Workshop on Implementation of EU regulations related to Newcastle disease, Avian influenza, West Nile and Mycoplasma
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Etiology, pathogenesis, clinical signs and diagnostics in different species, sampling and laboratory methods for detection of West Nile virus and disease

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West Nile fever aetiology: West Nile virus (WNV)

- Member of the *Flaviviridae* family, *Flavivirus* genus, Japanese encephalitis virus serocomplex
  - relative of the Yellow fever virus, Dengue virus, Tick-borne encephalitis virus, etc.
  - +ssRNA genome, ~ 11,000 base
  - 3 structural and 7 non-structural proteins
  - stem-loop structures at the UTR regions
  - icosahedral capsid
  - enveloped
  - relatively weak resistance (heat, detergents)
  - strong antigen
    - the E glycoprotein is the neutralizing Ag
    - one serotype
    - cross-reactions with other flaviviruses
• WNV genome
• **WNV genetic diversity**
  – Two main genetic lineages

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**Lanciotti et al.**
• **WNV genetic diversity**
  – Two main genetic lineages
    • lineage 1: worldwide distributed
      – topotype strain: Eg101 (Egypt, 1951)
      – three clades (~ geographic distribution)
      – virulence–variants
    • lineage 2: limited distribution
      – topotype strain: B956 (Uganda, 1937)
      – Sub-Saharan Africa and Madagascar
      – strain Q3574-5 isolated in 1968 from a migrating Barred Warbler (*Sylvia nisoria*) on Cyprus (Watson et al. 1972)
- **WNV genetic diversity**
  - Two main genetic lineages
    - lineage 1: world-wide distributed
    - lineage 2: limited distribution
      - topotype strain: B956 (Uganda, 1937)
      - Sub-Saharan Africa and Madagascar
      - emergence in central Europe (Hungary) in 2004
      - emergence in Russia (Volgograd) in 2007
      - differences in virulence!
  - Further genetic lineages
    - lineage 3: Rabensburg strain
      - first isolation in the Czech Republic, from *Cx. pipiens*
      - reisolation in 1999 (*Cx. pipiens*) and in 2006 (*Ae. rossicus*)
      - no Rabensburg strain associated cases reported in the area
      - limited pathogenicity in mice
• **WNV genetic diversity**
  - Two main genetic lineages
    - lineage 1: world-wide distributed
    - lineage 2: limited distribution
  - Further genetic lineages
    - lineage 3: Rabensburg strain
    - lineage 4: LEIV-Krnd88-190
      - isolated in Russia (Caucasus) in 1998
      - isolated from ticks (*Dermacentor marginatus*)
      - virulence is unknown
• WNV genetic diversity
  – Two main genetic lineages
    • lineage 1: worldwide distributed
    • lineage 2: limited distribution
  – Further genetic lineages
    • lineage 3: Rabensburg strain
    • lineage 4: LEIV-Krnd88-190 (Lvov et al., 2004)
    • lineage 5: Indian isolates (Bondre et al., 2007)
    • lineage 6: Malaysian isolate (Scherret et al., 2001)
    • lineage 7: Spanish isolate (Vazquez et al., 2010)
WNV ecology

• Transmission cycle
  – natural hosts of WNV are wild birds
    • several species are susceptible (USA: 371 bird species)
    • frequent subclinical infections
      – varying length of viraemia
      – varying level of virus titre in the blood (amplifying host)
      – transient shedding with faeces and body fluids
    • certain species are more vulnerable
      – USA: 62% of the WNV positive dead birds were American crows and Blue jays
  – main vectors are mosquitoes
    • several species are potential carriers
      – USA: 62 WNV positive mosquito species reported
      – >98% of the positive pools are of *Culex spp.*
      – in Europe the principal vector is usually *Culex pipiens*
        » ornithophillic
        » overwinters in adult form
        » gradation in late summer and autumn
      – the extrinsic period is significantly influenced by the weather
infected mosquitoes

