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Rural Industries Research and Development Corporation

Systematic pathotyping of Australian Marek's disease virus isolates

Final Report of RIRDC Project UNE-83J

A report for the Rural Industries Research and Development Corporation

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Foreword

Marek's disease (MD) remains one of the most important diseases of chickens worldwide with an estimated economic impact of $US1-2 billion. After a prolonged outbreak of Marek's disease between 1991 and 1996 in eastern Australia new vaccines were introduced into Australia to vaccinate layers and breeders and routine *in ovo* vaccination of broilers with HVT vaccine was introduced for the first time. Routine vaccination of broilers in the USA industry has been associated with an increase in virulence of the Marek's disease virus. These viruses not only cause more severe MD, they also overcome the protective effects of vaccines. This has meant that after a period of time vaccines such as HVT have become ineffective and have had to be replaced by new vaccines or vaccine combinations. In the USA a method for categorizing MD virus (MDV) isolates into different classes based on their ability to overcome the protective effects of vaccines has been developed. This groups MDVs into various 'pathotypes' on the basis of MDV-induced lesions in vaccinated and unvaccinated chickens.

As Australia has now embarked on a mass HVT-vaccination approach to controlling MD in broilers it is important that we assess the range of MDV pathotypes currently circulating in our poultry industry and develop methods that will enable us to both track increases in pathogenicity and respond quickly when they occur. This was the focus of this project.

The key findings of the project are that:

a) Isolation and growth of MDV in cell culture is improved if chicken kidney cells rather than chicken embryo kidney and chicken embryo fibroblasts are used. Isolation is further improved if clinical specimen material is inoculated directly to freshly trypsinised primary cells, rather than to monolayer cultures of the same cells in growth medium. Under these conditions it is unnecessary to undertake a medium change at 3-4 days.
b) A higher isolation rate is achieved if primary infective material is first amplified in chickens and fresh spleen material then used to inoculate cell cultures.
c) Nevertheless, the majority of MDV field isolates fail to grow to high titre in cell culture, or fail to produce typical cytopathic effects in cell culture without extensive passage.
d) Pathotyping in specific pathogen free (SPF), maternal antibody (mab)-free chickens provides a sensitive means of ranking MDV isolates on virulence.
e) Virulence in unvaccinated chickens is not strongly related to the ability to induce disease in HVT-vaccinated chickens. Thus pure virulence and "vaccine resistance" appear to different traits.
f) Australian MDV isolates vary widely in virulence with several falling into the very virulent (vvMDV) category. Highly pathogenic strains induce severe and permanent immunosuppression, marked early paralysis and mortality between days 9-20 post-challenge (especially in males) and a high incidence of lymphoid tumours in unvaccinated SPF chickens. Vaccination with HVT provided variable levels of protection against this with the level of protection not closely related to virulence in unvaccinated chickens.
g) Measurements made as few as 14 days post-challenge (well before any MD tumour lesions appear) correlate very well with final pathotyping measurements made at day 56 post-challenge. This offers a real opportunity for shorter, cheaper and ethically more acceptable, pathotyping of MDVs.
h) There is little evidence of a systematic increase in virulence in MDV in Australia over the last decade although our power to detect such a trend is low given the small number of isolates tested.
i) There is significant polymorphism in the sequence of the MDV Meq gene, a key gene involved in the ability of MDV to induce lymphoid tumours. This may eventually be linked to virulence or be used as a genetic marker for a given isolate.

The project outcomes should be of use to all levels of the Australian poultry industry. They confirm that while current MD vaccination programs are providing relatively good control of MD, there are isolates against which HVT provides only limited protection. This indicates that there is sufficient diversity amongst Australian MDVs in 'HVT-resistance' that selection pressure for it in the face of widespread MD vaccination in broilers is likely to occur. The project has provided researchers and policy-makers with

improved tools for isolation of MDV and monitoring of MDV pathogenicity. It has also provided a stock of current MDV isolates stored in various forms. The project has also demonstrated that isolation and growth of MDV on cell culture is much more difficult and expensive than originally anticipated.

In terms of industry policy the project results suggest that ongoing monitoring of MDV pathogenicity is warranted as the period of widespread broiler vaccination extends. Methods that reduce reliance on vaccination to control MD should also be supported. For the purposes of monitoring pathogenicity consideration should be given to developing methods of viral propagation and titration that do not rely on cell culture, but rather use chickens themselves to grow the virus. This approach is greatly facilitated by the presence of modern molecular methods for differentiation and quantification of MDV.

This project was funded from industry revenue which is matched by funds provided by the Australian Government

This report is an addition to RIRDC's diverse range of over 1500 research publications. It forms part of our Chicken Meat R&D sub-program which aims to

- develop the necessary technologies to define and control endemic and emerging infectious and non-infectious diseases and develop strategies and methods for rapid recognition and control of emergency animal diseases
- develop nutritional strategies to improve nutrient utilisation through the optimisation of gut health and manipulation of nutrient constituents
- develop and disseminate enhanced on-farm and processing plant food safety programs and develop through-chain strategies for control of Campylobacter and other food safety pathogens
- improve public awareness of safe handling of chicken meat products
- identify objective measures of bird welfare, evaluate welfare issues and address identified problem areas by the development of strategies that enhance bird welfare
- assist industry to develop and implement a national 'whole of industry' biosecurity program
- undertake regular assessment of consumer perceptions of industry practices and products
- quantify resource use across the industry and identify opportunities for more efficient resource and waste product management
- establish and facilitate adoption of performance-based environmental criteria based on acceptable farming practices
- identify and quantify the environmental impacts of the industry and investigate and develop practical technologies and management practices to minimise the impact of environmental emissions
- commission domestic and international benchmarking studies which investigate differentials in total costs of production
- investigate the impact of the regulatory operating environment on industry competitiveness
- provide a 'clearing house' function for international research in relevant fields
- identify and support relevant technology transfer, training and networking opportunities for both industry personal and the industry's supporting the R&D community

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Peter O'Brien
Managing Director

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Excellent technical support on this project was provided by Julie Cooke and Nadeene Clarke at RMIT, and Sue Burgess and Paul Reynolds at UNE. At UNE, Experiments 2 and 4 formed part of the Master of Rural Science program of Mr Zahid Hussain and we are grateful for his inputs into these experiments. Zahid was supported by a Poultry CRC student scholarship. Katrin Renz, a PhD student at UNE also contributed significantly to the execution of Experiment 5 and has done most of the Meq gene sequencing. Julie Cooke and Brian Meehan contributed the sections on MDV isolation methods and Katrin Renz drafted the Meq sequencing report. We are grateful for these inputs.

Poultry CRC project 03-17 was closely aligned with this project, particularly in its support of virus isolation and growth in cell culture and it is impossible to completely separate the activities of the two projects in this area.

The isolator facility at the University of New England used for this project was established with funding from UNE and the Australian Research Council in addition to Baiada and the RIRDC through this project. The Queensland Department of Primary Industries and Fisheries provided the original isolators for which we are grateful.

Abbreviations

AECL	Australian Egg Corporation Limited
ANOVA	Analysis of variance
ARC	Australian Research Council
BAC	Bacterial artificial chromosome. A means of enabling bacteria to express a complete viral genome. These can be fully infective and pathogenic but remain completely stable as they are cloned into a bacterim.
Bursa	Bursa of Fabricius
CAV	Chicken infectious anaemia virus
CEF	Chicken embryo fibroblasts
CKC	Chicken kidney cells
CPE	Cytopathic effects (in cell culture)
d	Day or days
HEPA	High Efficiency Particulate Air. Refers to filters designed to remove 99.97% of all airborne pollutants 0.3 microns or larger from the air.
HVT	Herpesvirus of Turkeys. Syn. Marek's disease virus serotype 3. Genus Mardivirus, subfamily alphaherpesvirus
Lϕ	Lymphocytes
Lymphoma	A cancer of lymphatic tissue, specifically of the lymphocytes.
mab	Maternal antibody
MD	Marek's disease
MDV	Marek's disease virus. Generally refers to MDV1.
MDV1	Marek's disease virus serotype 1. Genus Mardivirus, subfamily alphaherpesvirus
MDV2	Marek's disease virus serotype 2. Syn. Gallid herpesvirus type 3 (GaHV-3). Genus Mardivirus, subfamily alphaherpesvirus

mMDV	Mild MDV. A pathotype under the USDA ADOL classification. MDV which induces mainly paralysis and nerve lesions. HVT provides good protection.
Pathogen	A disease-producing organism
Pathogenicity	The capacity of a pathogen to cause disease. Syn. Virulence
Pathotype	A sub-specific classification of a pathogen based on its pathogenicity for a specific host(s). In the case of MDV it usually refers to a system of classification developed at the USDA ADOL involving the pathoypes m, v, vv and vv+ (Witter, 1997).
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
pc	Post challenge
PCR	Polymerase chain reaction (conventional, end point form)
pfu	Plaque forming units
PI	Protective index. (%MD in Sham-vaccinated chickens – %MD in HVT-vaccinated chickens) ÷ (%MD in Sham-vaccinated chickens) x 100
pv	Post vaccination
qPCR	Real-time quantitative PCR
RIRDC	Rural Industries Research and Development Corporation
RMIT	The Royal Melbourne Institute of Technology (RMIT University).
RTD-PCR	Real-time PCR
SPF	Specific pathogen free
Syn.	Synonym
UNE	The University of New England
USDA ADOL	United States Department of Agriculture Avian Diseases and Oncology Laboratory
Virulence	The ability of an infectious agent to induce disease. Syn. Pathogenicity.
vMDV	Virulent MDV. A pathotype under the USDA ADOL classification. MDV which causes low levels of mortality by day 56pc, but induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT provides good protection.
vvMDV	Very virulent MDV. A pathotype under the USDA ADOL classification. MDV which causes moderate levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT is only partially protective but HVT/MDV2 vaccines provide a high level of protection.
vv+MDV	Very virulent plus MDV. A pathotype under the USDA ADOL classification. MDV which causes high levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/MDV2 are only partially protective.
VR	Virulence rank (100 – PI)

Contents

Executive Summary

What is the report about?

This report is a final report of RIRDC research project UNE-83J. It details our findings on a project aimed at defining the virulence of the strains of Marek's disease virus currently circulating in the Australian Poultry Industry. One aspect of virulence is the ability to overcome the protection offered by current vaccination programs and this project investigated this for vaccination with HVT. The report also details the methods used to isolate MDV from the field and to test it for virulence and makes recommendations in this area.

Who is the target audience?

The report should be of interest to industry veterinarians, technical services managers and consultants, chicken health researchers, industry R&D administrators and vaccine manufacturers. It is written in a scientific manner and is not directed at individual producers.

Background

Marek's disease (MD) remains one of the most important diseases of chickens worldwide with an estimated economic impact of $US1-2 billion (Morrow and Fehler 2004). Caused by a cell-associated herpesvirus, MD includes a wide spectrum of pathology ranging from severe immunosuppression, acute early mortality, paralysis, and development of lymphoid tumours depending on the virulence of the MD virus (MDV) strain and a range of host factors. Since 1970 it has been controlled primarily with live vaccines. After a prolonged outbreak of Marek's disease between 1991 and 1996 in eastern Australia new vaccines were introduced into Australia to vaccinate layers and breeders and routine *in ovo* vaccination of broilers with HVT vaccine was introduced for the first time.

In the USA there has been a steady increase in the virulence of MDV over time, possibly associated with routine vaccination of broilers and extensive re-use of litter in that country. The more virulent strains not only cause more severe MD and new pathological syndromes, they also overcome the protective effects of vaccines. This has meant that after a period of time vaccines such as HVT have become ineffective and have had to be replaced by new vaccines or vaccine combinations. In the USA a method for categorizing MDV isolates into different classes based on their ability to overcome the protective effects of vaccines has been developed. This groups MDVs into various 'pathotypes' on the basis of MDV-induced lesions in vaccinated and unvaccinated chickens.

As Australia has now embarked on a mass HVT-vaccination approach to controlling MD in broilers it is important that we assess the range of MDV pathotypes currently circulating in our poultry industry and develop methods that will enable us to both track increases in pathogenicity and respond quickly when they occur. This was the focus of this project.

Objectives

The broad aim of the project was to improve our understanding of the evolution in virulence of MDV in Australia and to provide up to date information to the industry on the current situation regarding MD virulence, upon which rational decisions on managing the disease can be based. Specific project objectives were to:

a) define the level of pathogenicity of current and previous Australian MDV isolates in SPF chickens using internationally recognised protocols;
b) define the extent to which vaccination with HVT vaccine protects against these;
c) improve our ability to characterise MDV isolates at a molecular level; and
d) determine the extent of immunosuppression induced by the most virulent recent isolates as immunosuppression is closely related to pathogenicity.

An implicit objective in the work was also to review and test existing methodology for the isolation and amplification of MDV and to evaluate methods used in pathotyping.

Methods used

The project involved collaboration between researchers at UNE and RMIT. The general approach was as follows:

- an initial call to industry for submission of infective material from suspect MD outbreaks with submission of soft tissues or feathers to RMIT or dust samples to UNE
- isolation of MDV on cell culture at RMIT or into chickens at UNE
- growth of MDV in cell culture and confirmation of freedom from contaminating vaccine strains or other chicken pathogens
- use of strains growing to high titre in pathotyping experiments in isolators at UNE using SPF chickens. New isolates would be tested against the reference strain MPF57. As far as possible the UNE experiments should follow the USDA ADOL method of Witter, (1997) although only HVT vaccine, rather than HVT and HVT/MDV2 bivalent vaccine would be used.

The project commenced on July 1, 2002 and finished on 31/12/2005. Samples totalling 533 were submitted to RMIT from the field, of which 238 were MDV1 positive on PCR. A total of 655 different isolations on cell culture were attempted of which 181 recorded some cytopathic effects. Only 4 of these MDVs grew to high titre in cell culture. However 17 different isolates were able to successfully infect chickens out of the 27 tested in chickens. Four major chicken experiments and several smaller experiments were conducted in the isolator facility at UNE. Due to the non-availability of suitable MDV isolates grown to high titre, several of the UNE pathotyping experiments were converted to screening experiments with an element of pathotyping, but also a significant element of virus isolation and amplification included in the design.

Key findings

In terms of our ability to isolate and grow MDV the main findings were that:

a) Isolation and growth of MDV in cell culture is improved if chicken kidney cells rather than chicken embryo kidney and chicken embryo fibroblasts are used. Isolation is further improved if clinical specimen material is inoculated directly to freshly trypsinised primary cells, rather than to monolayer cultures of the same cells in growth medium. Under these conditions it is unnecessary to undertake a medium change at 3-4 days.
b) A higher isolation rate is achieved if primary infective material is first amplified in chickens and fresh spleen material then used to inoculate cell cultures.
c) Nevertheless, the majority of MDV field isolates fail to grow to high titre ($>10^4$ pfu/ml) in cell culture, or fail to produce typical cytopathic effects in cell culture without extensive passage. Only 4 new isolates grew to high titre from 238 MDV1-positive submissions between 2002 and 2006.

In terms of our ability to characterise the virulence of local isolates of MDV the main findings were that:

d) Pathotyping in specific pathogen free (SPF), maternal antibody (mab)-free chickens provides a sensitive means of ranking MDV isolates on virulence.
e) Australian MDV isolates vary widely in virulence with several falling into the very virulent (vvMDV) category. Highly pathogenic strains induce severe and permanent immunosuppression, marked early paralysis and mortality between days 9-20 post-challenge (especially in males) and a high incidence of lymphoid tumours in unvaccinated SPF chickens. Vaccination with HVT provided variable levels of protection against this with the level of protection not closely related to virulence in unvaccinated chickens.
f) Measurements made as few as 14 days post-challenge (well before any MD tumour lesions appear) correlate very well with final pathotyping measurements made at day 56 post-challenge. This offers a real opportunity for shorter, cheaper and ethically more acceptable pathotyping of MDVs.
g) There was little evidence of a systematic increase in virulence in MDV in Australia over the last decade although the power to detect such change was not great within the project.

h) There is significant polymorphism in the sequence of the MDV Meq gene, a key gene involved in the ability of MDV to induce lymphoid tumours. This may eventually be linked to virulence or be used as a genetic marker for a given isolate.

Implications

In terms of the virulence of current MDV isolates the work has confirmed that very virulent MDVs against which HVT provides limited protection are circulating within industry. However such strains were reported in Australia more than a decade ago (McKimm-Breschkin *et al.* 1990) and inclusion of two pathogenic strains from the early 1990s, MPF57 and Woodlands-1 (De Laney *et al.* 1995; De Laney *et al.* 1998) in experiment 4 did not reveal any major difference in virulence of these isolates compared with more recent isolates. However, given the switch to widespread vaccination of broiler chickens with HVT during the late 1990s it would be naive to assume that ongoing evolution in virulence, as documented in the USA, is unlikely to occur here. For this reason, and because virus isolation problems prevented wider testing of isolates under the present project, we recommend ongoing surveillance of MDV virulence by industry.

With regard to the methodology of isolating MDV, amplifying it, certifying it free of contaminants and using it in formal pathotyping experiments, the project has made considerable progress. While the methodology of MDV isolation in cell culture was refined and improved at RMIT, success rates in growing the virus to high titre ($>10^4$ pfu/ml) were extremely low, and growth on cell culture effectively became a barrier to testing the virulence of the bulk of current MDV isolates. Similar problems in the past were overcome by using chicken-derived infective material in challenge experiments (McKimm-Breschkin *et al.* 1990) and we suggest a return to these approaches with effort devoted to standardizing them for use in formal challenge experiments with known doses of infective virus. The recent advent of specific PCR assays for identification and enumeration of different MDV serotypes greatly facilitates this approach, as infection in chickens can be detected very early using molecular methods, and challenge material thus titrated in birds so that defined doses can be used (ie multiples of Chicken Infective $Dose_{50}$).

With regard to the methodology of pathotyping MDV, use of maternal antibody negative SPF chickens and a fixed 500pfu challenge dose of MDV provided sensitive ranking of isolates by virulence. It remains to be determined if such rankings are retained when the same isolates are used in maternal antibody positive commercial chickens.

Experiment 4 demonstrated that there is no clear association between the virulence of MDV isolates in unvaccinated SPF chickens, and the ability of such isolates to overcome the effects of HVT-vaccination. This indicates that "virulence" and "vaccine resistance" may be separate traits rather than belonging to the one continuum as suggested by the ADOL pathotyping method. The main implication of this is that all formal pathotyping experiments should include both vaccinated and unvaccinated treatments.

The marked association between early measures of MDV virulence around day 14pc and measures based on subsequent induction of gross MD tumours offers the prospect of very short pathotyping experiments, particularly those used for screening purposes. These could be as short as 14 days post-challenge if immunosuppressive or viral load end points are used, or up to 3 weeks if induction of the early mortality syndrome is included in the end points.

Recommendations

In order to maintain and improve Australia's readiness to deal with future MD problems two broad recommendations are made. These are aimed primarily at policy makers within the industry.

1. Given the low success rate of isolating and growing MDV in cell culture, the expense and time involved in such an approach and the resources devoted to it over the last decade, alternative

approaches to the isolation, amplification and storage of MDV isolates should be developed and standardized. In general these should replace cell culture with isolation and dose titration in SPF chickens with infectivity determined by PCR +/- outcomes when titrated in chickens rather than cell culture. Such methods should allow the rapid isolation and amplification of large numbers of field isolates and remove the artificial barrier of growth in cell culture to inclusion of isolates in pathotyping studies.

2. Given the wide variation in virulence observed during the project, and the failure of HVT to provide adequate protection against several isolates in Experiment 4 ongoing surveillance of MDV virulence is recommended. This should be facilitated by the development of low cost, effective methods. Such a scheme might include:
    - Screening of isolates for pathogenicity in unvaccinated SPF chickens. This could also serve as a viral amplification step and test for freedom from contaminants.
    - Amplification and titration of infective material from high virulence isolates in SPF chickens.
    - Formal pathotyping of the most virulent isolates in experiments using commercial chickens and current vaccination protocols.
    - At some stage importation of USA reference strains or BACs derived from such strains should be considered to allow a direct comparison of US and Australian isolates.

Introduction

Background to the project.

MD is a ubiquitous, complex, lymphoproliferative disease of chickens caused by a cell-associated herpesvirus in the genus *Mardivirus* subfamily *Alphaherpesviridae*. There are many excellent reviews of the disease (eg. (Osterrieder *et al.* 2006; Witter and Schat 2003) and monographs (Davison and Nair 2004; Hirai 2001; Payne 1985a). Prior to the introduction of the turkey herpesvirus (HVT) vaccine in 1970, MD was the major economic threat to modern poultry production causing paralysis and lymphoid tumours in many organs including the skin. However the HVT and subsequent vaccines have never provided complete protection against MD and the disease has always remained important. The MD virus (MDV) causes immunosuppression and impaired performance well before the appearance of lymphoproliferative lesions (Islam *et al.* 2002; Morimura *et al.* 1995; Payne 1985b) and this is perhaps the aspect of major importance in broiler chickens due to their short lifespan relative to the incubation period for the disease. For this reason the majority of broiler chickens in the USA and Australia are now vaccinated against MD prior to hatching using *in ovo* vaccination at days 17.5-18.5 of incubation (Islam *et al.* 2001b; Ricks *et al.* 1999).

MD viruses comprise 3 speces the genus *Mardivirus* which were once conventionally differentiated by serotyping and known as MDV serotypes 1, 2 and 3 (Table 1). In this report we will retain the older serotype and HVT nomenclature for simplicity.

Table 1. Current classification of MDVs. Current specific names taken from Osterrieder *et al.* 2006.

Serotype	Current specific name	Attributes
MDV1	GaHVT-2 (Gallid Herpesvirus type 2)	Cause Marek's disease, primarily in chickens. Are oncogenic and spread efficiently between birds. Attenuated strains used as vaccines
MDV2	GaHVT-3 (Gallid Herpesvirus type 3)	Non-oncogenic MDV of chickens. Does not cause disease. Spreads efficiently between birds. Used in vaccines, particularly in combination with HVT.
MDV3 or HVT (herpesvirus of turkeys)	MeHV-1 (Meleagrid herpesevirus type 1)	Non-oncogenic herpesvirus of Turkeys. Does not spread effectively between chickens. Widely used as a vaccine, alone or in combination.

The isolation and propagation of MDV *in vitro* has been well reviewed (Schat 2005; Schat and Purchase 1998; Sharma 1998). MDV is strongly cell associated and is most easily isolated from blood (typically 0.2ml), PBL or splenocytes (2 x 10^6 cells), or dispersed tumour cells. Free virus is more difficult to obtain but can be done so from feather tips or spleen. Permissive cell cultures are considered the best substrate for virus isolation with differentiation between serotypes obtained using the indirect immunofluorescent test with monoclonal antibody. Chicken kidney cells (CK) and duck embryo fibroblast (DEF) cultures are most suitable for isolation of MDV1 while MDV2 and HVT grow well in chick embryo fibroblast (CEF) cultures which are the most convenient substrate to use. MDV1 may not always grow well initially in CEF but may adapt following growth in more permissive cells. However in Australia CEF were found to be the equal of CK for the isolation of one MDV isolate, and chick embryo kidney cells (CEK) were also found to support the growth of MDV1 (De Laney *et al.* 1995; De Laney *et al.* 1998). The different MDV serotypes all cause typical cytopathic effects in infected cells but only experienced observers can distinguish between the serotypes reliably on this basis. MDV may also be isolated in chicken embryos or in chickens although cell culture is generally preferred.

A feature of MDV1 has been a steady increase in virulence over time, marked by changes in the nature and severity of Marek's disease itself, and by the ability of the virus to overcome the protective effects of vaccination (Figure 1; Reviews: (Payne 2004; Witter *et al.* 2005).

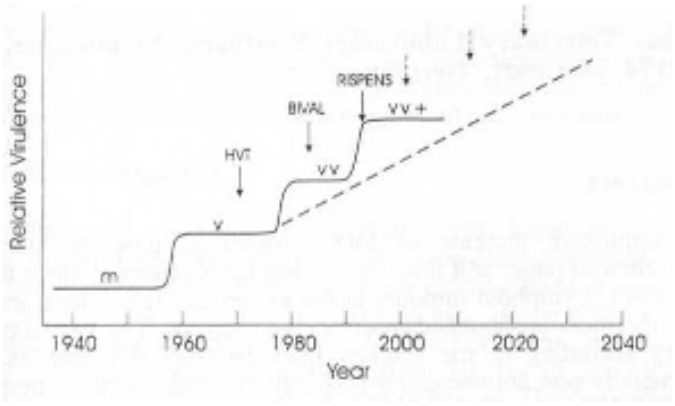

Figure 1. Stepwise evolution of virulence of MDV isolates: past history and future predictions. There appears to be a relationship between the introduction of new vaccines and the development of more virulent pathotypes (Witter 1998).

In the USA a formal pathotyping scheme for MDVs was developed at the USDA Avian Diseases and Oncology Laboratory in Michigan (Witter, 1997). The basic method uses challenge groups of 17 genetically susceptible chickens in isolators (Strain 15x7). The chickens contain maternal antibody against MDV (mab+) and are either left unvaccinated, or are vaccinated with 2000pfu of HVT or HVT/SB1 bivalent vaccine (HVT/MDV2) at hatch. Five days later chickens are challenged with 500pfu of the MDV isolate under test and mortality, immune organ atrophy and gross pathology monitored until the termination of the experiments at day 56pc. New isolates are ascribed a pathotype class on the basis of comparative lesions with reference viruses of each pathotype. The pathotype classifications are as follows:

- mMDV. Mild MDV. Induces mainly paralysis and nerve lesions with little or no mortality in pathotyping experiments. HVT provides good protection. The predominant pathotype in "classical" MDV. Classification based on lack of significantly lower pathogenicity than JM/102/W in HVT-vaccinated chickens.
- vMDV. Virulent MDV. Causes low levels of mortality by day 56pc, but induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT provides good protection. Reference US strain is JM/102/W. Classification based on lack of significant difference from JM/102/W in HVT-vaccinated chickens.
- vvMDV. Very virulent MDV. Causes moderate levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT is only partially protective but HVT/MDV2 vaccines provide a high level of protection. Reference US strain is MD5. Classification based on lack of significant difference from MD5 in HVT/SB1-vaccinated chickens.
- vv+MDV. Very virulent plus MDV. Causes high levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/MDV2 are only partially protective. Classification based on significantly higher pathogenicity than MD5 in HVT/SB1-vaccinated chickens.

