CHAPTER 2.1.10.

JAPANESE ENCEPHALITIS

SUMMARY

Description of the disease: *Japanese encephalitis virus* (*JEV*) *is a member of the genus* Flavivirus *in the family* Flaviviridae *and causes encephalitis, principally in horses and humans. JEV also infects pigs where it causes abortions and stillbirths. JEV is maintained in nature between mosquitoes, pigs and water birds. The major vector of JEV throughout most of Asia is* Culex tritaeniorhynchus, *however other species may be locally important. Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread in the environment. The disease has been observed in large parts of Asia and recently in the western Pacific region. In horses, the infection is usually inapparent. Affected horses show clinical signs that include pyrexia, depression, muscle tremors, and ataxia. In pigs, abortions and stillbirths can occur when pregnant sows are infected with JEV for the first time. Infected pregnant sows usually show no clinical signs.*

Identification of the agent: *For virus isolation, brain material is collected from sick or dead horses that have demonstrated the clinical signs of encephalitis. Isolation procedures include the inoculation of mice and cell cultures. A suspension of brain tissue is inoculated intracerebrally into 2–4-day-old mice. If the mice show neurological signs followed by death within 14 days, then virus identification can be carried out by cell culture. The virus can also be isolated in primary cell cultures made from chicken embryos, porcine or hamster kidney cells and established cell lines such as African green monkey kidney* (*Vero*)*, baby hamster kidney* (*BHK-21*)*, or mosquito* (*C6/36*) *cells. Identification of the virus isolated in mice or in tissue cultures is confirmed by serological or nucleic acid detection methods such as reverse-transcription polymerase chain reaction assay.*

Serological tests: *Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing Japanese encephalitis in diseased individuals. The assay methods include virus neutralisation* (*VN*), *haemagglutination inhibition* (*HI*)*, complement fixation* (*CF*)*, and enzyme-linked immunosorbent assay* (*ELISA*). *There is serological cross reactivity with other flaviviruses, such as West Nile virus, which can confuse the diagnosis. The plaque reduction VN test is the most specific and can be used to differentiate JEV infection from other flavivirus infections. Because of the cross-neutralisation within the Japanese encephalitis serocomplex, serological investigations should include related co-circulating flaviviruses tested in parallel.*

Requirements for vaccines: *Two types of vaccines are commercially available in several Asian countries for humans and animals. For horses, inactivated vaccines prepared from infected mouse brains or in cell cultures have been used. For pigs, inactivated and live-attenuated vaccines are available.*

A. INTRODUCTION

Japanese encephalitis (JE) is a disease caused by a mosquito-borne flavivirus that elicits clinical signs of encephalitis in infected humans and horses and can be fatal (Fenner *et al*., 1992; Hoke Jr & Gingrich, 1994). However infections in humans and horses usually result in subclinical infection. JE virus (JEV) also causesreproductive failure in sows, leading to abortion, stillbirths or fetal mummification, though infected pregnant sows usually demonstrate no clinical signs and the infection does not affect the future pregnancies (Williams *et al.,* 2012).

JEV is maintained in nature among mosquitoes, wild birds and pigs. Pigs act as important amplifiers of the virus, and birds can also be involved in its amplification and spread. The principal vector of JEV is *Culex tritaeniorhynchus* in most parts of Asia. Other culicine mosquitoes also play a role as vectors. Because of low

titres and short duration of viraemia, humans and horses do not transmit viruses to biting mosquitoes and are considered as dead-end hosts. JEV is widespread in eastern, south-eastern and southern Asian countries and has recently spread to western India and to the western Pacific region including the eastern Indonesian archipelago, Papua New Guinea and Northern Australia (Mackenzie *et al.*, 2007).

JEV belongs to the genus *Flavivirus* in the family *Flaviviridae*. JEV is the type member of the Japanese encephalitis serocomplex, along with several important zoonotic viruses including West Nile virus (see chapter 2.1.24), St Louis encephalitis virus and Murray Valley encephalitis virus. Only a single serotype of JEV has been identified, although antigenic and genetic differences among JEV strains have been demonstrated by several techniques including complement fixation, haemagglutination inhibition, neutralisation tests using polyclonal or monoclonal antibodies (Ali & Igarashi 1997; Banerjee, 1986; Hale & Lee, 1954; Hasegawa *et al*., 1994; Kimura-Kuroda & Yasui, 1986) and oligonucleotide fingerprints of viral RNA (Banerjee & Ranadive, 1989; Hori *et al.*, 1986). Envelope (E) gene analysis was shown to be a good representative of the phylogenetic analysis of JEV. To date, five genotypes of JEV have been described based on phylogenetic analysis of the viral E gene (Solomon *et al*., 2003; Uchil & Sachidanandam, 2001; Williams *et al*., 2000).

