

CHAPTER 3.3.9.

MAREK'S DISEASE

SUMMARY

Description and importance of disease: Marek's disease (MD) is a lymphomatous and neuropathic disease of gallinaceous birds caused by an alphaherpesvirus, designated Marek's disease virus (MDV).

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.

In chickens, classical 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 younger 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 gonads, 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.

Detection and identification of the agent: Infection by MDV is detected by virus isolation and the demonstration of viral nucleic acid, antigen or antibodies. 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. Virus isolation is performed 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 alphaherpesvirus 2 (serotype 1), Gallid alphaherpesvirus 3 (serotype 2) and Meleagrid alphaherpesvirus 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 18–19 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.

A. INTRODUCTION

1. Description and impact of the disease

Marek's disease (MD) (Davison & Nair, 2004; Nair *et al.*, 2020) is a lymphoproliferative disease of gallinaceous birds caused by Marek's disease virus (MDV). Birds are infected by inhalation of contaminated dust from the poultry houses, and, following complex pathogenic pathways, the virus is shed from the feather follicle of infected birds. MD can occur at any time, beginning at 3–4 weeks of age or older, sometimes even well after the onset of egg production. During the early phases of the disease, atrophy of the bursa of Fabricius and the thymus are detected which often remains unnoticed. MD is associated with several distinct clinicopathological syndromes. In the classical neurological 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 lymphoproliferative 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% incidence 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 lymphoproliferative form, birds are dull, lose condition and show signs of immunosuppression. Another syndrome is characterised by vasogenic oedema of the brain resulting in transient paralysis is increasingly recognised with MD induced by the more virulent strains of the virus.

In its classical neurological 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 examination are the sciatic, brachial and sometimes vagal nerves. However, other nerves can also be affected. 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. Microscopic examination of the nerves reveals lymphoid infiltration which can vary from mild inflammatory lesions to marked lymphomatous infiltration. In certain neurological forms, similar lesions can be detected in the brain and the eye resulting in typical clinical signs. Tumours typical of other forms of MD are sometimes present in this form of MD, most frequently as small, soft, grey tumours in the gonads, liver, kidney, heart and other tissues.

In the lymphoproliferative form, the typical finding is a multicentric lymphoma with involvement of the liver, gonads, spleen, kidneys, lungs, proventriculus and heart. Grossly, the lymphomatous lesions can appear as distinct white masses or as marked ill-defined enlargement of the affected organ, mostly the liver, spleen and proventriculus. Histopathology reveals typical lymphoid proliferation. Lymphomas can also arise in the skin around the feather follicles and in the skeletal muscles. The eye can be similarly affected usually presenting with lymphomatous infiltration of the iris. Upon microscopic examination, lymphoid infiltrations can also be detected in other parts of the eye.

2. Nature and classification of the pathogen

MDV is a highly cell-associated alphaherpesvirus of the genus *Mardivirus*. The genus includes three species (serotypes) designated as Gallid alphaherpesvirus 2 (serotype 1), Gallid alphaherpesvirus 3 (serotype 2) and Meleagrid alphaherpesvirus 1 or herpesvirus of turkeys (HVT) (serotype 3). Serotype 1 includes all the virulent strains, which are further divided into pathotypes that include mild (m)MDV, virulent MDV (vMDV), very virulent MDV (vvMDV) and very virulent plus MDV (vv+MDV). Some attenuated vaccine strains also belong to serotype 1. 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 is a double-stranded DNA virus of approximately 160–180 kb in length. The structure is similar to other alphaherpesviruses with unique short and long sequences that are both flanked by inverted repeat sequences. Whole genome sequences are available for a number of MDV strains that have been used for both diagnostic and research purposes.

3. Zoonotic potential and biosafety and biosecurity requirements

MDV is not considered to be a zoonotic pathogen, as nearly all experimental data indicate that mammalian cells or animals cannot be infected with MDV (reviewed by Schat & Erb, 2014). Biosecurity, however, is an important component for prevention of MD in poultry, in combination with vaccination and improvements in host genetic

resistance. Limiting early MDV exposure of a newly hatched flock is crucial for maximum efficacy of vaccination programmes, and the failure to limit early exposure is likely to be the leading cause of vaccination failures. Laboratory manipulations should be performed with appropriate biosafety and containment procedures as determined by biological risk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*).

4. Differential diagnosis

MD diagnosis requires differentiation from other avian tumour virus diseases, such as lymphoid leukosis and reticuloendotheliosis, as well as non-virus-induced tumours, and some non-tumour lesions. 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 leukosis, in which the lymphomatous infiltrations are composed of uniform lymphoblasts. Another important difference is that, in lymphoid leukosis, 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 leukosis as they are in MD. The greatest difficulty comes in distinguishing between lymphoid leukosis 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 the 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 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 leukosis 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 considered 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.

