

CHAPTER 2.3.8.

DUCK VIRUS HEPATITIS

SUMMARY

Hepatitis in ducks can be caused by at least three different viruses. The more common and internationally widespread is duck hepatitis virus (DHV) type I, a member of the Picornaviridae in the new genus Avihepatovirus, which causes a highly lethal, acute, contagious infection in ducklings under 6 weeks of age and, frequently, under 3 weeks of age. It does not occur in older birds. This infection is often referred to simply as duck virus hepatitis. Three genotypes that may also be serotypes of DHV type I have been identified and designated duck hepatitis A virus (DHAV) types 1, 2 and 3.

DHV type II has been reported in the United Kingdom only. It occurred in ducklings from 10 days to 6 weeks of age, and caused pathological changes similar to those of DHV type I. From electron microscopy and molecular studies it is a member of the Astroviridae and is known as duck astrovirus type 1 (DAstV-I).

DHV type III has been reported only in the United States of America. It causes similar liver lesions in young ducklings, but is less virulent than DHV type I. From molecular investigations it is a member of the Astroviridae and designated duck astrovirus type II (DAstV-II). It is considered distinct from DAstV-I.

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.

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

Serological tests: *Serological tests have little value in the diagnosis of the acute infections caused by DHV types I, II and III.*

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

Requirements for vaccines: *DHV type I 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 DHV type-I-susceptible day-old ducklings.*

Ducklings susceptible to DHV type I may also be passively protected with a chicken egg yolk antibody preparation.

DHV type III 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

Duck hepatitis (DH) is caused by at least three different small RNA viruses, namely duck hepatitis virus (DHV) types I, II and III. The most common is DHV type I. The genome sequences of five strains of DHV type I have been released on public databases (strain-DRL-62 – DQ219396, strain-R85952 – DQ226541, strain 03D – DQ249299, strain H – DQ249300 & strain 5886 – DQ249301) (Kim *et al.*, 2006; Tseng *et al.*, 2007).

Analyses of these sequences show them to be closely related picornaviruses, but unrelated to any members of the currently defined genera. It has therefore been proposed that they should form a novel genus within the *Picornaviridae* (Kim *et al.*, 2006; Tseng & Tsai, 2007). It has also been suggested that DHV type I be subdivided and renamed as two additional genotypes and possibly serotypes have been detected; thus duck hepatitis A virus type 1 (DHAV-1 the original DHV type I), DHAV-2 and DHAV-3 have been created (Wang *et al.*, 2008). DHAV-2 is also documented as N-DHV (Tseng & Tsai, 2007) and DHAV-3 has been described (Kim *et al.*, 2007a). DHAV-2 was originally isolated from a mule duckling and a gosling but mixed DHAV-1 and DHAV-2 infections are common in Taiwan (Tseng & Tsai, 2007). All the DHAV-3 isolates are from Korea (Kim *et al.*, 2007a).

The International Committee on Taxonomy of Viruses have created a new genus *Avihepatovirus* in the family *Picornaviridae*. This genus contains the species, *Duck hepatitis A virus*, which includes three (sero)types – duck hepatitis virus I (DHV-I) and two new duck hepatitis virus (N-DHV) genotypes; the three types would be known as *duck hepatitis A virus* (DHAV): DHAV-1, DHAV-2 and DHAV-3.

Until recently DHV type I had only been associated with causing disease in mallard and Pekin ducklings but it has now been reported to cause pancreatitis and encephalitis in Muscovy ducks (Guerin *et al.*, 2007).

DHV type II is an astrovirus also known as duck astrovirus-1, (DAstV-1), and DHV type III is now classified as an astrovirus, duck astrovirus-2 (DAstV-2), distinct from DAstV-1) (Todd *et al.*, 2009).

These viruses, which cause acute infections, should not be confused with *duck hepatitis B virus*, an *Avihepadnavirus* classified in the same group as mammalian hepatitis B virus. The significance of this infection for the duck is not fully understood.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the agent

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

Limited information on the pathogenicity of DHAV-2 and DHAV-3 indicates that the clinical presentation is similar to DHAV-1 but 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).

DHV type I causes a highly contagious infection of ducks. It is of no known public health significance. 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.

The clinical and pathological observations are highly indicative of DHV type I infection. The virus 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. DHV type I is resistant to this treatment.

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

1.1.1. Confirmatory procedures

- i) By subcutaneous or intramuscular inoculation of the isolate into ducklings between 1 and 7 days of age that are susceptible to DHV type I. The characteristic clinical disease should follow, with deaths occurring within 18–48 hours of inoculation, often in less than 24 hours. The ducklings should show the gross pathology attributable to DHV type I. Virus should be re-isolated from the livers.
- 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 later, whereas chicken embryos are more variable and erratic in their response and usually take 5–8 days to die. 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, the greenish colour of the allantois is more pronounced, and both the liver lesions and stunting become more evident.
- iii) 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.

