CHAPTER 2.3.7.

DUCK VIRUS ENTERITIS

SUMMARY

Duck virus enteritis (DVE) or duck plague is an acute contagious infection of ducks, geese and swans (order Anseriformes) caused by an alpha-herpesvirus. Diagnosis is based on a combination of assessing the clinical signs, gross pathology and histopathology supported by identification of the virus by either isolation or polymerase chain reaction.

Identification of the agent: The virus may be isolated from the liver, spleen and kidneys of birds dying from this infection. Virus may be recovered by infecting susceptible ducklings, in which the disease can be reproduced; by inoculating embryonated Muscovy duck eggs on the chorioallantoic membrane; or by inoculating primary cell cultures of duck embryo or Muscovy duck embryo origin. The identity of the virus can be confirmed by neutralisation tests using specific antiserum to inhibit pathological changes in the duck embryos or the cytopathological effects in the cell cultures, or by direct or indirect immunofluorescence tests on infected cell cultures. Alternatively the viral DNA may be detected by the polymerase chain reaction from the oesophagus, liver and spleen of DVE virus infected birds as well as from Muscovy duck embryos or cells used for virus isolation.

Serological tests: Immunological tests have little value in the diagnosis of acute infection. Serum neutralisation tests in ovo and in vitro have been used to monitor exposure to the virus in wildfowl.

Requirements for biological products: A live attenuated virus vaccine is available to control DVE in birds over 2 weeks of age. Ducks are vaccinated subcutaneously or intramuscularly for active immunity. Vaccine virus is not thought to spread from vaccinated to unvaccinated stock. An inactivated vaccine has been reported to be efficacious in laboratory tests, but has not been developed or licensed for large-scale use.

A. INTRODUCTION

Duck virus enteritis (DVE) is an acute, sometimes chronic, contagious virus infection that occurs naturally only in ducks, geese and swans, all members of the family Anatidae of the order Anseriformes. The disease is a potential threat to commercially reared, domestic and wild waterfowl. The aetiological agent, anatid herpesvirus-1 or DVE virus (DEV), is a member of the Alphaherpesvirinae subfamily of the Herpesviridae. DVE may also be referred to as duck plague, anatid herpes, eendenpest, entenpest and peste du canard. The infection has not been reported in other avian species, mammals or humans.

In domestic ducks and ducklings, DVE has been reported in birds ranging from 7 days of age to mature breeders. In susceptible flocks the first signs are often sudden, high and persistent mortality with a significant drop in egg production in laying flocks. In domestic ducks the incubation period ranges from 3–7 days. Mortality usually occurs 1-5 days after the onset of clinical signs and is often more severe in susceptible adult breeder ducks. In chronically infected partially immune flocks only occasional deaths occur. Recovered birds may be latently infected carriers and may shed the virus in the faeces or on the surface of eggs over a period of years (Richter & Horzinek, 1993; Shawky & Schat, 2002). DVE limited solely to Muscovy ducks has been observed in the USA (Campagnolo et al., 2001; Davison et al., 1993).

