AUSTRALIAN POULTRY CRC

FINAL REPORT

Program 2A

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Executive Summary

This project aimed to demonstrate the usefulness of molecular technologies such as quantitative real-time PCR for improving our understanding and control of important endemic diseases of poultry. The project used Marek's disease as a case in point.

One strand of the project aimed to isolate field strains of Marek's disease virus (MDV), adapt them to cell culture and eliminate contaminants. Significant improvements in methodology implemented in 2004 produced good results and a set of 5 challenge MDV strains for use in pathotyping experiments. However overall there was a low overall rate of recovery of MDV in cell culture from the 755 submissions of infective material (25%) and an even lower proportion of isolates that grew to a titre of 100pfu/ml or greater (4.4%). Only 6 isolates (<1%) grew to the titre of 10^4 pfu or greater required for effective use in formal pathotyping experiments. The reasons for this are unclear and it is recommended that the causes be formally determined.

The pathotyping strand of the project thoroughly characterised 5 isolates in formal pathotyping experiments based on the USDA ADOL international model. The isolates were pathotyped in commercial broiler and layer chickens, complementing work on another project using the same viruses and protocols in SPF chickens. The results of these experiments suggest that the most pathogenic MDVs in Australia are of the very virulent (vv) classification rather than the vv+ classification commonly seen now in the USA and elsewhere. This suggests that the evolution of virulence of MDV in Australia lags behind that of the USA and some other countries although increases in virulence and vaccine failure can be expected in the future.

The measurement of MDV viral counts in spleen and dust during the pathotyping experiments proved highly informative with MDV load in spleen, and to a lesser extent, shed dander, being very highly correlated with subsequent MD incidence on a group basis. MDV load in spleen was a powerful early predictor of future MD incidence from 7 days post challenge. This confirmed work on other projects in SPF chickens and indicates that very short 7-14 day challenge experiments can be performed which will predict either the virulence of the MD strains being tested, or the efficacy of the control measures (e. vaccines) under test. Early immune organ weights were less accurate predictors, particularly in maternal antibody (mab) positive commercial chickens. Mab was also protective against the early mortality/paralysis syndrome observed with the most virulent isolates in mab negative SPF chickens.

The comparative lack of isolates for formal pathotyping experiments led to innovative experiments looking at alternative infective materials (eg infective dust, splenocytes and blood) and methods of accurately determining the infectivity of such materials, so that doses could be accurately determined. Dose response experiments with dust and infective cell culture material demonstrated that a bird infective dose 50 (BID_{50}) can be calculated from the proportion of infected chickens at different doses using the presence of MDV in spleen at days 10-12 after challenge as the end point (detected by qPCR). This work opens the way for methods of isolating, testing for contamination, bulking, titrating for infectivity and use in fixed dose challenge experiments of MDV strains without recourse to cell culture. It is recommended that such methods be optimised and formalised into standard protocols and that some 20 current isolates of MDV be screened for pathogenicity using the short pathotyping experiment model. Ideally international reference isolates would be included in the test.

The final strand of the project was on epidemiology. A major field survey of broiler 80 farms across Australia investigated levels of MDV in shed dust and in chicken spleens, and CAV in serum, at various ages. MDV was shown to be far from ubiquitous, being detectable in only 50% of farms, with a strong seasonal prevalence. A range of important risk factors and protective factors for the presence of MDV was identified. Interestingly, using wood shavings as litter was associated with significantly reduced MDV counts, a finding warranting further investigation. There was a very high incidence of CAV positive flocks, but surprisingly many flocks were not 100% positive. This, with the small sample size (5 chickens/shed) precluded the drawing of strong conclusions as to the risk factors for CAV. Other work in the epidemiology strand showed that MDV infected dander remains infective for chickens for up to 2 years, at 6 storage temperatures ranging from -80˚C to 37˚C, with a lower level of infectivity at warmer temperatures. This emphasises the importance of good cleanout in managing MDV. A third aspect of the epidemiology work involved optimisation of a sophisticated epidemiological model of MD within a shed of chickens. The model has not been adopted by industry but has been a valuable research tool.

Contents

Introduction

This project is essentially about demonstrating the capacity of new molecular tools, principally quantitative real-time PCR for improving our understanding and control of poultry diseases, using Marek's disease as a case study. The original proposal extended to many other diseases and involved collaboration with CSIRO and UMelb, but those aspects of the project were removed and mainly run as separate projects. However the broad original project title remained unchanged.

The rationale for the project is built upon the following set of facts, principles and ideas.

1. Modern intensive poultry production is totally dependent upon adequate control of infectious disease. Disease control and loss of production induced by disease constitute major costs for the industry.
2. Disease diagnostics based upon the detection of specific sequences of DNA or RNA in pathogens offer significant potential advantages over other diagnostic methods, particularly for viruses;
    - Easier collection and storage of diagnostic samples than those requiring isolation, culture and identification of the pathogen.
    - Ease and accuracy of differentiation between different strains, serotypes or even pathotypes of the organism
    - Speed and cost. Molecular methods are much more rapid and potentially much cheaper than culture and pathology-based methods. In some cases a combination of methods is required.
    - The ability to create multiplex tests that test for a number of pathogens or strains of a pathogen simultaneously.
    - With quantitative PCR, ease of quantification of pathogen load in tissues or environmental samples.
3. Despite widespread use in human and veterinary medicine, and in plant pathology, the use of molecular methods in the routine diagnosis and monitoring of disease in Australian poultry remains limited;
    - The range of available diagnostic tests available is limited.
    - Some laboratories do not have the required equipment and skills.
    - Some of the new tests require further testing to enable accurate interpretation of the results.
    - In some cases there has been insufficient collaboration/interaction between those developing tests and potential end users of the tests.
4. The level of control achieved for the major endemic diseases of poultry in Australia varies widely and is currently sub-optimal for a number of major endemic diseases including Marek's disease and Infectious Bronchitis in some instances.
5. At least part of the breakdown in control seen in these instances is due to pathogen evolution and change in the face of widespread use of vaccination. In some cases pathogen-host-vaccine interactions lead to major disease outbreaks such as the MD outbreak between 1992-97. To prevent such catastrophic breakdowns in control there is a need for ongoing monitoring of pathogens in current host systems (bird strains and vaccination regimens).
    - This requires systematic collection of pathogens from the field and taking them through to the point where they can be used effectively in challenge models. This includes guaranteeing freedom from contaminating pathogens.
    - Virulence testing and vaccine protection experiments using animal challenge experiments is a slow and expensive activity. This project includes work of this nature, but also work proposing a molecular alternative to such experiments.
    - More widespread routine disease monitoring systems in industry based on cheaper and more rapid molecular tests offers significant potential to track changes in pathogen virulence.

6. Cheaper, more accurate tests for disease diagnosis, particularly those that enable quantification of the infecting organism offer the prospect of greatly improved understanding of disease dynamics in the host (pathogenesis) or in host populations (epidemiology). Because the tests are cheaper and in many cases more informative than existing tests, the volume of information available on disease behaviour both in experiments and in the field will increase dramatically. The potential then exists to begin modelling this formally, to initiate the development of predictive models of disease behaviour for incorporation into DSS (Decision Support Systems). This proposal aims to initiate such work for Marek's disease.

In summary the development and effective application of specific, real-time fully quantitative PCR methods offers a considerable opportunity to the Australian poultry industry.

- Rapid diagnosis during disease outbreaks.
- Differentiation of vaccinal and wild strains in the same sample, useful in identifying breakdown of vaccinal protection.
- Quantitative PCR could revolutionise routine monitoring of pathogen levels in birds or the environment for diseases such as MD by tracking pathogen number as well as presence.
- Quantitative PCR is also a very powerful tool for examining the epidemiology of disease within and between flocks. Quantitation of virus previously was a prohibitive cost in such studies.
- Molecular markers for pathogenicity may reduce the requirement for pathotyping in birds.
- Molecular methods are also useful for tracking phylogenetic relationships between different isolates of a pathogen.

Full interpretation of the outcomes of this project, and capitalisation upon them is best achieved if they are considered in the light of the outcomes of several linked projects that ran concurrently with this project, at least for part of its life. These projects and the areas of linkage with the present project include:

- ARC Linkage Project 0211607 (2002-2004) Molecular approaches to solving current and emerging problems in the epidemiology and diagnosis of Marek's disease in Australia. *Australian Research Council (ARC) - Linkage* (Walkden-Brown SW, Groves PJ, Young PL, Islam AF, Cheetham BF, Underwood GJ)
- Development of the molecular tools underpinning the present project (Islam *et al.* 2006a; Islam *et al.* 2004; Renz *et al.* 2006)
- Development of animal research methodologies underpinning the present project (Islam and Walkden-Brown 2007; Islam *et al.* 2007a; Islam *et al.* 2007b; Islam *et al.* 2006b)

RIRDC/AECL Project UNE-83J (2002-2005). Systematic pathotyping of Australian Marek's disease virus isolates. *Rural Industries Research and Development Corporation* Walkden-Brown SW, Groves PJ, Tannock GA, Cheetham BF, Islam AF

- Isolation of new MDV strains and improvement of MDV isolation in cell culture
- Pathotyping of MDV in SPF chickens (Walkden-Brown *et al.* 2007)

RIRDC Project RMI-12J (2002-2005). Molecular evaluation of responses to vaccination and challenge by Marek's disease viruses. *Rural Industries Research and Development Corporation* Tannock GA, Tan J, Cooke J

- Improvement of MDV isolation in cell culture (Tan *et al.* 2007)

Objectives

The original broad objectives of the project were:

1. To assist with the systematic collection of current field strains of MDV and CAV, clear the isolates of contaminating organisms, adapt to cell culture if necessary, and characterise them at the molecular, cell culture and *in vivo* level (RMIT).
2. To characterise current field strains *in vivo* in pathotyping experiments in commercial chickens and to seek alternatives to full pathotyping experiments, based on molecular measurements during early infection (UNE).
3. To conduct epidemiological studies using the new molecular methods and initiate formal modelling of the epidemiology of key diseases such as MD (Zootechny/UNE).
4. To facilitate interaction and exchange of ideas and methods between virologists, molecular biologists, epidemiologists and veterinarians in the common pursuit of improved disease monitoring and control in the Australian poultry industry.

Following an External Review of the project in August 2005 a revised set of objectives and milestones was agreed upon and these are summarised below. All *in vivo* work and CAV work at RMIT was removed from the project with UNE taking on the *in vivo* work with MD originally slated to happen at RMIT. However, due to the major effort on cell culture of MDV occurring at RMIT there was no alteration to the budget or its distribution between institutions. Two pathotyping experiments at UNE were removed for lack of new MDV isolates to test, and instead a new KPI for the project introduced at this stage relating to the determination of infectious dose 50 for infective dust samples.

OUTCOME 6: Improved diagnostics and disease surveillance through the application of molecular technology.

Output 6.1: **Isolation, adaptation, testing for contaminants and maintenance of stocks of Marek's disease virus and *in vivo* characterisation.**

Milestone 6.1.1a: Isolation of field strains of MDV, adaptation to cell culture and elimination of contaminants (RMIT).

Key Performance Indicators:

1. Regular submissions of field samples to RMIT. Minimum of 20 field submissions per annum. (Dec 31 2004/05/06). KPIs have been met to date.
2. Development and validation of improved MDV isolation methods based on CK cells rather than CEF (Nov 04). Met.
3. Four new MDV isolates clean and bulked for challenge experiments. (Nov 04) Met March 05.
4. Provision of source viruses for potential new vaccines as required. Available but not utilised to date.
5. Bulking up of virus for use in challenge experiments for this project as follows:
    - Back-passage, re-isolation and bulking of reference strains and selected new strains for second round of pathotyping experiments (May 06)
    - Four more new MDV isolates clean and bulked for challenge experiments (May 06)

Milestone 6.1.1b: Preliminary characterisation of MD isolates in vivo (RMIT/UNE).

Key Performance Indicators:

Verification of infectivity and freedom from contaminants of new isolates.

1. Freedom from contaminants of new isolates verified *in vivo*.
2. Infectivity of new isolates verified *in vivo*:
    - Four new MDV isolates tested *in vivo* (Nov 04) Met Mar 05
    - Four more new MDV isolates tested *in vivo* (June 06)

Note. **These KPI depend upon regular submission of field material to RMIT and successful growth of MDV in culture.**

Milestone 6.1.1c: Characterisation of recent MDV isolates in commercial strains of broiler and layer chickens (UNE).

Key Performance Indicators:

1. Pathotyping of the most virulent recent isolates in current strains of broiler and layer chickens using MPF57 as the reference strain completed:
    - Broiler pathotyping experiment 1 completed. Two new MDV isolates pathotyped in Cobb broilers (June 05). Met
    - Layer pathotyping experiment 1 completed. Two new MDV isolates pathotyped in ISA layer (June 05). Met Aug 05.
    - Broiler pathotyping experiment 2 completed. Two new MDV isolates pathotyped in one broiler strain (Sept 06).
    - Layer pathotyping experiment 2 completed. Two new MDV isolates pathotyped in one layer strain (Dec 06).
2. Assessment of protection provided by HVT and HVT-MDV2 bivalent vaccines against new isolates completed. (Dec 05, 06) Met for 05.
3. Assessment of evolution of virulence in Australian MDV isolates and implications for industry vaccination protocols. (Dec 06)

Note. **These KPI are totally dependent upon output 6.1 from RMIT**

Milestone 6.1.2a: Validation of novel measurements to assist in pathotyping MDV strains (UNE).

Key Performance Indicators:

1. Value of quantitative-PCR using real-time PCR technology to measure the relative abundance of MDV in host tissues and exhaust dust from isolators as a predictor of pathogenicity determined. (Dec 05, 06). Met for 05.
2. Development of simpler, cheaper and/or more informative measures to determine the pathogenicity of MDV.
    - Comparison of conventional (USA) and novel pathotyping measurements in broiler Expt 1 (June 05). Met.
    - Comparison of conventional and novel pathotyping measurements in layer Expt 1 (June 05). Met (Dec 2005).
    - Ongoing refinement of measurements used in subsequent pathotyping experiments. Met.

Note. **These KPI are totally dependent upon output 6.1 from RMIT**

Output 6.2: Epidemiological studies and modelling for Marek's disease and CAV infection.

Milestone 6.2.1: Field epidemiology study (Zootechny/UNE/RMIT).

Key Performance Indicators:

1. Large cross sectional survey of 80 broiler farms from all Australian companies.
    - Study design completed and agreements with participating companies and institutions in place (Nov 04). Met.
    - Study completed on farms (Jan 06). Will be met.
    - Lab analysis of samples completed. This will include MDV2 if funds permit. (June 06).
2. MDV quantified in environment (shed dust) and host (chicken spleens) at UNE (June 06).
3. CAV assessed by serology at RMIT (June 06).
4. Detailed information compiled on risk factors for, and production consequences of, MDV and CAV infections alone or in combination in the field (Sept 06).

Milestone 6.2.2a: Enabling research into MDV to provide inputs into a simulation model (UNE /RMIT/Zootechny)

Key Performance Indicators:

1. Method for titrating MDV in birds rather than cell culture developed. This will enable infectivity of poultry dust to be determined. (Nov 04) Met
2. Determination of the effect of temperature on the MDV content and infectivity of poultry dust (Dec 05). Met for storage up to 90 days
3. Assistance with development of decision support tools for MD. Provision of critical information required for modelling MDV which is not available in the literature or from past experiments. Met.
4. Subsequent KPI's will develop as the project develops and gaps in knowledge appear.
   - Determine infectious dose 50 (ID50) for infective dusts (Sept 06).

Milestone 6.2.2b: Modelling of MDV epidemiology (UNE/Zootechny/AusVet).

Key Performance Indicators:

1. A deterministic model of MD behaviour and consequences in broiler flocks developed:
   - Conceptual approach to model finalised (Oct 03) Met.
   - Literature review and development of functioning model completed (March 04). Met.
   - Ongoing model evaluation, validation and modification. (Ongoing)
2. Use of the model:
   - by researchers to identify critical links in understanding of MDV and priorities for research. Met. Very useful for this purpose to date.
   - by senior technical personnel in industry as a decision support tool to test scenarios relating to MD control. Spreadsheet version of model currently with industry people for evaluation.
   - as a teaching aid for students. Not used for this purpose to date.
   - Delivery of functional epidemiological model of a broiler flock in a shed that will predict MD outcomes (immunosuppression, performance reduction, mortality etc) based on a range of host, environment/management and pathogen factors input into the model (April 06).

Methodology

Overview of project work and locations

The original plan of conducting a significant amount of *in vivo* work at RMIT was not possible due to the closure of their isolator facility. Thus in broad terms the following work was carried out by the different project collaborators:

1. RMIT (Tannock, Cooke, Meehan)
    - All cell culture work, including isolation of MDV in cell culture
    - CAV serology for the field epidemiological study
    - Assistance with design of titration of infectivity studies
2. UNE (Walkden-Brown, Reynolds, Islam, Cheetham, Gao)
    - All project *in vivo* work in chickens including
        - Pathotyping experiments
        - Isolation of MDV in SPF birds from various tissues including dust samples
        - Preparation and storage (or submission to RMIT) of infective spleen, blood and dust samples
        - Titration of infectivity in chickens
        - Duration of infectivity of MDV under different storage conditions
    - Coordination and implementation of the field epidemiological study
    - qPCR of MDV in various tissues/materials from all in vivo experiments and the field epidemiological study
    - Epidemiological modeling (Gao)
3. Zootechny Pty Ltd (Groves)
    - Design and analysis of the field epidemiological survey
    - Assistance with *in vivo* experiments including final post mortem examination
    - Assistance with epidemiological modeling at UNE

The experimental chapters contain descriptions of methodology, particularly Chapter 1 which was a methodological study. However the general methods deployed during the project are described below.

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 lymphocyte (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 earlier RIRDC research projects] 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 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 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.

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 (Plate 1), 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.

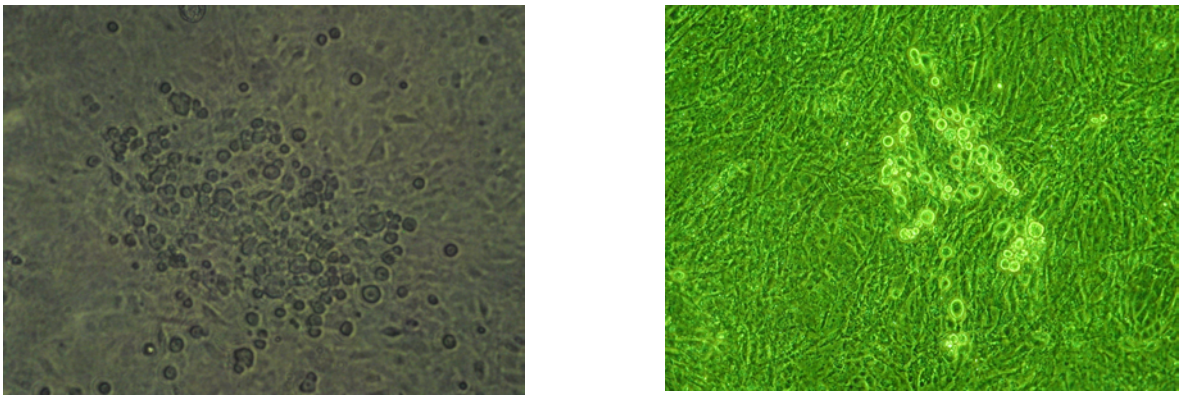

Plate 1. Plaques of MDV isolates FT158 (left) and MPF57 (Right)

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. Challenge material was 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

Pathotyping and other *in vivo* experiments were conducted in a 24-isolator facility at UNE the facility. The isolators are housed in a biological 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 2 and 3.

Plate 2. 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 3. 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 37˚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 4). 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 4. 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 5). 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).

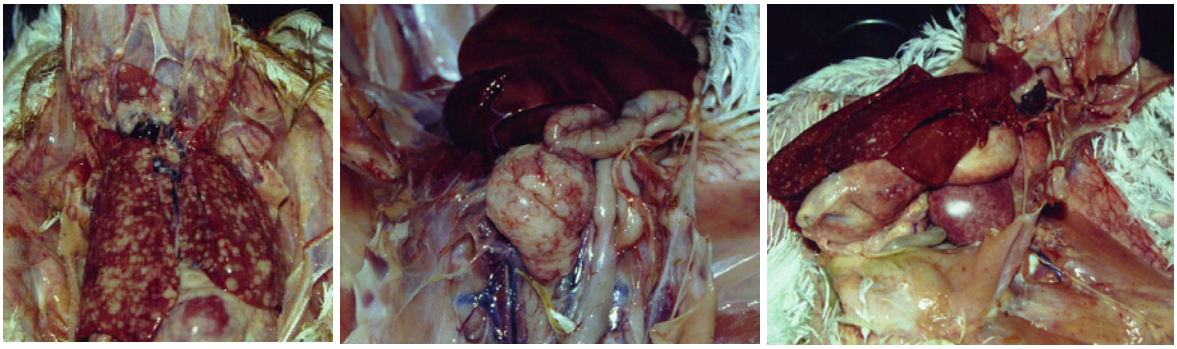

Plate 5. Typical gross Marek’s disease lymphomas of the liver (left), ovary (middle) and liver and spleen (right).

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.

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Authorship of Experimental Chapters

The experimental chapters listed below were analysed and written up by the named authors. Where other people were involved in the experimental work, but not the write up, they are acknowledged in the chapter and in the Acknowledgements section.

Chapter 1. MDV isolation and growth in cell culture
Julie Cooke, Brian Meehan, Greg Tannock

Chapter 2. MD04-C-PT2. Virus isolation from dust and other infective material in SPF chickens
Steve Walkden-Brown

Chapter 3. MD05-C-PT1. 1st pathotyping experiment in broiler chickens
Steve Walkden-Brown

Chapter 4. MD05-C-PT3. 1st pathotyping experiment in layer chickens
Steve Walkden-Brown

Chapter 5. MD06-C-VI5. *In vivo* propagation of MDV strains for pathogenicity screening and subsequent propagation in cell-culture
Steve Walkden-Brown

Chapter 6. MD06-C-BID1. Determination of the infective dose 50 (ID_{50}) of Marek's disease virus (MDV) in feather dust
Steve Walkden-Brown and Fakhrul Islam

Chapter 7. MD06-C-BID2. Determination of the ID_{50} of Marek's disease virus in infective cell-culture material
Steve Walkden-Brown

Chapter 8. MD06-C-PT4. 2nd pathotyping experiment in broiler chickens: recent isolates in Cobb and Ross strains
Steve Walkden-Brown

Chapter 9. MD04-C2-DUST. Effect of temperature on MDV content and infectivity of poultry dust
Fakhrul Islam

Chapter 10. Epidemiological survey of MDV incidence and risk factors
Peter Groves

Chapter 11. Development of a deterministic epidemiological model of MD in broiler chickens
Steve Walkden-Brown with papers from Zhanhai Gao.

Chapter 1: MDV isolation and growth in cell culture

Project Objectives

"To assist with the systematic collection of current field strains of selected key poultry viral pathogens, clear the isolates of contaminating organisms, adapt to cell culture if necessary and characterize them at the molecular, cell culture and in vivo level." (Project 3-17 proposal August 2003).

Strategy One

"Isolation, adaptation, removal of contaminant viruses from field samples from Marek's problem flocks and the maintenance of stocks of MDV and CAV."

Background

It was shown by Churchill & Biggs (1967) that Marek's Disease Virus (MDV) could be isolated from infected birds using chicken kidney (CK) cell cultures. The cytopathic effect (CPE) was produced when intact viable cells were inoculated and the CPE produced spread through the monolayer by cell-to-cell contact to form a visible plaque. Witter (1967) used a range of different primary cells to propagate MDV and found that chicken bone marrow cells were the most susceptible to the JM strain with kidney, liver and lung being less susceptible. Different chicken embryo cells were also investigated and spleen, liver and kidney cultures were more susceptible to MDV infection than fibroblast or heart cell cultures (Witter, 1967).

During the 1970's it was shown that after primary isolation in susceptible cells such as CK, duck embryo fibroblast (DEF) and chicken embryo kidney (CEK), serotype 1 viruses could be adapted and maintained in chicken embryo fibroblast (CEF) cells. DeLaney continued this work at RMIT during his PhD in the 1990's. RMIT continued with this protocol and isolated MDV1 initially in CEK's and then adapted them to CEF's.

Prior to the initiation of this research project, MDV cell culture isolation was primarily performed in CEK's at RMIT University. This isolation procedure had been successfully used in earlier studies. (DeLaney, 1995, 1998)

A chronological breakdown of background activities at RMIT University are as follows:

2002:

As part of the RIRDC Project (UNE83J), 237 field samples (spleen, blood) were submitted by industry (79% Inghams P/L) for MDV isolation and characterization.

Of these 237 field samples, 122 were MDV1 PCR positive and 82 produced CPE at about passage 5 (CEK- P0, P1 then CEF-P2 onwards). None of the 82 samples attained titres of >100pfu/mL.

The methodology for the characterisation of MDV isolates was agreed to by RIRDC Marek's Steering Committee in June, 2003 and is shown in Appendix 1.

The methodology used for Flock Sampling and information retrieved as part of sample collection is shown at Appendix 2. The information contained in this form allowed industry and the farmers collecting the samples to follow a protocol specific for our needs and stressed the importance of sample integrity. It also gave some additional background data such as location, vaccination type, age of birds and morbidity/mortality rates which could be used to provide an overall snapshot of Marek's disease status in Australia.

2003:

CRC project commenced. 32 field samples were submitted from the field. 55 samples (32 new, 23 old) were placed onto cell culture (CEK/CEF) in 2003. Whereas 14 of the new samples were MDV1 PCR positive only 7 elicited CPE at passage 5 and of these 7 isolated only two isolates attained a titre of 100-1000pfu/mL.

Methodology:

Cell Culture (CEF/CEK):

Preparation of cell culture involves removing organs aseptically from freshly euthanased animals or from embryonating eggs. During this project chicken embryo fibroblasts, chicken embryo kidney and chicken kidney cells were used to isolate MDV1 wild - type from field samples.

Chicken Embryo Fibroblast Cell Culture: [RMIT MDS Method 1.0]

CEF cell culture is suitable for the growth of MDV, especially vaccine strains. Ten day old embryos were removed from the egg and the head, feet and abdominal contents discarded. Embryos were rinsed with PBS and then forced through a 10mL syringe. Trypsin solution was added and the flask stirred for 10 minutes at 37^{o}C. This was repeated twice with the supernatant collected after each trypsinisation and kept at 4^{o}C. The supernatant was then filtered and centrifuged for 8 minutes at 1200rpm at 4^{o}C. Packed cells are removed and added to a small amount of growth medium prior to a cell count being performed. Flasks and plates are seeded at concentrations according to their size and incubated in 5%CO_2 at 37^{o}C. A confluent monolayer was attained after approximately 24-36 hours.

Chicken Embryo Kidney Cell Culture: [RMIT VES Method 8.0; Tannock, 1985]

Embryos were extracted after firstly removing the head. The embryo is then pinned face down and cut from the tail along each side of the vertebra. The backbone is cleared from the intestines and organs so the kidneys are exposed. Kidneys are removed using forceps and placed in tube containing citrate saline solution. The kidneys were again washed; excess citrate saline removed and placed in a clean petri dish. They were then chopped finely, transferred to trypsinizing solution using 10mL syringe and left overnight at 4^{o}C. On Day 2, the cells were shaken, filtered and centrifuged at 700g for 5 minutes. The pellet was added to a 10mL tube with PBS and centrifuged as above.

The packed cell volume is estimated and a dilution of 1/200 is prepared in growth medium which was subsequently dispensed into plates or flasks and incubated in 5% CO_2 at 37^{o}C. A confluent monolayer was attained after 24-36 hours.

Lymphocyte (LØ) Isolation: [RMIT MDS Method 14.0 and 13.0]

Samples arriving at RMIT were either heparinised blood samples or spleens transported in phosphate buffered saline (PBS).

Field samples of heparinised blood from vaccinated and unvaccinated flocks were transported to RMIT at 4^{o}C. The blood was diluted with an equal volume of PBS and 7mL of the mixture layered onto 3mL of Ficoll-Paque in a 10mL centrifuge tube. The tube was centrifuged at 400g for 35minutes at 4^{o}C. The narrow band of LØ's that formed at the interface of the Ficoll-Paque and the upper plasma fraction was removed with a pasteur pipette and washed in cold PBS before suspending in cell freezing medium (CFM – MEM/HEPES, 15%FCS, 10%DMSO, $NaHCO_3$ and antibiotics). The concentration of LØ's was adjusted to 2x10^{6}cells/mL in CFM. After preparation, the suspensions were immediately inoculated onto freshly prepared cells or slowly frozen and stored at -80^{o}C until fresh primary cell cultures were available.

Spleen samples from vaccinated and unvaccinated flocks were also transported to RMIT at 4°C. Each spleen was placed in a clean weigh boat with 1mL of chilled PBS. Using sterile forceps and scalpels the outer membrane was removed and the spleen macerated into a thick homogenous liquid. A further 5mL of PBS was added and then the homogenate allowed settling before filtering through a 40um filter and making the final volume of 10mL. The cell suspension was then centrifuged at 1200rpm for 6 minutes at 4°C. The supernatent was decanted and the pellet resuspended in 7mL of PBS. This was then layered onto 3mL Ficoll-Paque and centrifuged at 400g for 35 minutes at 4°C. The narrow band was removed and the LØ concentration was adjusted to $2x10^6$cells/mL in CFM. LØ's were inoculated immediately onto freshly prepared cells, an aliquot taken for DNA isolation and the remainder slowly frozen prior to storage at -80°C.

PCR analysis: [RMIT MDS Method 11.0]

DNA from blood and spleen cells was extracted using Qiagen Blood Mini Kit and Qiagen DNeasy Tissue Kit respectively. DNA was extracted according to the manufacturers' instructions.

PCR was used to ascertain the MDV status of each sample received at RMIT. Following initial cell culture inoculation only MDV1 positive PCR isolates were subsequently passed in cell culture. The presence of contaminants such as MDV vaccine (HVT) and CAV was also determined using a virus-specific PCR assay. MDV1 viruses were detected using primers specific for the 132bp tandem repeat within the short terminal and internal inverted sequences, TR_L and IR_L respectively (Silva, 1992). HVT detection was performed using primers specific for the gB gene which lies within the U_L region of the genome (Aminev, 1998).

All reactions were performed in 25uL volumes and contained the following final concentrations of 0.2mM dNTP mix, 1.5mM $MgCl_2$, 0.2uM primers (forward and reverse), 1 x unit Amplitaq DNA polymerase, 10x reaction buffer and 5uL DNA template. The PCR amplifications were performed on the Perkin Elmer Gene Amp PCR System 2000. Following the initial pre-incubation at 94°Cfor 3 minutes, the DNA was amplified during 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. A final elongation step at 72°C for 5 minutes completed the PCR reaction.

The CAV PCR was performed using primers specific for the VP2 gene (Tan, PhD thesis RMIT, 2002). The reactions were performed in 25uL volume containing 0.2mM dNTP mix, 2.0mM $MgCl_2$, 0.2uM of each forward and reverse primers and 1 x unit Amplitaq DNA polymerase, 10x reaction buffer and 5uL DNA template. Each reaction consisted the pre-incubation of 95°C for 3 minutes and of 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. The elongation of 72°C for 5 minutes completed the reaction. Agarose gel electrophoresis was used to visualise the PCR products. 15uL of PCR product was separated through a 2% agarose gel. The gel then stained with ethidium bromide, visualized by UV light and photographed.

IP determination: [RMIT MDS Method 8.0]

Detection and serotyping of MDV can be achieved by an immunological test using MDV specific monoclonal antibodies and a secondary enzyme labeled antibody. The positive virus-specific immunoreaction is characterised by a reddish-brown precipitate in the infected cells and assessed using a conventional inverted light microscope.

Isolates producing distinct viral plaques after passaging were grown in 24 well plates. At day 6, cells are washed with PBS then fixed with 80% acetone. Specific monoclonal antibody, H19, was then added to detect any wild-type virus present. The monoclonal antibody H19 reacts with pp38 antigen of the wild - type MDV1's but not the CVI988 vaccine strain (Lee 1983, 2005). A green counter stain was used to provide contrast with the virus-specific staining. This staining technique allows us to distinguish between isolates that may have contained the CVI988 vaccine virus and the ones that only contained the wild-type MDV1.

CAV PCR check:

The pathogenesis of MDV can be greatly influenced by other immunosuppressive viral or bacterial infections. CAV causes clinical and subclinical disease in chickens and is known to impair T cell response to MDV (Schat, 2003). The interaction between MDV and CAV required RMIT to ensure the isolates from the field and grown in cell culture were free of CAV. Although it had been shown that CAV doesn't grow in monolayer cell cultures prepared from various tissues of chickens (Yuasa, 1979) and at RMIT (S. Cunningham, PhD Thesis, 1998) a final CAV PCR was performed following propagation of the field isolates to confirm the absence of the CAV in the MDV1 cell culture isolates.

Archiving and storage

Storage of LØ preparations and viruses were initially placed in -80^{o}C. This slowly froze the samples preventing damage to the cells by ice crystals. Samples were then placed into LN2 until required.

Reference MPF57 preparations for challenge experiments

MPF57, isolated in the 1990's, has been used as a reference preparation for Marek's Disease projects throughout Australia. Originally isolated in CEK cells, it was adapted to CEF's by David DeLaney at RMIT.

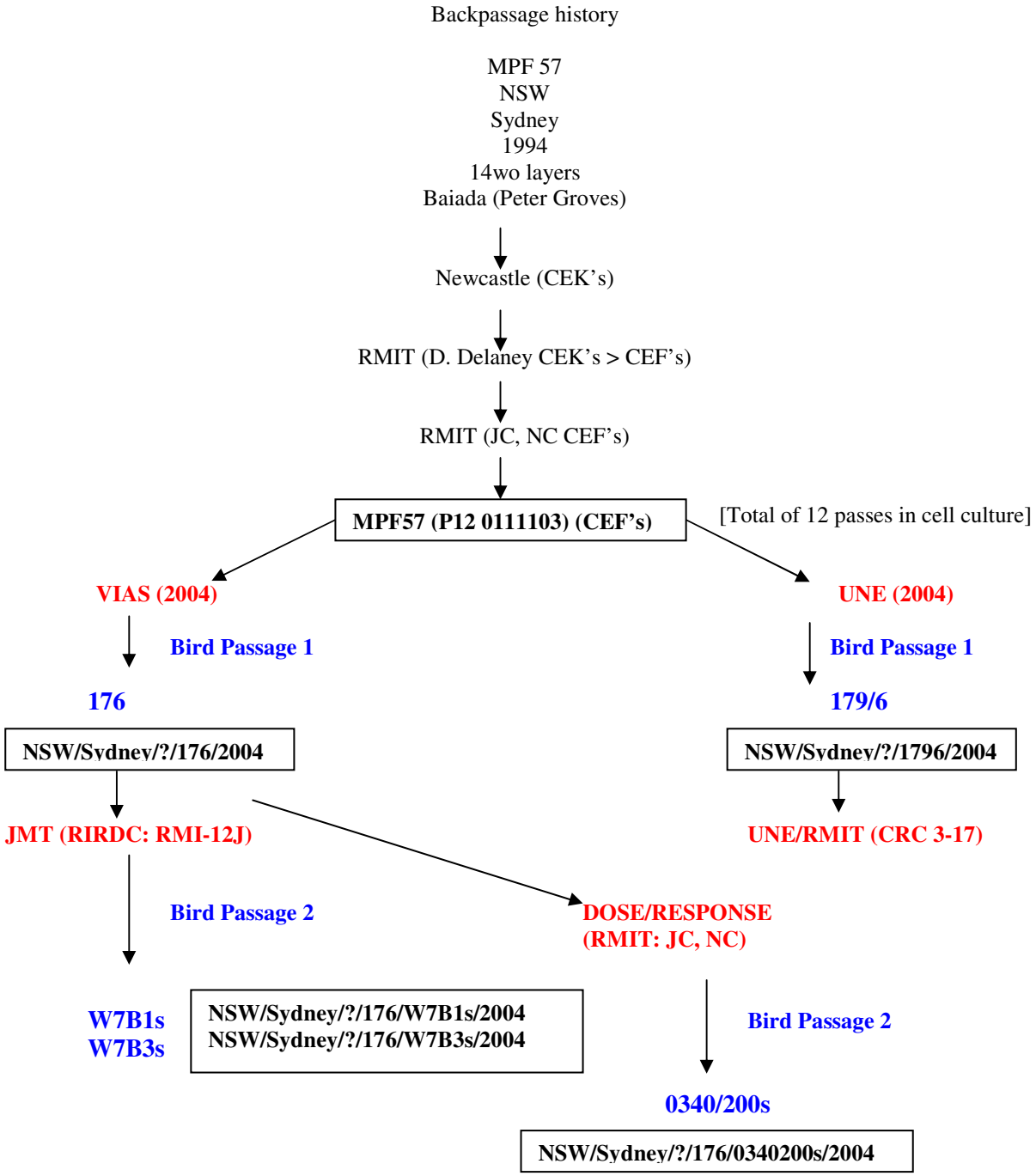
Passage history of this reference has been kept since isolation. The preparation, used by various projects, has been used at P12. This facilitated the generation of the volumes of inocula at the titres required whilst keeping the chance of attenuation to a minimum.

MPF57 (P12 011103) ($TCID_{50}$=3.85 propagated in CEFs), was used in 2004 as part of this project at UNE. Spleens were returned to RMIT for isolation of the chicken passaged material and subsequently re-isolated in CK cells. This MPF57 grew very well to a titre of 20,270pfu/mL and has been used by UNE as a control. All subsequent isolation or re-isolation experiments at RMIT University since 2004 have been performed on CKs. The complete history of MPF57 passages and backpassages over time is show in Figure 1.1.

The work, both overseas and at RMIT University, on cell types and their use in the isolation of MDV1 supported the use of CEK's as the primary cell if CK's weren't a viable option. RMIT access to SPF eggs was the most cost effective option and figures prior to 2004 suggested not a great deal of difference between their use and CK's. DeLaney had shown that isolating in CEK's and then later passaging in CEF's gave the required titres of WDS1 and MPF57 (DeLaney, PhD Thesis, RMIT, 1998).

Previous work with MPF57, when being used as the reference preparation, used 50pfu/dose. This dosage is only 1/10 the dosage adopted in this project. After many attempts at achieving the 500pfu/dose for both the reference and the new isolates in CEK/CEF the use of CK's was considered. Discussions were held at the RIRDC Marek's Steering Committee meeting (June, 2003) and shortly after Dr Tan started his work with cell optimization. The results obtained from his study and the extra funding provided, RMIT started using CK's as the primary cell for the isolation of MDV1 viruses in 2004.