B. DIAGNOSTIC TECHNIQUES

Table 1. *Test methods available for the diagnosis of Japanese encephalitis 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 identification[1]						
Virus isolation	–	–	–	+++	–	–
Antigen detection	+	+	+	+	+	–
Real-time RT-PCR	++	++	++	++	++	–
Detection of immune response						
HI	++	+++	++	+++	+++	+++
CFT	+	+	+	+	+	+
ELISA	++	++	++	++	++	++
VN (PRNT)	+	++	+	+++	++	++
IFAT	+	+	+	+	+	+

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; n/a = not applicable.
Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
RT-PCR = reverse-transcription polymerase chain reaction; HI = haemagglutination inhibition; CFT = complement fixation test; ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation; PRNT: plaque reduction neutralisation test; IFAT = indirect fluorescent antibody test.

The definitive diagnosis of JE in horses, or in cases of reproductive failure in sows, depends on the isolation or detection of the causal virus in neurological specimens. The isolation rate of virus from diseased or dead horses is usually very low, which may be due to the instability of the virus under certain environmental conditions, and also to the presence of antibody in infected animals. Clinical, serological and pathological findings are of

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

assistance in diagnosis. Diagnosis is also possible by the detection of specific IgM and IgG antibodies in cerebrospinal fluid by enzyme-linked immunosorbent assay (ELISA) methods (Burke *et al*., 1982). Viral nucleic acid has been detected in the brain of infected horses by reverse-transcription polymerase chain reaction (RT-PCR) (Lian *et al*., 2002; Lam *et al.,* 2005).

In horses, the specimens collected for virus isolation or detection (nucleic acid or antigen) are portions of the corpus striatum, cortex or thalamus of the brain. Blood and spinal cord samples can also be used. In fetuses, stillborns or neonates of sows, virus may be isolated or detected from brain, spleen, liver or placental tissues. All materials should be refrigerated immediately after collection and frozen to –80°C if specimens are to be stored for more than 48 hours. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*), to prevent the risk of human infection. Humans may be infected by direct contact of infectious material with broken skin or mucous membranes, accidental parenteral inoculation or aerosol. Diagnosticians collecting samples should also take the appropriate precautions. A human vaccine is available and at risk field veterinarians and laboratory workers should be vaccinated.

1. Identification of the agent

Tissue samples are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 µg/ml) and penicillin (100 units/ml). The calf serum should be free from antibodies to JEV. The suspension is centrifuged at 1500 ***g*** for 15 minutes, and the supernatant fluid is removed for testing. Virus isolation in cell culture can use primary cultures of chicken embryo, African green monkey kidney (Vero), baby hamster kidney (BHK) cells, or the C6/36 mosquito cell line (a cloned cell line from *Aedes albopictus*). Homogenates of specimens, such as brain and blood taken from animals suspected of being infected are inoculated onto the cell cultures. Unlike vertebrate cells, JEV does not normally cause cytopathic effect (CPE) in C6/36 cells. Therefore, confirmation may require further culture in vertebrate cells and/or detection of viral antigen or RNA. Monoclonal antibodies specific to flavivirus and JEV can also be used to identify the virus in fixed infected cell monolayers using the indirect fluorescent antibody test (Lian *et al.,* 2002).

For virus isolation using mice, 0.02 ml is inoculated intracerebrally into 2- to 4-day-old mice. The inoculated mice are kept under clinical observation for 14 days. No clear clinical signs may develop, but anorexia becomes evident by the disappearance of the white milk spot on the abdomen. The skin then changes colour from pinkish to dark red, and convulsions develop immediately before the mice die. Severely ill mice should be euthanised. Brains of dead and euthanised mice are collected and stored at –80°C for confirmation by RT-PCR or a further passage in mouse brain or cell culture.

