

MAREK'S DISEASE

SUMMARY

Marek's disease (MD) is a lymphomatous and neuropathic disease of domestic fowl caused by an alphaherpesvirus, designated Marek's disease virus (MDV), belonging to the genus Mardivirus.

Diagnosis is made on clinical signs and gross or microscopic lesions. Definitive diagnosis must be made by diagnosing the disease (tumour), not the infection. Chickens may become persistently infected with MDV without developing clinical disease. Infection by MDV is detected by virus isolation and the demonstration of viral nucleic acid, antigen or antibodies.

MD is prevented by vaccination with monovalent or multivalent live virus vaccines belonging to various species or serotypes. Vaccines are given as injections either at hatch or, increasingly, in ovo at 17–19 days of incubation.

In chickens, MD can occur at any time, beginning at 3–4 weeks of age or older, sometimes even well after the onset of egg production. Clinical signs observed are paralysis of the legs and wings, with enlargement of peripheral nerves, although nerve involvement is sometimes not seen, especially in adult birds. MDV strains of higher virulence may also cause increased mortality in young birds of 1–2 weeks of age, especially if they lack maternal antibodies. Depending on the strain of MDV, lymphomatous lesions can occur in multiple organs such as the ovary, liver, spleen, kidneys, lungs, heart, proventriculus and skin. Tumours produced by MDV may also resemble those induced by retroviral pathogens such as avian leukosis virus and reticuloendotheliosis virus and their differentiation is important. Compared with the uniform cell populations observed in lymphoid leukosis, MD lymphomas consist of pleomorphic lymphoid cells of various types.

Identification of the agent: *Under field conditions, most chickens become infected with MDV during the first few weeks of life and then carry the infection throughout their lives, often without developing overt disease. The infection is usually detected by co-cultivating live buffy coat cells on monolayer cultures of chicken kidney cells or chicken/duck embryo fibroblasts, in which characteristic viral plaques develop within a few days. MDV belongs to the genus Mardivirus that includes three species (serotypes) designated as Gallid herpesvirus 2 (serotype 1), Gallid herpesvirus 3 (serotype 2) and Meleagrid herpesvirus 1 or herpesvirus of turkeys (HVT) (serotype 3). Serotype 1 includes all the virulent strains and some attenuated vaccine strains. Serotype 2 includes the naturally avirulent strains, some of which are used as vaccines. Antigenically related HVT is also used as vaccine against MD, and, more recently, as a recombinant viral vaccine vector. MDV genomic DNA and viral antigens can be detected in the feather tips of infected birds using polymerase chain reaction (PCR) and the radial immunoprecipitation test, respectively. These molecular diagnostic tests can be used for differentiating pathogenic and vaccine strains.*

Serological tests: *Antibodies to MDV develop within 1–2 weeks of infection and are commonly recognised by the agar gel immunodiffusion test, or the indirect fluorescent antibody test.*

Requirements for vaccines: *MD is prevented by vaccinating chickens in ovo at 17–19 days of incubation, or at day of hatch. Attenuated variants of serotype 1 strains of MDV are the most effective vaccines. Serotype 2 strains may also be used, particularly in bivalent vaccines, together with HVT. Serotype 1 and 2 vaccines are only available in the cell-associated form. Live HVT vaccines are widely used and are available both as cell-free (lyophilised) and cell-associated ('wet') forms. Bivalent vaccines consisting of serotypes 1 and 3 or trivalent vaccines consisting of serotypes 1, 2, and 3 are also used. The bivalent and trivalent vaccines have been introduced to combat the very virulent strains of MDV that are not well controlled by the monovalent vaccines.*

Vaccination greatly reduces clinical disease, but does not prevent persistent infection and shedding of MDV. The vaccine viruses may also be carried throughout the life of the fowl although shedding is not common.

A. INTRODUCTION

Marek's disease (MD) (Davison & Nair, 2004; Schat & Nair, 2013; Sharma, 1998) is a disease of domestic fowl (chickens) caused by a herpesvirus of the genus *Mardivirus*. The genus includes three species (serotypes) designated as Gallid herpesvirus 2 (serotype 1), Gallid herpesvirus 3 (serotype 2) and Meleagrid herpesvirus 1 or herpesvirus of turkeys (HVT) (serotype 3). Serotype 1 includes all the virulent strains and some attenuated vaccine strains. Serotype 2 includes the naturally avirulent strains, some of which are used as vaccines. Antigenically related HVT is also used as vaccine against MD, and, more recently, as a recombinant viral vaccine vector.

