

DUCK VIRUS HEPATITIS

SUMMARY

Duck virus hepatitis (DVH) is typically associated with an acute, contagious infection in susceptible ducklings less than 6 weeks of age and frequently under 3 weeks of age. It does not occur in older birds. The disease, DVH, has traditionally been subdivided into types I, II and III.

DVH type I can be caused by at least three different genotypes of duck hepatitis A virus (DHAV) virus, a member of the genus Avihepatovirus, of the family Picornaviridae. The most pathogenic and widespread is DHAV type 1 (DHAV-1), which was formerly designated as duck hepatitis virus type 1. DHAV-2 and DHAV-3 are two additional genotypes within the genus Avihepatovirus that have subsequently been identified as additional aetiological agents of DVH in ducklings.

DVH type II is caused by duck astrovirus type 1 (DAstV-1), a member of the Astroviridae family. AstV-1 has been reported primarily in the United Kingdom. It has been reported in ducklings from 10 days to 6 weeks of age, and causes pathological changes similar to those of DHAV-1.

DVH type III is caused by duck astrovirus type 2 (DAstV-2), a member of the Astroviridae. It is considered distinct from DAstV-1 and has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHAV.

Diagnosis of hepatitis in ducklings is based on the characteristic disease pattern in the flock, gross pathological changes, the recovery of virus from dead ducklings, and the reproduction of the disease in susceptible ducklings.

The causal viruses of DVH are not considered zoonotic.

Identification of the agent: *It is not possible to distinguish among DVH types I, II and III on the basis of clinical findings and pathology, but distinctions can be made based on the responses of ducklings, embryonated eggs and cell cultures to the isolated viruses. Alternatively, DHAV RNA may be detected by a one-step reverse-transcriptase polymerase chain reaction (RT-PCR) from duckling liver, and also from allantoic fluid and embryo liver from inoculated duck eggs. Molecular tests have also been described for the detection of DAstV-1 and DAstV-2.*

Serological tests: *Serological tests have little value in the diagnosis of the acute infections caused by DHAV, DAstV-1 and DAstV-2.*

Serum neutralisation tests in ovo have been used with all three viruses and in-vitro tests have been developed for DHAV-1. These tests have been used for virus identification, assay of immune responses to vaccination and epidemiological surveys.

Requirements for vaccines: *DHAV-1 infections can be controlled by the use of live attenuated virus vaccines and an inactivated virus vaccine. They are administered to breeder ducks to confer passive immunity to ducklings. Live attenuated virus vaccines may also actively immunise DHAV-1susceptible day-old ducklings.*

Ducklings susceptible to DHAV-1 may be passively protected with a chicken egg yolk antibody preparation.

DAstV-2 infections can be controlled by the use of a live attenuated virus vaccine given to breeder ducks to confer passive immunity to ducklings.

A. INTRODUCTION

The disease, duck virus hepatitis (DVH) has traditionally been categorised as DVH types I, II or III. It is caused by at least three different small RNA viruses that are of no known public health significance.

1. DVH type I (DHAV-1, DHAV-2, DHAV-3)

DVH type I is caused by the virus species, *Duck hepatitis A virus* (DHAV) an *Avihepatovirus* within the *Picornaviridae* family. The International Committee on Taxonomy of Viruses created the novel genus, *Avihepatovirus*¹. This genus contains the species, *Duck hepatitis A virus* (DHAV). Three antigenically unrelated genotypes have been identified, DHAV-1, DHAV-2 and DHAV-3 (Kim *et al.*, 2006; Tseng & Tsai, 2007; Wang *et al.*, 2008). The most prevalent and internationally widespread is DHAV-1. Until recently DHAV-1 had only been associated with disease in mallard and Pekin ducklings, but it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (Guerin *et al.*, 2007). DHAV-2 is also documented as N-DHV (Tseng & Tsai, 2007). DHAV-2 was originally isolated from a mule duckling and a gosling in Chinese Taipei (Tseng & Tsai, 2007). Mixed DHAV-1 and DHAV-2 infections are common in Chinese Taipei (Tseng & Tsai, 2007). DHAV-3 isolates have been reported from Korea (Rep. of) (Kim *et al.*, 2007a) and China (People's Rep. of) (Fu *et al.*, 2008). The limited information on the pathogenicity of DHAV-2 and DHAV-3 indicates that the clinical presentation is similar to DHAV-1.