While the ADOL system is well established it is expensive and difficult to implement outside the USA because of its specific requirements for a single strain of susceptible chicken and reference strains of MDV. To overcome the need for the 15x7 chicken, Witter *et al.* (2005) proposed a method that could be used in SPF chickens of different strains, but which still required the reference pathotype strains. These have since been made available from the American Type Culture Collection.

The epidemiology of MD infection is well reviewed by (Calnek and Witter 1997). MDV is shed in a cell-associated form in sloughed cells of the feather follicle epithelium in productively infected birds, and the virus is spread from bird to bird by inhalation of infective feather dust. Spread and severity of the disease involves interaction between the resistance status of the host (both genetic and acquired), pathogenicity of the challenge strain, and the magnitude of challenge. In a flock situation the resistance status of the host and the magnitude of challenge are closely linked because, as host

resistance declines (eg with declining vaccine efficacy or introduction of a more susceptible chicken strain) viral replication and shedding from birds increases leading to subsequent increase in challenge to flockmates or subsequent placements. This can lead to explosive outbreaks of MD as was experienced in Australia during the early and mid 1990s.

This Australian outbreak has been well summarised (Jackson 2000). Relaxation of Australian quarantine protocols in 1990 saw the importation of new strains of both layer and broiler chickens from 1992 onwards and their complete dominance of the Australian industry due to improvements in efficiency. However, between 1992 and 1997 MD wreaked havoc in both the layer and broiler industries as conventional Australian vaccines and vaccination programs failed to control MD in the imported genotypes with mortalities in the range of 20-40% of birds being common in layers and breeder flocks (Cumming *et al.* 1998; Groves 1995). The problem in layers and broiler breeders was brought under control by the importation in 1997 of seed for the Rispens CVI988 serotype-1 vaccine and MD remains well controlled by this vaccine at present. Broilers had not traditionally been vaccinated against MD in Australia, but during 1992-97 clinical MD was appearing in birds from 35 days onwards associated with reduced flock productivity, typically around 8 points (0.08) in FCR, and increased intercurrent disease. These problems were generally responsive to vaccination with HVT and in 1996 Baiada Poultry Pty. Ltd. imported two Embrex® machines for *in-ovo* vaccination of broiler eggs, a move followed subsequently by the other Australian companies. This, coupled with the production from 1997 onwards of high titre cell-associated HVT vaccine, helped to bring the immediate problem in broilers under control, although at considerable cost. However, HVT vaccine which had been first introduced in 1970, had broken down many years earlier in the USA and other countries, and appears destined to do so in Australia. The process appears to be well on its way with vvMDV strains, against which HVT confers only partial protection, first identified in Australia in 1985 (McKimm-Breschkin et al. 1990) and isolated from subsequent outbreaks of MD in vaccinated birds (De Laney *et al.* 1995; Zerbes *et al.* 1994) . These findings are consistent with evolution of Australian MD viruses towards greater pathogenicity in the face of HVT vaccination. This process is likely to have accelerated since the introduction of blanket HVT vaccination of broilers in the late 1990s.

If MDV is evolving towards greater virulence in Australia in the face of blanket vaccination, as it appears to be doing, the industry needs to be pro-active in monitoring these developments and reacting to them, to prevent a re-occurrence of the situation it faced in the 1990s. This project aims to contribute to this by providing ongoing surveillance of the pathogenicity of current isolates of MDV and improving our ability to grow them and characterise them at a molecular level.

Experimental approach and timelines

The project was a collaboration between UNE and RMIT. The general approach was as follows:

- call to industry for submission of infective material from suspect MD outbreaks with submission of soft tissues or feathers to RMIT or dust samples to UNE
- isolation of MDV on cell culture at RMIT or into chickens at UNE
- growth of MDV in cell culture and confirmation of freedom from contaminating vaccine strains or other chicken pathogens
- use of strains growing to high titre in pathotyping experiments at UNE using SPF chickens. New isolates would be tested against the reference strain MPF57. As far as possible the UNE experiments should follow the USDA ADOL method of Witter, (1997) although only HVT vaccine, rather than HVT and HVT/MDV2 bivalent vaccine would be used.

The project commenced on July 1, 2002 and finished on 31/12/2005. Samples totalling 533 were submitted to RMIT from the field, of which 238 were MDV1 positive on PCR. A total of 655 different isolations on cell culture were attempted of which 181 recorded some cytopathic effects. Only 6 MDVs grew to high titre in cell culture. In the end 17 different isolates were able to successfully infect chickens out of the 25 or so tested in chickens. Four major chicken experiments and several smaller experiments were conducted in the isolator facility at UNE. Due to the non-availability of suitable MDV isolates grown to high titre, several of the UNE pathotyping experiments were converted to screening experiments with an element of pathotyping, but also a significant element of virus isolation and amplification included in the design.

General materials and methods

Processing of field samples

Field samples were forwarded by courier from both industry and UNE to RMIT University. Upon receipt, samples were logged in the "Specimen Reception Log" and given a unique ID for future reference. Consignments of samples which generally comprised of either spleen, feathers or a mixture of both were subjected to initial visual and temperature inspection to confirm sample integrity prior to storage and subsequent sample processing in the following manner. Any deviation from accepted criteria was logged accordingly.

Feather samples were given RMIT codes and subsequently archived at -80C in the "ziplock" bags they were initially submitted in by UNE prior, to any subsequent processing for cell culture isolation of MDV. Whereas for submitted spleen clinical samples, a lymphocytes (LØ's) preparation was obtained from individual or pooled spleens following homogenisation and Ficol Paque® density gradient centrifugation [RMIT – MDS: Method 13.0]. The LØ's, which formed a "layer" following centrifugation were subsequently removed and either frozen away at -80 ºC prior to long-term storage in liquid nitrogen, or used immediately for cell culture isolation of MDV. For all new submissions, an aliquot of each LØ preparation [200 µl] was checked for the presence of MDV1 by PCR [RMIT – MDS: Method 11.0]. Only samples that were confirmed to be PCR positive were used for subsequent cell culture isolation.

MDV isolation on cell culture including virus handling and storage

Earlier work at RMIT University [conducted as part of the previous RIRDC UNE12J and on-going Poultry CRC 03-17 research projects] have utilised qPCR and quantitative virus isolation techniques to monitor the growth characteristics of MDVs in a range of chicken cell types such as chicken embryo fibroblasts [CEFs]; chicken embryo kidney [CEK] and 2-week-old SPF chicken kidney [CKs] cells. This earlier work has shown that chicken kidney cells [CKs] are the best cell line for both the propagation and isolation of MDV strains. Accordingly, the use of CKs was adopted for all subsequent virus isolation experiments conducted as part of this research project. The optimisation and refinement of the cell culture isolation procedure used was presented at the RIRDC Marek's Disease Steering Committee Group Meeting – May 2004 and included in the RIRDC UNE 83J report dated 19th December, 2003.
Accordingly, the use of CKs for the isolation and propagation of MDV has been in routine use at RMIT University since the start of 2004.

For virus isolation experiments, CK cells were derived weekly following CO_2 euthanasia and kidney derivation from approximately 20 x 2 week old SPF chicks (SPAFAS AUST) according to RMIT - MDS Method 15.0 (Schat & Purchase, 1998 and subsequently adapted by Kristy Jenkins, CSIRO following personal correspondence with Prof. Schat). Following organ derivation and disruption using activated trypsin versene [ATV], CK cell preparations at a concentration of 1:200 of packed cell volume following centrifugation were used for the cell culture isolation of MDV from the processed clinical specimens prepared earlier.

Of particular significance, earlier work at RMIT University [conducted as part of the RIRDC, RMI 12J Research Project] has shown that virus isolation rates can be increased following infection of CK cells in suspension as opposed to the infection of monolayer cultures. Accordingly, this "co-infection" protocol using freshly trypsinised CKs was adopted for all subsequent cell culture isolation experiments conducted as part of the later stages of the present work programme. As described previously, this refinement was documented in earlier Project Progress Reports.

In brief, the protocol used was as follows: The LØ's preparations obtained from the submitted field samples were inoculated onto 24-well plates containing freshly prepared CK's [200 µl of individual LØ preparation into 1 ml of CK cells per well using 12 wells [replicates] per sample]. The plates were incubated for 24 hrs at 37 ºC; 5% CO_2 prior to the media being discarded and replenished.

Following the change in cell culture media, the inoculated CK cultures were subsequently incubated at 37 ºC; 5% CO_2 for 7 days and monitored for cytopathic effect [CPE] every 2-3 days. All inoculated cell cultures were then repassed following disruption of the cell monolayer and inoculated onto freshly trypsinised CK preparations. This procedure was repeated on a weekly basis.

Following plaque visualisation using an inverted microscope, the cultures that exhibited high levels of CPE were repassed into larger flasks and a small amount retained as a contingency for future use prior to storage long-term under liquid nitrogen to facilitate any future repassage *in vitro* or *in vivo* that may be required.

The on-going process of repassing infected CK preparations into fresh cells was continued until the CPE attained the greatest level at the lowest passage possible. Although additional cell culture propagation has been shown to increase CPE, significantly this is also associated with a commensurate drop in the pathogenicity of the resultant virus pool. Indeed, to obviate such decreases in pathogenicity and to facilitate the generation of virus pools of high infectivity, a re-iterative series of back-passage *in vivo* followed by limited cell culture is generally required. Consequently, to retain pathogenicity it is considered imperative that a seed stock system is recommended to keep passage levels consistent and low and not passed excessively in cell culture (Witter *et al*., 2005). In addition, aliquots of low passage virus pools were retained as "seed virus lots" for future experimentation.

The harvesting of infected CK preparations was performed at day 5 following final passage with viruses harvested following disruption using a cell scraper and homogenisation of the cell monolayer using a pipette, prior to being aliquoted into cell freezing medium. Resultant virus pools were subsequently stored at -80 ºC prior to long-term storage under liquid nitrogen.

Titration of MDV in cell culture and calculation of pfu

Although the levels of infectious virus can be gauged visually - both during cell culture passage and prior to harvesting of virus pools - quantitative determinations of infectious titre [plaque forming units per ml; pfu / ml] are required to compensate for the decrease in infectious titre normally associated with the inactivation of the virus both during and following initial harvesting and storage. The determination of the infectious virus titre is also of paramount importance for the standardisation of any virus pool to be used as an experimental inoculum. The titration of MDV virus pools was performed in the following manner.

Preparations of freshly trypsinised CK cells were seeded onto a series of 9, 60 mm gridded petri dishes at a cell density of 1:200 of packed cell volume per sample. To facilitate virus plaque visualisation, the titre of each stored virus pool was determined at three different dilutions. Following thawing of the virus pool at 37 ºC, dilutions of 10^{-1}, 10^{-2} and 10^{-3} in cell culture media were made for each virus pool and triplicate aliquots [1 ml] of each dilution were subsequently added to the freshly trypsinised CK cell preparations in each gridded Petri dish.

The gridded Petri dishes were subsequently incubated at 37 ºC at 5% CO_2 for 6 days following which time the number of plaques was enumerated. The infectious titre of each virus pool - expressed as pfu / ml –was calculated using the average of the three replicates.

Virus handling at UNE

Frozen infective material containing MDV was shipped from RMIT to UNE in dry shippers able to maintain samples at -196˚C for a week or so. Samples generally arrived the day after despatch. On arrival at UNE samples were transferred into liquid N_2 with each ampoule cross-checked against the delivery note and logged in the virus storage log. Prior to use ampoules were moved to the isolator facility in portable liquid N2 canisters. Samples were thawed in a 37˚C water bath immediately before use and diluted in media supplied by RMIT. The timing of thawing and use was recorded and all thawed material was used within 30minutes of thawing. Dilutions were calculated using a purpose-designed dilution calculator in a spreasdsheet and checked by two individuals each time. Vaccine

viruses were treated the same way but were diluted using the manufacturer's diluent and according to their instructions.

Infective dust samples were sent to UNE either at room temperature or in chilled eskys with other material. At UNE it was stored at either 4˚C or -20˚C before being used as challenge material. Dust collected from isolator exhausts was similarly treated.

Infective fresh spleen material was frequently sent from UNE to RMIT. Spleens were collected as aseptically as possible with the capsule intact and rinsed with sterile PBS prior to being put in individual 5-20ml tared sterile bottles which were weighed with the spleen in them. After transfer to the laboratory spleens were once again rinsed, pooled by treatment and dispatched in sterile PBS to RMIT by overnight express in esky's containing freezer blocks.

UNE isolator facility

This project partially supported the establishment of a 24-isolator facility at UNE that was formally opened by the Vice-Chancellor of the UNE on October 14, 2002. Pathotyping experiments were conducted in the facility. The isolators are housed in a PC2 laboratory under constant negative pressure and with all outgoing air HEPA filtered. Each isolator has a length of 2.05m, width of 0.67m and height of 0.86m with a stainless steel frame. The floor is 2.5mm stainless steel (304 2b) with 12.7mm holes punched out with centres 17.45mm apart staggered providing 49% open area. This is critical to enable housing of chickens from day-old to adult without faecal accumulation on the floor (Thanks to Dr Gordon Firth for providing these specifications originally). Isolators are positive-pressure soft-bodied with disposable plastic linings, gauntlets and gloves, disposed of after every experiment. Isolators are provided with temperature-controlled HEPA-filtered air via a central air supply system and air is scavenged from each isolator via a series of scavenger ducts and HEPA filtered on exit. Both inlet and outlet air supplies are under manual control via a variable speed controller, giving complete control over air-flow and isolator pressures. The automated airflow control system originally installed was complex and unreliable and were discarded in favour of this manual system. Isolators are individually fitted with heat lamps under separate thermostatic control, automatic waterers and feeders. The entire feed supply for each experiment is loaded into a large feed hopper for each isolator and sealed for the duration of the experiment. Temperature in each isolator is monitored constantly via a datalogger and displayed on a computer screen in the facility. The entire facility has automated power backup via a 13KVA generator. At the time of writing 9 major experiments have taken place in the facility without breakdown of biosecurity or other major problems. Photographs of the facility are included in plates 1 and 2.

Plate 1. Interior of isolator facility at UNE showing 24 isolators and main air inlet duct. This carries HEPA filtered, heated air to each isolator. Note the green feed hopper above each isolator.

Plate 2. Exterior of the isolator facility at UNE showing the plant room on the right and the main isolator facility in the middle with the air extraction and filtration system next to the people.

Chicken vaccination and challenge protocols

Experimental chickens were manually vaccinated subcutaneously in the loose skin at the top of the neck using recommended doses of vaccine and diluent. Vaccines were thawed at 36˚C in a water bath and used within 30min of thawing. Disposable 1ml syringes with 21G needles were used.

Challenge with cell culture material, splenocytes or blood was via the intra-abdominal route in 200ul dispensed using disposable 1ml syringes with 21G needles.

Challenge with infective dust was either by intra-tracheal insufflation of weighed amounts (typically 1-5mg) of dust, or by penning chickens in a corner of the isolator on paper sheets and dispersing known amounts of dust over the chickens. Chickens were kept in the corner on the paper for 2 hours before being released to the rest of the isolator. Initially insufflation was performed using a commercial dust insufflator but it was subsequently found that using a 2.5ml syringe with a blunted 18G needle was easier to use and as effective (Plate 3). No adverse effects of insufflation were observed in any of the groups so treated.

Chicken management in the isolators

Isolator temperatures were set at 34˚C for the first two days and are then decreased by 1˚C every 2nd day until a temperature of 22˚C was reached. Feed and water was provided *ad libitum*. Chickens were initially placed on paper and have a scratch tray containing feed, and an ice tray filled with water. Feed for the SPF chickens was commercial layer starter (Ridley Ag Products) provided for the first 2 weeks followed by layer grower feed for the remaining period of the experiment (generally 61 days). Faeces accumulate under the floor for the duration of the experiment. Water spillage was collected and drained from the isolator via a water-filled U tube. Lighting was initially 24hr light (days 1-2) followed by 12L:12D lighting set with an automatic timer.

Plate 3. Intratracheal insufflation of dust.

MD lesion detection and scoring

Standard post-mortem examination was carried out for all dead and euthanized chickens (Bermudez and Stewart-Brown 2003). Carcasses were checked for nodular lesions on the skin. Breast and thigh muscles were inspected for discrete lymphoid tumours or diffuse infiltration. The thymus was inspected for atrophy and scored 0-3 in ascending order of severity (0 = normal, 3 = complete or almost complete atrophy). After opening the carcass, the liver, spleen, gonads, kidney, proventriculus, mesenteries, gastro-intestinal tract, heart, lungs were examined for gross enlargement and discrete or diffuse MD lesions (Plate 4). The bursa of Fabricius was examined and scored for atrophy as for the thymus. Tumorous enlargement of the thymus and bursa of Fabricius were recorded as gross MD lesions. The sciatic nerve and plexus were examined for enlargement, change of colour or loss of striations, or asymmetry in size. Histopathological confirmation of lesions was not carried out. However unchallenged controls were always present for comparative purposes.

The protective index (PI) provided by HVT for each challenge strain was calculated as:

(%MD in Sham-vaccinated chickens – %MD in HVT-vaccinated chickens) ÷ (%MD in Sham-vaccinated chickens) x 100 (Sharma and Burmester 1982)

where %MD is the percentage of birds "at risk" of exhibiting MD lesions, in which lesions are present. This is generally the population of chickens alive at the time the first gross MD lesion is detected.

Virulence rank (VI) was calculated as100 – PI (Witter 1997).

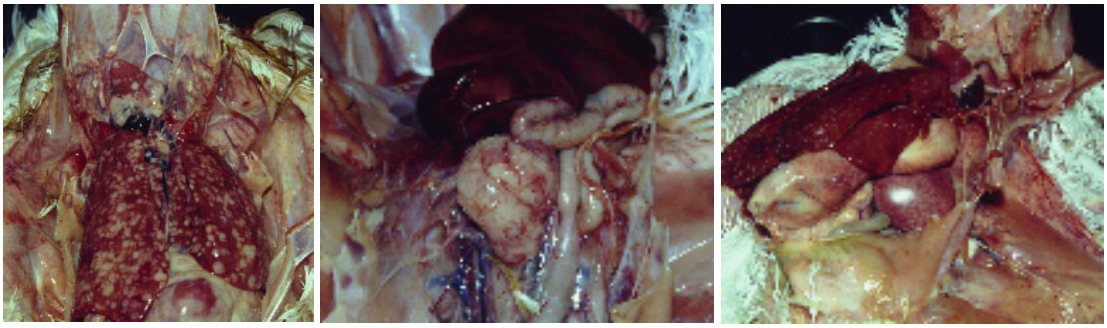

Plate 4. Typical gross Marek's disease lympomas of the liver (left), ovary (middle) and liver and spleen (right).

DNA extraction and qPCR for MDV differentiation and quantification

At UNE DNA was extracted either from 10mg of spleen tissue or 5mg of dust using DNeasy kits (Qiagen Pty Ltd, Victoria, Australia). Extracted DNA was quantified at 260nm using a spectrophotometer (Bio-Rad, SmartSpec TM300). MDV and HVT were quantified in a fixed amount of 25ng of extracted DNA using a real-time quantitative polymerase chain reaction (qPCR) assay on a Rotorgene 3000 real-time PCR machine (Corbett Research, Sydney, Australia) as described previously (Islam *et al.* 2004). Absolute quantification as described by (Islam *et al.* 2006a) was provided by the use of a full standard curve for each assay. The intra-assay co-efficient of variation was calculated from duplicates of each sample and inter-assay co-efficient of variation calculated from a quality control sample included in each assay run. Samples, standards and quality controls were assayed in duplicate with samples stratified across assays to remove individual assay effects.

Meq gene sequencing

The methods for this are included in the section outlining the results of this work below.

Results

Summary of field submissions and MDV isolation at RMIT

A summary of the number of field submission to RMIT is shown in Table 2. For logistic reasons - specifically to facilitate the use of fresh clinical samples for virus isolation experiments and given the requirement for the pre-ordering of SPF chicks in advance of anticipated industry / UNE sample submissions - in most instances a MDV1 PCR was performed retrospectively following the initiation of cell culture isolation procedures. Following retrospective PCR analyses, the cell culture propagation of all samples that were shown to be MDV1 PCR negative were terminated.

The fact that the number of isolates placed unto cell culture exceed the number of PCR positives in each year is a reflection of the greatest use being made of any available CK cells for the propagation of earlier PCR positive MDV1 isolates and represent a very considerable additional effort made by staff at RMIT University.

Of the MDV isolates that grew in cell culture only 4 grew to sufficiently high titre to use in pathotyping experiments (Experiment 4) while several others grew sufficiently to be infective for chickens when tested at UNE (Table 3). In addition to the 4 new isolates which grew to high titre, two older isolates, previously grown in cell culture were also successfully amplified in cell culture during the project (MPF 57 and Woodlands1).

Table 2. Summary of the MDV-suspected samples received at RMIT 2002-2005.

Year	Number received from industry	Number MDV1 pcr positive	CPE positive	Number of isolates placed onto cell culture.
2002	257	127	91	257
2003	38	15	12	52
2004	220	89	45	258
2005	18	7	33	88
TOTAL	533	238	181	655

Details of all MDV isolates used in the project

Of 27 MDV isolates used to challenge chickens at UNE, 17 were successful. These are detailed in Table 3.

Table 3. Details of all MDV isolates which successfully infected chickens during the course of the project (sorted by date of origin).

Name of the virus	Origin	Year of origin	Laboratory submitted to	Type of bird strain	Vaccination history
MPF 23 P3 (CEK)	Victoria	Mid 1980's	TGAL Parkville	Unknown	Unknown
Woodlands-1	SE Queensland	1992	RMIT	Broiler breeder	Bivalent (serotype 2 and 3)
MPF57 original	NSW	1994	RMIT	Layer	unknown
MPF132/5 (179/8)	NSW	2001	RMIT	Broilers	unknown
FT158	Northern NSW	2002	RMIT	Broilers breeder	Rispens CVI988
02LAR (179/3)	Victoria (Mornington P)	2002	UNE - dust	Broilers	Unvaccinated
02NOV	Victoria (Mornington P)	2002	UNE - dust	Broilers	Unvaccinated
04CRE (179/2)	NSW (Sydney)	2004	UNE - dust	Layers pullets 6 wo	Rispens CVI988
MPF 164/6	WA	2003	RMIT	Layers	Rispens/HVT
04KAL	SA	2004	UNE - dust	SPF-UNE	Unvaccinated
04OWE	SA	2004	UNE - dust	SPF-UNE	Unvaccinated
MPF 176/734o,734s,94	MPF57 B1	2004	RMIT	Broilers-UNE	Unvaccinated
MPF 179/2	04CRE B1	2004	RMIT	SPF UNE	Unvaccinated
MPF 179/6	MPF57 B1	2004	RMIT	SPF UNE	Unvaccinated
MPF 189/8	QLD	2004	RMIT	Broilers	Unvaccinated
MPF 192/1	SA	2004	RMIT	Broilers	Unvaccinated
MPF 192/4&10	SA	2004	RMIT	Broilers	Unvaccinated
MPF 199/3	SA	2004	RMIT	Broilers	Unvaccinated
MPF 199/9	SA	2004	RMIT	Broilers	Unvaccinated
MPF 210/1s	FT158 B1	2005	RMIT	Broilers UNE	Unvaccinated
MPF 210/2s	02LAR B1	2005	RMIT	Broilers UNE	Unvaccinated
MPF 212	05JUR B1	2005	RMIT	Layer cockerels UNE	Unvaccinated
O5JUR	NSW (Sydney)	2005	UNE-Dust	Layers 77wo	Rispens CVI988
W7B1S	MPF57 B2	2004	RMIT	SPF-UNE	

MPF 57 B1: One passage through chickens before re-isolation in CK's.
MPF57 B2: Two passages through chickens with re-isolation

UNE Experiment 1. MD03-R-PT1. 31/7/03-30/9/03. "Pathotyping of new MDV isolate 163/10".

This experiment was the first pathotyping experiment run on this project and it proved unsuccessful. The objective was to pathotype new field isolates of MDV and compare their virulence with that of a reference strain MPF-57. The decision to run with the experiment despite uncertainty of supply of new isolates was made at the Marek's Disease Steering Committee meeting on 10 June, 2003 and represented a calculated risk taking into account SPF chicken availability, isolator facility availability and likely availability of new isolates. Subsidiary objectives of the experiment were

- Confirm feasibility of working with SPF chicks flown up from Melbourne to Armidale.
- Confirm isolator facility capacity to prevent MDV cross infection between isolators over long term experiments.

Experimental design and methods

For the pathotyping experiment a 2 x 3 factorial (6 treatment combinations) design was used with 3 replicates (isolators) of each combination.

- Two vaccination statuses (sham or 8000pfu of cell-associated HVT vaccine sc at hatch)
- Three viral challenge treatments (Sham, MPF 57 - reference, MPF 163/10 - new isolate)

The experiment had approval of the UNE Animal Ethic committee (AEC02/100). A total of 234 SPF chickens were used sourced from SPAFAS, Melbourne. The line is called the SPAFAS Australia Bird and is a continuation of the CSIRO Hyline White Leghorn (HWL) line. This line of chickens has been shown to be relatively resistant to Marek's disease (McKimm-Breschkin *et al.* 1990). Chickens arrived from Melbourne at about 5pm on 31/07/2003. Half of the chickens were vaccinated with 8000pfu of caHVT vaccine sc on arrival (The Marek's Co, BN H02308, Titre 9540 x 10^3 pfu/ampoule) and permanently marked by toe-web cutting. The other half was sham-vaccinated with diluent only. The chickens were placed in 9 isolators (28 chickens in each) so that equal numbers of vaccinated and sham-vaccinated chickens were present in each isolator (14 vaccinated and 14 sham-vaccinated in each isolator).

At day 5 (05/08/2003), chickens (approximately 84) from three isolators (Isos 7, 11 and 13) were challenged i.p. with 100pfu of MPF 57 (RMIT BN P12 230603, titre 630.9 $TCID_{50}$/ml or 435.4 pfu/ml) and those from another three isolators (Isos 6, 12 and 14) with 100pfu of MPF 163/10 (RMIT BN P5 100703, titre 4786 $TCID_{50}$/ml or 3302 pfu/ml). Inoculum volume for challenge viruses was 250µl. Chickens of three other isolators (Isos 8, 10 and 18) were sham-challenged with 250µl of diluent.

At day 14 post-challenge (19/08/2003), 9-10 chickens from each treatment combination (total = 56) were euthanased and body weight and the weight of thymus, bursa and spleen was recorded. Relative weights of lymphoid organs (organ weight/body weight) were evaluated for the determination of pathotype of the viruses as described (Calnek *et al.* 1998). A sub-set of samples was analysed by normal PCR to confirm presence of challenge virus.