MPF57 CHALLENGE VIRUS:



Old Nomenclature	RMIT Accession Number	New Nomenclature	Cell Culture Passage History
MPF57B1	179/6	NSW/Sydney/?/1796/2004	MPF57 at P12 011103, total passage in cell culture
MPF57B1	176	NSW/Sydney/?/P12 011103/176/2004	
MPF57B2	W7B1s	NSW/Sydney/?/P12/176/W7B1s/2004	
MPF57B2	W7B3s	NSW/Sydney/?/176/W7B3s/2004	

Figure 1.1: History of MPF57 backpassages and proposed complete nomenclature system.

Method optimisation on CRC Project 03-17

During the course of this research project, methods for the optimisation of MDV isolation were evaluated.

Cell Type Optimisation

Isolation and recovery of MDV using different primary cell culture types was optimised with the help of QPCR. This work, funded by the RIRDC, conducted in 2003, was presented to RIRDC in May 2004 and reported in the CRC progress report June 2004. (See Fig. 1.2a and 1.2b)(Tan, 2007)

A real time PCR was used to investigate the growth kinetics of MDV in primary chicken cells and establish the optimal method for the isolation of field isolates. This was done by comparing the growth of vaccine and challenge MDV in three different types of chicken cells.

Similar growth curves were observed with different MDV vaccine and challenge strains in the different chicken cells. The MDV copy number of each strain increased linearly from day 1 to day 10. This suggests that propagation of MDV in cell culture can be monitored using the DNA copy number increases. This study also found that there was a linear correlation between viral copy number and infectious titre (Tan, 2007).

Although the growth of MDV challenge virus (WDS1) is possible in all three types of cell culture, the highest DNA copy number was observed in CK cells. MDV DNA copy number in CK cells was significantly higher than those for CEF cultures and slightly higher than that observed using CEK cultures [CK ($10^{8.5}$/well) followed by CEK ($10^{7.8}$/well) and then CEF ($10^{6.4}$/well)].

In addition, the effect of different inoculation methods, propagation conditions and harvesting procedures were also investigated. It was found that the highest DNA copy numbers were observed when freshly trypsinised cells were infected into a suspension of CK cells prior to monolayers forming. Using freshly trypsinised CK cultures, yields were 6-150 fold higher than those obtained by infection of monolayer cultures. Increases were also observed for CEK and CEF cells using freshly trypsinised cells. Media changing after 24 hours was found not to produce a significant increase in MDV copy number and harvesting at day 6 or 7 was optimal before the monolayers started to deteriorate.

Optimisation of cell type and harvesting

The results of the growth characteristics of WDS1 in CK, CEF and CEK were presented by Julie Cooke at the RIRDC Meeting May 2004.

The conclusions were as follows:

- CK cells were demonstrated to be better for the isolation of MDV1
- Infection of freshly trypsinised cells were demonstrated to increase the titre
- Changing the media post infection had no effect
- Optimal harvest time of CK cultures was at day 6 post infection

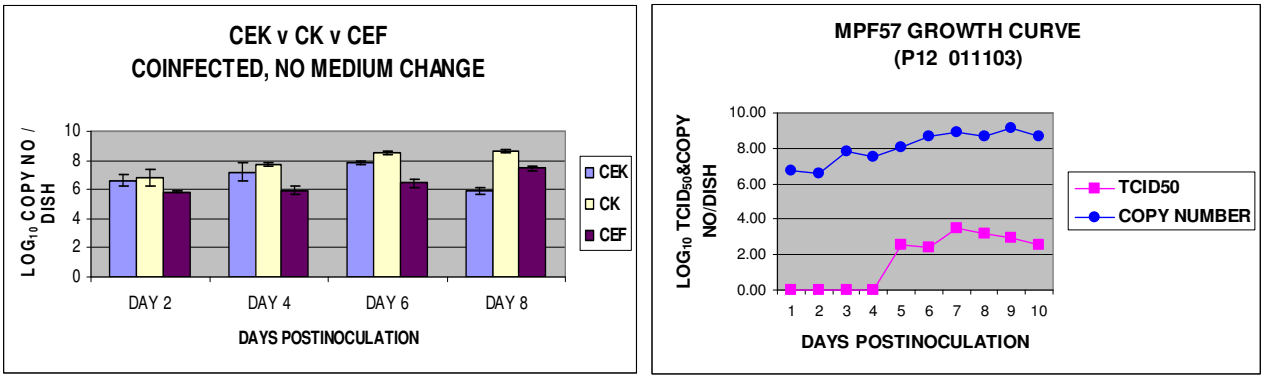


Figure 1.2a, b: Cell Type and Harvest Time optimisation.

Concurrently, this work was corroborated by K.A.Schat and presented at the Marek's Disease Symposium in Oxford in July 2004.

In a subsequent publication, Schat (2005) reported that CK cells was the preferred substrate for isolation of MDV-1 over CEF and CEK cells as determined by counting the plaques on the cell monolayers after inoculation of vv and vv+ MDV. In addition, Schat also found that although CEK cells can be used for virus isolation, continuous passage on CEK cells resulted in minimal increases or even decreases in virus yield.

Therefore, both K.A. Schat and RMIT in 2004 had evidence that CK's provided a better medium for isolation of MDV field isolates and the Project Management Team agreed for RMIT to use CK's for the isolation and propagation of field isolates.

Specifically, a revised isolation procedure using CK's has been implemented at RMIT University since 2004.

2004:

A revised flowchart was constructed taking into account the dust samples received by UNE from industry (Baiada P/L), (Appendix 3).

Chicken Kidney Cell Culture: [Tannock, 1985; Schat &Purchase,1998]

Two week old SPF chickens were euthanased by CO_2 inhalation. The kidneys were removed and washed twice in PBS. The PBS was decanted and warm trysinizing solution added. It was shaken for ~30 seconds, allowed to settle and the supernatant discarded. Warm trysinizing solution, was again added and stirred for 3minutes at 37oC before also being discarded. The kidney cells and fresh trypsinizing solution was stirred for 5minutes at 37^{o}C. The supernatant was then decanted into a chilled flask containing 10%v/v newborn serum. The step was repeated a further 5-8 times and then the collection filtered. The cell suspension was centrifuged at 400g for 5minutes and then diluted to 1/200 of the packed cell volume in growth medium (10% newborn serum). CK cells were then placed into plates or flasks, rocked gently and incubated in 5%CO_2 at 37^{o}C. A complete monolayer was attained within 48 hours.

Repassing of MDV viruses in CK cells:

Fresh CK cells were prepared and new flasks seeded. The same day, viruses that had been growing for 6 days were washed with warm PBS and cells scraped free using a cell scraper. The virus/cells were then transferred into the fresh cells using a cannulae and syringe. After 24-36 hours the media was changed with fresh growth medium. The process was repeated 6 days later after microscopic inspection of the cells and enumeration of plaques.

During 2004, 225 new field samples were received by RMIT from industry (84% Inghams P/L) and another 70 older samples and reference viruses were reisolated on CK cells. 13 isolates grew to >1000pfu/mL while 6 isolates fulfilled the requirements of pathotyping protocols requiring 500pfu/dose (~2500pfu/mL) and were used by UNE in the pathotyping experiment. Two of these 6 isolates were originally isolated in chickens at UNE from field dust samples.

In November 2004, pathotyping experiment using the 6 isolates in SPF birds commenced. (Experiment MD04-R-PT2, Walkden-Brown et al., 2006). In 2005, the most pathogenic viruses were used in experiments in layers and broilers (Chapters 3 and 4, this report).

At that time is was readily apparent that the number of new field isolates growing to adequate titre initially anticipated would not be achieved and this was detailed in CRC Progress Report in June 2004 (Risk Management).

2005:

In an attempt to increase the number of high titre field isolates, a range of isolates grown in CK cells to low passage at RMIT were sent to UNE for *in vivo* passage through chickens. This procedure had been used previously to expand the quantity of virus and increase the titre of available virus pools. Also, dust samples received by UNE were used to inoculate SPF chickens to amplify any virus present. Spleens from the above birds were then sent back to RMIT for re-isolation and passaging in CK cells.

Of the 26 viruses that produced CPE during 2005, 4 grew to titres between 100-1000pfu/mL.

Two consignments arriving late in 2005, one of which got delayed 24hr due to the courier error, were processed and placed in -80°C ready for isolation in 2006.

2006:

Fresh spleen material from MDV infected SPF chickens received from UNE in late 2005 were isolated in early 2006. One consignment was passed in CK's to P6 and the other consignment to P4.

21 of 27 isolates produced CPE. These isolates produced titres that ranged from 17-680pfu/mL (Table 1.1).

At a Project 3-17 meeting held in Jan 2006 it was agreed that there was 'little confidence' that a further back-passage in chickens would improve titres. A new proposal that MDV content of cultures could be determined by titration in chickens and then pathotyping experiments run based on fixed multiples of BID50 (bird infective dose 50) was discussed. It was agreed that this be performed in SPF chickens with a protocol drafted by UNE and agreed to by RMIT (Chapter 7).

RMIT sent the 21 P4/P6 isolates to UNE to be used in the BID50 experiment MD06-C-BID2.

In addition, RMIT would place the 16 (P4 and P6) isolates through another round of CK cells (8 weeks) to see if the titres could be increased. Of the 16 isolates repassed, only 2 had titres that increased significantly (Table 1).

Table 1.1 Growth of MDV on CK culture at RMIT in 2006. Infective material was splenocytes harvested from infected chickens at UNE in experiments MDO5-R-PT3 and MDO6-C-V15 and stored prior to use.

ISOLATES	*CK's: February-April (MDO5-R-PT3 and MDO6-C-V15)*				*CK's: July-August*	
	PASSAGE	Pfu/mL	PASSAGE	Pfu/mL	PASSAGE	Pfu/mL
02LAR			*P6 020406*	*60*		
04KAL	*P4 020406*	*140*			P10 210806	62
164/6			*P6 020406*	*70*		
FT158			*P6 020406*	*17*	P12 210806	10
23	*P4 020406*	*60*				
MPF57	*P4 020406*	*80*				
176			*P6 020406*	*27*	P12 210806	126
179/6			*P6 020406*	*300*	P12 210806	33
189/8	*P4 020406*	*90*	P6 020406	60		
192			*P6 020406*	*140*	P12 210806	1250
199	*P4 020406*	*200*	P6 020406	110	P10 210806	34
210/1S	*P4 020406*	*100*	P6 020406	50		
210/2S	*P4 020406*	*70*	P6 020406	30		
212			*P6 020406*	*210*	P12 210806	55
W7B1s			*P6 020406*	*110*	P12 210806	33
WDS1	P4 020406	600	*P6 020406*	*680*	P12 210806	2515

Note: Shaded isolates didn't show plaques after 5 weeks and were discarded.

Table 1.2 Summary of the isolation and adaptation in different cell types; 2002-2006.

YEAR	Number of isolates inoculated onto cells.	Number of PCR +ve (MDV1)	Number of isolates with CPE (@ ~P5)	Number of titred isolates. > 100pfu/mL (pfu range)
RIRDC 2002 – [CEK's (Passage 0 and 1) then CEF's (P2 onwards)]	237	122	82	0
RIRDC / CRC 2003 – [CEK's (Passage 0 and 1) then CEF's (P2 onwards)]	55	14	7	x2 (100-1,000pfu/mL)
CEK/CEF TOTALS **% OF TOTAL SAMPLES INOCULATED** **% OF CPE POSITIVE @ P5**	**292**	**136** **46.6%**	**89** **30.5%**	**2** **0.7%** **2.2%**
2004 – [CK's – coinfected onto cells passage 0 onwards]	293	158	49	x6 (>10,000pfu/mL) x7 (1,000-10,000pfu/mL) x1 (100-1,000)
2005 – [CK's – coinfected onto cells passage 0 onwards]	127	116	26	x4 (100-1,000pfu/mL)
2006 – [CK's – coinfected onto cells passage 0 onwards]	43	43	30	x2 (1,000-10,000pfu/mL) x11 (100-1,000pfu/mL)
CK TOTALS **% OF TOTAL SAMPLES INOCULATED** **% OF CPE POSITIVE @ P5**	**463**	**317** **68.5%**	**105** **22.7%**	**31** **6.7%** **30.0%**
OVERALL TOTALS **% OF TOTAL SAMPLES INOCULATED** **% OF CPE POSITIVE @ P5**	**755**	**453** **60.0%**	**194** **25.7%**	**33** **4.4%** **17.0%**

Discussion

Increases in the number of isolates of high titre MDV-1 from Australian field samples were achieved by inoculation to suspensions of CK cells in growth medium rather than to monolayer cultures of first CEK and then CEF. The increase was from 0% in 2002 and 2003 to 26.5 and 15.3% in 2004 and 2005. These increases are not reflected in the proportion of samples being PCR-positive and having the capacity to react with Mab H19 following passage in cell culture. The relatively low isolation rate can be explained, in part, from the fact many samples were sourced from other parts of Australia and may have been subject to losses in infectivity due to difficulties in sample collection, storage and transportation. However, the clear improvement in the isolation rate during 2005 and 2006 suggests selection of high titre strains following the introduction of (a) CK culture and (b) the inoculation of clinical material to freshly trypsinised cell suspensions (Tan, 2007).

Clearly, being PCR–positive and having the capacity to react with Mab H19 following passage in cell culture are not reliable predictors of the capacity of MDV isolates to grow to levels that are suitable for use in challenge studies. This problem is particularly acute with MDVs, which are highly labile and cell-associated, and long-term storage is only possible in liquid nitrogen. Improvements in the recovery of high titre strains by inoculation to CK cell suspensions should be useful in the maintenance of a representative repository of field strains that could be compared with earlier isolates over several years (Tan, 2007).

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Chapter 2: Experiment 1. MD04-C-PT2

"Virus isolation from dust and other infective material in SPF chickens"

Start: 8/6/04 Completion: 3/8/04. AEC: UNE AEC04/095

Introduction

Due to a shortage of new MDV isolates growing in cell culture, this experiment was designed 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. RIRDC/AECL Project UNE 83-J was also operating at this time and also required new MDV isolates. This experiment was therefore a joint experiment of UNE-83J and the Poultry CRC project 03-17 conducted by Mr Zahid Hussain, the holder of a Poultry CRC scholarship for a Master of Rural Science degree at UNE. A report on this experiment is also included in the final Report of Project UNE-83J. This is the only one of the 7 major chicken experiments carried out under CRC Project 03-17 which was shared with another project and reported for that project.

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 was conducted at the University of New England isolator facility. 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 2.1 and further details of the origins of the viruses can be found in Table 2.2.

Table 2.1: **Experiment 1. 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 LP0211607, RIRDC project UNE 83-J and Poultry CRC project 03-17 were assayed for MDV-1 using real-time qPCR and a selection of positive samples were selected for use (Table 2.1). 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 2.2). 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 2.2. Experiment 1. 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 2.3. Survival analysis revealed significant differences (p=0.007) in the pattern of mortality between treatments in survival to day 56pc (Figure 2.1). 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 2.1). 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 2.1, Right panel).

Table 2.3. Chicken numbers and mortality during Experiment 1.

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**

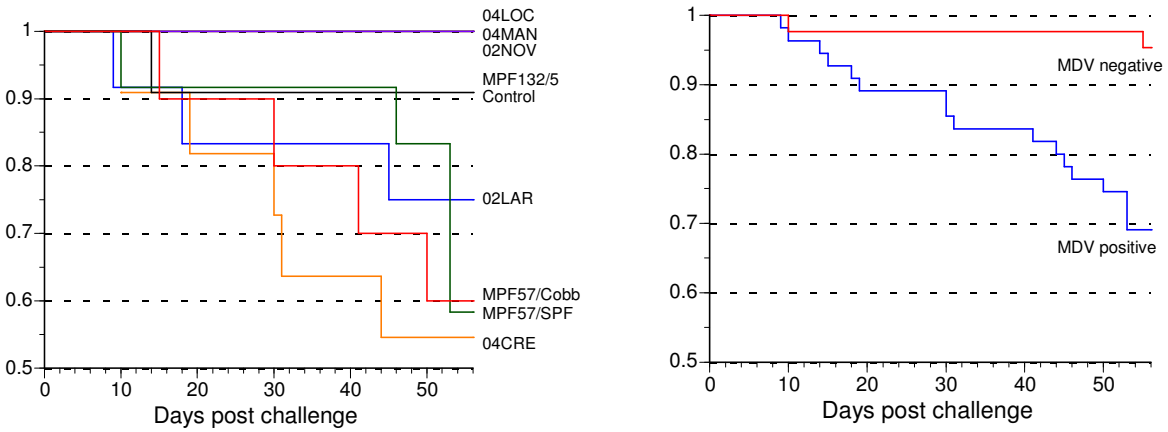

Figure 2.1. Survival patterns in Experiment 1 showing the effects of treatment (Left panel, P=0.007) and pooling of treatments according to whether or not MDV was detected in the treatment group or not (Right panel, P=0.001). The MDV positive group included 04CRE, MPF57/SPF and MPF57/Cobb and O2LAR while 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 2.3). 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 2.3. Distribution of MD lesions by treatment and chicken survival status in Experiment 1. 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 2.2) and Sex on final bodyweight in the SPF chicken treatments at day 56pc. Males were significantly heavier than females (759 v 609g , P<0.0001).

Serology at day 56pc.

This is summarised in Table 2.4. 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 2.4.

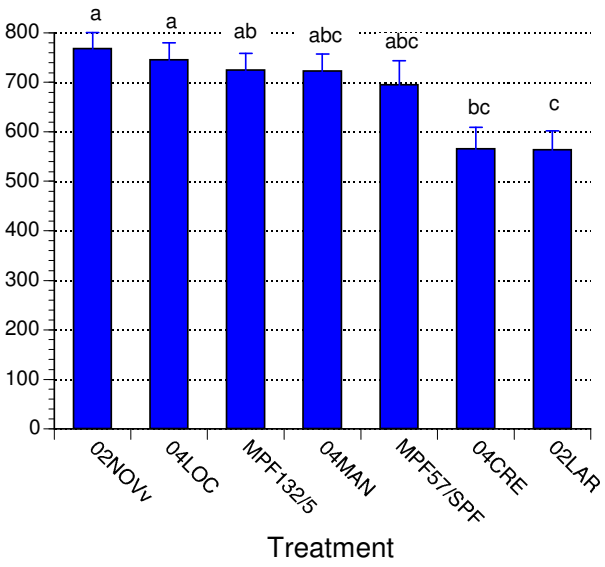

Figure 2.2. Mean (LSM±SE) final body weights in Experiment 1. Columns not sharing a common letter in the superscript are significantly different ($p<0.05$).

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 or eliminating contaminating organisms. 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 Chapter 6 for a dose response study with dust infection. 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.

Table 2.4. 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)

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.

Chapter 3: Experiment 2. MD05-C-PT1

"1st pathotyping experiment in broiler chickens"

Start: 3/3/05 **Completion: 3/5/05** **AEC: UNE AEC04/006**

Introduction

With the availability of new isolates of MDV grown to high titre at RMIT the pathotyping strand of the project could finally commence. The availability was due to a combination of isolation of MDV directly from dust into SPF chickens at UNE, and amplification of low titre material from RMIT in SPF chickens at UNE under RIRDC Project UNE 83-J, and improvements in cell culture methodology at RMIT (See Chapter 1). The experiments in SPF chickens provided fresh infective spleen material being sent to RMIT resulting in good success rates for growing of MDV in cell culture.

Our goal was to take the most virulent recent isolates identified each year and evaluate them for pathogenicity against the reference strain MPF57 in commercial broilers and layer chickens. The approach was to use an adaptation of the USDA ADOL pathotyping method described in the Methodology Section. This involves measuring MD incidence in unvaccinated chickens and those vaccinated with HVT and HVT/MDV2 bivalent vaccines and determining vaccinal protective index. Another goal was to investigate novel methods of pathotyping MDV using qPCR determination of viral load.

New MDV isolates 02-LAR and FT158 were identified in early 2005 as the most pathogenic recent MDV isolates in an RIRDC 8-J experiment in SPF chickens (MD04-R2-PT2). Both were more pathogenic than MPF57 the current reference strain in HVT-vaccinated SPF chickens.

The specific hypotheses under test were:

1. Pathotyping rankings derived from SPF chickens will be the same in commercial Cobb broiler chickens (02LAR>FT158>MPF57).
2. Thymus and bursal weights at days 7 and 14 will be good predictors of pathogenicity without marked differences between the two times.
3. MDV load in spleen at days 7 and 14 will be good predictors of pathogenicity without marked differences between the two times.
4. Viral shedding of MDV in dander will be positively associated with pathogenicity
5. Birds challenged with MDV of higher pathogenicity will have a higher incidence of mortality and *E. coli* lesions after challenge with *E. coli* than birds challenged with MDV of lower pathogenicity and this will be a more sensitive test of MDV pathogenicity than immune organ weights.

The pathotyping aspects of the project were led and implemented by Dr Steve Walkden-Brown while a UNE PhD student Mr Aminul Islam working on ARC project LP0211607 was heavily involved in the execution of the experiment and made a large number of additional measurements, unrelated to the main objectives of the CRC project.

Materials and methods

The experiment was conducted at the University of New England isolator facility. It involved 648 chickens in a complete 3x4 factorial design with two replicates using 24 positive pressure isolators (3x4x2=24). The two experimental factors were:

- Vaccine virus (3) ie. Sham, HVT and bivalent (HVT/SB1) @8000pfu/bird. Chickens were vaccinated sc in 200ul diluent on the day of hatch (day 0). Sham-vaccination was with diluent only.
- Challenge virus (4) ie. Sham, MPF57 (reference strain), 02-LAR and FT158 @500pfu/bird. Challenge was intra-abdominal at day 5 post-vaccination. Sham-challenge was with diluent only.

02-Lar and FT158 were identified in early 2005 as the most pathogenic recent MDV isolates in an RIRDC experiment in SPF chickens (MD04-R2-PT2). Both were more pathogenic than MPF57 the current reference strain in HVT-vaccinated SPF chickens. The bivalent vaccine used in the present experiment was the new Bioproperties Vaxsafe SBH bivalent HVT/MDV2 vaccine. Details of the viruses used are shown in Table 3.1.

Table 3.1. Viruses used in Experiment 2.

Viral isolate	Batch number	Dose used	Year of isolation	Place of origin	Flock of origin	Vaccination history
MPF57 B1 (179/6)	P7 200904	500pfu[a]	1994	NSW (Sydney)	Layer (14wo IsaBrown)	Unknown
02LAR (179/3)	P6 120904	500pfu[a]	2002	Victoria (Mornington P)	Broilers	Unknown
FT158	P7 260904	500pfu[a]	2002	Northern NSW	Broiler breeder	Rispens
HVT vaccine FC126	H02308	8000pfu	?	Bioproperties - USA		
Vaxsafe SBH vaccine SB1/HVT FC126	SBH4101	8000pfu combined	?	Bioproperties - USA		

[a]Titrated on CK cells at RMIT.

The experimental chickens were female Cobb500 broiler chickens from Baiada's Kootingal Hatchery. The chickens were unvaccinated but came from a Rispens-vaccinated parent flock (65 wo, Linwood 1). Chickens were fed *ad lib* on commercial starter then broiler finisher (Ridley Agricultural Products, Tamworth). Chicks were exposed to 12:12 light dark in positive pressure isolators. There were 27 chickens/isolator placed initially (54 per treatment combination). The experiment started on the day of hatch (day 0, 3/3/05) and was terminated on 3/5/05 (day 61, day 56 post challenge).

At both days 7 and 14 pc six chickens per isolator were removed and liveweight, and immune organ weights (bursa, thymus and spleen) were determined.

For determination of MDV1, HVT and MDV2 load in dander, samples were collected weekly from the dust deposits at the 90 degree bend in the exhaust air outlet duct of each isolator as described in the General Materials and Methods section.

At day 49 pc all chickens were challenged with pathogenic *E. coli* (strain E3, O untypable, H28 (Ginns *et al.* 1998) kindly provided by Prof. Glenn Browning, University of Melbourne. A dose of approx 1.2 x 10^8 cfu per chicken was administered as a coarse spray to all the chickens in the isolator in approx 75 ml of diluent per isolator.

All chickens dying during the experiment were weighed and examined post-mortem for gross MD lesions as described in the General Materials and Methods section.

At day 56 pc the experiment was terminated and all surviving chickens were euthanased, weighed examined for gross MD lesions and had immune organ weights determined. Diagnosis of MD was by 3 operators Drs Peter Groves, Fakhrul Islam and Steve Walkden-Brown.

Selected spleen samples from days 7, 14 and 56 pc were subject to DNA extraction and seorype-specific qPCR assay for MDV1, HVT or MDV2 as described in the General Materials and Methods section.

Feed conversion efficiency (FE) was calculated as (Total weight of feed consumed – 5% spillage)/(Total weight of live or dead chicken removed from each isolator-initial weight of chickens placed in isolator). Adjustement to a common weight of 3kg (approx final weight of the chickens) or 2.45kg (industry standard) was achieved by reducing FI by 0.03 for each 100g weight above the standard (x the proportion of chicken weight removed from the isolator at the end of the experiment).

Statistical analysis. Analyses were performed with JMP6 (SAS Institute Inc. 2006). Where the chicken was the experimental unit measured discrete data (mortality, presence of MD) were analysed using a generalized linear model with a binomial link function (logistic) fitting the effects of vaccination, challenge treatment and their interaction. Mortality data were also analysed using survival analysis (Kaplan-Meier method). Continuous data (eg bodyweight) were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction directly. MDV load in spleen was log transformed prior to analysis testing the effects of day post challenge, vaccination and challenge treatment. MDV load in dust was square root transformed prior to analysis and data are presented as back-transformed least squares means and standard errors. Vaccinal protective index (PI) based on the presence of gross MD lesions alone, was calculated as (%MD in unvaccinated chickens - %MD in vaccinated chickens) / (%MD in unvaccinated chickens). Virulence rank (VR) was calculated as 100-PI (Witter 1997). Only chickens at risk of MD were included in the calculation (ie alive at the time of the first MD case). For these data (PI and VR) which could only be measured on a whole isolator, data were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction. For this analysis there were only 2 measures per treatment combination (two isolators per treatment combination). For continuous variables least squares means and standard errors are presented.

Results

Application of treatments

All vaccination treatments were successfully applied and maintained, as determined by MDV serotype-specific real-time qPCR (Tables 3.2 and 3.3). Levels of vaccinal virus in spleen and dust are shown later in the results.

Table 3.2 Summary of number of chicken spleens positive for vaccine virus at different times post-vaccination. Only sham-challenged birds tested.

Vaccine	qPCR test	Number of chickens positive to qPCR test of spleen (%)		
		Day 12 post-vacc.	Day 19 post-vacc.	Day 61 post-vacc.
HVT	HVT	10/10 (100%)	7/10 (70%)	15/20 (75%)
Bival	HVT	7/8 (87.5%)	8/8 (100%)	
	MDV2	3/8 (37.5%)	8/8 (100%)	17/18 (94.4%)

MDV1 challenge was also successful for each of the challenge viruses with the proportion of spleen samples positive for MDV1 at days 7, 14 and 56 post challenge shown in Table 3.3. Due to the sampling method used at day 7 and to a lesser extent, day 14, there was slight contamination of spleens. This was evidenced by some low-level positive spleen samples in the sham challenge treatments which were shown to be negative for MDV1 in repeated MDV1 analysis of isolator exhaust dust. Given that spleens were collected at post mortem of large numbers of birds in a contaminated environment, this is not surprising. MDV1 spleen data for days 7 and 14 were therefore adjusted by subtracting the highest MDV1 value from sham challenged chickens from all MDV1 values.

Table 3.3 Summary of number of chicken spleens positive for MDV1 challenge virus at different times post-vaccination. Data are adjusted for low level contamination of spleens during collection at days 7 and 14.

Vaccine	Challenge	Number of chickens positive for MDV1 test of spleen (%)		
		Day 7 post-chall.	Day 14 post-chall.	Day 56 post-chall.
Sham	MDV1	15/15	26/30	35/35
HVT	MDV1	12/30	22/30	53/53
Bival	MDV1	9/30	20/30	53/55
Sham	Sham	0/10	0/10	

Weekly dust samples were collected from 5 sham-challenged isolators (2 HVT-vaccinated, 2 Bivalent-vaccinated and 2 sham-vaccinated). In week 1 trace amounts of MDV1 were detected in 4/5 isolators but all 5 isolators were subsequently negative for MDV from weeks 2-8, the end of the experiment. This confirmed unequivocally that effective isolation was achieved, and also served as a reminder of

the specificity of the qPCR assay. The faint positives at day 7 are appear to be remnants of inactivated virus remaining on the surface of the exhaust tubing following cleanup and disinfection.

Mortality/Survival

Mortality at the start of the experiment was high and a mixed bacterial infection killed 43 chickens on day 1 pc. This was brought under control by water medication with amoxycillin. Total mortality to the end of day 1 pc was 64 chicks or 9.9%. The next mortality occurred on day 5 pc and this and all subsequent mortality is detailed in Table 3.4. The first MD lesions were detected at day 27 pc.

There were significant effects of both vaccination and challenge treatment (P<0.0001) for both total mortality and for mortality with MD lesions. There was also significant interaction between these effects (P<0.0001), mainly because vaccination had no effect on mortality in sham-challenged chickens. Regarding vaccination, significantly more sham-vaccinated chickens (36.2%) died than those vaccinated with either HVT (9.6%) or Bivalent vaccine (10.2%) with no difference between the latter two treatments. The same was true for mortality with MD lesions, with values of 32.8%, 4.4% and 5.1% respectively. Regarding challenge, significantly fewer sham-vaccinated chickens died (2.2%) than those challenged with MDV (21.2%, 28.2±3.4% and 23.9% respectively for, MPF57, FT158 and 02LAR). There were no differences between the virus isolates. The same was true for mortality with MD lesions, with the respective values being 0%, 17.7%, 24.7% and 14.8%.

Table 3.4. Mortality from day 2 post challenge of the experiment. Includes all chickens eligible to die after day 1 pc (n), excluding chickens sacrificed at days 7 and 14 for sample collection.

Vaccine	Challenge virus	n	Total mortality (%)	Mortality with MD lesions (%)
Sham	02LAR	27	37.0	33.3
Sham	FT158	29	55.2	51.7
Sham	MPF57	28	50.0	50.0
Sham	Sham	32	6.3	0.0
HVT	02LAR	29	17.2	6.9
HVT	FT158	27	14.8	11.1
HVT	MPF57	27	7.4	0.0
HVT	Sham	32	0.0	0.0
Bival	02LAR	32	18.8	6.3
Bival	FT158	29	13.8	10.3
Bival	MPF57	30	6.7	3.3
Bival	Sham	27	0.0	0.0

Survival analysis similarly revealed no difference between the two vaccines under test, or the 3 viruses, with significant effects due to the sham-vaccination and sham-challenge treatments only (Figure 3.1).

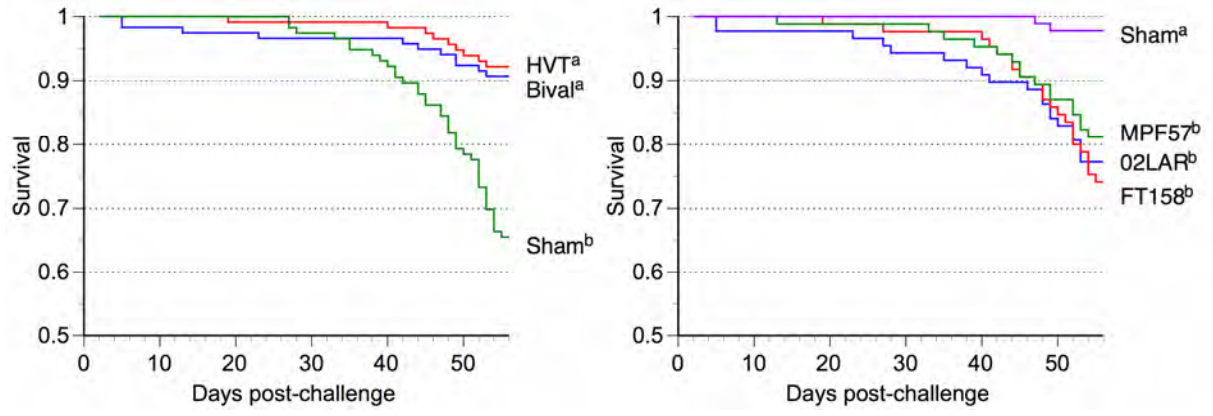

Figure 3.1. Survival analysis of the effects of vaccination (left plot) and challenge treatment (right plot) on survival of eligible chickens to day 56 pc. Treatments not sharing a common letter in the superscript differ significantly (P<0.05).

Incidence of MD vaccinal protective index and pathotype rank.

The combined incidence of gross MD lesions in chickens that died with MD or had MD lesions on post mortem after sacrifice at day 56 is summarized by treatment in Table 3.5. Amongst MDV-challenged chickens there was a significant effect of vaccination (P<0.0001) but not challenge virus (P=0.20) on the percentage of chickens with MD with significant interaction between these effects (P=0.03). Sham-vaccinated birds had a significantly higher incidence of MD (85.7%) than birds vaccinated with HVT (26.8%) or Bivalent vaccine (27.6%) with no difference between the two vaccines. For the challenge viruses 02LAR, FT158 and MPF57 the overall incidence of MD was 42.3%, 54.8% and 42.9% respectively (P=0.20). In isolator 8 which was not challenged with MDV, but was vaccinated with Bivalent vaccine, 2 chickens were identified as MD lesion-positive at the day 56 post mortem, on the basis of minor liver lesions. However dust from this isolator was negative for MDV1 at weeks 7 and 8 post challenge, so the lesions are likely to be false positives. There was a higher than usual incidence of bacterial infection and lesions in this experiment, which may have been responsible. This isolator has no bearing on calculations of protective index or virulence.

Table 3.5. Incidence of MD to day 56 pc, vaccinal protective index and virulence rank by challenge virus and vaccination treatment.

Variable	Vaccination treatment	MDV challenge			
		Sham	MPF57	02LAR	FT158
MD incidence (%)	Sham	0 (0/32)	96.4[a] (27/29)	70.4[b] (19/27)	89.7[ab] (26/29)
MD incidence (%)	HVT	0 (0/32)	14.8[a] (4/27)	31.0[a] (9/29)	34.6[a] (9/26)
MD incidence (%)	Bival	0 (0/27)*	17.2[a] (5/29)	27.6[a] (8/29)	37.9[a] (11/29)
Prot. Index (%)	HVT		84.6	56.0	61.4
Prot. Index (%)	Bival		82.2	60.8	57.7
Vir. Rank (%)	HVT		15.4	44	38.6
Vir. Rank (%)	Bival		17.8	39.2	42.3

[ab] Means within rows not sharing a common letter in the superscript differ significantly (P<0.05)
* Two probable false positives are coded negative. See text for details.

With regards PI there was a significant effect of challenge virus (P=0.04) but not vaccine used (P=0.99, HVT 67.2±4.7%, Bivalent 67.0±4.7%) with no interaction between these effects (P=0.84). For the challenge viruses, challenge with 02LAR and FT158 was associated with significantly lower protection than challenge with MPF57 (58.2±5.7%, 59.9±5.7% and 83.2±5.7% respectively P=0.04). AS VR is derived from PI the analysis of VR produced the same level of significance for effects the effect of challenge virus (P=0.04) being due to MPF57 having a significantly lower virulence rank than FT158 and 02LAR. Interestingly however, MPF57 induced the highest level of MD lesions in unvaccinated chickens.

Distribution of MD lesions

The overall distribution of MD lymphomas and the average severity of the lesions (score 0-3 with 3 most severe) are shown in Table 3.6. The mean severity score was 1.75 and this was significantly higher in sham-vaccinated chickens (1.84) than those vaccinated with Bival (1.40) with those vaccinated by HVT (1.70) intermediate (P=0.029). The mean number of organs with lymphomas in MD positive chickens was 2.25. Challenge virus did not affect this significantly (p=0.389; 1.91, 2.19 and 2.21 for MPF57, 02LAR and FT158 respectively), but there was a strong trend towards an effect of vaccination (P=0.064) with higher numbers of organs affected in sham-vaccinated (2.41) than Bival (2.05) or HVT (1.87) vaccinated chickens. The specific contrast between the vaccinated (both groups pooled) and sham vaccinated treatments was significant ((P<0.022).

Table 3.6. Overall distribution of MD lymphomas in different organs, and average severity of the lesions (score 0-3). Numbers relate to a total of 118 chickens with MD lymphomas.

Organ	n with MD lesions in organ	Incidence of lesions (% of total cases)	Mean severity score
Ovary	81	68.6	1.9
Liver	66	55.9	1.6
Spleen	44	37.3	1.7
Heart	21	17.8	1.6
Lung	16	13.6	1.9
Thymus	14	11.9	1.6
Kidney	7	5.9	2.3
Muscle	4	3.4	1.5
Eye	3	2.5	1.7
Mesenteries	3	2.5	2.0
Bursa	2	1.7	1.5
Skin	2	1.7	1.5
Feather follicle	1	0.8	1.0
Pancreas	1	0.8	1.0

E. coli lesions

E. coli challenge appeared mild and there was no overt mortality associated with challenge. Of the 310 chickens alive at day 50, one day after challenge with *E. coli*, only 53 (17.1%) showed signs of colibacillosis on post mortem over the next 7 days post challenge. Of these chickens 38 had a severity score of 1, 9 had a score of 2 and only 4 had a score of 3. The incidence of *E. coli* lesions was significantly influenced by the presence of MD with a significantly higher incidence of lesions in chickens with MD lesions (23.7%) than in those without lesions (13.4%) (P=0.021).

Chicken bodyweight

The overall effects of vaccination and challenge on bodyweight at days 7, 14 and 56 pc are shown in Figures 3.2 and 3.3 respectively.

At day 7 pc there were significant effects of vaccination treatment (P=0.006) and challenge treatment (P=0.003) with no significant interaction between these (P=0.489). There was also a significant effect of isolator nested within treatment combination (P<0.001). HVT vaccinated chickens were significantly lighter (221±6 g) than either sham- (245±6 g) or Bival-vaccinated (247±6 g) chickens (Figure 3.2). Surprisingly sham-challenged chickens were the lightest (218±8 g) being significantly lighter than those challenged by 02LAR (250±7 g) or FT158 (253±7 g). Those challenged by MPF57 had intermediate weights (229±7 g) and did not differ from the other treatments (Figure 3.3).

At day 14 pc the mean weight of all chickens was 538±7 g. The effects of vaccination (P=0.160, Figure 3.2) and challenge (P=0.437, Figure 3.3) were not significant and there was no significant interaction between these effects (P=0.102). The effect of isolator nested within treatment combination was the only significant factor in the model (P=0.003).