Detection of antigen from infected mouse brains can be performed using sucrose/acetone-extracted antigen prepared as described in Section B.2.b.1. This antigen is checked for its ability to agglutinate the red blood cells (RBCs) of 1-day-old chickens or of geese at different pH levels between pH 6.0 and 7.0, at intervals of pH 0.2, according to the method described (Clarke & Casals, 1958). Briefly, RBC suspensions of 1/24 dilution are prepared in the diluent with different pH values. In a 96-well plate with a U-shaped bottom, 25 µl volumes of the extracted antigen are diluted serially. Then, 25 µl of the diluted RBCs is added to each well. The plate is incubated at 37°C for 1 hour, and the haemagglutination result is read. If the antigen is able to haemagglutinate red blood cells, it is used in a haemagglutination inhibition (HI) test using a JE virus antiserum.

To detect JE virus RNA from clinical specimens, from vertebrate cells showing CPE or from mouse brains of infected mice, RT-PCR can also be employed using appropriate primers specific for JEV (Chung *et al*., 1996; Jan *et al*., 2000; Lian *et al*., 2002; Tanaka, 1993, Williams *et al*., 2001). Recently a new nucleic acid detection method, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of JEV RNA was reported (Parida *et al*., 2006). Other RT-PCR methods have been described for human diagnosis, although there is little published data on nucleic acid detection methods in veterinary applications.

2. Serological tests

Serological tests are useful to determine the prevalence of infection in an animal population, the geographical distribution of the virus, and the degree of antibody production in vaccinated horses. If serology is to be used for the diagnosis of infection or disease in domestic animals or wildlife, it should be remembered that in an endemic area prior infection with the virus may have occurred. When testing horses and pigs, consideration should also be given to vaccination status when interpreting positive serology results. Maternal antibody can also persist in pigs for up to 8 months. Antibody assay is a useful technique for determining the prevalence of infection in an animal population, and also for diagnosing JE in diseased horses or pigs. The assay methods include virus neutralisation (VN), haemagglutination inhibition (HI), complement fixation (CF), and enzyme-linked immunosorbent assay (ELISA). Diagnosis requires a significant rise in antibody titre in paired sera collected during the acute and convalescent phases (for example a four-fold rise in VN titre). The specificity of each serological test should also be considered. A latex agglutination test to detect swine antibodies to Japanese encephalitis has recently been

described (Xinglin *et al*., 2002). An ELISA for antibodies to a nonstructural protein (NS1) of JEV can be used to differentiate antibodies following natural infection from those induced by inactivated vaccines.

In some regions of the world, there is a need to carry out additional tests for related viruses before an unequivocal diagnosis of Japanese encephalitis can be made. For example, in Australia Murray Valley encephalitis and West Nile-Kunjin virus occur; these viruses are members of the JEV serocomplex and are antigenically closely related to JEV. Recent expansion of the distribution of West Nile virus in North America, where St Louis encephalitis virus was known to be endemic, further illustrates the flexibility of flaviviruses to adapt to new environments. The presence of antibody to these other flaviviruses can make serological diagnosis of Japanese encephalitis difficult. There is some cross reactivity with other flaviviruses on all the tests; the plaque reduction VN test is the most specific, especially if a 90% neutralisation threshold is used.

2.1. Virus neutralisation (plaque reduction neutralisation test)

2.1.1. Cell culture

African green monkey-derived Vero cells (ATCC No. CCL-81) are recommended to propagate viruses and for use in the plaque reduction neutralisation test (PRNT).

The Vero cells are cultured in a complete alpha minimal essential medium (α-MEM) supplemented with fetal bovine serum (FBS) and antibiotics. To prepare the cells for PRNT in a 24-well format, use the following protocol:

i) Verify that the Vero cells are in the log phase (approximately 2×10^7 cells in 175 cm^2 flask) with greater than 95% viability.

ii) Add 1.0×10^4 cells ~ 5×10^4 cells to each well of the plate; the cells are maintained at 37°C in a 5% CO_2 incubator for 2 or 3 days.

A confluent monolayer of cells should be prepared 2–3 days before the course of the assay, because cell monolayer is critical for plaque forming and to evaluate accurate results.

2.1.2. Virus strain and propagation

The JEV strain (KV1899 strain or Nakayama strain) is routinely used for PRNT.

The conditions for virus preparation should be standardised with the use of an appropriate multiplicity of infection (MOI: 10^{-2} to 10^{-3}).

2.1.3. Reagents

i) α-MEM supplemented with 2–5% FBS and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). For the PRNT, a low final concentration of FBS ranging from 2 to 5% should be used for virus and cell growth, and for dilution of samples.

ii) 4% agarose stock solution (dissolved in distilled water). To prepare overlay medium for plaque formation, agarose solutions are typically used at 1–2% final concentrations.

iii) 0.1% neutral red solution. To visualise the plaque, a vital dye such as neutral red is added to the overlay medium. Neutral red is cytotoxic at high concentrations and light sensitive, therefore a low dye concentration is recommended. Dissolve the neutral red powder in distilled water at a concentration of 0.1 % (w/v) and, after autoclave, store the solution in light-tight container at 4°C until use.