The remaining chickens were kept into the isolators to day 56 post-challenge (day 61 of age). Feed and water were provided *ad libitum*. During the experimental period, any dead chickens were post-mortemed to ascertain the cause of death and to record gross MD lesions. At day 56 post challenge (30/09/03), all surviving chickens were euthanased and examined post-mortem for gross MD lesions.

Statistical analysis. Continuous data variables were analysed by analysis of variance after fitting a general linear model including the effects of Challenge (MPF57, MPF163/10, sham), Vaccination (HVT, Sham) and Sex (Male, Female), interactions between these, and the effect of isolator nested within challenge group. Interactions with a p value >0.2 were removed from the model. Least squared means and standard errors of the mean are presented. Data were analysed using JMP 5.1 (SAS Institute Inc., NC, USA).

Results.

Mortality

Fifteen chickens (6.4%) died during the experiment, most of them during the early days of experiment following vaccination and challenge (Table 4). There was no significant effect of vaccine or challenge on the mortality of chickens. No chickens that died during the course of experiment exhibited gross MD lesions.

Table 4. Experiment 1. Weekly mortality of chickens (initial placement of 234 birds).

Week	No of dead birds	Percentage
1	11	4.70%
2	3	1.28%
3	1	0.43%
4 - 8	0	0.0%
Total	15	6.41%

Detection of challenge virus

Detection of MPF57 only occurred late in the experiment (>d40 pc) and challenge with MPF163/10 appears to have been unsuccessful as birds were not conclusively positive to this virus at any time during the experiment (Table 5).

Table 5. Experiment 1. Summary of MDV1 detection by qPCR and standard PCR during the experiment.

Challenge	Vaccinated	Sample	Day PC	MDV1 Test	Pos/Total	Comments
MPF 163/10	No	Spleen	14	qPCR	0/6	
MPF 57	No	Spleen	14	qPCR	0/4	One weak positive below lowest standard
MPF 57	No	PBL	35	qPCR	0/5	One weak positive below lowest standard
MPF 163/10	Mixed	Isolator dust	40	PCR	1/3	Very weak positive
MPF 57	Mixed	Isolator dust	40	PCR	3/3	Clear viral shedding
MPF 163/10	No	Spleen	56	qPCR	0/12	
MPF 57	No	Spleen	56	qPCR	7/12	
Sham	No	Spleen	56	qPCR	0/10	

Live weight and lymphoid organ weights at day 14 post-challenge.

Body weight was significantly affected by Sex (P<0.0001) although the effects of Challenge (p=0.08, Table 6) and isolator within Challenge (P=0.09) approached significance. Males were significantly heavier than females (LSM 238 v 207 g). Surprisingly it was the sham-challenged birds that were tended to be lightest, followed by MPF57 and MPF163/10 (LSM 214, 221 and 232 g respectively).

Table 6. Experiment 1. Least squared means for body weight and relative immune organ weights at day 14 post challenge. Bird numbers were 9 or 10 per treatment combination (total n=56)

Variable	Sham-challenge		MPF57		MPF163/10	
	Sham-vac.	HVT	Sham-vac.	HVT	Sham-vac.	HVT
Body weight (g)	213±7.2	215±7.6	221±8.0	223±7.8	234±9.0	232±7.6
Rel. thymic wt. (%BW)	0.35±0.04	0.40±0.04	0.29±0.04	0.30±0.04	0.32±0.05	0.38±0.04
Rel. bursal wt. (%BW)	0.46±0.04	0.47±0.05	0.41±0.05	0.38±0.05	0.47±0.07	0.44±0.05

Rel. splenic wt. (%BW)	0.14±0.03	0.20±0.03	0.19±0.03	0.21±0.03	0.15±0.03	0.24±0.03

Relative thymic weight was not affected by Challenge (p=0.11), Vaccination (p=0.18) or Sex (p=0.72) although there was a trend towards lower weights with MPF57 challenge and in unvaccinated chickens (Table 6). Relative bursal weight was not affected by Challenge (p=0.35), Vaccination (p=0.70) or Sex (P=0.12) although there was a trend towards larger bursa in males than females (0.47 v 0.40 %BW P=0.12). Relative spleen weight was affected by Vaccination only (P=0.007, Table 6) with HVT-vaccinated birds having a significantly greater relative spleen weight than sham-vaccinated birds (LSM 0.22 v 0.15, respectively). The effects of Challenge (P=0.51) and Sex (P=0.23) were not significant and there was no significant interaction between effects.

Body weight, bursal weights and MD lesions at day 56 post challenge.

For bodyweight at d56pc there was a significant effect of Vaccination (P=0.04, LSM 995g and 960g for HVT and sham respectively) and Sex (P<0.0001, LSM 1073 and 882 for male and female respectively) but not Challenge (P=0.77) or isolator within challenge ((P=0.97). There was a trend towards interaction between the effects of Vaccination and Challenge (P=0.07) on live weight due to a protective effect of HVT only in the MPF57 challenge treatment (Table 7).

For relative bursal weight there was a significant effect of Vaccination (P=0.0007, LSM 0.387 and 0.337 %BW for HVT and sham respectively) but not Challenge (P=0.18) or Isolator (P=0.97). There was a strong trend towards a sex difference (LSM male 0.375, female 0.349 %BW, P=0.07) and interaction between the effects of Vaccination and Sex (p=0.07) with the effect of vaccination being greatest in females. The thymus and spleen were not weighed in this experiment.

Table 7. Experiment 1. Least squared means for body weight and relative bursal weight at day 56 post challenge. Bird numbers ranged between 21 to 32 per treatment combination (total n=162).

Variable	Sham-challenge		MPF57		MPF163/10	
	Sham-vac.	HVT	Sham-vac.	HVT	Sham-vac.	HVT
Body weight (g)	988±21	985±22	930±15	1016±20	960±21	985±24
Rel. bursal wt. (%BW)	0.35±0.02	0.37±0.02	0.32±0.02	0.37±0.02	0.35±0.02	0.41±0.02

Only 4 chickens showed gross MD lesions on post-mortem at day 56 following euthanasia. All were from MPF 57 challenge group. There were not enough MD-positive chickens to calculate virulence rank or protective index of the challenge virus. The proportion of MD lesion-positive chickens for each challenge treatment was 0/49 for MPF 163/10, 4/48 (8.3%) for MPF 57 and 0/51 for the Sham-challenged group.

Discussion and Conclusions

Clearly the MPF57 challenge resulted in active MDV infection. However the consequences of the infection were extremely mild relative to previous experience with commercial broiler chickens (Islam *et al.* 2001b; Islam *et al.* 2002) or SPF chickens (De Laney *et al.* 1998). We feel that the most probable cause of this is infection with a very low initial dose of MPF57 (well below the 100pfu target infection dose). With regards MPF163/10, the data are indicative of a complete absence of challenge, or challenge with a vanishingly small amount of virus. At this time MDV was being grown on CEF and it was proving difficult to grow MDV. Both challenge viruses plus (MPF 57 BN P12 070703) were sent together to UNE and handled in unison. They were shipped from RMIT in a dry shipper on 29/7/03 and arrived at UNE on 30/7/03 in frozen condition and were moved into a liquid N2 dewar on 31/7/03. The viruses were used in the experiment on the morning of 5/8/03. Unused virus from batches MPF 163/10 BN P5 100703 and MPF 57 BN P12 070703 were returned to RMIT in October, but no virus could be grown from them.

The experiment was a disappointment as it did not fulfil its main objective of pathotyping recent isolates of MDV. However it did demonstrate that SPF chickens could safely be sent from Melbourne to Armidale for use in such experiments. It also showed that the isolator facility was functional and prevented cross infection. Results of organ weight measures were in the expected direction given the

low level of challenge obtained. The experiment was also a strong impetus for change in the cell culture methodology at RMIT to enable easier and more reliable production of new isolates.

Experiment 2. MD04-C-PT2. 8/6/04-3/8/04. "Virus isolation from dust and other infective material in SPF chickens"

With the continuing shortage of new isolates growing in cell culture, this experiment was planned to isolate MDV in chickens, have the virus grow to high titre in the birds and then send infective spleen material to RMIT for inoculation of cell cultures. By this time MDV was being detected readily in dust samples from the field using qPCR at UNE and a decision was made to attempt to infect chickens with this dust in addition to using low titre cell culture material from RMIT. Australian Poultry CRC project 03-17 had commenced by this time and also required new MDV isolates. This experiment was conducted by Mr Zahid Hussain the holder of a Poultry CRC scholarship for a Master of Rural Science degree at UNE. For these reasons this experiment is a joint experiment of UNE-83J and the Poultry CRC project 03-17. The specific objectives of the experiment were to:

a) develop an intra-tracheal insufflation method for infecting chickens with chicken dust
b) isolate recent Australian MDV-1 strains from chicken dust samples collected from commercial farms
c) determine whether any other poultry diseases are transmitted with dust infection
d) assist with amplification of field MDV strains for subsequent use in formal pathotyping studies
e) allow preliminary screening of isolates for pathogenicity
f) compare the effects of MPF 57 in SPF chickens and commercial broiler chickens.

Experimental design and methods

The experiment had Animal Ethics approval number UNE AEC04/095. It started on the 8th of June, 2004 (hatch date, day 0) and finished on the 3rd of August, 2004. A completely randomised design was used with 9 treatment groups each in their own isolator with no replication at the isolator level. The treatments involved day 0 challenge of SPF chickens with infective material from 6 potential new MDV isolates, challenge of SPF and Cobb broilers with a reference MDV strain (MPF 57) and one unchallenged control group. These are detailed in Table 8 and further details of the origins of the viruses can be found in Table 3

Table 8: Experiment 2. Description of the experimental treatments. The total number of chickens is 124.

Challenge MDV	Origin	Challenge material	Vaccination history	MDV1 copy number/mg dust	Chickens challenged	Chicken strain	Dose/bird
04LOC	NSW	Dust	HVT	2.7×10^4	13	SPF	2mg
04CRE	NSW	Dust	Rispens	5.26×10^5	14	SPF	2mg
02LAR	Vic	Dust	Nil	1.45×10^6	15	SPF	2mg
02NOV	Vic	Dust	Nil	2.97×10^5	14	SPF	2mg
04MAN	Vic	Dust	HVT	8.0×10^3	12	SPF	2mg
MPF132/5	NSW	CEF	Nil	-	15	SPF	50pfu
MPF57	NSW	Dust	Nil	5.44×10^6	14	SPF	2mg
MPF57	NSW	Dust	Nil	5.44×10^6	15	Cobb	2mg
Control		Nil	NA	NA	12	Cobb	NA

Each treatment comprised 12-15 chickens placed a single positive pressure isolator. Dust samples from broiler flocks in NSW and Victoria submitted to UNE as part of ARC project LP0211607and RIRDC project UNE 83-J were assayed for MDV-1 using real-time qPCR and a selection of positive samples were selected for use (Table 8). Dust collected from isolator exhausts from a previous experiment (ARC project experiment MDO3-A6-ISO) using the reference challenge virus MPF57 was used as a positive control. This challenge virus was applied to both SPF chickens and to commercial Cobb broiler chickens to determine the relative MD-susceptibility of the two types of chicken. One

cell-culture adapted isolate MPF 132/5 from RMIT was also included in the experiment. This was the only cell-culture adapted recent MDV isolate available in Australia at the time.

Dust samples were stored at -20˚C until use and some had been stored for more than 2 years. Chickens were infected with 2mg dust per bird on day 0 as described in the General Materials and Methods section. One sample (04Loc) was not in an appropriate form for insufflation, having apparently become damp and then forming hard lumps. It was therefore made up in a solution containing penicillin (10,000 units/ml), Streptomycin sulphate (10,000 µ gm/ml) and amphotericin-B (25µ gm/ml), and a 100µl of this solution was used to inoculate each bird intra-abdominally (2mg dust in 100 µl/bird). Chicks challenged with MPF 132/5 were administered 50pfu in 200ul intra-abdominally.

Two to three chickens per isolator were sacrificed at day 16 pc and spleens assayed for MDV1 to verify successful MD challenge. Dust samples were also collected from isolator exhaust ducts on day 14pc. DNA was extracted from each dust and spleen sample and MDV was quantified using real time qPCR. Blood samples were collected at day 56pc (5 birds/isolator) and plasma stored to test for antibody against 18 chicken pathogens (standard chick inoculation test serology) at the University of Melbourne. At day 56pc all surviving birds were euthanased and weighed individually. Spleens were sent to RMIT for virus propagation on cell culture.

Statistical analysis. Continuous data variables were analysed by analysis of variance after fitting a general linear model including the effects of Challenge treatment, Sex, interactions between these effects and the effect of isolator nested within challenge group. Interactions with a p value >0.2 were removed from the model. Significant differences amongst means were determined using Tukey's HSD test. Least squared means and standard errors of the mean are reported. Categorical data such as mortality or MD incidence were analysed using contingency table analysis and the Pearson chi-square statistic and Fisher's exact test in the case of 2-way tables. Mortality data were also subject to survival analysis using the product-limit (Kaplan-Meier) method. Data were analysed using JMP 5.1 (SAS Institute Inc., NC, USA). A significance level of $P \leq 0.05$ is used throughout.

Results

Detection of MDV and confirmation of infection

The presence of MDV1 was only consistently detected in 4 challenge treatments, MPF57 (SPF and Cobb), 02LAR, 04CRE and MPF 132/5 (Table 9). These data clearly demonstrate that these 5 treatments were successfully challenged with MDV while the remaining 4 treatments were not. The trace values for MDV1 in isolator exhaust dust at d14pc in 04LOC, 02NOV and 04MAN may reflect contamination during collection or trace amounts remaining from the original dust challenge with material known to contain MDV. Similarly 5 of 31 d56pc spleens from the 04LOC, 02NOV and 04MAN treatments had low positive values which is suggestive of contamination rather than infection. It is difficult to control external contamination of spleen samples during collection in a post-mortem room full of MDV.

Table 9. Experiment 2. Detection of MDV infection by various means by treatment. At day 56pc 6-11 spleens per treatment were assayed for MDV1 using qPCR.

Isolator	Treatment	qPCR isolator dust (Day 14) (VCN/mg dust)	qPCR spleen d5pc (mean VCN/10^6 host cells)	Gross MD lesions	MDV Serology (d56pc)	Growth of MDV from d56 spleens at RMIT
1	04LOC	222*	3*	-	-	-
2	04CRE	5,615	24,972	+	+	+
3	04LAR	832	15,448	+	+	+
4	02NOV	249*	11*	-	-	-
5	04MAN	262*	58*	-	-	-
6	MPF57/SPF	53,926	22,500	+	+	+
7	Control	Not tested	0	-	-	Not done

8	MPF132/5	2,911	12,695	+	+	+
9	MPF57/Cobb	53,065	Not tested	+	+	Not done

* Trace values only in a small number of chickens so mean is very low.

Mortality and MD Lesions

Chickens numbers and mortality by treatment are detailed in Table 10. Survival analysis revealed significant differences (p=0.007) in the pattern of mortality between treatments in survival to day 56pc (Figure 2). Mortality was greatest in the treatments later shown to be infected with MDV1 (04CRE, MPF57/SPF and MPF57/Cobb and O2LAR) and the survival pattern for these treatments grouped was significantly different from those not infected with MDV (P=0.001, Figure 2). There was a tendency towards a higher mortality rate in females than males (24% v 11%, P=0.09), particularly during the latter stages of the experiment. The first MD lesions were detected at day 41pc in the MPF57/SPF treatment. Interestingly there was substantial mortality associated with MDV infection prior to the detection of the first gross lesions (Figure 2, Right panel).

Table 10. Chicken numbers and mortality during Experiment 2.

Isolator No	Treatment	Chicken number							Mortality rate (%)
		Total	Killed d16	Killed d56	Removed for other studies	Early (<d5pc) or accidental mortality	Effective chicken no (eligible to die)	Mortality	
1	04LOC	13	2	10		1	10	0	0.0
4	02NOV	14	3	11			11	0	0.0
5	04MAN	13	2	10			11	0	0.0
7	Control	12		10		1	11	1	9.1
8	MPF132/5	15	3	10		1	11	1	9.1
3	04LAR	15	3	9			12	3	25.0
9	MPF57/Cobb	15		7	3		12	5	41.7
6	MPF57/SPF	13	3	6		1	9	4	44.4
2	04CRE	14	3	6			11	5	45.5
Total		**124**	**19**	**79**	**3**	**4**	**98**	**19**	**19.4**

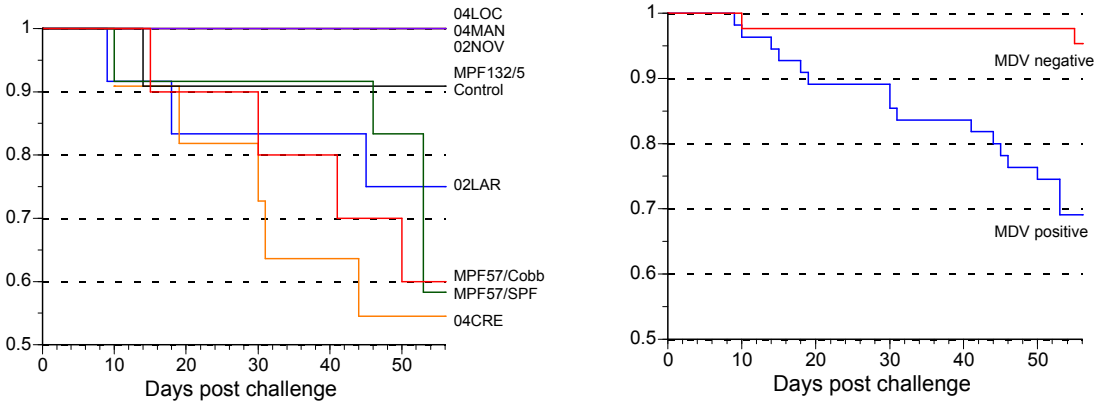

Figure 2. Survival patterns in Experiment 2 showing the effects of treatment (Left panel, P=0.007) and pooling of treatments according to whether or not MDV was detected in the treatment group or not (Right panel, P=0.001). The MDV positive group included 04CRE, MPF57/SPF and MPF57/Cobb and O2LAR while the MDV negative group included Control, 02NOV, 04LOC and 04MAN.

The overall incidence of MD lesions ranged from 27.3-58.3% in the chickens in the treatments with MDV infection confirmed (Table 11). There were no significant differences between these treatments and only the MPF57/Cobb treatment had significantly higher mortality than the control group. This is due to the very low numbers of chickens in the experiment.

Table 11. Distribution of MD lesions by treatment and chicken survival status in Experiment 2. Only chickens given the opportunity to die or express MD lesions after day 4pc are included.

Treatment	n	Chickens dying by day 56pc		Surviving chickens to d56pc		Total with MD lesions	Total without MD lesions	%MD
		MD lesions	No MD lesions	MD lesions	No MD lesions			
Control	11	0	1	0	10	0	11	0.0% [a]
02NOV	11	0	0	0	11	0	11	0.0%[a]
04LOC	10	0	0	0	10	0	10	0.0% [a]
04MAN	10	0	0	0	10	0	10	0.0% [a]
04CRE	11	0	5	3	3	3	8	27.3% [ab]
MPF132/5	11	0	1	3	7	3	8	27.3% [ab]
MPF57/SPF	10	2	2	2	4	4	6	40.0% [ab]
02LAR	12	1	2	4	5	5	7	41.7% [ab]
MPF57/Cobb	12	4	1	3	4	7	5	58.3% [b]
Total	**98**	**7**	**12**	**15**	**64**	**22**	**76**	**22.4%**

[ab]Means not sharing a common letter in the superscript are significantly different (P<0.05)

Bodyweight at day 56pc.

There were significant effects of Treatment (P<0.001, Figure 3) and Sex on final bodyweight in the SPF chicken treatments at day 56pc. Males were significantly heavier than females (759 v 609g , P<.0001).

Serology at day 56pc.

This sis summarised in Table 11. All samples (pooled sample from 5 chickens per treatment) were negative for all poultry pathogens tested for in the standard CIT (chick inoculation test) except for the MDV results shown in Table 9.

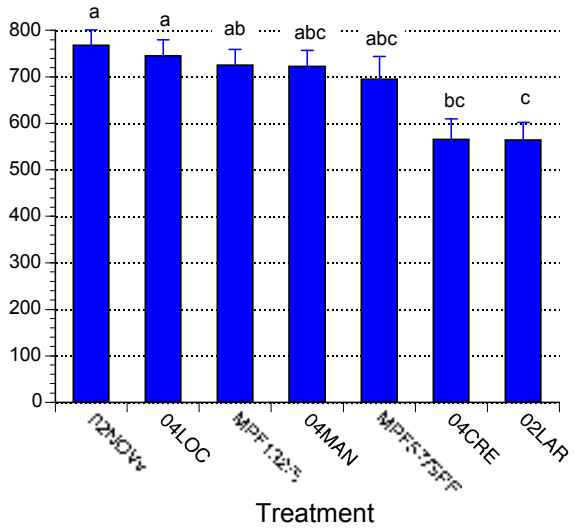

Figure 3. Mean (LSM±SE) final body weights in Experiment 2. Columns not sharing a common letter in the superscript are significantly different (p<0.05).

Discussion and conclusions

This experiment has shown that intra-tracheal insufflation with infective dust, is an effective way of challenging chickens with MDV. No pathogens other than MDV were transmitted, despite the fact that the dusts were collected from commercial chickens. This is a preliminary finding that suggests

that the unique means of transmission of MDV may mean that this mode of infection is a method for reducing

Table 12. Experiment 2. Results of serology (standard CIT test at University of Melbourne) from pooled plasma samples at day 56.

Pathogen	Test used	Results
Avian adenovirus (Gp 3) EDS, NDV	HI	All negative
Marek's disease virus	AGP&IFA	*Positive/Negative
Big liver & spleen, H.enteritis, AIV, Avian adenovirus 1	AGP	All negative
MG, MS, SP	RSA	All negative
AE, IB, AL, ILT, Avian reovirus, CAV, IBD	ELISA	All negative
Reticuloendotheliosis virus	ELISA/IFA	All negative

*Positive - 04Cre, 02Lar, MPF57, and MPF 132/5. Negative - 02Loc, 02Nov, 04Man, Neg control)

or eliminating contaminating organisms. However, infection was dose related, and only dusts containing more than 5x 10^5 virus copies per mg dust were successful. However one dust (02NOV) with an initial dust load of 2.97 x 10^5 VCN/mg dust was subsequently shown to be able to infect chickens when a higher dose of dust was used (see Experiment 3b below). MPF132/5 a CEK preparation from RMIT also grew well in chickens and induced tumours although it appeared to be of lower pathogenicity than some of the other MDVs used.

Amplification of these viruses in chickens was successful and fresh spleen samples from infected treatments all yielded MDV that grew to high titre in CK cell culture at RMIT. This suggests that a route of primary isolation in chickens (using dust or other materials), followed by growth in cell culture has promise as a means of isolating new MDV strains.

There was good agreement between tests in terms of identifying infected and non-infected treatments although the sensitivity of the qPCR method appears to mean that some false positives will occur, due to contamination during sample collection.

The isolates tested did appear to differ consistently in their effects on the host, with MPF57, 04CREand 02LAR having more adverse effects than MPF132/5. However it should be noted that initial challenge dose rates and routes of administration varied between treatments, the treatments were not replicated and only small numbers of chickens were used. When the same dose of MPF57 dust was used to infect commercial Cobb broiler chickens as well as SPF chickens, there was little difference in MDV viral load in dust or the chickens, in mortality rates or in the incidence of MD lesions. This is somewhat surprising given that the Cobb broiler chicks contain maternal antibody against MDV and the SPF chickens do not. The SPF SPAFAS Australia birds are derived from the CSIRO HWL line which has been shown to be comparatively resistant to MD (McKimm-Breschkin *et al.* 1990). These results would support that finding.

Experiments 3a), 3b) and 3c). "Virus isolation from dust and other infective material"

These were smaller animal experiments conducted between or alongside the major experiments.

Experiment 3a)

This consisted of three dust challenge treatments each in an isolator without replication at the isolator level. The MDV-positive dust from broiler farms were collected during 2004 and were stored at -20˚C until the experiment started. Six unvaccinated SPF chickens were used for each dust treatment. Dust samples were assayed by qPCR for MDV1 before inoculation (Table 13).

The dust samples were inoculated intra-tracheally (5mg/chicken) at day 0 as described previously. Birds were housed in the isolator facility from day 0 to day 56pc and mortality and MD lesion data was collected as described for Experiment 2. At the end of the experiment (day 56) spleen samples were collected and sent to RMIT overnight to inoculate on to chick kidney cell culture.

Table 13. Origin and MDV content of dust samples used in Experiment 3a).

Sample name	Origin	Vaccination history	MDV1 copy number/mg dust	Chickens challenged	Chicken strain	Dose/bird
04KAL	SA, 2004	Unvacc.	2.81×10^5	6	SPF	5mg
04BAK	SA, 2004	Unvacc.	1.35×10^5	6	SPF	5mg
04OWE	SA, 2004	Unvacc.	1.11×10^6	6	SPF	5mg

Only one chicken died during the experiment. This was from the 04OWE treatment and it exhibited gross MD lesions. Post-mortem at day 56pc revealed that 3 out of 5 remaining birds in this treatment had gross MD lymphomas of the ovary and kidney liver and spleen. There were no gross MD lesions in birds challenged with 04KAL and 04BAK. Spleen tissue from 04OWE was positive to qPCR for MDV1 but spleen from the other treatments was negative.

These data support the findings of the previous experiment, with the most infective dust successfully infecting chickens. However increasing the dose to 5mg dust/chicken did not result in successful infection in dusts containing less than 5×10^5 VCN/mg dust.

Experiment 3b)

The main objective of the third experiment was to test whether an increased dose of MDV-positive chicken dust can induce the infection when it had failed previously. The dust samples which failed to induce MDV infection in Experiment 2 were used to infect commercial unvaccinated male layer cockerels (IsaBrown) using a higher dose (5mg rather than 2mg). Dust samples 02NOV and 04LOC were selected as they both had higher initial viral copy numbers than 04MAN. Only four male cockerel birds were used per treatment. Chicks were inoculated with 10mg of dust by insufflation at day 0 and were reared in a multi-tier brooder in a sealed climate controlled room in the animal house until 10day pc. All birds were then euthanased, spleens were collected aseptically and sent overnight to RMIT for virus isolation on kidney cell culture.

There was no mortality in either treatment. Spleens from 02NOV were positive by qPCR whereas those from 04LOC were negative. Viral propagation in cell culture at RMIT was also successful for 02NOV but not for 04LOC although 02NOV did not grow to high titre.