Survivor bodyweight at d56 pc was significantly affected by vaccination treatment (P=0.002), but not challenge treatment (p=0.079) with no interaction between these effects (P=0.225). The effect of isolator was also non-significant (P=0.691). Chickens vaccinated with bivalent vaccine had significantly higher BWT (3150±48g) than sham-vaccinated chickens (2878±61g) with HVT-vaccinated chickens (2997±49g) being intermediate and not differing significantly the others (Figure 3.2).

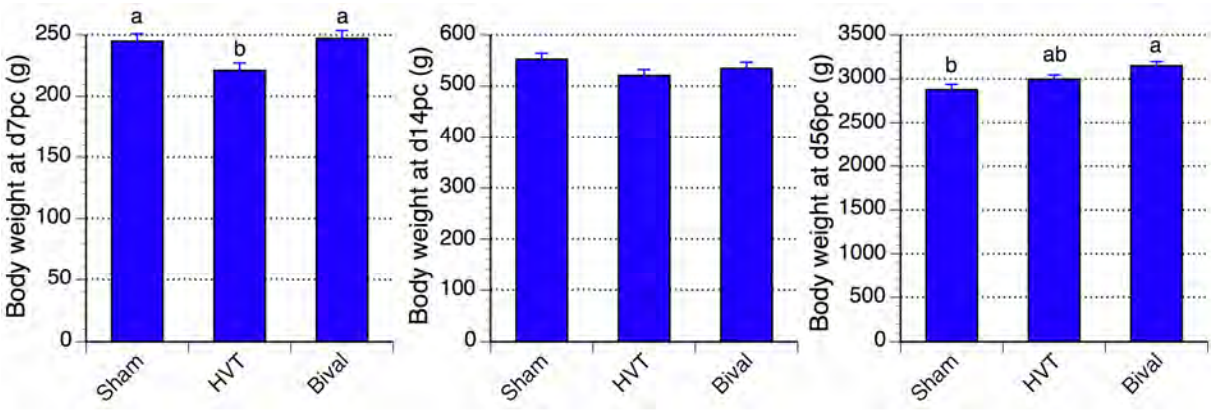

Figure 3.2. Chicken bodyweight (LSM±SEM) at days 7, 14 and 56 pc by vaccination treatment. All challenge treatments included (including sham-challenge). Columns not sharing a common letter in the superscript differ significantly (P<0.05). Where there are no letters there is no significant difference

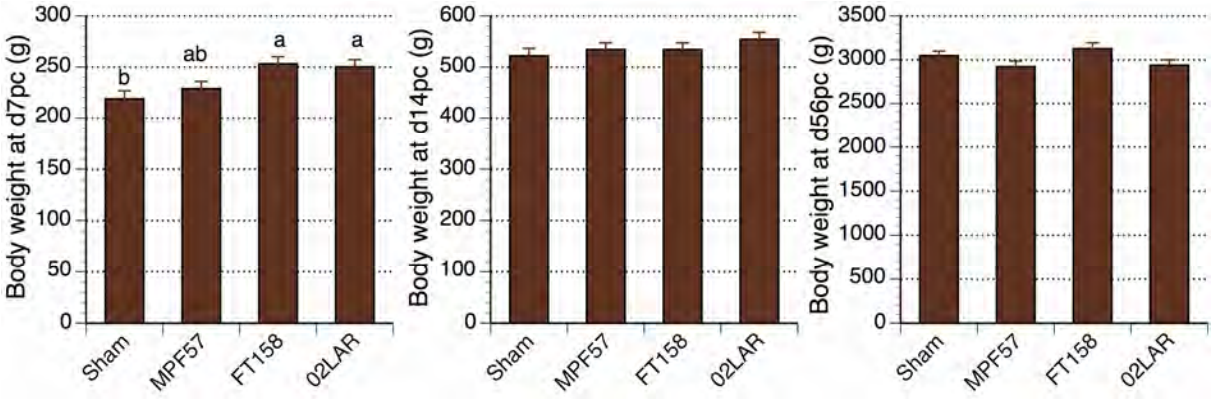

Figure 3.3. Chicken bodyweight (LSM±SEM) at days 7, 14 and 56 pc by MDV challenge treatment. All vaccination treatments included (including sham-vaccination). Columns not sharing a common letter in the superscript differ significantly (P<0.05). Where there are no letters there is no significant difference

At day 56 pc, surviving chickens with MD lesions weighed significantly less (P<0.001) than those without MD lesions (2849±89g v 3084±34g). On the other hand, chickens with *E. coli* lesions did not differ in weight from those without (2993±82 v 3057±30 g respectively, P=0.411).

Feed conversion efficiency (FE)

Mean unadjusted FE for the 24 isolators at d56 pc (day 61 of age) was 2.264±0.024 (range 2.060-2.455). Mean FE adjusted to 3kg bird weight was 2.259±0.009 (range 2.007-2.505). Mean FE adjusted to 2.45kg bird weight was 2.124±0.012 (range 1.857-2.354).

There was no effect of vaccination (P=0.86), challenge treatment (P=0.61) or interaction between these effects (P=0.24) of FE adjusted to 3kg with a similar result for analysis of unadjusted FE or FE adjusted to 2.45kg.

Immune organ weights

The overall effects of vaccination and challenge virus on the relative weights (organ weight as a % of bodyweight) of thymus, bursa and spleen at days 7, 14 and 56 pc are shown in Figures 3.4 and 3.5.

Thymus. There was significant protective effect of vaccination against thymic atrophy was observed at both day 7 pc (P=0.043) and day 14 pc (P=0.003) with no difference between the vaccines (Figure 3.4). There was significant interaction between the effects of vaccination and challenge at both dates with greater protective effects of vaccination seen in chickens challenged with 02LAR and FT158, than sham-challenged chickens or those challenged with MPF57. By day 56 pc the thymus was reduced to 30-40% of its relative size at the earlier ages, and there was no effect of vaccination. There was no significant overall effect of challenge virus on relative thymic weight at any age, although at days 7 and 14 pc there was a trend towards higher values in sham-vaccinated chickens (Figure 3.5). At

day 7 pc there was a significant linear reduction in thymic weight in unvaccinated chickens that were Sham challenged (0.25±0.02) or challenged with MPF57 (0.23±0.02), FT158 (0.19±0.2) and 02LAR (0.17±0.02). This effect was not evident at day 14 pc.

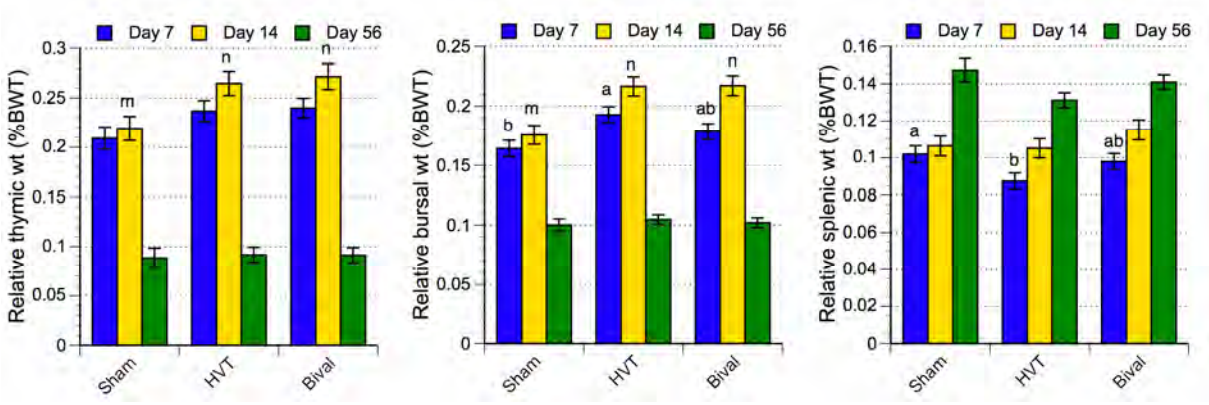

Figure 3.4. Relative weights (LSM±SEM) of thymus (left), bursa of Fabricius (centre) and spleen (right) at days 7, 14 and 56 pc by vaccination treatment. All vaccination treatments included (including sham-vaccination). Columns of the same day of measurement (ie colours) not sharing a common letter in the superscript differ significantly (P<0.05). Where there are no letters there is no significant difference.

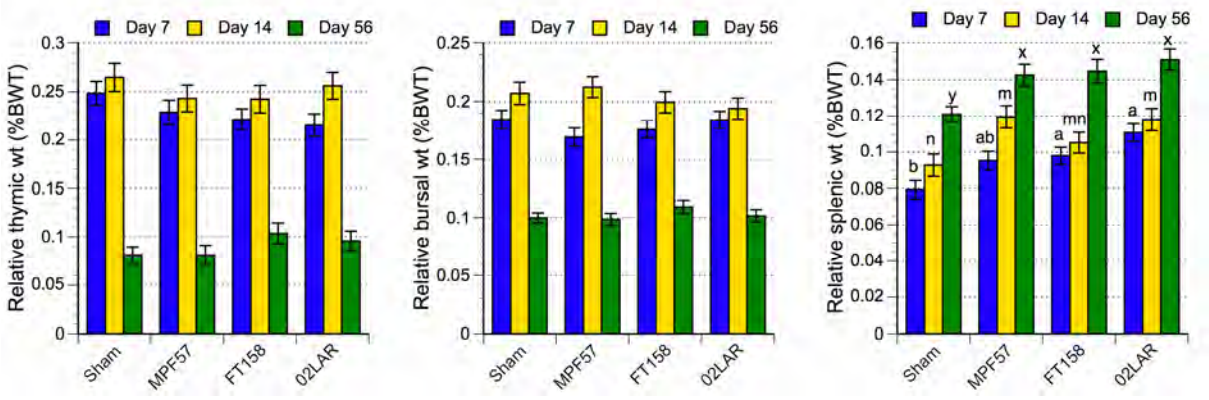

Figure 3.5. Relative weights (LSM±SEM) of thymus (left), bursa of Fabricius (centre) and spleen (right) at days 7, 14 and 56 pc by MDV challenge treatment. All vaccination treatments included (including sham-vaccination). Columns of the same day of measurement (ie colours) not sharing a common letter in the superscript differ significantly (P<0.05). Where there are no letters there is no significant difference.

Bursa of Fabricius. Again there was significant protective effect of vaccination against atrophy observed at both day 7 pc (P=0.006) and day 14 pc (P<0.0001) with no difference between the vaccines (Figure 3.4). Unlike the thymus, there was no significant interaction between the effects of vaccination and challenge although at both days 7 and 14 pc it was clear that the effects of vaccination were only seen in chickens challenged MDV and not in sham-challenged chickens. By day 56 pc the bursa was reduced to about half of its relative size at the earlier ages, and there was no effect of vaccination. There was no significant overall effect of challenge virus on relative bursal weight at any age (Figure 3.5).

Spleen. Unlike the thymus and bursa, infection with MDV is generally associated with increases in spleen size (splenomegaly). Thus sham-vaccinated chickens, unprotected against MDV1 tended to have larger relative spleen weights than vaccinated chickens (Figure 3.4) although the overall effect of vaccination only reached statistical significance at day 7 pc (P=0.006). As with the thymus, there was significant interaction between the effects of vaccination and challenge at day 7 pc (P=0.041) with a strong trend towards interaction at day 14 pc (P=0.010) with vaccination resulting in increased spleen size in sham-challenged chickens, but decreased size in chickens challenged with MDV1. Unlike the other immune organs, the relative size of the spleen increased throughout the experiment (Figure 3.4). The spleen was the most sensitive of the immune organs to the effect of challenge treatment (Figure 3.5) with a highly significant overall increase in relative spleen size following challenge with MDV1 at all ages (day 7 pc P<0.0001; day 14 pc P=0.004; day 56 pc P<0.0001). In each case there was no

difference between challenge viruses with the difference due to differences between MDV1 challenged and Sham-challenged chickens.

MDV1, HVT and MDV2 load in spleen

The load of MDV1 in spleen cells was significantly affected by day post challenge (P<0.0001), and vaccination (P<0.001), with significant interaction between the effects of day post challenge and vaccination (P=0.001, Figure 3.6 left) and day post challenge and challenge virus (P=0.022, Figure 3.6 right). The overall effect of challenge virus was not significant (P=0.09) and neither was the interaction between the effects of challenge virus and vaccination (P=0.214).

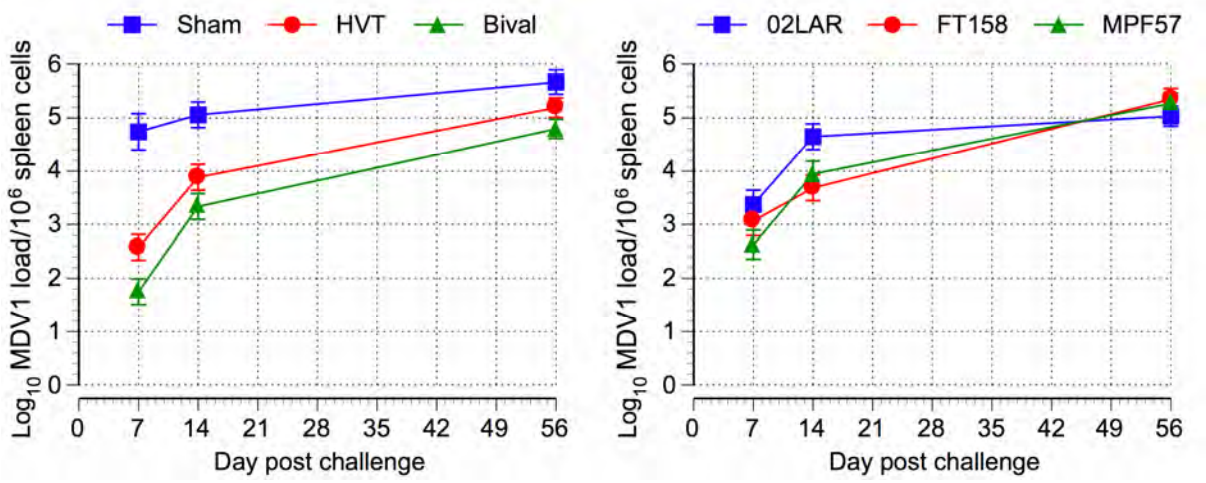

Figure 3.6. Load of MDV1 (LSM±SEM) in spleen of chickens at days 7, 14 and 56 post challenge, by vaccination treatment (left) or challenge virus (right). (See Table 3.2 for numbers of chickens).

Over all treatments Log_{10}MDV1 load per10^6 spleen cells increased significantly between day 7 pc (3.02±0.16) and day 14pc (4.09±0.14) and between these and day 56 (5.22±0.11). Sham-vaccinated chickens had higher overall MDV load (5.16±0.16) than those vaccinated with HVT (3.88±0.13) which in turn had significantly higher MDV load than those vaccinated with the bivalent vaccine (3.29±0.13). The significant interaction between the effects of day post challenge and vaccination was due to a decrease in the difference between vaccination treatments over time. In sham-vaccinated chickens MDV levels in spleen were comparatively high throughout the experiment whereas for the two vaccinated treatments MDV loads were initially low at day 7 pc, rising sharply at day 14 pc and then more slowly to day 56 pc (Figure 3.6 left panel). The significant interaction between the effects of day post challenge and challenge virus was due to different profiles in MDV load over time (Figure 5.6 right panel). Challenge with 02LAR induced the highest loads at days 7 and 14 but there was little increase between days 14 and 56. FT158 and MPF57 induced more gradual increases over time and induced arithmetically higher loads than 02LAR at day 56 pc despite having lower values initially.

HVT load in spleen cells of vaccinated but not challenged birds, was not influenced by vaccine type (HVT or Bivalent) at day 12, 19 or 61 post-vaccination. However there was a significant effect of day post vaccination, with higher values observed at day 61pv than days 19 or 12 (P=0.002; Figure 3.7). Least squares means (±SE) for log_{10}HVT load/10^6 spleen cells were 4.82±0.13, 4.19±0.13 and 4.23±0.12 respectively.

MDV2 load in spleen cells of bivalent vaccinated but not challenged birds, was significantly affected by day post vaccination. As with HVT higher values were observed at day 61pv than at days 19 or 12 (P=0.001; Figure 3.7). Least squares means (±SE) for log_{10}MDV2 load/10^6 spleen cells were 4.56±0.11, 3.81±0.16 and 3.76±0.27 respectively.

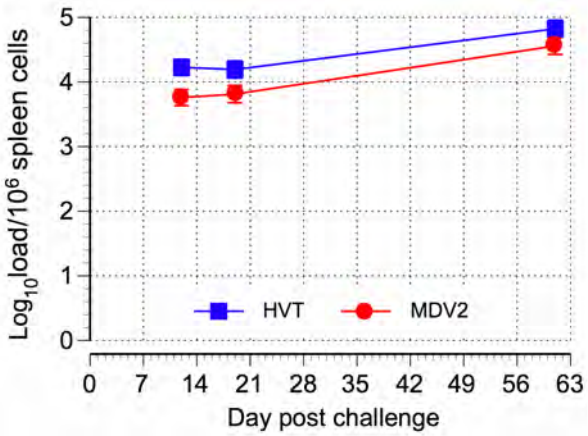

Figure 3.7. Load of HVT and MDV2 (LSM±SEM) in spleen of chickens at days 12, 19 and 61 post vaccination. Chickens vaccinated by both HVT and Bivalent vaccine are included as there was no effect of vaccine. Birds negative for either virus were excluded from the analysis to meet the assumptions of ANOVA (See Table 3.2 for numbers of chickens). All chickens were sham-vaccinated.

MDV, HVT and MDV2 load in isolator dust

A brief description is provided here taken from the paper of Islam and Walkden-Brown (2007).

MDV1 appeared in isolator exhaust dust from day 7 pc, increased markedly between days 14-28 and then plateaued until day 56. It was significantly affected by vaccination (P =0.002) and days post-challenge (P <0.0001) with significant interaction between these effects (P<0.0001, Fig. 3.8). However, the effect of challenge with three isolates of MDV1 (P =0.91) and its interaction with dpc (P = 0.083) were not significant (Fig. 3.8). The interaction between challenge virus and vaccination was also not significant (P = 0.257). Dander from sham-vaccinated chickens had significantly higher MDV1 load than that of HVT and bivalent-vaccinated chickens with this effect being most marked between days 14 and 28 (Fig. 3.8).

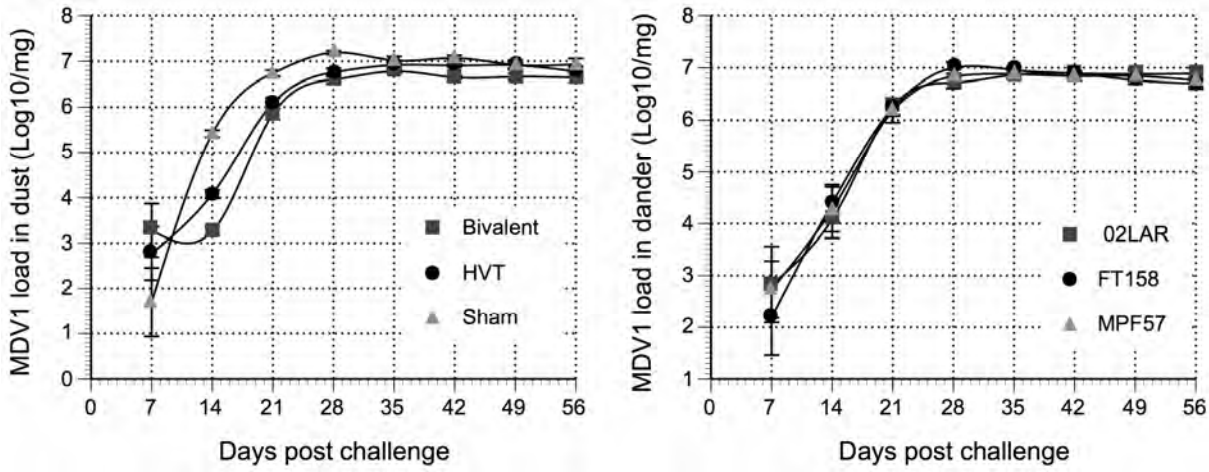

Figure 3.8. Load of MDV1 (LSM±SEM) in isolator exhaust dust at weekly intervals throughout the experiment. Only chickens challenged with MDV1 included. The overall effects of vaccination treatment (left) and challenge virus (right) are shown. Lines are smoothed splines.

HVT was present in isolator dust from day 7 post vaccination onwards in chickens vaccinated with HVT or Bivalent vaccine. Surprisingly the effect of challenge with MDV1 was highly significant (P <0.0001) with sham-challenged chickens having HVT loads in dust nearly two logs lower than that found in chickens challenged with MDV1 (Figure 3.9). There were no significant differences due to the different challenge viruses used. MDV load in dust peaked between days 21 and 28 post vaccination. The effect of vaccine type was significant (P = 0.031) with slightly higher overall values in chickens vaccinated with HVT (160,221 copies/mg dander) than those vaccinated with the bivalent vaccine (110,789 copies/mg dander)

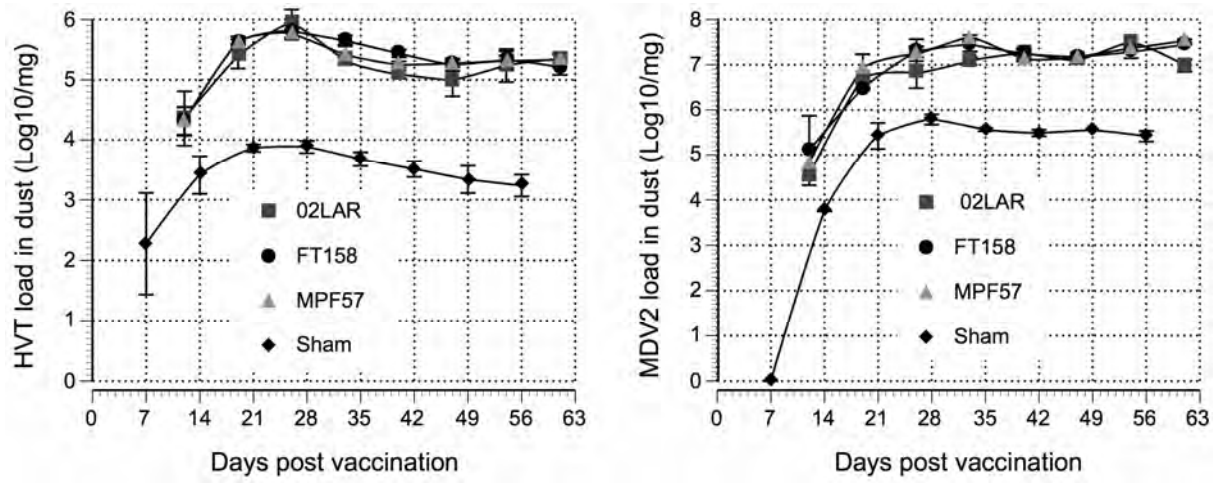

Figure 3.9. Load (LSM±SEM) of HVT (Left panel) and MDV2 (Right panel) in isolator exhaust dust at weekly intervals throughout the experiment showing the effect of challenge virus. The left panel includes chickens vaccinated with HVT and Bivalent vaccine, the right panel those vaccinated with Bivalent vaccine alone.

MDV2 was not present in dander sampled at day 7 post vaccination but was present from day 12 onwards. As for HVT, the effect of challenge treatment was highly significant (P<0.0001) as were the effects of days post vaccination and its interaction with challenge treatment (P< 0.001, Fig. 3.9). There were no significant differences due to the different challenge viruses used. In MDV1- challenged groups, MDV2 load in dander increased sharply between days 12 and 19 post vaccination and then slightly between 19 and 26 dpv after which little change occurred. In sham-challenged chickens a similar pattern was observed but at greatly reduced levels. In sham-challenged groups, MDV2 loads in dander also sharply increased between days 12-19 dpv and then slightly between days 19-26 post vaccination before stabilizing thereafter.

In some chickens all three MDVs were present, namely those vaccinated with Bivalent vaccine and challenged with MDV1. In these samples the level of the different serotypes of MDV varied significantly (P<0.001), and they also varied significantly over time, with significant interaction between these two effects (P <0.0001). Overall viral load of MDV2 (12.628 x 10^6/mg dust) was significantly higher than that of MDV1 (2.331 x 10^6/mg dust) which in turn was significantly higher than that of HVT (0.210 x 10^6/mg dust). The interaction between dpc and MDV is shown in Fig. 3.10 with clear differences in the pattern of viral load over time for the different MDV serotypes.

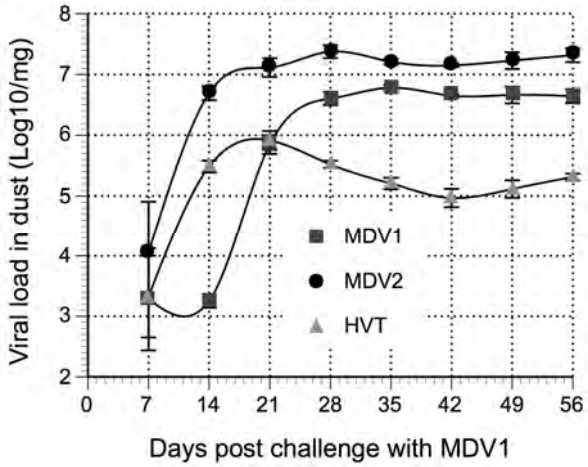

Figure 3.10. Load (LSM±SEM) of MDV1, HVT and MDV2 in isolator exhaust dust from chickens vaccinated with Bivalent vaccine and challenged with MDV1 (n=6 isolators).

Prediction of MD incidence

MDV load in spleen and isolator exhaust dust

MDV1 load in spleen was significantly positively associated with the ultimate incidence of MD in each isolator at days 7, 14 and 56 pc, with the strongest relationship evident at day 7 (Figure 3.11).

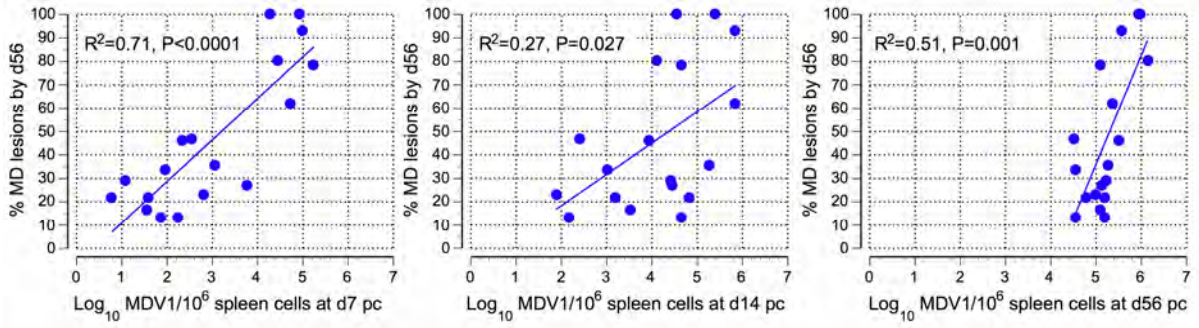

Figure 3.11. Association between MDV1 load in spleen at days 7, 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions) in chickens challenged with MDV1. Each point represents one isolator. The line is a linear regression curve.

At d7 pc, Log_{10} MDV1 in spleen accounted for 71% of the total variation between isolators in the incidence of MD.

MDV1 load in isolator exhaust dust at day 7 pc was significantly negatively associated with MD incidence by day 56 pc, but at days 14 and 21 there was strong positive association between MDV1 in dust and MD incidence (Figure 3.12). The strongest association was at day 21, but the greatest range in MDV1 load values at day 14. The positive association remained strong at day 28 pc (R^2=0.55. P<0.001) but from days 35 to 56 there was no association at all between MDV1 load in isolator dust and ultimate incidence of
MD.

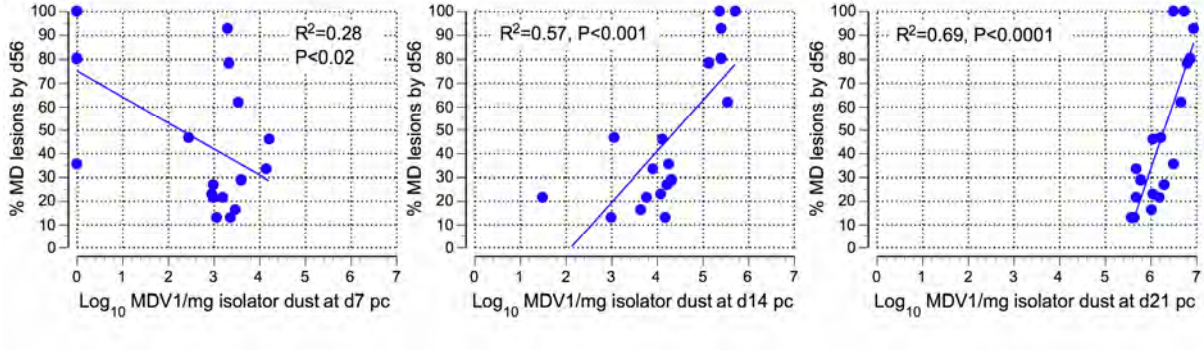

Figure 3.12. Association between MDV1 load in isolator exhaust dust at days 7, 14 and 21 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions) in chickens challenged with MDV1. Each point represents one isolator. The line is a linear regression curve.

HVT and MDV2 load isolator exhaust dust

HVT and MDV2 were not measured in the spleens of chickens challenged with MDV1. However they were measured in isolator exhaust dust of vaccinated, challenged chickens weekly from d7 to d56 pc. There was no significant association between the mean level of either vaccinal virus in dust and the incidence of MD, and no trend towards an association. The direction of association was not consistent for either vaccine virus.

Relative immune organ weight

There was a significant negative association between relative thymic weight at days 7 and 14 pc, and the incidence of MD by day 56 pc, although the association was not particularly strong (Figure 3.13). Interestingly, by day 56 pc, the direction of the association had reversed although it was not significant (Figure 3.13).

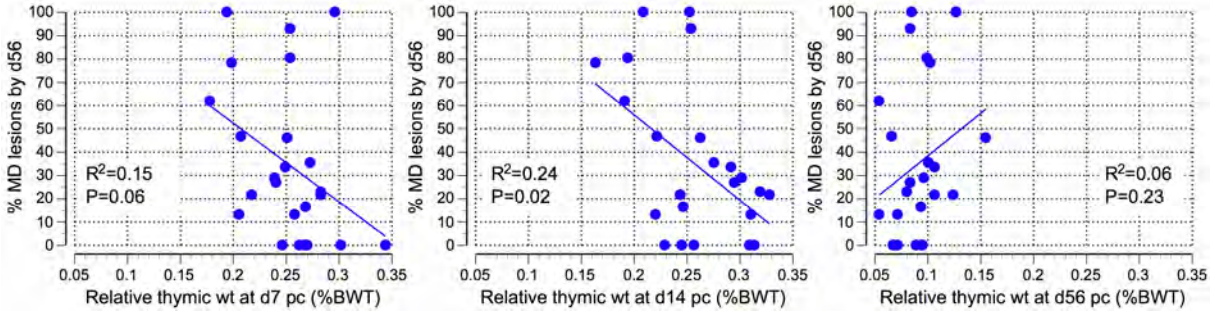

Figure 3.13 Association between relative thymic weight (% of bodyweight) at days 7, 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator. Sham-challenged isolators are included. Chickens with tumours of the thymus are excluded. Relative thymic weights are adjusted for the effect of operator. The line is a linear regression curve.

The situation was similar for relative bursal weight, was a significant negative association between relative bursal weight at days 7 and 14 pc, and the incidence of MD by day 56 pc and a non-significant relationship in the reverse direction at day 56 pc (Figure 3.14).

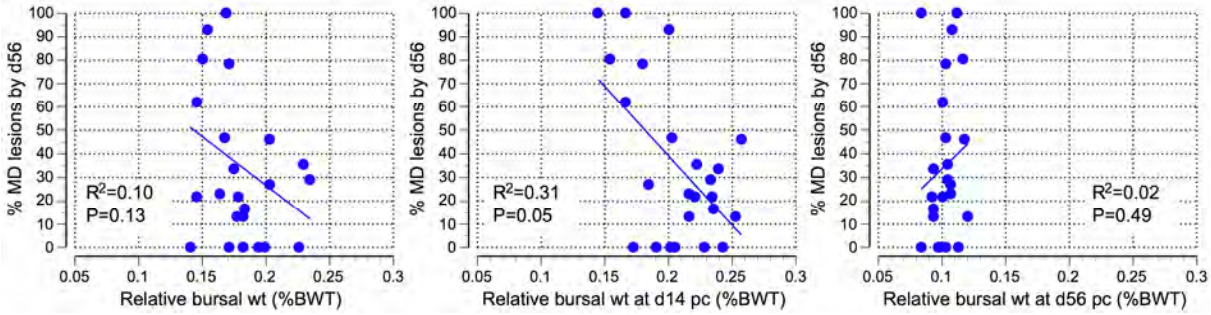

Figure 3.14 Association between relative bursal weight (% of bodyweight) at days 7, 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator. Sham-challenged isolators are included. Chickens with tumours of the bursa are excluded. Relative bursal weights are adjusted for the effect of operator. The line is a linear regression curve.

Relative splenic weight was significantly and positively associated with the incidence of MD by day 56 pc throughout the experiment as evidenced by significant relationships at days 7, 14 and 56 pc (Figure 3.15). In general the associations were stronger than for bursal or thymic weight.

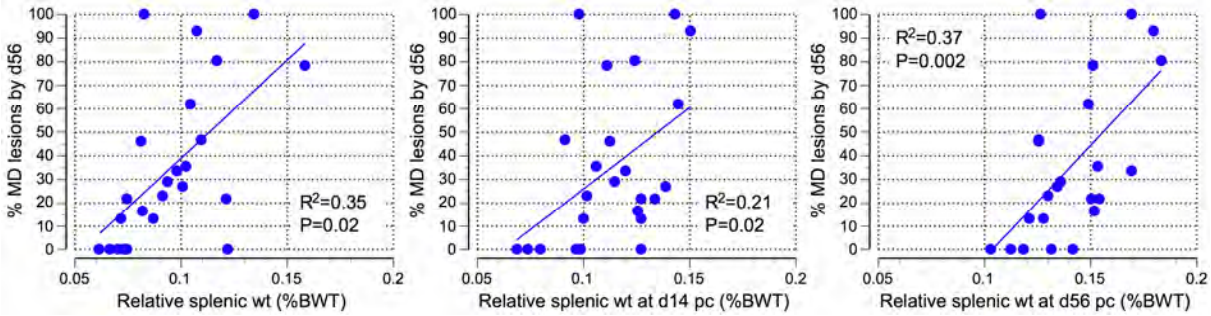

Figure 3.15 Association between relative splenic weight (% of bodyweight) at days 7, 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator. Sham-challenged isolators are included. Chickens with tumours of the spleen are excluded. Relative splenic weights are adjusted for the effect of operator. The line is a linear regression curve.

Use of K-means cluster analysis of relative thymic, splenic and bursal weights of individual birds to identify patterns of association between the 3 organs enabled more accurate early prediction of MD than any individual organ measurement but R^2 values increased to approximately 0.4 - 0.5 depending on the settings used. The system adopted uses a 3 point scale and essentially differentiates chickens with relatively large spleens and relatively small bursal and thymic weights (putatively MD positive; score 1), those with the reverse (putatively MD negative; score 3) with those being intermediate being scored 2.

Use of the data from all 3 variables in stepwise regression equations also produced more accurate prediction of MD% than individual measurements at days 7 and 14 pc, but not day 56 pc. Equations are given below.

Day 7 pc. d56MD% = 26.5 + (765.2*Rel splenic wt) - (256.0*Rel thymic wt). R^2=0.43

P values of 0.004 and 0.095 for the two coefficients respectively. Relative bursal wt was dropped from the model with a P value of 0.80

Day 14 pc. d56MD% = 115.2 + (625.1*Rel splenic wt) - (282.2*Rel thymic wt) - (378.0*Rel bursal wt). R^2=0.57

P values of 0.011, 0.026 and 0.037 for the three coefficients respectively.

Day 56 pc. d56MD% = -99.6 + (960.8*Rel splenic wt). R^2=0.37

P value of 0.002 for the coefficient. Relative bursal wt (P=0.79) and Relative thymic weight (P=0.74) were dropped from the model.

E. coli lesion severity score

Lesion scores following *E. coli* challenge at day 49 pc were weakly positively associated with MD incidence at day 56 (Figure 3.16).

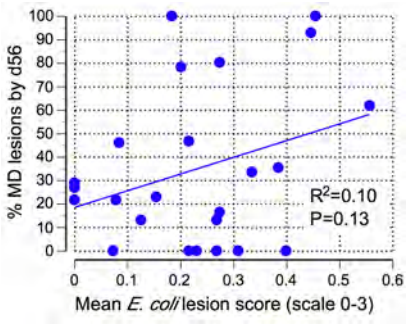

Figure 3.16 Association between mean *E. coli* lesion score (0-3 in ascending order of severity) following challenge at day 49 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator. Sham-challenged isolators are included. The line is a linear regression curve.

Brief discussion and conclusions

The experiment was successfully implemented although there was some low level contamination of spleen samples collected at post-mortem in a contaminated environment, and also some apparent residual DNA contamination of equipment which showed up in day 7 dust samples from some control isolators, but not thereafter. This is the first pathotyping experiment in Australia using bivalent vaccine as per the USDA system, and the first experiment anywhere in which quantitative profiling of all three MDV serotypes in spleen and shed dander has been done.

The three viruses used appeared broadly similar, causing significant MD-associated mortality (33-50%) and MD (70-96%) in unvaccinated chickens. MPF57 induced the highest level of MD in unvaccinated chickens (96.4%), but the lowest level in vaccinated chickens (16.1%) with the result that it had a significantly higher protective index (83.2%) than either 02LAR (58.2%) or FT158 (59.9%). This raises the issue of whether "virulence" (the ability to induce disease) and "vaccine resistance" (the ability to induce disease in a vaccinated host) are the same trait. The USDA ADOL method ascribes pathotype to the latter, and it is often taken to mean virulence. Certainly the lower "pathotype" of MPF57 was not evident in the viral load data in spleen or dust, with the 3 viruses showing very similar profiles during the experiment. However with regards to hypothesis 1, the overall ranking of the MDV1 isolates for pathogenicity (VR) was as in SPF chickens, although there was no effective difference between 02LAR and MPF57.

The HVT and the bivalent vaccine produced the same level of protection against these challenge viruses overall, although the bivalent vaccine resulted in significantly lower load of MDV1 in spleen throughout the experiment, and in dust at day 14 pc. Based on the levels MD-induced mortality and the levels of vaccinal protection obtained with bivalent vaccine (58-82%) the viruses are likely to fall in the v to vv category on the USDA ADOL system. The absence of reference strains for comparison,

and the different host system used make it impossible to be definitive about this. However it is clear that the isolates were not vv+ MDV.