2.1.4. Preparation of overlay medium

The composition of the first overlay medium:

Reagents	*Amount*
α-MEM containing antibiotics	14 ml
FBS (5% final concentration)	1 ml
4% agarose stock solution	5 ml
Total volume	20 ml

The composition of the second overlay medium:

Reagents	*Amount*
α-MEM containing antibiotics	12.8 ml
FBS (5% final concentration)	1 ml
4% agarose stock solution	5 ml
0.1% neutral red solution	1.2 ml
Total volume	20 ml

All amounts and volumes are given on a 24-well plate format. Combine reagents immediately prior to use. Be sure to keep the overlay medium at 42°C prior to adding to wells.

2.1.5. Viral plaque assay

To achieve accurate measurement, the appropriate virus dose for challenge should be determined before performing PRNT. Therefore, the target number of plaques can be determined by viral plaque assay.

For viruses belong to *Flaviviridae*, a two-overlay method is mostly used for this assay.

Test procedure (24-well plate format)

i) Prepare 90–100% confluent monolayer cells in a 24-well format.

ii) Prepare a 7-log serial dilution (10^{-1} to 10^{-7}) of the clarified JEV stock in a complete α-MEM. To do this, sequentially dilute 0.2 ml of the viral stock in 1.8 ml of medium in microtube.

iii) After labelling the plates, discard the medium from each well and immediately replace with 0.1 ml of the appropriate virus dilution. As a negative control, add a complete medium without virus.

iv) Incubate the cells with virus for 1 hour at 37°C in a CO_2 incubator.

v) Following 1 hour's incubation, discard medium containing virus from the wells and replace with 0.5 ml of agarose containing first overlay medium.

vi) Allow agarose overlay to be hardened for 1 hour at room temperature, and incubate the plates upside down to minimise water condensation in the wells in a 37°C incubator for 48 hours to allow virus plaques to develop.

vii) Add 0.5 ml of the second overlay medium containing 0.1% neutral red to each well and allow agarose overlay to be harden for 1 hour in the light-tight incubator.

viii) Incubate the plates upside down in a CO_2 incubator at 37°C for 48 hours to allow the cells to be maximally stained.

ix) Count the plaques by naked eyes and calculate the titre of viral stock. The titre can be calculated by using the following formula.

Titre (plaque-forming units [PFU]/ml) = number of plaques × dilution factor × 1 ml of inoculum per well.

2.1.6. PRNT of swine antiserum to JEV

Test procedure

i) Prepare 90–100% confluent monolayer cells in a 24-well format.

ii) Prepare serial two-fold or four-fold dilutions of test sera and positive and negative control sera. The test sera should be heat-inactivated at 56°C for 30 minutes before the course of the assay. All test sera for assay should be initially ten-fold diluted by a complete α-MEM prior to making two-fold diluents.

Test group allotted by serum type:

a) Swine sera collected from unvaccinated pigs;

b) Swine sera collected from vaccinated pigs;

c) Immuno-positive swine serum against JEV;

d) Negative serum: FBS or immuno-negative swine serum against JEV.

iii) Prepare 200 PFU/0.1 ml of virus dilutions. The virus plaque dose in diluent should be previously determined by the viral plaque assay. 20 PFU/0.1 ml of virus dilution should be also prepared for the comparison (used as cut off value of virus titre).

iv) Add an equal volume of the serum dilution to the diluted virus stock for a final virus concentration of approximately 100 PFU/0.2 ml. In case of 20 PFU/0.1 ml of virus dilution, the final concentration of virus stock will be at 10 PFU/0.2 ml.

v) Incubate the plate containing the mixture of serum and virus for 1 hour at 37°C. After incubation, transfer 0.1 ml of the mixture to the cell.

vi) Following 1 hour's incubation, discard medium containing virus from the wells and replace with 0.5 ml of agarose containing first overlay medium.

vii) Allow agarose overlay to harden for 1 hour at room temperature, and incubate the plates upside down to minimise water condensation in the wells in a CO_2 incubator at 37°C for 48 hours to allow virus plaques to develop.

viii) Add 0.5 ml of the second overlay medium containing 0.1% neutral red to each well and allow agarose overlay to harden for 1 hour in the light-tight incubator.

ix) Incubate the plates upside down in a CO_2 incubator at 37°C for 48 hours to allow the cells to be maximally stained.

x) Count the plaques by naked eyes and calculate the titre of viral stock.

xi) Calculate the average number of plaques in the serum-free control wells and determine the pfu threshold at 50% and 90% reduction levels: 50% reduction = 0.5 × pfu/well (no serum) 90% reduction = 0.1 × pfu/well (no serum)

xii) Calculate the 50% and 90% end-point titre for the test sera, being the dilution of serum closest to the reduction level relative to average plaque number in the serum free control wells.