This small experiment that the infectivity of dust is dose responsive. By increasing the challenge dose five-fold (from 2-10mg dust/chicken) a dust with relatively low initial viral content in dust (2.97×10^5 VCN/mg dust in the case of 02NOV) were able to induce infection.

Experiment 3c) (MD05-R-VI4, 28/6/05-18/7/05).

The main objective of this experiment was to passage or back passage 14 sets of MDV-infective material in chickens to amplify MDV and assist with growth to high titre in cell culture. Most of the

material (13/14 samples) came from RMIT in the form of either chicken lymphocytes or stored cell culture material. The experiment included the most pathogenic Australian isolate from the 1980s (McKimm-Breschkin *et al.* 1990). Also included were dust samples collected from a Sydney farm in May 2005 reporting a MD problem. The isolates used in the experiments are listed in Table 14.

All samples from RMIT were transported to UNE in a dry shipper and arrived in good condition on 30/6/05. The dust sample arrived at UNE on 19/5/05 and was stored at 4°C until use. The experiment commenced on the 28th of June 2005 and ended on 18th of July 2005 on which date fresh spleen samples were sent to RMIT.

Unvaccinated mab positive layer cockerels were used, nine chickens per treatment. Chickens were infected with each viral strain at 6 days of age either intra-abdominally or by insufflation in the case of the dust sample. At day 10 post-infection, one wing feather from each chicken was collected from each treatment for quantification of MDV on a pooled treatment basis. Only seven treatments were positive for MDV in feather tips (Table 15). At day14 post-challenge, chickens were euthanized for harvesting spleen samples. Fresh spleen samples from the seven MDV positive treatments were shipped to RMIT overnight for separation of splenocytes and cryopreservation or inoculating onto cell culture.

Table 14. Name and origin of the viral isolates passaged or re-passaged into chickens in Experiment 3c.

No.	Viral Strain	Source	Type	Challenge dose/bird (ul)*
1	MPF 21/3 P4 280605	RMIT	CEF	111
2	MPF 23 P3	RMIT	CEK	111
3	MPF 118/10 P3	RMIT	CEK	111
4	MPF 164/6	RMIT	Lymphocytes	222
5	MPF 182/2	RMIT	Lymphocytes	111
6	MPF 187/9	RMIT	Lymphocytes	111
7	MPF 189/8 P2 280605	RMIT	CEF	111
8	MPF 192/1	RMIT	Lymphocytes	167
9	MPF 192/8 P3 280605	RMIT	CEF	111
10	MPF 195/4	RMIT	Lymphocytes	167
11	MPF 199/3	RMIT	Lymphocytes	167
12	MPF 199/9 P2 280605	RMIT	CEF	167
13	MPF 200/6	RMIT	Lymphocytes	167
14	05JUR dust (Sydney May 2005)	Baiada	Dust	10mg

*Undiluted raw material.

Table 15. MDV titres in feather tips of chickens of the treatments positive to MDV1 by qPCR

Isolate	MDV load in FFE (VCN/10^6 host cells)
MPF23 P3	6,000
MPF 164/6	29,200
MPF 189/8 P2 280605	84,500
MPF 192/1	1,832,300
MPF 199/3	4,399,400
MPF 199/9 P2 280605	113,400
05JUR dust, Sydney 20/5/05, ISA brown pullets d77, Rispens-vaccinated.	37,000

Experiment 4. MDO4-R-PT2. "Pathotyping of recent Australian MDV-1 isolates in sham- and HVT-vaccinated SPF chickens" 18/11/04 – 18/1/05

Introduction.

The problems with growing up new isolates of MDV appeared to have been solved by a combination of changes in methodology at RMIT involving culture on chicken kidney cells, and initial isolation or amplification in chickens at UNE prior to submission to RMIT as shown in Experiment 2. In that experiment one older virus (MPF57) and 3 new isolates (02LAR, MPF132/5 and O4Cre) were shown to be oncogenic and free of other contaminating pathogens in SPF chickens. Each of these viruses grew to high titre at RMIT when fresh spleen material from the experiment was sent to RMIT. Meanwhile at RMIT another older isolate (Woodlands1) and a new isolate (FT158) had been successfully grown to high titre. The present experiment had the objective of pathotyping these viruses in SPF chickens.

The main hypothesis under test was that recent MDV isolates would be more virulent than the older isolates MPF57 and Woodlands1 that were isolated in the early 1990s. It was also hypothesised that that increased virulence will be indicated by more severe reduction in immune organ weights (thymus and bursa), earlier and more severe induction of mortality, a higher proportion of MD lesions, and a lower protective index for HVT.

Mr Zahid Hussain, led the execution of the experiment although it was funded exclusively by RIRDC/AECL project UNE 83-J.

Experimental design and methods.

The experiment had a 2x7 factorial design with

- 2 Vaccination treatments HVT (caHVT 8000pfu s.c. at hatch) and SHAM (diluent s.c. at hatch) and
- 7 Challenge treatments (Sham, MPF57, MPF132/5, 04CRE, 02LAR, FT158 and Woodlands1, (Table 16) all administered intra-abdominally at day 5 at a dose of 500pfu.

Table 16. Details of the viruses used in Experiment 4. Historical details can be found in Table 3.

Virus	Back passage in Expt 2?	Year of Origin	Batch no	Viral titre (pfu/ml)	MDV copy number per 10^6 cells
MPF57	Yes (179/6)	1994	P6-140904	20,270	6.09×10^6
MPF132/5	Yes (179/8)	Pre-2000	P5-050904	147,000	1.53×10^6
04Cre	Yes (179/2)	2004	P8-260904	26,000	1.01×10^6
02Lar	Yes (179/3)	2002	P6-120904	9,833	6.73×10^6
FT158	No	2002	P7-260904	11,000	1.06×10^6
Woodlands1	No	1992	P14-310804	19,000	5.87×10^6
HVT-FC126			MC HO20308	4,770,000	

Each treatment combination had 3 replicates in separate isolators. HVT and sham treatments were in the same isolator, identified by toe web cuts as HVT does not spread laterally between chickens. Treatments were allocated to isolators at random with stratification for location within the facility. The experiment commenced on 18th Nov 2004 (day 0) and ended on 18th Jan, 2005 (day 61, or day 56pc). The AEC approval number was UNE 04/177.

Four hundred and eighty-two SPF chickens (SPAFAS Australia line, ex CSIRO Hyline White leghorn line) were used in 21 isolators with 24-25 birds in each isolator and 36 birds in each treatment combination except the sham-challenge treatment for which treatment numbers were 24 and 26 for HVT and sham-vaccinated respectively. This was due to a shortfall in birds available from SPAFAS relative to those ordered, due to a power failure during incubation. This also resulted in very poor quality chicks.

At day 13pc 9 chickens per treatment combination (6 birds/isolator) were sacrificed for determination of immunosuppression by relative immune organ weight (bursa, spleen, and thymus) as described by(Calnek *et al.* 1998). All birds dying during the experiment were examined post mortem for gross MD lesions as described in the General Materials and Methods section. HVT protective index (PI) and Virulence rank for each challenge strain was calculated as described in the same section.

Data for normally distributed or transformed continuous variables was investigated and analysed by ANOVA following the fitting of appropriate general linear models using. The effects of Vaccination, Challenge treatment, Sex, and their interactions were fitted with removal of interaction terms with a P value below 0.2. The effect of isolator nested within vaccination and challenge treatment was also fitted and retained where significant. Significant differences amongst means were determined using Tukey's HSD test. Categorical data such as mortality or MD incidence were analysed using contingency table analysis and the Pearson chi-square statistic and Fisher's exact test in the case of 2-way tables. Mortality data were also subject to survival analysis using the product-limit (Kaplan-Meier) method. Data were analysed using JMP 5.1 (SAS Institute Inc., NC, USA). A significance level of $P \leq 0.05$ is used throughout.

Results

Successful application of treatments

Infection with MDV was successful for all MDV challenge treatments and absence of virus in sham-challenge treatments was confirmed by qPCR of day 56 spleens.

Mortality and MD lesions

Due primarily to the very poor quality of the chickens supplied (acknowledged by SPAFAS and discounted) there was significant mortality in the first 12 days of the experiment (to d6pc) with mortality of 40/482 birds (8.3%). The first bird dying with gross MD lesions was observed at day 34pc.

Survival and mortality analysis included 314 chickens eligible to die after d6pc - the 127 chickens removed at day 14pc were not included. Overall mortality rates are summarized in Table 17 and were significantly influenced by both challenge treatment and vaccination status. The challenge viruses 02LAR, O4CRE, Woodlands1 and FT158 induced high levels of mortality (36-53% overall) while MPF57 and MPF132/5 induced lower rates of mortality (13-21%). Only 1/30 chickens in the sham-challenged control group died.

Table 17. Mortality rate by challenge virus and vaccination in Experiment 4.

Treatment	Vaccinated			Sham-vaccinated			Overall		
	n	Died	%Mort	n	Died	%Mort	n	Died	%Mort
02Lar	24	4	16.7^{ab}	27	23	85.2^{a}	51	27	52.9^{a}
04 Cre	16	2	12.5^{abc}	23	14	60.9^{a}	39	16	41.0^{a}
Woodlands	27	9	33.3^{a}	25	12	48.0^{ab}	52	21	40.4^{a}
FT 158	22	3	13.6^{abc}	23	13	56.5^{ab}	45	16	35.6^{ab}
MPF132/5	27	3	11.1^{abc}	25	8	32.0^{bc}	52	11	21.2^{b}
MPF57	23	0	0.0^{c}	22	6	27.3^{c}	45	6	13.3^{bc}
Sham	13	1	7.7^{bc}	17	0	0.0^{d}	30	1	3.3^{c}
Total	152	22	14.5^{x}	162	76	46.9^{y}	314	98	31.2

Survival analysis revealed significant effects of Challenge ($P<0.001$) and Vaccination ($p<0.001$) but not Sex ($P=0.6$) on the pattern of mortality (Figure 4). The mortality in the challenge treatments showed a distinct pattern of sudden early mortality between days 11-15pc followed by a period of low mortality to around day 34pc followed by a further surge in mortality associated with the presence of gross lymphomas. The first MD tumour was detected at day 34 pc, and of the 65 chickens dying thereafter 59 had clear MD tumours, 4 were "suspicious" (eg. diffusely enlarged spleen) and only 2

birds did not exhibit gross MD lesions. The early mortality was associated with marked paralysis and other neural signs and many of these chickens were euthanased after prolonged prostration and dehydration. There were no gross MD lymphomas, although thymic and bursal atrophy is a feature. This early syndrome is consistent with the acute paralysis syndromes reported to be induced by highly pathogenic MDV in the USA (Gimeno *et al.* 1999; Witter *et al.* 1999).

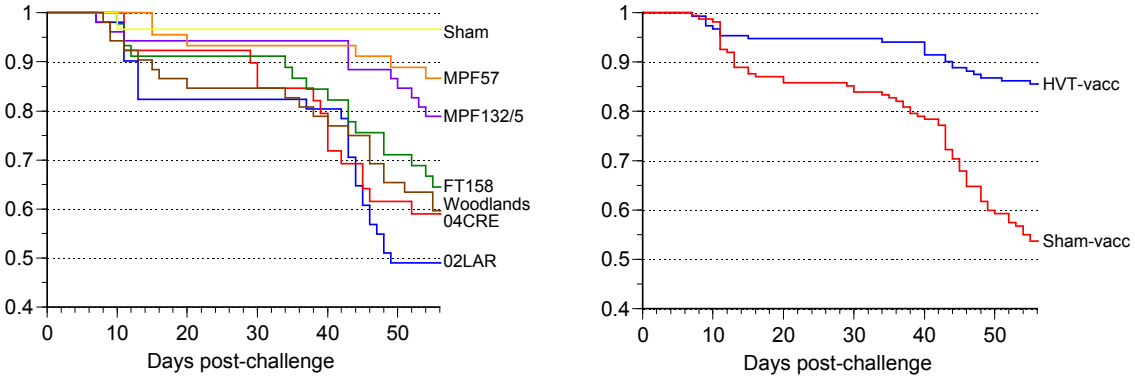

Figure 4 Survival patterns in Experiment 4 showing the effects of Challenge (Left panel, P<0.001) and Vaccination treatments (Right panel, P<0.001).

All the overall effect of sex on survival was not significant, males died at a greater rate than females between days 11-16pc (15/221 males v 5/21 females P=0.03) during the early paralysis syndrome. Conversely females died at a greater rate than males between days 34-55pc (40/147 females v 23/133 males) (Figure 5). This indicates a significant sex difference in susceptibility to the early paralysis and the later oncongenic forms of MD.

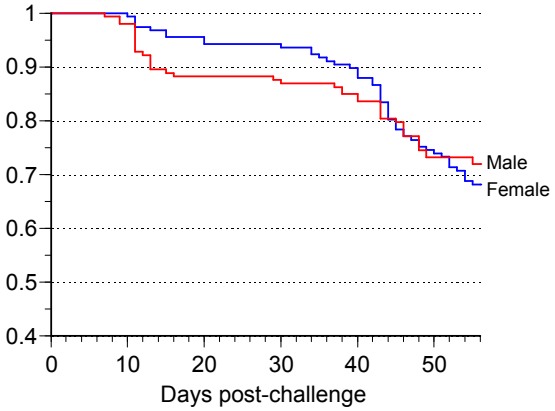

Figure 5. Survival patterns in Experiment 4 showing the overall effect of Sex (P=0.6).

Percentage MD, vaccinal protective index and virulence rank.

The definition of these variables is dependant on the definition of the "population at risk of developing gross MD lesions" Table 4.3 below summarizes the data for birds at risk being those alive at the time of detection of the first MD gross lesions (day 34pc).

Combining gross MD lesions in dead and euthanased birds resulted in 137 birds with MD lesions out of a population at risk of 281 chickens. The distribution of these chickens by vaccination status and challenge virus is shown in Table 18 with both effects being highly significant (P<0.001). Of 144 vaccinated chickens 42 (29.2%) had MD lesions compared to 95 of 137 (69.3%) of unvaccinated chickens (P<0.0001).

When the incidence of MD in unvaccinated and vaccinated chickens is plotted in a scatter plot (Figure 6) it is clear that for one group of viruses (02LAR, FT158, Woodlands1 and 04CRE) the incidence of

MD in unvaccinated chickens is positively associated with the incidence in vaccinated chickens, but for two MPF132/5 and MPF57 the association is negative, ie they induce a high level of lesions in unvaccinated chickens but vaccination with HVT provides good protection against MD lesions.

Table 18. Experiment 4. MD lesion occurrence, protective index and virulence rank by challenge treatment with the population at risk defined as that alive at the time of the first detection of gross MD lesions (day 34pc). Total population size is 281 (137 with MD, 144 without).

Challenge	HVT-vaccinated				Unvaccinated				Overall			
	MD	NoMD	n	MD%	MD	NoMD	n	MD%	MD	NoMD	n	MD%
02LAR	14	10	24	58.3^{a}	17	1	18	94.4^{a}	31	11	42	73.8^{a}
FT158	11	11	22	50.0^{ab}	17	3	20	85.0^{ab}	28	14	42	66.7^{a}
Woodlands	11	12	23	47.8^{ab}	17	4	21	81.0^{abc}	28	16	44	63.6^{a}
MPF132/5	3	21	24	12.5^{bc}	18	7	25	72.0a^{bc}	21	28	49	42.9^{bc}
04CRE	4	12	16	25.0^{bc}	9	8	17	52.9^{c}	13	20	33	39.4^{bc}
MPF57	0	23	23	0.0^{c}	16	3	19	84.2^{ab}	16	26	42	38.1^{c}
Sham	0	12	12	0.0^{c}	0	17	17	0.0^{d}	0	29	29	0.0^{d}
Total	43	101	144	29.9^{x}	94	43	137	68.6^{y}	137	144	281	48.8

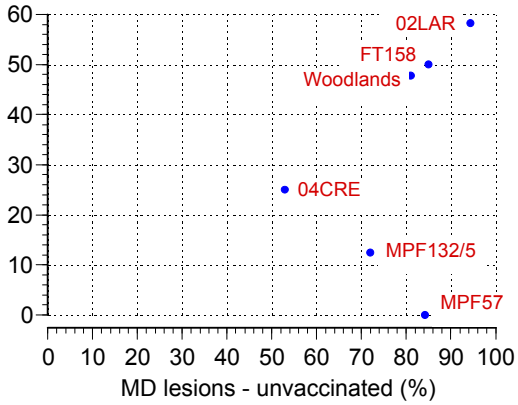

Figure 6. Scatter plot of incidence of MD lesions in unvaccinated and HVT-vaccinated chickens by challenge virus in Experiment 4.

Regarding the effect of sex, 87 (63.5%) of the MD-positive chickens were female and 50 (36.5%) were male (P<0.001). This confirms the greater susceptibility of females to the oncogenic form of the disease.

The data in Figure 6 is reflected in the protective index against MD lesions provided by vaccination with HVT (Table 19). Protective index ranged from 100% for MPF57 to 38.2% for 02LAR. Using the ADOL definition of virulence rank (1-PI), virulence rank ranged from 0 to 61%.

Table 19. Incidence of MD lesions, protective index provided by HVT-vaccination and virulence rank by challenge treatment in experiment 4.

Challenge virus	%MD - Unvacc	%MD - Vacc	Protective index	Virulence rank
MPF57	84.2	0	100.0	0
MPF132/5	72.0	12.5	82.6	17.4
04CRE	52.9	25.0	52.8	47.3
FT158	85.0	50.0	41.2	58.8
Woodlands	81.0	47.8	40.9	59.1
02LAR	94.4	58.3	38.2	61.8

Anatomical distribution of MD tumours

Amongst chickens exhibiting MD lesions, gross tumour lesions were most commonly found in the ovary of females, testis of males followed by the liver, kidney and proventriculus. Apart from the sex effect on the gonads, no major effects of MDV isolate, vaccination status or sex, were evident (Table 20)

Table 20. Anatomical distribution of MD lesions in chickens exhibiting gross lesions in Experiment 4.

Organ	Percentage of chickens with MD lesions showing lesions in each organ				
	Unvacc (n=94)	HVT-Vacc (n=43)	Female (n=87)	Male (n=50)	Overall (n=137)
Ovary	52.9	59.1	92.0		92.0*
Liver	39.4	22.7	36.8	38.0	37.2
Kidney	22.1	9.1	14.9	28.0	19.7
Proventriculus	15.4	4.5	11.5	16.0	13.1
Testis	13.5	13.6		40.0	40.0*
Spleen	12.5	11.4	13.8	8.0	11.7
Muscle	4.8	2.3	5.7	2.0	4.4
Heart	3.8	6.8	5.7	4.0	5.1
Bursa	2.9	2.3	2.3	4.0	2.9
Lung	1.9	0.0	2.3	0.0	1.5
Eye	1.0	0.0	1.1	0.0	0.7
Thymus	1.0	15.9	3.4	10.0	5.8
Gizzard	0.0	2.3	1.1	0.0	0.7
Skin	0.0	4.5	0.0	4.0	1.5

* Ovary and testis data for females and males only, respectively

Body weight, relative immune organ weights and MDV1 load in spleen at day 13pc.
At day 13 post-challenge 9 chickens from each challenge x vaccine combination were euthanased, weighed and had the weights of the thymus, bursa and spleen recorded (total n=126). Treatment effects are summarized in Figure 7.

Body weight was significantly affected by vaccination status and MDV challenge treatment ($P<0.001$) with significant interaction between these effects ($P<0.001$). The effect of sex was also significant (Males 162±2.8, Females 149±3.1 $P<0.001$). There were no significant interactions with sex.

Relative spleen weight was significantly affected by MDV challenge treatment ($P<0.004$) and sex ($P=0.015$) with significant interaction between the effects of vaccination status and sex ($P=0.01$). No other effects or interactions were significant although the effect of vaccination approached significant ($p=0.07$). MDV-challenged chickens had larger spleens than sham-challenged chickens and female chickens had larger relative spleen weight than males (0.211 v 0.188 respectively, $P=0.015$). The interaction between the effects of vaccination status and sex was manifest as a reduction in relative spleen weight in HVT-vaccinated females, but not males.

Relative thymus weight was significantly affected by vaccination status and MDV challenge treatment ($P<0.001$) with significant interaction between these effects ($P=0.003$). No other effects or interactions were significant although there was a trend towards higher relative thymus weight in females than males ($P=0.01$). The significant effects can be visualized in Figure 7c. Thymus weight was reduced by all challenge viruses in unvaccinated chickens, with extreme thymic atrophy evident for Woodlands, FT158 and 02LAR. For many birds, no thymic tissue could be detected. HVT-vaccination generally provided good protection against thymic atrophy induced by all challenge strains.

Relative bursa weight showed very similar trends as relative thymic weight. It was significantly affected by vaccination status and MDV challenge treatment ($P<0.001$) with significant interaction between these effects ($P<0.001$). No other effects or interactions were significant. The significant effects can be visualized in Figure 7d. Bursa weight was reduced by all challenge viruses in unvaccinated chickens, with marked atrophy evident for the Woodlands, FT158 and 02LAR strains. HVT-vaccination generally provided good protection against bursal atrophy.

MDV1 load ($\log_{10}$VCN/10^6 spleen cells) was significantly affected by vaccination status and MDV challenge treatment ($P<0.001$) with no interaction between these effects (Figure 8a). No other effects

or interactions were significant. MDV1 load was significantly lower in HVT-vaccinated than unvaccinated chickens (2.9±0.24 v 4.5±0.26 P<0.0001). Challenge treatment also significantly affected MDV1 load (Figure 8b). As with Experiment 1 there appeared to be a problem with aseptic collection of spleen samples which showed basal level contamination with MDV1 (87 VCN/10^6 spleen cells).

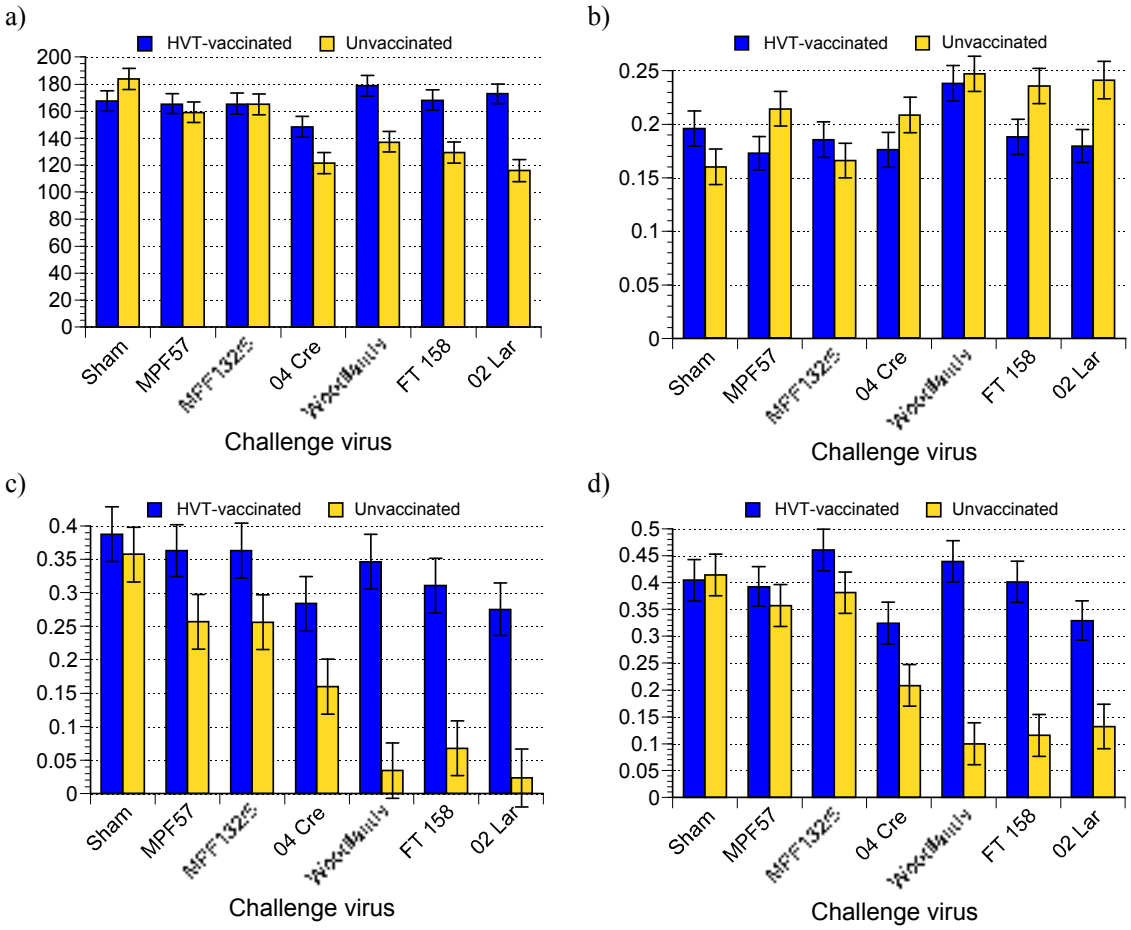

Figure 7. Experiment 4. Least square means (±SEM) for a) body weight b) relative spleen weight c) relative thymic weight and d) relative bursal weight for experimental chickens at d13pc.

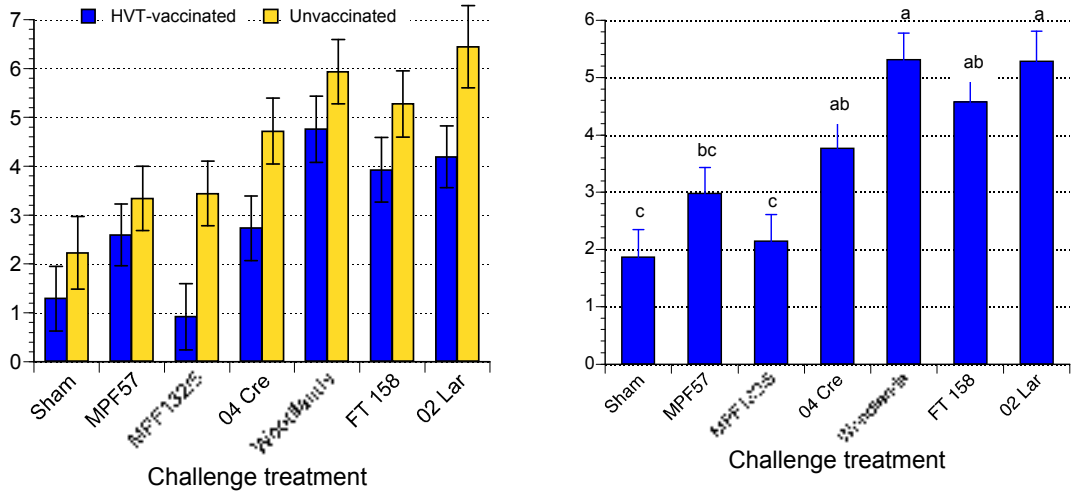

Figure 8. Experiment 4. Least square means (±SEM) for MDV load (log_{10} VCN/million host cells) in spleen at d13pc by challenge treatment and vaccination (Left panel) or by challenge treatment alone (right panel). Means not sharing a common letter in the superscript differ significantly (P<0.05).