The MDV1 isolates used induced none of the early paralysis/mortality syndrome observed when they were used in SPF chickens and neither did they cause the severe immune organ atrophy observed in SPF chickens (Walkden-Brown *et al.* 2006). These differences are almost certainly due to the presence of maternal antibody against MDV in the current experiment, which is not present in SPF chickens (Chubb and Churchill 1969). Maternal antibody is implicated in another observation in this experiment, namely that sham vaccinated chickens had a lower level of shedding of MDV at day 7 than vaccinated chickens (Figure 3.8) and that in fact at day 7 there was a significant negative association between the level of MDV1 shedding in challenged chickens and the subsequent incidence of MD (Figure 3.12). Similar results have been observed in an earlier experiment (Islam *et al.* 2007) and a possible explanation is a sparing effect of vaccinal virus on the inactivation of MDV1 by maternal antibody. In chicks challenged only with 500pfu of MDV1, the only target for mab is this virus, while for those also vaccinated with 8000pfu of vaccinal virus at day 0, it is possible that much of the mab has formed immune complexes with vaccinal virus prior to challenge, thus providing a sparing effect on the challenge virus.

Measurement of bird performance revealed few major effects of challenge or vaccination. Bivalent vaccinated chickens were heavier at day 56 pc than HVT or sham-vaccinated chickens, but there were no significant effects at days 7 or 14. Similarly there was no effect of challenge or vaccination on feed conversion efficiency. These effects are confounded to some extent by the death of many chickens (presumably lighter) in the more susceptible treatments. In two separate experiments in Cobb broiler chickens challenged at days 0 or 3 of age/post vaccination with MPF57 and weighed weekly up to the end of the experiment at day 35 pc (Islam *et al.* 2002) observed bodyweight reductions due to MDV1 challenge at days 28 and 35 pc but vaccination was not protective against this.

The late *E. coli* challenge used in this experiment (day 49 pc) confirmed the more definitive findings of (Islam *et al.* 2002) that challenge with MDV greatly increases susceptibility to colibacillosis but extends the finding from day 28 to day 49 post challenge, a period during which chickens with MD are in a period of permanent immunosuppression (Calnek 2001).

The ability to quantify MDV1 and the vaccinal viruses in spleen and feather dander is a significant advance and this experiment both demonstrates the utility of these measurements, and validates them in the sense that the findings are broadly consistent with expectations. Key observations are:

a) MDV load in spleen in unvaccinated chickens increased by only 1 log between days 7 and 56, whereas in feather dander, shedding is extremely low at day 7, increases rapidly to day 28 by more than 5 logs, and declines slightly thereafter. Thus MDV load in dander appears to lag considerably behind that is spleen in addition to having a quite different pattern of increase.

b) The suppressive effects of vaccination on MDV load are:

   - Greater in spleen than in feather dander
   - Most pronounced in the first few weeks after infection
   - At day 7 in feather dander, vaccination actually enhances rather than suppresses shedding of MDV.

c) Challenge with MDV1 greatly increases the shedding rate of vaccinal virus, a finding that is now published separately (Islam and Walkden-Brown 2007).

One of the aims of the experiment was to examine methods of predicting MD outcomes other than through the running of a full pathotyping experiment.

By far the best predictor was the level of MDV1 in spleen or dust shed from isolators. The level of MDV1 in spleen at day 7 pc had a very strong relationship with subsequent MD incidence (R^2=0.71). For reasons that are not clear, the relationship was much weaker 7 days later, being non-significant with an R^2=0.27. However at day 56 pc the relationship was again significant and strong (R^2 0.51), suggestive of a role for this variable at any age as a predictor of future MD. With regards isolator exhaust dust, strong relationships were seen at weeks 2-4 post-challenge (Figure 3.12), but a poor relationship was evident at day 7 for reasons discussed above. Measurement of MDV1 load in spleen

or dust in groups of birds in the first few weeks prior to challenge appears to have excellent potential to predict MD outcomes and replace full pathotyping experiments. These measurements can be made prior to the onset of clinical MD, offering significant ethical advantages. Interestingly, in vaccinated chickens, the level of vaccinal virus in isolator exhaust dust bore no relationship to final MD outcome. This is due to the novel finding in this experiment that challenge with MDV1 greatly increases the shedding rate of vaccinal virus.

Unfortunately the strong association between MDV1 load and subsequent incidence of MD did not hold for relative immune organ weights. While these are a sensitive measure of immunosuppression and MD outcomes in SPF chickens without maternal antibody (Calnek *et al.* 1998; Walkden-Brown *et al.* 2006) in commercial birds with commercial antibody their efficacy is much reduced due to the immune organ sparing actions of maternal antibody. No single immune organ provided accurate prediction of MD incidence although spleen enlargement had the most consistent association. Nevertheless, by combining the measurements of 2 or 3 organ weights in stepwise regression equations, predictive power approaching that of MDV1 load in spleen and dander was achieved at days 7 and 14 pc. Unfortunately, the labour required to measure thymic weight in experiments such as these, precludes its use as a practical means of early prediction of MD incidence. Apart from being labour intensive, there are also significant operator effects observed for thymic weight which do not occur for spleen or bursa.

Based on the observations of (Islam *et al.* 2002) that susceptibility to *E. coli* infection was highly dependant on MD status, it was thought that challenge with *E. coli* as a demonstration of functional immunosuppression, may be a more sensitive marker of MD infection status than immune organ weights. However lesions were only seen in a comparatively small proportion of challenged birds (17.1%) and the association between mean *E. coli* lesion score and MD incidence was non-significant (P=0.13) although in the in the expected direction. Given this poor relationship, the availability of better indicators and the ethical and other practical problems associated with *E. coli* challenge, it should be discarded as a potential detector of MD status.

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Chapter 4: Experiment 3. MD05-C-PT3

"1st pathotyping experiment in layer chickens"

Start: 9/6/05 **Completion: 9/8/05** **AEC: UNE AEC05/076**

Introduction

With the successful completion of the 1st pathotyping experiment in commercial broilers, the next experiment was to test the same viruses and concepts in commercial layer chickens. This would complete a round of experiments in SPF, broiler and layer chickens using the same batches of challenge and vaccinal viruses under the same experimental conditions. Unfortunately there was insufficient stock of the challenge virus FT158, so it was substituted with 04CRE, an MDV isolate from Sydney initially isolated from dust into SPF chickens at UNE then grown to high titre in cell culture at RMIT.

The specific hypotheses under test were:

1. Pathotyping rankings (virulence rank) will be in the same order as those derived from experiments in SPF and commercial Cobb broiler chickens (02LAR>MPF57).
2. Patterns of MDV load in spleen and dander over time will be similar to those observed in Cobb broiler chickens.
3. Thymus bursal and spleen weights at day 14 will be moderately good predictors of pathogenicity.
4. MDV load in spleen at day 14 will be good predictors of pathogenicity.
5. The rate of MDV shedding in dander will be positively associated with pathogenicity.

The pathotyping aspects of the project were led and implemented by Dr Steve Walkden-Brown. A UNE PhD student, Ms Katrin Renz was also heavily involved in the execution of the experiment and made a large number of additional measurements, unrelated to the main objectives of the CRC project. Paul Reynolds was also a key person involved in the implementation of the project and Dr Fakhrul Islam and others assisted on the major sampling days.

Materials and methods

The experiment involved 535 chickens in a 3x3 factorial design with two replicates using 18 positive pressure isolators (3x3x2=18) with an additional two isolators acting as unchallenged controls (total isolators = 20, approx 27 birds/isolator). The two experimental factors were:

Vaccine virus (3) ie. Unvaccinated, HVT and Bivalent (HVT/SB1) vaccine @8000pfu/bird. Chickens were vaccinated sc in 200ul diluent on the day of hatch (day 0). Unvaccinated chickens were sham-vaccinated was with diluent only.

Challenge virus (3) ie. MPF57 (reference strain), 02-LAR and 04CRE @500pfu/bird. Challenge was intra-abdominal at day 5 post-vaccination. The unvaccinated controls were sham-challenged with diluent only.

04CRE was used in this experiment, as available stocks of FT158 were insufficient. 04CRE was isolated from 6 week-old IsaBrown layer pullets from an MD outbreak in Sydney in 2004. It was of intermediate pathogenicity (HVT PI of 52.8%) in an RIRDC experiment in SPF chickens (MD04-R2-PT2). Details of the viruses used are shown in Table 1.

The experimental chickens were female IsaBrown commercial layer chickens from Baiada's Kootingal Hatchery. The chickens were unvaccinated but came from a Rispens-vaccinated parent flock (Dungowan 1).

Chickens were fed *ad lib* on commercial layer starter then finisher (Ridley Agricultural Products, Tamworth). Chicks were exposed to 12:12 light dark in positive pressure isolators. There were 27

chickens/isolator placed initially (54 per treatment combination). The experiment started on the day of hatch (day 0, 9/6/05) and was terminated on 9/8/05 (day 61, day 56 post challenge).

At day 14 pc six chickens per isolator were removed and liveweight, and immune organ weights (bursa, thymus and spleen) were determined. At day 28 pc all chickens were beak trimmed within their isolators to alleviate feather pecking problems which had developed.

For determination of MDV1, HVT and MDV2 load in dander, samples were collected weekly from the dust deposits at the 90 degree bend in the exhaust air outlet duct of each isolator as described in the General Materials and Methods section.

All chickens dying during the experiment were weighed and examined post-mortem for gross MD lesions as described in the General Materials and Methods section.

At day 56 pc the experiment was terminated and all surviving chickens were humanely sacrificed, weighed examined for gross MD lesions and had immune organ weights determined. Diagnosis of MD was by 3 operators Drs Steve Walkden-Brown, Fakhrul Islam and Justin Bailey (BVSc).

Selected spleen samples from day 14 were subject to DNA extraction and seorype-specific qPCR assay for MDV1, HVT or MDV2 as described in the General Materials and Methods section.

Table 4.1. Viruses used in Experiment 3

Viral isolate	Batch number	Dose used	Year of isolation	Place of origin	Flock of origin	Vaccination history
MPF57 B1 (179/6)	P7 200904	500pfu[a]	1994	NSW (Sydney)	Layer (14wo IsaBrown)	Unknown
04CRE (179/2)		500pfu[a]	2004	NSW (Sydney)	Layers 6wo pullets	Rispens
02LAR (179/3)	P6 120904	500pfu[a]	2002	Victoria (Mornington P)	Broilers	Unknown
HVT vaccine FC126	H02308	8000pfu	?	Bioproperties - USA		
Vaxsafe SBH vaccine SB1/HVT FC126	SBH4101	8000pfu combined	?	Bioproperties - USA		

[a]Titrated on CK cells at RMIT.

Statistical analysis. Analyses were performed with JMP6 (SAS Institute Inc. 2006). Where the chicken was the experimental unit measured, discrete data such as mortality (died/survived), presence of MD (positive/negative) were analysed using a generalized linear model with a binomial link function (logistic) fitting the effects of vaccination, challenge treatment and their interaction. Differences between main effects were tested by contrasts within the model while differences between individual treatment groups were tested by individual Chi-square tests. Mortality data were also analysed using survival analysis (Kaplan-Meier method).

Continuous data (eg bodyweight) were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction directly. MDV1 load in spleen or dander was log transformed [Log_{10} (x + 1)] prior to fitting the effects of day post challenge, vaccination, challenge treatment and their interactions in a general linear model. Relative immune organ weights (thymus, bursa and spleen) were expressed as % of bodyweight and analysed using a similar model but with the effect of operator/dissector fitted, and day post challenge removed (Separate analysed were used for each dpc). Birds with tumours of the given organ were excluded from the analysis. Vaccinal protective index (PI) based on the presence of gross MD lesions alone, was calculated as (%MD in unvaccinated chickens - %MD in vaccinated chickens) / (%MD in unvaccinated chickens). Virulence rank (VR) was calculated as 100-PI (Witter 1997). Only chickens at risk of MD were included in the calculation (ie alive at the time of the first MD case). For these data (PI and VR) which could only be measured on a whole isolator, data were analysed using a general linear model fitting the effects of vaccination, challenge treatment and their interaction. For this analysis there were only 2 measures per treatment combination (two isolators per treatment combination). For continuous variables least squares means and standard errors are presented.

Results

Application of treatments

All vaccination treatments were successfully applied and maintained, as determined by MDV1 serotype-specific real-time qPCR (Table 4.2). Levels of vaccinal virus in spleen and dust are shown later in the results.

In day 14 pc spleen samples 02LAR and MPF57 induced uniformly high proportions of MDV1 positive chicks (83-100%) but this was not the case with 04CRE which induced 92% positives in unvaccinated chickens but on 66.7% and 25% positives in chickens vaccinated with HVT or Bivalent vaccine respectively (Table 4.2). There was some background contamination of samples as in the previous experiment, and this was corrected for by subtraction of the maximum background level from all sample values. On control chicken had higher than background levels of infection despite no subsequent evidence of MDV1 in that isolator as determined by weekly qPCR of dust and absence of clinical or pathological signs of MD. HVT was detected in 13/13 vaccinated chickens challenged by the different MDV1s. No assays for MDV2 were done on spleen samples.

Table 4.2 Summary of number of chicken spleen samples positive for MDV1 and HVT at d14 pc.

Vaccine	Challenge	qPCR test	Number of chickens positive to qPCR test of spleen (%)
Sham	02LAR	MDV1	12/12 (100%)
Sham	04CRE	MDV1	11/12 (91.7%)
Sham	MPF57	MDV1	12/12 (100%)
Sham	Sham	MDV1	1/12* (0%)
HVT	02LAR	MDV1	12/12 (100%)
HVT	04CRE	MDV1	8/12 (66. 7%)
HVT	MPF57	MDV1	11/12 (91.7%)
Bival	02LAR	MDV1	12/12 (100%)
Bival	04CRE	MDV1	3/12 (25%)
Bival	MPF57	MDV1	10/12 (83.3%)
HVT	All 3	HVT	13/13 (100%)

* 3 spleens contained trace MDV1 contamination. All values in the experiment were adjusted by subtracting the maximum contamination amount. One sample had greater than trace amounts despite no subsequent evidence of infection in the isolator.

Weekly dust samples were collected from the 2 sham-challenged unvaccinated isolators. As in Experiment 2, trace amounts of MDV1 were detected occasionally with no evidence of infection in the isolators. Trace levels were detected in both isolators at days 7 and 14, but they were subsequently negative for MDV from weeks 2-8 apart from traces in one of the isolators at weeks 6 and 7. Dust data are corrected by subtracting this trace contamination from all values in the experiment. In all of the isolators containing chicks vaccinated with HVT or bivalent vaccine HVT detected was detected at each weekly sampling and the same was true for MDV2 in isolators vaccinated with bivalent vaccine.

Mortality/Survival

Fifteen of 535 eligible chickens (2.8%) died of miscellaneous causes (mostly bacterial infections) up to d5 pc (ie day 10 of experiment). The next mortality occurred on d22 pc and this and all subsequent mortality is included in the analysis of mortality with the exception of 7 chickens which were either removed from the experiment for other uses or died of causes unrelated to MD (2 removed, 2 died due to cannibalism, 2 due to post-beak-trimming haemorrhage and one asphyxiated in a gauntlet). Also excluded were 120 chickens sacrificed at day 14 pc. Thus of the 393 eligible chickens at risk of death from d6 pc onwards, 69 died up to d56 pc (17.6%) while the remainder were sacrificed on this date. The first MD lesions were detected at d22 pc and 63/69 chickens with eligible mortality had MD lesions. Mortality by treatment is summarised in Table 4.3.

There were significant effects of both vaccination ($P<0.0001$) and challenge treatment ($P=0.003$) on total mortality with no interaction between these effects ($P=0.27$). Sham-challenged chickens were excluded from the analysis as they were not part of the factorial design. Regarding vaccination, significantly more unvaccinated chickens (38.4%) died than those vaccinated with either HVT (13.0%) or Bivalent vaccine (8.2%) with no difference between the latter two treatments. Regarding

challenge, no unvaccinated chickens died (excluded from the analysis) than those challenged with 02LAR had a significantly higher mortality rate (28.3%) than those challenged with 04CRE (16.2%) or MPF57 (13.3%).

Table 4.3 Mortality between days 6 and 56 post-challenge of the experiment. Includes all chickens eligible to die after day 5 pc, excluding chickens sacrificed at day 14 for sample collection or which were removed or had accidental or non-MD related causes of death.

Vaccination	Challenge	n	Total mortality n (%)	Mortality with MD lesions (%)
Sham	02LAR	37	18 (48.6%)	17 (45.9%)
Sham	04CRE	39	12 (30.8%)	11 (28.2%)
Sham	MPF57	36	13 (36.1%)	12 (33.3%)
Sham	Sham	36	0 (0%)	0 (0%)
HVT	02LAR	41	11 (26.8%)	9 (21.9%)
HVT	04CRE	40	4 (10.0%)	4 (10.0%)
HVT	MPF57	42	1 (2.4%)	1 (2.4%)
Bival	02LAR	42	5 (11.9%)	5 (11.9%)
Bival	04CRE	38	3 (7.9%)	3 (7.9%)
Bival	MPF57	42	2 (4.8%)	1 (4.8%)
All	All	393	69 (17.6%)	63 (16.0%)

Survival analysis similarly produced similar results revealed with significant differences between the patterns of mortality of unvaccinated chickens compared to chickens vaccinated with HVT or bivalent vaccine (P vaccines under test, or the 3 viruses, with significant effects due to the sham-vaccination and sham-challenge treatments only (Figure 4.1).

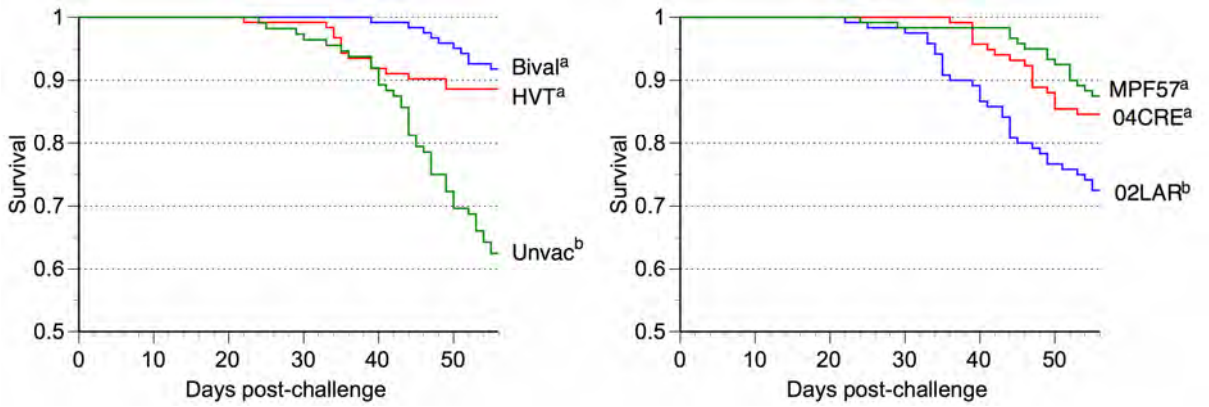

Figure 4.1. Survival analysis of the effects of vaccination (left plot) and challenge treatment (right plot) on survival of eligible chickens to day 56 pc. Treatments not sharing a common letter in the superscript differ significantly (P<0.05).

Incidence of MD, vaccinal protective index and virulence rank.

The combined incidence of gross MD lesions in the 393 at risk chickens that died with MD or had MD lesions on post mortem after sacrifice at day 56 is summarized by treatment in Table 4.4. Amongst MDV1-challenged chickens there was a significant effect of vaccination (P<0.0001) and challenge virus (P<0.0001) on the percentage of chickens with MD with no interaction between these effects (P=0.67). Unvaccinated birds had a significantly higher incidence of MD (70.5%) than birds vaccinated with HVT (41.5%) which in turn had significantly higher incidence of MD than those vaccinated with Bivalent vaccine (24.6%). For the challenge viruses 02LAR induced a higher overall incidence of MD (57.5%) than MPF57 (45.8%) which in turn was higher than that induced by 04CRE (30.8).

Analysis of PI (%) by isolator replicate revealed a significant effect of vaccine virus (P=0.03) but not challenge (P=0.09) with no interaction between these effects (P=0.70). Bivalent vaccine induced significantly higher protection (67.2±6.9) than HVT vaccine (43.8±6.9). While the challenge viruses

did not differ significantly there was a strong trend for 04CRE to have higher PI (73.1±8.5%), than MPF57 (48.4±8.5%) and 02LAR (45.1±8.5) and indeed a specific contrast comparing 04CRE against the other two challenge viruses was significant (P=0.03).

Table 4.4. Incidence of MD to day 56 pc, vaccinal protective index (PI%) and virulence rank (VR%) by challenge virus and vaccination treatment.

Variable	Vaccination treatment	MDV1 challenge				Overall (Excl Sham chall)
		Sham	MPF57	02LAR	04CRE	
MD (%)	Sham	0 (0/36)	69.4^{axy} (25/36)	83.8^{ax} (31/37)	59^{ay} (23/39)	70.5 (79/112)^{a}
MD (%)	HVT		42.9^{bx} (18/42)	61.0^{bx} (25/41)	20.0^{bz} (8/40)	41.5 (51/123)^{b}
MD (%)	Bival		28.6^{bx} (12/42)	31.0^{cx} (13/42)	13.2^{bx} (5/38)	24.6 (30/122)^{c}
MD (%)	All	0 (0/36)	45.8 (55/120)^{y}	57.5 (69/120)^{x}	30.8 (36/117)^{z}	
PI (%)	HVT		38.3	27.2	66.1	
PI (%)	Bival		58.9	63.1	77.7	
VR (%)	HVT		61.7	72.8	33.9	
VR (%)	Bival		41.1	36.9	22.3	

^{xy} MD Incidence. Means within rows not sharing a common letter differ significantly (P<0.05)
^{ab} MD Incidence. Means within columns not sharing a common letter differ significantly (P<0.05)

As VR is derived from PI the analysis of VR produced the same level of significance for effects as PI. The main finding is that the VR of 02LAR (54.9±8.5%) and MPF57 (51.6±8.5%) combined was significantly higher than that of 04CRE (26.8±8.5%).

Distribution, number and severity of MD lesions

The overall distribution of MD lymphomas and the average severity of the lesions (score 0-3 with 3 most severe) are shown in Table 4.5. The mean number of organs with lymphomas in MD positive chickens was 2.56. This was significantly affected by vaccination (P=0.04) with significantly more organs affected in unvaccinated chickens (2.84±0.16) than those vaccinated with HVT (2.14±0.22). Bivalent-vaccinated chickens were intermediate (2.56±0.28) and did not differ from the other groups. Challenge virus did not affect the number of organs involved (P=0.26; 2.68±0.28, 2.63±0.18 and 2.22±0.20 for 04CRE, 02LAR and MPF57 respectively).

Table 4.5. Overall distribution of MD lymphomas in different organs, and average severity of the lesions (score 0-3). Numbers relate to a total of 160 chickens with MD lymphomas.

Organ	n with MD lesions in organ	Incidence of lesions (% of total cases)	Mean severity score
Ovary	127	79.4	2.08
Liver	76	47.5	1.76
Spleen	75	46.9	1.65
Kidney	40	25.0	1.93
Heart	21	13.1	2.00
Eye	15	9.4	1.67
Lung	12	7.5	2.08
Sciatic nerve	10	6.3	1.30
Mesenteries	8	5.0	1.88
Thymus	7	4.4	2.43
Proventriculus	5	3.1	2.20
Muscle	4	2.5	2.00
Skin	4	2.5	2.00
Pancreas	4	2.5	2.75

The mean severity score was 1.85 with no overall influence of vaccination (P=0.11) or challenge (P=0.08) although there was significant interaction between these effects (P=0.03). The significant interaction revealed that vaccination had little effect on MD severity in chickens challenged with

02LAR, with increasing effects of vaccination when challenged with 04CRE and particularly MPF57 (Figure 4.2). Overall there were non-significant trends towards lower severity in HVT but not Bivalent vaccinated chickens, and in chickens challenged with MPF57 rather than 02LAR or 04CRE.

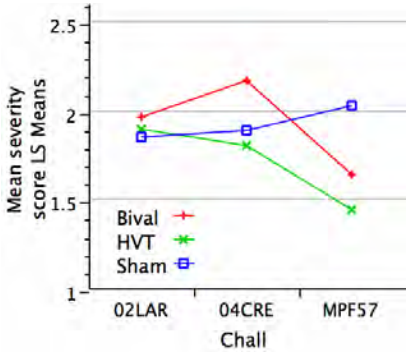

Figure 4.2. Interaction plot showing the differential effect of vaccination on the MD lesion severity score induced by the different challenge viruses.

Bodyweight and immune organ weights

Statistical analysis of these variables used the complete 3 x 3 factorial design (3 vaccination and 3 challenge treatments in combination) and excluded the "control" sham challenged and unvaccinated treatment. However the means and standard errors for this treatment are presented and included in the figures for reference.

Bodyweight. At d14 pc mean bodyweight was 160±1.7 g with no significant overall effect of vaccination (P=0.38) or challenge (P=0.54), However there was significant interaction between these effects (P=0.03) due to a different response to vaccination in 04CRE compared with the other challenge strains. In birds challenged with 04CRE bodyweight was depressed relative to unvaccinated whereas in birds challenged with the other two strains BWT was higher than in unvaccinated birds (Figure 4.3). The mean weight in sham challenged control chickens that were not included in the analysis was 172±4.1g, higher than that of any other treatment.

At day 56 pc the situation was quite different with significant overall effects of vaccination (P=0.02) and challenge (P<0.001) with no significant interaction between these effects (P=0.11) (Figure 4.3). Unvaccinated chickens were significantly lighter (744±13 g) than those vaccinated with either HVT (795±10 g) or bivalent vaccine (795±9 g). Chickens challenged with 02LAR were significantly lighter (741±11 g) than those challenged with either MPF57 (795±10 g) or 04CRE (797±10 g). The mean weight in unchallenged control chickens that were not included in the analysis was 873±14g, considerably higher than that of any other treatment.

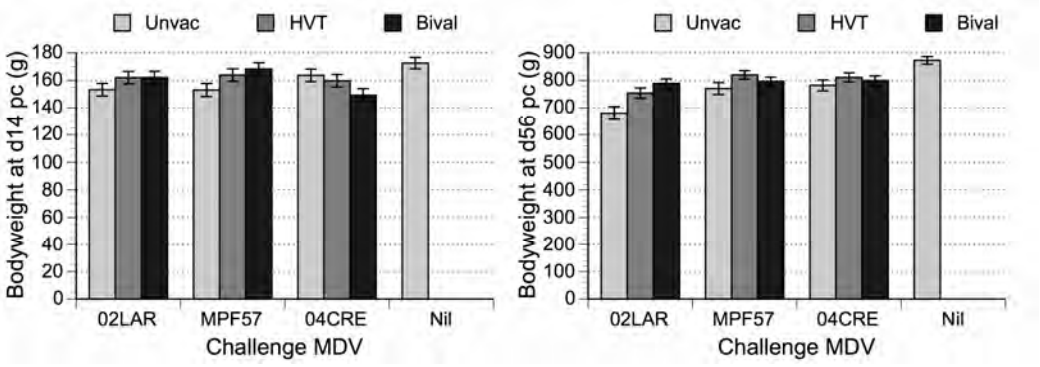

Figure 4.3. Interaction plot showing the effects of MDV1 challenge and vaccination on chicken bodyweight (LSM±SEM) at days 14 pc (Left panel) and 56 pc (right panel).

Thymus. At day 14 pc the overall mean relative thymic weight was 0.350±0.009 %BW. It was significantly influenced by vaccination (P=0.01), and operator (P<0.001), but not challenge (P=0.22) with no significant interaction between these effects (Figure 4.4). Unvaccinated chickens had significantly lower relative thymic weight (0.32±0.01 %BW) than those vaccinated with HVT (0.37+0.01 %BW) or bivalent vaccine (0.37+0.01 %BW). The operator effect was entirely due to the

number of lobes dissected (Figure 4.5A) with no operator effect on mean lobe weight (P=0.65). The mean relative thymic weight in sham challenged control chickens that were not included in the analysis was 0.39±0.03 %BW, which was amongst the higher values. In an effort to reduce the time and labour required to dissect the thymus out of each chicken thymuses were also scored for atrophy on a 0-3 scale (3= severe atrophy). There was a strong linear association between thymus weight and atrophy score (R2=0.61, P<0.001) as shown in Figure 4.5B.

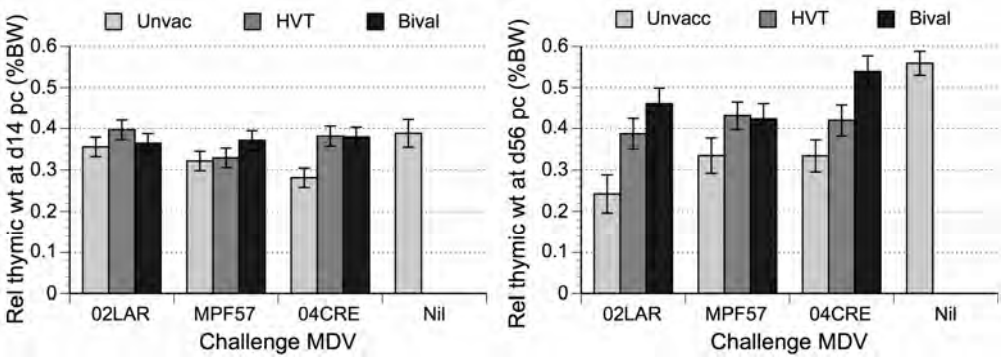

Figure 4.4. Interaction plot showing the effects of MDV1 challenge and vaccination on relative thymic weight (LSM±SEM) at days 14 pc (Left panel), and 56 pc (right panel).

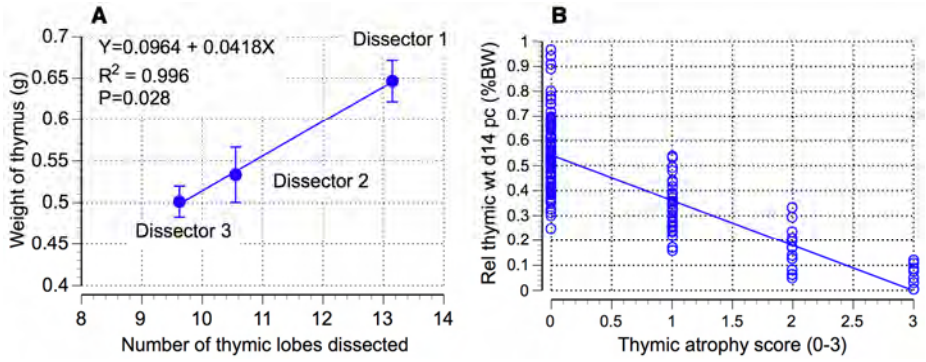

Figure 4.5. Association between mean relative thymic weight and mean number of lobes dissected by each operator at day 14 pc (A), and association between mean relative thymic weight and mean thymic atrophy score (0-3) at day 14 pc (B).

At day 56 pc, the overall mean relative thymic weight was 0.419±0.013 %BW and treatment effects were more marked. It was again significantly influenced by vaccination (P<0.001), and operator (P<0.005), but not challenge (P=0.11) with no significant interaction between these effects (Figure 4.4). Unvaccinated chickens had significantly lower relative thymic weight (0.30±0.02 %BW) than those vaccinated with HVT (0.41+0.02 %BW) or bivalent vaccine (0.47+0.02 %BW). There was a non significant trend for 02LAR to have a lower thymic weight than 04CRE (P=0.11). The mean relative thymic weight in sham challenged control chickens that were not included in the analysis was 0.56±0.03 %BW, which was the highest value amongst the treatment groups. The value was considerably higher than for the same value as the same group had at day 14 pc, indicating that relative thymic weight had increased substantially over this period in unchallenged birds.

Bursa of Fabricius. At day 14 pc the overall mean relative bursal weight was 0.377±0.008. There were significant effects of vaccination (P<0.001), but not challenge (P=0.29) or operator (P=0.61) and there was no significant interaction between these effects (Figure 4.6).

Unvaccinated chickens had significantly lower relative bursal weight (0.31±0.01 %BW) than those vaccinated with HVT (0.40+0.01 %BW) or bivalent vaccine (0.41+0.01 %BW). The mean relative thymic weight in sham challenged control chickens that were not included in the analysis was 0.40±0.02 %BW, which was amongst the higher values although not the highest (Figure 4.6).

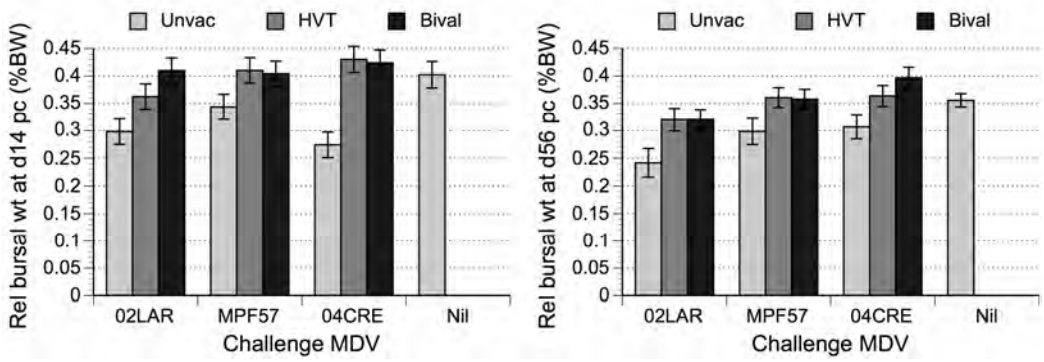

Figure 4.6. Interaction plot showing the effects of MDV1 challenge and vaccination on relative bursal weight (LSM±SEM) at days 14 pc (Left panel), and 56 pc (right panel).

At day 56 pc the overall mean relative bursal weight was 0.345±0.006, only slightly lower than the day 14 pc value. There were significant effects of vaccination (P<0.001) challenge (P=0.002) and operator (P=0.008) with no significant interaction between these effects (Figure 4.6). Unvaccinated chickens again had significantly lower values (0.28±0.01 %BW) than those vaccinated with HVT (0.35±0.01 %BW) or bivalent vaccine (0.36±0.01 %BW). Chickens challenged with 02LAR also had significantly lower values (0.29±0.01 %BW) than those challenged with 04CRE (0.36±0.01 %BW) or MPF57 (0.34±0.01 %BW). The mean relative bursal weight in sham challenged control chickens that were not included in the analysis was 0.36±0.01%BW, which was among the higher value amongst the treatment groups, and slightly lower than the value at day 14 pc indicating, as with relative thymic weight, that relative bursal weight was relatively constant over this period in unchallenged birds.

Spleen. As in Expt 2 the splenomegaly induced by challenge with MDV1 was in clear evidence (Figure 4.7). However, unlike the situation in broilers, the relative spleen weight of unchallenged control chickens remained constant between day 14 pc (0.212±0.012 %BW) and day 56 pc (0.210±0.005 %BW) in much the same way as thymic and bursal weights.

At day 14 pc the overall mean relative weight of spleen (excluding those classed as tumorous) was 0.228±0.004 %BW. This was significantly affected by vaccination (P<0.001) challenge (P=0.02) and operator (P<0.001) with no interaction between these effects (Figure 4.7). Unvaccinated chickens had significantly larger relative splenic weights (0.25±0.01 %BW) than those vaccinated with HVT (0.21±0.01 %BW) or bivalent vaccine (0.23±0.01 %BW). Chickens challenged with 04CRE had significantly higher values (0.24±0.01 %BW) than those challenged with MPF57 (0.22+0.01 %BW) with those challenged with 02LAR being intermediate (0.23±0.01 %BW) and not differing from the other two.

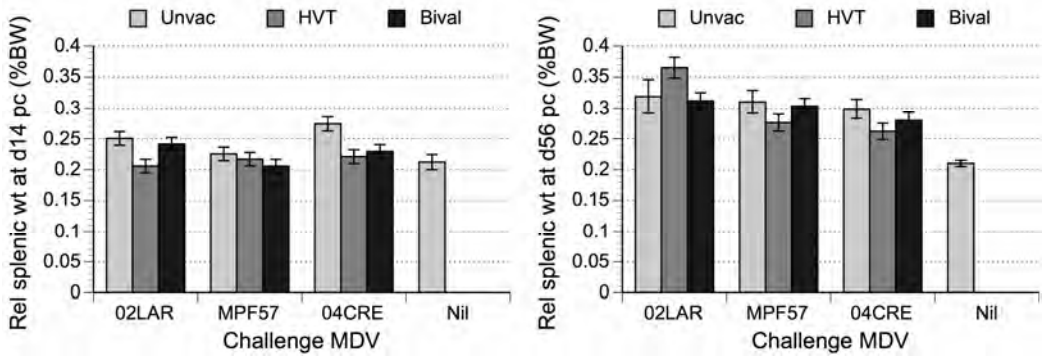

Figure 4.7. Interaction plot showing the effects of MDV1 challenge and vaccination on relative splenic weight (LSM±SEM) at days 14 pc (Left panel), and 56 pc (right panel).

By day 56 pc there was no longer an overall effect of vaccination treatment (P=0.75) although the effects of challenge virus (P=0.001) and operator (P=0.03) remained significant. There was significant interaction between the effects of vaccination and challenge virus (P=0.03) due to a differential effect of HVT vaccine for the 3 viruses (elevated relative splenic weight in birds challenged with 02LAR, but reduced relative splenic weight in chickens challenged with 04CRE and MPF57) (Figure 4.7).

Chickens challenged with 02LAR had significantly higher relative splenic weights (0.33±0.01 %BW) than those challenged with either MPF57 (0.30±0.01 %BW) or 04CRE (0.28±0.01 %BW).

MDV1 and HVT load in spleen

The load of MDV1 in spleen cells at day 14 pc was significantly affected by vaccination (P<0.0001) and challenge virus (P<0.0001) with significant interaction between these effects (P=0.02) (Figure 4.8). Unvaccinated chickens had significantly higher $\log_{10}$ MDV1 load/10^6 spleen cells (6.67±0.25) than those vaccinated with HVT (4.32±0.25) which in turn had significantly higher load than those vaccinated with bivalent vaccine (3.19±0.25). Chickens challenged with 02LAR (5.41±0.25) and MPF57 (4.85±0.25) had higher loads than those challenged with 04CRE (2.91±2.5) (Figure 4.8).

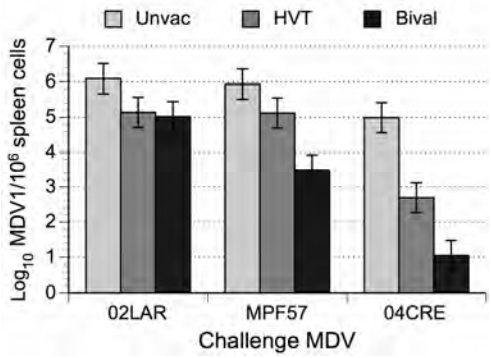

Figure 4.8. Load of MDV1 (LSM±SEM) in spleen of chickens at day 14 post challenge, by vaccination treatment and challenge virus. (See Table 4.2 for numbers of chickens).