DHAV causes a highly contagious infection of ducks. The disease is an acute, rapidly spreading, often fatal virus infection of young ducklings. It usually affects ducklings under 6 weeks of age and often much younger. The clinical disease is characterised by lethargy and ataxia followed by opisthotonos and death. Ducklings lose their balance, fall on their sides and kick spasmodically prior to death. The whole disease sequence is rapid and can take as little as 1–2 hours. Practically all mortality in a flock will occur within 3–4 days, with most deaths on the second day. Gross pathological changes appear chiefly in the liver, which is enlarged and displays distinct petechial and ecchymotic haemorrhages. Spleen enlargement and swelling of the kidneys with some congestion of renal blood vessels may also be apparent. Microscopic changes in the liver are characterised by extensive hepatocyte necrosis and bile duct hyperplasia, together with varying degrees of inflammatory cell response and haemorrhage.

2. DVH type II (duck astrovirus type 1 [DAstV-1])

DVH type II is caused by the virus, duck astrovirus-1 (DAstV-1), in the family *Astroviridae* (Gough *et al.*, 1985; Koci & Schultz-Cherry, 2002). Virions have the typical astrovirus morphology and are 28–30 nm in diameter.

Infection of ducklings with DAstV-1 has only been reported from the United Kingdom (Asplin, 1965; Gough *et al.*, 1985). It is an acute, fatal infection of ducklings producing clinical and pathological signs similar to DHAV. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages with both punctate and confluent bands in the liver, swollen pale kidneys with congested blood vessels, and enlarged spleens. The alimentary tract is often empty although the small intestine may contain mucus, and haemorrhagic areas are occasionally seen. Petechial haemorrhages are occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHAV infections; the extent of bile duct hyperplasia may be somewhat greater than with DHAV.

3. DVH type III (duck astrovirus type 2 [DAstV-2])

The causal virus of DVH type III is classified as duck astrovirus-2 (DAstV-2), distinct from DAstV-1 (Todd *et al.*, 2009). DAstV-2 has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHAV-1 (Haider & Calnek, 1979; Toth, 1969). DAstV-2 causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology induced by DAstV-2 is also similar to DHAV infection. The liver surface is pale and mottled with many red bands and some petechial haemorrhages. The spleen is paler, but not noticeably enlarged, and the kidneys may show patchy congestion.

1 http://www.ictvonline.org/taxonomyHistory.asp?taxnode_id=20140971&taxa_name=Avihepatovirus

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of duck hepatitis A virus (DHAV) 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 identification²						
Virus isolation	+	++	+	++	++	–
Antigen detection	+	++	+	++	++	–
RT-PCR ^a	++	+++	+++	+++	++	–
Detection of immune response						
ELISA	++	++	++	–	+	++
VN	+++	+++	+++	–	+	+++

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method, in some cases may need further validation regarding use in specimens from live animals e.g. faeces; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

^aThe use of RT-PCR (reverse-transcription polymerase chain reaction) is most frequently reported for testing clinical suspect samples; typically livers. Use of RT-PCR for detection of DHAV using faecal specimens requires further validation. ELISA = enzyme-linked immunosorbent assay; VN = virus neutralisation.

Table 2. Test methods available for the diagnosis of duck astrovirus type 1 (DAstV-1) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Virus isolation	+	++	+	++	++	–
Antigen detection	++	++	++	++	++	–
RT-PCR ^a	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	++	+	++	–	+	++

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method, in some cases may need further validation regarding use in specimens from live animals e.g. faeces; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

^aThe use of RT-PCR is most frequently reported for testing clinical suspect samples; typically livers. Use of RT-PCR for detection of DHAV using faecal specimens requires further validation.

RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

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

Table 3. Test methods available for the diagnosis of duck astrovirus type 2 (DAstV-2) and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
Virus isolation	+	++	+	++	++	–
Antigen detection	++	++	++	++	++	–
RT-PCR	+++	+++	+++	+++	+++	–
Detection of immune response						
ELISA	++	+	++	–	+	++

Key: +++ = recommended method, validated for the purpose shown; ++ = suitable method, in some cases may need further validation regarding use in specimens from live animals e.g. faeces; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose.

RT-PCR = reverse-transcription polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay.

1. Identification of the agent

1.1. DVH type I (DHAV-1, DHAV-2, DHAV-3)

The clinical and pathological observations in ducklings can be highly indicative of DHAV infection. DHAV can readily be recovered from liver tissue by homogenisation as a 20% (w/v) suspension in buffered saline. The suspension is clarified, and can then be treated further (if desired) with 5% chloroform (v/v) for 10–15 minutes at ambient temperature. DHAV is resistant to this treatment.