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

Feature	Marek's disease	Lymphoid leukosis	Reticuloendotheliosis*
<i>Microscopic lesions</i>			
Peripheral nerves	Yes	No	Infrequent
Liver tumours	Often perivascular	Focal or diffuse	Focal
Spleen	Focal/multifocal in layers or diffuse in broiler breeders	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
Detection and identification of the agent^(a)						
Histopathology	–	–	–	+++	+	–
Virus isolation	–	–	–	+	–	–
Antigen detection	–	–	–	+	–	–
PCR	–	–	–	++	+	–
Real-time PCR	–	–	–	+++	+	–
LAMP	–	–	–	++	+	–
Detection of immune response						
AGID	–	–	–	–	+	+
IFA	–	–	–	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;

+ = suitable in very limited circumstances; – = not appropriate for this purpose.

PCR = polymerase chain reaction; LAMP = Loop-mediated isothermal amplification;

AGID = agar gel immunodiffusion; IFA = indirect fluorescent antibody.

^(a)A combination of agent detection methods applied on the same clinical sample is recommended.

1. Detection and identification of the agent

1.1. Cell culture for 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. While this is more commonly used for PCR-based diagnosis, feather tips can also be useful for preparing cell-free MDV. 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.

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 HVT (serotype 3), if it is present as a result of vaccination. With experience, cytopathic effects and 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.

1.2. Antigen detection – immunolabelling techniques

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 (ideally blood 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.

Antigen detection can be especially helpful in differentiating a MD tumour from other lymphoid tumours. In addition to confirming the tumour as T-cell lymphoma, immunolabelling with monoclonal or polyclonal antibodies can be used to confirm expression of Meq, which is the primary MDV oncogene (Ahmed *et al.*, 2018; Gimeno *et al.*, 2014). MDV and HVT cell culture plaques may also be identified as such using specific antibodies raised in chickens. Monoclonal antibodies may be used to differentiate serotypes (Lee *et al.*, 1983).

1.3. Molecular methods – detection of nucleic acids

The 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 using specific primer sets that amplify

specific MDV genes (Table 3). Real-time PCR to quantify MDV genome copies has also been described (Baigent *et al.*, 2005; Gimeno *et al.*, 2005; Islam *et al.*, 2004). MD lymphomas have relatively high levels of MDV DNA compared with latently infected tissues and can be differentiated using defined cycle threshold ratio cut-off levels (Gimeno *et al.*, 2005). 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; Zhu *et al.*, 1992) have been described. Different studies have also described the differentiation of oncogenic and vaccine strains (Baigent *et al.*, 2016; Gimeno *et al.*, 2014; Renz *et al.*, 2006) by real-time PCR using specific primer and probe sets (Table 4). Real-time PCR tests to distinguish virulent MDV-1 strains from CVI-988 (Rispens) vaccine are based on a single nucleotide polymorphism in the pp38 gene. Because of this constraint, there can be low-level cross reactivity between the virulent MDV-specific probe and CVI-988 (Rispens) DNA. This can be overcome by inclusion of CVI-988 (Rispens) DNA as a negative control in the PCR: in this case the reaction threshold must be set above the amplification signal of this CVI-988 (Rispens) negative control. Consideration must also be given to the possibility of the emergence of virulent MDV strains with a similar mutation but one that cannot be distinguished from CVI-988 (Rispens). For the emerging MDV gene-deleted vaccines (Zhang *et al.*, 2017), the tumorigenic strain can be differentiated from the vaccine strain by PCR for the specific gene deletion fragment. PCR may also be used to quantitate virus load in tissues (Baigent *et al.*, 2005; Bumstead *et al.*, 1997; Burgess & Davison, 1999) 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 (5' → 3')	Product size	Reference
pp38	Fwd: GTG-ATG-GGA-AGG-CGA-TAG-AA Rev: TCC-GCA-TAT-GTT-CCT-CCT-TC	225 bp	Cao <i>et al.</i> , 2013
gB	Fwd: CGG-TGG-CTT-TTC-TAG-GTT-CG Rev: CCA-GTG-GGT-TCA-ACC-GTG-A	66 bp	Gimeno <i>et al.</i> , 2005
Meq	Fwd: GAG-CCA-ACA-AAT-CCC-CTG-AC Rev: CTT-TCG-GGT-CTG-TGG-GTG-T	1.41 kb	Dunn <i>et al.</i> , 2010