Birds are infected by inhalation of contaminated dust from the poultry houses, and, following a complex life cycle, the virus is shed from the feather follicle of infected birds (Baigent & Davison, 2004). MD can occur at any time, beginning at 3–4 weeks of age or older, sometimes even well after the onset of egg production. MD is associated with several distinct pathological syndromes, of which the lymphoproliferative syndromes are the most frequent and are of the most practical significance. In the classical form of the disease, characterised mainly by the involvement of nerves, mortality rarely exceeds 10–15% and can occur over a few weeks or many months. In the acute form, which is usually characterised by visceral lymphomas in multiple organs, disease incidence of 10–30% in the flock is not uncommon and outbreaks involving up to 70% can occur. Mortality may increase rapidly over a few weeks and then cease, or can continue at a steady or slowly falling rate for several months. In the acute form, birds are often severely depressed and some may die without showing signs of clinical disease. Non-neoplastic disease involving brain pathology with vasogenic oedema resulting in transient paralysis is increasingly recognised with MD induced by the more virulent strains of the virus.

In its classical form, the most common clinical sign of MD is partial or complete paralysis of the legs and wings. The characteristic finding is enlargement of one or more peripheral nerves. Those most commonly affected and easily seen at post-mortem are the vagus, brachial and sciatic plexuses. Affected nerves are often two or three times their normal thickness, the normal cross-striated and glistening appearance is absent, and the nerve may appear greyish or yellowish, and sometimes oedematous. Lymphomas are sometimes present in the classical form of MD, most frequently as small, soft, grey tumours in the ovary, and sometimes also in the lungs, kidneys, heart, liver and other tissues. 'Grey eye' caused by iridocyclitis that renders the bird unable to accommodate the iris in response to light and causes a distorted pupil is common in older (16–18 week) birds, and may be the only presenting sign.

In the acute form, the typical finding is widespread, diffuse lymphomatous involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Sometimes lymphomas also arise in the skin around the feather follicles and in the skeletal muscles. Affected birds usually have enlarged peripheral nerves, as is seen in the classical form. In younger birds, liver enlargement is usually moderate in extent, but in adult birds the liver may be greatly enlarged and the gross appearance identical to that seen in lymphoid leukaemia, from which the disease must be differentiated. Nerve lesions are often absent in adult birds with MD.

The heterogeneous population of lymphoid cells in MD lymphomas, as seen in haematoxylin-and-eosin-stained sections, or in impression smears of lymphomas stained by May–Grünwald–Giemsa, is an important feature in differentiating the disease from lymphoid leukaemia, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukaemia, gross lymphomas occur in the bursa of Fabricius, and the tumour has an intrafollicular origin and pattern of proliferation. In MD, although the bursa is sometimes involved in the lymphoproliferation, the tumour is less apparent, diffuse and interfollicular in location. Peripheral nerve lesions are not a feature of lymphoid leukaemia as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukaemia and forms of MD sometimes seen in adult birds in which the tumour is lymphoblastic with marked liver enlargement and absence of nerve lesions. If post-mortems are conducted on several affected birds, a diagnosis can usually be made based on gross lesions and histopathology. However there are other specialised techniques described. The expression of a Meq biochemical marker has been used to differentiate between MD tumours, latent MDV infections and retrovirus-induced tumours (Schat & Nair, 2013). The procedure may require specialised reagents and equipment and it may not be possible to carry out these tests in laboratories without these facilities. Development of a number of polymerase chain reaction (PCR)-based diagnostic tests has allowed rapid detection of pathogenic MD virus (MDV) strains (Schat & Nair, 2013). Other techniques, such as detection by immuno-fluorescence of activated T cell antigens present on the surface of MD tumour cells (MD tumour-associated surface antigen or MATSA), or of B-cell antigens or IgM on the tumour cells of lymphoid leukaemia can give a presumptive diagnosis, but these are not specific to MD tumour cells.