Clinical signs and gross pathology associated with a DVE outbreak vary with the species, immune status, age and sex of the affected birds, and with the virulence of the virus. Similarly, as infection progresses within a flock, more clinical signs are typically observed. In breeder ducks the range of signs include ‘sudden deaths’, photophobia associated with partially closed, pasted eye-lids, polydipsia, loss of appetite, ataxia, and nasal discharge. Birds often have ruffled feathers, watery diarrhoea and soiled vents. Sick birds may maintain an upright stance by using their wings for support, but their overall appearance is one of weakness and depression. In ducklings 2–7 weeks of age, losses may be lower than in older birds and the signs associated with DVE include dehydration, loss of weight, conjunctivitis and serous ocular discharge, a blue colouration of the beaks and blood-stained vents.
At necropsy, adult ducks that have died are typically in good body condition. In mature males, prolapse of the penis may occur. In mature females, haemorrhages may be observed in ovarian follicles. The gross lesions are characterised by vascular damage, with tissue haemorrhages, free blood in the body cavities and intestinal lumen and a range of lesions affecting the digestive tract mucosa. These latter lesions progress with the course of the disease and include initial mucosal haemorrhages and eruptions and intense annular congestion, leading to pseudo-membranous or diphtheritic mucosal lesions. Necrotic degenerative changes are evident in the lymphoid and parenchymatous organs. In the liver this manifests as irregularly distributed pinpoint haemorrhages and white foci giving a speckled appearance. In ducklings lesions of the lymphoid tissues tend to be more prominent than visceral haemorrhages. Collectively, these lesions are pathognomonic for DVE. The pathology and histopathology of DVE in white Pekin ducks has been reviewed (Sandhu & Metwally, 2008). Microscopic lesions are characterised by vascular damage and its consequences in visceral organs. Eosinophilic intranuclear inclusions and cytoplasmic inclusions in epithelial cells of the digestive tract are typically present. Birds that recover from natural infection are suggested to be immune to re-infection, but latency (in the trigeminal ganglion) and reactivation of virus is recognised.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

Primary isolation of the virus is best achieved from samples of liver, spleen or kidney tissue, which have been homogenised in buffered saline containing antibiotics and clarified by low-speed centrifugation (1800 g). Isolation may be attempted by inoculating such homogenates into cell cultures, ducklings or duck embryos.

a) Cell cultures

Cell culture is reported as the method of choice for isolation of DVE virus, but may not always be successful. If attempted, isolations may be made in primary duck embryo fibroblasts (DEF) (Wolf et al., 1976) or, preferably primary Muscovy duck embryo fibroblasts (MDEF) (Gough & Alexander, 1990; Kocan, 1976). Muscovy duck embryo liver (MDEL) cells are thought to be even more sensitive (R.E. Gough, pers. comm.). Cell monolayers grown in Eagle’s minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin are washed with serum-free MEM and then inoculated with the clarified sample homogenate suspected to contain DVE virus. After incubation for 1 hour at 37°C to allow for virus adsorption, the cultures are maintained on MEM containing 2% FCS, 2 mM glutamine, and 0.17% sodium bicarbonate and gentamicin, and incubated in an atmosphere containing 5% CO₂. The cytopathic effect (CPE) is characterised by the appearance of rounded clumped cells that enlarge and become necrotic 2–4 days later. Cultures should be stained with a fluorescent antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.d). Cells can also be fixed and then stained with haematoxylin and eosin to show syncytial formation, intranuclear inclusions and marked cytoplasmic granulation. It has been reported (Burgess & Yuill, 1981) that the isolation of DEV in MDEF cells is favoured by incubation at temperatures between 39.5°C and 41.5°C. However, an elevated temperature does not appear to be essential for isolation, which is often carried out at 37°C. More than one passage in cell culture may be necessary to isolate the virus. This virus isolation method in cell cultures may be modified to a plaque assay by overlaying the cell monolayer with maintenance medium containing 1% agarose. As the virus can be cell associated, sequential passagings should be carried out by trypsinising potentially infected cells and replanting them, as well as inoculating fresh cells with infected culture supernatant from the previous passage.

b) Ducklings

Inoculation of live ducklings should be used only when no other methods are available for meaningful diagnosis. The requirements of the OIE Terrestrial Animal Health Code (Chapter 7.8 Use of animals in research and education) must be followed. When inoculated intramuscularly, 1-day-old susceptible ducklings die within 3–12 days; uninoculated ducklings, housed separately, should be maintained as controls at the same time. Muscovy ducklings (Cairina moschata) are more susceptible than white Pekin ducklings. Both macroscopic and microscopic lesions typical of DVE should be seen on post-mortem examination. The diagnosis may be confirmed either by vaccinating ducklings against DVE and challenging them subsequently with the virus isolate or by immunofluorescence. However, virulent strains of the virus exist, against which the vaccine may be ineffective (Kisary & Zsak, 1983). In the author’s experience of natural infections occurring in Muscovy ducks, this method of virus isolation has proved more sensitive than cell culture methods.

c) Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. Before harvest, inoculated embryonated eggs should be chilled at 4°C for 4 hours or overnight to kill the embryos before further manipulations. The embryos may die, showing
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characteristic extensive haemorrhages 4–10 days after inoculation. Two to four serial blind passages of the homogenised CAMs may be necessary before isolation can be effected. This method is not as sensitive as that using susceptible day-old ducklings.

