SUMMARY

Eastern and Western equine encephalomyelitis viruses belong to the genus Alphavirus of the family Togaviridae. Alternate infection of birds and mosquitoes maintain these viruses in nature. The disease occurs sporadically in horses and humans from mid-summer to late autumn. Horses and humans are tangential dead-end hosts. The disease in horses is characterised by fever, anorexia, and severe depression. In severe cases, the disease in horses progresses to hyperexcitability, blindness, ataxia, severe mental depression, recumbency, convulsions, and death. Eastern equine encephalomyelitis (EEE) virus infection in horses is often fatal, while Western equine encephalomyelitis (WEE) virus can cause a subclinical or mild disease with less than 30% mortality. EEE and WEE have been reported to cause disease in poultry, game birds and ratites. Sporadic cases of EEE have been reported in cows, sheep, pigs, deer, and dogs.

Identification of the agent: A presumptive diagnosis of EEE or WEE can be made when susceptible horses display the characteristic somnolence and other signs of neurological disease in areas where haematophagous insects are active. There are no characteristic gross lesions. Histopathological lesions can provide a presumptive diagnosis. EEE virus can usually be isolated from the brain and sometimes other tissues of dead horses, however WEE virus is rarely isolated. EEE and WEE viruses can be isolated from field specimens by inoculating newborn mice, embryonating chicken eggs, cell cultures, or newly hatched chickens. The virus is identified by complement fixation (CF), immunofluorescence, or plaque reduction neutralisation (PRN) tests. EEE and WEE viral RNA may also be detected by reverse-transcription polymerase chain reaction methods.

Serological tests: Antibody can be identified by PRN, haemagglutination inhibition (HI), CF tests, or IgM capture enzyme-linked immunosorbent assay.

Requirements for vaccines and diagnostic biologicals: EEE and WEE vaccines are safe and immunogenic. They are produced in cell culture and inactivated with formalin.

A. INTRODUCTION

Eastern equine encephalomyelitis (EEE) and Western equine encephalomyelitis (WEE) viruses are members of the genus Alphavirus of the family Togaviridae. The natural ecology for virus maintenance occurs via alternate infection of birds and ornithophilic mosquitoes. Clinical disease may be observed in humans and horses, both of which are dead-end hosts for these agents. EEE has been diagnosed in Quebec and Ontario in Canada, central and eastern regions of the United States of America (USA), the Caribbean Islands, Mexico, and Central and South America. Disease caused by the WEE virus has been reported in the western USA and Canada, Mexico, and Central and South America (13, 16, 24). Highlands J virus, antigenically related to WEE virus, has been isolated in eastern USA. Although Highlands J virus is generally believed not to cause disease in mammals, it has been isolated from the brain of a horse dying of encephalitis in Florida (7).

Even though the mortality is lower for WEE, the clinical signs of EEE and WEE can be identical. The disease caused by either virus is also known as sleeping sickness. Following an incubation period of 5–14 days, clinical signs include fever, anorexia, and depression. A presumptive diagnosis of EEE or WEE virus infection in unvaccinated horses can be made if the characteristic somnolence is observed during the summer in temperate climates or the wet season in tropical and subtropical climates, when the mosquito vector is plentiful. However, a number of other diseases, such as West Nile virus and Venezuelan equine encephalomyelitis (chapters 2.1.20 and 2.5.14, respectively), produce similar clinical signs and the diagnosis must be confirmed by the described diagnostic test methods. WEE virus infection in horses is often observed over a wide geographical area, e.g. sporadic cases over 1000 square miles. EEE virus infections are usually observed in limited geographical areas.
Isolated events of high mortality in captive-raised game birds, primarily pheasants, chukars, aquarium penguins, and quail have been traced to WEE, EEE, or Highlands J virus infection (13, 16, 19). Most encephalomyelitis infections in domestic fowl are caused by EEE virus and occur on the east coast states of the USA. The virus is introduced by mosquitoes, but transmission within the flocks is primarily by feather picking and cannibalism. Both EEE and WEE viruses have caused a fatal disease in rats. Haemorrhagic enteritis has been observed in emus infected with EEE and WEE viruses, and morbidity and mortality rates may be greater than 85%. Highlands J and EEE viruses have been found to produce depression, somnolence, decreased egg production, and increased mortality in turkeys (5). EEE virus has been reported to cause disease in cows (11, 14), sheep (1), pigs (3), white-tailed deer (18), and dogs (4).