Nerve lesions and lymphomatous proliferations induced by certain strains of reticuloendotheliosis virus (REV) are similar, both grossly and microscopically, to those present in MD. Although REV is not common in the majority of chicken flocks, it should be borne in mind as a possible cause of lymphoid tumours; its recognition depends on virological and serological tests on the flock. REV can also cause neoplastic disease in turkeys, ducks, quail and other species. Another retrovirus, designated lymphoproliferative disease virus (LPDV), also causes lymphoproliferative disease in turkeys. Although chicken flocks may be seropositive for REV, neoplastic disease is rare. The main features in the differential diagnosis of MD, lymphoid leukosis and reticuloendotheliosis are shown in Table 1. Peripheral neuropathy is a syndrome that can easily be confused with the neurological lesions caused by MDV. This is not very common but its incidence may be increasing in some European flocks (Bacon *et al.*, 2001). There are no recognised health risks to humans working with MDV or the related herpesvirus of turkeys (HVT) (Schat & Erb, 2014).

Table 1. Features useful in differentiating Marek's disease, lymphoid leukosis and reticuloendotheliosis

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis*
Age	Any age. Usually 6 weeks or older	Not under 16 weeks	Not under 16 weeks
Signs	Frequently paralysis	Non-specific	Non-specific
Incidence	Frequently above 5% in unvaccinated flocks. Rare in vaccinated flocks	Rarely above 5%	Rare
<i>Macroscopic lesions</i>			
Neural involvement	Frequent	Absent	Infrequent
Bursa of Fabricius	Diffuse enlargement or atrophy	Nodular tumours	Nodular tumours
Tumours in skin, muscle and proventriculus, 'grey eye'	May be present	Usually absent	Usually absent
<i>Microscopic lesions</i>			
Neural involvement	Yes	No	Infrequent
Liver tumours	Often perivascular	Focal or diffuse	Focal
Spleen	Diffuse	Often focal	Focal or diffuse
Bursa of Fabricius	Interfollicular tumour and/or atrophy of follicles	Intrafollicular tumour	Intrafollicular tumour
Central nervous system	Yes	No	No
Lymphoid proliferation in skin and feather follicles	Yes	No	No
Cytology of tumours	Pleomorphic lymphoid cells, including lymphoblasts, small, medium and large lymphocytes and reticulum cells. Rarely can be only lymphoblasts	Lymphoblasts	Lymphoblasts
Category of neoplastic lymphoid cell	T cell	B cell	B cell

*Reticuloendotheliosis virus may cause several different syndromes. The bursal lymphoma syndrome is most likely to occur in the field and is described here.

B. DIAGNOSTIC TECHNIQUES

Table 2. Test methods available for the diagnosis of Marek's disease 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						
Histo-pathology	n/a	n/a	n/a	+++	+	–
Virus isolation	n/a	n/a	n/a	+	–	–
Antigen detection	n/a	n/a	n/a	+	–	–
Real-time qPCR	n/a	n/a	n/a	++	+	–
Detection of immune response						
AGID	n/a	n/a	n/a	–	+	+
IFA	n/a	n/a	n/a	–	+	+

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.
 qPCR = quantitative polymerase chain reaction; AGID = agar gel immunodiffusion; IFA = Indirect fluorescent antibody.

1. Identification of the agent

1.1. Virus isolation

Infection by MDV in a flock may be detected by isolating the virus from the tissues of infected chickens. However, the ubiquitous nature of MDV must be taken into consideration and the diagnosis of MD should be based on a combination of MDV isolation or detection of the genome and the occurrence of clinical disease. Commonly used sources are buffy coat cells from heparinised blood samples, or suspensions of lymphoma cells or spleen cells. When these samples are collected in the field, it is suggested that they be transported to the laboratory under chilled conditions but not frozen. As MDV is highly cell associated, it is essential that these cell suspensions contain viable cells. The cell suspensions are inoculated into monolayer cultures of chicken kidney cells or duck embryo fibroblasts (chicken embryo fibroblasts (CEF) are less sensitive for primary virus isolation). Serotype 2 and 3 viruses (see Section C.2.1 *Characteristics of the seed*) are more easily isolated in CEF than in chicken kidney cells. Usually a 0.2 ml suspension containing from 10^6 to 10^7 live cells is inoculated into duplicate monolayers grown in plastic cell culture dishes (60 mm in diameter). Inoculated and uninoculated control cultures are incubated at 37°C in a humid incubator containing 5% CO₂. Alternatively, closed culture vessels may be used. Culture medium is replaced at 2-day intervals. Areas of cytopathic effects, termed plaques, appear within 3–5 days and can be enumerated at about 7–10 days.

