

DUCK VIRUS ENTERITIS

SUMMARY

Description and importance of the disease: 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 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 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 vaccines: 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 alphaherpesvirus-1* or DVE virus (DVEV), is a member of the *Alphaherpesvirinae* subfamily of the *Herpesviridae*, genus *Mardivirus*. DVE may also be referred to as duck plague, anamid 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 to 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. Specific digestive mucosal lesions may be found in the oral cavity, oesophagus, caeca, rectum and cloaca. Lesions undergo alterations as the disease progresses, from initial macular surface haemorrhages, to yellow-white crusty plaques, then green superficial scabs. Lesions may coalesce and be covered with a diphtheritic membrane. 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

Table 1. Test methods available for the diagnosis of duck virus enteritis 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 identification¹						
Virus isolation	+	–	++	+++	+	–
Antigen detection	+	+	++	++	+	–
Real-time PCR,	++	++	++	+++	++	–
Conventional PCR,	++	++	++	+++	++	–
LAMP	++	++	++	+	++	–
Detection of immune response²						
Microtitre plate VN	+++	+++	+++	+	+++	+++
VN in duck embryos	+++	+++	+++	++	+++	+++
ELISA	+	+	+	+	++	+

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method but may need further validation; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = purpose not applicable.
 PCR = polymerase chain reaction; LAMP = loop mediated isothermal amplification
 ELISA = enzyme-linked immunosorbent assay; VN = Virus neutralisation

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

2 Antibody response of little value in acute DVE infection. Humoral response may be low in natural infection and antibodies may be short lived.

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 or duck embryos.

1.1. Cell cultures

Cell culture is reported as the method of choice for isolation of DVEV, 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. 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 DVEV. 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 labelled antibody conjugate using a direct or indirect method to identify the virus (see Section B.1.3). 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 DVEV 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 passaging 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.

1.2. Duck embryos

Primary virus isolations can be made by inoculation on to the chorioallantoic membrane (CAM) of 9–14 day embryonated Muscovy duck eggs. The inoculated embryos should be monitored daily and those that die within 72 hours should be removed. Virus may be harvested from embryos that survive for 72–120 hours. 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 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.

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

1.3. 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 DVEV in duck embryo cell cultures has been described (Dardiri & Hess, 1968). A microtitre assay using primary MDEF or MDEL cells can be used. Provided a hyperimmune antiserum of sufficiently high titre is used, a fluorescent antibody test (direct or indirect) for DVEV 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 DVEV 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 DVEV antigen in formalin-fixed, paraffin-embedded sections of liver and spleen from experimentally infected birds has been described (Islam *et al.*, 1993). 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 DVEV. Immunoelectron microscopy has been developed recently using DVE hyperimmune serum (Pearson & Cassidy, 1997).

1.4. Nucleic acid recognition methods: polymerase chain reaction

Several protocols for the detection of DVEV by conventional polymerase chain reaction (PCR) have been reported (Hansen *et al.*, 1999; 2000; Plummer *et al.*, 1998; Pritchard *et al.*, 1999). Real-time

quantitative PCR protocols for the detection of DVEV have also been reported (Qi *et al.*, 2009; Wu *et al.*, 2011a; Yang *et al.*, 2005).

A loop-mediated isothermal amplification (LAMP)-based method for the detection of DVEV DNA has been published (Ji *et al.*, 2009; Woźniakowski & Samorek-Salamonowicz, 2014). Primers have been identified that are able to amplify DNA from DVEV present in various tissues, including oesophagus, liver and spleen, from an original outbreak and after passage from Muscovy duck embryos. Tissues are preferred to cloacal swabs as DVEV shedding may be intermittent in infected waterfowl.

The following is an example protocol for conventional PCR for detection of DVEV (Hansen *et al.*, 2000); other protocols exist.

1.4.1. Extraction of viral DNA

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

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.
- 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.

1.4.2. Test method

Lower reaction mixtures for the DVEV 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 wax at 80°C, as recommended by the manufacturer, and stored at 4°C for 1–2 months.

PCR primers for DVEV 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 DVEV 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 DVEV 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.

1.4.3. 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 DVEV 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 stains should preferably be used to visualise PCR products). 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.

1.4.4. 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 DVEV known DNA control indicates the DVEV 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 DVEV 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.

1.4.5. Real-time PCR

The following is an example protocol for a real-time PCR for detection of DVEV (*Yang et al;* 2005); other protocols exist.

PCR primers targeting a 124-bp fragment of the DVE DNA polymerase gene

Primer 1 sequence: 5'-CTC-TAC-GCA-GCT-TTT-GAC-GAT-TT-3' (forward)

Primer 2 sequence: 5'-AGA-AAC-ATA-CTG-TGA-GAG-TGA-CGA-3' (reverse)

The labelled probe (5'-CCT-CCT-CCT-CGC-TGA-GTG-GCA-TCC-3') is complementary to a 24 bp region between the upstream and downstream primer pair labelled with 6-carboxy-fluorescein at the 5' end and 6-carboxy-tetramethyl-rhodamine at the 3' end.