Multivariate analysis of relative thymic, bursal and spleen weights simultaneously was informative. Cluster analysis (K-Means clusters) was able to group animals into two clusters (Table 21) with good indirect evidence that these predict MD infection (Figure 9).

Table 21 K-Means cluster analysis of relative thymic, bursal and spleen weights at day 13pc in Experiment 4, grouping individual chickens into one of two clusters. The association with MDV copy number in spleen is also shown.

Cluster	n	Rel Bursa wt (%BW)	Rel spleen wt (%BW)	Rel thymus wt (%BW)	Log_{10}MDV load in spleen (VCN/10^6 cells)	P value
1	88	0.412±0.092	0.180±0.043	0.345±0.107	2.93±0.25	
2	37	0.122±0.090	0.240±0.057	0.049±0.066	5.24±0.29	<0.0001

Figure 9 Example parallel plots of relative bursal weight, relative spleen weight and relative thymic weight coded following cluster analysis. Cluster 2 appears to be predictive of MDV infection by a variety of tests. Each line represents data from a single chicken.

Body weight and relative immune organ weights at day 56pc

At day 56 post-challenge all remaining surviving chickens were euthanased, weighed and had the weights of the thymus, bursa and spleen recorded (total n=216). Treatment effects are summarized in Figure 10. In general effects were similar to those seen at day 13.

Body weight was significantly affected by vaccination status and MDV challenge treatment (P<0.001) with significant interaction between these effects (P<0.001) (Figure 10a). The effect of sex was also significant (P<0.001). There were no significant interactions with sex. All challenge viruses reduced body weight in sham-vaccinated birds with HVT-vaccination providing good protection against this. Males were significantly heavier than females (821 v 701g P<0.001).

Birds with MD lesions in the spleen were excluded from analysis of relative spleen weight (n=8). Relative spleen weight was significantly affected by MDV challenge treatment (P<0.0001) vaccination (P=0.0002) and sex (P=0.0001) with significant interaction between the effects of challenge virus and vaccination status (P=0.024) (Figure 10b). No other effects or interactions were significant. MDV-challenged chickens had larger spleens than sham-challenged chickens (particularly for MPF57 and Woodlands) and sham-vaccinated chickens had larger spleens than vaccinated chickens although this varied significantly between challenge treatments (Figure 10b). Female chickens had larger relative spleen weight than males (0.339 v 0.271 respectively, P=0.0001).

Birds with MD lesions in the thymus were excluded from analysis of relative thymic weight (n=9). Relative thymus weight was significantly affected by vaccination status (P<0.0001) and MDV challenge treatment (P=0.001) with significant interaction between these effects (P<0.0001) Figure 10c). There was also a significant interaction between the effects of challenge virus and sex (P=0.05). Thymus weight was reduced by all challenge viruses in unvaccinated chickens, with marked thymic atrophy evident for Woodlands, FT158 and 02LAR. The interaction between vaccination status and

challenge treatment was due to greater relative thymus weight in vaccinated birds for all challenge treatments except the sham-challenge group. The interaction between challenge virus and sex was due to higher relative thymus weight in females for all treatments except for 04Cre and 02Lar where the reverse was true.

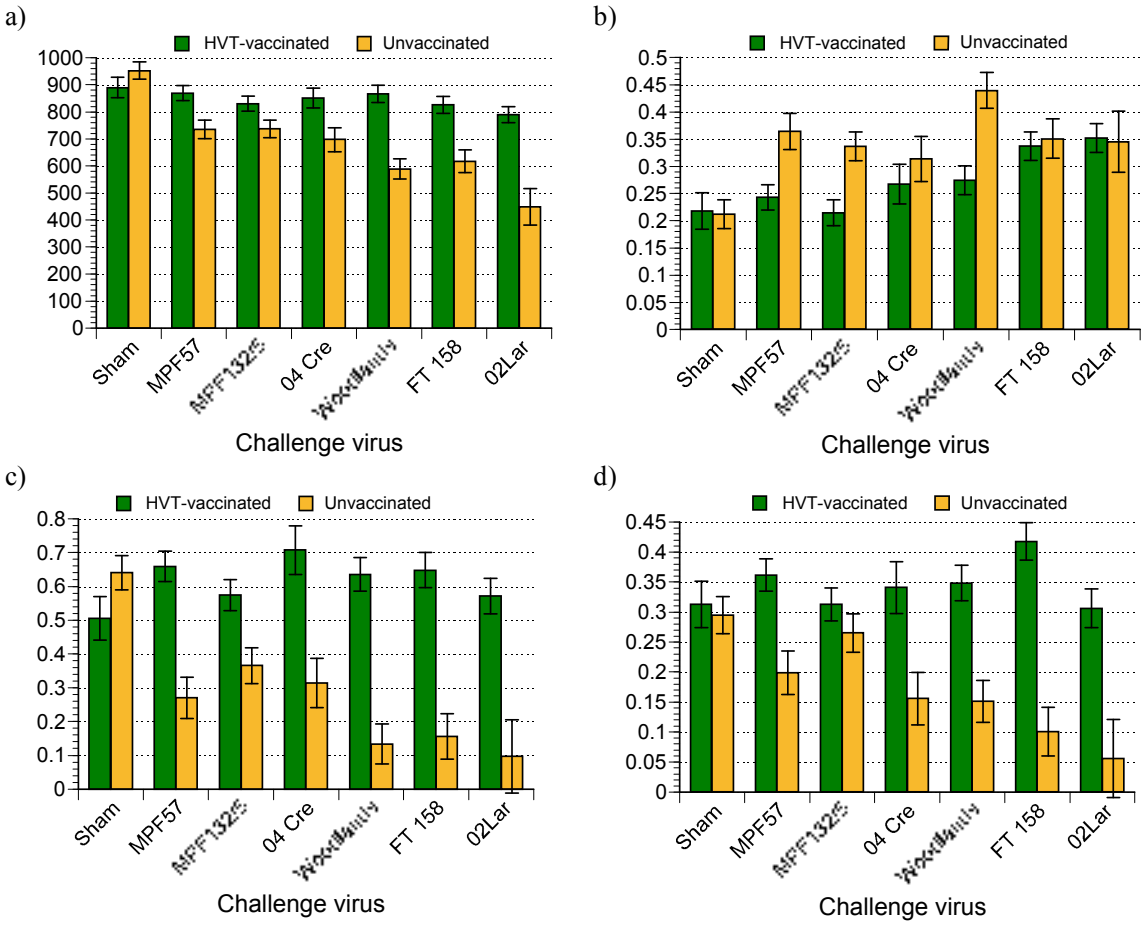

Figure 10. Least square means (±SEM) for a) body weight b) relative spleen weight c) relative thymic weight and d) relative bursal weight of chickens in Experiment 4 at day 56pc.

Birds with MD lesions in the bursa were excluded from analysis of relative bursal weight (n=4). Relative bursa weight showed similar trends as relative thymic weight. It was significantly affected by vaccination status (P<0.0001) with significant interaction between the effect of vaccination and challenge virus (P<0.001) (Figure 10d). No other effects or interactions were significant although the effects of challenge virus (P=0.08) and sex (P=0.09, F>M) approached statistical significance. The interaction between vaccination status and challenge treatment was due to greater relative bursal weight in vaccinated birds for all challenge treatments except the sham-challenge and MPF132/5 groups.

Multivariate analysis of relative thymic, bursal and spleen weights simultaneously was again interesting. Cluster analysis (K means clusters) was able to group animals into two clusters with highly significant (P<0.0001) association with gross MD lesions (Table 22).

Table 22 K-means cluster analysis of relative thymic, bursal and spleen weights grouping individual chickens into one of two clusters. The association with presence of MD lesions is shown, as is the significance of the association (Contingency table chi square analysis).

Cluster	Rel Bursa wt (%BW)	Rel spleen wt (%BW)	Rel thymus wt (%BW)	MD Lesions	No MD lesions	P value
1	0.348±0.119	0.260±0.089	0.633±0.227	21 (14%)	128 (86%)	

2	0.095±0.085	0.390±0.189	0.159±0.156	39 (78%)	11 (22%)	<0.0001

MDV in isolator dust

Isolator exhaust dust was collected from one isolator per challenge treatment between days 17 and 42 during the experiment. There was an increase in MDV load in dust over time in most treatments with a tendency for more virulent isolates to produce early and steeper increases in MDV load (Figure 11).

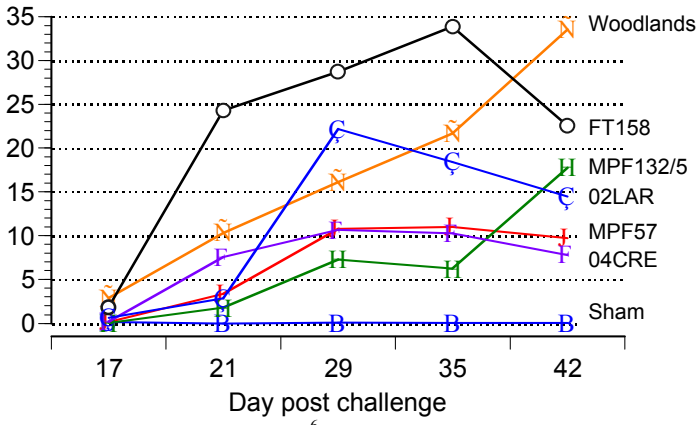

Figure 11 MDV1 load in isolator exhaust dust (10^6 VCN/mg dust) during experiment 4. Data were for one isolator per treatment only so no standard error bars are included.

Association between variables and early prediction of virulence rank

Correlation coefficients for the different measurements in the experiment are presented in Table 23. In general Day 13pc measures correlated well with final virulence rank, generally better than the same measures made at day 56pc (with the exception of bodyweight). In particular, day 13 thymus and bursal relative weights were strongly negatively correlated with virulence rank while MDV load in spleen and was positively correlated with VR. Bodyweight at d13pc had a non-significant negative association with VR while spleen size at d13 tended to be positively associated with virulence.

Brief discussion

The experiment was successfully implemented. The viruses under test showed a considerable range in pathogenicity with good agreement between rankings based on relative immune organ weights and those based on the presence of gross MD lesions. MDV viral load in spleen at day 13pc was also a good indicator of subsequent pathogenicity. The most pathogenic viruses, as assessed by PI and virulence rank were the recent isolates 02LAR and FT158 and the older isolate Woodlands1, isolated more than 10 years earlier. The relatively new isolates MPF132/5 and 04CRE were of lower pathogenicity. The standard Australian challenge virus MPF57 had the lowest pathogenicity of all the viruses under test. Although it was highly pathogenic in unvaccinated chickens, inducing MD lesions in 84% of at-risk unvaccinated chickens, vaccination with HVT provided complete protection against these lesions. We have observed complete protection with HVT previously with this strain in commercial broilers, although protection of 70-80% is more typical ((Islam *et al.* 2001b; Islam *et al.* 2005b). Given these findings, the provisional virulence ranking of vv ascribed to this virus by (De Laney *et al.* 1998) cannot be supported. In the USDA pathotyping system (Witter 1997) isolates ascribed the vv ranking, typically have PI with HVT of 0-50%. Although direct comparisons with the USDA pathotyping system cannot be made from this experiment due to differences in host genotype, the absence of maternal antibody, the absence of reference MDV strains of defined pathotype, and differences in the calculation of PI, the viruses in this experiment fall into two broad categories viz:

- Lower virulence (~ v ranking in USDA system). MPF57 and MPF132/5. These viruses typically cause relatively little early mortality, have mild suppressive effects on thymus

and bursa at d13pc and while there is a high incidence of MD tumours in sham-vaccinated birds (72-84%) HVT-vaccination provides good protection against all of these effects.

- Higher virulence (~ vv ranking in USDA system). 02Lar, FT158, Woodlands1, O4Cre. These viruses cause early mortality syndrome with some neural signs and have marked suppressive effects on thymus and bursa at day 13pc. However HVT provides good protection against these effects. On the other hand, protection against gross MD tumours by day 56pc is poor, in the range 38-53%.

Table 23 Experiment 4. Matrix of Pearson correlation coefficients (r) for different variables measured at days 13 and 56pc. Correlations are between group least square means for each challenge group (7 challenge groups, df=5). Significant values are marked in bold. Immune organ weights refer to relative weights expressed as %BW. Viral load in spleen at day 13pc is expressed as Log_{10} VCN/million host cells. VR = Virulence rank. Significant P values for a two tailed test with df=5 for P<0.05, <0.01 and <0.001 are 0.88, 0.96 and 0.99 respectively.

Variable	d13 BW	d13 Thymus	d13 Bursa	d13 Spleen	d13 LogVCN/mhc	d56 BW	d56 Bursa	d56 Spleen	d56 Thymus	% Mort	% MD	VR
d13 BW	1	0.80	0.84	-0.34	-0.66	0.68	0.72	-0.58	0.44	-0.84	-0.62	-0.75
d13 Thymus	0.80	1	**0.95**	-0.75	**-0.96**	**0.92**	0.87	**-0.90**	0.85	**-0.97**	**-0.91**	**-0.96**
d13 Bursa	0.84	**0.95**	1	-0.73	**-0.94**	0.79	0.84	-0.79	0.70	**-0.91**	-0.76	**-0.93**
d13 Spleen	-0.34	-0.75	-0.73	1	**0.89**	-0.61	-0.53	0.84	-0.73	0.64	0.71	0.74
d13Log_{10} VCN/mhc	-0.66	**-0.96**	**-0.94**	**0.89**	1	-0.85	-0.83	**0.92**	-0.86	**0.89**	0.87	**0.91**
d56 BW	0.68	**0.92**	0.79	-0.61	-0.85	1	**0.90**	**-0.89**	**0.94**	**-0.91**	**-0.96**	-0.82
d56 Bursa	0.72	0.87	0.84	-0.53	-0.83	**0.90**	1	-0.71	0.79	**-0.90**	-0.76	-0.78
d56 Spleen	-0.58	**-0.90**	-0.79	0.84	**0.92**	**-0.89**	-0.71	1	**-0.93**	0.80	**0.96**	0.81
d56 thymus	0.44	0.85	0.70	-0.73	-0.86	**0.94**	0.79	**-0.93**	1	-0.78	**-0.95**	-0.75
%Mort	-0.84	**-0.97**	**-0.91**	0.64	**0.89**	**-0.91**	**-0.90**	0.80	-0.78	1	0.85	**0.94**
%MD	-0.62	**-0.91**	-0.76	0.71	0.87	**-0.96**	-0.76	**0.96**	**-0.95**	0.85	1	0.83
VR	-0.75	**-0.96**	**-0.93**	0.74	**0.91**	-0.82	-0.78	0.81	-0.75	**0.94**	0.83	1

The relationship between thymic weight at day 13pc and key indicators of subsequent pathogenicity at day 56pc are shown graphically in Figure 12.

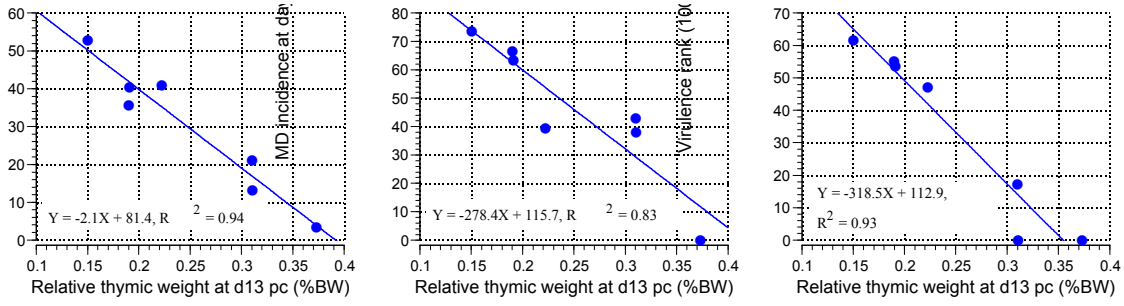

Figure 12 Linear regression between relative thymic weight at day13pc and various measures of pathogenicity at day 56pc. Each point represents the overall least squared mean for a challenge treatment (7 treatments). All associations are significant (P<0.05).

In the present experiment, early immunosuppression as measured by relative bursal and thymic weights was closely aligned to virulence, as reported by (Calnek *et al.* 1998). Interestingly this

relationship was still in evidence at day 56pc in the present experiment. At day 13 there was also evidence that the more virulent viruses caused greater splenomegaly than the less virulent viruses. The combination of reduced thymus and bursal weight combined with elevated splenic weight appeared to be a good predictor of future MD at day 13pc using K-mean cluster analysis, and also a good predictor of actual MD at day 56pc. (Calnek *et al.* 1998), like us, were working with antibody negative SPF chickens. (Witter 1997) working with maternal antibody-positive chickens reported a weak but significant relationship between bursal and thymic atrophy and virulence rank but did not consider these to be good indicators of virulence. In the present experiment, viral load in spleen at day 13pc, was also a significant predictor of virulence rank, suggesting that virulence is associated with increased rates of viral replication, as reported by others (Yunis *et al.* 2004). Taken together the data on early measurements suggest that in maternal antibody negative SPF chickens, early measures of pathogenicity at day 13-14pc (prior to the onset of any gross MD lesions) are likely to correlate well with final estimates based on induction of lesions.

It is difficult to compare the results of the present experiment directly with those of the most comprehensive work of this kind in Australia previously, that of McKimm-Breschkin et al., (1990). Different chicken strains, virus challenge protocols, and HVT vaccines and doses make the results difficult to compare. Nevertheless it is clear that some of the more virulent viruses in that study (MPF23, MPF15) produced results comparable with the more virulent viruses in the present experiment. Vaccination with HVT (250-1000ffu, probably cell-free) provided no protection at all against MD gross lesions when challenged with MPF23 the most virulent virus in the that study. MPF23 could possibly be in the same league as 02Lar in the present experiment although the differences in vaccine type and dose between experiments make this speculative.

In summary, this experiment provides good evidence that current isolates of MDV vary widely in pathogencity. However there is no clear evidence that the most virulent recent isolates are more pathogenic than older isolates obtained more than a decade ago. It also provides strong evidence that virulence can be predicted well by measurements as early as day 13pc, and introduces two new such measures, MDV load in spleen measured by qPCR, and a cluster score based on K-means cluster analysis of relative thymic, bursal and splenic weights.

Experiment 5. MDO4-R-PT3. "In vivo isolation of current Marek's disease virus isolates and screening for pathogenicity" 20/10-05 – 12/12/05

Introduction

After an initial experiment (MD03-R-PT1) in which there was failure of adequate challenge Experiments 2 (MD04-C-PT2) and 4 (MD04-R-PT2) in SPF chickens enabled the pathotyping of 6 isolates of MDV namely (MPF57, Woodlands1, 02LAR, 04CRE, FT158 and MPF 132/5). Several of these viruses have subsequently been used in Poultry CRC pathotyping experiments in commercial chickens.

This final pathotyping experiment was to pathotype 6 new isolates in SPF chickens but in the absence of any further new isolates successfully grown to high titre at RMIT the experiment was instead designed as a screening experiment in which a wide range of infective material (21 new samples) from recent MDV outbreaks or submissions was screened in SPF chickens with the objectives of:

a) Identifying which of the isolates grows rapidly in birds and induce immunosuppression and tumours.

b) Providing quality material with high viral titre for use at RMIT for subsequent virus isolation and growth on cell culture. This was very successful after experiment 2, providing 4/6 of the isolates used in the subsequent experiment (MD05-R2-PT2).

Ms Katrin Renz, a PhD student at UNE was heavily involved in the execution of the experiment although it was funded exclusively by RIRDC/AECL project UNE 83-J.

Experimental design and methods

The experiment tested 21 samples of stored infective MDV material at RMIT for infectivity and pathogenicity in SPF chickens. Each sample was used to infect 19-20 chickens in a single isolator with one uninfected isolator remaining as a control. One sample (MPF23 stored blood) was tested at 3 concentrations so in total there 24 isolators and 470 chickens. There was no replication at the isolator level and all chickens were unvaccinated. The chickens were SPAFAS Australia SPF white leghorns (ex CSIRO HWL line) air-freighted from Melbourne.

The experiment started on 20/10/05 (day 0) and finished on 12/12/05 (day 53, day 48pc). The AEC approval number was UNE 05/172.

At day 5 (day 0 pc) chickens were challenged with the different MD infective materials as shown in Table 24. For liquid materials (cell culture, lymphocytes, blood) material was thawed from liquid nitrogen at 37˚C in a water bath before pooling and intraabdominal inoculation with or without dilution with growth media.

For challenge with dust using the dust-box method, chickens were placed in a custom made cardboard box (32x25cm), with a transparent roof so that chickens could be observed while handling (Plate 5). Each chicken had 3mg of dust dispersed near the nostrils using a 5ml syringe and a blunt 18g needle. 100mg of dust was then introduced into the box at the completion of individual infections. A further five 100mg aliquots were introduced into the box at 20 minute intervals. Chickens were disturbed within boxes at 5-minute intervals throughout to circulate dust. Ports on the boxes were be sealed with filter material to prevent the escape of dust. Two hours after the initial circulation of 100mg, (20 minutes after last circulation) chicks were transferred to isolators, taking great care to prevent cross-contamination. A different infection box was used for each isolate infected this way.

Plate 5. Dust infection box (left) and detail showing initial individual chick infection (right). Photos from a different experiment using coloured birds.

Initially chickens in isolators 19 and 21 received the same treatment mixture. However on day 20 (d15pc) the surviving birds in this treatment were pooled into isolator 21. Isolator 19 was dismantled, disinfected, re-built and stocked with 14 control chickens (7 each from Isolators 2 and 15). These chickens were then infected intra-tracheally as described in the General Materials and Methods section with 10mg of infective dust from isolate FT158 collected during experiment MD04-R-PT2. These chickens were therefore infected 15 days after the other treatments. The next 4 control chickens (2 from each isolator) were transferred to isolator 3 in which numbers were becoming low. These chickens would have faced challenge 16 days after their cohorts in isolator 3.

Chickens were offered food and water ad libitum with 12L:12D lighting. All birds dying during the experiment were examined post mortem for gross MD lesions as described in the General Materials and Methods section. Thymic and bursal atrophy were also scored in this experiment for all dead and the euthanized chickens (0, no atrophy, 1, mild atrophy, 2 moderate atrophy, 3, severe atrophy or complete ablation of organ. On day 52 (d47pc) 5 chickens from each isolator were blood sampled and plasma retained to do chick inoculation test serology should it be required. At day 53 (d48pc) all surviving chickens were euthanized and similarly examined post mortem for gross MD lesions and thymic and bursal atrophy. In some treatments where MD had clearly not been induced, only a subset of birds was examined post mortem.

Data for normally distributed or transformed continuous variables was investigated and analysed by ANOVA following the fitting of appropriate general linear models using. The effects of Challenge treatment, Sex, and Operator (where relevant) and their interactions were fitted with removal of interaction terms with a P value below 0.2. Significant differences amongst means were determined using Tukey's HSD test. Categorical data such as mortality or MD incidence were analysed using contingency table analysis and the Pearson chi-square statistic and Fisher's exact test in the case of 2-way tables. Mortality data were also subject to survival analysis using the product-limit (Kaplan-Meier) method. Data were analysed using JMP 5.1 (SAS Institute Inc., NC, USA). A significance level of $P \leq 0.05$ is used throughout

Table 24. Details of the MDV isolates used in Expt 5. Historical details can be found in Table 3.

Isolator	Isolate	Batch No/details	Immediate source	Challenge dose/bird	Year of origin	State
1	W7BIS (MPF57 B2)	260904, P8, CK	RMIT	0.2ml	1994	NSW
2	Control					
3	MPF199/3 &9	190705, LØ	RMIT	0.33ml (pooled)	2004	SA
4	MPF57 B1 (MPF179/6)	200904 P7, CK Backpass at UNE	RMIT	0.2ml	1994	NSW
5	MPF23	Whole blood KR 080805	UNE	0.4ml	mid-1980s	VIC
6	MPF176/734 o MPF176/734s MPF176/94 (MPF57 B1)	030504, P4 CK 030504, P4 CK 060404, Ovary, LØ	RMIT	0.33ml (pooled)	1994	NSW
7	MPF210/2s (02LAR B1)	040505, LØ Backpass at UNE	RMIT	0.2ml	2002	VIC
8	MPF164/6	190705, LØ Backpass at UNE	RMIT	0.2ml	2003	WA
9	MPF212 (05JUR B1)	190705, LØ Backpass at UNE	RMIT	0.2ml	2005	NSW
10	MPF210/1s (FT158 B1)	040505 Backpass at UNE	RMIT	0.2ml	2002	NSW
11	05JUR	Field dust. May 2005.	UNE	Dust box, 33mg, VCN $3.74x10^5$/mg	2005	NSW
12	MPF179/6 (MPF57 B1)	040804 Spleen LØ Backpass at UNE	RMIT	0.2ml	1994	NSW
13	04OWE	Dust (one pass in MD04-R-PT2, Nov 2004)	UNE	Dust box, 33mg, VCN $4.25x10^4$/mg	2004	SA
14	MPF189/8	190705 Spleen LØ Backpass at UNE of 280605 P2.	RMIT	0.2ml	2004	QLD
15	Control	Sham challenge with diluent only				
16	MPF23	Whole blood KR 080805	UNE	0.1ml	mid-1980s	VIC
17	MPF179/2 (04CRE B1)	040804 Spleen LØ Backpass at UNE	RMIT	0.2ml?	2004	NSW
18	Woodlands-1	310804, P14, CK	RMIT	0.3ml	1992	QLD
19*	FT158	Dust (one pass in MD04-R-PT2, Nov 2004)	UNE	Dust intra-tracheal 10mg	2002	NSW
20	05JMJ	Field dust, August 2005	UNE	Dust box, 33mg, VCN $1.12x10^5$/mg	2005	SA
21	MPF192/4 MPF192/10 MPF192/1	050705, P3, CK 130305, P6, CK 190705 LØ Backpass at UNE of 280904	RMIT	0.38ml	2004	SA
22	MPF23	Whole blood KR 080805	UNE	0.025ml	mid-1980s	VIC
23	02LAR	Dust (one pass in MD04-R-PT2, Nov 2004)	UNE	Dust box, 33mg, VCN $1.05x10^5$/mg	2002	VIC
24	04KAL	Dust (one pass in MD04-R-PT2, Nov 2004)	UNE	Dust box, 33mg, VCN $3.29x10^4$/mg	2004	SA

* Challenge 15 days after other birds. See text for details.