Another, less commonly used source of MDV for diagnostic purposes is feather tips, from which cell-free MDV can be extracted. Tips about 5 mm long, or minced tracts of skin containing feather tips, are suspended in an SPGA/EDTA (sucrose, phosphate, glutamate and albumin/ethylenediamine tetra-acetic acid) buffer for extraction and titration of cell-free MDV (Calnek *et al.*, 1970). The buffer is made as follows: 0.2180 M sucrose (7.462 g); 0.0038 M monopotassium phosphate (0.052 g); 0.0072 M dipotassium phosphate (0.125 g); 0.0049 M L-monosodium glutamate (0.083 g); 1.0% bovine albumin powder (1.000 g); 0.2% EDTA (0.200 g); and distilled water (100 ml). The buffer is sterilised by filtration and should be at approximately pH 6.5.

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

This suspension is sonicated and then filtered through a 0.45 µm membrane filter for inoculation on to 24-hour-old chicken kidney cell monolayers from which the medium has been drained. After absorption for 40 minutes, the medium is added, and cultures are incubated as above for 7–10 days.

Using these methods, MDV of serotypes 1 and 2 may be isolated, together with the HVT (serotype 3), if it is present as a result of vaccination. With experience, plaques caused by the different virus serotypes can be differentiated fairly accurately on the basis of time of appearance, rate of development, and plaque morphology. HVT plaques appear earlier and are larger than serotype 1 plaques, whereas serotype 2 plaques appear later and are smaller than serotype 1 plaques.

MDV and HVT plaques may be identified as such using specific antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes (Lee *et al.*, 1983).

1.2. Antigen detection

A variation of the agar gel immunodiffusion (AGID) test used for serology (see below) may be used to detect MDV antigen in feather tips as an indication of infection by MDV. Glass slides are prepared with a coating of 0.7% agarose (e.g. A37) in 8% sodium chloride, containing MDV antiserum. Tips of small feathers are taken from the birds to be examined and are inserted vertically into the agar, and the slides are maintained as described below. The development of radial zones of precipitation around the feather tips denotes the presence in the feather of MDV antigen and hence of infection in the bird.

1.3. Polymerase chain reaction (PCR)

Genomes of all three serotypes have been completely sequenced (Afonso *et al.*, 2001; Lee *et al.*, 2000). PCR tests have been developed for the diagnosis of MD. Real-time quantitative PCR (qPCR) to quantify MDV genome copies has also been described (Abdul-Careem *et al.*, 2006; Baigent *et al.*, 2005; Islam *et al.*, 2004). In addition, PCR tests that enable differentiation of oncogenic and non-oncogenic strains of serotype 1 MDV, and of MDV vaccine strains of serotypes 2 and 3 (Becker *et al.*, 1992; Bumstead *et al.*, 1997; Handberg *et al.*, 2001; Silva, 1992; Zhu *et al.*, 1992) have been described. Two methodologies have also been described for differentiation of oncogenic and non-oncogenic strains of serotype 1 MDV by qPCR (Baigent *et al.*, 2016; Gimeno *et al.*, 2014). PCR may also be used to quantitate virus load in tissues (Baigent *et al.*, 2005; Bumstead *et al.*, 1997; Burgess & Davison, 1999; Reddy *et al.*, 2000) or differentially detect MDV and HVT in the blood or feather tips (Baigent *et al.*, 2005; Davidson & Borenshtain, 2002). A modification of the PCR test, designated LAMP (loop-mediated isothermal amplification), has also been used for the detection and differentiation of MDV serotypes (Wozniakowski *et al.*, 2013).