EEE virus causes severe disease in humans with a mortality rate of 30–70% and a high frequency of permanent sequelae in patients who survive. WEE is usually mild in adult humans, but can be a severe disease in children. The fatality rate is between 3 and 14%. Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be performed at containment level 3 (see Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities). It is recommended that personnel be immunised against EEE and WEE viruses (22). Precautions should also be taken to prevent human infection when performing post-mortem examinations on horses suspected of being infected with the equine encephalomyelitis viruses.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

The most definitive method for diagnosis of EEE or WEE is the isolation of the viruses. EEE virus can usually be isolated from the brains of horses, unless more than 5 days have elapsed between the appearance of clinical signs and the death of the horse. EEE virus can frequently be isolated from brain tissue even in the presence of a high serum antibody titre. WEE virus is rarely isolated from tissues of infected horses. Brain is the tissue of choice for virus isolation, but the virus has been isolated from other tissues, such as the liver and spleen. It is recommended that a complete set of these tissues be collected in duplicate, one set for virus isolation and the other set in formalin for histopathological examination. Specimens for virus isolation should be sent refrigerated if they can be received in the laboratory within 48 hours of collection; otherwise, they should be frozen and sent with dry ice. A complete set of tissues will allow the performance of diagnostic techniques for other diseases. For isolation, a 10% suspension of tissue is prepared in phosphate buffered saline (PBS), pH 7.8, containing bovine serum albumin (BSA) (fraction V; 0.75%), penicillin (100 units/ml), and streptomycin (100 µg/ml). The suspension is clarified by centrifugation at 1500 g for 30 minutes.

EEE and WEE viruses can be isolated in a number of cell culture systems. The most commonly used cell cultures are primary chicken or duck embryo fibroblasts, continuous cell lines of African green monkey kidney (Vero), rabbit kidney (RK-13), or baby hamster kidney (BHK-21). Isolation is usually attempted in 25 cm² cell culture flasks. Confluent cells are inoculated with 1.0 ml of tissue suspension. Following a 1–2-hour absorption period, maintenance medium is added. Cultures are incubated for 6–8 days, and one blind passage is made. EEE and WEE viruses will produce a cytopathic change in cell culture. Cultures that appear to be infected are frozen. The fluid from the thawed cultures is used for virus identification.

The newborn mouse is also considered to be a sensitive host system. Inoculate intracranially one or two litters of 1–4-day-old mice with 0.02 ml of inoculum using a 26-gauge 3/8 inch (9.3 mm) needle attached to a 1 ml tuberculin syringe. The inoculation site is just lateral to the midline in the midportion of one lateral hemisphere. Mice are observed for 10 days. Mice that die within 24 hours of inoculation are discarded. From 2 to 10 days postinoculation, dead mice are collected daily and frozen at −70°C. Mouse brains are harvested for virus identification by aspiration using a 20-gauge 1 inch (2.5 cm) needle attached to a 1 ml tuberculin syringe. A second passage is made only if virus cannot be identified from mice that die following inoculation.

The chicken embryo is considered to be less sensitive than newborn mice when used for primary isolation of EEE and WEE viruses. Tissue suspensions can be inoculated by the yolk-sac route into 6–8-day-old embryonating chicken eggs. There are no diagnostic signs or lesions in the embryos infected with these viruses. Inoculated embryos should be incubated for 7 days, but deaths usually occur between 2 and 4 days post-inoculation. Usually only one passage is made unless there are dead embryos from which virus cannot be isolated. Newly hatched chickens are susceptible and have been used for virus isolation. If this method is used, precautions must be taken to prevent aerosol exposure of laboratory personnel, as infected birds can shed highly infectious virus.

