

CHAPTER 2.1.7.

JAPANESE ENCEPHALITIS

SUMMARY

Definition 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 and pigs. The major vector of JEV is a culicine mosquito, *Culex tritaeniorhynchus*. 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.

Description of disease: 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, diagnosis by serology should be confirmed, preferably by virus isolation.

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 causes stillbirths and abortions in pigs, though infected pregnant sows usually demonstrate no clinical signs and the infection does not affect the future pregnancies.

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, 2005).

JEV belongs to the genus *Flavivirus* in the family Flaviviridae. JEV constitutes Japanese encephalitis serocomplex along with several important zoonotic viruses that include West Nile virus (see chapter 2.1.20), St Louis encephalitis virus, Murray Valley encephalitis virus and Kunjin 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

The definitive diagnosis of Japanese encephalitis in horses depends on the isolation of the causal virus. 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 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).

The specimens collected for virus isolation are portions of the corpus striatum, cortex or thalamus of the brain of affected horses. The virus can also be isolated from blood and spinal cord samples. All materials should be refrigerated immediately after collection and frozen to -80°C if specimens are to be stored for more than 48 hours. Any potentially infected materials must be handled following containment level 3 procedures (see Chapter 1.1.3 *Biosafety and biosecurity in the veterinary microbiology 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

Samples of brain and spinal cord are homogenised in a 10% suspension in buffered saline, pH 7.4, containing calf serum (2%) or bovine serum albumin (0.75%), streptomycin (100 $\mu\text{g}/\text{ml}$) and penicillin (100 units/ml). The calf serum should be free from Japanese encephalitis antibodies. The suspension is centrifuged at 1500 *g* for 15 minutes, and the supernatant fluid is removed for inoculation: 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 a further passage.

To identify the virus, sucrose/acetone-extracted antigen is prepared from the infected mouse brains of a second passage in mice 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 Japanese encephalitis antiserum.

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*) may be used for virus isolation. The specimens, such as brain and blood taken from animals suspected of being infected, and the brain suspension from mice after inoculation, are inoculated onto the cell cultures. Monoclonal antibodies specific to flavivirus and Japanese encephalitis virus are used to identify the virus in the indirect fluorescent antibody test (Lian *et al.*, 2002). RT-PCR assay can also be used for identification of JEV in clinical specimens or cell culture fluid using appropriate primers specific for JEV (Chung *et al.*, 1996; Jan *et al.*, 2000; Lian *et al.*, 2002; Tanaka, 1993). 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 the disease in individual horses, it should be remembered that horses in an endemic area may have been inapparently infected with the virus or may have been immunised with a vaccine. Antibody assay is a useful technique for determining the prevalence of infection in a horse population, and also for diagnosing JE in diseased individuals. 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. 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 antigenically closely related viruses of Murray Valley encephalitis and Kunjin virus occur. 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.

a) Virus neutralisation

The plaque reduction test using chicken embryo primary cultures, African green monkey kidney (Vero) cells or baby hamster kidney (BHK) cells is sensitive and the most specific serological procedure available. The cross reaction with other flaviviruses is minimal; however, if an animal has a high titre to another flavivirus, such as West Nile virus, there may be a low cross-neutralising antibody titre to JEV.

Japanese encephalitis virus (Nakayama strain or JaGAR-01 strain) is propagated in cell culture. The supernatant of virus-infected cell culture is stored in aliquots at -80°C and is used for virus neutralisation test.

• Test procedure

- i) Dilute the test sera in 1/10 dilution in culture medium and then inactivate sera for 30 minutes in a water bath at 56°C .
- ii) Make twofold serial dilutions of the inactivated sera in cell culture medium in test tubes.
- iii) Dilute stock virus in cell culture medium to make 200 plaque-forming units (PFU)/0.2 ml.
- iv) Mix one volume of each diluted serum with an equal volume of diluted virus in test tubes. Positive and negative control sera are included in each test. Culture medium is also included for the virus control.
- v) Incubate for 90 minutes in a water bath at 37°C .
- vi) Add 200 μl of the virus/serum mixture to each well of a preformed monolayer of BHK-21 or Vero cells in 6-well culture plates.
- vii) Incubate the plates in a CO_2 atmosphere for 90 minutes at 37°C .
- viii) Remove the inoculum and add 1 ml of overlay medium (1.5% carboxymethyl cellulose, 1% fetal calf serum in Eagle's medium).
- ix) Incubate the plates in a CO_2 atmosphere for 4–6 days at 37°C .
- x) After removing the culture fluid, fix the plates in a solution containing 2.5% potassium dichromate, 5% glacial acetic acid and 5% formalin for 30 minutes at room temperature. Wear rubber gloves when handling the fixing solution.
- xi) Stain the plate in 0.1% crystal violet solution for 30 minutes at room temperature.
- xii) Discard the stain and rinse the cells with tap water.
- xiii) Air dry the cells and count the plaques.