Table 3. Example PCR primer sets used for identification of MDV

MDV specificity	Primer set	Product size	Reference
pp38	Fwd: 5'-GTG-ATG-GGA-AGG-CGA-TAG-AA-3' Rev: 5'-TCC-GCA-TAT-GTT-CCT-CCT-TC-3'	225 bp	Cao <i>et al.</i> , 2013
gB	Fwd: 5'-CGG-TGG-CTT-TTC-TAG-GTT-CG-3' Rev: 5'-CCA-GTG-GGT-TCA-ACC-GTG-A-3'	66 bp	Gimeno <i>et al.</i> , 2005
Meq	Fwd: 5'-GAG-CCA-ACA-AAT-CCC-CTG-AC-3' Rev: 5'-CTT-TCG-GGT-CTG-TGG-GTG-T-3'	1.41 kb	Dunn <i>et al.</i> , 2010

2. Serological tests

The presence of antibodies to MDV in non-vaccinated chickens from about 4 weeks of age is an indication of infection. Before that age, such antibodies may represent maternal transmission of antibody via the yolk and are not evidence of active infection.

Viruses, antigens and antisera can be obtained from commercial suppliers or from OIE Reference Laboratories for Marek's Disease (see Table in Part 4 of this *Terrestrial Manual*), but international standard reagents have not yet been produced.

2.1. Agar gel immunodiffusion

There is no test suitable for certifying individual animals prior to movement, but the AGID test is employed commonly to detect antibody. The test is conducted using glass slides coated with 1% agar in phosphate buffered saline containing 8% sodium chloride. Adjacent wells are filled with antigen or serum and these are incubated in a humid atmosphere at 37°C for 24 hours for diffusion to take place; positive sera show reactions of identity with known positive serum and antigen. The antigen used in this test is either disrupted MDV-infected tissue culture cells or an extract of feather tips, or skin containing feather tracts obtained from MDV-infected chickens. The cell culture antigen is prepared by propagating MDV in chicken kidney cells or chicken embryo fibroblast cells. When cytopathic effect is confluent, the cells are detached from the culture vessel and suspended in culture medium or phosphate buffered saline without tryptose phosphate broth (presence of tryptose phosphate broth may produce non-specific precipitin lines) at a concentration of about 1×10^7 cells/ml. This suspension is then freeze-thawed three times and used as antigen.

2.1.1. Test procedure

- i) Make a 1% solution of agar in 8% sodium chloride by standing the mixture in a boiling water bath.
- ii) Either a microscope slide or a Petri dish can be used and the agar is poured to a thickness of 2–3 mm.
- iii) Cut holes in the agar using a template with a centre well and 6 wells spaced at equal distance around the centre well. The diameter of wells should be approximately 5.3 mm, and the wells should be about 2.4 mm apart. A template with cutters is commercially available.
- iv) The antigen is placed in the centre well and the standard antiserum is placed in alternate exterior wells. Serum samples to be tested are placed in the remaining three wells so that a continuous line of identity is formed between an unknown sample that is positive and the known positive control sera.
- v) Incubate the slide for 24 hours at 37°C in a humid container and read the results over a lamp in a darkened room.

2.2. Indirect fluorescent antibody

The indirect fluorescent antibody test demonstrates the ability of a test serum to stain MDV plaques in cell cultures (Silva *et al.*, 1997; Spencer & Calnek, 1970). These tests are group specific and more sensitive than the AGID test.

2.2.1. Test procedure

- i) Prepare MDV antigen in cell culture dishes or 96-well microtitre plates.
- ii) Fix cells with acetone–alcohol mixture for 10 minutes then air dry. Plates may be held in refrigerator until ready to be stained or frozen for longer periods of time.
- iii) Wet surface of plate with PBS, discard PBS, then add serum at multiple dilutions (1:5, 1:10, 1:20, 1:40). Incubate in water bath or incubator at 37°C for 30–60 minutes.
- iv) Discard serum, wash plates three times with distilled water followed by three washes with PBS, blot.
- v) Add fluorescein-labeled affinity purified antibody to chicken IgG. Incubate in water bath or incubator at 37°C for 30–60 minutes.
- vi) Repeat washing, then plates read plates immediately using fluorescent microscope

2.3. Other tests

A virus neutralisation test for the ability of a serum to neutralise the plaque-forming property of cell-free MDV can also be employed. However, this test is more suitable for research purposes than for routine diagnostic use. Enzyme-linked immunosorbent assays (ELISA) for detecting MDV antibodies are available (Cheng *et al.*, 1984; Sharma, 1998; Zelnik *et al.*, 2004).