EEE or WEE viruses can be identified in infected mouse or chicken brains, cell culture fluid, or amnionic-allantoic fluid by complement fixation. A 10% brain suspension is prepared in veronal (barbitone) buffer; egg and cell culture fluids are used undiluted or diluted 1/10 in veronal buffer. The fluid or suspension is centrifuged at 9000 g for 30 minutes, and the supernatant fluid is tested against hyperimmune serum or mouse ascitic fluid prepared against EEE and WEE viruses using a standard CF procedure (21). The CF test requires the overnight incubation at 4°C of serum-antigen with 7 units of complement. Virus can be identified in cell culture by direct
immunofluorescent staining. The less commonly used method of virus identification is the neutralisation test, as outlined below.

Reverse-transcription polymerase chain reaction (RT-PCR) methods to detect EEE, WEE and VEE viral nucleic acid in mosquitoes and vertebrate tissues have been described, although few have been extensively validated for mammalian samples (8, 9, 12, 23). A multiplex PCR method was developed to expedite differential diagnosis in cases of suspected EEE or West Nile arboviral encephalomyelitis in horses (6). The assay has enhanced speed and sensitivity compared to cell culture virus isolation and has been used effectively in the USA during several recent arbovirus seasons. Recently, a combination of an RT-PCR with an enzyme-linked immunosorbent assay (ELISA: RT-PCR-ELISA) was reported as a method to identify alpha-viruses that are pathogenic to humans (25).

Antigen-capture ELISA has been developed for EEE surveillance in mosquitoes. This can be used in countries that do not have facilities for virus isolation or PCR (2). Immunohistochemical procedures for diagnosis of EEE have also been described (15).

2. Serological tests

Serological confirmation of EEE or WEE virus infection requires a four-fold or greater increase or decrease in antibody titre in paired serum samples collected 10–14 days apart. Most horses infected with EEE or WEE virus have a high antibody titre when clinical disease is observed. Consequently, a presumptive diagnosis can be made if an unvaccinated horse with appropriate clinical signs has antibody against only EEE or WEE virus. The detection of IgM antibody by the ELISA can also provide a presumptive diagnosis of acute infection (17). The plaque reduction neutralisation (PRN) test or, preferably, a combination of PRN and haemagglutination inhibition (HI) tests is the procedure most commonly used for the detection of antibody against EEE and WEE viruses. There may be cross-reactions between antibody against EEE and WEE virus in the CF and HI tests. CF antibody against both EEE and WEE viruses appears later and does not persist; consequently, it is less useful for the serological diagnosis of disease.

a) Complement fixation

The CF test is frequently used for the demonstration of antibodies, although the antibodies detected by the CF test may not persist for as long as those detected by the HI or PRN tests. A sucrose/acetone mouse brain extract is commonly used as antigen. The positive antigen is inactivated by treatment with 0.1% beta-propiolactone.

In the absence of an international standard serum, the antigen should be titrated against a locally prepared positive control serum. The normal antigen, or control antigen, is mouse brain from uninoculated mice similarly extracted and diluted.

Sera are diluted 1/4 in veronal buffered saline containing 1% gelatin (VBSG), and inactivated at 56°C for 30 minutes. Titrations of positive sera may be performed using additional twofold dilutions. The CF antigens and control antigen (normal mouse brain) are diluted in VBSG to their optimal amount of fixation as determined by titration against the positive sera; guinea-pig complement is diluted in VBSG to contain 5 complement haemolytic units-50% (CH50). Sera, antigen, and complement are reacted in 96-well round-bottom microtitre plates at 4°C for 18 hours. The sheep red blood cells (SRBCs) are standardised to 2.8% concentration. Haemolysin is titrated to determine the optimal dilution for the lot of complement used. Haemolysin is used to sensitise 2.8% SRBCs and the sensitised cells are added to all wells on the microtitre plate. The test is incubated for 30 minutes at 37°C. The plates are then centrifuged (200 g), and the wells are scored for the presence of haemolysis. The following controls are used: (a) serum and control serum each with 5 CH50 and 2.5 CH50 of complement; (b) CF antigen and control antigen each with 5 CH50 and 2.5 CH50 of complement; (c) complement dilutions of 5 CH50, 2.5 CH50 and 1.25 CH50; and (d) cell control wells with only SRBCs and VBSG diluent. These controls test for anticomplementary serum and anticomplementary antigen, activity of complement used in the test, and integrity of the SRBC indicator system in the absence of complement, respectively.