2.2. Haemagglutination inhibition

The HI test is widely used for the diagnosis of Japanese encephalitis, but has cross-reactivity with other flaviviruses. For this test, the sera must first be treated with acetone or kaolin, and then adsorbed with homotypic RBCs to remove any nonspecific haemagglutinins in the test sera. The RBCs of geese or of 1-day-old chickens are used at the optimum pH (see the table below). The optimal pH is dependent on the JEV strain used. The test should be conducted with the treated sera and 8 units of standard antigen; this is commercially available in some countries.

2.2.1. Haemagglutination (HA)

i) Preparation of virus antigen

1. Sucrose–acetone extraction of antigen from infected suckling mouse brains (SMB)

a) Homogenise infected SMB with 4 volumes of 8.5% sucrose.

b) Add the homogenate drop-wise to 20 times its volume of cold acetone.

c) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

d) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.

e) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

f) Pool the sediment with cold acetone in a single tube.

g) Centrifuge (500 ***g*** for 5 minutes), then remove the supernatant.

h) Spread the sediment inside the tube and vacuum dry for 1–2 hours.

i) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.

j) Centrifuge (8000 ***g*** for 1 hour, 4°C). The supernatant is ready for use.

2. Infected fluid of Aedes albopictus, clone C6/36, cell line

a) Harvest the infected fluid after incubation of the infected cultures at 28°C for 1 week.

b) Centrifuge (1000 ***g*** for 15 minutes). The supernatant is ready for use.

ii) Preparation of goose red blood cells

1. Solutions

a) Acid-citrate-dextrose (ACD)

11.26 g sodium citrate ($Na_3C_6H_5O_7.2H_2O$); 4.0 g citric acid ($H_3C_6H_5O_7.H_2O$); 11.0 g dextrose ($C_6H_{12}O_6$); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.

b) Dextrose-gelatine-veronal (DGV)

0.58 g veronal (Barbital); 0.60 g gelatine; 0.38 g sodium veronal (sodium barbital); 0.02 g (0.026 g) $CaCl_2$ (for $CaCl_2.2H_2O$); 0.12 g $MgSO_4.7H_2O$; 8.50 g NaCl; 10.0 g dextrose; distilled water to a final volume of 1000 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes (five times stock volume is easier to prepare).

2. Blood collection

1.5 ml of ACD + 8.5 ml of blood (0.5 ml of ACD + 2.8 ml of blood).

3. Washing (sterile)

a) Total blood + 2.5 volume of DGV. Centrifuge (500 ***g*** for 15 minutes), then remove the supernatant.

b) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.

c) Centrifuge (500 ***g*** for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).

d) Transfer the final RBC suspension to a flask with aluminium foil cover.

4. Adjusting the RBC concentration

a) 0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).

b) Read the optical density $(OD)_{490}$ in a spectrophotometer with 10 mm tube.

c) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD_{490}. (Final volume = Initial volume × absorbance OD_{490}/0.450.)

d) Store the RBC stock in a refrigerator for up to 1 week.

e) Before use, resuspend the RBCs gently and dilute 1/24 in virus-adjusting diluent (VAD).

iii) Antigen dilution

1. Stock solutions (should be kept at 4°C)

a) 1.5 M NaCl

87.7 g NaCl and distilled water to a final volume of 1000 ml.

b) 0.5 M boric acid

30.92 g H_3BO_3 and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down).

c) 1 N NaOH

40.0 g NaOH and distilled water to a final volume of 1000 ml.

d) Borate saline (BS), pH 9.0

80 ml 1.5 M NaCl, 100 ml 0.5 M H3BO3, 24 ml 1.0 N NaOH, and distilled water to a final volume of 1000 ml.

e) 4% bovine albumin

4 g bovine albumin fraction V (Armour), 90 ml BS, pH 9.0, adjust pH to 9.0 with 1 N NaOH, and BS, pH 9.0, to make a final volume of 1000 ml.