The presence of DHAV is usually confirmed by one or more of the procedures listed in Section B.1.1.1.

1.1.1. Confirmatory procedures

- i) By inoculation of primary cultures of duck embryo liver (DEL) cells, which are particularly sensitive (Woolcock, 1986). Dilutions of the liver homogenate containing DHAV type 1 cause a cytopathic effect (CPE), which is characterised by cell rounding and necrosis. When overlaid with a maintenance medium containing 1% agarose (w/v), the CPE gives rise to plaques approximately 1 mm in diameter.
- ii) By inoculation of serial dilutions of the liver homogenate into the allantoic sac of embryonated duck eggs (10–14 days) or chicken eggs (8–10 days). Duck embryos die between 24 and 72 hours post-inoculation, whereas chicken embryos are more variable and erratic in their response and death usually occurs 5–8 days post-inoculation. Gross pathological changes in the embryos include stunting and subcutaneous haemorrhages over the whole body, with oedema particularly of the abdominal and hind limb regions. The embryo livers may be red and yellowish, swollen and may show some necrotic foci. In embryos that take longer to die, a greenish-yellow colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.

1.1.2. Immunological tests

Antigenic variation among DHAV isolates inducing hepatitis in ducklings exists. Serologically, there is no cross neutralisation between DHAV-1 and DHAV-2 (Tseng & Tsai, 2007) and limited cross neutralisation between DHAV-1 and DHAV-3 (Kim *et al.*, 2007a). A variant, DHAV-1a, isolated in the United States of America (USA) only partially reacts with the classical DHAV-1 virus in cross serum neutralisation tests (Sandhu *et al.*, 1992; Woolcock, 2008b). Other variants have been reported from India and Egypt, but nothing further is known about them. Reports of disease in Muscovy ducks from France (Guerin *et al.*, 2007; Sandhu *et al.*, 1992; Woolcock, 2008b) also suggest a greater diversity among DHAV than was originally thought.

Immunological tests have not been used extensively for the routine identification of DHAV infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DVH types II and III infections become more widespread. The tests that have been described for DHAV-1 (Chalmers & Woolcock, 1984; Woolcock, 1986; 1991; 2008a) include:

- i) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHAV-1-specific hyperimmune serum diluted between 1/5 and 1/10. The mixtures are allowed to react at room temperature for 1 hour and are then inoculated (0.2 ml) subcutaneously into susceptible ducklings, also via the allantoic cavity (0.2 ml) of embryonated duck eggs and on to primary DEL cell monolayer cultures. Controls in each case consist of the virus isolate mixed with control serum.

1.1.3. Nucleic acid recognition methods

Several publications on the molecular structure of DHAV have been published emphasising genotypic variation among isolates (Tseng *et al.*, 2007; Wang *et al.*, 2008). Based on phylogenetic analyses, DHAV known isolates are subdivided into 3 genotypes (Wang *et al.*, 2008). DHAV-1 is the most prevalent genotype (Asplin, 1965; Ding & Zhang, 2007; Jin *et al.*, 2008; Kim *et al.*, 2006; Liu *et al.*, 2008). DHAV-2 has been reported in Chinese Taipei (Tseng & Tsai, 2007). DHAV-3 has been reported in South Korea (Kim *et al.*, 2007a; 2008) and China (Fu *et al.*, 2008).

A one-step reverse-transcriptase polymerase chain reaction (RT-PCR) assay using primers to the conserved 3D gene has been described for DHAV-1 (Kim *et al.*, 2007b). Anchun *et al.* 2009 also report an RT-PCR to detect Chinese isolates, but it is not clear whether these are DHAV-1 or DHAV-2. The development of a one-step real-time Taqman RT-PCR assay again based on primers to a conserved region in the 3D gene has been reported (Yang *et al.*, 2008), but it is also not clear whether this is for DHAV-1 or DHAV-2; this report does not provide a clear step-by-step protocol used for the method they developed. Several multiplex RT-PCR tests have been developed for the simultaneous detection and differentiation of DHAV genotypes.

- i) Polymerase chain reaction

This method has been extracted from Kim *et al.* (2007b). It is based on primers specific to amplifying a region of the 3D gene of DHAV-1.