- xiv) Estimate the serum dilution that reduces the number of plaques by 50% or more of the control without serum. The validity of the test is confirmed when the mean number of plaques in the control wells without serum is within 50 to 150 plaques.

b) 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.

- **Haemagglutination (HA)**

- **Preparation of virus antigen**

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

- i) Homogenise infected SMB with 4 volumes of 8.5% sucrose.
- ii) Add the homogenate drop-wise to 20 times its volume of cold acetone.
- iii) Centrifuge (500 **g** for 5 minutes), then remove the supernatant.
- iv) Resuspend the sediment with the same volume as above of cold acetone, and keep in an ice bath for 1 hour.
- v) Centrifuge (500 **g** for 5 minutes), then remove the supernatant.
- vi) Pool the sediment with cold acetone in a single tube.
- vii) Centrifuge (500 **g** for 5 minutes), then remove the supernatant.
- viii) Spread the sediment inside the tube and vacuum dry for 1–2 hours.
- ix) Dissolve the dry sediment with saline: 0.4 volume of original homogenate.
- x) 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*

- i) Harvest the infected fluid after incubation of the infected cultures at 28°C for 1 week.
- ii) Centrifuge (1000 **g** for 15 minutes). The supernatant is ready for use.

- **Preparation of goose red blood cells**

1. *Solutions*

Acid-citrate-dextrose (ACD): 11.26 g sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$); 4.0 g citric acid ($\text{H}_3\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$); 11.0 g dextrose ($\text{C}_6\text{H}_{12}\text{O}_6$); distilled water to a final volume of 500 ml. Autoclave at 10 lb (1.7 unit pressure) for 10 minutes.

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 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$); 0.12 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 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)*

- i) Total blood + 2.5 volume of DGV. Centrifuge (500 **g** for 15 minutes), then remove the supernatant.
- ii) Resuspend the sedimented RBCs in three volumes (total blood) of DGV.
- iii) Centrifuge (500 **g** for 15 minutes), then remove the supernatant. Repeat steps 2 and 3 twice more (total four spin cycles).
- iv) Transfer the final RBC suspension to a flask with aluminium foil cover.

4. *Adjusting the RBC concentration*

- i) 0.2 ml of the RBC suspension + 7.8 ml of 0.9% NaCl (1/40 dilution).
- ii) Read the optical density (OD)₄₉₀ in a spectrophotometer with 10 mm tube.
- iii) Adjust the RBC stock so that 1/40 dilution gives 0.450 of OD₄₉₀. (Final volume = Initial volume × absorbance OD₄₉₀/0.450.)
- iv) Store the RBC stock in a refrigerator for up to 3 weeks.
- v) Before use, resuspend the RBCs gently and dilute 1/24 in virus-adjusting diluent (VAD).

• **Antigen dilution**

1. Stock solutions (should be kept at 4°C): *1.5 M NaCl*: 87.7 g NaCl and distilled water to a final volume of 1000 ml; *0.5 M boric acid*: 30.92 g H₃BO₃ and hot distilled water to a final volume of 700 ml (dissolve boric acid and cool down); *1 N NaOH*: 40.0 g NaOH and distilled water to a final volume of 1000 ml; *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; *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. Twofold serial dilution of antigen with BABS on U-bottom microtitre plate.