The mean HVT load in spleen ($\log_{10}$HVT load/10^6 cells) of 13 HVT-vaccinated birds challenged with MDV1 was 4.34±0.13.

MDV1, HVT and MDV2 load in isolator dust

This work was led and executed by PhD student Katrin Renz.

Shedding of MDV1 in dander was detected from 7 dpc onwards with a mean of $10^{3.5}$ viral copy numbers/ mg dust. The amount of MDV1 shed in isolator dust increased rapidly between 7 and 21dpc after which it continued to slowly increase to $10^{6.5}$ – $10^{7.2}$ viral copy numbers/ mg dust between 42 and 56 dpc (Figure 4.9). MDV1 load in dust varied significantly due to the effects of vaccine (P<0.0001) and days post challenge (P<0.0001) with significant interaction between these effects (P<0.0001, Figure 4.9).

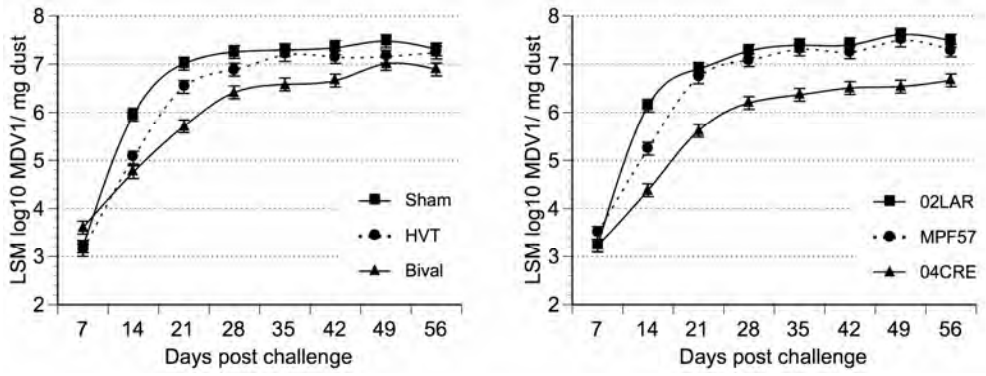

Figure 4.9. Load of MDV1 (LSM±SEM) in isolator exhaust dust at weekly intervals throughout the experiment. Only chickens challenged with MDV1 are included. The overall effects of vaccination treatment (left) and challenge virus (right) are shown.

Unvaccinated chickens had significantly higher levels of Log_{10} MDV1 in dander (6.60±0.05) than those vaccinated with HVT (6.29±0.05) which were in turn significantly higher than those vaccinated with Bivalent vaccine (5.95±0.10). The effect of MDV1 challenge isolate was also highly significant (P<0.0001) with significant interaction between challenge isolate and days post challenge (P<0.0001,

Figure 4.9). Chickens challenged with 02LAR (6.68±0.05) had a significantly higher overall Log_{10} MDV1 load in dander than those challenged with MPF57 (6.48±0.05) which in turn had a significantly higher load than those challenged with 04CRE (5.69±0.05) (Figure 4.9).

HVT could be detected in all vaccinated chickens (HVT or Bivalent) from 12 post vaccination (7 dpc). The mean initial amount of HVT in isolator dust was $10^{5.2}$ viral copy numbers/ mg dust and increased rapidly to a peak at day 19 pc (approx. $10^{6.8}$ viral copy numbers/mg dust) after which it plateaued at around 10^6 viral copy numbers/mg dust which is shown in Figure 4.10. There was a significant effect of days post vaccination (P<0.0001) but no significant effect of challenge (P=0.65) or vaccine type (P=0.97) on the amount of HVT in isolator exhaust dust. The interaction between these two effects was also not significant (P=0.64).

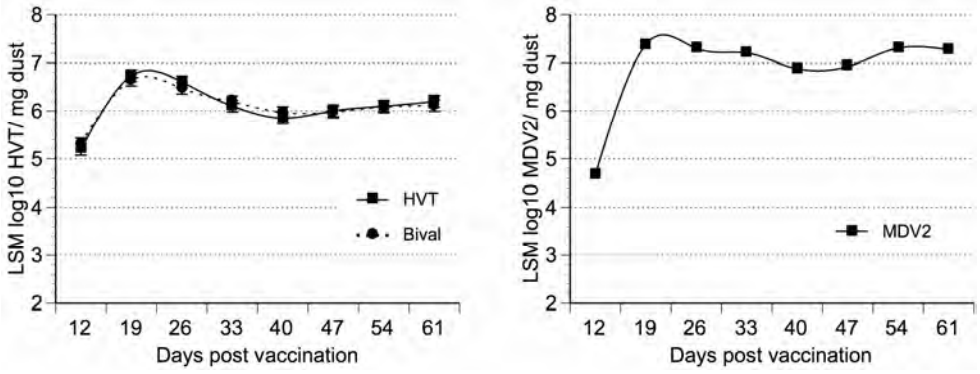

Figure 4.10. Load (LSM±SEM) of HVT (Left panel) and MDV2 (Right panel) in isolator exhaust dust at weekly intervals throughout the experiment showing the effect of challenge virus. The left panel includes chickens vaccinated with HVT and Bivalent vaccine, the right panel those vaccinated with bivalent vaccine alone.

MDV2 in chickens vaccinated with bivalent vaccine was significantly affected by day post vaccination (P<0.0001) but not challenge treatment (P=0.64) with no significant interaction between them (P=0.63). MDV2 was present from the 1st measurement at day 12 pv, onwards. Initial load at this time was $10^{4.7}$ per mg dust, increased remarkably to a peak of $10^{7.4}$ /mg dust at day 19 pv after which MDV2 load plateaued at around 10^7 viral copy numbers/mg dust (Figure 4.10).

In some chickens all three MDVs were present, namely those vaccinated with bivalent vaccine and challenged with MDV1. In these samples the level of the different serotypes of MDV varied significantly (P<0.0001), and they also varied significantly over time, with significant interaction between these two effects (P <0.0001) (Figure 4.11).

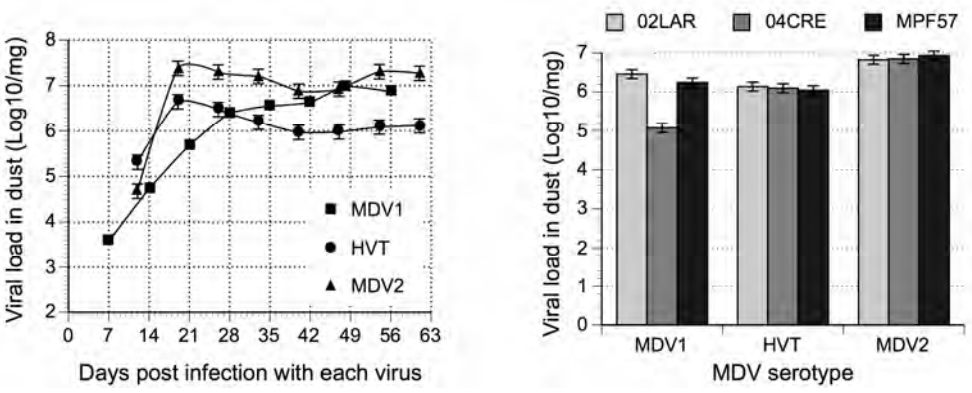

Figure 4.11. Left Panel. Interaction between the effects of MDV serotype and dpc on viral load in isolator exhaust dust from chickens vaccinated with Bivalent vaccine and challenged with MDV1 (LSM±SEM) (n=6 isolators). Right panel. Interaction between the effects of MDV serotype and challenge MDV on viral load in isolator exhaust dust from the same chickens (LSM±SEM) (n=6 isolators).

Overall viral load of MDV2 ($10^{6.87}$/mg dust) was significantly (P<0.05) higher than that of MDV1 ($10^{5.93}$/mg dust) and HVT ($10^{6.10}$/mg dust) which did not differ from each other. The interaction between dpc and MDV serotype (P<0.0001) is shown in Fig. 4.11 (left panel) with clear differences in the pattern of viral load over time for the different MDV serotypes. The interaction between MDV serotype and challenge virus (P<0.0001) is shown in Fig. 4.11 (right panel) demonstrating that the

level of HVT and MDV2 was not influenced by the challenge virus, whereas the level of MDV1 was significantly influenced by challenge virus with significantly lower MDV1 in chickens challenged with 04CRE relative to the other two challenge viruses.

Prediction of MD incidence

MDV1 load in spleen and isolator exhaust dust

MDV1 load in spleen at day 14 pc was significantly positively associated with the ultimate incidence of MD in each isolator, accountiong for 64-71% of the total variation between isolators in the incidence of MD (Figure 4.12).

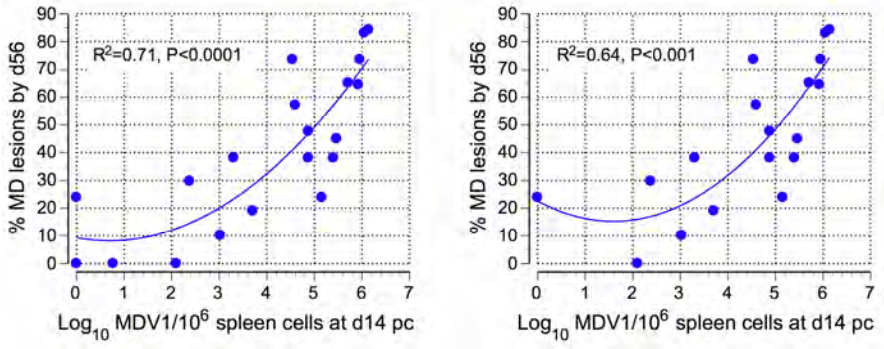

Figure 4.12. Association between MDV1 load in spleen at days 14 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator and the line is a 2nd degree polynomial fit. The left panel includes two sham-challenged isolators (each with 0% MD) while the right panel excludes these.

MDV1 load in isolator exhaust dust on days 14, 21, 28, 35, 42 and 49 were significantly positively associated with MD incidence by day 56 pc (R2 0.48-0.66), but at day 7 pc no relationship was evident with a trend towards a negative association (Figure 4.13). The strongest associations were at days 14 and 21.

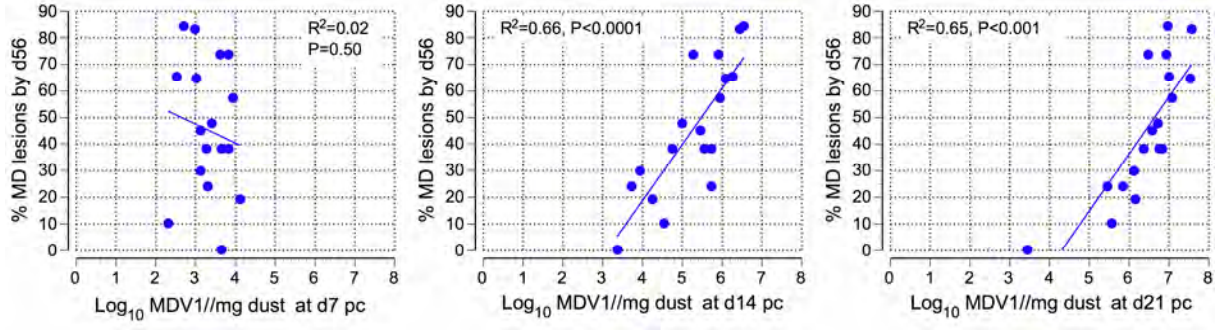

Figure 4.13. Association between MDV1 load in isolator exhaust dust at days 7, 14 and 21 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions) in chickens challenged with MDV1 (sham challenged isolators excluded). Each point represents one isolator. The line is a linear regression curve.

HVT and MDV2 load in isolator exhaust dust

HVT and MDV2 load in isolator exhaust dust of vaccinated, challenged chickens weekly from d7 to d56 pc. There was no significant association between the mean level of HVT in dust and the incidence of MD, and no trend towards an association. The direction of association was not consistent over time. For MDV2, despite only 6 isolators being involved, significant associations between MDV2 in dust and subsequent incidence of MD were observed. In the middle part of the experiment (days 28-42 pc) there was a negative association between MDV2 and %MD (R^2 range 0.32-0.83) whereas at days 49 and 56 pc the association was positive (R^2 0.62 and 0.67 respectively) (Figure 4.14).

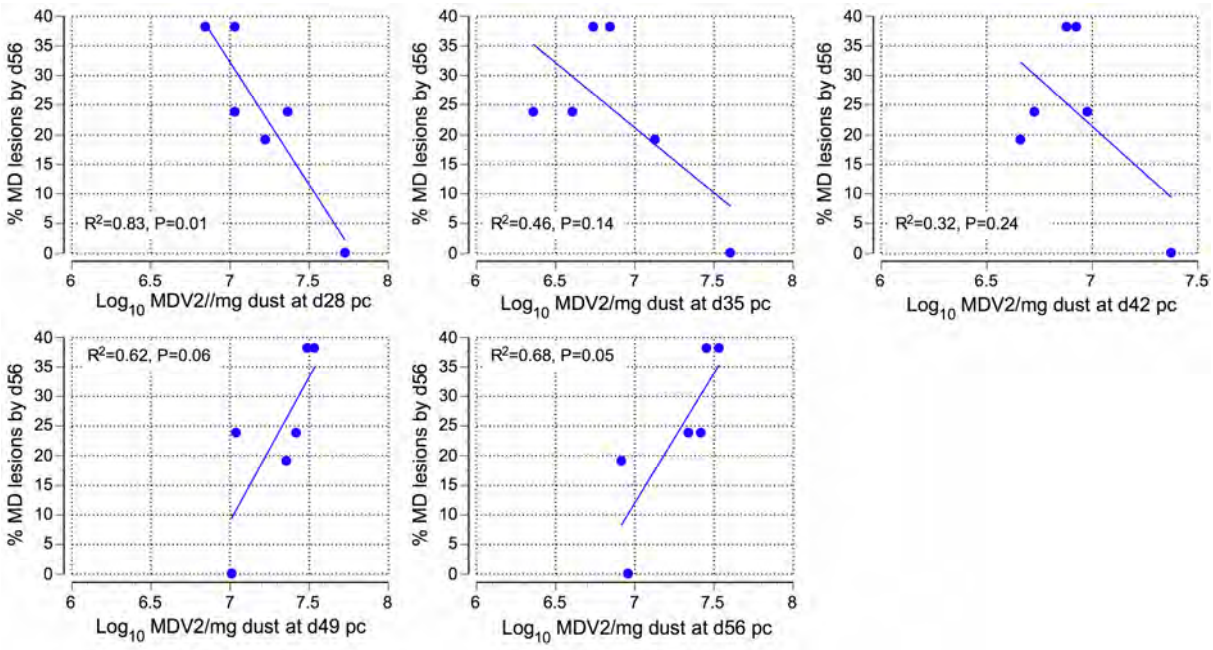

Figure 4.14. Association between MDV2 load in isolator exhaust dust at days 28, 35 and 42 pc (top panels) 49 and 56 (lower panels) and the incidence of MD by day 56 pc (% birds with gross MD lesions) in chickens challenged with MDV1 (sham challenged isolators excluded). Each point represents one isolator. The line is a linear regression curve. Note the change in direction of the association between the top and bottom panels.

Relative immune organ weight

At day 14 there was a non-significant negative association between relative thymic weight and the incidence of MD by day 56 pc (P=0.08) but by day 56 pc the association was very strong, for both relative thymic weight and thymic atrophy score (Figure 4.15).

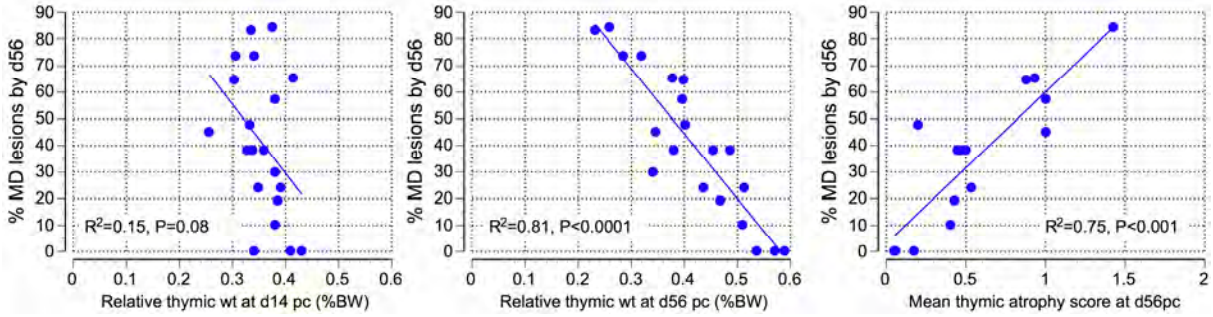

Figure 4.15 Association between relative thymic weight (% of bodyweight) at days 14 and 56 pc (left panels), thymic atrophy score (right panel) and the incidence of MD by day 56 pc (% birds with gross MD lesions). Each point represents one isolator. Sham-challenged isolators are included. Chickens with tumours of the thymus are excluded. Relative thymic weights are adjusted for the effect of operator. The line is a linear regression curve.

The situation was similar for relative bursal weight except that there was a significant negative association at both days 14 and 56 pc (Figure 4.16).

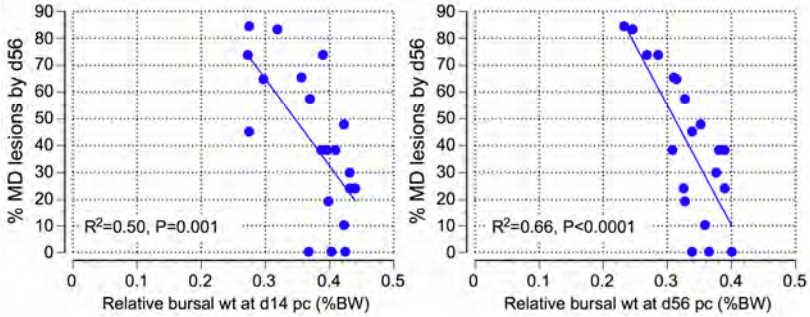

Figure 4.16 Association between relative bursal weight (% of bodyweight) at days 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Further details are as per Figure 4.15.

Unlike the other immune organs, relative splenic weight was positively associated with the incidence of MD although this was only significant at day 56 pc (Figure 4.17). However the associations were weaker than for bursal or thymic weight.

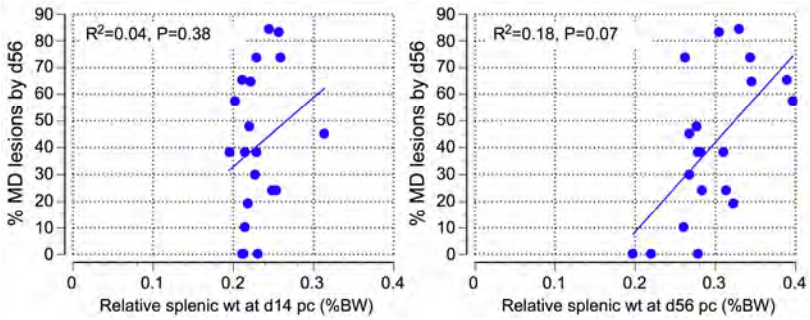

Figure 4.17 Association between relative splenic weight (% of bodyweight) at days 14 and 56 pc and the incidence of MD by day 56 pc (% birds with gross MD lesions). Further details are as per Figure 4.15.

Use of K-means cluster analysis of relative thymic, splenic and bursal weights of individual birds to identify patterns of association between the 3 organs did not enable more accurate early prediction of MD than individual organ measurement with an R^2 values of 0.38 (P=0.006) for a 2 cluster analysis. The two clusters essentially differentiate chickens with relatively large spleens and relatively small bursal and thymic weights with those having the reverse pattern.

Use of the data from all 3 relative immune organ weights at day 14 pc variables in stepwise regression equations did not improve the overall fit with a single variable, relative bursal weight, proving the sole predictor of value.

Brief discussion and conclusions

The experiment was successfully implemented and provided some very interesting contrasts with the previous experiment in broilers. The host background clearly is clearly an important determinant of MD incidence and vaccinal efficacy.

In the layer chickens there was clear differentiation of the 3 challenge viruses with 02LAR inducing significantly more MD (57.5%) than MPF57 (45.8%) which in turn induced significantly more MD than 04CRE (30.8%). There were many significant correlated measures that also differentiated the 3 viruses in the same order, including MDV1 load in spleen at day 14 pc, MDV1 load in isolator exhaust dust from day 14 pc onwards and immune organ weights at day 56 pc. Many of these offer good prospects for early prediction of subsequent MD incidence. As with the effects of challenge virus, the effects of vaccination treatment were also far greater in layer chickens than they were in the broilers. There was clear differentiation of HVT and Bivalent vaccines, with the latter providing significantly higher levels of protection. The differences between the two experiments in the levels of MD induced and protection provided by vaccines are summarised in Figure 4.18 which emphasises the differences between the two host systems.

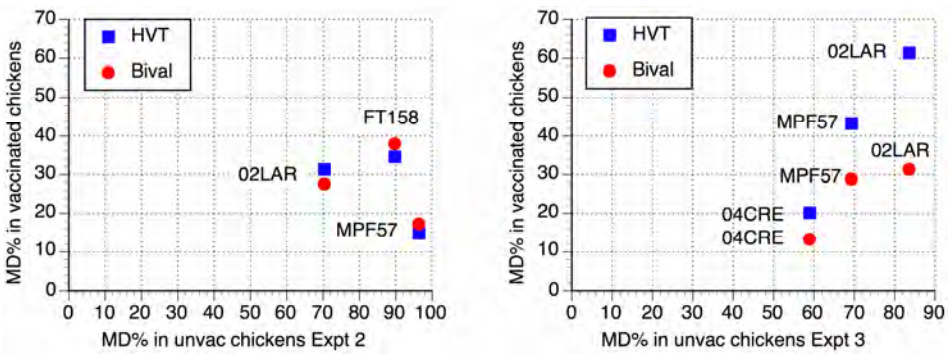

Figure 4.18 Association between MD incidence (%MD) induced in Cobb broilers (Expt 2) and Isa Brown Layers (Expt 3, this expt) either unvaccinated, or vaccinated with HVT or bivalent vaccine.

Based on the levels MD-induced mortality and the levels of vaccinal protection obtained with bivalent vaccine (59-78%) the viruses are likely to fall in the v to vv category on the USDA ADOL system.

There was little difference in tissue trophism between the two experiments with the first 3 organs (ovary, liver, spleen) being ranked 1-3 in both chicken genotypes. However in the second experiment (layers) there were considerably more heart lymphomas, and fewer kidney lymphomas, than in the broilers.

As was the case in broilers, none of MDV1 isolates induced the early paralysis/mortality syndrome observed in SPF chickens (Walkden-Brown *et al.* 2006). However, unlike the broilers more severe MDV-induced immune organ atrophy observed in the layer chickens, particularly at day 56 pc. This was only partially protected against by vaccination, with bivalent vaccine offering greater protection than HVT. Interestingly the relative thymic and bursal weights in unchallenged layers at day 56 pc are 3-5 fold greater than those in broiler chickens at the same age, however there is no evidence that this compromises the ability of broilers to resist MDV infection. Relative splenic weights are also greater but by less than 2-fold. In this experiment, the major source of variation between operators in thymic dissection was identified, the number of lobes removed by each dissector.

Bird performance as determined by bodyweight at d56 pc was significantly affected by MDV1 challenge and vaccination in directions consistent with the level of MD induced. These effects were again clearer in the layers than they had been in broilers.

MDV1 load in spleen at day 14 pc (Figure 4.8) appeared to provide a very accurate reflection of subsequent MD status and MDV1 shedding with the effects of vaccination and challenge MDV1 clearly demarcated at this early stage. This was reflected in a strong association between this variable, and MD% at day 56 pc for each individual isolator (Figure 4.12).

As was observed in broilers, unvaccinated chickens unexpectedly had a lower level of shedding of MDV1 at day 7 than vaccinated chickens (Figure 4.9) and there was a non-significant negative association between the level of MDV1 shedding in challenged chickens and the subsequent incidence of MD (Figure 4.13). Similar results have been observed in an earlier experiment (Islam *et al.* 2007) so the observation has now been made in 3 experiments and explanation of a sparing effect of vaccinal virus on the inactivation of MDV1 by maternal antibody (as described in the discussion to Chapter 3) appears likely.

The measurement of MDV1 in isolator exhaust dust proved revealing in this experiment. Once again the effects of vaccination and challenge virus were clearly expressed from day 14 onwards. These effects were much greater in the layer chickens than the broilers with clear demarcation between each of the 3 vaccine treatments, and each of the 3 MDV1 challenge treatments (Figure 4.9). This is the most powerful demonstration to date of the utility of this very simple measure which integrates information from a number of birds in a single sample. The overall level of MDV1 and MDV2 in dander, and the patterns of shedding over time, were broadly consistent with what was observed in broilers although levels of MDV1 were slightly higher in the layer experiment. While the pattern of HVT load in dander was also broadly similar it peaked earlier in layers and at a level nearly 1 log higher than that seen in broiler chickens.

Key observations relating to MDV1 load in isolator exhaust dust are:

a) The suppressive effects of vaccination on MDV1 load are:
    - Far greater in spleen (1-3.8 logs) than in feather dander (1-1.2 logs)
    - Are most pronounced between days 21-42, but are evident throughout the experiment after day 7 (Figure 4.9).
    - At day 7 pc, vaccination probably enhances rather than suppresses shedding of MDV1.
b) MDV1 load in dander was in proportion to virulence with the most virulent virus (02LAR) inducing the highest levels of shedding. Differences in shedding between challenge viruses were greatest at day 14 but were sustained throughout the experiment (Figure 4.9)

c) Unlike MDV1, HVT and MDV2 load in dander decreases after days 19-26 post vaccination with a subsequent increase again around day 54 post vaccination (Figure 4.10).

One of the aims of the experiment was to examine methods of predicting MD outcomes other than by running a full pathotyping experiment.

By far the best predictor was the level of MDV1 in spleen or dust shed from isolators, the same as was observed in broilers. The level of MDV1 in spleen at day 14 pc had a strong relationship with subsequent MD incidence (R^2=0.71). With regards isolator exhaust dust, strong relationships were seen at days 14-49 post-challenge (Figure 4.13), but a poor relationship was evident at day 7 for reasons discussed above. Measurement of MDV1 load in spleen or dust in groups of birds in the first few weeks prior to challenge once again appears to have excellent potential to predict MD outcomes and replace full pathotyping experiments. These measurements can be made prior to the onset of clinical MD, offering significant ethical advantages.

The level of vaccinal virus in dander proved uninformative in broilers for predicting future MD status, and the same was true for HVT in the present experiment. This was thought to be due to the fact that challenge with MDV1 greatly increases the shedding rate of vaccinal virus as shown in Expt 2 (not tested in Expt 3). However in the current experiment, the level of MDV2 in dust was significantly associated with subsequent MD incidence, with an inverse relationship at days 28-42 pc but a positive relationship at days 49 and 56 pc.

Associations between relative immune organ weights and MD incidence were much stronger than were observed in the broiler experiment, particularly for thymus and bursa at day 56 pc (Figure 4.15 and 4.16). The associations were not strong for spleen at either d14 or d56 pc, or thymus at d14 pc but relative bursal weight at d14 pc was significantly negatively associated with future MD incidence at d56 pc. This indicates that even in maternal antibody-positive chickens measurement of bursal weight as an indicator of future MD status may have merit, particularly in layer type chickens. Estimation of thymic atrophy score at day 56 pc provided approximately the same level of predictive power as actual measurement of thymic weight (Figure 4.15)

The main findings of this experiment are that:

a) Host genotype can significantly influence the expression of MD in both vaccinated and unvaccinated chickens and can significantly affect virulence ranks.

b) Commercial IsaBrown layer chickens enable a more “sensitive” ranking of MDV1 isolates than commercial Cobb broiler chickens.

c) Measurement of MDV1 in spleen or dust at around day 14 pc is the most accurate predictor of subsequent MD status, with measurement in dust being considerably more convenient and inexpensive.

d) Operator variation in measurement of thymic weight is mostly associated with the number of lobes actually found and removed, and simple thymic atrophy scores are as efficient as actual thymic weights for estimation of subsequent MD status of groups of chickens.

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Chapter 5: Experiment 4. MD06-C-VI5

"*In vivo propagation of MDV strains for pathogenicity screening and subsequent propagation in cell-culture*"

Start: 17/1/06 **Completion: 27/2/06** **AEC: UNE 05/178**

Introduction

Having formally pathotyped several isolates of MDV1 (MPF57, 02LAR, FT158, 04CRE) in commercial broiler and layer chickens and in SPF chickens under RIRDC project UNE-83J our next task was to pathotype a new round of isolates in commercial broilers and layers again. However due to difficulties in growing new MDV1 isolates to sufficient titre for use in pathotyping experiments at a challenge dose of 500pfu/chicken efforts were made to back-passage infective material in chickens to amplify it for further growth in cell culture. Such back-passage in chickens is commonly used to boost the growth of MDV1 in cell culture (Schat and Purchase 1998).

Just prior to this experiment we had recently completed a similar large experiment (MD05-R-PT3) to screen 24 samples of infective MDV1 material in SPF chickens for subsequent MDV1 amplification in cell culture (See (Walkden-Brown *et al.* 2006) for a detailed description of this experiment). This experiment was very successful with 14 isolates producing tumours *in vivo*, 8 from isolates which were new from the field (summary in Table 5.1). Unfortunately, the spleen samples sent from UNE to RMIT for propagation of MDV1 arrived at RMIT a day late (two nights in transit rather than 1). As a result, there was a risk that those viruses might not grow in the cell culture. At UNE we had stored some infective materials (splenocytes) for further characterization and we also had stored dust samples from all candidate isolates. As it would be several months before the next cell culture run at RMIT it was therefore decided to immediately conduct another experiment to screen isolates and produce further fresh spleen material for isolation of MDV1 at RMIT. At the same time the opportunity was taken to test other methods of infection of chickens with MDV1. Thus within the overall experiment were nested 2 trials with the following main objectives:

Trial 1. To generate fresh infective materials from the stored dust samples for RMIT to propagate MDVs in chicken kidney cell culture

Trial 2. To compare stored whole blood and splenocytes as infective materials for isolate MPF23

This experiment was led by Dr Steve Walkden-Brown with assistance from Dr Fakhrul Islam, Katrin Renz. and Paul Reynolds. Katrin Renz took particular responsibility for Trial 2.

Materials and methods

The whole experiment took place at the UNE isolator facility, using 13 isolators placed with 194 SPF chickens (Charles River, Australia. Ex CSIRO HWL line). All infective dust material came from the previous RIRDC experiment (MD05-R-PT3) and was collected at days 36-48 pc in that experiment. Chickens arrived at UNE on the day of hatch (day 0, 17/1/06), were left unvaccinated and were challenged at 2 days of age on 19/1/06. The experiment ended on 27/2/06 on day 41 (day 39 post challenge).

Experimental design – Trial 1

The experiment was a completely randomised design of 12 treatment groups (isolators) without replication as summarised in Table 5.2. Chickens from trials 2 and 3 could also contribute to the objectives of this trial by providing infective material to send to RMIT.

At day 2 of age chickens in the trial were penned in a corner of their isolator for 2 hours and two bags of isolator dust containing the isolate of interest were emptied and dispersed over them. Air flow through the isolator was reduced to a minimum during this period. The bags were then torn apart and

Table 5.1. Summary of results of experiment MD05-R-PT3 in SPF chickens. Isolates are sorted in order of ability to induce MD tumours. This experiment provided the resource material for the present experiment.

Isolator	Treatment	n	% MD (tumours only)	%MD (including thymic and bursal atrophy)	%Mortality to day 49 pc	Splenocytes stored at UNE	Comments
7	MPF 210/2s	19	89.5	100	52.6	Y	
18	Woodlands 1	19	88.9	94.7	36.8	Y	
8	MPF 164/6	20	72.2	100	40.0	Y	
10	MPF 210/1s	19	72.2	78.9	15.8		
12	MPF 179/6	19	62.5	94.7	47.4	Y	
3	MPF 199/ 3&9	22	60	90.9	72.7		6 added later in expt
4	MPF57	20	60	95	30.0		
1	W7BIS	20	55.6	75	20.0	Y	
14	MPF 189/8	19	50	100	73.7		
21	MPF 192/1,4,10	37	37.5	89.2	56.8		
6	MPF 176/734o,734s,94	20	26.3	57.9	15.0	Y	
9	MPF 212	20	21.1	80	5.0		
19	FT158	14	14.3	57.1	21.4		Dust chall at day 20
23	02LAR	18	11.1	77.8	33.3	Y	
24	04KAL	19	0	84.2	5.3		
13	04OWE	19	0	0	0		
20	05JMJ	19	0	8.33	0		
11	05JUR	20	0	0	0		
2&15	Control	21	0	0	0		
5,16,22	MPF23	57	0	0	0		
17	MPF 179/2	17	na	na	0		

Table 5.2 Treatment allocation and distribution of chickens in each isolator by trial.

Serial no	Trial	Isolator	Challenge MDV1	MDV1/mg dust ($x10^6$)	n	Challenge mode	Comments
1	1	6	MPF 210/2s	30.02	14	Dust aerosol	
2	1	7	MPF 189/8	6.59	16	Dust aerosol	UNE back up
3	1	8	MPF FT158	19.16	14	Dust aerosol	
4	1	9	MPF 164/6	28.72	14	Dust aerosol	
5	1	10	02 LAR	5.55	14	Dust aerosol	
6	1	11	04KAL	0.04	16	Dust aerosol	UNE back up
7	1	12	MPF 210/1s	30.75	16	Dust aerosol	UNE back up
8	1	14	MPF57	4.26	14	Dust aerosol	
9	1	15	MPF 199/3&9	10.56	16	Dust aerosol	UNE back up
10	1	18	Woodlands	26.67	14	Dust aerosol	
11	1	19	W7BIS	21.61	14	Dust aerosol	
12	1&2	20	Nil		18	No challenge	
16	2	16	MPF 23		14	Whole blood or splenocytes IA	
Total					194		

left in the isolator until they disintegrated many days later. Dust was collected between 1-7/12/05 and 7-12/12/05 from an isolator infected with the given isolate in experiment MD05-R-PT3. It was stored at 4˚C in the paper vacuum bags in which it was collected (sealed in plastic bags) in early Dec 2005 until used in the experiment.

Experimental design – Trial 2

In the absence of infective dust material for the 1980's highly pathogenic isolate MPF23 (McKimm-Breschkin *et al.* 1990) we used stored infective spleen and blood material from earlier experiments. Whole blood was collected on 18/7/05 at the end of experiment MD05-R-VI4 (Walkden-Brown *et al.* 2006) a small experiment in off sex layer cockerels in which infection with MPF23 was achieved. Splenocytes were extracted from spleen samples collected on 8/8/05 from a follow-up small experiment (MD05-R1-MPF23) in which MPF23 material from MD05-R-VI4 proved infective, again in commercial male layer cockerels. Both the blood and splenocytes were cryopreserved in 10% DMSO and stored in liquid nitrogen. On day 2, chickens in isolator 16 were challenged intra-abdominally with 250ul of whole blood (n=7, LI) or 200ul of a splenocyte preparation containing 2 x 10^7 cells/ml (n=7, LO).

Management and measurements

All chickens dying during the experiment were weighed and examined post-mortem for gross MD lesions as described in the General Materials and Methods section. The thymus and bursa on dead chickens was scored 0-3 for thymic atrophy (3 = extreme atrophy)

At 39 dpc the experiment was terminated and all surviving chickens were humanely sacrificed, weighed, examined for gross MD lesions and had immune organ weights determined. Thymic and bursal atrophy scores were also recorded. Diagnosis of MD was by 3 operators Drs Steve Walkden-Brown, Fakhrul Islam and Aminul Islam. Prior to sacrifice, selected chickens were blood sampled to store plasma for CIT test if necessary. Spleens from successfully challenged treatments were sent to RMIT (split between two couriers) with some retained at UNE for splenocyte extraction and storage.

Statistical analysis

Analyses were performed with JMP6 (SAS Institute Inc. 2006). Mortality data were analysed using survival analysis (Kaplan-Meier method).

Continuous data (eg bodyweight) were analysed using a general linear model fitting the effect challenge treatment. Relative immune organ weights (thymus, bursa and spleen) were expressed as % of bodyweight and analysed similarly but with the effect of operator/dissector fitted. Birds with tumours of the given organ were excluded from the analysis. For continuous variables least squares means and standard errors are presented.

Results

Trial 1. Group dust challenge

Mortality/Survival

Two chickens died at 3 dpc and a further 2 at 7 dpc of abdominal bacterial infections. Six chickens were removed from the control isolator at 12 dpc and were thus excluded from the experiment. These 10 chickens were excluded from the analysis leaving 166 eligible birds in the mortality analysis. From days 13-15 pc there was a rash of paralysis and mortality, characteristic of the MD-associated early mortality syndrome observed in previous experiments in SPF chickens ((Walkden-Brown *et al.* 2006) (Figure 5.1). This mortality was associated with extreme thymic atrophy. The first chicken to die with MD tumours was at 24 dpc.

Of the 166 chickens at 7 dpc 36 (21.7%) died prior to 39 dpc, of which 6 had MD tumours (3.6%). Mortality by challenge treatment is summarised in Table 5.3. Numbers of chickens dying varied significantly between treatments (P=0.05) with MPF57 and 02LAR standing out with the highest mortality levels.

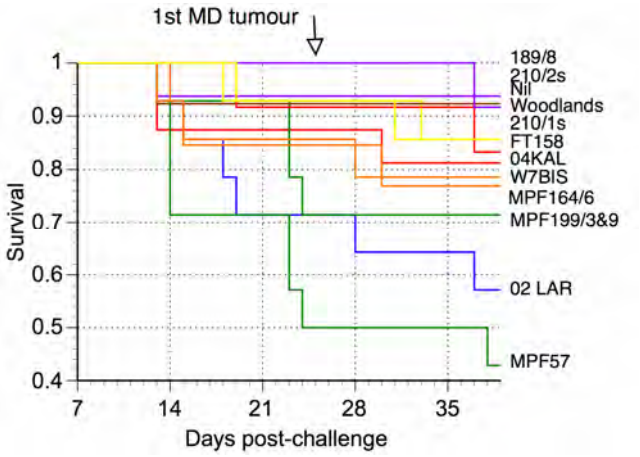

Figure 5.1. Survival analysis of the effect of challenge MDV1 on survival of eligible chickens from after 7 dpc to 39 dpc. Labels are in order of descending final survival rate and may not line up exactly with the appropriate line. The timing of the first MD tumour at 24 dpc is shown with the arrow. Survival patterns differed significantly (P=0.02).