- ii) Detection of DHAV-I from duck and chicken embryo organs and nucleic acid extraction

Supernatants prepared from duckling livers infected with DHAV-1 are collected and filtered (0.2 µm). The allantoic cavities of each of five 11-day-old duck and 9-day-old chicken embryonated eggs are inoculated with 0.2 ml viral supernatant. The allantoic fluid and liver samples are collected from embryos inoculated with two reference strains and each liver minutes, the supernatants are treated with the a suitable viral DNA/RNA extraction kit following the manufacturer's instructions. The nucleic acids are used for one-step RT-PCR. After measuring RNA, the samples are stored at –20°C.

- iii) Oligonucleotide primers

DHAV-1 ComF (5'-AAG-AAG-GAG-AAA-ATY-[C or T]-AAG-GAA-GG-3') and

DHAV-1 ComR (5'-TTG-ATG-TCA-TAG-CCC-AAS- [C or G]-ACA-GC-3')

Flanked by a 467 bp DNA sequence in the 3D gene.

Alternative primers proposed by Fu *et al.* (2008) are:

Antisense 501-519: 5'-CCT-CAG-GAA-CTA-GTC-TGG-A-3'

Sense 270-285: 5'-GGA-GGT-GGT-GCT-GAA-A-3'

- iv) One-step RT-PCR

Times and temperatures should be optimised according to reagents or kits used. The following are given as examples. The one-step RT-PCR is conducted using a suitable kit containing 1 U reverse transcriptase, 2.5 mM dNTPs, 2.5 U DNA polymerase, and RT-PCR buffer (50 mM Tris/HCl and 75 mM KCl). In addition, the following components are included in the reaction: 4 µl (50 ng) RNA or DNA template, 1 µl (10 pmol/µl) of each

specific primer (DHAV-1 ComF and DHAV-1 ComR), and diethyl pyrocarbonate (DEPC)-treated dH₂O to a total reaction volume of 20 µl.

A T-gradient thermal cycler is used for one-step RT-PCR. Reverse transcription is performed at 45°C for 30 minutes, after which the enzyme is inactivated at 94°C for 5 minutes. PCR amplification is conducted using an initial denaturation for 20 seconds at 94°C; followed by 40 cycles of annealing for 30 seconds at 52°C, extension for 30 seconds at 72°C, and denaturation for 20 seconds at 94°C; and a final extension for 5 minutes at 72°C. Reaction products are stored at 4°C.

v) Detection of one-step RT-PCR products

PCR products (10 µl) are separated by electrophoresis (100 V) in horizontal 1.5% agarose gels and Tris-acetate buffer (40 mM Tris-acetate, 1 mM ethylenediamine tetra-acetic acid). Gels are stained with nucleic acid stain (0.5 µg/ml), visualised under ultraviolet or blue light, and photographed.

vi) Interpretation of results

A DNA fragment of 467 bp is amplified by one-step RT-PCR using RNA extracted from the livers of ducklings infected with reference DHAV-1 strains. Negative control RNA is obtained from an uninfected duckling liver and does not amplify under the same conditions.

1.2. DVH type II (DAstV-1)

For DAstV-1 detection, homogenised liver suspensions can be evaluated by electron microscopy for DAstV-like particles.

DAstV-1 may be recovered in 20% (w/v) homogenised liver suspensions in buffered saline. This can be used to inoculate:

- i) Embryonated chicken or duck eggs, either via the amniotic cavity or yolk sac. These may respond erratically, after four passages, but no deaths may be seen during earlier passages. Embryos take 6–10 days to show evidence of infection; when this occurs there is stunting with green necrotic livers.

Growth of DAstV-1 in primary chicken embryo liver cell cultures has been reported (Baxendale & Mebatsion, 2004); plaque formation was detected at 5 days post-infection after 4 or 5 serial passages. Cell culture techniques are not routinely used as propagation of DAstV-1 in tissue culture typically does not result virus propagation to sufficient levels for diagnostic tests.

1.2.1. Immunological tests

Immunological tests have not been employed routinely for detection of DAstV-1 antigen. However, a neutralisation assay has been applied (Gough *et al.*, 1985) for DAstV-1 identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

1.2.2. Molecular characterisation

The complete genome of DAstV-1 consists of 7752 nucleotides and 3 ORF, ORF1a, ORF 1b and ORF2 (Chen *et al.*, 2012).

Confirmation of the identity of putative DAstV-1 isolates can be made using RT-PCR followed by nucleic acid sequence determination and analysis of the amplified fragment (Todd *et al.*, 2009). This degenerate primer based RT-PCR amplifies approximately 434 nt in ORF 1b. Since this RT-PCR method can amplify other duck atroviruses in samples, sequence analysis of the amplified product is required.