2. Antigen diluent

0.4% bovine albumin/borate saline (BABS): 10 ml 4% bovine albumin, pH 9.0, and 90 ml BS, pH 9.0.

3. Serial dilution

Two-fold serial dilution of antigen with BABS on U-bottom microtitre plate.

iv) Addition of goose red blood cells

1. Stock solutions

1.5 M NaCl

0.5 M Na_2HPO_4: 70.99 g Na_2HPO_4 (for Na_2HPO_4, 12 H_2O: 179.08 g), and distilled water to a final volume of 1000 ml.

1.0 M NaH_2PO_4: 138.01 g $NaH_2PO_4.H_2O$ (for Na_2PO_4, $2H_2O$: 156.01 g), and distilled water to a final volume of 1000 ml.

2. Working solution: virus adjusting diluent (VAD)

VAD (pH)	1.5 M NaCl	0.5 M Na_2HPO_4	1.0 M NaH_2PO_4	
6.0	100	32	184	
6.2	100	62	160	Add distilled
6.4	100	112	144	water to a
6.6	100	160	120	final volume of
6.8	100	192	104	1000 ml
7.0	100	240	80	

Values of VADs are not the pH of each VAD, but the pH after each VAD is mixed with an equal volume of BABS, pH 9.0.

3. Procedures

a) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).

b) Add 25 µl of diluted RBCs to each well on microtitre plate containing diluted antigen (25 µl/well).

c) Incubate at 37°C for 30 minutes, then read the result.

++ Complete agglutination (uniformly thin pellicle of RBCs following the curvature of the well bottom)

\+ Partial agglutination (a ring associated with a rough or thinner pellicle)

± Minimal agglutination (a button on a thin or scattered pellicle)

– Negative agglutination (clearly defined button with no RBC film)

End point is the last dilution (highest dilution) in which ++ or + is observed.

Titre: the reciprocal of the end point dilution.

2.2.2. Haemagglutination inhibition

i) Preparation of test sera

1. Blood collection and separation of the sera

a) Incubate blood specimen at 37°C for 1 hour and then at 4°C overnight. If the test must be performed immediately, incubating the sample for 2–3 hours at 37°C can replace the overnight incubation.

b) Centrifuge (2000 ***g*** for 15 minutes) to separate the serum from the clot.

c) Heat inactivate at 56°C for 30 minutes.

d) Store at –20°C if not processed immediately.

2. 2-mercaptoethanol treatment (perform this step when IgM antibody titres should be determined)

a) Place 50 µl of the sera into two small test tubes.

b) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.

c) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. Acetone extraction

a) Add 2.5 ml of cold acetone to serum in a test tube. Cap with rubber stoppers, mix well and extract for 5 minutes in an ice bath.

b) Centrifuge cold (1500 ***g*** for 5 minutes), then remove the supernatant.

c) Repeat steps i and ii once more.

d) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.

e) Add 0.5 ml of BS, pH 9.0, to each tube. Apply rubber stoppers. Dissolve the sediment overnight at 4°C to make 1/10 dilution of the sera.

4. Kaolin extraction as an alternative to acetone extraction

a) 25% acid-washed kaolin in BS, pH 9.0.

b) 1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.

c) Extract at room temperature for 20 minutes with occasional shaking.

d) Centrifuge (1000 ***g*** for 30 minutes). The supernatant is 1/10 dilution of the sera.

5. Adsorption with goose RBCs

a) To each treated serum add 1/50 volume of packed goose RBCs.

b) Adsorb for 20 minutes in an ice bath.

c) Centrifuge (800 ***g*** for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).

ii) Haemagglutination inhibition test

1. Primary haemagglutination titration of antigen

Dilute the antigen to make 8 units/50 µl.

2. Serial two-fold dilution of test sera on microtitre plate

a) Serum–antigen reaction

Add 25 µl of diluted antigen into each well containing diluted test sera. Place the remainder of the antigen in empty wells and incubate at 4°C overnight or 1 hour at 37°C.

3. Secondary haemagglutination titration of the antigen

a) Serially dilute the prepared antigen (8 units/50 µl) two-fold in a 25 µl system.

b) Add 25 µl of BABS to each well to make 50 µl/well.

4. Addition of goose RBCs

a) Dilute RBC stock (1/24) in VAD.

b) Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.

c) Incubate at 37°C for 30 minutes then read the result.