Table 4. Example PCR primer and probe sets used for differentiating virulent and vaccine strains using real-time PCR

Real-time PCR Target	Sequence (5' → 3'): 5' reporter and 3' quencher for probes	Product size	Reference
Virulent MDV-specific pp38	SNP FP: GAG-CTA-ACC-GGA-GAG-GGA-GA SNP RP: CGC-ATA-CCG-ACT-TTC-GTC-AA Probe CCC-ACT-GTG-ACA-GCC (5' FAM, 3' BHQ1)	99 bp	Baigent <i>et al.</i> , 2016
CVI-988 (Rispens)-specific pp38	SNP FP: GAG-CTA-ACC-GGA-GAG-GGA-GA SNP RP: CGC-ATA-CCG-ACT-TTC-GTC-AA Probe CCC-ACC-GTG-ACA-GCC (5' FAM, 3' BHQ1)	99 bp	Baigent <i>et al.</i> , 2016
MDV-2 DNA Pol gene	Pol FP: AGC-ATG-CGG-GAA-GAA-AAG-AG Pol RP: GAA-AGG-TTT-TCC-GCT-CCC-ATA Probe CGC-CCG-TAA-TGC-ACC-CGT-GAC-T (5' FAM, 3' BHQ1)	100 bp	Renz <i>et al.</i> , 2006
HVT sORF-1 gene	sORF1 FP: GGC-AGA-CAC-CGC-GTT-GTA-T sORF1 RP: TGT-CCA-CGC-TCG-AGA-CTA-TCC Probe AAC-CCG-GGC-TTG-TGG-ACG-TCT-TC (5' FAM, 3' BHQ1)	77 bp	Renz <i>et al.</i> , 2006

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 the WOAHA Reference Laboratory for Marek's Disease¹, 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 (PBS) 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 CEF. 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) Pour the agar to a thickness of 2–3 mm on either a microscope slide or a Petri dish.
- 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) Add the antigen in the centre well and the standard antiserum in alternate exterior wells. Add the serum samples to be tested to 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 serum.
- 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 immunofluorescence test

The indirect immunofluorescence 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.

1 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

- iv) Discard serum, wash plates three times with distilled water followed by three washes with PBS, blot.
- v) Add fluorescein-labelled affinity purified antibody to chicken IgG. Incubate in water bath or incubator at 37°C for 30–60 minutes.
- vi) Repeat washing, then 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; Zelnik *et al.*, 2004).

C. REQUIREMENTS FOR VACCINES

1. Background

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 18–19 days 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; Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985). Protocols are given in the British Pharmacopoeia Monograph 589, and 9CFR, Part 113 (9CFR, 2016). The guidelines in this *Terrestrial Manual* are intended to be general in nature and may be supplemented with national and regional requirements.

2.1. Characteristics of the seed

2.1.1. Biological characteristics of the master seed

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 (Rispons) strains. 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 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; Veterinary Services Memorandum 800.65²). 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, fowl pox virus, infectious bronchitis virus, infectious bursal disease virus, infectious laryngotracheitis virus, influenza type A virus, MDV, *Mycoplasma gallisepticum*, *M. synoviae*, Newcastle disease virus, reticuloendotheliosis virus, *Salmonella* spp., and turkey rhinotracheitis virus. Requirement for freedom from chicken anaemia virus varies between different countries. 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. Some countries, including the USA, do not have an official definition of SPF duck flock. 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. 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 (or by *in ovo* route for an *in ovo* label claim) 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.

2.1.3. Validation as a vaccine strain

The vaccine strain shall be non-pathogenic for chickens as shown by the inoculation of 50 MD susceptible SPF egg/chickens with a 10× field dose by the route of vaccination(s) planned. At

2 https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_65.pdf

120 days of age, the birds are evaluated for MD lesions. If lesions are found, the seed is considered unsatisfactory.

The master seeds are tested for purity, identity, and extraneous agents and are approved for use in vaccine production following satisfactory testing.

The serotype 2 MDVs used in vaccines function in a synergistic fashion with HVT protecting against the milder challenges and the bivalent/trivalent combinations with serotypes 1 and 2 protecting against more virulent challenges.

An effective titre of each serotype of MDV is established in a vaccination-challenge study.

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 (derived from SPF embryonated eggs) as the substrates.

2.2.2. Requirements for ingredients

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. When serotypes are combined for a product, the product is titred in serotype-specific fashion to confirm a sufficient quantity of each serotype.

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 (9CFR, 2016; Ministry of Agriculture, Fisheries and Food, UK, 1990; Thornton, 1985) and in Chapter 1.1.9 *Tests for sterility and freedom from contamination of biological materials intended for veterinary use*.