- i) RT-PCR detection of DAstV-1 (method extracted from Todd *et al.*, 2009)

Extract viral RNA from 200 µl samples using a suitable RNA extraction kit. Degenerate oligonucleotide primers are used with a suitable One-Step RT-PCR kit. Forward primer 5'-GAY-TGG-ACI-MGI-TAY-GAY-GGI-ACI-ATI-CC-3' and reverse primer 5'-YTT-IAC-CCA-CAT-ICC-RAA-3 amplifies a fragment of approximately 434 nt, which corresponds to nucleotides 3799 to 4233 in the genome of G4260 (accession number: AB033998). The

RT-PCR conditions were initial denaturation, 94°C for 5 minutes, then 45 cycles of denaturation, 94°C for 1 minute; annealing, 45°C for 1 minute; and extension, 72°C for 90 seconds followed by a final extension step, 72°C for 5 minutes. Examine the amplified product by electrophoresis in a 1% agarose gel, treated with nucleic acid stain and visualised by ultraviolet or blue light transillumination. Sequencing and sequence comparison of other ORF1b sequences of astroviruses should be performed.

1.3. DVH type III (DAstV-2)

DAstV-2 can be recovered from homogenised liver suspensions and is resistant to treatment with 5% chloroform. The virus can be isolated by:

- i) Inoculating the isolate on to the chorioallantoic membrane (CAM) of 10-day-old embryonated duck eggs. The response is erratic, but some embryo mortality always occurs within 7–10 days. The membranes assume a dry crusty appearance, beneath which they are oedematous. The embryos may be stunted and oedematous with skin haemorrhages. The liver, kidneys and spleen are enlarged.

Attempts to cultivate the virus in hens' eggs have not been successful.

Attempts to induce a CPE with the virus in tissue cultures have not been successful, but the virus has been detected by direct immunofluorescence in experimentally infected DEL and duck embryo kidney (DEK) cell monolayer cultures (Haider & Calnek, 1979).

1.3.1. Molecular characterisation

DVH type III has now been identified as an astrovirus (DAstV-2) by nucleic acid sequence data. It is considered distinct from DVH type II (DAstV-1) (Todd *et al.*, 2009). The RT-PCR and sequencing molecular method described for detection of DAstV-1 can also be used to identify DAstV-2 (Todd *et al.*, 2009).

2. Serological tests

These antibody detection tests are not useful for diagnosis as the clinical disease is too acute for detection of early infection. Serological tests are useful for virus identification, epidemiological disease investigations and titration of antibody response to vaccination. A comparative study assessing DHAV-1 virus neutralisation (VN), enzyme-linked immunosorbent assay (ELISA) and agar gel immunodiffusion (AGID) tests, suggest that VN and ELISA have comparable sensitivity but the sensitivity of AGID was very low (Zhao *et al.*, 1991). A VP1-ELISA utilising the VP1 of DHAV-1 as a coating antigen has been described for the detection of DHAV-1 antibodies in sera. In comparison with VN, the VP1-ELISA had a reported specificity and sensitivity of 92.5% and 96.7%, respectively (Lui *et al.*, 2010). The level of detectable antibodies after DAstV-1 challenge were shown to be low. While an indirect ELISA expressing the C-terminal ORF2 protein of DAstV-1 has been reported, the exact sensitivity and specificity of this test is unclear.

2.1. ELISA for DHAV-1 Detection (procedure extracted from Zhao *et al.*, 1991)

Add 100 µl of coating antigen diluted in carbonic acid-buffered saline (0.5 M, pH 9.6) to each well of an ELISA plate. Incubate overnight at 4°C. Wash plate with 0.01 M PBS (pH 7.4) containing 0.05% Tween-20 (PBST) three times. Add 100 µl of each test serum diluted in PBST containing 0.1% bovine serum albumin (PBST-BSA) to duplicate wells in the sensitised plate. Incubate for 2 hours at 37°C in a humid chamber. Wash plate as described above. Add 100 µl of rabbit anti-duck IgG HRP conjugate diluted in PBST-BSA to each well. Incubate for 2 hours at 37°C. Wash plate as described above. To each well add 100 µl substrate solution (24.3 ml of 0.1 M citric acid + 25.7 ml of 0.2 M phosphate + 50 ml of distilled water, mix and add 40 mg o-phenylenediamine-free base or dihydrochloride, as well as 40 µl of 30% H₂O₂, and use immediately). After 30 minutes, add 25 µl 2.5 M H₂SO₄ to each well. Read absorbance at 490 nm with an ELISA reader. Alternative chromogens may be used in a suitable diluent with appropriate detection systems.