Results

Mortality and MD lesions.

Twelve chickens died up to day 6 (day 1pc, 12/470=2.6%) and are not included further in the analysis. The next chicken to die was at day 10pc and this chicken and all others dying beyond this date are included. Four chickens were badly decomposed when found dead and a diagnosis of presence or absence of gross MD lesions could not be made. The first MD tumour was detected at day 18pc.

Survival and mortality analysis included 458 eligible chickens. Overall mortality rates by treatment and sex are summarized in Table 25 with both having significant effects on overall mortality. Most of the chickens challenged with infective wet material from RMIT exhibited mortality whereas there was no mortality or very limited mortality in the dust challenge treatments and the challenge with MPF23.

Table 25. Mortality rate by day 48pc by treatment and sex and vaccination in Experiment 5.

Effect of treatment				Effect of sex*			
Treatment	n	Died	%Mort	Sex	n	Died	%Mort
MPF 189/8	19	14	73.7	F	187	46	24.6[a]
MPF 199/ 3&9	22	16	72.7	M	189	64	33.9[b]
MPF 192/1,4,10	37	21	56.8	Overall	376	110	29.3
MPF 210/2s	19	10	52.6				
MPF 179/6	19	9	47.4				
MPF 164/6	20	8	40.0				
Woodlands 1	19	7	36.8				
02LAR (dust)	18	6	33.3				
MPF 57	20	6	30.0				
FT158 (dust)	14	3	21.4				
W7BIS	20	4	20.0				
MPF 210/1s	19	3	15.8				
MPF 176/734 etc	20	3	15.0				
04KAL (dust)	19	1	5.3				
MPF 212	20	1	5.0				
04OWE (dust)	19	0	0.0				
05JMJ (dust)	19	0	0.0				
05JUR (dust)	20	0	0.0				
MPF 179/2	17	0	0.0				
MPF23 25ul	19	0	0.0				
MPF23 100ul	18	0	0.0				
MPF23 400ul	20	0	0.0				
Control	21	0	0.0				
Overall	458	112	24.5				

* Not all chickens from all treatments were post mortemed so sex was not determined for all chickens in the experiment.

Survival analysis revealed significant effects of Treatment (P<0.001) and Sex (p=0.03) on the pattern of mortality (Figure 13). Once again there was clear evidence of an acute mortality syndrome between days 10 and 22 with severe mortality during this period (>40%) in 3 treatments (189/8, 199/3&9 and 192/1,4&10). Thymic and bursal atrophy was recorded in this experiment and was severe during this early mortality period with mean scores of 2.85 and 2.14 respectively (maximum score is 3). Of the 52 chickens dying during this period for which sex could be ascertained, 31 (60%) were male and 21 (40%) were female (P<0.05). This supports the observation in experiment 4 that male chickens are more susceptible to the MD acute mortality syndrome than female chickens. Although the first MD tumour was detected at day 18pc, tumours were only detected in 5 chickens prior to day 33 after which mortality was dominated by MD tumours (35/45 deaths with MD tumour, 78%). For several treatments there was little or no early mortality syndrome, but significant later losses associated with MD tumours (Figure 13).

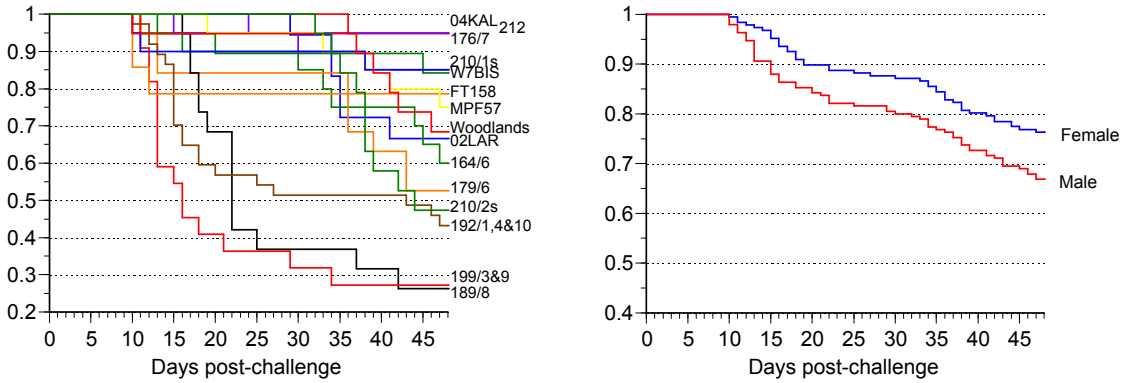

Figure 13. Survival patterns in Experiment 5 showing the effects of Treatment (Left panel, P<0.001) and Sex (Right panel, P=0.03). There was no mortality in the Control treatment or treatments 04OWE, 05JMJ, 04JUR, 179/2 and MPF23 so they are excluded.

Incidence of MD

Chickens were classified as MD positive one of two ways.

a) Exhibiting gross MD lesions. The population at risk was defined as that alive at day 18pc, the day of detection of the first MD lesion and subsequently subjected to post mortem examination (n=336).

b) Exhibiting gross MD lesions and/or having a mean atrophy score of 3 or more. The mean atrophy score is the sum of the thymic and bursal atrophy scores. The population at risk was defined as that alive at day 10pc, the day of first mortality with thymic or bursal atrophy and subsequently subjected to post mortem examination (n=378).

As no vaccinated chickens were included in the experiment, protective index and virulence rank could not be calculated. However the incidence of mortality and MD is an indication of virulence in unvaccinated chickens, bearing in mind that challenge dose rate was not standardized in this experiment.

Using the presence of gross lesions as the criterion for detecting MD, 125/336 chickens (37.2%) were MD-positive during the experiment or following euthanasia at day 48pc. There were highly significant effects of Treatment (P<0.001, Table 26) and Sex (P=0.002, Table 27). The incidence of MD ranged from 0% (Control, MPF23, 05JUR, 05JMJ, 04OWE and 04KAL) to 89.5% for MPF210/2s (=02LAR B1) with Woodlands-1 also inducing 88.9% MD lesions. MD lesions were detected in 14 treatments. The effect of Sex was manifest as a significantly higher incidence of gross lesions in female (44.1%) than male (30.3%) overall (Table 27).

Using an atrophy score >3 and/or the presence of gross lesions as the criteria for detecting MD, 262/378 chickens (69.3%) were MD-positive during the experiment or following euthanasia at day 48pc. There was a significant effect of Treatment (P<0.03, Table 26) but not Sex (P=0.46, Table 27). The incidence of MD ranged from 0% (Control, MPF23, 05JUR and 04OWE) to 100% for MPF210/2s MPF 164/6 and MPF189/8. Interestingly the 04KAL treatment which had an MD incidence of 0% based on gross tumours, had an incidence of 84.2% when atrophy score was included as a criterion. Similar large increases were observed for the dust challenge treatments 02LAR and FT158. The higher incidence of MD in females, observed when gross lesions were the sole criterion effect is no longer present when atrophy score is included (Table 27).

Anatomical distribution of MD tumours

Amongst chickens exhibiting MD lesions, gross tumour lesions were most commonly found in the ovary of females, testis of males followed by the liver, spleen, kidney and muscle. There was no effect of sex apart from its effect on the gonads (Table 28). There was little evidence of major effects of different MDV isolates apart from a significantly lower incidence of MDV in the testis (Table 29)

Table 26. Experiment 5. Incidence of MD by Challenge treatment with MD cases defined either by the presence of gross MD lesions alone, or by the presence of thymic and bursal atrophy (combined score >3) and/or the presence of gross lesions. Chickens from treatment MPF179/2 were not examined post-mortem as there was no mortality in this treatment and no evidence of illness in the birds.

	MD lesions only			MD lesions + Atrophy		
Treatment	n	MD Pos	%MD	n	MD Pos	%MD
MPF 210/2s	19	17	89.5	19	19	100.0
Woodlands 1	18	16	88.9	19	18	94.7
MPF 164/6	18	13	72.2	20	20	100.0
MPF 210/1s	18	13	72.2	19	15	78.9
*MPF 179/6	16	10	62.5	19	18	94.7
MPF 199/3&9	10	6	60.0	22	20	90.9
*MPF57	20	12	60.0	20	19	95.0
*W7BIS	18	10	55.6	20	15	75.0
MPF 189/8	12	6	50.0	19	19	100.0
MPF 192/1,4,10	24	9	37.5	37	33	89.2
*MPF 176/pool	19	5	26.3	19	11	57.9
MPF 212	19	4	21.1	20	16	80.0
FT158 (Dust)	14	2	14.3	14	8	57.1
02LAR (Dust)	18	2	11.1	18	14	77.8
04KAL (Dust)	19	0	0.0	19	16	84.2
04OWE (Dust)	19	0	0.0	19	0	0.0
05JMJ (Dust)	12	0	0.0	12	1	8.3
05JUR (Dust)	12	0	0.0	12	0	0.0
MPF23 100ul	10	0	0.0	10	0	0.0
Control	21	0	0.0	21	0	0.0
Total	336	125	37.2	378	262	69.3

* Various back passages of MPF57

Table 27. Experiment 5. Incidence of MD by Sex with MD cases defined either by the presence of gross MD lesions alone, or by the presence of thymic and bursal atrophy (combined score >3) and/or the presence of gross lesions.

Sex	MD lesions only			MD lesions + Atrophy		
	n	MD Pos	%MD	n	MD Pos	%MD
Female	172	77	44.8[a]	188	133	70.7[a]
Male	163	48	29.4[b]	187	127	67.9[a]
Overall	335	125	37.3	375	260	69.3

[ab]Means within columns not sharing a common letter in the superscript are significantly different ($p<0.05$)

Table 28. Anatomical distribution of MD lesions by Sex in chickens exhibiting gross lesions in Experiment 5.

Organ	Female			Male		
	n	MD Lesions	%Lesions	n	MD Lesions	%Lesions
Ovary*	77	63	81.8	48	0	0
Testes*	77	0	0	48	29	60.4
Liver	77	31	40.3	48	25	52.1
Spleen	77	29	37.7	48	19	39.6
Kidney	77	21	27.3	48	13	27.1
Muscle	77	14	18.2	48	8	16.7
Heart	77	7	9.1	48	6	12.5
Lungs	77	7	9.1	48	9	18.8
Proventriculus	77	6	7.8	48	5	10.4
Thymus	77	5	6.5	48	4	8.3
Eye	77	1	1.3	48	0	0
Mesentery	77	1	1.3	48	0	0.0
Bursa	77	0	0	48	3	6.3

* Ovary and testes data for females and males only, respectively

Table 29. Experiment 5. Anatomical distribution of MD lesions by Challenge treatment in chickens exhibiting gross lesions in Experiment 5. Only treatments where 9 or more birds exhibited MD lesions are included. Figures for ovary and testis apply only to chickens of the relevant sex.

Organ	Incidence of MD lesions in each organ (%) (Numbers of male and female chickens in brackets)							
	MPF210/ 2s (6F, 11M)	Woodland s1 (10F,6M)	MPF164/ 6 (5F,8M)	MPF210/ 1s (9F,4M)	MPF57 (8F,4M)	MPF179/ 6 (7F,3M)	W7BIS (7F,3M)	MPF192/ 1 etc (6F,3M)
Ovary*	100	100	100	67	100	100	71	100
Testes*	100	67	50	100	0	67	67	67
Liver	52.9	50	53.8	38.5	16.7	70	60	55.6
Spleen	23.5	37.5	46.2	61.5	25	60	50	44.4
Kidney	41.2	25	23.1	53.8	16.7	40	20	22.2
Muscle	29.4	25	23.1	7.7	8.3	30	10	22.2
Heart	11.8	6.3	30.8	0	16.7	30	10	0
Lungs	5.9	18.8	7.7	23.1	25	10	20	11.1
Proventriculus	11.8	6.3	15.4	7.7	8.3	20	10	0
Thymus	0	12.5	0	15.4	8.3	0	10	11.1
Eye	5.9	0	0	0	0	0	0	0
Mesentery	5.9	0	0	0	0	0	0	0
Bursa	11.8	0	0	0	8.3	0	0	0

***** Figures for ovary and testis apply only to chickens of the relevant sex.

Body weight and relative immune organ weights at day 48pc.

At day 48 post-challenge all remaining surviving chickens (348) were euthanased and a subset were weighed and had the weights of the thymus, bursa and spleen recorded (total n=266). Treatment effects are summarized in Figure 14.

Body weight was significantly affected by challenge treatment (P<0.0001, Figure 14a) and Sex (P<0.0001) with significant interaction between these effects (P=0.018). Challenge treatments MPF23, 05JUR, 04OWE, 05JMJ, MPF210s, MPF176/734oetc, FT158, 02LAR and MPF57 did not differ significantly from the control chickens (control LSM = 714±22g) with all other treatments significantly reducing final bodyweight. Males were significantly heavier than females (641 v 561g P<0.0001). The significant treatment x sex interaction was due to four challenge treatments in which final weights of females were greater than those of males (MPF210/2s, MPF189/88, Woodlands1 and MPF212).

Birds with MD lesions in the spleen were excluded from analysis of relative spleen weight (n=27). Relative spleen weight was significantly affected by challenge treatment (P<0.0001, Figure 14b) and Sex (P<0.0001) with no significant interaction between these effects. MDV-challenged chickens generally had had larger spleens than sham-challenged controls however the following challenge treatments did not differ from the controls: 05JMJ, 04OWE, 05JUR, MPF23, MPF189/8 and MPF199/3&9, Figure 14b). Male chickens had larger relative spleen weight than females (0.584 v 0.504 %BW respectively, P<0.0001).

Birds with MD lesions in the thymus were excluded from analysis of relative thymic weight (n=4). Relative thymic weight was significantly affected by challenge treatment (P<0.0001, Figure 14c) and Sex (P=0.011) with no significant interaction between these effects. MDV-challenged chickens generally had had lower relative thymic weight than sham-challenged controls, in many cases markedly so with very little thymus present in some treatments (Figure 14c). However the following challenge treatments did not differ from the controls: 04OWE, 05JMJ, MPF23 and 05JUR, Figure 14c). Female chickens had higher relative thymic weights than males (0.310 v 0.256 %BW respectively, P=0.011).

Birds with MD lesions in the bursa were excluded from analysis of relative bursal weight (n=2). Relative bursal weight was significantly affected by challenge treatment (P<0.0001, Figure 14d) but not Sex (P=0.85). There was no significant interaction between these effects. MDV-challenged

chickens generally had had lower relative bursal weight than sham-challenged controls, in many cases markedly so, although the level of bursal atrophy was not as great as that seen for the thymus (Figure 14d). The same challenge treatments did not differ from the controls as for relative thymic weight, namely 05JMJ, 04OWE, 05JUR and MPF23, Figure 14d). There was no difference between the sexes in relative bursal weights (0.212 v 0.214 %BW for males and females respectively, P=0.85).

Three operators did the post-mortems and dissections. The effect of operator was non significant for relative thymic weight (p=0.01) and relative bursal weight (P=0.07), but was significant for relative spleen weight (P=0.03).

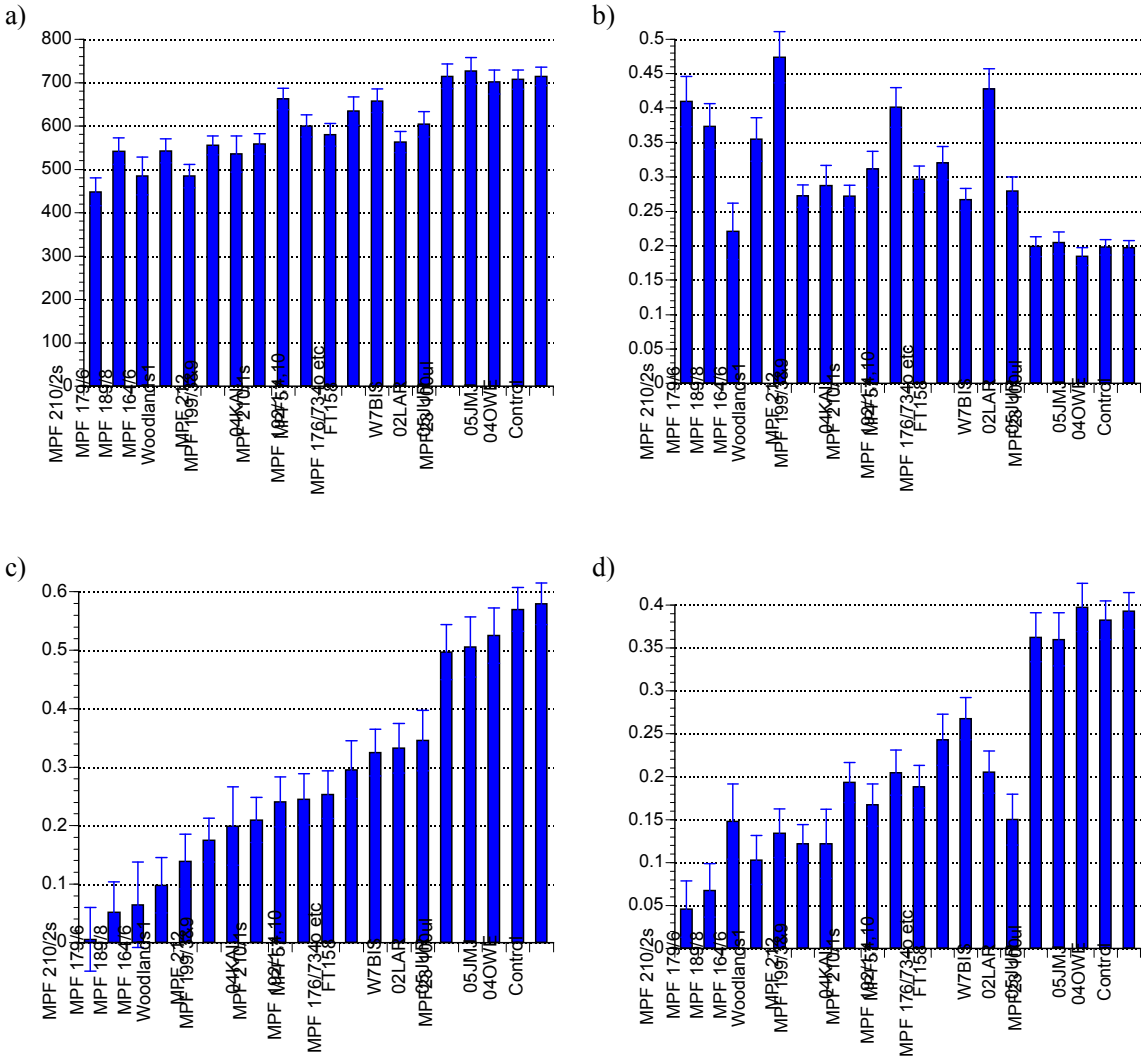

Figure 14. Least square means (±SEM) for a) body weight b) relative spleen weight c) relative thymic weight and d) relative bursal weight of chickens at day 48pc in Experiment 5. Data for relative spleen weight are back-transformed means from the $\log_{10}$ scale.

Visual scoring of thymic and bursal atrophy clearly differentiated groups of chickens with different relative weights of these organs, but there was considerable overlap (Figure 15). Least squared means for the organ weights for each atrophy score are presented in Table 30, as is the relationship between TA and BA score and the percentage of chickens exhibiting MD lesions.

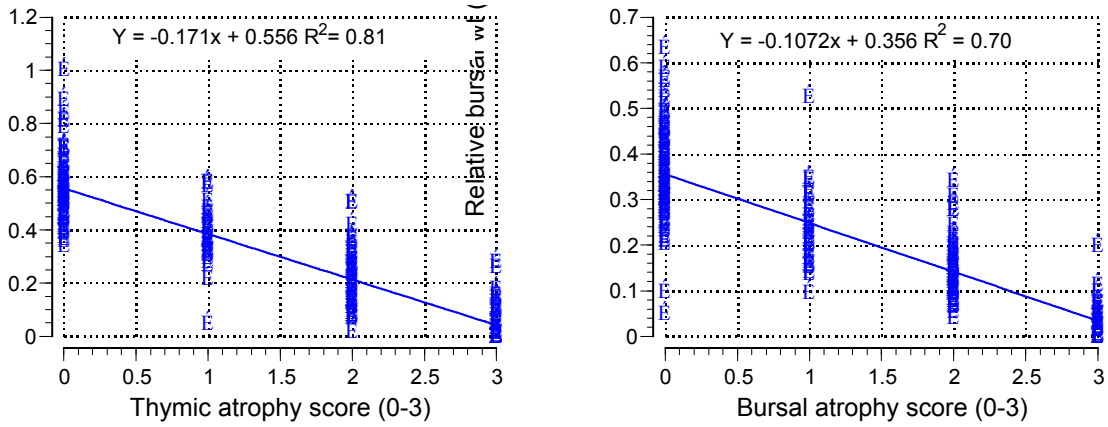

Figure 15. Linear regression of relative thymic and bursal weights at day 48pc on atrophy scores for each organ in experiment 5. Both relationships are highly significant (P<0.0001). Chickens with tumours in either organ are excluded.

Table 30. Mean and SEM for relative thymic and bursal weights by atrophy score for each organ in experiment 5. Also shown is the percentage of chickens with each score exhibiting gross MD lesions.

Atrophy score	Relative thymic weight (%BW)			%MD	Relative bursal weight (%BW)			%MD
	n	Mean	SE		n	Mean	SE	
0	90	0.560[a]	0.011	5.6[a]	104	0.360[a]	0.008	6.7[a]
1	39	0.381[b]	0.017	25.6[b]	42	0.232[b]	0.012	33.3[b]
2	64	0.216[c]	0.013	40.0[b]	81	0.141[c]	0.009	44.4[b]
3	72	0.041[d]	0.012	57.5[c]	39	0.040[d]	0.012	65.0[c]

[abc] Means within columns not sharing a common letter in the superscript are significantly different (p<0.05)

For each increase in thymic and bursal atrophy score there was a significant increase in mean relative thymic and bursal weight. There was also an increase in the incidence of MD tumours with each increase in score. The increase in MD was significant for each increment in score except between scores 1 and 2.

Multivariate analysis of relative thymic, bursal and spleen weights simultaneously was again interesting. Cluster analysis (K means clusters) was able to group animals into two clusters with a highly significant (P<0.0001) association with gross MD lesions (Table 31).

Table 31. K-means cluster analysis of relative thymic, bursal and spleen weights at day 48pc in Experiment 5, grouping individual chickens into one of two clusters. The association with incidence of MD lesions is also shown (p<0.0001).

Cluster	n	Relative thymus weight (%)	Relative spleen weight (%)	Relative bursal weight (%)	%MD Lesions
1	114	0.518	0.232	0.357	5.26[a]
2	124	0.153	0.357	0.128	39.67[b]

The overall atrophy score (sum of TA and BA scores) was also significantly associated with the presence of MD lesions, although the association was strongest at the extremes of the scoring range (Table 32). Overall atrophy score was also significantly associated with TA and BA, and the mean K-means cluster value (Table 32). The relationships are represented visually in Figure 16.

Table 32. Mean incidence of MD lesions, relative immune organ weights and K-means cluster scores by atrophy score (sum of TA and BA) at day 48pc in Experiment 5.

Atrophy score	n	%MD Lesions	Body weight (g)	Rel. thymic wt (%BW)	Rel. spleen wt (%BW)	Rel bursal. wt (%BW)	Mean cluster score
0	78	3.8[a]	708	0.564[a]	0.212[c]	0.385[a]	1.00[c]
1	26	17.9[b]	678	0.420[b]	0.283[bc]	0.297[b]	1.16[c]
2	19	43.5[c]	612	0.375[b]	0.356[ab]	0.227[bc]	1.62[b]
3	22	23.1[bc]	582	0.247[c]	0.316[b]	0.206[c]	1.83[ab]
4	32	47.6[c]	570	0.216[c]	0.320[b]	0.166[cd]	1.86[a]
5	28	50.0[c]	569	0.068[d]	0.336[b]	0.117[d]	2.00[a]
6	32	64.9[c]	477	0.031[d]	0.426[a]	0.038[e]	2.00[a]

[abc] Means within columns not sharing a common letter in the superscript are significantly different ($p<0.05$)

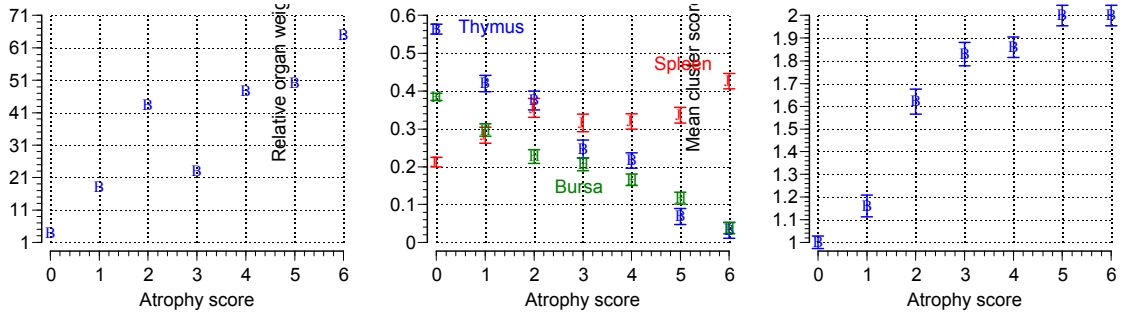

Figure 16. Scatter plots of mean incidence of MD lesions, relative immune organ weights (±SEM) and K-means cluster scores (±SEM by atrophy score (sum of TA and BA) at day 48pc in Experiment 5.

Association between variables

Correlation coefficients for the different measurements made at day 48pc are presented in Table 33.

Table 33. Experiment 5. Matrix of Pearson correlation coefficients (r) for different variables measured at day 48pc. Correlations are between group least square means for each challenge group (20 challenge groups, df=18). All chickens with MD tumours of thymus, bursa or spleen were omitted from the organ weight analyses. All but one values of r is statistically significant. Values of r over 0.85 are marked in bold. Significant P values for a two tailed test with df=18 for P<0.05, <0.01 and <0.001 are 0.44, 0.56 and 0.68 respectively. BWT = bodyweight, RTW, RBW and RSW = Relative thymic, bursal and spleen (backtransformed) weight, TA, BA and AS = thymic, bursal and total atrophy score, Cluster = mean cluster value, %Mort = % Mortality, %MD = % chickens with MD lesions, %MD+AS = % chickens with MD lesions and/or AS>2.