C. REQUIREMENTS FOR VACCINES

1. Background

1.1. Rationale and intended use of the product

Control of MD is essentially achieved by the widespread use of live attenuated vaccines (Nair, 2004, Schat & Nair, 2013). Commercial biological products mainly used in the control of MD are the cell-associated live virus vaccines. Lyophilised cell-free vaccines are rarely used. Marek's disease vaccines are injected subcutaneously into day-old chicks after hatch or *in ovo* at the 17th to 19th day of embryonation (Sharma, 1999).

2. Outline of production and minimum requirements for conventional vaccines

The requirements for producing vaccines are outlined below, and in Chapter 1.1.8 *Principles of veterinary vaccine production*, but other sources should be consulted for further information on the procedures (Code of Federal Regulations title 9 [9CFR], 2016; European Pharmacopoeia, 1997a and 1997b; Merieux *et al.*, 1974; Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985). Protocols are given in the British Pharmacopoeia Monograph 589, and 9CFR, Part 113 (CFR, 2016). The guidelines in this *Terrestrial Manual* are intended to be general in nature and may be supplemented by national and regional requirements.

2.1. Characteristics of the seed

2.1.1. Biological characteristics

Viruses of the MDV group are classified under three serotypes – 1, 2, and 3 – on the basis of their antigenic relatedness.

i) Serotype 1

This includes all the pathogenic strains of the virus, ranging from strains that are very virulent plus (e.g. 648A), very virulent (e.g. Md/5, Md/11, Ala-8, RB-1B), virulent (e.g. HPRS-16, JM GA), mildly virulent (e.g. HPRS-B14, Conn A) and finally to weakly virulent (e.g. CU-2, CVI-988). These strains may be attenuated by passage in tissue culture, with loss of pathogenic properties but retention of immunogenicity, to provide strains that have been used as vaccines. Those that have been used commercially include attenuated HPRS-16 and CVI-988 (Rispen) strains. Attenuated variants of the very virulent strains have been used in experimental vaccines to protect against the variant form of acute MD caused by the very virulent strains. Md11/75C/R2/23 is one such strain (Witter, 2001) licensed for use in the United States of America. Serotype 1 vaccines are prepared in a cell-associated ('wet') form that must be stored in liquid nitrogen.

ii) Serotype 2

This includes naturally avirulent strains of MDV (e.g. SB-1, HPRS-24, 301B/1, HN-1), and several of these have been shown to provide protection against virulent strains. The SB-1 and 301B/1 strains have been developed commercially and used, particularly with HVT, in bivalent vaccines for protection against the very virulent strains. Serotype 2 vaccines exist only in the cell-associated form.

iii) Serotype 3

This contains the strains of naturally avirulent HVT (e.g. FC126, PB1), which are widely used as a monovalent vaccine, and also in combination with serotype 1 and 2 strains in bivalent or trivalent vaccines against the very virulent strains of MDV. HVT may be prepared in a cell-free form as a freeze-dried (lyophilised) vaccine or in a cell-associated ('wet') form.

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

The substrates used for commercial vaccine production are primary chicken embryo fibroblasts (CEF) derived from specific pathogen free (SPF) flocks or duck embryo fibroblasts. CEF from SPF flocks are preferred to duck cells because more is known about chicken-embryo-transmitted pathogens and methods for their detection.

Methods for testing SPF flocks for freedom from infection are available (Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985). SPF chicken flocks should be free from avian adenoviruses, including egg-drop syndrome 76 virus, avian encephalomyelitis virus, avian leukosis virus (subgroups A, B and J), avian nephritis virus, avian reoviruses, avian rotaviruses, chicken anaemia virus, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, Newcastle disease virus, reticuloendotheliosis virus, *Salmonella* spp., and turkey rhinotracheitis virus. Freedom from other infections may also be required as they become recognised.

SPF duck flocks should be free from avian adenoviruses, avian reoviruses, *Chlamydia*, duck virus enteritis, duck virus hepatitis types I and II, influenza type A virus, Newcastle disease virus, *Pasteurella* (now *Riemerella*) *anatipestifer*, REV, and *Salmonella* infections. Freedom from other infections may also be required as they become recognised.