2.2. Virus neutralisation

DHAV, DAstV-1 and DAstV-2 have been used in VN tests *in ovo*. Their success depends on the expression of the virus in the assay system used; with DAstV-1 and DAstV-2, this can be a problem. *In-vitro* tests have been developed for DHAV-1; these include a plaque reduction assay and a microtitre assay (Woolcock, 1986; 1991). The plaque reduction assay may be performed using either primary DEK or DEL cells. Primary cell culture monolayers are prepared in Eagle's minimal essential medium

(MEM) containing 5–10% fetal calf serum (FCS), 2 mM glutamine, 0.17% sodium bicarbonate and gentamicin. Trypsinised cells are seeded into 5 cm diameter Petri dishes, then incubated at 37°C in a 5% CO₂ atmosphere. Monolayers should be nearly confluent at 24–48 hours post-seeding. The monolayers are washed twice with serum-free MEM or Hank's balanced salt solution to remove all traces of FCS before infecting with DHAV-1. Equal volumes of DHAV-1 suspended in serum-free MEM, adjusted to 200 plaque-forming units (PFU) per 0.1 ml, are mixed with equal volumes of serially diluted duck sera (twofold dilutions in MEM). The serum samples should be heat inactivated at 56°C for 30 minutes before testing. The virus/serum mixtures are incubated at 37°C for 1 hour; then 0.1-ml aliquots are added to the confluent cell monolayers, three dishes per dilution. The plates are left for 30 minutes at room temperature (20–22°C), then overlaid with agarose maintenance medium (MEM containing 2% chicken serum and 0.1–0.2% FCS to which agarose had been added to a final concentration of 1% [w/w]). The plates are then placed at 37°C in a 5% CO₂ atmosphere. The number of plaques produced is recorded after 48 hours' incubation. Plaques may be observed using an oblique light source, or alternatively monolayers may be fixed with 10% formal-buffered saline and stained with 1% crystal violet. Serum antibody titres are expressed as the reciprocal of the highest serum dilution that reduces the plaque count by 50%.

A microtitre neutralisation assay may be performed using primary DEK cells. Serial twofold dilutions of each serum sample (heat-inactivated) are prepared in 50 µl of serum-free Eagle's basal medium (BME) in microtitre plates. Approximately 10^{2.0} TCID₅₀ (50% tissue culture infective dose) of DHAV-1 in 50 µl of BME is added to each well and the mixtures are allowed to react at 37°C for 1 hour. Primary DEK cells are suspended in BME supplemented with 10% tryptose phosphate broth, 2 mM L-glutamine, 0.17% sodium bicarbonate and 2–4% chicken serum, and are adjusted to contain 3 × 10⁵ cells/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 fixed with 10% formal-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 4 log₂ is considered to be negative.

These neutralisation tests have been used to assay humoral immune responses to vaccination and for epidemiological surveys, as well as for virus identification.

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

DHAV-1 can be controlled by the use of a live attenuated virus vaccine. This is given to breeder ducks so that immunity is transferred via the yolk to newly hatched birds. Live vaccine virus can also be used to actively immunise newly hatched DHAV-1-susceptible ducklings (Crighton & Woolcock, 1978). An inactivated DHAV-1 vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHAV-1; progeny from these breeders have maternal immunity (Woolcock, 1991). Ducks may also be passively protected by inoculation of antibodies in chicken egg-yolk.

The development and evaluation of a vaccine to protect ducklings against DHAV-3 in Korea has been described (Kim *et al.*, 2009). The methods used are comparable to those described in this text for DHAV-1.

An attenuated live virus DVH type II (DAstV-1) vaccine has been used to protect ducklings only under experimental conditions (Gough *et al.*, 1985).

DAstV-2 infections have been controlled by the use of attenuated live virus vaccines given to breeder ducks, so that the immunity is transferred via the yolk sac to the hatching ducklings.

2. Outline of production and minimum requirements for conventional vaccines

2.1. Characteristics of the seed

2.1.1. Biological characteristics

The DHAV-1 virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times; the DHAV-1 virus vaccine seed used in the USA for live and inactivated vaccines has been passaged 84–89 times.