1.1.2. Immunological tests

Such tests have not been used extensively for the routine identification of DHV type I infection. Various virus neutralisation (VN) assays have been described, which may assume greater significance if DHV types II and III infections become more widespread. The tests that have been described (Chalmers & Woolcock, 1987; Woolcock, 1986; 1991; 2008a) include:

- i) Passive subcutaneous immunisation of 1–7-day-old ducklings susceptible to DHAV type 1 with 1–2 ml specific hyperimmune serum or specific egg yolk antibody. These ducklings are then challenged intramuscularly or subcutaneously, 24 hours later with at least $10^{3.0}$ LD₅₀ (50% lethal dose) of the virus isolate. A control group of uninoculated ducklings is similarly challenged. Identification of infection is based on 80–100% survival in the passively immune ducklings and 80–100% mortality in the controls.
- ii) 1–7-day old DHAV type-1-susceptible and DHAV type 1 maternally immune ducklings are challenged intramuscularly or subcutaneously with at least $10^{3.0}$ LD₅₀ of the virus isolate. Identification is based on 80–100% losses in the susceptible ducklings and 80–100% survival in the maternally immune ducklings.
- iii) Serial tenfold dilutions of the virus isolate are mixed with equal volumes of DHAV type-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.

There is evidence for antigenic variation among DHV type I isolates. A variant, DHV type Ia, isolated in the United States of America (USA) only partially reacts with the classical type I 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. Recent reports of disease in Muscovy ducks from France (Guerin *et al.*, 2007; Sandhu *et al.*, 1992; Woolcock, 2008b) and two new genotypes (DHAV-2 and DHAV-3) that may also be serotypes (Kim *et al.*, 2007a; Tseng & Tsai, 2007; Wang *et al.*, 2008) suggest a greater diversity among DHV type I than was originally thought.

1.1.3. Nucleic acid recognition methods

Several publications on the molecular structure of DHV type I have been published emphasising genotypic variation among isolates, particularly from China (Ding & Zhang, 2007; Fu *et al.*, 2008; Jin *et al.*, 2008; Liu *et al.*, 2008), Chinese Taipei (Tseng *et al.*, 2007; Tseng & Tsai, 2007) and Korea (Kim *et al.*, 2006; 2008).

A one-step reverse transcriptase-PCR assay using primers to the conserved 3D gene has been described for DHV-I (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 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.

i) Polymerase chain reaction

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

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

Supernatants prepared from duckling livers infected with DHV type I 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 sample is ground in a tissue grinder and phosphate buffered saline is added to make 10% suspensions. Liver sample suspensions and allantoic fluid are centrifuged at 2000 *g* for 30 minutes, the supernatants are treated with the Viral Gene-spin™ viral DNA/RNA extraction kit following the manufacturer's instructions (iNtRON Biotechnology, Seongnam, Korea). The nucleic acids are used for one-step RT-PCR. After measuring RNA concentrations using the NanoDrop ND-1000 (NanoDrop, Wilmington, DE), the samples are stored at –20°C.

iii) Oligonucleotide primers

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

DHV-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.

iv) One-step RT-PCR

The one-step RT-PCR is conducted using the Maxime RT-PCR PreMix kit (iNtRON Biotechnology). The 20-µl reaction mixtures contain 1 U of OptiScript reverse transcriptase, 2.5 mM dNTPs, 2.5 U i-Star*Taq* 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 (DHV-1 ComF and DHV-1 ComR), and DEPC-treated dH₂O to a total reaction volume of 20 µl.

A T-gradient thermal cycler (Biometra, Gottingen, Germany) 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. Reactions 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 (iNtRON Biotechnology) and Tris-acetate buffer (40 mM Tris-acetate, 1 mM ethylenediamine tetra-acetic acid). Gels are stained with ethidium bromide (0.5 µg/ml), visualised under ultraviolet 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 DHV type I strains. Negative control RNA is obtained from an uninfected duckling liver and does not amplify under the same conditions.

1.2. DHV type II (duck astrovirus type 1 [DAstV-1])

DHV type II, also named duck astrovirus type 1, infection of ducks 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 DHV type I. Affected birds may show signs of polydypsia and usually die within 1–2 hours of appearing sick.

Gross pathological changes include multiple haemorrhages, 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 also occasionally seen on the heart. Histologically, changes in the liver are similar to those seen in DHV type I infections; the extent of bile duct hyperplasia may be greater than with DHV type I, but this is relative. DHV type II has astrovirus-like morphology and virions are 28–30 nm in diameter. It is classified in the family Astroviridae as duck astrovirus 1 (DAstV-I) (Gough *et al.*, 1985; Koci & Schultz-Cherry, 2007).

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

- i) Susceptible ducklings, in which the response can be variable. A mortality rate of up to 20% may occur within a period of 2–4 days. The gross pathology is similar to that observed in field cases (Gough *et al.*, 1985). This is in contrast to the findings with DHAV type 1 infection, which is more virulent and rapid in its effect.
- ii) 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 DHV-II 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.