Seed virus must be free from the agents listed for SPF flocks and from other contaminants that may be acquired in the laboratory. A vaccine strain derived from turkeys must also be free from LPDV and haemorrhagic enteritis virus.

The ability of the master seed virus – and derived virus at the limit of the passage range used to produce vaccinal virus (usually not more than five tissue culture passages) – to protect against MD must be determined. Standardised protection tests are published but requirements may vary according to the relevant regulatory authority. They involve vaccination of MD-susceptible SPF chickens at 1 day of age and challenge with sufficient virulent MDV 5–8 days later to cause at least a 70–80% incidence of MD in unvaccinated chickens. Two types of tests are used. In the protection index test, a single field dose (1000 PFU) (plaque-forming units) of vaccine is given and the incidence of MD in vaccinated birds is compared with that in unvaccinated birds. Protective indices should be greater than 80, i.e. vaccinated birds should show at least 80% reduction in the incidence of gross MD, compared with unvaccinated controls.

A PD₅₀ (50% protective dose) test may also be used, involving the inoculation of five four-fold serial dilutions of vaccine virus selected to provide protection above and below the 50% level, followed by challenge 8 days later to determine the PD₅₀ value. The assays are conducted using a standard reference vaccine for comparison. The PD₅₀ may be as low as 4 PFU, but higher values can be obtained depending on the vaccine strain, whether cell-free or cell-associated and the presence or absence of maternal antibodies in the test chickens. On the basis of the PD₅₀ test, it has been suggested that the minimum vaccine field dose should be the greater of two values: 10³ PFU or 100 PD₅₀.

Extensive field trials of a new vaccine strain in the presence of field challenge should be conducted, using different breeds of birds of varying MDV maternal antibody status, to ensure efficacy and persistence of immunity. Experience suggests that vaccinal immunity, once acquired, is lifelong.

2.2. Method of manufacture

2.2.1. Procedure

Vaccines against MD are prepared from live attenuated strains belonging to the 3 serotypes using CEF as the substrates.

2.2.2. Requirements for substrates and media

Substrate cells are seeded into flat-bottomed vessels for stationary incubation, or into cylindrical vessels for rolled incubation. Media commonly used are Eagle's minimal essential medium, or 199 medium, buffered with sodium bicarbonate and supplemented with 5% calf serum. Incubation is at 37°C for 48 hours.

For cell-associated vaccine, cultures are infected with production HVT or MDV seed-virus stock, in cell-associated form, which is usually two passages beyond the master seed stock. Cultures are incubated for 48 hours (depending on the vaccine strain) then the infected cells are harvested by treating the washed cell sheet with an EDTA/trypsin solution to allow the cells to begin to detach. The flasks are then returned to the incubator (37°C) to allow complete detachment. The cells are subjected to low-speed centrifugation, and then resuspended in the freezing mixture consisting of cell growth medium containing 7.5–15% dimethylsulphoxide (DMSO), and held at 4°C or dispensed immediately into the final vaccine containers, usually glass ampoules, which are flame sealed and frozen in liquid nitrogen.

Cell-free lyophilised vaccine may be prepared from HVT, but not from MDV strains. For the production of this form of vaccine, HVT-infected cultures are incubated for 72 hours, infected cells are detached from the vessel as described above, or scraped from the walls of the vessel. The cells are suspended in a small volume of growth medium, centrifuged, and resuspended in a buffered stabiliser solution containing 8% sucrose, but free from protein to prevent frothing. The cell suspension is sonicated to release virus, the cell debris is removed, the suspension is diluted with a complete stabiliser – such as SPGA – filled into the final containers, and lyophilised.

The dilution rate for both cell-associated and cell-free vaccines is based on previous experience, as is the number of doses required per container, because the virus content of the harvested material cannot be assayed prior to filling the final containers. The virus content of the finished product can subsequently be added to the label.

2.2.3. In-process controls

For optimal results in preparing cell-associated vaccine, a slow rate of freezing (1–5°C per minute) and rapid thawing are essential. The infectivity titre of the infected cells, and hence the number of doses per ampoule, are determined after filling the ampoules. Similarly for cell-free vaccine, the virus content of the final suspension, and hence the number of doses per container, is determined after filling.