The DAstV-1 virus vaccine seed originated from an isolate attenuated by 25 serial passages in embryonated chicken eggs (Asplin, 1965), and has been employed only experimentally under field conditions (R.E. Gough, pers. comm.).

The DAstV-2 vaccine seed has been attenuated by 30 serial passages in embryonated duck eggs inoculated via the CAM.

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

All seed viruses should be shown to be free from extraneous viruses that are pathogenic for ducks, chickens or turkeys. The seeds should be free from all microbiological and fungal contamination.

The identity of the virus type should be confirmed by a VN test conducted with specific antiserum by a constant-serum/varying-virus method. In the case of DHAV-1 and DAstV-1, the tests are performed in embryonated chicken eggs; with DAstV-2 the tests are done in embryonated duck eggs. The antiserum should reduce the titre of the respective virus by at least $10^{2.0}$ ELD₅₀ (50% embryo lethal dose).

2.2. Method of manufacture

2.2.1. Procedure

DHAV-1 and DAstV-1 viruses are treated similarly. The vaccine is produced in 9- to –10-day-old specific pathogen free (SPF) embryonated chicken eggs inoculated via the allantoic route, and incubated at 37°C. Most embryo deaths occur within 2–3 days in the case of DHAV-1, but with DAstV-1, the deaths do not occur until 6–10 days after inoculation, although they are harvested at 3–5 days for maximum virus yield. The embryo harvests are homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below. Subsequently, they may be stored satisfactorily between –20°C and –40°C. DHAV-1 attenuated vaccine is also available as a lyophilised preparation that may be stored at 2–8°C. The reconstituted vaccine may be used with or without the incorporation of aluminium hydroxide in the diluent.

In the case of inactivated DHAV-1 vaccine, the embryo harvests are homogenised and clarified by low-speed centrifugation and then further purified by treatment with chloroform (final concentration 10% [v/v]). This preparation is then inactivated with freshly prepared binary ethylenimine (BEI). The inactivated virus is then blended with a suitable adjuvant; 0.2 % (v/v) formalin is added as a preservative (Woolcock, 1991).

The DAstV-2 vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DAstV-2 and incubated at 37°C. Most embryo deaths occur between 6 and 10 days. Eggs containing dying embryos, together with their CAMs, are harvested and homogenised in buffered saline and clarified by low-speed centrifugation. The preparation is diluted as appropriate and dispensed into vials, which are preferably frozen rapidly at –70°C or below.

2.2.2. Egg yolk antibody

Virulent DHAV-1 prepared from duckling livers or attenuated virus may be used to hyperimmunise SPF chickens for egg-yolk antibody production. Eggs are collected from the hyperimmunised birds and stored at 4°C until time of production. The yolks are separated, pooled and blended with an antifoaming agent. The mixture is diluted with buffered saline containing no more than 0.2% (v/v) formalin as a preservative. The dispensed product is stored at 4°C and has a shelf life of 1 year. Tests are carried out for sterility in the usual way for the absence of contaminants.

2.2.3. Requirements for substrates and media

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

2.2.4. In-process controls

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

2.2.5. 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

A group of 1- to 3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of DHAV-1 and DAstV-1), or subcutaneously (in the case of DAstV-2), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHAV-1 vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on egg-yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

iii) Batch potency

For DHAV-1 and DAstV-1 viruses, the virus titre of the vaccine should be determined in 9–10-day-old embryonated chicken eggs inoculated into the allantoic cavity and incubated at 37°C. The immunogenicity, of the vaccine for ducklings susceptible to DHAV-1 and DAstV-1 virus can be assessed by inoculating subcutaneously a minimum of $10^{3.3}$ ELD₅₀ per duckling of the vaccine virus and challenging subcutaneously 72 hours later with $10^{3.0}$ LD₅₀ per duckling of virulent DHAV-1 and DAstV-1 (Crighton & Woolcock, 1978). At least 80% of the vaccinated birds should survive. In the case of DHAV-1, at least 80% of the controls should die and in the case of DAstV-1, a 20% mortality in the controls is more realistic.

The immunogenicity of the inactivated vaccine is considered to be satisfactory if a four-fold or greater increase in neutralising antibody titre can be demonstrated following administration to ducklings that have been previously primed with live attenuated DHAV-1.

For DAstV-2, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated onto the CAM. Immunogenicity tests in ducklings have proved difficult because of the variable pathogenicity of the challenge virus for ducklings.