Table 5.3. Mortality between days 7 and 39 post-challenge of the experiment in descending order. Includes all chickens eligible to die after day 7 pc, excluding 6 chickens removed from the experiment at day 12 pc.

Chall Virus	n	Survived No MD	Survived MD	Died No MD	Died MD	%Mort	%Mort with MD
MPF57	14	3	3	7	1	57.1	7.1
02LAR	14	3	5	5	1	42.9	7.1
MPF 164/6	14	8	2	3	1	28.6	7.1
MPF 199/3&9	13	6	4	2	1	23.1	7.7
W7BIS	14	5	6	3	0	21.4	0.0
04KAL	16	5	8	3	0	18.8	0.0
FT158	12	8	2	1	1	16.7	8.3
MPF 210/1s	14	9	3	1	1	14.3	7.1
Woodlands 1	14	10	2	2	0	14.3	0.0
Nil	12	11	0	1	0	8.3	0.0
MPF 210/2s	13	6	6	1	0	7.7	0.0
MPF 189/8	16	9	6	1	0	6.3	0.0
Total	166	83	47	30	6	21.7	3.6

Incidence of MD tumours

Of the 142 chickens alive at the time of the 1st MD tumour at 24 dpc, 53 (37.3%) had MD tumours on post mortem examination following death or sacrifice on day 39 pc. In the absence of maternal antibody in these chickens, early mortality syndrome (without MD tumours) and severe immune organ atrophy was evident, so an alternative case definition for MD was also employed, namely the presence of tumours and/or a combined thymic and bursal atrophy score of 4 or greater out of 6. Using this case definition 98/166 (59%) of chickens after 12 dpc exhibited MD. These data are summarized by challenge virus in Table 5.4 and Figure 5.2. The absence of MD in the nil challenge control group as defined by either criterion confirmed that isolation between treatments was effectively achieved.

All challenge groups except the unchallenged controls exhibited tumours and atrophy of the bursa and thymus indicating successful challenge with the dust. The incidence of MD was significantly affected by challenge virus (P<0.0001). Separation of means statistically is difficult, and given the non-standard dose of MDV1 used in the experiment it is decided not to fully identify out the significance of each mean comparison. Unexpectedly there was a significant (P=0.04) negative association between the level of MDV1 load in the challenge dust used and the incidence of MD tumours at 39 dpc (Figure 5.2, right panel).

Table 5.4. Incidence of MD calculated on the basis of immune organ atrophy (total score >3/6) and presence of tumours, or solely on the presence of tumours. Data sorted in descending order of tumour incidence.

Challenge virus	MD cases from 13 dpc including both tumours and atrophy score>3			MD cases from 24 dpc including only tumours		
	Eligible (n)	MD (n)	MD (%)	Eligible (n)	MD (n)	MD (%)
02LAR	14	12	85.7	10	6	60.0
04KAL	16	12	75.0	14	8	57.1
210/2s	13	9	69.2	12	6	50.0
MPF57	14	12	85.7	8	4	50.0
W7BIS	14	10	71.4	12	6	50.0
199/3&9	13	11	84.6	11	5	45.5
189/8	16	10	62.5	15	6	40.0
210/1s	14	6	42.9	13	4	30.8
164/6	14	8	57.1	11	3	27.3
FT158	12	3	25.0	11	3	27.3
Woodlands	14	5	35.7	13	2	15.4
Nil	12	0	0.0	12	0	0.0
Total	166	98	59.0	142	53	37.3

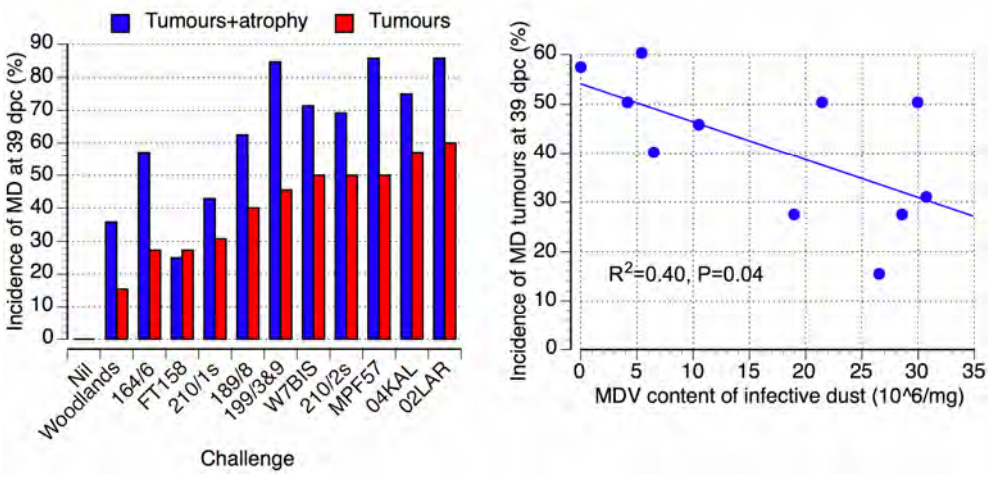

Figure 5.2. Incidence of MD by 39 dpc calculated on the basis of immune organ atrophy (total score >3/6) and presence of tumours, or solely on the presence of tumours (Left panel). Data sorted in descending order of tumour incidence. The right panel illustrates the relationship between MDV1 content in the infective dust used, and the incidence of MD tumours at day 59 of the experiment.

Bodyweight and immune organ weights at 39 dpc

Bodyweight (P<0.001) and the relative weights of thymus (P<0.0001), bursa (P<0.0001) and spleen (P=0.007) (expressed as a % of BW) were all significantly affected by challenge virus, with relative thymic weight also significantly affected by operator (P<0.0001) without interaction between these effects (Figure 5.3). Means separation is complex and can best be judged on the basis of the standard error values in Fig 5.3. MDV1 challenge resulted in comparatively large reductions in the relative weights of thymus and spleen and moderate increases in the relative weight of the spleen. Bodyweight was reduced in challenged chickens overall.

Thymic and bursal atrophy scores, alone or in combination were also significantly (P<0.0001) affected by challenge virus with TA score also affected by operator (P=0.02) emphasising the need for standardisation between operators. An index of immune organ effects calculated at (relative thymic weight + relative bursal weight – relative spleen weight) was also significantly affected by challenge virus (P<0.0001) and operator (p<0.001) (Figure 5.3).

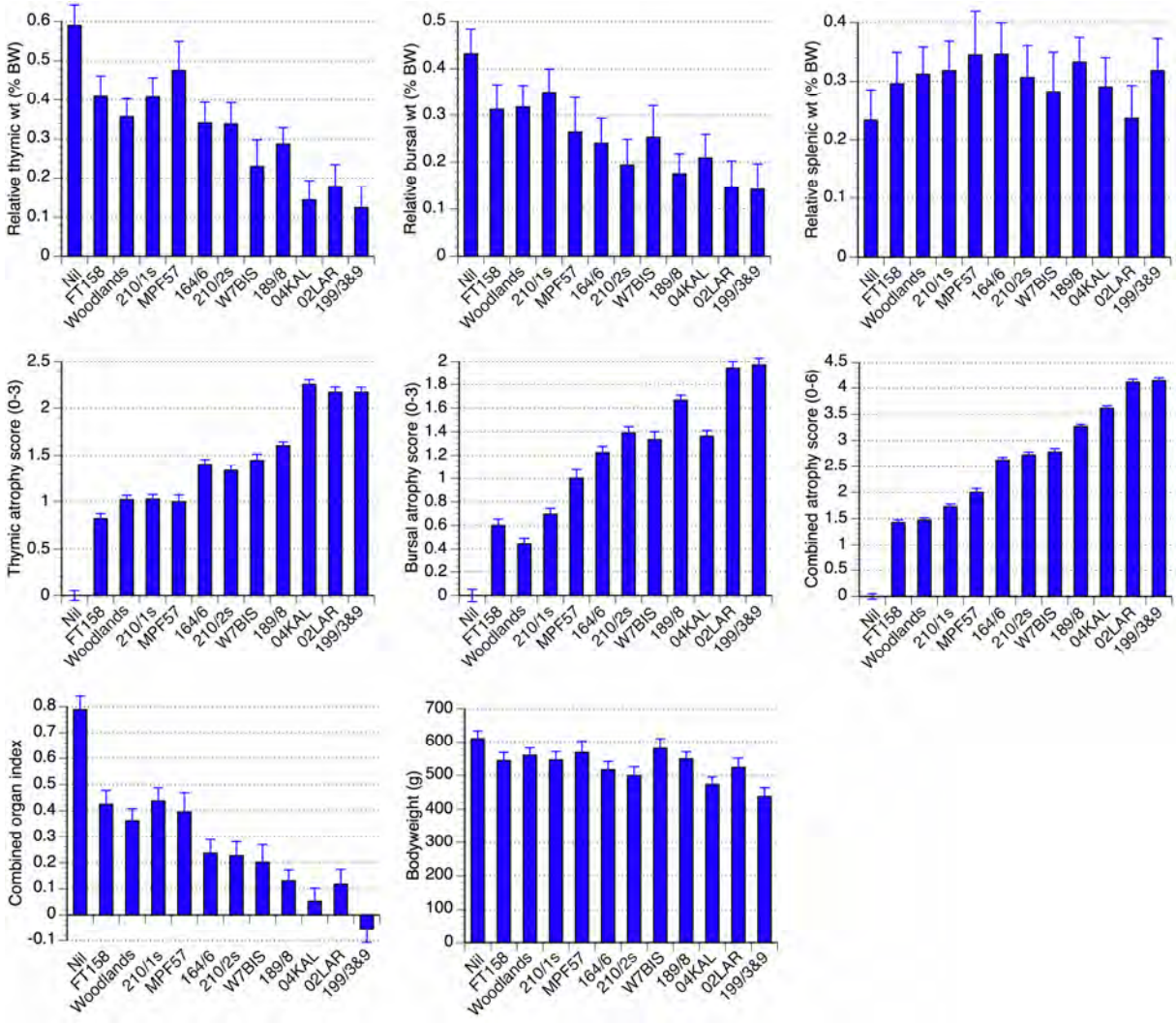

Figure 5.3. Least squares mean (±SEM) relative immune organ weights (%BW), bodyweight, and immune organ atrophy scores and index values at 39 dpc. Group sizes range from 5 to 14 (total n = 117). Data sorted in ascending order of combined atrophy score. Chickens with tumours of the relevant organ are excluded.

Association between key variables

In general there was a very high level of association amongst the immune organ measurements and their derivatives and the incidence of MD although relative spleen weight was a notable exception (Table 5.5). MD incidence on a group basis was best predicted by bursal atrophy score or relative weight alone or in combination with thymic atrophy score (Table 5.5). Bodyweight had significant positive correlations with relative thymic and bursal weight and organ index, and significant negative associations with atrophy scores for thymus and bursa.

Growth of MDV1 from spleens

Unlike the case with Experiment 1 (MD04-C-PT2) in SPF chickens, which produced high titres of MDV1 in cell culture, the fresh spleens from this experiment produced MDV1 which grew to only modest titres at RMIT (Chapter 1, Table1.1) which were insufficient for use in pathotyping experiments. Despite the success of overnight courier delivery of the fresh spleens from this experiment, there was no apparent difference in the success rate of MDV1 growth from these spleens compared to those from RIRDC experiment MD05-R-PT3 which were delayed in reaching RMIT (Chapter 1, Table 1.1).

Table 5.5. Correlation matrix of MD incidence (MD%) and various correlates of it involving immune organ measures or scores. Correlation is between least squares mean values for each challenge treatment. There are 12 treatments in total.

Variable	%MD tumours	%MD incl atrophy	BWT (g)	Rel thymic wt	Rel bursal wt	Rel splenic wt	TA score	BA score	Atrophy score	Organ index
%MD tumours	1	***0.92***	-0.53	***-0.72***	***-0.82***	0.10	**0.81**	***0.83***	***0.84***	***-0.78***
%MD incl atrophy	***0.92****	1	-0.57	***-0.72***	***-0.87***	0.29	***0.83***	***0.88***	***0.87***	***-0.83***
BWT (g)	-0.53	-0.57	1	***0.74***	***0.72***	-0.26	***-0.78***	**-0.68**	***-0.75***	***0.79***
Rel thymic wt	***-0.72***	***-0.72***	***0.74***	1	***0.83***	-0.01	***-0.96***	***-0.84***	***-0.93***	***0.95***
Rel Bursal wt	***-0.82***	***-0.87***	***0.72***	***0.83***	1	-0.21	***-0.90***	***-0.97***	***-0.96***	***0.94***
Rel Splenic wt	0.10	0.29	-0.26	-0.01	-0.21	1	0.11	0.15	0.13	-0.27
TA score	***0.81***	***0.83***	***-0.78***	***-0.96***	***-0.90***	0.11	1	***0.90***	***0.98***	***-0.96***
BA score	***0.83***	***0.88***	**-0.68**	***-0.84***	***-0.97***	0.15	***0.90***	1	***0.97***	***-0.92***
Atrophy score	***0.84***	***0.87***	***-0.75***	***-0.93***	***-0.96***	0.13	***0.98***	***0.97***	1	***-0.97***
Organ index	***-0.78***	***-0.83***	***0.79***	***0.95***	***0.94***	-0.27	***-0.96***	***-0.92***	***-0.97***	1

*Critical values of r (two tailed) for df=10 are 0.576, 0.708 and 0.824 for P = 0.05, 0.01 and 0.001 respectively. Values with P<0.05 are **bolded**, P<0.01, ***bolded and italicised***, p<0.001 ***bolded, italicised and underlined***.

Trial 2. Challenge using stored blood or splenocytes containing MPF23

Mortality and MD incidence

This was only a very small experiment with 14 chickens in a single isolator, 7 challenged with stored infective blood and 7 with stored infective splenocytes. Nevertheless it produced clear results with both methods of infection proving successful. Challenge with blood induced a single mortality at 12 dpc with the classical signs of early mortality syndrome induced by MDV1 (paralysis, very severe thymic and bursal atrophy). The next mortality was at 28 dpc and associated with the presence of MD tumours. Mortality was rapid subsequently, particularly in the group challenged with blood (Figure 5.4, Table 5.6).

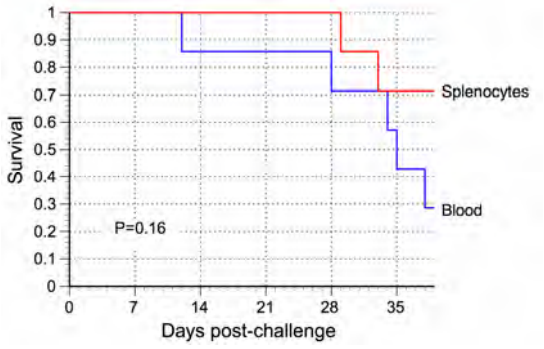

Figure 5.4. Patterns of survival in Trial 2. Chickens were challenged with MPF23 in stored blood or splenocytes. Differences were non-significant due to the small numbers of chickens in each treatment.

Table 5.6. Summary of mortality (with and without MD tumours) and incidence of MD in Trial 2.

Challenge treatment	n	Mortality	Mortality %	Mortality with MD	Mortality with MD%	Chickens at risk of MD tumours	Chickens with MD tumours by day 39 pc	Incidence of MD (% eligible birds)
Blood	7	5	71%	4	57%	6	5	83%
Splenocytes	7	3	43%	3	43%	7	7	100%
Total	14	8	57%	7	50%	13	12	92%

Bodyweight and immune organ weights at 39 dpc

Analysis of these measurements in the Trial 2 chickens compared with the unchallenged (Nil) treatment revealed significant influences of treatment for each variable measured (Figure 5.5). Challenge with MPF23 infected splenocytes significantly reduced bodyweight, relative thymic weight and relative bursal weight compared to the Nil challenge treatment (P<0.0001). Challenge with MPF23 in blood tended to have a non-significant effect in the same direction (Figure 5.5). In the case of relative splenic weight the reverse was true, with MPF23 challenge with either blood or splenocytes inducing a significant (P<0.0001) increase in size relative to the Nil challenge group.

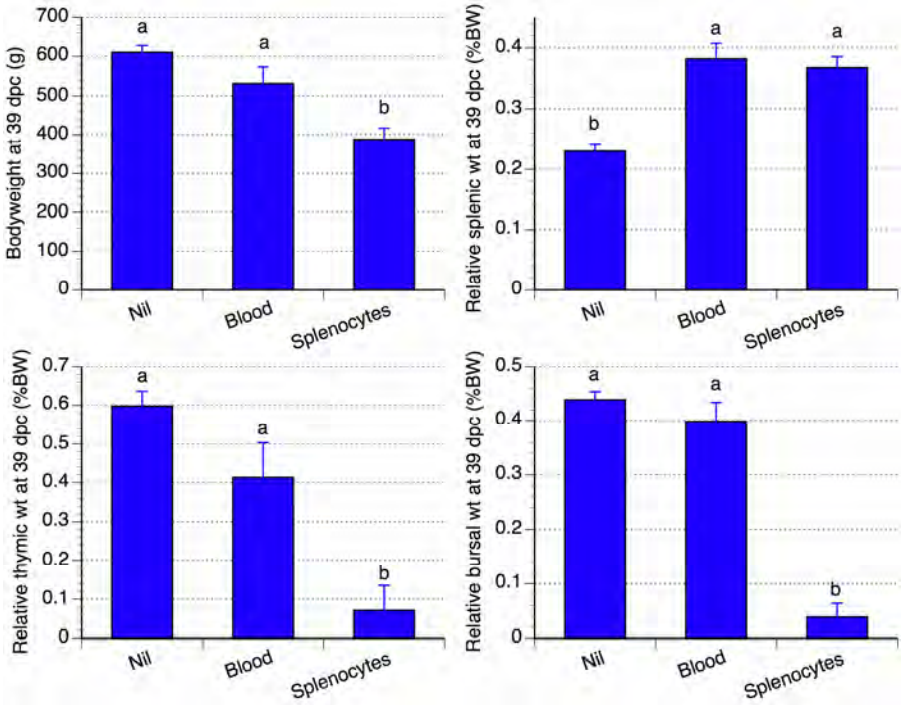

Figure 5.5. Trial 2. LS mean (±SEM) bodyweight (g), and relative immune organ weights (%LW) at 39 dpc. Group sizes were from 2 (Blood), 4 (Splenocytes) and 11 (Nil). Columns not sharing a letter differ significantly (P<0.05).

Brief discussion and conclusions

The experiment was successfully implemented with both trial 1 and trial 2 meeting their objectives.

In trial 1, dust infection by simple exposure to dust from a previous isolator experiment in SPF chickens produced successful infections with MDV for all 11 isolates tested with the induction of tumours in each case. The overall level of MD tumours induced was higher than that seen in Experiment 1 (MD04-C-PT2) in which infective dust was administered to individual chickens via the intra-tracheal route. While intra-tracheal infection uses less material and allows more quantitative challenge using a defined dose, it is very difficult and labour intensive, and simple exposure to dust should be the preferred method of infection if the infective dose is not critical.

As observed in other experiments in maternal-antibody negative SPF chickens, infection with most isolates was marked by the presence of the early mortality syndrome well before the onset of tumours. This syndrome, which will be described in detail in the General Discussion, is associated with marked atrophy of immune organs and neurological signs including paralysis, tremors and ticks. The pronounced impact of infection with MDV on immune organ weights in maternal antibody negative chickens was also demonstrated in this experiment, with the thymus unable to be found in some chickens at 39 dpc. Measures of thymic and bursal atrophy were strongly associated with the incidence of MD tumours and scores rather than actual weights had similar or stronger associations suggesting that the tedious work of dissecting out the thymus may be adequately replaced by thymic atrophy

scoring. Including atrophy scores as a measure of MD as done by (Witter 1997) increased the number of MD cases by 51% overall with some variation between MDV1 isolates. However it is difficult to determine what level or measure of atrophy is sufficient to constitute a case of MD.

Unfortunately the fresh infective spleen material produced in this experiment did not result in MDV1 growth to high titre in cell culture at RMIT. This differs from the very successful growth of many of the same MDV1 isolates to high titre on CK cells at RMIT following the provision of fresh spleen material from the similar experiment 1 (MD04-C-PT1). While recovery of MDV1 was adequate, the viruses did not grow to high titre on CK cells (Table 1.1, Chapter 1) and did not differ in recovery rate or titre from spleen material containing the same viruses from RIRDC experiment MD05-R-PT3 which was run just before the present experiment, but for which the spleen samples were delayed for an extra day during transit to RMIT. This clearly shows that fresh spleen material is relatively tolerant to delays in shipment (up to 48hrs). However, why the same isolates that grew so well out of fresh spleen material from experiment MD04-C-PT1 failed to do so subsequently from fresh spleen material from the present experiment and MD05-R-PT3 is unknown.

MPF23 was the most virulent MDV1 isolate from the 1980s in the study of McKimm-Breschkin *et al.* (1990) and it was gratifying that we were able to infect chickens with this isolate using stored infective blood and splenocytes from small experiments in which this isolate had been amplified in chicks from stored infective material at RMIT. While infective blood produced higher mortality rates and early MD incidence, chickens challenge with infective splenocytes, ultimately had 100% MD tumours and more marked immunosuppression (as determined by immune organ atrophy) than those challenged with infective blood, suggesting that the use of splenocytes provided a more uniform challenge. These challenge materials which have been widely used previously but have been criticised because the infectivity of challenge doses is not generally titrated and defined. The ability to measure the MDV content of these materials in a quantitative way using qPCR may make the use of these materials feasible, should titrated cell culture material not be available. Titration of the material in chickens to determine the infectivity of the material is also possible and is the subject of the next two chapters.

Despite being some 20 years old, MPF23 demonstrated a significant capacity to induce MD and severe immunosuppression, inducing the highest incidence of MD in the experiment and, in the case of splenocyte challenge, the most severe thymic and bursal atrophy. While direct comparisons between challenge treatments cannot be made due to the absence of specified challenge doses, and different routes of infection, it is clear that MPF23 remains a highly pathogenic MDV. Unfortunately it failed to grow at RMIT and is not included in any further experimentation in the project.

In conclusion this study has demonstrated that simple exposure of chickens to infective MDV1 dust in their isolators is a highly effective means of infection, and that properly cryopreserved infective blood and splenocytes are also highly effective. These materials have potential either as stored reservoirs of infective material or as alternate challenge materials in the absence of cell culture grown and titrated material. Should they be used for the latter purpose, means of determining the infectivity of the material and development of standard dose rates is required. This is the subject of the next two chapters.

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Chapter 6: Experiment 5. MD06-C-BID1

"Determination of the infective dose 50 (ID_{50}) of Marek's disease virus (MDV) in feather dust"

Start: 4/5/06 **Completion: 20/5/06** **AEC: UNE AEC06/059**

Introduction

Despite the production of highly infective fresh spleen material in SPF chickens from a wide range of isolates in experiments MD05-R-PT3 and MD06-C-VI5 at the end of 2005 and early 2006, MDV1 was still unable to be grown to sufficient titre for use in pathotyping experiments. Therefore, in order to meet the pathotyping obligations of the project we needed to consider alternative means of challenge.

In Chapter 2 (Experiment MD04-C-PT2) we had demonstrated the feasibility of challenging individual chickens with an intratracheal dose of MDV1-infective dust. However, for an effective challenge system based on dust to be developed, the infectivity of the dose delivered needs to be defined. For example, for cell culture derived infective material a pfu score based upon a $TCID_{50}$ is used.

This experiment set out to determine the feasibility of titrating the infectivity of dust containing MDV1 by dose titration in live chickens to determine the infective dose 50 (ID_{50}).

The main objectives of the experiment were:

1. To test whether titration of dust MDV1 infectivity is possible using a chicken bioassay.
2. To determine the BID_{50} (bird infective dose 50) of two dust samples, one of which is used in the study of MDV1 decay in infectivity at various temperatures over two years.

The execution of this experiment was led by Dr Fakhrul Islam with assistance from Paul Reynolds and Dr Steve Walkden-Brown.

Materials and methods

A total of 100 unvaccinated male IsaBrown layer cockerels from a commercial parent flock were used in this experiment. Parents had been vaccinated against MD with Rispens CVI988 (serotype 1 MDV) so the chicks contained maternal antibody directed against MDV1. The experiment utilized a two x five factorial design with two infective dust samples and five doses of dust (10, 5, 2.5, 1.25, 0.65mg per chicken). There were 10 treatment combinations (Table 6.1) with 10 chickens per treatment. An additional 10 chickens were not challenged with dust and remained as controls to detect any spread of infection between groups. Chickens were housed in a 4-deck brooder in a climate-controlled room in the UNE animal house and offered layer starter feed and water *ad libitum*.

Dust was inoculated to the chickens at day 6 of age by intra-tracheal insufflation under fume hoods in a separate laboratory. Chickens were sacrificed at day 16 of age (day 10 post-infection, pre infective) for harvesting of spleens. Whole spleens were weighed individually and MDV1 content was quantified using real-time qPCR as described in the General Materials and Methods.

Dust samples. The two challenge dust samples used were:

- MPF210/2s a back passage of 02LAR (collected from isolator 7 of the experiment MD05-R-PT3) having MDV1 content of 500 million (500,353,840 or $10^{8.70}$) per mg of dust.
- MPF57, collected from the experiment MD04-A6ISO. This is the same sample as that used for the MDV1 infectivity decay experiment (Chapter 9) having a viral copy of 6.3 million (6,336,616 or $10^{6.80}$) per mg of dust. The dust used in this experiment had been stored at -80°C for two years.

Statistical analysis. Spleen weight in the factorial design was analysed by ANOVA fitting the effects of challenge dust, dust dose and their interaction. Log_{10} transformed MDV1 (Log_{10} VCN+1)

content in spleen was also analysed by ANOVA testing the effects of viral strain and dust dose. Significant differences between individual means were determined using Tukey's HSD test. Chickens positive to qPCR for MDV1 were coded as positive and a dose response curve determined using logistic regression. The BID_{50} was then determined by solving the infectivity response equation for 50% infected. All analyses were conducted using JMP 6.0 (SAS Institute, NC, USA).

Table 6.1. Treatment allocation for the experiment.

Dust	Treatment	No of Chickens	Pen	Toe-mark
Dust 210/2s	10mg	10	8	LORI
	5mg	10	9	LORO
	2.5mg	10	10	RIRO
	1.25mg	10	11	LILORI
	0.625mg	10	12	LILORO
Dust MPF57	10mg	10	13	RIROLO
	5mg	10	14	RIROLI
	2.5mg	10	15	RIROLILO
	1.25mg	10	16	Black -head
	0.625mg	10	17	Black-body
Nil (Control)	0.00	10	18	Red- head

Results

Confirmation of application of treatments

Uninfected control chickens remained negative for MDV1 in spleen (10/10). For MPF210/2s and MPF57, 50% and 70% chickens were infected at the lowest dose (0.625mg) respectively. Infectivity increased to 100% for MPF210/2s at the second dose (1.25mg) onward and for MPF57 at 4th dose (5mg) (Fig 6.2A).

Spleen weight

Analysis of the overall effects of challenge on spleen weight, including the control chickens, revealed that challenge with dust markedly increased spleen size (P<0.001) with no difference between the two challenge dusts (Figure 6.1A)

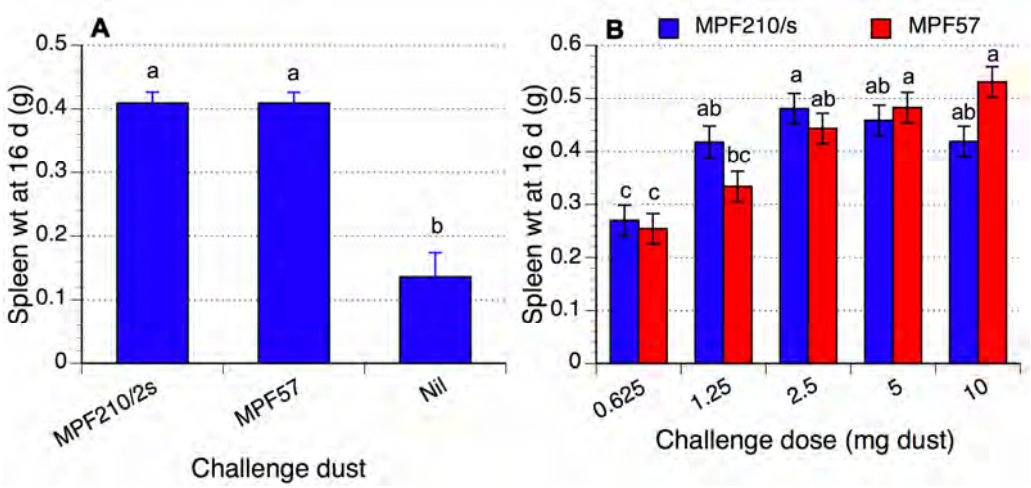


Figure 6.1. A. Overall effect of challenge with dust containing MDV1 on spleen weight (LSM±SEM) at 16 days of age (10 days post challenge) and **B.** interaction between the effects of challenge dust and dose of dust administered. Columns not sharing a common letter differ significantly (P<0.05)

Analysis of spleen weights excluding the controls revealed a significant overall effect of dust dose (P<0.0001), but not challenge virus (P=0.99), with significant interaction between these effects (P=0.01) (Figure 6.1B). The interaction was due to a steady increase in spleen size with increasing

dust dose for the MPF57 dust (low MDV1 content) whereas for the MPF210/s dust (high MDV1 content) maximal responses were observed at the 2.5mg dose.

MDV1 load in spleen

No MDV1 was detected in the spleens of chickens that were not challenged with dust (n=10), demonstrating the absence of spread of MDV1 within the 10-day post-infection period. On the other hand high proportion of chickens (89/99, 90%) challenged with infective dust were positive for MDV1 (Figure 6.2A). The proportion increased with increasing dose, but only at between the lowest two doses after which the proportion of chickens infected was 90-100%.

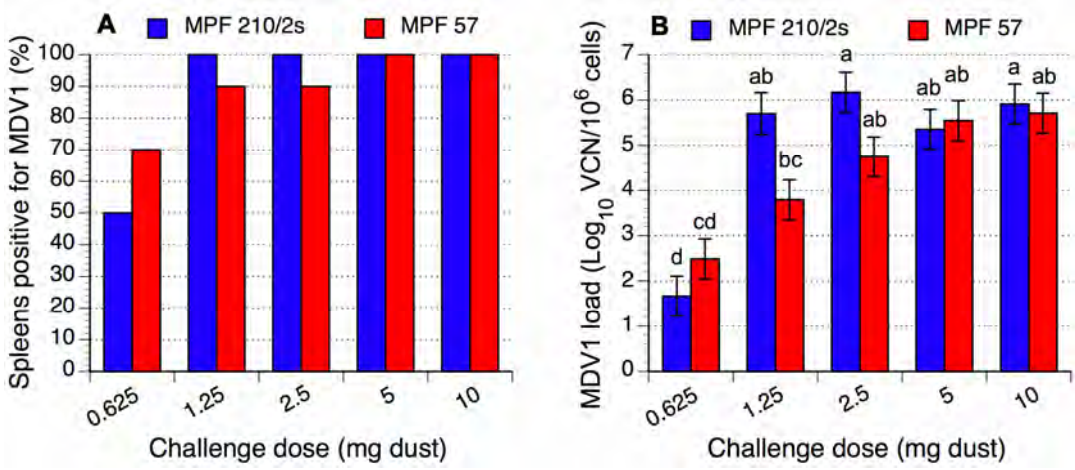


Figure 6.2. MDV1 in spleen. **A.** The proportion of chickens with spleens positive for MDV1 10 days post-challenge with various doses of infective dust. **B.** The effects of challenge dust and dose of dust administered on Log_{10}MDV1 load in spleen weight (LSM±SEM) at 10 days post-challenge. Columns not sharing a common letter differ significantly (P<0.05).

Quantitative determination of MDV1 load in spleen provided a more sensitive discrimination between treatment effects (Figure 6.2B). There was a significant overall effect of challenge dose (P<0.001) and a strong trend towards higher load in chickens challenged with MPF210/2s (P=0.075). Once again there was significant interaction between the effects of challenge dose and challenge dust (P<0.02). This was due to a strong positive association between challenge dose and MDV1 load with challenge dust MPF57 (particularly over the first 4 doses) whereas for challenge dust MPF210/2s there was a rapid increase in load between challenge doses 0.625 and 1.25mg, with little change thereafter (Figure 6.2B).

The treatment effects on MDV1 load in spleen may be influenced by the proportion of negative chickens in each treatment group (ie zero values). However analysis of MDV1 load excluding chickens which were negative, showed a very similar pattern (Figure 6.3).

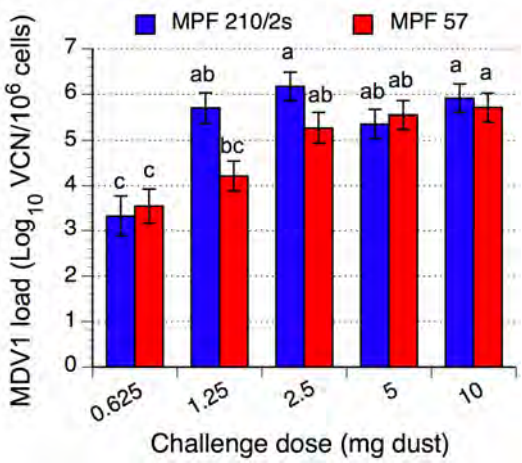


Figure 6.3. The effects of challenge dust and dose of dust administered on Log_{10}MDV1 load in spleen weight (LSM±SEM) at 10 days post-challenge. Zero values (uninfected chickens) have been removed for this analysis. Columns not sharing a common letter differ significantly (P<0.05).

This indicates that treatment effects were mainly on the load of MDV1 in the spleen of individual chickens, rather than on the proportion of infected chickens in each treatment.

Association between variables

The increase in spleen size associated with dust challenge may represent a non-specific response to increasing doses of dust, or a specific response to MDV1 which has been shown in many experiments to increase spleen size. Fitting a general linear model testing the effects of both dose of dust and MDV1 load in spleen on the weight of the spleen 10 days post challenge revealed that the effect of dust dose approached significance (P=0.06) whereas the effect of MDV1 load was highly significant (P<0.001). The two sources of variation accounted for 18% and 36% of the observed variation in spleen size respectively. Clearly the specific action of MDV1 is the major factor responsible for the increases in spleen size associated with increased challenge dose (Figure 6.4A).

As the MDV1 content of the challenge dusts was determined by qPCR prior to challenge, the different challenge doses could be expressed in terms of copies of MDV1 rather than in mg of dust. The load of MDV1 in spleen 10 days after challenge could then be related to initial challenge with MDV1. As is shown in Figure 6.4B there was a highly significant association, but it was dependant upon the challenge virus. For each of the challenge viruses 37% of the variation in MDV1 load in spleen 10 days after challenge was explained by the initial challenge dose of MDV1 in dust, determined by qPCR. The slope of the relationship was the same for both challenge viruses (2.7) but the X-axis intercepts were very different ($10^{5.56}$ for MPF57 and $10^{7.31}$ for MPF210/2s) respectively, a difference of 1.75 logs. This suggests that the infectivity of MPF57 is considerably higher than that of MPF210/2s. Interestingly the MPF57 sample had been stored for 2 years at -80˚C prior to use. This appears to have been associated with no significant loss of infectivity. It may seem from the earlier figures that MPF210/2s was the virus with higher infectivity, but rather it appears that its 1.75 logs lower infectivity was more than offset by it having 1.9 logs higher initial content of MDV1 in the challenge dust.

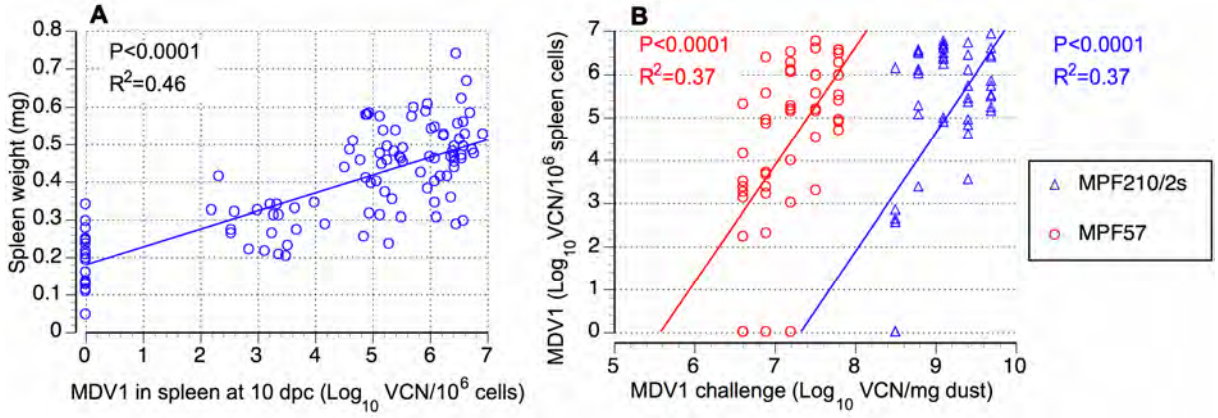


Figure 6.4. A. Association between spleen weight 10 days after challenge and $\log_{10}$ MDV1 load in spleen cells at the same time. **B**. Association between $\log_{10}$ MDV1 load in spleen cells 10 days after challenge and $\log_{10}$ MDV1 load in the challenge dust. In both cases each point represents an individual chicken and the lines are linear regressions.

Estimation of BID_{50}

The range of challenge doses used did not provide a good spread of infectivity on either side of 50% so accurate estimation of BID_{50} using traditional methods (eg Reed and Muench method, logistic regression) was not possible. By chance the minimum infectivity of the dust MPF210/2s was 50% at a dose of 0.625mg. This equates to 3.13 x 10^8 million viral genome copies ($10^{8.5}$) and may be a crude approximation of a $BID_{50.}$ For MPF57, the minimum level of infectivity was 70%. While it is not advisable to extrapolate backwards to determine the BID_{50}, doing so based upon a 2nd degree polynomial fit between the 0.625mg dust (3.9 x 10^6 VCN) and the 5mg dust (3.15 x 10^7 VCN) doses, provides a BID_{50} estimate of 2 x 10^6 VCN ($10^{6.3}$). This suggests a difference in infectivity of 2.19 logs in favour of MPF57.

A much more robust approach would appear to one based upon the quantitative relationships shown in Figures 6.2B and 6.4B rather than quantal relationships shown in Figure 6.2A. These could be used in two ways. Firstly they could be used alter the definition of "infection", in this case by changing the definition from the current one which is the threshold of detection of MDV1 in spleen (around 200 copies per 10^6 cells) to a higher standardized number. This could then be applied to individual chickens which would then be scored + or – accordingly, and standard quantal assay methods could be used. This has the effect of shifting the level of infectivity downwards if values around 50% are not achieved. For example if a case definition of MDV1 in spleen at Log4 or higher 10 days post challenge is used, the resultant data set for infectivity is as shown in Figure 6.5. This has produced an even spread of values around 50% infection for MPF57 with some improvement in the data for MPF210/2s also.

