italy. *Equine Vet. J.*, **32**, 31–35.

- DANIS K., PAPA A., PAPANIKOLAOU E., DOUGAS G., TERZAKI I., BAKA A., VRIONI G., KAPSIMALI V., TSAKRIS A., KANSOUZIDOU A., TSIODRAS S., VAKALIS N., BONOVAS S. & KREMASTINOY J. (2011). Ongoing outbreak of West Nile virus infection in humans, Greece, July to August 2011. *Eurosurveillance*, **16**, pii: 19951.
- DAVIS B.S., CHANG G.J., CROPP B., ROEHRIG J.T., MARTIN D.A., MITCHELL C.J., BOWEN R. & BUNNING M.L. (2001). West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses *in vitro* a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. *J. Virol.*, **75**, 4040–4047.
- DI GENNARO A., LORUSSO A., CASACCIA C., CONTE A., MONACO F. & SAVINI G. (2014). Serum neutralization assay can efficiently replace plaque reduction neutralization test for detection and quantitation of West Nile virus antibodies in human and animal serum samples. *Clin. Vaccine Immunol.*, **10**, 1460–1462.
- EIDEN M., VINA-RODRIGUEZ A., HOFFMANN B., ZIEGLER U. & GROSCHUP M.H. (2010). Two new real-time quantitative reverse transcription polymerase chain reaction assays with unique target sites for the specific and sensitive detection of lineages 1 and 2 West Nile virus strains. *J. Vet. Diagn. Invest.*, **22**, 748–753.
- FROST M.J., ZHANG J., EDMONDS J.H., PROW N. A., GU X., DAVIS R., HORNITZKY C., ARZEY K.E., FINALAISON D., HICK P., READ A., HOBSON-PETERS J., MAY F.J., DOGGETT S.L., HANIOTIS J., RUSSELL R.C., HALL R.A., KHROMYKH A.A. & KIRKLAND P.D. (2012). Characterization of virulent West Nile virus Kunjin strain, Australia, 2011. *Emerg. Infect. Dis.*, **18**, 792–800.
- HALL R.A., SCHERRET J.H. & MACKENZIE J.S. (2001). Kunjin Virus. *Ann. N. Y. Acad. Sci.*, **951**, 153–160.
- HAYES C.G. (1989). West Nile fever. In: *The Arboviruses: Epidemiology and Ecology*, Vol. 5, Monath T.P., ed. CRC Press, Boca Raton, Florida, USA, 59–88.
- JOHNSON D.J., OSTLUND E.N., PEDERSEN D.D. & SCHMITT B.J. (2001). Detection of North American West Nile virus in animal tissue by a reverse transcription-nested polymerase chain reaction assay. *Emerg. Infect. Dis.*, **7**, 739–741.
- JOSEPH S., WERNERY U., TENG J.L., WERNERY R., HUANG Y., PATERIL N.A., CHAN K.H., ELIZABETH S.K., FAN R.Y., LAU S.K., KINNE J., WOO P.C. (2016). First isolation of West Nile virus from a dromedary camel. *Emerg. Microbes Infect.*, **5**, e53.
- LANCIOTTI R.S., KERST A.J., NASCI R.S., GODSEY M.S., MITCHELL C.J., SAVAGE H.M., KOMAR N., PANELLA N.A., ALLEN B.C., VOLPE K.E., DAVIS B.S. & ROEHRIG J.T. (2000). Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J. Clin. Microbiol.*, **38**, 4066–4071.
- MACKENZIE J.S. & WILLIAMS D.T. (2009). The zoonotic flaviviruses of southern, south-eastern and eastern Asia, and Australasia: the potential for emergent viruses. *Zoonoses Public Health*, **56**, 338–356.
- MORALES M.A., BARRANDEGUY M., FABBRI C., GARCIA J.B., VISSANI A., TRONO K., GUTIERREZ G., PIGRETTI S., MENCHACA H., GARRIDO N., TAYLOR N., FERNANDEZ F., LEVIS S. & ENRIA D. (2006). West Nile virus isolation from equines in Argentina, 2006. *Emerg. Infect. Dis.*, **12**, 1559–1561.
- NATIONAL INSTITUTES OF HEALTH (NIH): NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES (NIAID) (2015). <https://www.nih.gov/news-events/news-releases/nih-funded-vaccine-west-nile-virus-enters-human-clinical-trials>
- OSTLUND E.N., ANDRESEN J.E. & ANDRESEN M. (2000). West Nile encephalitis. *Vet. Clin. North Am., Equine Pract.*, **16**, 427–441.
- OSTLUND E.N., CROM R.L., PEDERSEN D.D., JOHNSON D.J., WILLIAMS W.O. & SCHMITT B.J. (2001). Equine West Nile encephalitis, United States. *Emerg. Infect. Dis.*, **7**, 665–669.
- PANTHIER R., HANNOUN C.L., OUDAR J., BEYTOUT D., CORNIOU B., JOUBERT L., GUILLON J.C. & MOUCHET J. (1966). Isolement du virus West Nile chez un cheval de Camargue atteint d'encéphalomyélite. *C.R. Acad. Sci. (Paris)*, **262**, 1308–1310.
- PLATONOV A.E., KARAN L.S., SHOPENSKAIA T.A., FEDOROVA M.V., KOLIASNIKOVA N.M., RUSAKOVA N.M., SHISHKINA L.V., ARSHBA T.E., ZHURAVLEV V.I., GOVORUKHINA M.V., VALENTSEVA A.A. & SHIPULIN G.A. (2011). Genotyping of West Nile fever virus strains circulating in southern Russia as an epidemiological investigation method: principles and results. *Zh. Mikrobiol. Epidemiol. Immunobiol.*, **2**, 29–37.