- i) The DVEV DNA extraction can be performed using a suitable tissue/cell DNA extraction kit or the extraction procedure described in Section B.1.4.1. The PCR can be performed using a suitable real-time PCR kit and PCR detection system and software.
- ii) The amplification is performed in a total of 25 µl PCR mixture containing 1 µl of DNA solution, 13PCR buffer, 10 mM MgCl₂, 0.4 mM dNTP, 0.2 µM concentration of each primer, 0.24 µM fluorogenic probe, and 1.25 U of Taq polymerase.
- iii) The PCR conditions consisted of:
 - a) one cycle of 5 minutes at 95 C
 - b) 40 two-step cycles of 5 seconds at 94°C and 20 seconds at 65°C.
- iv) If quantification is desired, the number of target copies in the reaction can be deduced from the threshold cycle (CT) values corresponding to the fractional cycle number at which the released fluorescence exceeds 15 times the standard deviation of the mean baseline emission.

1.5. Strain variation

Although strains of DVEV 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 DVE infections, but assays based on serum neutralisation in embryonated eggs and cell cultures have been used to monitor antibodies following exposure to DVE in wild waterfowl. The humoral response to natural infection with DVEV 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 DVEV 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 DVEV 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 DVEV; a NI of 1.75 or greater was considered to be evidence of prior exposure to DVEV (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} TCID₅₀ (50% tissue culture infective dose) of DVEV 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 × 10⁵ 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 DVEV (Docherty & Franson, 1992). 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.

Immunochromatographic (ICS) strip tests have been developed for DVE antibody detection and may have promise for use in the field (Shen *et al.*, 2010). The ICS strip test is based on membrane chromatography and

uses recombinant UL51 protein as the capture antigen. This test is reported to have a sensitivity comparable to ELISA and much higher than VN tests. A dot-ELISA and passive haemagglutination tests have been reported for the detection of DVE antibodies but the sensitivity and specificity of these tests are moderate (Malmarugan & Sulochana, 2002). Several indirect ELISAs (Wu *et al.*, 2011b) have been described for the serological detection of DVE. Indirect ELISA using the entire DVEV virion as coated antigen has been described for DVE antibody detection and is commercially available (Xuefeng *et al.*, 2007). Several indirect ELISAs that use recombinant DVE proteins that act as coating antigen have also been developed. Wu *et al.* described an indirect ELISA using a recombinant UL55 protein of DVE expressed in *E. coli* (UL55-ELISA). Compared with a commercial indirect ELISA based on whole DVE virions and VN tests, the UL55-ELISA was found to be intermediate in sensitivity and specificity (Wu *et al.*, 2011b). Another indirect ELISA using thymidine kinase fusion protein expression in *E. coli* as coating antigen was reported to detect post-vaccine DVE antibodies 5 days earlier compared with conventional assays (Wen *et al.*, 2010). While ELISAs based on recombinant DVE proteins are reported to be rapid, simple and more economical alternatives for DVE serological detection, further testing of their stability is needed before widespread use.

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.

1.1. 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

2.1. Characteristics of the seed

2.1.1. 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- to 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

2.1.2. 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 $10^{1.75}$ ELD₅₀ (50% embryo lethal dose).

2.2. Method of manufacture

2.2.1. 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.

2.2.2. Requirements for substrates and media

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

2.2.3. In-process controls

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

2.2.4. Final product batch tests

i) Sterility and purity

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

ii) Safety

Safety tests in target animals are not required by many regulatory authorities for the release of each batch. Where required, standard procedures are generally conducted using fewer animals than are used in the safety tests required for the relevant regulatory approval.

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.

iii) Batch potency

The virus titre of the vaccine should be determined in 9- to 11-day-old embryonated chicken eggs inoculated on to the CAM and incubated at 37°C. The vaccine should contain a minimum of $10^{3.0}$ ELD₅₀ 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 later with virulent DVEV. 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.

2.3. Requirements for authorisation

2.3.1. 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.

i) Target and non-target animal safety

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

ii) Reversion-to-virulence for attenuated/live vaccines

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

- iii) Environmental consideration
None.

2.3.2. Efficacy requirements

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

2.3.3. 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

3.1. Vaccines available and their advantages

Research has been published on the development and efficacy of recombinant DVE vaccines in SPF ducks. In 2011, Liu *et al.* reported the use of a DVEV-vectored live bivalent vaccine in which the haemagglutinin gene of H5N1 avian influenza virus was inserted between the unique short (US) 7 and US8 genes of the DVEV genome. This bivalent vaccine was reported to be efficacious against both DVEV and H5N1 avian influenza viral infection in SPF ducks under experimental conditions (Liu *et al.*, 2011). DVE vaccines based on biotechnology show promise under experimental conditions but are currently not commercially available for widespread use.

3.2. Special requirements for biotechnological vaccines, if any

None.

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NB: FIRST ADOPTED IN 1989; MOST RECENT UPDATES ADOPTED IN 2018.