disseminated infection in mosquitoes

transmitting mosquitoes

ratio
days after feeding

Kilpatrick et al., PloS Path. 2008. 4/6, e1000092
Transmission cycle of WNV

- natural host of WNV are wild birds
- main vectors are mosquitoes
- tick are also potential vectors
- several other vertebrates are susceptible hosts
  - mammals (humans, horses), reptiles (alligators), amphibians (frogs)
  - incidental hosts – the mosquitoes’ choice
  - dead-end hosts – viraemia is lower than the mosquito infection threshold
- possible non-vectorial transmission
  - iatrogenic – blood transfusion, organ transplantation
  - intrauterine, lactogenic – reported in humans
  - accidental – necropsy, laboratory infections
  - peroral
    - scavenger birds – carrion feeding (i.e. caws)
    - birds of prey (i.e. goshawks)
    - virus shedding by faeces (common coots, grebes?)
WNV pathogenesis

- local multiplication → viraemia
- ~ 80% of the infections remain subclinical
- ~ 20% acute febrile illness – West Nile fever
- ~ 1-0.1% neuroinvasive infection – West Nile encephalitis
- neuroinvasiveness: tumour necrosis factor-α, toll-like receptors
- neuron apoptosis, inflammatory processes
- genetic markers of the virus may influence neuroinvasiveness
- antibodies emerge on 7-11. days post infection
- chronic infections are rare
  - shedding of WNV RNA in urine of humans several years after infection
  - detection of WNV RNA in brain tissue of birds years after initial infection
WNV – Clinical signs

- Animals
  - Birds
    - Frequent subclinical infections
    - Vulnerable species: raptors, corvids, passeriformes (?)
    - Poultry: goose
    - Encephalitis, weakness, inappetite, death
  - Mammals
    - Typically horses
    - Less frequently dogs, cats, sheep and others
    - Experimentally mice

- Humans
  - Incubation period of 2-15 days
  - West Nile fever:
    - Fever, headache
    - Nausea, vomiting
    - Rash, lymphadenopathy
  - Rarely hepatitis, myocarditis, pancreatitis
  - West Nile encephalitis
WNV encephalitis in humans

- Meningitis: Fever, nuchal rigidity, CSF pleocytosis
- Encephalitis: Altered mental status

- **Tremor (~94%)**
  - Sometimes associated with other viruses
  - Static / kinetic; sometimes with movement
  - Occasionally disabling

- **Myoclonus (~63%)**
  - Upper extremity, facial involvement most frequent
  - Nocturnal myoclonus
  - Both tremor and myoclonus – onset generally > 5 days following initial symptoms

- **Parkinsonism (~68%)**
  - Cogwheel rigidity, bradykinesia, postural instability
  - Rest tremor not observed
  - Seen both in encephalitis and meningitis cases

Sejvar et al., CDC
Acute flaccid paralysis (rare)
- Relatively young; lack of premorbid conditions
- May have absence of fever, headache
- Clinical hallmarks:
  - Onset during acute infection
  - Asymmetry of weakness
  - Absence of sensory changes
  - Elevation of CSF protein and WBC
- Multiple alternative diagnoses (stroke, GBS, myopathy)
- Syndrome actually localized to spinal anterior horn cells - resultant poliomyelitis
- Recognition could limit unnecessary diagnostic procedures, treatment
- Little or no improvement short-term

Rhabdomyolysis – acute destruction of skeletal muscle cells
- Infrequent manifestation of viral infection
- September 2002 – rhabdomyolysis reported in Chicago WNV patients: 14 cases
- Trauma, medication effect unlikely; further studies to assess association

Flaccid paralysis with sensory symptoms
- Neuropathic pain, causalgia, paresthesias
- Peripheral neuropathy, polyradiculopathy
- Optic neuritis
- Acute demyelinating encephalomyelitis (ADEM)
- Prenatal WNV infection with CNS developmental abnormalities

Sejvar et al., CDC
WNV encephalitis in humans

Clinical outcome data

• Fatality rates
  – 10% fatality rate in CNS disease
  – Elderly, immunosuppressed
  – Independent risk factors unknown

• Long-term outcomes in NYC:
  – >50% with continued impairment at 1 year
  – Only 37% considered fully recovered

• Follow-up telephone query data
  – Persistent / chronic headache
  – Concentration, memory difficulties
  – Overwhelming fatigue
  – Persistence of tremor, parkinsonism