When firms use different MDV serotypes and HVT constructs in manufacturing, serials may be screened for the presence of other contaminating serotypes and constructs.

ii) Safety

Ten doses of vaccine or a quantity of diluent equivalent to 10 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 of each serotype per bird will be achieved.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 *Characteristics of the seed* and Section C.2.2 *Method of manufacture*) should be submitted to the relevant authorities. The manufacturing information should be consistent with the production of the serial used in the efficacy study. Testing information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume. This testing may be confirmed by a regulatory authority laboratory.

In-process controls are part of the manufacturing process in the form of cell substrate testing and working seed testing to ensure the purity and identity of the seeds and cells.

2.3.2. Safety requirements

i) Target and non-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 (or embryonated eggs when using the *in-ovo* route) 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.

Three serials are evaluated in a field safety study to evaluate the product prior to approval for use under field conditions. Pharmacovigilance evaluates the product post-licensure.

Recombinant seeds should be tested in non-target animals to ensure no unexpected changes in virulence had resulted from the gene insertion.

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

No increase in virulence should occur during at least four 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 CVI-988 (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. The CVI-988 (Rispens) strain must be field safety tested alone or it must be marketed in combination with the other Marek's serotypes as the other serotypes may attenuate the CVI-988 (Rispens).

2.3.3. Efficacy requirements

One group of SPF eggs or chickens receives the vaccine at a minimum dose at a minimum age. Other chickens or eggs from the same hatch serve as controls. The non-vaccinated challenged group serve as challenge controls. Another group is neither vaccinated nor challenged to serve as test controls. If a bivalent or trivalent vaccine is being evaluated, a group is vaccinated with HVT. This group should not be well protected to validate the virulence of the challenge. Challenge occurs at 5–8 days of age and the challenge culture must be carefully handled to preserve virulence. The birds are observed until 7 weeks of age. Birds lost during this time or birds evaluated at the end of the study, are evaluated for grossly observable Marek's disease lesions. For a satisfactory test, at least 80% of the vaccinates must remain free of grossly observable lesions.

A titre is associated with the efficacy study. From this titre, a titre for serial release and a throughout dating titre are assigned based on test variation and titre loss over time. For bivalent or trivalent products, two or three titres are assigned to the efficacy.

A test for duration of immunity may be carried out on the product. Such immunity is apparently lifelong for Marek's disease vaccines but must be supported by data to be included on the labelling. Preservatives may be 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. If duration of immunity data are not created, the labelling must specify that the duration of immunity is not known.

2.3.4. Duration of immunity

As part of the regulatory approval procedure, the manufacturer may demonstrate the duration of immunity (DOI) of a given vaccine by either challenge or alternative test at the end of the claimed period of protection. In the case of Marek's disease vaccines it is a vaccination–challenge study design. The level of immunity at the short-term study described above and at the claimed duration should be similar. If it is demonstrated/approved, it may be included on the labelling as opposed to a statement that the duration is not known.

2.3.5. Stability

Tests for stability are carried out on representative batches of vaccine to show that titre is maintained during the stated shelf life of the vaccine – a real-time stability study. 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 increase the virus content of the vaccine to compensate for some loss of titre during storage but must maintain a titre above the throughout dating titre. The throughout dating titre is set based on the titre of the efficacy serial. Appropriate diluting fluids are provided for use with cell-associated and freeze-

dried vaccines. The stability of reconstituted vaccine over a 2-hour period is tested as part of the serial or batch release testing. The diluted virus is held on ice for 2 hours to mimic field use.

3. Vaccines based on biotechnology

3.1. Vaccines available and their advantages

Genetically engineered recombinant vaccines (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. They can also offer the *in ovo* route for other antigens. 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 recombinant vaccines

For HVT constructs expressing other antigens, the potency test is a two-part assay. First, the HVT backbone is titred. Second, the protein expression from the resulting plaques is assayed. The foreign gene expression should be equal to or greater than the HVT titre. Combining two HVT constructs in a single vaccine is not acceptable due to interference. However, the combination of more than one antigen in a single HVT construct is allowed. For recombinants, specific studies to support no changes to the tissue tropism, non-target animal safety, environmental stability, and shed and spread are conducted to support environmental release of the vaccine. Additionally, for duration of immunity claims, the duration of the Marek's disease protection and the protection provided by the inserted gene must be measured for the claim.

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NB: There is a WOAHP Reference Laboratory for Marek's disease (please consult the WOAHP Web site: <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>).

Please contact the WOAHP 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 2023.