Potency tests on yolk antibody are done by determining the neutralising index (NI) for the product in embryonated hens' eggs using the constant-yolk/varying-virus method. A minimum NI of $10^{3.0}$ is considered to be satisfactory. The efficacy of the product is determined by inoculating a group of susceptible ducklings with the recommended dose of egg yolk antibody. A second group is left untreated. After 24 hours, each group is challenged with virulent DHAV-1. The product is adjudged efficacious if at least 80% of the treated ducklings survive and at least 80% of the controls die.

2.3. Requirements for authorisation

2.3.1. Safety requirements

A group of 1–3-day-old ducklings susceptible to the type of virus concerned, should be inoculated subcutaneously or intramuscularly (in the case of DHAV-1 and DAstV-1), or

subcutaneously (in the case of type DAstV-2), with the attenuated vaccine at ten times the recommended dose, and kept under observation for between 10 and 21 days for any adverse reactions. Attenuated live vaccines should be stable and not revert to virulence on back passages in susceptible ducklings.

A safety test on the inactivated DHAV-1 vaccine is performed by inoculating the recommended dose (0.5 ml) intramuscularly into a group of day-old ducklings; no adverse effects should be observed during the period of testing.

Safety tests on yolk antibody are done by inoculating 1 ml subcutaneously into each of a group of ducklings, which are then kept under observation for 3 days for signs of adverse effects.

i) Target and non-target animal safety

The vaccines and egg yolk are intended solely for use to protect ducklings against DVH and to immunise breeder ducks so that maternal antibodies may be transferred to progeny.

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

Reversion to virulence on serial passage in ducklings has been reported (Woolcock & Crighton, 1979; 1981).

iii) Environmental consideration

None.

2.3.2. Efficacy requirements

i) For animal production

In the case of newly hatched ducklings, attenuated live DHAV-1 replicates rapidly and results in an immunity within 48–72 hours of vaccination. This immunity persists throughout the susceptible period of life (Crighton & Woolcock, 1978). However in ducklings protected by vaccination of their parents, the level of maternally derived immunity decreases over the first 2 weeks of life, but such ducklings can be actively re-immunised with attenuated virus given subcutaneously or orally at about 7–10 days of age (Hanson & Tripathy, 1976; Tripathy & Hanson, 1986). Alternatively, the immunity can be enhanced by the administration of either specific hyperimmune serum or egg yolk antibody prepared from eggs laid by chickens actively hyperimmunised against DHAV-1.

Breeder ducks primed with live DHAV-1 at 12 weeks of age and then given, intramuscularly, a single dose of inactivated DHAV-1 vaccine at 18 weeks of age should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

ii) For control and eradication

Breeder ducks given live attenuated DHAV-1 vaccine two or three times at 12, 8 and 4 weeks before coming into lay, and breeder ducks given live attenuated DAstV-2 vaccine twice at 12 and 4 weeks before coming into lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHAV-1 vaccine and every 6 months with DAstV-2 vaccine after the onset of lay. DHAV-1 attenuated vaccine can also be supplied as a lyophilised preparation that is blended with a diluent containing aluminium hydroxide, just before administration. This is given at 7 weeks of age with a second dose 2 weeks before onset of lay. This should provide maternally immune progeny throughout a complete laying cycle. No information on the use of DAstV-1 vaccine in breeder ducks is available.

Live attenuated DHAV-1 and DAstV-1 vaccine given subcutaneously or intramuscularly to 1-day-old ducklings protects against the disease for the duration of their susceptibility. No information is available on the use of DAstV-2 vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHAV-1 at 12 weeks of age and then given a single dose of inactivated DHAV-1 vaccine intramuscularly at 18 weeks of age, should produce maternally immune progeny through a complete laying cycle (Woolcock, 1991).

Egg-yolk antibody offers passive immunisation in the face of an outbreak. The duration of its efficacy is short-lived.

2.3.3. Stability

Aqueous preparations of live attenuated DHAV-1, DAstV-1 and DAstV-2 vaccines when stored frozen at -70°C or lower should remain stable for at least 1 year. Once thawed these vaccines should be held at 4°C and used within 1 week. Live lyophilised vaccines may be stored at $2-8^{\circ}\text{C}$ and should retain their potency for at least 1 year.

The inactivated DHAV-1 vaccine is blended with adjuvant and can be stored at 4°C for at least 20 months without loss of immunogenicity.

Egg-yolk antibody can be stored for up to 1 year at 4°C .

3. Vaccines based on biotechnology

Not applicable at present.

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NB: FIRST ADOPTED IN 1989. MOST RECENT UPDATES ADOPTED IN 2017