Embryonated chicken eggs are not very susceptible to infection with field strains of DEV. The virus can nevertheless be adapted to chicken embryos by serial passages. Pekin duck embryos vary in their susceptibility to strains of DVE virus.

d) Immunological methods

Serological methods used to confirm the identity of newly isolated virus include neutralisation assays performed in either embryonated eggs or cell cultures. A plaque assay for DEV in duck embryo cell cultures has been described (Dardiri & Hess, 1968). In the author's laboratory a microtitre assay using primary MDEF or MDEL cells is used. Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody test (direct or indirect) for DEV in DEF, MDEF or MDEL cells is the next most sensitive assay after isolation in 1–9-day old ducklings (Erickson et al., 1974). A reverse passive haemagglutination test for DEV has been described (Deng et al., 1984), but it is reported to be less sensitive than immunofluorescence and plaque assays. An avidin–biotin–peroxidase method of immunoperoxidase staining to detect DEV antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (Islam et al., 1983). The identity of the virus may also be confirmed by negative stain electron microscopy, but this alone is not positive confirmation that the herpesvirus observed is DVE virus. Immunoelectron microscopy has been developed recently using DVE hyperimmune serum (Pearson & Cassidy, 1997).

e) Nucleic acid recognition methods

Detection of DVE virus by polymerase chain reaction (PCR), including quantitative real-time PCR, has been reported (Hansen et al., 1999; 2000; Plummer et al., 1998; Pritchard et al., 1999; Qi et al., 2009; Wu et al., 2011; Yang et al., 2005). Primers have been identified that are able to amplify DNA from DVE virus present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. The following is an example protocol for PCR methods for detection of DVE virus; other protocols exist. Recently, a LAMP-based nucleic acid amplification method for the detection of DVE DNA has been published (Ji et al., 2009).

- PCR method

This DNA extraction procedure can be used on disrupted cell suspensions from DEV-infected tissue culture, 10% ground tissue suspensions, or cloacal swab material in transport medium. This method is used to prepare duck plague DNA for the known positive PCR controls.

- Extraction of viral DNA

Note: All product transfers in steps i to v are performed in a biological safety cabinet.

i) For a 10% ground tissue suspension, add 400 µl to a 1.5 ml microfuge tube and microfuge at 16,000 g for 5 minutes. Transfer the supernatant to a new tube and go to step ii.

ii) For tissue culture suspensions and cloacal swab material, add 400 µl of the sample, or supernatant from step i above, to a 1.5 ml tube and microfuge at 16,000–20,000 g for 45 minutes to pellet the virus.

iii) Discard the supernatant and resuspend the pellet with 200 µl of Tris/ethylene diamine tetra-acetic acid (EDTA) buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA).

iv) Add 10 µl of a 5 µg/µl proteinase K solution to give a final concentration of 0.2 µg/µl, mix thoroughly, and incubate at 56°C for 1 hour.

v) Add 25 µl of 10% sodium dodecyl sulfate (SDS) solution to give a final SDS concentration of 1%, mix thoroughly, and incubate at 37°C for 1 hour.

vi) Add 15 µl of 5 M NaCl to give a final concentration of 0.3 M and mix thoroughly.

vii) Add 300 µl of fresh phenol buffered with Tris/HCl, pH 8.0, to the tube, and mix by inverting 50 times.

viii) Microfuge the tube at 16,000 g for 5 minutes and transfer the top aqueous phase (sample) to a new tube.