1.2.1. Immunological tests

Immunological tests have not been employed routinely as the serological response to infection of both ducklings and duck embryos is poor. However, a neutralisation assay has been applied (Gough *et al.*, 1985) for virus identification by inoculating chicken embryos via the amniotic cavity with constant-serum/varying-virus mixtures.

Cross protection tests have been performed in 2–4-day old ducklings (Gough *et al.*, 1985); these are inoculated with antisera to types I or II, then challenged 3 days later with the virus isolate. This technique could distinguish DHV type II from types I and III.

1.2.2. Polymerase chain reaction

Confirmation that DHV-II is an astrovirus has been made using nucleic acid sequence data (Todd *et al.*, 2009).

1.3. DHV type III (duck astrovirus type 2 [DAstV-2])

DHV type III has been reported in the USA only. Losses of up to 20% occur in ducklings immune to DHV type I (Haider & Calnek, 1979; Toth, 1969). DHV type III causes an acute infection of young ducklings with clinical signs similar to those seen in type I infections.

The gross pathology is also similar to type I 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.

The virus 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 intramuscularly into susceptible ducklings. The mortality rate may reach 20% with 60% morbidity. No deaths occur in the first 24 hours and all losses ensue between day 2 and day 4 after inoculation. Intravenous inoculation is more effective; type III infection is less virulent than type I.

- ii) 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. Polymerase chain reaction

DHV type III has now been identified as an astrovirus (DAstV-2) by nucleic acid sequence data. It is considered distinct from DHV type II (DAstV-1) (Todd *et al.*, 2009).

2. Serological tests

These do not apply to diagnosis as the clinical disease is too acute.

All three DHV types have been used in virus neutralisation tests *in ovo*, but their success depends on the expression of the virus in the assay system used; with type II and III viruses this can be a problem. *In-vitro* tests have been developed for DHV type I; 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 DHV type I. Equal volumes of DHV type I 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 DHV type I 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

DHV type I 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 DHV type-I-susceptible ducklings (Crighton & Woolcock, 1978). An inactivated DHV type I vaccine is also effective when administered to breeder ducks that have been primed with live vaccine or previously field exposed to live DHV type I; 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 DHV type I (also named DHAV-1).

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

DHV type III 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 type I virus vaccine seed used most commonly in Europe is derived from an isolate passaged in embryonated chicken eggs 53–55 times; that in the USA for live and inactivated vaccines has been passaged 84–89 times.

The type II 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 type III 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 types I and II viruses, the tests are performed in embryonated chicken eggs; with type III virus 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

DHV types I and II viruses are treated similarly. The vaccine is produced in 9–10-day-old 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 DHV type I, but with type II, 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. DHV type I 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 DHV type I 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 an adjuvant such as LES-STM¹; 0.2 % (v/v) formalin is added as a preservative (Woolcock, 1991).

The type III vaccine is prepared in 10-day-old SPF duck eggs inoculated via the CAM with attenuated DHV type III 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 DHV type I 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 of biological materials for sterility and freedom from contamination may be found in the chapter 1.1.9.

ii) Safety

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 types I and II), or subcutaneously (in the case of type III), 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 DHV type I 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.

iii) Batch potency

For DHV types I and II 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 type I or II 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 DHV virus type I or II (Crichton & Woolcock, 1978). At least 80% of the

1 A preparation of *Salmonella typhimurium* (STM), a B cell mitogen, in a lipid emulsion system (LES). Available from Ribi Immunochem Research, Hamilton, Montana 59840, USA.

vaccinated birds should survive and, in the case of type I, at least 80% of the controls should die; in the case of type II, 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 DHV type I.

For type III virus, the titre of the vaccine should be determined in 10-day-old embryonated duck eggs inoculated on to 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 DHV type I virus. 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 types I and II), or subcutaneously (in the case of type III), 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 DHV type I 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 DHV 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 DHV type I 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 DHV type I.

Breeder ducks primed with live DHV type I at 12 weeks of age and then given, intramuscularly, a single dose of inactivated type I 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 DHV type I vaccine two or three times at 12, 8 and 4 weeks before coming in to lay, and breeder ducks given live attenuated DHV type III vaccine twice at 12 and 4 weeks before coming in to lay should produce passively immune progeny throughout a breeding season. However, it is usually recommended to revaccinate every 3 months with DHV type I vaccine and every 6 months with DHV type III vaccine after the onset of lay. DHV type I 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 DHV type II vaccine in breeder ducks is available.

Live attenuated DHV type I or type II 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 DHV type III vaccine to actively immunise 1-day-old ducklings.

Breeder ducks primed with live DHV type I at 12 weeks of age and then given a single dose of inactivated DHV type I 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 DHV type I, II and III 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°C and should retain their potency for at least 1 year.

The inactivated DHV type I 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

3.1. Vaccines available and their advantages

None

3.2. Special requirements for biotechnological vaccines, if any

Not applicable.

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