Serum HI titre: the reciprocal of the highest dilution of the test sera showing complete inhibition of HA.

5. Interpretation of the results

Four-fold difference between the titre in the acute and convalescent sera is considered to be a significant rise or fall and is diagnostic of infection with a virus antigenically related to that used in the test.

2.3. Complement fixation

Complement fixation (CF) is sometimes used for serological diagnosis. The antigen for this test is extracted with acetone/ether from the brains of inoculated mice.

2.3.1. Antigen preparation

i) Extract and weigh the brains of the inoculated dead mice.

ii) Add to the brains 20 volumes of cold acetone, kept at –20°C, and homogenise.

iii) Centrifuge the suspension at 5000 ***g*** for 5 minutes at 4°C, and remove the supernatant.

iv) Add to the pellet the same volume of cold acetone as used in step ii above, and mix well.

v) Extract with acetone by keeping the pellet at –20°C for 20 minutes, and repeat the centrifugation described in step iii above.

vi) Repeat steps iv and v.

vii) Repeat steps iv and v, but this time use cold acetone/ether (equal volume mixture).

viii) Repeat steps iv and v twice using cold ether.

ix) Remove the supernatant by aspirator and spread the pellet over the centrifuge tube.

x) Vacuum dry for 1–2 hours.

xi) Dissolve the pellet in cold saline (2 ml/g of brain) and keep at 4°C overnight.

xii) Centrifuge at 5000 ***g*** for 1 hour. The supernatant is the antigen.

2.3.2. Test procedure

i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.

ii) Serially dilute the sera two-fold in a 96-well microtitre plate (25 µl).

iii) Add 25 µl of 4 units of antigen and mix by vibration.

iv) Add 50 µl of 2 units of complement (pooled fresh guinea-pig serum).

v) Mix by vibration and incubate at 4°C for 18 hours.

vi) Leave the microtitre plate at room temperature for 15 minutes.

vii) Add 25 µl of sensitised sheep RBCs to each well.

viii) Mix by vibration and incubate at 37°C for 30 minutes, then read the result.

ix) The highest dilution of test sera showing no haemolysis is the titre of the sera by CF test. A rise or drop of four-fold or more in the titre is considered to be significant.

2.4. Enzyme-linked immunosorbent assay

Various ELISA formats have been used to detect antibodies to JEV in horses and pigs. An epitope-blocking ELISA using a JEV-specific monoclonal antibody has been reported that can detect IgG in pigs (Pant *et al.,* 2006; Williams *et al.,* 2001) and horses (Lam *et al.,* 2005), although antibodies to closely-related flaviviruses can still cross-react). An IgM capture ELISA has been reported for testing pig sera (Pant *et al.,* 2006). An indirect ELISA for prevalence studies of JEV antibody in pigs has also been described (Yang Dongkun *et al.,* 2006). These assays have been used for sero-prevalence studies and for diagnostic investigations. Conventional serological methods cannot differentiate antibodies induced by natural infection from those induced by vaccination. To detect antibodies induced by natural infections but not those induced by inactivated vaccines, an ELISA method that detects antibodies against non-structural 1 (NS1) protein of JEV, which is induced only by infection, has been developed (Konishi *et al.,* 2004).

C. REQUIREMENTS FOR VACCINES

**NB: THIS SECTION WAS ADOPTED IN 2010
AND IS CURRENTLY BEING CONSIDERED FOR REVISION**

Two types of vaccines are commercially available in several Asian countries for humans and animals. For humans, inactivated vaccines prepared from infected mouse brains have been used for many years. An inactivated vaccine derived from Vero cell culture was licensed in 2009 in Japan. A live attenuated vaccine prepared in cell cultures has been used mainly in China (People's Rep. of).

The vaccine for Japanese encephalitis in horses is prepared by formalin-inactivation of a virus suspension derived from infected mouse brains or cell cultures. For pigs, both inactivated and live-attenuated vaccines derived from cell cultures are used in Japan.

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.

1. Background

1.1. Rationale and intended use of the product

Inactivated vaccines have been used to protect horses from encephalitis and possible subsequent death caused by JEV infection. In pigs, both inactivated and live attenuated vaccines have been used to protect pregnant sows from stillbirth.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Beijing-1 strain of JEV is used for vaccine production for humans in Japan. Other strains of JEV are also used for horses and pigs. The virus strains of inactivated vaccines must be lethal for 3-week-old mice when inoculated intraperitoneally, and must be able to grow in a primary culture of porcine kidney or susceptible cell lines. The virus strain for live attenuated vaccine must be lethal for 2-day-old mice when inoculated intracerebrally but shows no viraemia when inoculated in 1-month-old piglets and does not infect fetuses when inoculated in pregnant sows of the first month of gestation. The viruses have the capacity to haemagglutinate the RBCs of geese, 1-day-old chickens or pigeons. The viruses must be able to be neutralised by a standard antiserum to JEV.