• Paralysis – no short-term improvement

Sejvar et al., CDC
WNV – Diagnosis

- Epizootiology, clinical signs
  - seasonal (midsummer to fall) ~ infected mosquitoes
  - suspicion if CNS signs are seen

- Laboratory diagnosis
  - direct virus detection
    - virus isolation
    - immunohistochemistry, *in situ* hybridisation
    - RT-PCR, qRT-PCR
  - virus serology
    - cross-reactions! – TBEV, Usutu virus
    - virus neutralisation, plaque-reduction neutralisation test (PRNT)
    - haemagglutination-inhibition, indirect immunofluorescence, ELISA

- Differential dg.: from febrile illnesses with CNS signs
## WNV – Laboratory diagnosis

- OIE World Organisation for Animal Health
- Chapter 2.1.20. West Nile fever
  - [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_WEST_NILE.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_WEST_NILE.pdf)

### B. Diagnostic Techniques

<table>
<thead>
<tr>
<th>Method</th>
<th>Population freedom from infection</th>
<th>Individual animal freedom from infection</th>
<th>Confirmation of clinical cases</th>
<th>Prevalence of infection – surveillance</th>
<th>Immune status in individual animals or populations post-vaccination</th>
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<tbody>
<tr>
<td>Nested RT-PCR</td>
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<td>Real time RT-PCR</td>
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<td>Isolation in tissue culture</td>
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<td>IgM capture ELISA</td>
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<td>Plaque reduction neutralisation</td>
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<td>Serum neutralisation</td>
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<td>Immunohistochemistry</td>
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*Table 1. Test methods available for the diagnosis of West Nile fever and their purpose*

Key: +++ = recommended method; ++ = suitable method; + may be used in some situations, but cost, reliability, or other factors severely limits its application; -- = not appropriate for this purpose.

Although not all of the tests listed as category +++ or ++ have undergone formal standardisation and validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.

RT-PCR = reverse-transcriptase polymerase chain reaction; IgM = immunoglobulin; ELISA = enzyme-linked immunosorbent assay.
WNV – Laboratory diagnosis

– OIE World Organisation for Animal Health
– Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2013
– Chapter 2.1.20. West Nile fever
  • [http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_WEST_NILE.pdf](http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.20_WEST_NILE.pdf)

– Sample collection:
  • For direct virus detection
    – Animals with clinical signs
      » anticoagulant-treated blood
      » cerebrospinal fluid
      » urine, faeces
    – Dead animals
      » brain tissue (hindbrain, medulla, spinal chord)
      » myocardium, spleen, kidney, lung, liver, lymph nodes
    – Mosquitoes
  
  • For serology
    – blood serum
    – cerebrospinal fluid
Identification of the agent

- *In vitro* and *in vivo* culture – virus isolation
  - suckling mouse brain
  - RK-13, Vero, PK-15, primary goose embryo fibroblast
  - embryonated egg – allantoic / amniotic cavity inoculation
  - usually successful in the early stage of the infection
  - in BSL3 laboratory

- Immunohistochemistry
  - monoclonal antibodies (?)
  - successful in ~ 50%, if death is within 1 week

- *In situ* hybridisation – more specific, less sensitive

- RT-PCR
  - Conventional RT-PCR
    » detection from brain and tissue samples
    » pan-flavi systems – broad spectrum
  - Nested RT-PCR
    » higher sensitivity, higher risk of contamination
    » PBMC, mosquito pools
  - Quantitative, real-time RT-PCR
    » TaqMan – more sensitive and specific

Determination of the nucleotide sequence of the amplicon is useful
WNV – Laboratory diagnosis

- Serology
  - ELISA
    - Competitive, IgG ELISA
      - host-independent
      - cross-reactive with other flaviviruses
      - not discriminative (vaccination)
      - for screening
    - Equine IgM capture ELISA
      - for clinical diagnosis
  - Indirect immunofluorescence, haemagglutination-inhibition
    - Usually in-house assays
    - less specific, less reliable, more cross-reactions
    - for screening or for clinical diagnosis (seroconversion)
  - Neutralisation assays
    - PRNT, microtitre VN assay
    - most specific, less sensitive, indicates protection
    - in BSL-3 lab, time-consuming
    - for validation of the ELISA / IFA / HAI results
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