1 Provided by Dr W.R. Hansen, US Geological Survey, Biological Resources Division, National Wildlife Health Center, 6006, Schroeder Road, Madison, WI 53711, USA. This procedure uses the following commercial items: GeneAmp PCR Reagent Kits containing dNTPs, 10× amplification buffer for hot start PCR, Taq DNA polymerase, Lambda PCR control reagents, and Ampliwax beads (Applied Biosystems), and a 100 base pair molecular size ladder (Invitrogen)
ix) Repeat the phenol extraction steps vii and viii once more.

x) Add 500 µl of ether to the tube, mix thoroughly, and microfuge at 16,000 g for 1 minute.

xi) Discard the top aqueous phase (ether) and repeat the ether extraction step (step x) once more.

xii) Heat the tube with the lid open at 56°C for about 15 minutes or until the smell of ether is gone.

xiii) Split the tube contents in two and add 2.25 times the sample volume of 100% ethanol to each tube, mix the tube contents by inverting the tube several times, and leave at room temperature (22°C) for 30 minutes.

xiv) Microfuge the tube at 16,000 g for 45 minutes and discard the supernatant.

xv) Add 200 µl of 70% ethanol to gently wash the pellet and then microfuge at 16,000 g for 15 minutes.

xvi) Discard the supernatant and dry the pellet at 56°C for 30–45 minutes with the tube lid open.

xvii) Resuspend the DNA in 30 µl distilled water that is RNAase and DNAase free.

xviii) Store the sample tube at 4°C until tested (few days) or at −20°C for long-term storage.

Polymerase chain reaction

Lower reaction mixtures for the DEV PCR and the lambda control are prepared in advance in a biosafety cabinet using the kit manufacturer’s recommended methods for a hot start PCR. The lower reaction mixture is dispensed into tubes, sealed with Ampliwax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DEV DNA-directed DNA polymerase gene

- Primer 1 sequence: 5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3' (forward)
- Primer 2 sequence: 5'-CAA-GGC-TCT-ATT-CGG-TAA-TG-3' (reverse)

i) The upper reaction mixture is prepared according to the kit manufacturer’s recommendations the day of the test, and distributed to each sample tube including DEV and lambda control tubes.

ii) Add 10 µl of DNA suspension from the stored sample tubes to the PCR lower reaction tubes with corresponding labels.

iii) Place known DEV DNA diluted to 1 pg/10 µl into one control tube and 10 µl of distilled water into the no DNA control tube. Add 10 µl of lambda DNA supplied in the kit and 10 µl of water to the corresponding lambda control tubes.

iv) Place all the tubes in a thermal cycler that is programmed as follows:

One cycle:
- Hold 94°C for 2 minutes
- Hold 37°C for 1 minute
- Hold 72°C for 3 minutes

35 cycles:
- Hold 94°C for 1 minute
- Hold 55°C for 1 minute
- Hold 72°C for 2 minutes

One cycle:
- Hold 72°C for 7 minutes
- Hold 4°C until stored

PCR tubes are stored at 4°C until the samples are examined for amplification products.

Electrophoretic analysis of PCR products

i) A fresh 1 × TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.3) is prepared from a 10× stock for agarose preparation and for use in the electrophoresis chamber.

ii) A 1% agarose solution is prepared in TAE buffer, heated to dissolve the agarose, and, when cool, poured into a gel former with a comb.

iii) The solidified gel is placed into the electrophoresis chamber and TAE running buffer is added.

iv) PCR test samples, including the DEV and lambda controls, are mixed 1/10 with 1 µl of loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol, 0.01 M Tris/HCl, pH 8.0, and 50% [v/v] glycerol) and 10 µl of each is added to individual wells of the gel. The 100 bp molecular size markers are added to each side of the gel.

v) Run the gel for 1 hour at 120 volts and then stain in a 1% ethidium bromide solution for 20 minutes (alternative, safer products may be used to visualise PCR products, e.g. RedSafe™ or GelRed™). De-
stain the gel for 45 minutes in deionised water and view the gel on a UV-illuminated light box. Photograph the gel to record results.