To avoid anticomplementary effects, sera should be separated from the blood as soon as possible. Positive and negative control sera should be used in the test.

b) Haemagglutination inhibition

The antigen for the HI test is the same as described above for the CF test. The antigen is diluted so that the amount used in each haemagglutinating unit (HAU) is from four to eight times that which agglutinates 50% of the RBCs in the test system. The haemagglutination titre and optimum pH for each antigen are determined with goose RBCs diluted in pH solutions ranging from pH 5.8 to pH 6.6, at 0.2 intervals.

Sera are diluted 1/10 in borate saline, pH 9.0, and then inactivated at 56°C for 30 minutes. Kaolin treatment is used to remove nonspecific serum inhibitors. Alternatively, nonspecific inhibitors may be removed by
acetone treatment of serum diluted 1/10 in PBS followed by reconstitution in borate saline. Sera should be absorbed before use by incubation with a 0.05 ml volume of washed packed goose RBCs for 20 minutes at 4°C.

Following heat inactivation, kaolin treatment and absorption, twofold dilutions of the treated serum are prepared in borate saline, pH 9.0 with 0.4% bovabumin. Serum dilutions (0.025 ml/well) are prepared in a 96-well round-bottom microtitre plate in twofold dilutions in borate saline, pH 9.0, with 0.4% bovabumin. Antigen (0.025 ml/well) is added to the serum. Plates are incubated at 4°C overnight. RBCs are derived from normal white male geese¹ and washed three times in dextrose/gelatin/veronal (DGV), and a 7.0% suspension is prepared in DGV. The 7.0% suspension is then diluted 1/24 in the appropriate pH solution, and 0.05 ml per well is added immediately to the plates. Plates are incubated for 30 minutes at 37°C. Positive and negative control sera are incorporated into each test. A test is considered to be valid only if the control sera give the expected results. Titres of 1/10 and 1/20 are suspect, and titres of 1/40 and above are positive.

c) Enzyme-linked immunosorbent assay

The ELISA is performed by coating flat-bottomed plates with anti-equine IgM capture antibody (17). The antibody is diluted according to the manufacture’s recommendations in 0.5 M carbonate buffer, pH 9.6, and 50 µl is added to each well. The plates are incubated at 37°C for 1 hour, and at 4°C overnight. Prior to use, the coated plates are washed three times with 200–300 µl/well of 0.01 M PBS containing 0.05% Tween 20. After the second wash, 200 µl/well of PBS/Tween/5% nonfat dried milk is added and the plates are incubated at room temperature for 1 hour. Following incubation, the plates are washed again, three times with PBS/Tween. Test and control sera are diluted 1/400 in 0.01 M PBS, pH 7.2, containing 0.05% Tween 20, and 50 µl is added to each well. The plates are incubated at 37°C for 90 minutes and then washed three times. Next, 50 µl of viral antigen is added to all wells. (The dilution of the antigen will depend on the source and should be empirically determined) The plates are incubated overnight at 4°C, and washed three times. Then, 50 µl of horseradish-peroxidase-conjugated monoclonal antibody (MAB) to encephalitis virus² is added. The plates are incubated for 90 minutes at 37°C and then washed six times. Finally, 50 µl of freshly prepared ABTS (2,2’-Azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)) substrate + hydrogen peroxidase is added, and the plates are incubated at room temperature for 15–40 minutes The absorbance of the test serum is measured 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 normal antigen.