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**, 264–274.

SCHMIDT J.R. & EL MANSOURY H.K. (1963). Natural and experimental infection of Egyptian equines with West Nile virus. *Ann. Trop. Med. Parasitol.*, **57**, 415–427.

SIRBU A., CEIANU C.S., PANCULESCU-GATEJ R.I., VAZQUEZ A., TENORIO A., REBREANU R., NIEDRIG M., NICOLESCU G., & PISTOL A. (2011). Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *EuroSurveill.* JAN 13, **16** (2).

SMITHBURN K.C., HUGHES T. P., BURKE A.W. & PAUL J.H. (1940). A neurotropic virus isolated from the blood of a native of Uganda. *Am. J. Trop. Med.*, **20**, 471–492.

SNOOK C.S., HYMANN S.S., DEL PIERO F., PALMER J.E., OSTLUND E.N. BARR B.S., DEROSCHERS A.M. & REILLY L.K. (2001). West Nile virus encephalomyelitis in eight horses. *J. Am. Vet. Med. Assoc.*, **218**, 1576–1579.

STEELE K.E., LINN M.J., SCHOEPP R.J., KOMAR N., GEISBERT T.W., MANDUCA R.M., CALLE P.P., RAPHAEL B.L., CLIPPINGER T.L., LARSEN T., SMITH J., LANCIOTTI R.S., PANELLA N.A. & MCNAMARA T.S. (2000). Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York. *Vet. Pathol.*, **37**, 208–224.

VAZQUEZ A., SANCHEZ-SECO M.P., RUIZ S., MOLERO F., HERNANDEZ L., MORENO J., MAGALLANES A., TEJEDOR C.G. & TENORIO A. (2010). Putative new lineage of West Nile virus, Spain. *Emerg. Infect. Dis.*, **16**, 549–552.

UNITED STATES DEPARTMENT OF AGRICULTURE (USDA) (2011). Animal and Plant Health Inspection Service, Veterinary Services Memorandum 800.112 Guidelines for Validation of *In Vitro* Potency Assays at: URL: http://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_112.pdf

WEINGARTL H.M., DREBOT M.A., HUBALEK Z., HALOUZKA J., ANDONOVA M., DIBERNARDO A., COTTAM-BIRT C., LARENCE J. & MARSZAL P. (2003). Comparison of assays for detection of West Nile virus antibodies in chicken sera. *Can. J. Vet. Res.*, **67**, 128–132.

WODAK E., RICHTER S., BAGÓ Z., REVILLA-FERNÁNDEZ S., WEISSENBOCK H. NOWOTNY N. & WINTER P. (2011). Detection and molecular analysis of West Nile virus infections in birds of prey in the eastern part of Austria in 2008 and 2009. *Vet. Microbiol.*, **149**, 358–366.

ZELLER H.G. & 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**, 147–156.

*

* *

NB: There are OIE Reference Laboratories for West Nile fever
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/scientific-expertise/reference-laboratories/list-of-laboratories/>).

Please contact the OIE Reference Laboratories for any further information on diagnostic tests, reagents and vaccines for West Nile fever.

NB: FIRST ADOPTED IN 2004 AS WEST NILE ENCEPHALITIS; MOST RECENT UPDATES ADOPTED IN 2018.