- Interpretation of the results

A 500 bp amplification band in the lambda control sample indicates the PCR ran successfully. A 446 bp band in the DEV known DNA control indicates the DEV primers are working. A 446 bp band in the unknown test sample indicates DVE viral DNA was present. No amplification products will be present in the DEV or lambda no DNA controls. If bands appear in these negative control products, cross-contamination occurred during the test set-up and the test must be repeated.

f) Strain variation

Although strains of DEV differ considerably in virulence, there is little reported evidence of serological variation.

2. Serological tests

Serological tests have little value in the diagnosis of acute DEV infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to DEV in wildfowl. The humoral response to natural infection with DVE virus is often low and antibodies may be short-lived (Docherty & Franson, 1992); it is assumed that cell-mediated immunity also plays a role in the infection (Richter & Horzinek, 1993). However, detection of neutralising antibodies to DVE virus in serum is possible. Virus neutralisation (VN) (Thayer & Beard, 1998) assays using a constant-serum/varying-virus method may be performed in chicken or duck embryos by using embryo-adapted virus, or in cell cultures. For laboratories lacking duck embryos, serological diagnosis is possible by virus neutralisation, using a chicken embryo fibroblast adapted DVE strain and primary chicken embryo fibroblasts (CEF). Neutralisation indices (NI) (Thayer & Beard, 1998) between 0 and 1.5 were detected in domestic and wild waterfowl that had not been exposed to DEV; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVE virus (Dardiri & Hess, 1967). Alternatively, sera may be screened using a constant-virus/varying-serum method. In the author’s laboratory a microtitre neutralisation assay using primary MDEF or DEF is used. Serial twofold dilutions of each serum sample (heat-inactivated at 56°C) are prepared in 50 µl of serum-free MEM in microtitre plates. Approximately $10^{2.0} \text{TCID}_{50}$ (50% tissue culture infective dose) of DVE virus in 50 µl of MEM is added to each well and the mixtures are allowed to react at 37°C for 1 hour. A suspension of primary MDEF or DEF in MEM supplemented with 2 mM L-glutamine, 0.17% sodium bicarbonate and 10% FCS, are adjusted to contain $3 \times 10^5$ cells per ml. Cells are next added to the plates at 100 µl per well and the plates are then incubated for up to 96 hours at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, cells are observed daily by light microscopy and finally fixed with 10% formol-buffered saline and stained with 1% crystal violet. The plates are read macroscopically. The titre for virus neutralising activity is expressed as the reciprocal of the highest dilution of serum at which a monolayer grew, i.e. there is no evidence of CPE and therefore complete virus neutralisation has occurred. A titre of less than 3 log₂ is usually considered to be negative. A VN titre of 8 or greater is considered to be significant and is evidence of exposure to DVE virus (7). VN antibody may also be detected using cell cultures by mixing sera at a single dilution, e.g. 1/10, with 100–200 TCID₅₀ virus and then testing inoculated cell cultures for non-neutralised virus by immunofluorescence. Although this method is not quantitative, it can be useful for screening large numbers of sera. These latter methods, using constant-virus/varying-serum, are much more economical on sera than the NI methods.

C. REQUIREMENTS FOR VACCINES

1. Background

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.

a) Rationale and intended use of the product

A live attenuated virus vaccine can be used to control DVE in birds over 2 weeks of age (Richter & Horzinek, 1993). The live vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducklings. Fattening or breeding ducks may be vaccinated subcutaneously or intramuscularly to produce an active immunity. Maternally-derived immunity in ducklings is reported to decline rapidly and progeny of breeders vaccinated with a live attenuated vaccine are fully susceptible.
A live attenuated vaccine propagated in a duck embryo fibroblast cell line has been reported to be successful (Mondal et al., 2010).