d) Plaque reduction neutralisation

The PRN test is very specific and can be used to differentiate between EEE and WEE virus infections. The PRN test is performed in duck embryo fibroblast, Vero, or BHK-21 cell cultures in 25 cm² flasks or six-well plates. Volumes listed are for flasks; the volume should be halved for wells in six-well plates. The sera can be screened at a 1/10 and 1/100 final dilution. Endpoints can be established using the PRN or HI test. Serum used in the PRN assay is tested against 100 plaque-forming units (PFU) of virus (50 PFU for six-well plates). The virus/serum mixture is incubated at 37°C for 75 minutes before inoculation on to confluent cell culture monolayers in 25 cm² flask. The inoculum is adsorbed for 1 hour, followed by the addition of 6 ml of overlay medium. The overlay medium consists of two solutions that are prepared separately. Solution I contains 2 X Earle’s Basic Salts Solution without phenol red,  4% fetal bovine serum, 100 µg/ml gentamicin, 200 µg/ml nystatin, 0.45% solution of sodium bicarbonate, and 0.002% neutral red. When duck embryo fibroblasts are used, Solution I also contains 6.6% yeast extract lactalbumin hydrolysate. 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 48–72 hours, and endpoints are based on a 90% reduction in the number of plaques compared with the virus control flasks, which should have about 100 plaques.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Inactivated vaccines against EEE and WEE viruses are available commercially. Attenuated EEE and WEE virus vaccines have not proven satisfactory. The vaccines licensed for use in the USA are prepared using the following combinations: EEE and WEE; EEE, WEE, and Venezuelan equine encephalomyelitis (VEE); and EEE and VEE. In addition, tetanus toxoid, inactivated influenza virus, and inactivated West Nile virus have been combined with

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¹ RBCs from adult domestic white male geese are preferred, but RBCs from other male geese can be used. If cells from female geese are used, there may be more test variability. It has been reported that rooster RBCs cause a decrease in the sensitivity of the test.
² Available from: Centers for Disease Control and Prevention, Biological Reference Reagents, 1600 Clifton Road NE, Mail Stop C21, Atlanta, Georgia 30333, United States of America.
EEE and WEE or EEE, WEE, and VEE. Early vaccines were produced from virus propagated in embryonating chicken eggs and inactivated with formalin. Current vaccines are prepared from virus propagated in cell culture, and inactivated with formalin (10) or monoethylamine.

1. **Seed management**
   
a) **Characteristics of the seed**

   Standard strains of EEE and WEE viruses that were isolated over 20 years ago have been used for vaccine production and have been proven to produce a protective immunity. Strains of EEE virus that differ antigenically and in molecular structure have been identified from different geographical regions. However, the North American and Caribbean isolates appear to be similar (26). Strains of WEE virus isolated from different countries have been found to be similar both by MAb testing and RNA oligonucleotide fingerprinting analysis (16). A recent well-characterised isolate from the country where the vaccine is to be used would be advantageous. Viruses that are selected must be immunogenic and replicate to high titres in cell culture.

b) **Method of culture**

   Primary chicken embryo fibroblasts and Vero cells have been used for propagation of viruses used for vaccine production. The fibroblasts should be prepared from specific pathogen free embryos. Other susceptible cell lines could also be used.

c) **Validation as a vaccine**

   If a cell line is used, the master cell stock is tested to confirm the identity of the cell line, species of origin, and freedom from extraneous agents. If primary cell cultures are used, a monolayer from each batch of each subculture should be tested for extraneous agents including bacteria, fungi, mycoplasma, and viruses. The master seed virus should also be tested to ensure freedom from bacteria, fungi, mycoplasma, and extraneous viruses (see Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials for a description of the method).

   The vaccines are administered by the intramuscular (in most cases) or intradermal route in the cervical region in two doses given 2–4 weeks apart. Annual revaccination is recommended. All foals vaccinated before 1 year of age should be revaccinated before the next vector season.