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

The seed virus must be free of contaminating bacteria, fungi, mycoplasmas and viruses. Tests of biological materials for sterility and freedom from contamination are found in chapter 1.1.9.

2.2. Method of manufacture

2.2.1. Procedure

The virus is grown in the brains of 3–4-week-old mice or in a monolayer cultures. The cultures should be tested to confirm that they do not contain adventitious agents (see chapter 1.1.9). The seed virus is inoculated intracerebrally into mice. The brains of those mice that show severe clinical signs of encephalitis are collected. These brains are homogenised in PBS, centrifuged at 1500 ***g*** for 30 minutes, and the supernatant fluid is processed as the virus suspension.

The seed virus is inoculated into cell cultures and the fluids are later harvested separately from each batch when virus replication is at its maximum. This fluid is filtered, or centrifuged at 1500 ***g*** for 30 minutes, and the supernatant fluid is processed as the virus suspension.

For inactivated vaccine, formalin (0.5%) is added to the suspension to inactivate any live virus; this is considered to be the 'undiluted virus suspension'. Adjuvant may be added to enhance its immunogenicity.

The passage levels should not exceed three more than the original virus and two more than the seed virus. It is recommended that the original and seed viruses be maintained below –70°C, or below 5°C after lyophilisation.

2.2.2. Requirements for substrates and media

Primary cell cultures for vaccine production must be obtained from healthy animals. Primary and line cells must be tested for and free from extraneous bacteria, fungi, mycoplasmas and viruses. Culture media, fetal bovine serum and supplements must be tested to confirm sterility.

2.2.3. In-process control

The virus suspension should be examined for bacterial and fungal contamination by culture techniques and for virus infectivity by intracerebral mouse inoculation or inoculation into cell cultures. The inactivated undiluted virus suspension should be re-examined for contamination by cell culture and by microscopy after staining, and should be checked by intracerebral mouse inoculation to ensure complete inactivation of the virus by the formalin.

2.3. Sterility

2.3.1. Final product batch tests

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

To test the inactivity of the final product, ten 3-week-old mice are inoculated intracerebrally with 0.03 ml of the product and observed daily. All mice tested should survive and show no encephalitis after 14 days' observation to ensure the complete inactivation of live virus.

The final product of inactivated and live attenuated vaccine must be tested for immunogenicity.

Inactivated vaccine: The product is diluted 1/10 in PBS. Thirty mice aged 2–3 weeks are inoculated intraperitoneally with 0.1 ml of the diluted product twice at 3-day intervals. There should be an equivalent uninoculated control group. Ten mice of each group are challenged intraperitoneally with ten-fold dilutions (1/10, 1/100 and 1/1000) of the appropriate virus such as Nakayama strain 8 days following the first inoculation, and observed for 14 days. The survival rate should be more than 40% in the immunised group and the mortality rate in the control group should be more than 90%. The titre of challenge virus should not be less than 10^3 LD_{50} (50% lethal dose) per 0.2 ml.

2.4. Requirements for authorisation

2.4.1. Safety requirements

The live attenuated vaccine shows no viraemia when inoculated in 1-month-old piglets and does not infect fetuses when inoculated in pregnant sows in the first month of gestation. For inactivated vaccine, ten 3-week-old mice are inoculated intracerebrally with 0.03 ml of the product and no death must be observed after 14 days.

2.4.2. Efficacy requirements

As JEV is maintained among vector mosquitoes, pigs and wild birds, control and eradication of JEV using vaccines is difficult. Vaccines are used to protect horses from encephalitis and pregnant sows from stillbirths.

2.4.3. Stability

The final product must be shown to be fully effective for 12 months when stored at 4°C.

3. Vaccines based on biotechnology

No biotechnology-based vaccine is currently available.

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NB: There is an OIE Reference Laboratory for Japanese encephalitis
(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/our-scientific-expertise/reference-laboratories/list-of-laboratories/).
Please contact the OIE Reference Laboratory for any further information on
diagnostic tests, reagents and vaccines for Japanese encephalitis