• **Addition of goose red blood cells**1. *Stock solutions**1.5 M NaCl*

0.5 M Na₂HPO₄: 70.99 g Na₂HPO₄ (for Na₂HPO₄, 12 H₂O: 179.08 g), and distilled water to a final volume of 1000 ml.

1.0 M NaH₂PO₄: 138.01 g NaH₂PO₄·H₂O (for Na₂PO₄, 2H₂O: 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 ₂ HPO ₄	1.0 M NaH ₂ PO 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*

- i) 1 volume of stock goose RBCs + 23 volumes of VAD (1/24 dilution).
- ii) Add 25 µl of diluted RBCs to each well on microtitre plate containing diluted antigen (25 µl/well).
- iii) Incubate at 37°C for 1 hour, 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.

- **Haemagglutination inhibition**

- **Preparation of test sera**

1. *Blood collection and separation of the sera*

- i) 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.
- ii) Centrifuge (2000 **g** for 15 minutes) to separate the serum from the clot.
- iii) Heat inactivate at 56°C for 30 minutes.
- iv) Store at –20°C if not processed immediately.

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

- i) Place 50 µl of the sera into two small test tubes.
- ii) Add 150 µl of 0.13 M 2-mercaptoethanol in PBS into one test tube, and 15 µl PBS into another tube.
- iii) Incubate at 37°C for 1 hour, then cool in an ice bath.

3. *Acetone extraction*

- i) 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.
- ii) Centrifuge cold (1500 **g** for 5 minutes), then remove the supernatant.
- iii) Repeat steps i and ii once more.
- iv) Spread the sediment inside tubes and vacuum dry at room temperature for 1 hour.
- v) 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*

- i) 25% acid-washed kaolin in BS, pH 9.0.
- ii) 1 volume of sera + 4 volumes of BS + 5 volumes of 25 % kaolin.
- iii) Extract at room temperature for 20 minutes with occasional shaking.
- iv) Centrifuge (1000 **g** for 30 minutes). The supernatant is 1/10 dilution of the sera.

5. *Adsorption with goose RBCs*

- i) To each treated serum add 1/50 volume of packed goose RBCs.
- ii) Adsorb for 20 minutes in an ice bath.
- iii) Centrifuge (800 **g** for 10 minutes). The supernatant is ready for the HI test (1/10 dilution).

- **Haemagglutination inhibition test**

1. *Primary haemagglutination titration of antigen*

Dilute the antigen to make 8 units/50 µl.

2. *Serial twofold dilution of test sera on microtitre plate*

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.

3. *Secondary haemagglutination titration of the antigen*

- i) Serially dilute the prepared antigen (8 units/50 µl) twofold in a 25 µl system.
- ii) Add 25 µl of BABS to each well to make 50 µl/well.

4. *Addition of goose RBCs*

- i) Dilute RBC stock (1/24) in VAD.
- ii) Distribute 50 µl into each well containing 50 µl of serum antigen mixture or secondary titration of antigen.
- iii) Incubate at 37°C for 1 hour 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.

c) **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.

• **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.

• **Test procedure**

- i) Heat-inactivate the test sera at 1/4 dilution in gelatin–veronal buffer.
- ii) Serially dilute the sera twofold 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 microtray 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.

d) **Enzyme-linked immunosorbent assay**

An indirect ELISA for prevalence studies of JE antibody in pigs has been described (Yang Dongkun *et al.*, 2006). 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). In human medicine, virus-specific IgM assays have proved useful for diagnosis.

C. REQUIREMENTS FOR VACCINES

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 1.1.6 *Principles of veterinary vaccine production*. The guidelines given here and in Chapter 1.1.6 are intended to be general in nature and may be supplemented by national and regional requirements.

1. Background

a) 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

a) Characteristics of the seed

i) *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.

ii) *Quality criteria (sterility, purity, freedom from extraneous agents)*

The seed virus must be free of contaminating bacteria, fungi, mycoplasmas and viruses. Tests for sterility and freedom from contamination of biological materials are found in chapter 1.1.7.

b) Method of manufacture

i) *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 1.1.7). 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.

ii) *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.

iii) *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.

a) **Sterility**iv) *Final product batch tests*

Tests for sterility and freedom from contamination of biological materials may be found in chapter 1.1.7.

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 tenfold 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 LD₅₀ (50% lethal dose) per 0.2 ml.

c) **Requirements for authorisation**i) *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.

ii) *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.

iii) *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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