2. **Method of manufacture**

   Details of the manufacture of vaccines currently on the market are not available. Consequently, the information provided here is intended only as background reference material on the vaccines and not as a method of manufacture. The virus and cell culture system should be selected so that a high virus titre, ≥10^6 TCID50 (50% tissue culture infective dose) per ml, is obtained in under 48 hours. Virus for vaccine production can be prepared from the supernatant fluid from infected cell cultures. The fluid is harvested when 70–100% of the monolayers have the characteristic cytopathic changes. The virus titre is determined by titration in cell culture or mice. The fluid is clarified by low speed centrifugation and filtered through gauze. The virus is inactivated by adding formalin to a final concentration of 1/2000 (0.05%) and holding at 37°C for 24 hours. Residual formaldehyde is neutralised by sodium bisulphite (10). The residual free formalin content in the inactivated vaccine should not exceed 0.2% formaldehyde.

3. **In-process control**

   Cultures should be examined daily for virus-induced cytopathic effect. The harvested virus should be tested for microbial contamination. The efficacy of the inactivation process should be checked by testing for viable virus.

4. **Batch control**

   a) **Sterility**

      Tests for sterility and freedom from contamination of biological materials may be found in Chapter 1.1.9.

   b) **Safety**

      The inactivated vaccine is safety tested by inoculating subcutaneously at least ten 6–12-hour-old chicks with 0.5 ml of the vaccine. The chickens are observed each day for 10 days for unfavourable reactions that are attributable to the vaccine (20). Safety testing can also be carried out by inoculating intracerebrally at least eight 1–4-day-old mice with 0.02 ml of the vaccine, and observing for 7 days. It is critical that safety tests be conducted on each lot of vaccine to insure that there is no residual virulent virus present.

   c) **Potency**
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Potency testing is performed by inoculating each of ten guinea pigs with either EEE or WEE virus, using one-half the horse dose on two occasions, 14–21 days apart, by the route recommended for the horse. Serum samples from each vaccinate and each control are tested 14–21 days after the second dose using the PRN test. The EEE titres should be $\geq 1/40$, and the WEE titres should be $\geq 1/40$ (20), using Vero cells. If duck embryo fibroblasts are used in the PRN test, the titres will be lower. An alternative potency test is to use intracerebral challenge, 14–21 days after the second vaccination. Each guinea pig is inoculated with 0.1 ml of virus containing 100 LD$_{50}$ (50% lethal dose). Simultaneous titration is carried out. In order for the vaccine to be approved, 80% of the guinea pigs must survive both viruses.

d) Duration of immunity

Comprehensive studies on duration of immunity are not available. An annual revaccination is recommended. Foals that are vaccinated before 1 year of age should be revaccinated before the next vector season.

e) Stability

The lyophilised vaccine is stable and immunogenic for 3 years if kept refrigerated at 2–7°C. After 3 years, vaccine should be discarded. The vaccines should be used immediately after reconstitution.

f) Preservatives

The preservatives used are thimerosal at a 1/10,000 dilution and antibiotics (neomycin, polymyxin amphotericin B and gentamicin).

g) Precautions (hazards)

Severe infection and death caused by EEE and WEE viruses have been reported in laboratory workers; therefore, any work with these viruses must be carried out at least in a containment level 3 laboratory (see Chapter 1.1.2) using biological safety cabinets, and it is recommended that personnel be immunised against EEE and WEE viruses (22).

Pregnant mares and foals under 2 weeks old should not be vaccinated.

5. Tests on the final product

a) Safety

See Section C.4.b.

b) Potency

See Section C.4.c.

REFERENCES


Chapter 2.5.5. – Equine encephalomyelitis (Eastern and Western)


NB: There is an OIE Reference Laboratory for Equine encephalomyelitis (Eastern and Western) (see Table in Part 3 of this Terrestrial Manual or consult the OIE Web site for the most up-to-date list: www.oie.int).