An inactivated vaccine has been reported to be as efficacious as modified live vaccine (Shawky & Sandhu, 1997). This vaccine has been tested only under laboratory conditions; it has not been tested on a large scale and is not licensed.

2. Outline of production and minimum requirements for conventional vaccines

a) Characteristics of the seed
   i) Biological characteristics

DVE vaccine can be prepared from a strain of the virus that has been attenuated by serial passage in embryonated chicken eggs. In the USA the vaccine strain seed was originally imported from Holland and has been serially passaged 41–46 times.

The seed virus should be prepared in 8–11-day-old specific pathogen free (SPF) embryonated chicken eggs by inoculating on to the CAM followed by incubation at 37°C. The seed may be stored at –70°C or lower in the form of a homogenate of the embryo CAM in buffered saline.

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

The seed virus should be shown to be free from extraneous viruses pathogenic to ducks, chickens and turkeys. It should also be free from bacterial, fungal and mycoplasmal contaminants.

The identity of the virus should be confirmed by a VN test conducted with specific antiserum using the constant-serum/varying-virus method. This test should be performed in embryonated chicken eggs. The antiserum should reduce the virus titre by at least $10^{1.75} \text{ELD}_{50}$ (50% embryo lethal dose).

b) Method of manufacture
   i) Procedure

The vaccine is produced in 8–11-day-old SPF embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. Most embryo deaths occur between 48 and 96 hours after inoculation. The embryos, their CAMs and chorioallantoic fluids are harvested, pooled and homogenised in buffered saline and clarified by low-speed centrifugation (1800 g). The preparation is diluted as appropriate, and a stabiliser is incorporated. It is then dispensed into vials and preferably frozen rapidly to –70°C or lower.

ii) Requirements for substrates and media

All reagents should be sterile and eggs obtained from a specific pathogen-free source.

iii) In-process controls

Any embryo dying within the first 24 hours of inoculation should be discarded as nonspecific deaths.

iv) Final product batch tests

   Sterility and purity

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

   Safety

A group of 1-day-old ducklings susceptible to DEV should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

   Batch potency

The virus titre of the vaccine should be determined in 9–11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0} \text{ELD}_{50}$ per dose at time of use.

The immunogenicity of the vaccine can be assessed in DVE-susceptible ducks or ducklings by inoculating the recommended vaccine dose intramuscularly and challenging intramuscularly 21 days
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later with virulent DVE virus. The vaccinated birds should survive challenge while unvaccinated control birds should die. This test should be carried out on the master seed but need not be done routinely on each vaccine batch produced. For release of subsequent batches, the titre of the virus should be a sufficient indication of vaccine potency.

c) Requirements for authorisation

i) Safety requirements

A group of 1-day-old ducklings susceptible to DVE should be inoculated subcutaneously or intramuscularly with the vaccine at 10 times the recommended dose, and observed for 7–14 days for any signs of adverse reactions.

Target and non-target animal safety

The vaccine is intended solely for use to protect ducklings and ducks against DVE virus.

Reversion-to-virulence for attenuated/live vaccines

There are no reports of reversion to virulence by the DVE vaccine.

Environmental consideration

None.

ii) Efficacy requirements

For animal production

Immunity in vaccinated ducks should last throughout a breeding season. Annual re-vaccination is recommended (Sandhu & Metwally, 2008).

For control and eradication

The vaccine virus is not thought to spread by contact from vaccinated to unvaccinated ducks, as the unvaccinated birds remain susceptible to infection.

iii) Stability

When stored at −70°C or lower the vaccine is stable for at least 1 year. Potency testing should be repeated after this time on an aliquot of vaccine to determine whether virus titre has been maintained. Once thawed the vaccine should not be refrozen, it should be maintained at 4°C in a refrigerator but for no longer than 1 week. Lyophilised vaccine should be stored at 4–8°C and used before the stated expiry date.

3. Vaccines based on biotechnology

a) Vaccines available and their advantages

None

b) Special requirements for biotechnological vaccines, if any

None

REFERENCES


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