Variable	BWT	RTW	RBW	RSW	TA	BA	AS	Cluster	% Mort	%MD	%MD +AS
BWT	1	**0.91**	**0.89**	-0.68	**-0.91**	**-0.96**	**-0.94**	**-0.91**	-0.77	-0.74	**-0.89**
RTW	**0.91**	1	**0.95**	-0.66	**-0.97**	**-0.96**	**-0.98**	**-0.96**	-0.74	-0.78	**-0.93**
RBW	**0.89**	**0.95**	1	-0.70	**-0.91**	**-0.96**	**-0.95**	**-0.95**	-0.73	-0.78	**-0.94**
RSW	-0.68	-0.66	-0.70	1	0.66	0.74	0.70	0.75	0.42	0.82	0.72
TA	**-0.91**	**-0.97**	**-0.91**	0.66	1	**0.96**	**0.99**	**0.94**	0.70	0.74	**0.92**
BA	**-0.96**	**-0.96**	**-0.96**	0.74	**0.96**	1	**0.99**	**0.96**	0.76	0.80	**0.95**
AS	**-0.94**	**-0.98**	**-0.95**	0.70	**0.99**	**0.99**	1	**0.96**	0.73	0.78	**0.95**
Cluster	**-0.91**	**-0.96**	**-0.95**	0.75	**0.94**	**0.96**	**0.96**	1	0.72	0.78	**0.98**
% Mort	-0.77	-0.74	-0.73	0.42	0.70	0.76	0.73	0.72	1	0.68	0.75
%MD	-0.74	-0.78	-0.78	0.82	0.74	0.80	0.78	0.78	0.68	1	0.77
%MD+AS	**-0.89**	**-0.93**	**-0.94**	0.72	**0.92**	**0.95**	**0.95**	**0.98**	0.75	0.77	1

All measurements were significantly associated with mortality rate to day 48pc apart from relative spleen weight (r=0.42). All were significantly associated with MD tumour incidence with relative spleen weight having the strongest association (r=0.82). Associations were negative for relative thymic and bursal weights and bodyweight, and positive for the other variables. Atrophy scores had

associations of similar strength as actual relative organ weights or combinations of these in the cluster analysis.

In Table 34 relationships between mortality/MD lesions, and atrophy scores measured over the whole experiment (ie chicken mortality included, not just d48pc chickens) is shown. Including all of the atrophy scores improves the relationships overall.

Table 34. Experiment 5. Matrix of Pearson correlation coefficients (r) between thymic, bursal and combined atrophy scores (TA, BA, AS) measured throughout the experiment and the incidence of total mortality (%Mort), gross MD lesions (%MD) and gross MD lesions and/or AS>2 (%MD+AS). Significant P values for a two tailed test with df=18 for P<0.05, <0.01 and <0.001 are 0.44, 0.56 and 0.68 respectively.

	TA whole expt	BA whole expt	AS whole expt	% Mort	%MD lesions	%MD+AS
TA whole expt	1	0.98	0.99	0.79	0.74	0.97
BA whole expt	0.98	1	0.99	0.81	0.76	0.97
AS whole expt	0.99	0.99	1	0.79	0.75	0.97
Mort%	0.79	0.81	0.79	1	0.68	0.75
%MD lesions	0.74	0.76	0.75	0.68	1	0.77
%MD+AS	0.97	0.97	0.97	0.75	0.77	1

Growth of viruses in cell culture.

On 12/12/05 pooled spleen samples from 17 different treatment groups in which MD was confirmed were sent to RMIT by overnight courier (TNT). Unfortunately the spleens did not arrive until the morning of 14/12/05 at which time they had a temperature of 14˚C. In response to this, UNE conducted another experiment (MD06-C-VI5) in Jan-Feb 2006 infecting chickens with 14 dust samples collected from Experiment 5. The results of MD06-C-VI5 are not presented as they fall under Australian Poultry CRC project 03-17. Spleens from MD06-C-V15 were split and sent by two different couriers (Toll and World Courier) to RMIT both of which were delivered overnight with samples arriving on the 28th Feb 2006. Following receipt at RMIT, samples from both experiments were subjected to a cycle of virus isolation in SPF CK cultures.

As of the 4th April 2006, the majority of samples received at RMIT that passed the initial selection criteria [21/29] had been successfully bulked-up following virus isolation in SPF CK cultures. However none of these viruses grew to a titre above 260 pfu/ml in 4-6 passages. A titre of 10^4pfu or greater is required to produce sufficient challenge virus for use in formal pathotyping experiments at a dose rate of 500pfu/ml. Further passages are planned and the underlying reasons for the low titres will be investigated.

Brief discussion

The experiment was successfully implemented and the isolates under test showed a wide range in pathogenicity in unvaccinated chickens. A number of new isolated from several Australian states exhibited high pathogenicity and induction of mortality and MD tumours at levels comparable with the most virulent current isolates such as 02LAR, Woodlands1, MPF57 and FT158. The new isolates with a similar level of pathogenicity include MPF 189/8 (Qld, 2004), MPF199/3&9 (SA, 2004), MPF192/1,4&10 (SA, 2004) and MPF 164/6 (WA, 2003).

While challenge dose was uncontrolled in this experiment, highly pathogenic viruses that induced high levels of MD in unvaccinated chickens in Experiment 4 (MD04-R-PT2) also tended to induce high levels in the present experiment, despite the use of various back-passages at unknown dose rates (Table 35). This suggests that the new isolates listed above are likely to be highly pathogenic once grown in cell culture, titrated and administered at a fixed dose.

In this experiment it was clear that cell culture material or lymphocytes from previous passages in chickens were good inocula, inducing MD successfully in all cases except MPF179/2. By contrast, challenge with infective dust via the box method was relatively unsuccessful with 05JUR, 04OWE and 05JMJ, failing to induce any evidence of infection with MDV, 04KAL inducing significant

immunosuppression but not MD lesions, and only 02LAR inducing both mortality (33.3%) and gross MD tumours (11.1%). On the other hand, late dust challenge with FT158 intra-tracheally on day 20 was clearly successful with 21.4% mortality and 14.3% MD lesions induced by day 33pc for this treatment (day 48pc for other treatments). The results of this experiment, together with those of Experiment 2, strongly suggest that box challenge with dust, at the doses used, is inferior to intra-tracheal challenge of individual birds. Challenge with whole frozen blood (with 10%DMSO) for the isolate MPF23 was unsuccessful despite earlier confirmation of infectivity of a different batch of blood from the initial back-passage of this virus at UNE. Fortunately we have since used the original blood in a different experiment and induced severe MD with this virus, the most virulent MDV isolate of the 1980s.

Table 35. Incidence of mortality and MD lesions in experiments 4 and 5 for four viruses included in both experiments. In experiment 5, 02LAR is MPF210/2s (02LAR/B1), Woodlands 1 is Woodlands 1, FT158 is MPF210/1s (FT158/B1) and MPF57 is the mean of MPF179/6, MPF57, W7BIS and MPF 176/343o etc., all of which are various back-passages of MPF57. Dust challenge treatments in Experiment 5 are not included.

Isolate	Experiment 4 – Unvaccinated, d56pc, challenge with 500pfu/bird		Experiment 5 – Unvaccinated d48pc, challenge pfu unknown	
	%Mortality	%MD	%Mortality	%MD
02LAR	85.2	94.4	52.6	89.5
Woodlands 1	48.0	81.0	36.8	88.9
FT158	56.5	85.0	15.8	72.2
MPF57	27.3	84.2	28.1	51.1

In this experiment, for the first time, thymic and bursal atrophy were scored in addition to having the organs weighed. This has major advantages in speed relative to dissecting out and weighing organs and then expressing them as a proportion of body weight. Once again in SPF, maternal antibody negative chickens, MDV induced profound atrophy of both the thymus and bursa, while most isolates induced splenomegaly. When treatment means were considered, thymic and bursal atrophy scores were as predictive of mortality or MD tumours as were the mean relative weights of these organs. Furthermore, the measurement of either of these organs (particularly the bursa) provided equal predictive value as complex measures such as combined atrophy score, or cluster analysis scores which take into account the relative weights of thymus, bursa and spleen. Dissection of thymus is a particularly difficult and time-consuming task, and it can no longer be recommended as part of routine pathotyping on the basis of these findings. A combination of thymic and bursal atrophy scores and spleen and bursal weights, which are all easily measured, is more likely to provide a useful adjunct to the presence of MD tumours.

The definition of a MD case in experiments such as this has been problematic. Our group had adopted a conservative approach to date, considering birds MD positive only if they exhibited gross tumours. This may be appropriate in commercial broiler chickens which are maternal ab-positive and exhibit little early MD-induced mortality (pre tumours) and limited immunosuppression as determined by thymic and bursal atrophy. However in all our major experiments in mab-negative SPF chickens we have noted significant MDV-induced mortality well before tumours appear. This mortality is associated with marked thymic and bursal atrophy. Furthermore many chickens at the end of the experiments remain free of MD tumours but exhibit thymic or bursal atropy to some degree. Undoubtedly this is compromising the chickens (r of >-0.9 with bodyweight) and is strongly associated with MDV infection. The USDA ADOL pathotyping system includes thymic and bursal atrophy in the definition of a MD-positive case (Witter, 1997) but is unclear on the extent of atrophy required to classify a chicken as MD-positive. In the present experiment, using a combined atrophy score of 3 or greater as a MD case threshold (with or without tumours) increased the number of MD cases from 125/335 with tumours (37.3%) to 260/375 (69.3%). The additional 135 cases (more than double) include a significant number not included in the “population at risk” for tumours, as they occurred well before the onset of tumours. Indeed, looking at Table 32 and Figure 16, a case could be made for inclusion of atrophy scores 2 and 1 in the definition of an MD case. Certainly the use of

tumours only as an indicator of Marek's disease in these experiments is too conservative and can no longer be recommended.

The point is relevant to pathotyping not only because in changes the number of MD cases and thus potentially protective index and virulence scores, but also because there is evidence in the present experiment that viruses may vary in the extent to which they immunosuppression and induce tumours. Some isolates had a tendency to kill many chickens early and induce marked immunosuppression but to have a relatively modest incidence of tumours (eg. MPF 189/8, 192/1,4&10) while others induced low levels of mortality but had a very high incidence of tumours (eg. 210/1s). Still others had very low levels of mortality and MD lesions but induced significant immunosuppression (eg. 04KAL). These effects are shown in Figure 17. It is conceivable that these are dose effects rather than true differences between viruses with the high early mortality pattern reflecting overwhelming challenge, and the late immunosuppression only representing low or late challenge with a moderately pathogenic virus. This needs to be established because if it is a characteristic of the virus, rather than the dose, the method of defining MD cases in pathotyping experiments will result in different rankings between viruses.

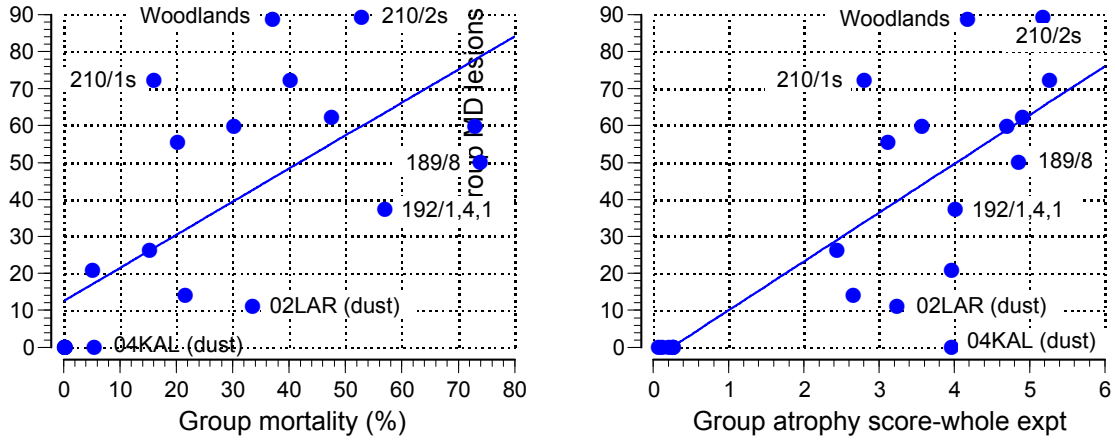

Figure 17. Relationship between treatment group means for MD mortality (%) and MD gross lesions (%) (Left panel) and Atrophy score and MD gross lesions (%) (Right panel) in Experiment 5. Selected points are labelled with the treatment group name.

The experiment has confirmed that back-passage into chickens is an effective way of amplifying MDV from cell culture or stored materials and suggests that if taken to the tumour stage, such amplification may prove useful as a screening test for pathogenicity.

Meq Gene sequencing

Introduction and objectives

The MDV genome contains two unique regions one long (U_L) and one short (U_S) each of which is flanked by internal and terminal repeat sequences (TR_L, IR_L, TR_S, IR_S) as shown diagrammatically in Figure 18. The MDV genome differs from other herpersviruses mostly in the repeat regions and mostly in the R_L region. Indeed the MDV genome codes for a number of proteins with no homology to any other herpesvirus proteins. These include Meq, pp38/pp24, pp14, vIL-8, SORF2, p7 and vLIP.

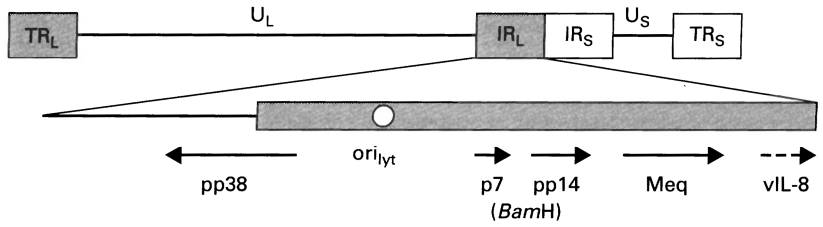

Figure 18. Structure of the MDV genome showing some of the unique genes in the IR_L region (Nair and Kung, 2004).

Meq is probably the principal oncogene in MDV (Nair and Kung 2004). The estimated size and mol. Wt. of the Meq protein is 339 aa and 40kDa for the gene (Jones *et al.* 1992). The Meq protein belongs the *fos/jun* family of transcriptional activators and like them it localizes to the nucleus and the nucleolus (Liu *et al.* 1997). There is considerable heterogeneity in Meq genes, but clear associations with pathogenicity have yet to be demonstrated. One clear finding is that the MDV serotype-1 attenuated vaccine strain CVI 988 has a 59aa insertion into the Meq protein in the proline-rich region of the protein, a feature shared with some low virulence isolates (JM10, MKT1). Another is that several vv+ strains have mutations in the proline rich region of the protein (Figure 19).

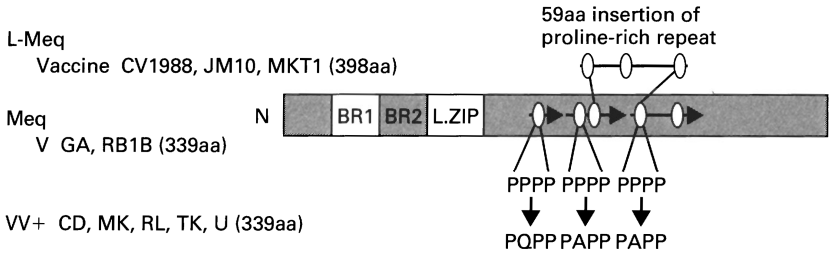

Figure 19. Natural variants of the Meq protein. Wild-type Meq, represented by the GA strain (Meq (339aa) carries two and a half of the proline-rich repeats (arrows) with multiple PPPP motifs (oval shape). L-Meq, represented by the vaccine strain CVI988, has a 59aa proline-rich repeat insert (top). The very virulent plus (vv+ MDV) isolates CD, MK, RL, TK and U carry mutations at the second position of the PPPP motif (Nair and Kung, 2004).

The objective of this initial study was to investigate whether there is a sequence variation in the Meq gene of Australian isolates and if so, to investigate whether the mutations in sequences correlate with virulence.

Materials and Methods

This work was carried out at UNE in Dr Brian Cheetham's laboratory. Much of the work was conducted by Katrin Renz a PhD student at UNE. Four Australian isolates of MDV1, namely MPF179/6 (MPF57B1), W7BIS (MPF57 B2), 02LAR and 04KAL, were obtained from feather tip

samples taken at 48 days post infection (dpi) from experimentally infected SPF- chickens (Experiment 5, MD05-R-PT3). Feather tips were stored in individual sealed plastic bags and kept at -20 degrees. DNA was extracted using the QIAamp DNA Kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions. Extracted DNA was used as template for a standard PCR to isolate the Meq gene from each sample. The Meq- specific primers used are shown in Table 36.

Table 36. Sequences of Meq-gene specific primers used in PCR amplification and sequencing.

Primers	Sequences	Location of primers in Md5 Meq	Expected size of amplified fragment
BCMD01	5'-TTCCGCACACTGATTCCTAG-3'	22-41	1159 bp
BCMD02	5'-TAGGGGAGAAGAAACATGGG-3'	1161-1180	

For each of the four strains, four individual reactions were set up to generate enough DNA for sequencing. PCR was performed in a 25 µl reaction mixture containing 1µmol of each primer, 1.8mM MgCl2, 0.2mM dNTP's, 10x reaction buffer (Fisher Biotec, Perth, Australia), 1 unit of Taq DNA polymerase and approx. 1ng of template DNA. Amplification was carried out over 35 cycles each consisting of 1.5min at 94°C, 1min at 60°C and 2min at 72°C, except for the initial 2 cycles in which the period at 94°C was extended to 5min. After the final cycle, the elongation phase at 72°C was extended to 10min with a consecutive step at 4°C for 5min. The amplified fragments were separated on an agarose gel (1%) and visualized by staining with ethidium bromide. The PCR products were then purified using the Wizard® DNA purification Kit (Promega, Madison, USA) according to the manufacturer's instructions. As Newcastle DNA demands a concentration of at least 100ng/µl for PCR products to be sequenced, the concentration of the DNA fragments was determined. Therefore, 2µl of purified DNA was run on a 1% agarose gel against a lambda standard with known concentration of DNA. Knowing the concentration of each of the DNA samples, aliquots of 10 µl of each sample as well as aliquots of the primer BCMD01 and BCMD02 were sent away for sequencing. The determination of the sequences of each of the samples was conducted by Newcastle DNA, University of Newcastle, Australia, using an Amersham Megabace 48 Capillary sequencer.

Results from sequencing were analysed using Chromas© 1.43 and Sequaid™ 3.70 software. The sequence of each of the samples was aligned against a reference Meq gene (1017 nucleotides long) from the MDV1 strain Md5 which has been completely sequenced (Tulman *et al.* 2000); Genbank accession number AF243438) to be able to detect changes in bases that might be of relevance. This MDV1 strain is classified as 'very virulent' according to the classification system suggested by Witter (1997). The exact position for the reference Meq gene started 101 bp before its start codon which is position 134766 of the Md5 genome and ends 102 bp after its stop codon which is position 135985 of the Md5 genome to ensure that the whole Meq gene sequence could be detected. Therefore, the reference region in total was 1219 nucleotides long containing the complete Meq gene and its flanking regions, but only changes within the start and stop codon were considered. The positions of primer sets as well as the positions where base changes occurred were referred to this 1219 nucleotides long region. As well, the results of each of the four samples were compared with the Rispens/ CVI988 Meq gene published by (Lee *et al.* 2000) which has the Genbank accession number AB033119.

Results

To detect the Meq gene in the four MDV1 strains, Meq- specific standard PCR was performed. A 1200bp fragment was detected which was about the expected size. The purified DNA of the four samples was run on an agarose gel together with a lambda standard of known concentration. The intensity of all four bands resembled the intensity of the first band of the lambda standard which is equivalent to 150ng DNA per µl. Purified DNA from all samples was then sent away to Newcastle DNA for sequencing, and Table 37 shows the differences that could be detected in all four samples compared to the Meq gene of the Md5 strain and CVI988. Interestingly, the base changes in all four Australian isolates are in the same position of the Md5 Meq gene. Within the four Australian samples themselves, there was an insert and a deletion as well as base mutations at several positions within the Meq sequence that could be detected thus making differentiation of these strains possible. When

comparing the four Australian isolates with CVI988, there were definite differences between CVI988 and the Australian isolates as well.

Table 37. Comparison of Meq gene sequence from four Australian isolates with Md5 and CVI988 (- = there is no equivalent base in the strain, ND = no results available yet).

GenBank access. No. strain reference	Position in MD5 Meq	AF243438 MD5 Tulman et al. (2000)	AB033119 CVI988 Lee et al. (2000)	04KAL	02LAR	MPF57	W7BIS
	312	G	G	T	T	T	T
	330	A	A	G	G	G	G
	331	A	A	C	C	C	G
	332	-	-	-	-	-	insert C
	340	A	A	C	A	C	C
	445	T	T	C	C	C	C
	627	C	C	A	C	ND	C
	628	C	C	A?	C	ND	C
	659	G	G	G	T	ND	G
	674	A	A	A	A	ND	deletion
	675	C	C	C	C	ND	deletion
	676	C	C	C	C	ND	deletion
	940	T	T	C	C	ND	ND
	949	T	C	C	C	ND	ND
	1060	C	T	T	T	ND	ND

However, sequencing results with the primers BCMD01 and BCMD02 were overlapping in base positions 500 to 700 of the reference Meq gene from Md5 and thus did not provide definitive results. Therefore, a second primer set needed to be designed to get results for this region. The second primer set used for this purpose is shown in Table 38. Unfortunately, the sequencing results with this primer set revealed that two sequences were present and therefore couldn't be analysed. The samples W7BIS and MPF57 did not basecall well with BCMD02 as again, there were two sequences present in the DNA. Therefore, results for theses two samples are incomplete. However, as far as the results with BCMD01 could be analysed, these two samples show the same base changes as 02LAR and 04KAL. In addition, W7BIS has a deletion of three consecutive bases compared with the Md5 Meq gene.

Table 38. Sequences of additional Meq gene-specific primers used for sequencing.

Primers	Sequences	Location of primers in Md5 Meq	Expected size of amplified fragment
BCMD07	5'-TGAACCTCCCATTTGCACTC -3'	536-555	126 bp
BCH315A	5'-AGCTGGGCGCAAAGTTCCTC -3'	642-661	

Discussion

The sequencing results revealed changes when compared to the Md5 Meq gene. As three consecutive bases encode one amino acid, a change of only one base in the sequence will change the amino acid that will be synthesized and consequently, the whole protein will change which finally may result in a change in virulence of the virus. Therefore, the aim of the first objective was achieved in that there are sequence variations in Australian strains of MDV1. However, further work needs to be done in order to be able to confirm results for parts of 02LAR and 04KAL. As well, the missing parts of the sequences of MPF57 and W7BIS have yet to be identified. The results mentioned above therefore should be regarded as preliminary results. The difficulties in getting two sequences in the DNA samples might be due to a second region within the long repeats of the MDV1 genome that encodes the Meq protein which may have a slightly different sequence. Tulman et al. (2000) reported the existence of a second region that synthesizes the Meq protein for the Md5 strain of MDV1. Therefore, it would be possible that the Australian stains do have this second region as well thus having two sequences present in the DNA samples, but more information is needed to confirm this presumption.

Considering the second and third objectives, besides the base changes, the Meq gene sequences of the four investigated Australian isolates of MDV1 share many equal bases with the Meq gene of the American strain Md5 which was classified as 'very virulent' according to the suggested classification system for MDV1 by Witter (1997). Therefore, the correlation between the base changes to the virulence of a specific strain could be considered a possibility. Consequently, the sequencing of the Meq gene of a specific strain of MDV1 could be helpful for differentiation of pathotypes. However, the current classification system as suggested by Witter (1997) is widely acknowledged and to be able to identify pathotypes using mutations in the Meq gene, further research, especially *in vivo*, needs to be done to establish a similar system. This work is ongoing at UNE. Even if no definitive relationship between Meq gene sequence and pathogenicity, the work will be useful for DNA fingerprinting of various MDV isolates.

General Discussion

Virus isolation and propagation in cell culture

Isolation of MDV from infective field material proved to be far more difficult than initially anticipated and the difficulty in growing new isolates to high titre on CEF delayed the early progress of the project considerably. These difficulties were partially overcome by a change to culture on CK cells and by amplification of infective material in chickens at UNE prior to isolation in cell culture. These changes allowed one pathotyping experiment with defined challenge doses of 500pfu/chicken to occur (Experiment 4).

The use of CK cells is recommended by some MD researchers in the USA (Schat 2005) while others, such as Dr Richard Witter's group at the ADOL, USDA use duck embryo fibroblasts which are impossible to access in SPF form in Australia. Both are recommended by the American Association of Avian Pathologists (Schat and Purchase 1998). Although earlier work in Australia (De Laney *et al.* 1995; De Laney *et al.* 1998) suggested little difference in MDV growth in CEF and CK, the results of this project and other related projects (eg. Poultry CRC 03-17) have demonstrated that most MDVs grow to approximately one log higher in CK than CEF after 4-6 passages. The change from CEF to CK greatly increased the costs and complexity of the cell culture work at RMIT. CEF are obtained from readily available SPF embryos and can be frozen for generation of secondary cultures whereas CK cells require hatching and SPF rearing to approx day 10 of age before extraction of kidneys for primary culture. CK cells do not freeze and secondary culture is not possible. Access to CK cells during the project was compromised by a national shortage of SPF chickens in 2004.

The use of back-passage in chickens during the virus bulking up stage was also recommended by both Drs Schat and Witter at a meeting with these researchers in Oxford in July 2004. This was something that we were already implementing with some success by this stage. In the present project only isolates FT158 and MPF132/5 grew to high titre ($>10^4$ pfu/ml) from primary infective material from the field. All other isolates required initial passage through chickens to reach high titre, and all isolates, required back-passage in chickens to create new batches of high titre material. A major disappointment in the project was the difficulty experienced in producing new batches of infective virus, even from isolates that had previously grown to high titre on CK cells. During the 3.5-year life of the project, no virus (including MPF57) was able to be re-grown to high titre in cell culture following initial growth to high titre. This necessitated a major shift in the deployment of project resources away from formal pathotyping of MD isolates, towards solving problems with the isolation and growth of MDV in cell culture.