2.2.4. Final product batch tests

Using immunofluorescence assay (IFA) with monospecific serum, checks should be carried out to show that the product is of the same specificity as the seed virus. This is best done using monoclonal antibodies.

i) Sterility/purity

Extensive testing is required of the materials used to produce the vaccine, and of the final product. Substrate cells should come from an SPF flock, in particular, free from vertically transmitted agents. Substances of animal origin used in the preparation of vaccines such as serum, trypsin, and bovine serum albumin, must be free from extraneous agents.

Batches of the final vaccine produced should be tested for freedom from contaminating bacteria, fungi, mycoplasma and the viruses listed for SPF flocks; tests for purity of the diluent should also be conducted. Suitable tests for the detection of extraneous agents at all stages of vaccine production are recommended by several official bodies (Ministry of Agriculture, Fisheries and Food, UK, 1990; 9CFR, 2016; Thornton, 1985) and in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

ii) Safety

Ten doses of vaccine or a quantity of diluent equivalent to two doses of vaccine should be inoculated into separate groups of 10–25 1-day-old SPF chickens. No adverse reactions should occur during a 21-day observation period.

With cell-associated vaccine, care is necessary to avoid injury from ampoules that may explode when they are removed from liquid nitrogen. Eye protection must be worn.

iii) Batch potency

The standard dose of each type of vaccine is 1000 PFU per chicken or egg but may be higher based on the titre used in the efficacy study. Virus content assays are conducted on batches of vaccine to ensure that the correct dose per bird will be achieved.

2.3. Requirements for authorisation

2.3.1. Safety requirements

i) Target animal safety

The master seed virus should be shown to be non-pathogenic for chickens by inoculating ten times the field dose into 1-day-old SPF chickens of a strain susceptible to MD, to ensure that it does not cause gross lesions or significant microscopic lesions of MD by 120 days of age. It should be noted that some vaccine strains of MDV and HVT may produce minor and transient microscopic nerve lesions.

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

No increase in virulence should occur during six serial passages of the vaccine strain in 1-day-old SPF MD-susceptible chickens. Ten times the field dose of vaccine is inoculated initially and then passaged by inoculation of heparinised blood at 5–7-day intervals, and tests for viraemia are run to check that virus is transferred at each passage. The birds receiving the final passage are kept for 120 days and should be free from MD lesions. However, some strains such as Rispens, may cause some mild MD lesions. The important observation is that the virulence should not change. This is a difficult test because the genetic resistance of the chickens fundamentally affects the apparent virulence of the virus, so does the type of inoculum. After successful completion of laboratory safety tests, the safety of the strain should be confirmed in extensive field trials.

2.3.2. Efficacy requirements

A test for duration of immunity is carried out on the seed virus only. Such immunity is apparently lifelong. Preservatives are not included in the vaccine or diluent. During use, reconstituted vaccine must be kept cool and cell-associated vaccine should be agitated to keep cells in suspension.

2.3.3. Stability

Tests for stability are carried out on six representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine. These tests should be conducted under the conditions of storage of the vaccine. The lyophilised product should have a shelf life of 12 months when stored at 2–8°C. Manufacturers may double the virus content of the vaccine to compensate for some loss of titre during storage. Appropriate diluting fluids are provided for use with cell-associated and freeze-dried vaccines. The stability of reconstituted vaccine over a 2-hour period should be tested.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Genetically engineered recombinant vaccines (Lee *et al.*, 2010; Reddy *et al.*, 1996) based on the existing live MD vaccines can offer simultaneous protection against other avian diseases, depending on the protective antigens engineered into the recombinant vaccine. A number of recombinant vaccines based on HVT vectors that induce protection against avian diseases such as avian influenza, infectious bursal disease, Newcastle disease and infectious laryngotracheitis are commercially available.

3.2. Special requirements for biotechnological vaccines, if any

None.

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NB: There are OIE Reference Laboratories for Marek's disease
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Marek's disease

NB: FIRST ADOPTED IN 1990. MOST RECENT UPDATES ADOPTED IN 2017.