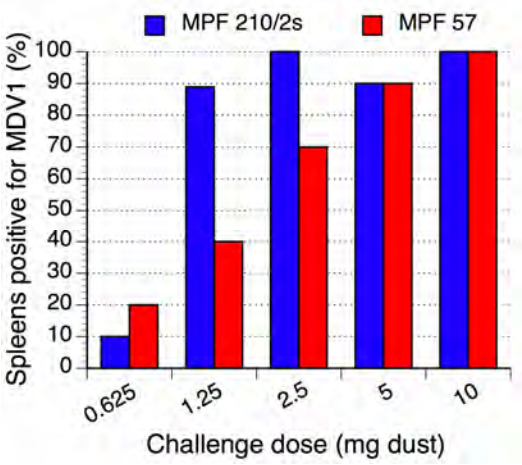


Figure 6.5. The proportion of chickens with spleens positive for MDV1 10 days post-challenge with various doses of infective dust. In this case the definition of "positive" is an MDV1 load of 10^4 or greater. This can be contrasted with Figure 6.2A in which the definition of "positive" is any positive value above the threshold of detection.

Logistic regression analysis of this data provides the estimates of BID_{50} shown in Table 6.3. Interestingly the difference in infectivity of the two viruses is close to that observed on the basis of the regression curves in Figure 6.4B.

Table 6.3. Estimates and 95% confidence intervals for BID_{50} of dusts containing MPF210/2s and MPF57 based on a definition of infection as a load of MDV1 in spleen 10 days after challenge of 10^4 or greater.

Virus	BID_{50} estimate (Log_{10} VCN)	Lower 95%CI	Upper 95% CI	R^2 of model	P value
MPF210/2s	8.67	8.44	8.83	0.48	<0.0001
MPF57	6.97	6.72	7.16	0.34	<0.0001
Difference	1.70				

A second way of using the quantitative data is simply to define a challenge dose based on the linear relationships described in Figure 6.4b. For example a "minimum infective dose" This could be defined as the dose providing a predicted level of MDV1 of some value close to the threshold of detection, 10 days after challenge. In our case, if we selected a threshold value of 200 VCN/10^6 spleen cells ($10^{2.3}$), the "minimum infective dose" of MPF210/2s and MPF57 would be as shown in Table 6.4. However because the size of the standard error increases at the extremities of a linear relationship, an alternative is simply to define a "standard challenge dose" to produce a higher level of MDV1. For example, the dose providing a mean level of MDV1 in spleen of 10^4 or 10^5 VCN/10^6 cells (Table 6.4).

Table 6.4. Estimates (with 95% CI) for the minimum infective dose (MID), standard challenge dose 1 (SCD1 and standard challenge dose 2 (SCD2) of dusts containing MPF210/2s and MPF57. MID is defined as a load of $10^{2.3}$ or greater, SC1 a load of 10^4 or greater and SC2 a load of 10^5 or greater (Log_{10} VCN/10^6 cells).

Virus	MID estimate (Log_{10} VCN) (95%CI range)	SCD1 estimate (Log_{10} VCN) (95%CI range)	SCD2 estimate (Log_{10} VCN) (95%CI range)	R^2 of model	P value
MPF210/2s	8.15 (7.45-8.5)	8.76 (8.43-8.95)	9.12 (8.94-9.32)	0.34	<0.0001
MPF57	6.41 (5.88-6.67)	7.03 (6.81-7.20)	7.40 (7.24-7.63)	0.37	<0.0001
Difference	1.74	1.73	1.72		

Brief discussion and conclusions

This proved to be a very useful and informative small study. Overall the main findings were:

1. Despite difficulties with it as a challenge material the infectivity of dust can be titrated and determined effectively in chickens.
2. The use of a quantitative end-point, such as viral load in spleen, provides greater flexibility and probably accuracy in determining infectivity, than a simple qualitative end point.
3. Based on MDV1 content in the dust measured by qPCR, different MDV1s may exhibit marked differences in their infectivity.
4. Spleen enlargement following challenge shows some potential as a crude bioassay of MD infectivity.
5. The presence of maternal antibody directed against MDV1 in the chickens used for the bioassay did not appear to detract from its efficacy.

These findings, in combination mean that it is now a practical proposition to used infective dust as a challenge material in formal pathotyping experiments with the challenge dose defined in terms of its infectivity eg. 10 x BID_{50}, 20 x MID (minimum infective dose) etc. We also have a tool to formally assess the infectivity of different dust samples containing MDV1.

Regarding finding 1 it is reassuring that the infectivity of dust appears to be dose related. This was clearly so in the case of MPF57, and less clearly in the case of MPF210/2s which had a very high initial load in the infective dust resulting in maximal levels of infectivity above the lowest dust dose. The high levels of infectivity achieved, excessive for the calculation of BID_{50}, could be reduced by reducing the duration of the bioassay to perhaps 6 or 7 days rather than 10. This is attractive from most perspectives. Despite the possibility of using dust as a challenge material, it has several drawbacks. Firstly handling and dose determination are difficult and cross contamination is a greater risk than with liquid suspensions. Secondly, intra-tracheal administration is technically demanding for large numbers of birds, and a relatively small range of doses is practically possible (the range used in this experiment). Thus, dusts of low MDV1 content are unlikely to be infective by this challenge method. Indeed in Experiment 1 (MD04-C-PT2) using a dust challenge dose of 2mg/bird it was observed that a MDV1 load of 10^5/mg dust or greater was a pre-requisite for success. Despite these drawbacks dust also has several advantages. It is a highly concentrated source of infective MDV1 which remains infective for long periods at easily accessible storage temperatures (4˚C, -20˚C) and even at room temperature. In many ways it is an ideal repository of infective material which can be titrated and stored for later use.

Regarding finding 2, the quantitative determination of MDV1 in the initial infective material and in the end point material (spleen in this case) is the crucial breakthrough which enables this bioassay for infectivity to be performed quickly and relatively inexpensively. The sensitivity of the method provides very early end point – potentially several days earlier than the 10 days used in this experiment. This is prior to any clinical disease, and therefore suffering in the chicks used, and prior to any shedding of infective virus, thus doing away with any requirement of isolation between groups. The quantitative nature of the end point also enables classical regression methodology to be used to define relationships between continuously distributed variables (eg infective dose of MDV1 and MDV1 load in spleen) and for end points to be refined or changed on a sliding scale. We now have evidence from a great many experiments that measures of viral load measured in a range of tissues are invariably strongly associated with classical end points for MD such as tumour induction and mortality, so our confidence in the measure is high.

Regarding finding 3, the large difference in infectivity between the two MDV1 isolates contained in the dusts was rather surprising. MPF57 had 60-fold higher infectivity than MPF210/2s despite more than 2 years storage prior to experiment. Some evidence that this was not an aberrant finding can be found in the results of Experiment 4 (Chapter 5, MD06-C-VI5) in which chickens were exposed to

highly infective dust in their isolators. In that experiment, with dusts sourced from a different experiment to those used in the present experiment, MPF210/2s infective dust had a MDV1 load of 30.02 x 10^6 VCN/mg dust while that of MPF57 had a load of 4.26 x 10^6 VCN/mg dust. Despite the advantage in initial challenge load, MPF210/2s induced tumours in only 7.7% of chickens, whereas MPF57 induced tumours in 57.1% of chickens. It may be argued that these differences relate to differences in virulence rather than infectivity, as we and others have shown that virulence is positively associated with MDV1 replication and viral load. This emphasizes the need for as early a measurement of infectivity as possible, so that the effects of infectivity and virulence are not confounded. These issues are not confined to the bioassay in chickens - the factors influencing MDV1 growth and induction of cytopathic change in cell culture are potentially complex, poorly understood and confound any measures of infectivity based on these methods.

Regarding finding 4, we have noted in earlier experiments that splenomegaly is a good marker for MDV1 load in spleen and for future MD outcomes. While potentially a non-specific response to infection, we have found it to be reliable marker for MDV1 in challenge experiments, despite the potential for other infections to be present. In the present experiment it was important to differentiate between spleen enlargement due to challenge with dust per se, and that due to the MDV1 content in dust. The analyses used show conclusively that the majority of the variation associated with spleen enlargement in this experiment was due to MDV1 rather than the amount of dust the chicken was challenged with. Bodyweight was not recorded in the present experiment and the usefulness of spleen would probably be even higher if relative spleen weight (%BW) was used, rather than absolute spleen weight. Nevertheless, MDV1 load in spleen or other tissues is likely to be a more specific and sensitive measure of MD status and is the preferable end point for these bioassays.

Regarding finding 5 it was reassuring to find that the presence of maternal antibody did not prevent high levels of infection and MDV1 replication in spleen, and associated splenic enlargement. The use of male layer cockerel chicks, which would otherwise be killed at hatch provides a cheap, readily available and effective resource animal for the bioassay.

In conclusion this experiment has confirmed the feasibility of objectively measuring the infectivity of dust samples containing MDV1. This combined with intra-tracheal insufflation with defined amounts of infective dust, provides an alternative defined-dose challenge model for MDV1. Possible improvements of the assay arise from this preliminary study, most notably the possibility of reducing the infection to detection period to 7 days.

Chapter 7: Experiment 6. MD06-C-BID2

"Determination of the ID_{50} of Marek's disease virus in infective cell-culture material"

Start: 1/6/06 **Completion: 14/6/06** **AEC: UNE AEC06/086**

Introduction

In order to run the second round of MDV1 pathotyping experiments infective material from new isolates was required. However we have had trouble in recent times growing MDV11 to high titre (>10^4 pfu/ml) in CKC culture. In contrast, in two recent experiments at UNE (MD05-R-PT3, Nov-Dec 2005 and MD06-C-VI5, Jan-Feb 2006) large numbers of MDV11 isolates grew well in SPF chickens inducing high levels of mortality and tumours.

MD05-R-PT3 used infective material from RMIT and dusts and MPF23 infectious material back-passaged at UNE from primary material supplied by RMIT (P3 CEK from Newcastle Uni). This experiment was Experiment 5 in the Final Report of RIRDC Project UNE 83-J and results are provided in detail there (Walkden-Brown *et al*. 2006). Fresh spleen samples (48 dpc) from this experiment were sent down to RMIT arriving on 14 Dec 2005 arriving after 2 nights in transit rather than one. This material was grown to passage 6 on CK at RMIT.

Because of this delay in transit, a second experiment in SPF chickens was undertaken at UNE, MD06-C-VI5 (Experiment 4, Chapter 5 in this report). Chickens were infected with isolator dust from the successful treatments in the previous experiment (MD05-R-PT3). Fresh spleen samples from 39 dpc) were sent to RMIT overnight successfully using two different couriers, arriving on 28/2/06. These samples were grown to passage 4 on CK at RMIT. It should be noted that these samples represent an additional back-passage in chickens over those from the earlier experiment.

By the end of April 2006, 11 viruses grown to comparatively low titre (17-680 pfu/ml) at RMIT when different back-passages of the same virus are grouped (Table 1). Titres and amounts of infective material were insufficient to run formal pathotyping experiments at any previously used dose (50-500pfu/chicken). Each experiment requires approximately 108 chickens to be challenged for each challenge virus – a total requirement of 216 infective doses from each batch of infective material.

Table 7.1. Summary of outcomes of CK cell culture runs at RMIT in Feb-April 2006 using spleen samples from 2 UNE experiments.

Original isolate and back-passage	Alternative name	MD06-C-VI5. Passage 4 in CK (2 extra back passages in chickens)			MD05-R-PT3. Passage 6 in CK (1 extra back passage in chickens)		
		Passage	pfu/ml	Volume	Passage	pfu/ml	Volume
MPF23 P3		P4 020406	60	20x1.8mL			
Woodlands1		P4 020406	600	6x1.8mL	P6 020406	680	15x1.8mL
MPF57		P4 020406	80	12x1.8mL			
MPF57 B1	176				P6 020406	27	2x1.8mL
MPF57 B1	179/6				P6 020406	300	20x1.8mL
MPF57 B2	W7B1s				P6 020406	110	12x1.8mL
FT158					P6 020406	17	12x1.8mL
FT158 BI	210/1S	P4 020406	100	6x1.8mL	P6 020406	50	6x1.8mL
02LAR					P6 020406	60	12x1.8mL
02LAR B1	210/2S	P4 020406	70	6x1.8mL	P6 020406	30	12x1.8mL
164/6					P6 020406	70	6x1.8mL
04KAL		P4 020406	140	12x1.8mL			
189/8		P4 020406	90	6x1.8mL	P6 020406	60	12x1.8mL
192 4&10					P6 020406	140	12x1.8mL
199 3&9		P4 020406	200	8x1.8mL	P6 020406	110	12x1.8mL
05JUR (B1)	212				P6 020406	210	12x1.8mL

It is possible that the samples in Table 1 have significant amounts of MDV11 in them, but the virus has failed to exhibit significant cytopathic effects on titration. A viral titration in chickens would ascertain this and may enable the material to still be used in pathotyping experiments on the basis of a formal dose rate based on multiples of a BID_{50} (bird infectious $dose_{50}$). This is the main objective of this experiment which included the most virulent MDV11 isolates from the 1980s, early 1990s and early 2000s. The detailed objectives were:

1. To determine if there is sufficient infective material in the low-titre cell culture material to run a full pathotyping experiment
2. To titrate infectivity of this material in chickens. This will require us to identify optimum end points and methods for titrating infectivity in chickens
3. To determine whether there is a correlation between *in vivo* and *in vitro* infectivity

The execution of this experiment was led by Dr Steve Walkden-Brown with assistance from Paul Reynolds and Dr Fakhrul Islam.

Materials and methods

The experiment had an 11 x 5 factorial design with the following factors and levels:

- Challenge MDV11. This comprised 11 MDV1s isolated from spleen of SPF chickens in experiments MD05-R-PT3 (Nov-Dec 2005) and MD06-C-VI5 (Jan-Feb 2006) and grown in CK at RMIT.
- Dilution. All challenge viruses were used at 5 dilutions of the original infective material supplied by RMIT. The dilution series was a 5-fold serial dilution from neat to 1:625 and equivalent to 200, 40, 8, 1.6 and 0.32 ul of the original infective material. The Woodlands 1 virus had one additional dilution included (1:3125).

Infective cell culture material was used undiluted at the highest concentration (170-200ul/chick depending on amount in the ampoule), then serially (5-fold) diluted as described above, in diluent sent up from RMIT (MEM + HEPES + $NaOH_2CO_3$+Antibiotic) with 200ul all subsequent dilutions used per chick. Chicks were inoculated intramuscularly into the breast muscle for 3 challenge viruses (MPF23, Woodlands 1 and MPF 179/6), and intra-abdominally for the remaining viruses. An uninfected control group was kept in the same brooder to test for cross infection. There were 57 treatment combinations, with approximately 6 chickens per treatment and 346 chickens used in total (Table 7.2).

Table 7.2. Overview of treatments and chick numbers

Brooder	Original virus and year of primary isolation	Code and back passage[A]	Batch	CK titration (pfu/ml)	Vol avail	Toe mark	No birds
1	MPF23 1980s	+2	P4 020406	60	20x1.8mL	LI	30
2	W'lands1 1992	+1	P6 020406	680	15x1.8mL	LO	36
3	MPF57 1994	179/6 B1+1	P6 020406	300	20x1.8mL	RI	30
4	02LAR 2002	+1	P6 020406	60	12x1.8mL	RO	30
5	FT158 2002	+1	P6 020406	17	12x1.8mL	LILO	30
6	MPF164/6 2003	+1	P6 020406	70	6x1.8mL	LIRI	30
7	04KAL 2004	+2	P4 020406	140	12x1.8mL	LIRO	30
8	Control					LORI	10
9	189/8 2004	+1	P6 020406	60	12x1.8mL	LORO	30
10	192 4&10 2004	+1	P6 020406	140	12x1.8mL	RIRO	30
11	199 3&9 2004	+1	P6 020406	110	12x1.8mL	LILORI	30
12	05JUR 2005	212 B1+1	P6 020406	210	12x1.8mL	LORORI	30
TOTAL							346

[A]Code of source virus in originating experiment and number of additional passages to present experiment

The 12 main treatment groups (11 MDV11s + control) were each maintained in a separate brooder cage in a multi-deck brooder in a single climate-controlled room in the UNE animal house. Birds in

each treatment were given a unique toe web mark and challenge doses within each challenge treatment were identified on by permanent marker. Layer chick starter and water were offered *ad libitum*.

The experimental chickens were male commercial IsaBrown layer cockerels from parents vaccinated with Rispens CVI988 vaccine so they were positive for maternal antibody homologous to MDV11. Chickens arrived at UNE on 1/6/06 (day -1), were challenged on day 0 and sacrificed on day 12 post-challenge for tissue measurements and recovery. The experimental dates, timing and measurements are shown in Table 7.3

Table 7.3. Timing and measurements

Date and Day	Activities
1/6/06 Thursday (day -1)	• Chickens transported to UNE from Tamworth Winton hatchery • Treatment marked by toe-web cutting. Chickens placed in brooder in controlled environment room in Animal House C and provided with food and water *ad libitum*
2/6/06 Friday (day 0)	• Chickens challenged ia with MDV11 as described above • Residual primary infective material kept for qPCR analysis
9/6/06 Friday (day 7)	• Check chick colour markings and re-mark as necessary
14/6/06 Wed (day 12)	• Chickens killed o Body weight o Spleen weighed and stored for qPCR for MDV11 o Thymic atrophy score (0-3) o Bursal weight and atrophy score (0-3)

Spleen samples were assayed for MDV11 load as described in the General Materials and Methods. Residual infective material at the neat, 1/25 and 1/625 dilutions was treated with Proteinase K, and assayed for MDV11 directly using 5ul of the material as template in the reaction.

Statistical analysis. Continuous variables in the factorial design was analysed by ANOVA fitting the effects of challenge treatment, challenge dose and their interaction. MDV11 load was log transformed (Log_{10} VCN+1) prior to analysis. Significant differences between individual means were determined using Tukey's HSD test. Data are generally presented as least squares means ± SE. Linear regression was used to test associations between continuously distributed variables. Chickens positive to qPCR for MDV11 were coded as positive and a dose response curve determined using logistic regression. The BID_{50} was then determined by solving the infectivity response equation for 50% infected. All analyses were conducted using JMP 6.0 (SAS Institute, NC, USA).

Results

Confirmation of application of treatments

The experiment was successfully implemented. Infection was successful in all challenge treatments except MPF164 and the control chickens (n=5) remained free of infection as determined by qPCR detection of MDV11 in spleen at 12 dpc. Five chickens of the 346 placed (1.4%) died prior to 12 dpc. Of the chickens not in the Control or MPF164 treatments, 110/301 (36.5%) were positive for MDV11.

MDV1 content of infective cell culture material used as challenge material

This is summarized in Table 7.4 and Figure 7.1. MDV1 1 was detected in all samples at dilutions of 1:1, 1:25 and 1:625. The dilution ratios were not reflected in the qPCR results with a greater than predicted reduction in MDV11 content with each dilution (Table 7.4). There was a weak positive relationship between titre calculated on CK culture and MDV11 content in the undiluted samples (P=0.25) and a stronger positive relationship between titre and MDV11 content in samples diluted 1:625 (P=0.03, Figure 7.1).

Table 7.4. Comparison of titre of samples used and MDV11 load determined by qPCR at 3 different dilutions.

Virus	Titre on CK (pfu/ml)	MDV11 load (VCN/reaction)		
		Neat	1/25	1/625
Woodlands	680	474,288	2,581	77
MPF57	300	208,454	1,366	8
05JUR	210	39,524	598	6
MPF192	140	110,564	305	6
04KAL	140	51,793	445	61
MPF199	110	49,322	230	5
MPF164	70	49,593	99	3
MPF189	60	431,479	316	20
02LAR	60	152,045	270	8
MPF23	60	83,663	10,734	9
FT158	17	431,717	254	26
Mean		189313	1563	21
Ratio relative to neat		1	121	9042

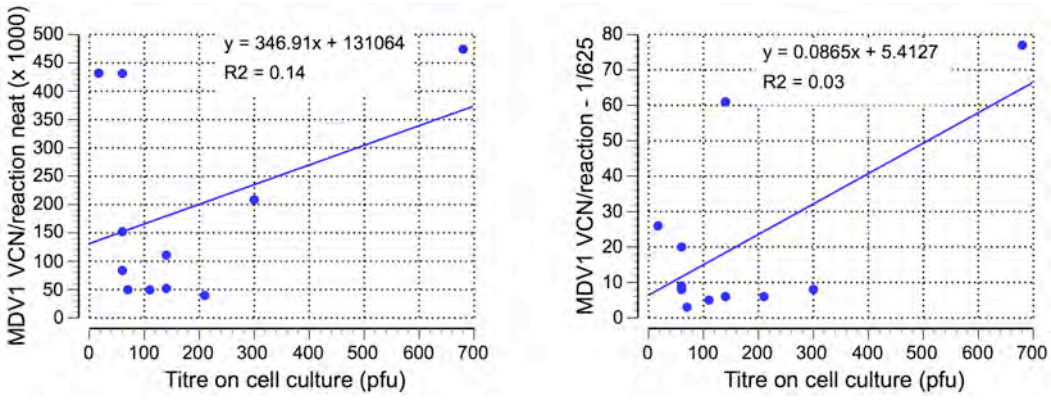


Figure 7.1. Linear regression of MDV11 load determined by qPCR in undiluted (A) and diluted 1:625 (B) samples on viral titre determined in CK culture.

Bodyweight and immune organ weights

Factorial analysis of chickens challenged with MDV11 showed that bodyweight was significantly affected by MDV11 isolate (P<0.0001), but not dilution factor (P=0.231) with no significant interaction between these effects (P=0.593). Relative spleen weight was significantly affected by MDV11 isolate and dilution factor (P<0.0001) with no interaction between these effects (P=0.345). Relative bursal weight was significantly affected by MDV11 isolate (P=0.024) but not dilution factor (P=0.378) with no significant interaction between these effects (P=0.177). Thymic atrophy score (0= no atrophy, 3 = severe atrophy) was significantly affected by MDV11 isolate (P<0.0001) and dilution factor (P=0.05) with no significant interaction between these effects (P=0.928).

As there were no significant interactions between plots the main effects of MDV11 isolate and dilution factor for these variables are shown in Figures 7.2 and 7.3 below.

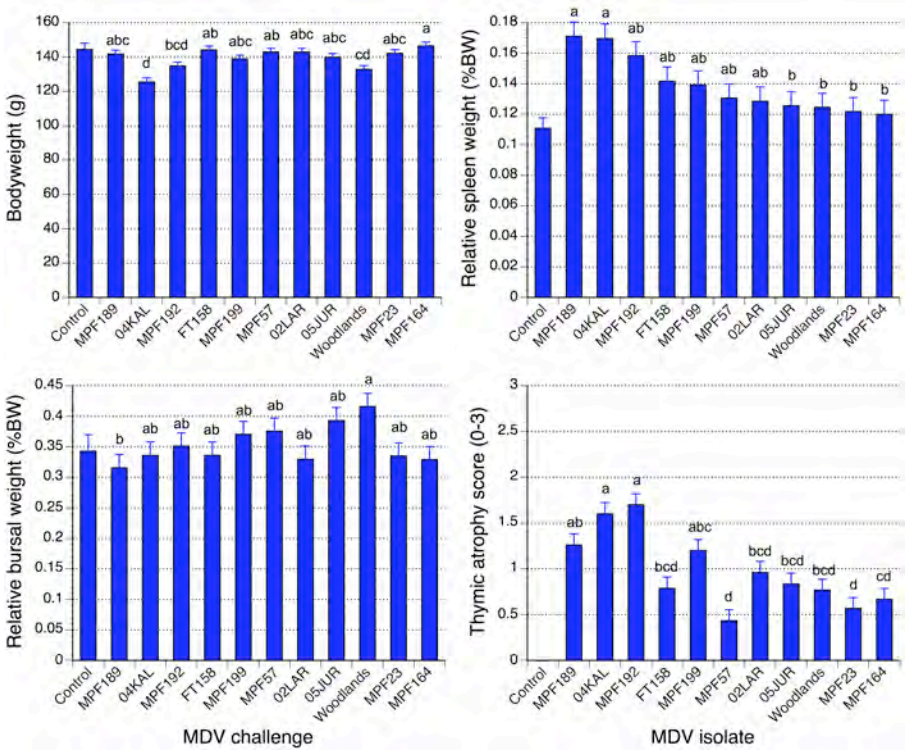


Figure 7.2 Main effect of MDV11 isolate on bodyweight (A), relative splenic (B) and bursal (C) weights and thymic atrophy score (D) of chickens 12 days after challenge with MDV11 infective material (LSM±SEM). Challenge isolates are sorted by relative splenic weight. Means not sharing a common letter are significantly different ($p<0.05$). Control chickens were not included in the factorial analysis and simple means and standard errors for this group are presented for reference.

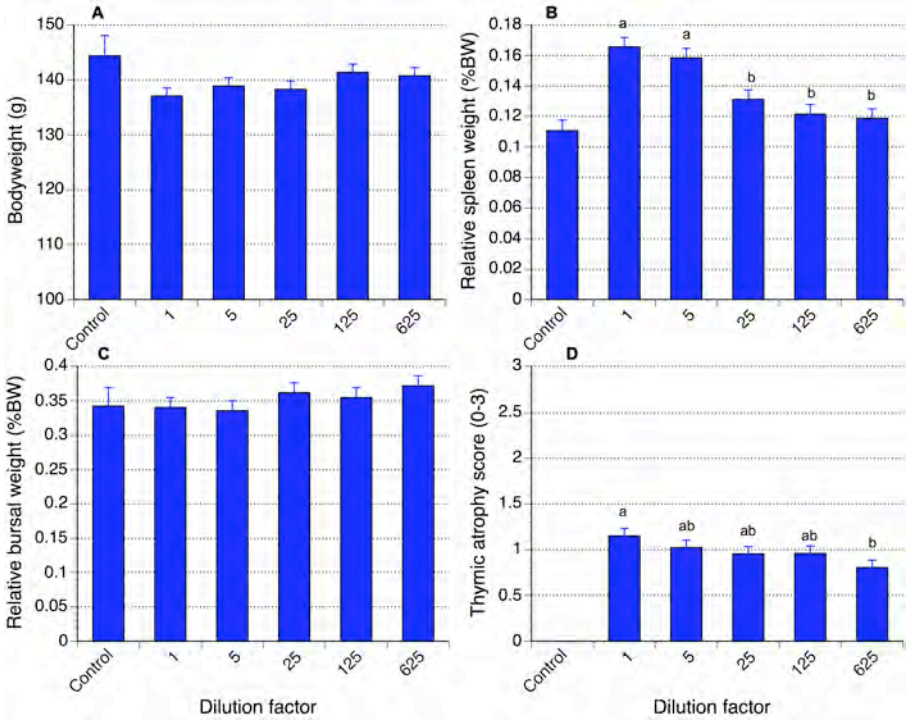


Figure 7.3 Main effect of dilution factor on bodyweight (A), relative splenic (B) and bursal (C) weights and thymic atrophy score (D) of chickens 12 days after challenge with MDV11 infective material (LSM±SEM). Means not sharing a common letter are significantly different ($p<0.05$). Control chickens were not included in the factorial analysis and simple means and standard errors for this group are presented for reference.

MDV11 load in spleen of infected chickens at 12 dpc

At 12 dpc MDV11 was detected in spleen of all groups except the controls (0/5) and the challenge group MPF 164 (0/30). Whether viewed in terms of proportion of chickens positive (Figure 7.4A) or the MDV11 load in spleen (Figure 7.4B) there were clear effects of challenge virus and dilution factor. The data underlying these figures is presented in Table 7.5. Of the chickens not in the Control or MPF164 treatments, 110/301 (36.5%) were positive for MDV11 and of those challenged with the undiluted infective material 37/60 (61.7% were positive).

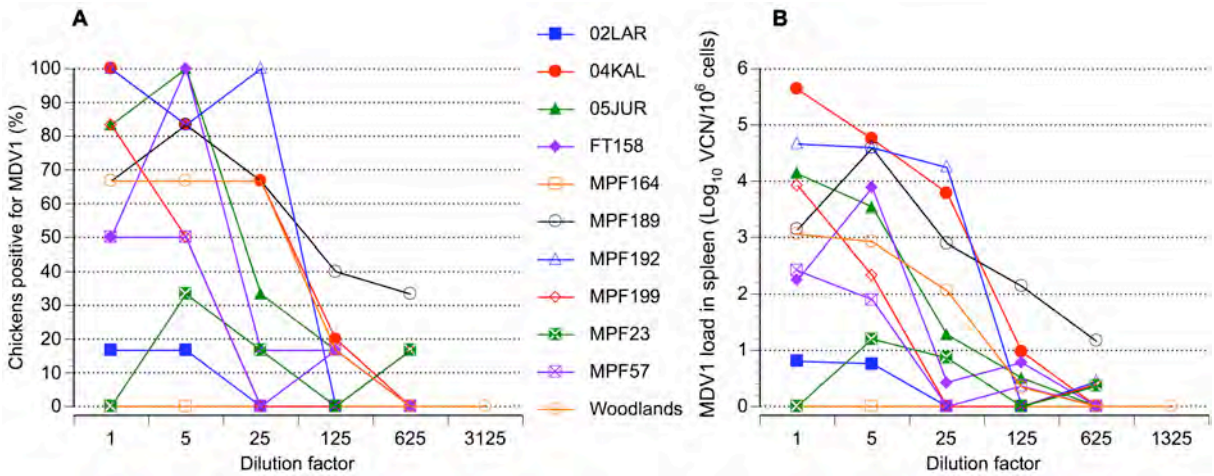


Figure 7.4 A. Percentage of chickens positive for MDV11 in spleen at 12 dpc by challenge group and dilution factor **B.** Mean load of MDV11 (log_{10} VCN/10^6 cells) at 12 dpc by challenge group and dilution factor.

Table 7.5. Percentage of chickens positive for MDV11 in spleen at 12 dpc and mean load of MDV11 (log_{10} VCN/10^6 cells) by challenge group and dilution factor at 12 dpc.

Challenge group	Chickens positive for MDV11 (%) (by dilution factor)						Log10 MDV11 load in spleen (VCN/10^6 cells) (by dilution factor)					
	1	5	25	125	625	3125	1	5	25	125	625	3125
MPF192	100	83	100	0	17		4.66	4.60	4.24	0.00	0.44	
04KAL	100	83	67	20	0		5.64	4.75	3.79	0.97	0	
05JUR	83	100	33	17	0		4.13	3.54	1.28	0.51	0	
MPF199	83	50	0	0	17		3.93	2.33	0	0	0.39	
MPF189	67	83	67	40	33		3.13	4.59	2.89	2.14	1.16	
Woodlands	67	67	67	17	0	0	3.06	2.92	2.06	0.34	0	0
FT158	50	100	17	17	0		2.25	3.88	0.43	0.78	0	
MPF57	50	50	0	17	0		2.42	1.90	0	0.36	0	
02LAR	17	17	0	0	0		0.80	0.76	0	0	0	
MPF23	0	33	17	0	17		0.00	1.20	0.86	0	0.36	
MPF164	0	0	0	0	0		0	0	0	0	0	

Leaving out the control and MPF 164 treatments and the 1/3125 dilution for the Woodlands treatment there was a significant effect of challenge virus ($P<0.0001$) and dilution factor ($P<0.0001$) with significant interaction between the two ($P=0.006$). The main effects of challenge virus and dilution factor shown in Figure 7.5. Overall mean MDV11 loads (Log_{10}) for the different challenge viruses varied from 0.31±0.31 for 02LAR to 3.02±0.33 for 05KAL. There was no effect of dilution between dilution factors 1 and 5, with decreases for each additional dilution after this (Figure 7.5B).

The effects of challenge virus and dilution factor on MDV11 load may be due to effects on the infection rate of chickens, effects on the growth of MDV11 in infected chickens, or both. Removal of all uninfected chickens from the analysis did not remove the significant effects of challenge (Figure 7.5C) or dilution factor (Figure 7.5D), but it did greatly the magnitude of the effects. There was a significant effect of challenge virus ($P=0.0004$) and dilution factor ($P=0.002$). Viral load tended to be highest in the viruses with the highest proportion of infected chickens and viral load declined with increasing dilution factor (Figure 7.5D). The latter indicates an effect of infective dose on viral load in spleen independent of the number of birds infected.

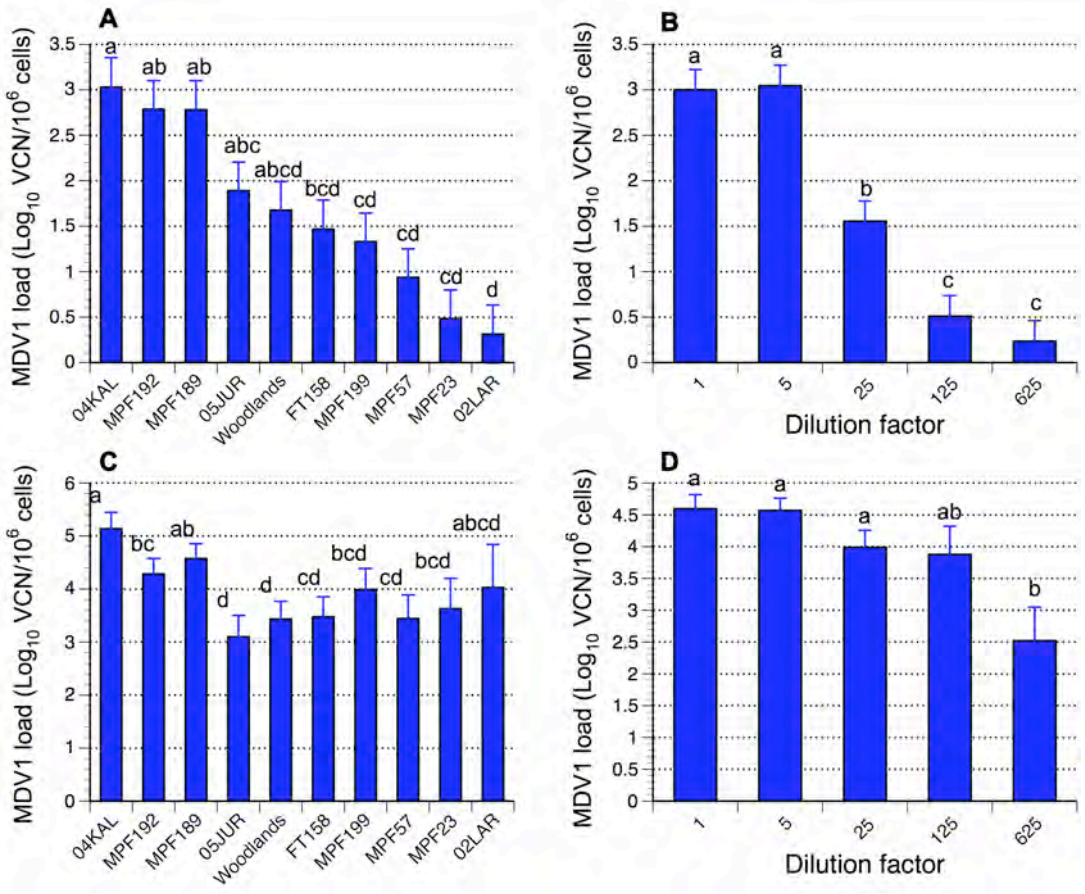


Figure 7.5. Least squares mean (±SEM) load of MDV11 in the spleen (log_{10} VCN/10^6 cells) of chickens 12 days after challenge with MDV11 infective material. Top panels show the results of analysis including zero counts, A. Effect of MDV11 isolate, B. effect of dilution factor of infective material. Bottom panels show the results of analysis excluding zero counts, C. Effect of MDV11 isolate, D. effect of dilution factor of infective material

Association between variables

As the initial MDV11 content of the infective material, its dilution rate and dose of administration are known it is possible to examine the association between the dose of MDV11 administered and the load of MDV11 in spleen at 12 dpc, collectively and for each virus. For two viruses (MPF23 and 02LAR) there was no significant association between the two given the very poor infectivity of the infective material for these viruses in this experiment. However there was a significant relationship overall, and for each of the remaining viruses individually (Figure 7.6, Table 7.7).

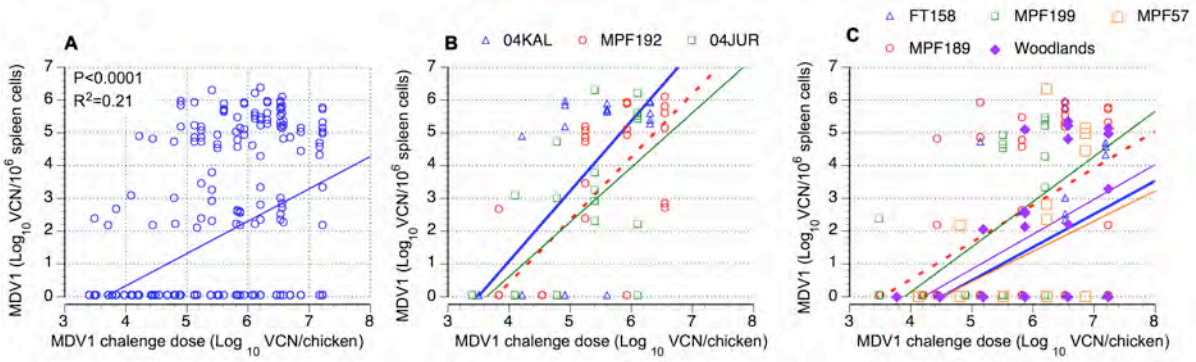


Figure 7.6. Linear regression of load of MDV11 in the spleen (log_{10} VCN/10^6 cells) of chickens at 12 dpc on the initial challenge dose of MDV11 as determined by qPRC. **A**) All viruses combined, **B**) three challenge viruses with the strongest association and steepest slope, **C)** the remaining viruses. See Table 7.6 for details.

Table 7.6. Details of the regression equations shown in Figure 7.7. Intercepts and slopes for the equation (Log_{10} VCN/10^6 spleen cells = Y axis intercept + Log_{10} challenge dose). The R^2 and P values are also provided as well as predicted challenge doses (Log_{10} VCN/10^6) to achieve mean spleen MDV11 loads of 2.3, 4 and 5 logs.