During this project, virus was amplified both in MDV mab-negative SPF chickens over long periods, and in off-sex commercial layer cockerels that were MDV mab positive over much shorter periods (typically 10 days). The success in isolation after passage in chickens was much higher for the former than the latter. This may be due to the effects of maternal antibody, because the longer period allows secondary infection between chickens and maximal viral titres amplification or that the larger spleen size in the older birds enables much greater recoveries of splenocytes than those from very young birds. In terms of viral replication we have shown that MDV load in PBL (Islam *et al.* 2006b) spleen cells (Islam 2006) or feather dander (Islam 2006) increases sharply to days 21-28pc, but does not increase greatly thereafter. If trying to amplify virus rapidly without detection of tumours perhaps day 28 would be optimal, with days 49-56 preferred if preliminary screening for oncogenicity is required. However the results of Experiments 4 and 5 strongly suggest that determination of bursal and/or thymic atrophy at the time of organ harvest is a good predictor of subsequent tumour induction in SPF mab negative chickens.

Our results indicate that primary infective material from the field which is celluar in nature (splenocytes, PBL, tumour material) may be inoculated directly (or after cryopreservation) on to cell culture and later amplified in chickens, or immediately cryopreserved for later amplification in chickens. The latter is probably more sensitive although this was not tested directly during the project.

A disadvantage would be the potential transmission of infective agents which would not survive in cell culture or could be readily eliminated in cell culture eg. CAV.

The project made significant progress in the area of isolation of MDV from field dust samples. MDV is naturally transmitted by the respiratory route (Carrozza *et al.* 1973) and a major reservoir of infection is infective feather dander, an important component of poultry dust. MDV is stable and survives for long periods in this form at room temperature (Blake *et al.* 2005; Carrozza *et al.* 1972) so submission of samples from the field to the laboratory and subsequent handling in the laboratory are considerably easier than with perishable soft tissues for which cell integrity is essential for MDV infectivity and which generally require cell dispersion/separation/counting prior to inoculation. In experiments 2, 3 and 5 intra-tracheal inoculation with 2-10mg of dust was successful in inducing infections in a high proportion of chickens, provided the dust contained a high MDV load ($5x10^5$ VCN/mg/dust or greater). In these experiments, no other avian pathogens were transmitted with the dust which is encouraging. Dust infection could probably also be used to screen out HVT in samples containing both MDV and HVT although this was not demonstrated in this project. We have shown that HVT is shed in significant quantities from vaccinated chickens (Islam *et al.* 2005a) but in lateral spread in young chickens does not appear to be significant (Cho and Kenzy 1975; Tink *et al.* 2005) suggesting that the shed virus is not infective.

We have previously shown that simply dispersing a large amount of infective dust (12-15g) in an isolator produces infection rates indistinguishable from intra-abdominal inoculation (Islam *et al.* 2001a) so in experiment 5 we attempted a modification of this method, by infecting chickens in a specially designed box, prior to transfer to isolators. Approximately 650mg of dust was used for these infections but they were unsuccessful in 3/5 cases indicating that larger amounts of dust and/or dust with a higher MDV content should be used. Intra-tracheal infection is unsuitable for infecting large numbers of chickens as it is time consuming and control of cross-infection is difficult when many different isolates are being used. Challenge at day-old is limited to 2-5mg of dust and is technically more difficult to perform. If challenge is delayed until around day 10, double the amount of dust can be administered and the method is much easier. Methods for dust infection other than individual chicken administration are preferred if the aim is simply to induce infections for amplification of virus.

Overall the project has shown that growing MDV to high titre in cell culture with the methods used, has a very low success rate, even for isolates that have previously grown to high titre. The difficulty in growing MDV in cell culture has been noted by other Australian researchers (De Laney *et al.* 1995; McKimm-Breschkin *et al.* 1990). On the other hand, chickens are readily infected with cell culture material or infective cells from chickens and, if viral load is sufficiently high, from infective poultry dust. Given the costs associated with isolation and growth in cell culture and the low success rate of this approach, consideration should be given to basing pathotyping work in the future on material that has been grown and titrated in SPF chickens rather than cell culture. This is likely to result in virtually any field isolate being able to be included in a pathotyping experiment, rather than the current situation where a very small subset of isolates can be included because of their tissue culture growth characteristics, or the success or failure of a given series of cell-culture runs. Dose rates based on chicken-infective doses are also likely to be more informative about actual infective dose rates than the current calculation of pfu on the basis of tissue-culture infective doses. The development of molecular methods for the differentiation and absolute quantification of MDV in chicken cells of many types (Baigent *et al.* 2005; Islam *et al.* 2006a; Islam *et al.* 2004; Renz *et al.* 2006) greatly facilitates this approach. Cell culture methods may have a role in removing contaminating organisms or allowing plaque picking to ensure strain purity but once clean virus is available it should be amplified and titrated in chickens rather than cell culture.

Early paralysis/mortality syndrome

An important finding of the project was the repeated induction of an early paralysis/mortality syndrome between days 9 and 20 after MDV challenge, usually with a peak in the first half of this period. This has not previously been reported in Australia. Affected chickens exhibited depression,

ataxia, altered head and wing carriage (including torticollis), progressing to marked paresis/paralysis, ventral recumbency, coma and death over 2-3 days (Plate 6).

Plate 6. Photographs of chickens exhibiting early paralysis syndrome induced by MDV, both in the isolator and following removal from it.

Diarrhoea was a feature in some cases. On post mortem examination, marked thymic and bursal atrophy were the most consistent findings. In some cases no trace of the thymus can be found. Most chickens were euthanized on ethical grounds prior to terminal coma or death but in small numbers in which intervention was delayed, death did occur. Therefore the syndrome appears not to be the well documented transient paresis/paralysis syndrome induced by MDV (Kornegay *et al.* 1983; Swayne *et al.* 1989) but rather the more severe acute paralysis syndrome associated with challenge with highly virulent MDV first detailed by (Witter *et al.* 1999). In our experiments the syndrome was observed only in the most pathogenic strains, and had a significantly higher prevalence in males than females. As challenge dose was uncontrolled in Experiment 5 it is unclear whether the massive mortality during this period (30-60%) induced by isolates 189/8, 199/3&9 and 192/1,4&10 was due to the pathogenicity of the viruses or very high challenge doses. It cannot be ruled out that these isolates have very high virulence since the back-passaged variants of the early challenge viruses, induced no greater early mortality in this experiment than in Experiment 4 when challenge doses were fixed at 500pfu/chicken. It is hoped that these apparently extremely virulent isolates grow well in cell culture and become available for formal pathotyping in Poultry CRC project 03-17. Samples for histological examination of 10 key organs were collected from cases of early mortality syndrome in experiment 4 but a funding request to RIRDC to examine these was declined.

Gross MD pathology

MDV isolates did not appear to have a major influence on the tissue trophism of MD lymphomas. The gonads, particularly the ovary, were the organs with the highest incidence of gross MD lymphomas, followed by the liver, spleen and kidney. Lymphomas are far more difficult to detect in testes than the ovary since the background colour of the testes is identical to that of lymphoma tissue (Plate 7), and bilateral enlargement of the testes due to lymphoma or lymphocyte infiltration is unlikely to scored as MD unless it is extreme. This may explain in part the much-observed predilection of the disease for females. However when other indices of MD such as immunosuppression or MDV load are examined, there is not the same consistent difference between sexes in favour of females.

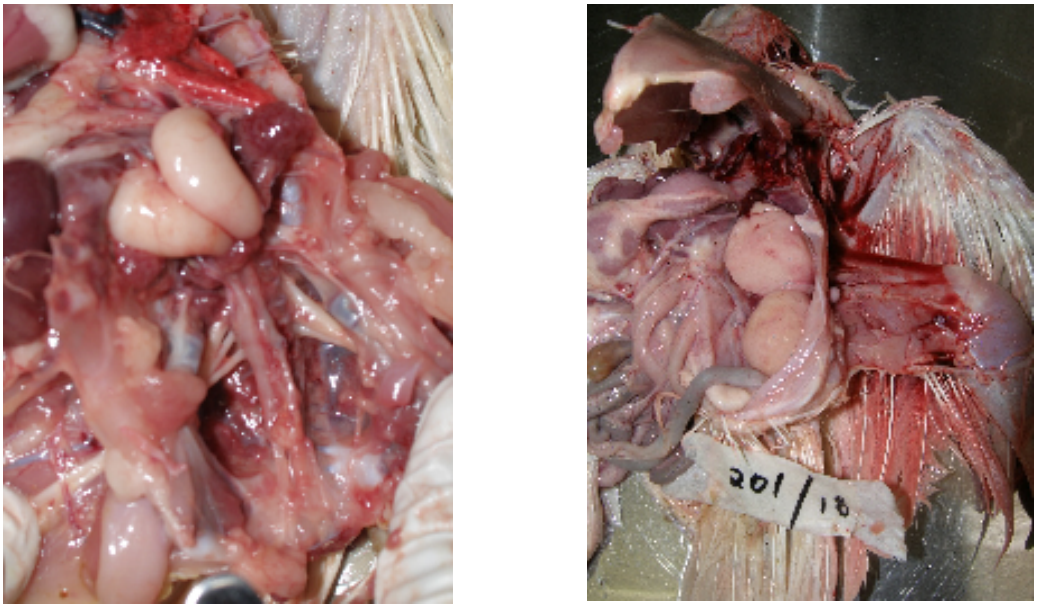

Plate 7. Chickens from experiment 5 showing lymphomas of the testis (left) and ovary and kidney (right).

Pathotyping methodology

MDV pathotyping experiments were effectively conducted in the UNE isolator facility with control of cross-infection demonstrated in each experiment. Using the presence or absence of gross MD lesions as the diagnostic criterion for MD resulted in clear differentiation between isolates, in both unvaccinated and HVT-vaccinated chickens. However it was clear that MDV was inducing early mortality prior to the onset of gross lesions, and that it was also inducing severe immunosuppression in some chickens in the absence of gross lesions. The extent of immunosuppression, as determined by thymic and bursal atrophy, was much greater than that observed in experiments in chickens with mab against MDV (Islam *et al.* 2002; Witter 1997) and more consistent with levels reported in mab-negative chickens (Calnek *et al.* 1998; De Laney *et al.* 1998). Inclusion of immunosuppressed and early mortality syndrome chickens in the calculation of MD incidence and PI takes into account these effects of MDV and increases the number of chickens included in the calculations, thus increasing the power to detect statistical differences between isolates. However, taking the example of Experiment 4, the inclusion of these additional chickens did not greatly influence the ranking of different isolates (Table 39). This situation may not hold in treatments with extreme early mortality without gross MD lesions, as seen in Experiment 5. Exclusion of these chickens from the calculation of MD incidence will definitely skew the estimation as these chickens, the most susceptible birds to MD, are excluded from the population at risk of gross MD lesions. This can be seen by comparing the viral rankings in Tables 25 and 26.

The pathotyping experiments provide strong support for the possibility of shorter pathotyping experiments in SPF chickens, using measures within 14 days of challenge to predict pathotype. In

experiment 4, day 13pc immune organ weights had strong predictive relationships with subsequent MD incidence, PI and virulence rank. They were also well correlated with day 56pc immune organ weights demonstrating that the immunosuppressive effects observed at day 13pc are permanent and likely to be good indicators of MD at all ages between. This was confirmed in Experiment 5 with organ weight data from day 48pc. In experiment 4, MDV load in spleen was also well correlated with virulence rank suggesting that it could have a role in early prediction of virulence. In experiment 5, subjective bursal and thymic atrophy scores were used for the first time (Plate 8) and on a group basis were shown to have equal predictive power as actual organ relative weights. Furthermore, either bursal or thymic scores alone were sufficient – combinations of the scores from both organs did not increase relationships with MD significantly.

Table 39. Incidence of MD and Protective Index in Experiment 4 calculated on the basis of presence of gross MD lesions only, or on the basis of gross lesions and/or early mortality and/or marked atrophy of the thymus or bursa.

Challenge virus	Gross lesions only (n=281)[1]		All indicators of MD (n=436)[2]	
	MD (%)	PI (%)	MD (%)	PI (%)
MPF57	38.1	100.0	29.7	100
MPF132/5	42.86	82.6	33.8	78.9
04CRE	39.4	52.8	52.6	61.0
FT158	66.7	41.2	65.6	62.2
Woodlands	63.6	40.9	64.2	57.9
02LAR	73.8	38.2	67.6	48.7

[1]Chickens with gross lesions only. 281 Eligible chickens from d34pc onwards
[2]Chickens with gross lesions and/or dying between days 10 and 20pc without a clear other cause of mortality, and/or having both thymic and bursal atrophy as defined as relative thymic and thymic weights lower than the mean minus 2 SDs of the value for the sham-challenged group. 436 eligible chickens from d10pc.

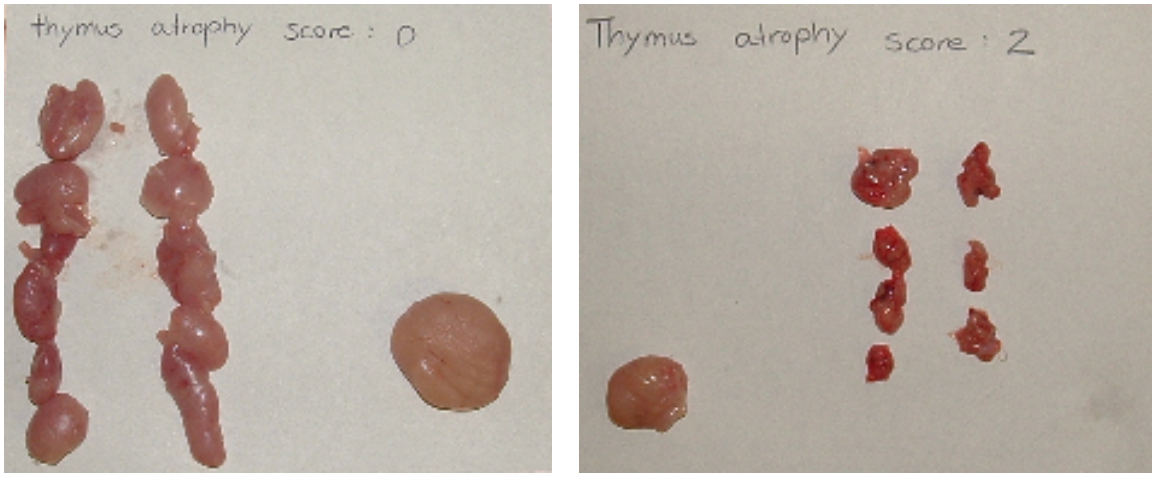

Plate 8. Example of thymus and bursa from chickens with thymic atrophy scores 0 (normal) and 2 at day 56pc. Scores are from 0-3 in ascending order of severity of atrophy. The association between thymic and bursal atrophy is also evident in these two samples.

These data suggest that short pathotyping experiments in SPF could be conducted over a period of 2 weeks following challenge. Measurements of MDV load in spleen and immune organ weights or atrophy scores should provide clear demarcation of MD viruses of different virulence. Such experiments may be particularly useful as screening experiments, particularly where ethical considerations do not permit death as an end point in experimentation. Work on Poultry CRC project 03-17 indicates that this is unlikely to hold for commercial chickens in which mab appears to provide good protection against the early immunosuppressive effects of MDV.

The relationship between the incidence of MD induced in unvaccinated chickens, and that induced in HVT-vaccinated chickens was poor (Figure 6) demonstrating that vaccinated treatments need to be included in pathotyping experiments. The reference strain, MPF57 stood out in this respect, being highly virulent in unvaccinated birds, inducing lesions in 84.2% of unvaccinated chickens but none in HVT-vaccinated chickens. Unvaccinated chickens were also completely protected against challenge with MPF57 using the wider criteria used in Table 31. This suggests that pure virulence and "vaccine resistance" may be different traits with the ADOL pathotype classifications skewed towards the latter.

Differences in virulence amongst Australian MDV isolates

The series of pathotyping experiments clearly demonstrated that Australian isolates of MDV show wide variation in their ability to induce MD in mab-negative SPF chickens, both unvaccinated and HVT-vaccinated. Experiment 4 was the only experiment in which challenge dose was standardized (500pfu/chicken) and in this experiment the incidence of MD lesions ranged from 53-94% of eligible unvaccinated chickens and 0-58% of eligible HVT vaccinated chickens. Using the ADOL virulence ranking system of Witter (1997) these viruses had PI ranging from 38.2% for 02LAR to 100% for MPF57 and conversely virulence ranks ranging from 0 to 61.8%. As discussed in Experiment 4, there was no evidence of systematic evolution in MDV virulence over the last decade or so, although only 6 viruses were tested. Of the two older isolates tested, one was in the low virulence grouping (MPF57) while the other was in the high virulence grouping (Woodlands 1). Similarly amongst the 4 recent isolates testes, one was in a low virulence grouping (MPF132/5), one was of intermediate virulence (04CRE) and two were in the high virulence group (FT158 and 02LAR). Interestingly the virus with the highest virulence rank (02LAR) was isolated from dust collected from an unvaccinated broiler farm with a history of poor performance, rather than from a flock with a defined MD problem or vaccine break.

As discussed in the Discussion section of Experiment 4, differences in pathotyping methods make direct comparisons between pathotyping experiments difficult. Some important differences between our experiment 4 and two other key pathotyping experiments are summarized in Table 40.

Table 40. Comparison of Experiment 4 with two other key pathotyping studies.

Variable	Experiment 4 – Present project	Witter (1997) USDA ADOL method.	McKimm-Breschkin et al., (1990) Australia
Chicken type	SPF, mab-negative, MD resistant	Line 151/7 mab-positive, MD susceptible	Various, mab negative
MDV challenge dose	500pfu	500pfu	Uncontrolled. 0.1ml blood or lymphocytes
HVT vaccine	Cell-associated	Cell-associated	Cell-free and cell-associated
HVT dose	8000pfu	2000pfu	250-1000 ffu
Vaccination age (d)	0	0	0
Challenge age (d)	5	5	5
MD case definition	Gross MD lesions.	Gross MD and/or bursal or thymic atrophy	Gross and histological tumours.
Virulence classification	Comparative approach. ADOL classifications inferred indirectly.	Based on comparison with "prototype" MDVs	Comparative approach. ADOL classifications inferred indirectly.

Nevertheless the viruses in this experiment fall into two broad categories viz:

- Lower virulence (~ v ranking in USDA system). MPF57 and MPF132/5. These viruses typically cause relatively little early mortality, have mild suppressive effects on thymus and bursa at d13pc and while there is a high incidence of MD tumours in sham-vaccinated birds (72-84%) HVT-vaccination provides good protection against all of these effects.

- Higher virulence (~ vv ranking in USDA system). 02Lar, FT158, Woodlands1, O4Cre. These viruses cause early mortality syndrome with some neural signs and have marked suppressive effects on thymus and bursa at day 13pc. However HVT provides good protection against these effects. On the other hand, protection against gross MD tumours by day 56pc is poor, in the range 38-53%.

It is difficult to directly compare the results of the present experiment directly with those of the most comprehensive work of this kind in Australia previously, that of McKimm-Breschkin et al. (1990) due to differences in methodology. Nevertheless it is clear that some of the more virulent viruses in that study (MPF23, MPF15) produced results comparable with the more virulent viruses in the present experiment. Vaccination with HVT (250-1000ffu, probably cell-free) provided no protection at all against MD gross lesions when challenged with MPF23 the most virulent virus in the that study. MPF23 could possibly be in the same virulence category as 02LAR although the differences in vaccine type and dose between experiments make this speculative. We have recently amplified MPF23 in chickens again, and if it grows in cell culture it will be available for use in pathotyping experiment under Poultry CRC project 03-17. This will provide a direct comparison of viruses isolated over a 20-year span and this will be a better indication of whether there has been a systematic increase in virulence over time.

Interestingly, in Experiment 5, in which challenge doses were not fixed and a range of infective material was injected into unvaccinated SPF chickens, 4 new isolates induced mortality and MD lesions comparable or greater than those induced by the hitherto most virulent isolates in these chickens, 02LAR, FT158, Woodlands 1 and MPF57. It will be interesting to see how these isolates perform in fixed challenge studies with the inclusion of vaccination treatments.

While no clear increase in MDV pathogenicity can be demonstrated from the limited pathotyping data available, it is clear that there are MD viruses circulating in Australia which are capable of inducing significant MD despite effective HVT-vaccination. In the field, with variable time and level of challenge, these viruses may induce frank MD, or more probably be associated with sub-clinical MD, immunosuppression, elevated intercurrent disease and poor performance. The generally good cleanout and biosecurity arrangements in Australia, together with limited litter re-use would assist with the latter expression. However should this situation change, or vaccination failure occur on individual farms, these strains of MDV have the capacity to induce severe MD.

Meq gene sequencing of MDV isolates

Initial work in this area has identified sequence variation that is likely to be useful as a marker for given MDV isolates. Relationships with pathogenicity and the identification of markers for pathogenicity cannot be established at this stage, but Ms Renz is likely to continue with this work as part of her PhD studies and more detailed and useful results should emerge from her work. The sequencing work is complicated by the presence of two copies of the Meq sequence in the MDV genome, with possible heterogeneity between the two copies. This is a finding not acknowledged by all other workers in the area.

Conclusions and recommendations

1. Cell-culture methods for the isolation of MDV1 remain laborious, expensive and provide a low yield of usable isolates for use in formal challenge experiments. Failure to grow in cell culture is an artificial barrier to pathotyping current MDV strains.
2. It is therefore recommended that support be given to optimising methods of MDV isolation, purification, amplification and titration in SPF chickens as an alternative. The recent development of molecular methods for the differentiation and absolute quantification of different MDV serotypes in chicken cells of many types greatly facilitates this approach.
3. Australian MDV1 isolates vary widely in the extent to which they induce MD in unvaccinated SPF chickens, indicating a wide range in virulence.

4. MDV virulence in such chickens can be readily gauged as early as 13 or 14 days post-challenge in unvaccinated SPF chickens by the extent of thymic or bursal atrophy. Additional measures such as splenic enlargement or MDV1 load in spleen or feather dander are also good correlates of virulence but add little over and above that provided by immune organ atrophy. Prospects are good for very short screening experiments for pathogenicity.
5. The most virulent strains of Australian MDV induce significant early paralysis and mortality between days 9 and 20 after challenge in mab negative, unvaccinated chickens. Male chickens are significantly more predisposed to this form of MD than females.
6. Virulence in unvaccinated chickens is not strongly related to the ability to induce disease in HVT-vaccinated chickens. Thus pure virulence and "vaccine resistance" appear to different traits.
7. The available data do not allow determination of whether there has been an increase in the virulence of MDV in recent years in Australia. Indeed there is no consistent trend in this direction in the data. However it should be noted that older isolates tend to be maintained or tested due to their virulence so they do not represent a random sample of the situation at the time.
8. Nevertheless there are several isolates that fall clearly into the vvMDV category. The project did not allow additional determination of the vv+MDV category, as vaccination treatments with HVT/MDV2 were not included.
9. There is significant polymorphism in the sequence of the MDV Meq gene, a key gene involved in the ability of MDV to induce lymphoid tumours. This may eventually be linked to virulence or be used as a genetic marker for a given isolate.
10. Given the wide variation in virulence observed during the project, and the failure of HVT to provide adequate protection against several isolates in Experiment 4, it would appear that the phenomenon of evolution of virulence is occurring in Australia, at least to some extent. Given the widespread use of HVT-vaccination in the Australian broiler industry, resistance to the effects of vaccination is likely to confer significant advantage to resistant strains and result in selection pressure in their favour.
11. Given 2. and 10. above it is recommended that methods for low cost routine monitoring of MDV virulence be investigated and implemented by the Australian Poultry Industry. Such a scheme might include:

- Screening of isolates for pathogenicity in unvaccinated SPF chickens. This could also serve as a viral amplification step and test for freedom from contaminants.
- Amplification and titration of infective material from high virulence isolates in SPF chickens.
- Formal pathotyping of the most virulent isolates in experiments using commercial chickens and current vaccination protocols.
- At some stage importation of USA reference strains or BACs derived from such strains should be considered to allow a direct comparison of US and Australian isolates.

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Plain English Compendium Summary

Project Title:	Systematic pathotyping of Australian Marek's disease virus (MDV) isolates
RIRDC Project No.:	UNE-83J
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Objectives	• To evaluate current Australian strains of MDV for their ability to induce disease and overcome the effects of vaccination. • To do this in a way that facilitates comparisons with similar studies overseas.
Background	In the USA there is clear evidence of evolution of MDV towards greater virulence (ability to induce disease) over time, possibly as a response to vaccination. A feature of this increased virulence is the successive failure of different vaccines against MDV to provide effective protection against the disease. Australia suffered a serious outbreak of MDV from 1991-1996 and part of the response to this has been to introduce routine *in-ovo* vaccination of broiler chickens with potential to facilitate such an evolution in virulence. Previous Australian studies had shown that there were highly virulent strains of MDV in the country.
Research	533 field samples were submitted for isolation of MDV between 2002 and 2005 with 655 isolations on cell culture attempted. Unfortunately isolation rates were low and only 6 isolates (4 new and 2 old) grew sufficiently for inclusion in formal pathotyping experiments while a further 11 grew sufficiently to infect chickens in screening experiments. These isolates showed a wide range of virulence in SPF (specific pathogen free) chickens with the more virulent strains inducing pathology typical of very virulent isolates overseas. A new early mortality syndrome induced by MDV was observed and detailed for the first time in Australia. There was no clear evidence of a trend towards increased virulence over the last decade although the number of isolates tested was relatively small. In unvaccinated SPF chickens, virulence was well predicted by a range of measurements on chickens as soon as 2 weeks after challenge.
Outcomes	• Confirmation of presence of very virulent MDV in Australia • No conformation of evolution in virulence over time. • Isolation of MDV in cell culture is proved impractical and isolation directly in chickens should be pursued instead. • New methods for screening for virulence in unvaccinated SPF chickens were developed. • Demonstration that virulence in unvaccinated chickens is not necessarily related to ability of MDV to overcome the effects of vaccination.
Implications	Improved understanding of current status of Australian MDV. HVT, the main vaccine used in the broiler industry, induces low levels of protection against several isolates of MDV. This suggests that some evolution in virulence is occurring in Australia and that ongoing monitoring for MDV virulence and vaccine reistance is worthwhile. The project has provided good data on how such ongoing monitoring might be conducted.
Publications	Hussain Z, Islam AMFM, Burgess SK, Reynolds PS, Walkden-Brown SW (2005) Isolation of Marek's disease virus from dust samples from commercial chicken farms. *Proc. Aust. Poult. Sci. Symp.* **17**, 100-104. More will follow.