Virus	n	Y Axis intercept	Slope	R^2	P Value	X axis intercept Y=2.3	X axis intercept Y=4	X axis intercept Y=5
All	305	-3.615467	0.9867553	0.21	<0.0001	5.99	7.72	8.73
04KAL	29	-7.473602	2.1393678	0.58	<0.0001	4.57	5.36	5.83
MPF192	30	-7.271393	1.9229391	0.56	<0.0001	4.98	5.86	6.38
05 JUR	30	-6.052482	1.6663386	0.50	<0.0001	5.01	6.03	6.63
MPF199	30	-5.379158	1.3795529	0.39	0.0002	5.56	6.79	7.52
FT158	30	-4.557914	1.0113469	0.24	0.0065	6.78	8.46	9.45
Woodlands	36	-4.480796	1.0642161	0.39	<0.0001	6.37	7.97	8.91
MPF57	30	-4.149462	0.9224503	0.24	0.0062	6.99	8.83	9.91
MPF189	30	-2.928023	0.9655618	0.14	0.0408	5.41	7.17	8.2

Overall correlations between body weight immune organ weights and scores and the MDV11 content in spleen at 12 dpc are shown in Table 7.7. The strongest overall association was between MDV11 content of spleen and relative spleen size and this is illustrated in Figure 7.7.

Table 7.7. Correlation coefficients (r) between key variables measured on individual chickens during the experiment. N=306, df 304. Values of r over 0.11, 0.15 and 0.19 are significant at the p<0.05, 0.01 and 0.001 level respectively.

Variable	Body Wt (g)	Thymus atrophy score	Bursal atrophy score	Rel bursal wt (%BW)	Rel spleen wt (%BW)	Log MDV11/10^6 spleen cells
Body Wt (g)	1					
Thymus atrophy score (0-3)	-0.34	1				
Bursal atrophy score (0-3)	-0.40	0.46	1			
Rel bursal wt (% BW)	0.11	-0.19	-0.31	1		
Rel spleen wt (%BW)	-0.21	0.29	0.35	-0.10	1	
Log MDV11/10^6 spleen cells	-0.24	0.35	0.43	-0.17	0.67	1
Log_{10} challenge dose (MDV11/chick)	-0.06	0.14	0.15	-0.15	0.31	0.45

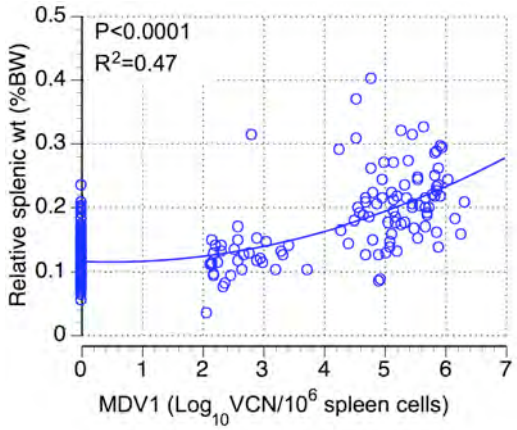


Figure 7.7. Association between relative splenic weight and the load of MDV11 in spleen at 12 days post challenge. The curve is a 2nd order polynomial

Titration of infectivity in chickens

Logistic regression of the ratio of positive birds against log_{10} dilution factor or challenge dose in pfu, or challenge dose in VCN of MDV11 showed a strong dose responsiveness of infectivity for 3 viruses (04KAL, MPF192 and 05JUR) and moderate dose responsiveness in four other viruses (MPF199, FT158, Woodlands, MPF57) enabling calculation of the ID_{50} for these viruses (Table 7.8).

Table 7.8. Determination of ID50 in different units using logistic regression of the ratio of MDV11-positive birds at day 12 (infected + or -) against the log_{10} of dilution, and individual chicken dose in Log_{10} pfu or Log_{10} MDV1 VCN. ID50 was calculated by inverse prediction from the logistic regression equation with 95% confidence intervals. The P value and R^2 of the regression models were the same and are provided.

Virus	% MDV11 positive chicks	P	R^2	ID_{50} (95% CI range)			ID_{50} doses/ml of stored material
				Dilution factor	pfu	Log_{10}VCN	
04KAL	57% (16/28)	<0.0001	0.5	34.8 (8.8-185)	0.80 (0.15-3.17)	4.77 (4.05-5.37)	174
MPF192	60% (18/30)	<0.0001	0.49	56.9 (14.7-259)	0.49 (0.11-1.85)	4.89 (4.23-5.46)	284
05JUR	47% (14/30)	<0.0001	0.44	18.9 (4.1-81)	2.07 (0.5-8.8)	4.89 (4.27-5.52)	95
MPF199	30% (9/30)	<0.0001	0.3	4.1 (0.131-18.7)	4.69 (1.1-154)	5.62 (4.99-7.14)	20
FT158	34% (10/29)	0.003	0.24	5.8 (0.06-36)	0.56 (0.09-43)	6.45 (5.67-8.33)	29
Woodlands	43% (13/30)	0.0027	0.21	12.9 (0.5-36)	10.62 (2.1-114)	6.17 (5.47-7.20)	65
MPF57	23% (7/30)	0.01	0.2	1.6 (0-8.8)	35.85 ($6.6-5.6 \times 10^{12}$)	6.70 (5.96-17.9)	8
02LAR	7% (2/29)	0.1	0.19	0.2	50.54	7.4	N/A
MPF189	59% (17/29)	0.085	0.08	N/A	N/A	5.29	N/A
MPF23	13% (4/30)	1	0	N/A	N/A	N/A	N/A

Correlation coefficients amongst the different measures of infectivity for the 8 viruses for which estimates of ID50 could be made are presented in Table 7.9 and presented visually in Figure 7.8. The only statistically significant associations were amongst the different measures of ID_{50} and between these and the percentage of positive chickens.

Table 7.9. Correlation coefficients amongst the different measures of infectivity for the 8 viruses for which estimates of ID_{50} could be made (see Table 7.6). For 6 df r values of 0.71, 0.83 and 0.91 are significant at P<0.05, P<0.01 and P<0.001 respectively.

Variable	Titre (pfu)	MDV11 Neat	MDV11 1/625	% positive chicks	ID_{50} (Dilution)	ID_{50} (pfu)
Titre on CK (pfu)	1					
MDV11 content Neat	0.49	1				
MDV11 content 1/625	0.62	0.51	1			
% MDV11 positive chicks	0.17	-0.15	0.36	1		
ID_{50} (Dilution factor)	-0.05	-0.32	0.11	**0.86**	1	
ID_{50} (pfu)	0.01	0.03	-0.26	**-0.84**	-0.55	1
ID_{50} (Log_{10}VCN)	0.03	0.51	-0.11	**-0.91**	**-0.77**	**0.82**

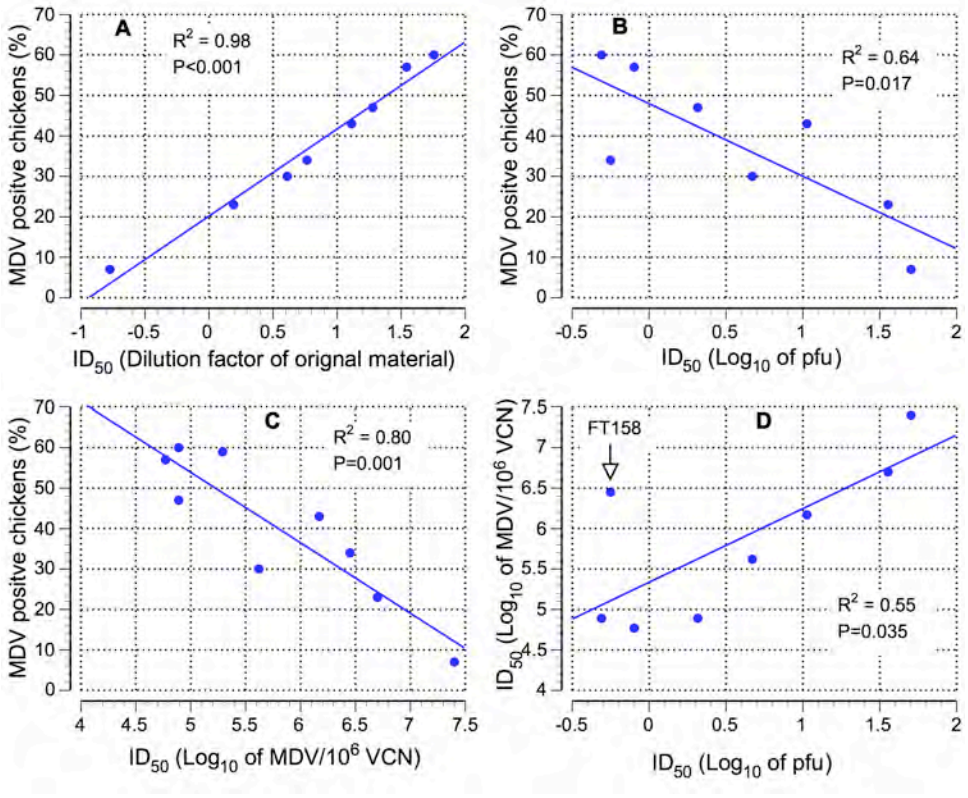


Figure 7.8. Association between some key measures of infectivity for the 8 challenge viruses for which ID50s were able to be calculated. Lines are linear regression fits.

Brief discussion and conclusions

The experiment was successfully implemented and met its main objective of determining whether there was sufficient infectivity in some of the low-moderate titre material from RMIT to conduct a full pathotyping experiment. Based on the outcomes of this experiment, the next pathotyping experiment was planned, based on using challenge doses of 04KAL, MPF192, 05JUR (MPF212) and Woodlands 1 based on equal multiples of the estimated BID_{50}.

Compared with the previous experiment (Chapter 6) the level of infectivity of the challenge material was considerably lower as evidenced by the MDV11 load in the challenge material, the overall percentage of chickens infected (36.5% v 90%) and the mean overall content of Log_{10}MDV11 in spleen at 10 dpc in the earlier experiment (4.7±0.2) and 12 dpc of the current experiment (1.62±0.13). The effect of challenge on spleen weights was also less in the current experiment. The reduced effects are almost certainly due to the very much lower Log_{10}MDV1 challenge per chicken in the present experiment (mean 5.30 range 3.40-7.23) compared with the previous experiment (mean 8.14, range 6.59-9.70)

Nevertheless, using the same methodology BID_{50} was able to be calculated with reasonable confidence for a number of viruses. For 3 viruses (04 KAL , MPF192 and 05JUR), clear challenge dose responses were obtained for both % infected chickens and mean load of MDV11 in spleen at 12 dpc. These viruses were characterised by dose-response R^2 above 0.4 for logistic regression of % infected over challenge dose and above 0.5 for linear regression of MDV11 load in spleen at 12 dpc over challenge dose. The slopes of the latter relationship were steep and similar for each virus (Figure 7.6B) resulting in a very similar estimate of BID_{50} in terms of challenge dose in Log_{10} MDV11 VCN/chicken (4.77-4.89, Table 7.6). For viruses with less clear dose responses and lower R^2, the precision of the estimate of BID_{50} fell sharply with very wide confidence intervals around the estimate. The estimate of BID_{50} also increased as the slope of the association decreased in all of these viruses.

This is likely to be due to the cell-associated nature of the infective material with infectivity "clumped" about a comparatively small number of infected cells rather than distributed evenly

throughout the infective material. When the number of viable infective cells is low, the serial dilution does not necessarily guarantee an even reduction in infectivity with infectivity perhaps depending on the presence or absence of a single infective viable cell in the challenge inoculum. This may explain while qPCR assay of the inoculated material found a more rapid decline in MDV11 copy number than than predicted by the dilution (Table 7.4). The association between poor estimates of BID_{50} and the elevation of the estimate as a consequence of this, means that such estimates are likely to be systematically biased upwards and should not be used. It is proposed that adequate estimates of BID_{50} are only obtained when the dose-response R^2 is 0.4 or above for logistic regression of % infected over challenge dose and 0.5 or above for linear regression of MDV11 load in spleen.

As in the previous experiment, this experiment showed that using a target response in terms of MDV11 load in spleen at a fixed time after challenge is also feasible for defining infectivity and would allow comparison between experiments. It has the advantage of being based up linear regression which is somewhat more available and better understood than logistic regression. It means that the assay for infectivity is a quantitative assay rather than a quantal assay. Table 7.10 below compares some estimates of infectivity between viruses in the two experiments.

Table 7.10. Different estimates of infectivity of challenge material in experiments MD06-C-BID1 and MD06-C-BID2 based on original challenge dose of MDV11 in (Log_{10} VCN). Estimates based on regression equations with reasonable R^2 values are bolded.

Experiment	MDV11 isolate	Challenge material	Route of challenge	BID_{50} (Log_{10} VCN)	MID[1] (Log_{10} VCN)	SCD[2] Log 4 (Log_{10} VCN)
MD06-C-BID1	MPF210/2s (02 LAR)	Isolator dust	Intra-tracheal	8.5*	**8.15 (7.45-8.5)**	**8.67 (8.44-8.83)**
MD06-C-BID1	MPF57	Isolator dust	Intra-tracheal	6.3*	**6.41 (5.88-6.67)**	**6.97 (6.72-7.16)**
MD06-C-BID2	04KAL	CK cells	Intra-abdom	**4.77 (4.05-5.37)**	**4.57 (4.14-4.90)**	**5.36 (5.03-5.81)**
MD06-C-BID2	MPF192	CK cells	Intra-abdom	**4.89 (4.23-5.46)**	**4.98 (4.58-5.31)**	**5.86 (5.52-6.37)**
MD06-C-BID2	05JUR	CK cells	Intra-abdom	**4.89 (4.27-5.52)**	**5.01 (4.64-5.47)**	**6.03 (5.56-6.95)**
MD06-C-BID2	MPF57	CK cells	Intra-muscular	6.70 (5.96-17.9)	6.99 (6.18-10.5)	8.83 (7.38-16.4)

[1]Minimum infective dose. That required to achieve a mean spleen Log_{10} VCN of 2.3 or higher.
[2]Standard challenge dose (log 4). That required to achieve a mean spleen Log_{10} VCN of 4 or higher.
* Estimates based on extrapolation as a spread of infectivity on either side of 0.5 was not achieved.

This data suggests the BID_{50} of good cellular infective material inoculated intra-abdominally is around $10^{4.9}$ copies of MDV11, while the BID_{50} of dander material inoculated intra-tracheally is considerably higher ($10^{6.3}$-$10^{8.5}$ copies of MDV11). The term MID is probably misleading as for the <u>average</u> viral load to reach the detectable threshold of around 200 VCN/10^6 cells (ie $10^{2.3}$) several chickens must exceed the threshold of infection. In fact the estimates of BID_{50} and MID are remarkably close and they probably approximate the same thing.

While measures of infectivity based on pfu and MDV11 load in the infective material tended to be significantly positively associated, the association was not always strong and varied between viruses. Given the importance of defining the MDV1 load in the original challenge material, in future, greater emphasis should be placed on more thorough determination of this (ie test several aliqots, not just one).

Conclusions.

1. Neither the initial pfu value of the infective material for the different isolates, nor its initial MDV11 content determined by qPCR, was significantly associated with infectivity of the material as determined by the % of chickens which were positive to MDV11 at 12 dpc. While this may be due to the comparatively narrow range of values tested, this emphasises the need to bioassay of infectivity in chickens as the ultimate measure of infectivity.

2. Titration of infectivity of infective cell-culture material in non-SPF chickens is feasible, rapid, cheap and practical using a range of methods as described above. However for it to be successful the following conditions should be met.
   - The original infective material should have its MDV1 load determined precisely
   - Preferably the infectivity of the undiluted material would have been tested in a preliminary experiment with few chickens (n=10) in which 50% or more birds are shown to be infected by 10 dpc. Such a test would have removed MPF164, MPF23 and 02LAR from the present experiment and saved the use of 90 chickens and a large amount of work.
   - As high challenge volumes as possible should be used in the dilution series and to infect chickens to reduce the risk of negative infections due to non-random distribution of infective cells in the material.
   - BID, MID or SCD estimates should always be provided with 95% CIs so that the precision of the estimate is available. For estimates based on regression relationship with an R^2 below 4 the precision of the estimate is likely to be insufficiently precise for determination of BID for use in a pathotyping experiment.
   - Increase in bird numbers from 6 to 10 chickens per dilution will produce estimates of BID_{50} of improved precision.
3. Based on the titration of infectivity in the present experiment, sufficient infective material is available to use 04KAL, MPF192, 05JUR (MPF212) and Woodlands 1 in pathotyping experiments with a challenge dose of approximately 6 x BID_{50}.

Walkden-Brown SW, Tannock GA, Cheetham BF, Islam AFMF, Groves PJ (2006) Systematic pathotyping of Australian Marek's disease virus isolates. Final Report of RIRDC Project UNE-83J. Rural Industries Research and Development CorporationFinal Report of RIRDC Project UNE-83J, Canberra.

Chapter 8: Experiment 7. MD06-C-PT4

"2nd pathotyping experiment in broiler chickens: recent isolates in Cobb and Ross strains"

Start: 16/10/06 Completion: 12/12/06 AEC: UNE 06/071

Introduction

Originally we were to pathotype recent MDV1 isolates in 2 broiler experiments and 2 layer experiments. The first experiment in broilers, MD05-C1-PT1 used Cobb broilers and tested 3 MDV1 isolates, namely MPF179/6 (MPF57 B2), 02LAR and FT158 given at 500pfu/chicken on day 5. Broilers were unvaccinated or vaccinated with HVT or bivalent HVT/MDV2 vaccine. It ran very successfully (Chapter 3).

We cannot test the isolates from the original batch in a new strain of broiler as originally intended due to unavailability of the original infective material (all used in experiments 2 and 3) or alternative isolates of sufficient titre. In response to this we have run 2 experiments to grow up new isolates in SPF chickens (MD05-R-PT3 and MD06-C-VI5, Chapter 5) before putting into cell culture at RMIT, and one experiment to titrate infective cell culture material from RMIT in mab positive commercial male cockerels (MD06-C-BID2, Chapter 7). This work has provided a number of isolates which are pathogenic, and which have now been titrated in chickens to determine the bird infectious dose 50 (BID_{50}).

Some of these isolates (04KAL, MPF192, MPF212 and Woodlands 1) will be used in the current experiment together with the original batch of MPF179/6 used in the earlier broiler experiment, but at reduced challenge dose of 200pfu/chicken. To enable chicken genotype comparison the experiment includes both the Ross and Cobb broiler strains in the single experiment. To get the two genotypes in a single experiment we have to sacrifice one vaccination treatment – the bivalent treatment, so only HVT vaccination is under test.

Hypotheses:

1. The broiler strains Ross and Cobb will not differ in susceptibility to the 5 MDV1 isolates whether unvaccinated or vaccinated with HVT.
2. HVT will only provide partial protection against MD.
3. The MDV1 isolates used will vary significantly in their ability to induce MD in unvaccinated and HVT-vaccinated chickens.
4. Pathotype rankings will not differ between chicken genotypes.
5. The order of rankings for pathogenicity will not differ between unvaccinated and HVT-vaccinated chickens (This has not been so in some earlier experiments).
6. Thymus atrophy scores, relative bursal weights and relative splenic weights at day 12 pc will be good predictors of pathogenicity.
7. MDV1 load in spleen at day 13 will be good predictor of pathogenicity.

Dr Steve Walkden-Brown led the execution of this experiment with assistance from Paul Reynolds and Dr Fakhrul Islam.

Materials and methods

The experiment used a 6x2x2 factorial design with two replicates using 24 isolators in the UNE isolator facility. The three experimental factors were:

- Challenge virus (6), Nil, MPF179/6 (reference strain MPF57 B2), Woodlands 1, 04KAL, MPF192, MPF212. Nil challenge were injected with diluent only.

- Vaccine virus (2), Unvaccinated and HVT. Unvaccinated chicks were injected with diluent only.
- Broiler strain (2), Cobb500 and Ross

The two replicates were achieved by having the sham and HVT chickens running together in the same isolator identified by toe marks. While this may lead to minor differences in the level of secondary challenge that the vaccination treatments are exposed to (higher in vaccinated, lower in unvaccinated) this is unlikely to be a major factor given the very limited effect that HVT vaccination has on the shedding of MDV1 (Islam and Walkden-Brown, 2007).

Birds and their management

Each treatment combination was replicated in two separate isolators. A total of 720 birds were used, with a total of 30 per treatment combination and 30 per isolator initially. The Ross and Cobb chicks used were unvaccinated (with any vaccine) commercial females hatched in the same batch at Baiada's Kootingal hatchery on 16/10/06. They both come from Rispens CVI988-vaccinated parent flocks. All chicks were fed *ad libitum* on the high-protein Ross diet kindly supplied by Bartter Enterprises Pt Ltd. They were on starter for approx 21 days and finisher thereafter. Lighting was 23L:1D for the first 2 days then12L:12D thereafter. The isolator air supply was heated to 34˚C initially reducing by 1˚C every second day until a setting of 21˚C was reached. Isolators were fitted with string bunches to peck at to reduce feather pick (mainly required for layer strains).

Vaccination. This was performed manually sc at UNE immediately on arrival (16/10/06) using 8000pfu/chicken administered sc in 0.2ml of diluent. The HVT vaccine used was Bioproperties caHVT vaccine [FC-126, Batch 4102 (1000 doses), Exp 04/2008, Titre 9315pfu/dose]. Vaccinated and unvaccinated chicks were identified by toe web marks (unvaccinated RIRO, vaccinated LILO) and placed in equal numbers in each isolator.

MDV1 Challenge. Challenge was on day 5 post-vaccination via the intra-abdominal route. A dose of 6 BID_{50}/chicken was used for each virus except MPF179/6 for which a dose of approx 200pfu chicken was used. Virus details are provided in Table 1. Virus was diluted in maintenance medium (MEM + HEPES + Antibiotics + NaHCO3 + 1.5% newborn calf serum) provided by RMIT.

supplemented with serum to 1.5%

Table 8.1. Details of viruses and challenge doses. All infective material comprised infective chick kidney cells.

MDV1 isolate/RMIT accession	Status[1]	Original field source	Year	[2]Most recent source (Expt)	Batch number	[3]Challenge dose (BID_{50}/bird)	Challenge dose (pfu/bird)
04KAL	New, B1	SA	2004	MD06-C-VI5	P4 020406	6	4.8
MPF192	New, B1	SA	2004	MD05-R-PT3	P6 020406	6	3.0
MPF212	05JUR B2	NSW	2005	MD05-R-PT3	P6 020406	6	13.3
Woodlands1	B1	QLD	1992	MD05-R-PT3	P6 020406	6	63
MPF179/6	MPF57 B2	NSW	1994	MD04-R-PT2	P7 200904	na	200

[1]B=Back passage. Add 1 more back passage in chickens for each virus except MPF179/6 as all were reisolated from SPF chickens in recent experiments at UNE (late 2005, early 2006)
[2]Experiment from which the virus was re-isolated from SPF chickens
[3]BID_{50} calculated using logistic regression (See Chapter 7).

Measurement and treatment schedule. This is summarized in Table 8.2 below.

Statistical analysis Continuous data (eg bodyweight) was analysed in a general linear model fitting the effects of vaccination, challenge treatment, breed and their interaction. In some cases (eg. relative spleen weights, MDV1 viral copy number), data were log transformed prior to analysis to better meet the assumptions of analysis of variance [$\log_{10}$ (VCN+1) for VCN so 0 is transformed to 0]. Mortality was analysed using survival analysis and treatment effects on the incidence of MD were tested using a generalized linear model with a binomial link function (logistic). Means were separated using Tukey's HSD test or by specific contrasts within the statistical model. Vaccinal protective index based on the presence of gross MD lesions alone, was calculated as (%MD in unvaccinated chickens -

%MD in vaccinated chickens) / (%MD in unvaccinated chickens). Only chickens at risk of MD were included in the calculation (ie alive at the time of the first MD case). FCR was calculated on an isolator basis as the total amount of feed consumed per isolator divided by the total weight of chickens per isolator (including deaths) less the initial weight placed in the isolator. Data are generally presented as least squares means and standard errors (LSM±SEM).

Table 8.2. Details of timing of treatments and measurements.

Date	Expt day	Day pc	Action
16/10/06	0	-5	Chicks arrive. Vaccinate sc in 200ul, toe mark, place 30 chicks per isolator.
21/10/06	5	0	Challenge. Intra-abdominal in 200ul.
3/11/06	18	13	Remove 8 birds/isolator (total 192). Sacrifice and weigh body, bursa, thymus and spleen. Atrophy scores. Retain spleen for qPCR.
25/11/06	40-41	35-36	Dust collection bags for each isolator except controls. Dust for qPCR. Settle plates in control isolators.
12/12/06	57	52	Sacrifice all remaining chickens, weigh body, bursa and spleen. Thymic atrophy score. PM for gross MD lesions and lesion scoring. Spleens from 10 unvaccinated controls for each breed.

Results

Confirmation of application of treatments

All vaccination and challenge treatments were successful, as determined by qPCR for MDV1 and the physical experimental results. Amongst nil challenge control chickens 16/16 were negative for MDV1 on qPCR at 13 dpc while the majority of chickens challenged with MDV1 were positive for each of the challenge isolates. No cases of MD tumors were observed in the nil challenge control group while there was a significant incidence of MD in the MDV1 challenge treatments.

Mortality/Survival.

Mean chick weight at delivery was 43.2g for Cobb and 38.2g for Ross. Mortality to 7 dpc (day 12 of the experiment) was 15/320 (4.7%) for Cobb and 21/320 (6.6%) for Ross birds respectively. This was due mainly to bacterial infections with some non-starters. Only mortality after 7 dpc is considered further. The early mortality ceased on 5 dpc, and the next mortality was on 9 dpc and this and all subsequent mortality is detailed in Table 8.3. There was a highly significant effect of vaccination ($p=0.0002$) and breed ($p=0.0003$) but not challenge treatment on mortality from days 8-52 pc. Mortality was significantly greater in Cobb (17.1%) than Ross (5.2%) birds and was greater in Unvaccinated (18.2%) than HVT-vaccinated chickens (4.2%).

Survival analysis similarly revealed highly significant effects of breed and vaccination (Figure 8.1). Chicken survival by challenge treatment in vaccinated and Unvaccinated chickens is shown in Figure 8.2. Challenge treatment had a significant effect on survival in Unvaccinated but not HVT-vaccinated chickens.

Table 8. 3. Chickens dying between days 7 and 52 pc of the experiment. This includes chickens euthanised for leg problems. It excludes the chickens sacrificed at day 13 pc for organ weights. MD cases are those with gross MD lesions.

Breed	Vaccine	Challenge	Eligible birds (n)	Deaths with MD	Deaths without MD	Total	Total mort (%)	Mort with MD (%)
Cobb	HVT	04KAL	21		2	2	9.5	0
		MPF179/6	20	2	2	4	20.0	10.0
		MPF192	21			0	0	0
		MPF212	20		1	1	5.0	0
		Sham	13		2	2	15.4	0
		Woodlands	21			0	0	0
	Sham	04KAL	22	3	2	5	22.7	13.6
		MPF179/6	22	3		3	13.6	13.6
		MPF192	19	3	1	4	21.1	15.8
		MPF212	21	3	3	6	28.6	14.3
		Sham	18		4	4	22.2	0
		Woodlands	22	7	3	10	45.5	31.8
	Overall	Overall	240	21	20	41	17.1	8.8
Ross	HVT	04KAL	22		1	1	5	0
		MPF179/6	19			0	0	0
		MPF192	22			0	0	0
		MPF212	21			0	0	0
		Sham	17			0	0	0
		Woodlands	20			0	0	0
	Sham	04KAL	19	1		1	5.3	5.3
		MPF179/6	20	5		5	25.0	25.0
		MPF192	19	1		1	5.3	5.3
		MPF212	21				0	0
		Sham	14				0	0
		Woodlands	19	3	1	4	21.1	15.8
	Overall	Overall	233	10	2	12	5.2	4.3
Overall	Overall	Overall	473	31	22	53	11.2	6.6

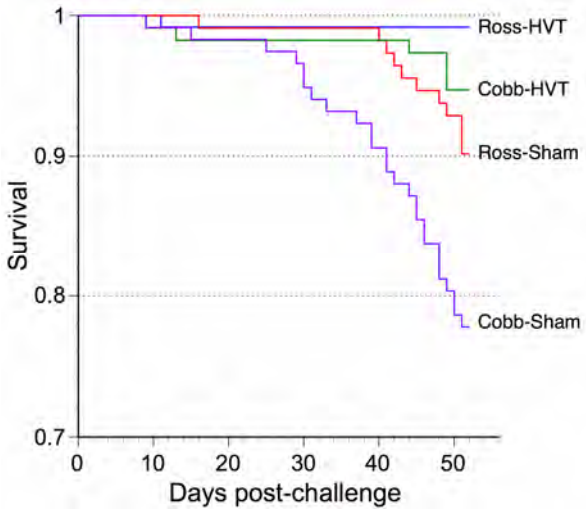


Figure 8.1. Survival analysis by breed and vaccination between days 8 and 52 pc. Both effects were highly significant ($p<0.001$). Based upon an eligible population of 473 chickens.

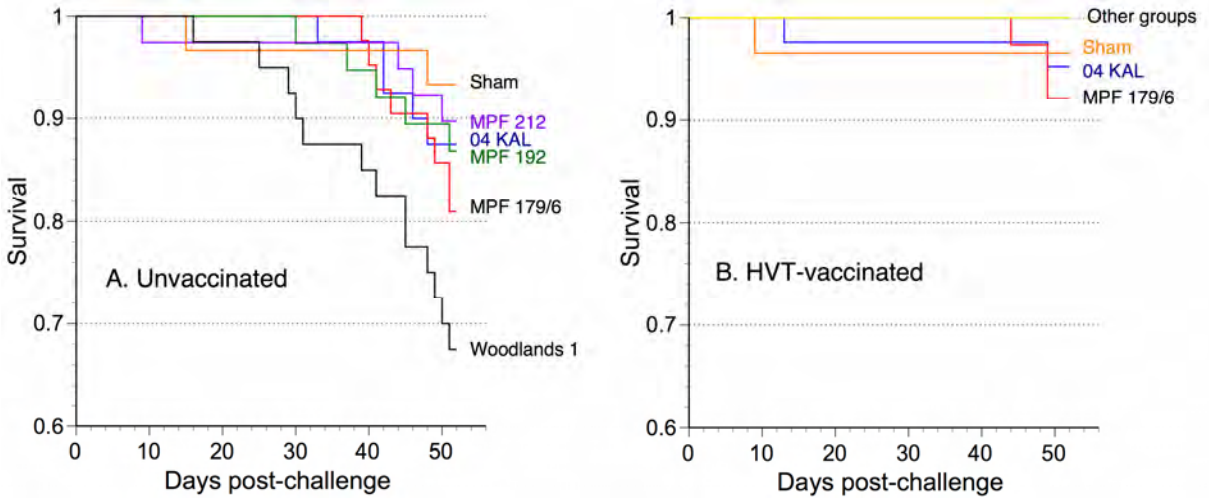


Figure 8.2. Survival analysis by vaccination status (A unvaccinated, B HVT-vaccinated) and challenge treatment, between days 8 and 52 pc. The effect of challenge treatment was significant in unvaccinated ($p<0.026$) but not HVT-vaccinated chickens. Based upon an eligible population of 473 chickens.

Chicken bodyweight and FCR

Cobb chickens were 13% heavier overall than Ross chickens on day 0 (43.2g v 38.2g respectively). At day 13 pc (18 days of age) they were 16% heavier than Ross (679 v 585g respectively, $p<0.0001$), but by the end of the experiment at day 52 pc (57 days of age) they were lighter but not significantly so (3010 v 3051g respectively $P=0.26$). Unvaccinated chickens were heavier than HVT-vaccinated chickens at day 13 pc (640 v 624g, $p=0.05$) but this was reversed by day 52 pc with Unvaccinated chickens being 6.2% lighter (2934 v 3127g $p<0.0001$). At day 52 pc the number of chickens per isolator was a highly significant factor affecting bird weights ($p=0.003$, higher bird number associated with lower weights) and so this was included as a covariate in the analysis and the means reported above are adjusted for this. Least squares mean weights for each challenge virus by vaccination combination are presented in Figure 8.3.

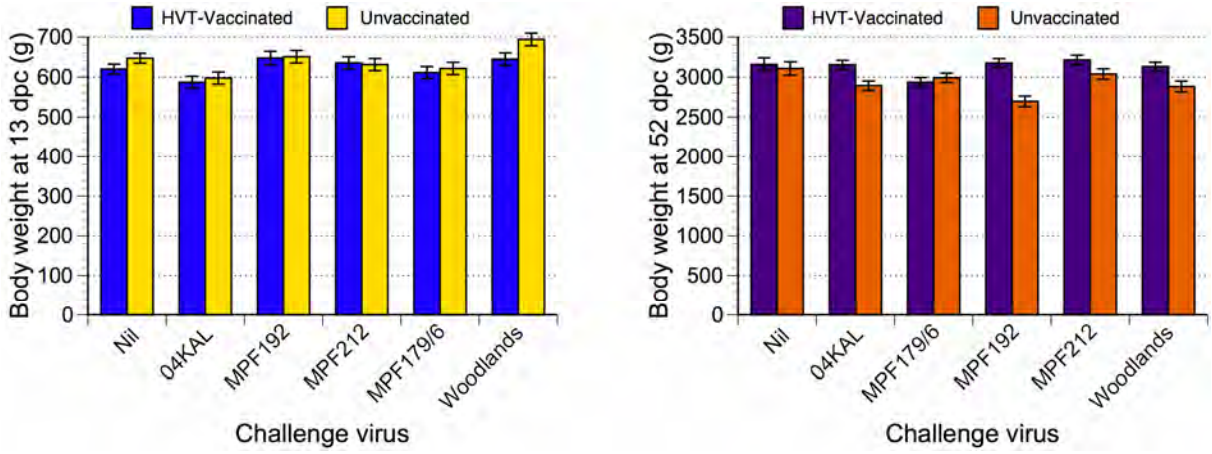


Figure 8.3. Mean least square mean bodyweight (±SEM) by challenge virus and vaccination on 13 (left) and 52 (right) dpc. Based upon total samples of 202 and 402 chickens respectively. Males are excluded.

Feed conversion ratio (feed consumed/weight gain), was significantly affected by bird numbers per isolator when fitted as a covariate ($p=0.016$). Higher densities were associated with higher FCR. Least squared means below are adjusted for this. FCR was significantly lower in Ross than Cobb (1.956 v 1.994 $p=0.02$). The effect of HVT vaccination could not be tested as vaccinated and unvaccinated chickens were in the same isolators. However there was a significant effect of challenge treatment ($p=0.005$, Figure 8.4).

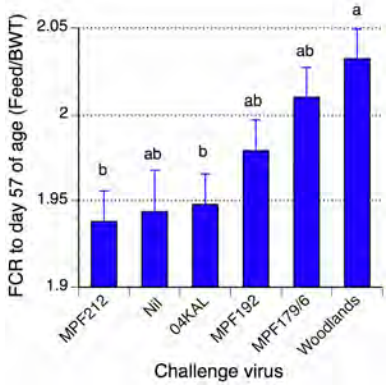


Figure 4. Mean least square mean FCR (±SEM) by challenge virus to day 52 pc (day 57 of age). Means without a common letter differ significantly ($p<0.05$).

Incidence of MD and vaccinal protective index.

The first case of MD was observed at day 29 pc. The combined incidence of gross MD lesions in chickens that died with MD or had MD lesions on post mortem at day 52 pc is summarized by treatment in Table 8.4.

Table 8.4. Incidence of MD lesions in vaccinated and unvaccinated chickens by challenge treatment and protective index. Includes 457 chickens eligible to have MD lesions (ie. those alive at the time of the first MD case on day 29 pc and excludes early culls due to leg problems and chickens sacrificed at day 13 pc for organ weights).

Breed	Challenge	Vaccination	n	MD lesions (n)	No MD lesions (n)	%MD	Protective index (%)
Cobb	Sham	HVT	11	0	11	0	
		Sham	15	0	15	0	NA
	MPF192	HVT	21	1	20	4.8	
		Sham	19	10	9	52.6	90.9
	MPF212	HVT	19	1	18	5.3	
		Sham	18	6	12	33.3	84.1
	Woodlands	HVT	21	3	18	14.3	
		Sham	20	14	6	70	79.6
	04KAL	HVT	20	2	18	10	
		Sham	21	8	13	38.1	73.8
	MPF179/6	HVT	19	4	15	21.1	
		Sham	22	13	9	59.1	64.3
	Overall	HVT	111	11	100	9.9	
		Sham	115	51	64	44.3	77.7
		Both	226	62	164	27.4	NA
Ross	Sham	HVT	17	0	17	0	
		Sham	14	0	14	0	NA
	MPF192	HVT	22	0	22	0	
		Sham	19	7	12	36.8	100
	MPF212	HVT	21	1	20	4.8	
		Sham	21	7	14	33.3	85.7
	Woodlands	HVT	20	1	19	5	
		Sham	18	10	8	55.6	91
	04KAL	HVT	21	1	20	4.8	
		Sham	19	9	10	47.4	89.9
	MPF179/6	HVT	19	1	18	5.3	
		Sham	20	10	10	50	89.5
	Overall	HVT	120	4	116	3.3	
		Sham	111	43	68	38.7	91.4
		Both	231	47	184	20.3	NA
Both	All	HVT	231	15	216	6.5	
		Sham	226	94	132	41.6	84.4
Total	All	Both	457	109	348	23.9	NA

Analysis of MD incidence in vaccinated and unvaccinated chickens challenged with MDV1 revealed significant effects of breed (p=0.035), challenge (p=0.035) and vaccination (p<0.0001) with no significant interaction between these effects. Cobb chickens had a higher incidence of MD than Ross (31% v 23.5%) and unvaccinated chickens had higher incidence than HVT-vaccinated chickens (47.7% v 7.4%). More chickens challenged with Woodlands1 (31.5%) and MPF179/6 (30.1%) had MD than those challenged with MPF212 (17.9%) with 04KAL (22.6%) and MPF192 (21.6) being intermediate. The two older viruses (Woodlands1 and MPF179/6) had a significantly higher incidence of MD (30.8%) than the other 3 newer viruses (20.7%) (p=0.002).

Within Unvaccinated chickens alone, there was no significant overall effect of either breed (p=0.367) or challenge virus (p=0.084) but the specific contrast between Woodlands1 (63.2%) and MPF212 (33.3%) was significant (p=0.009). Once again more chickens challenged with the two older viruses (Woodlands1 and MPF179/6) had a significantly higher incidence of MD (58.8%) than the other 3 newer viruses (40.2%) (p=0.002).

Within HVT-vaccinated chickens alone, there was a significant overall effect of breed (p=0.05) but not challenge virus (p=0.36). Cobb chickens had a higher incidence than Ross (9.9 v 3.3%). Once again more chickens challenged with the two older viruses (Woodlands1 and MPF179/6) had a significantly higher incidence of MD (11.3%) than the other 3 newer viruses (4.8%).

The incidence of MD in unvaccinated chickens was positively associated with the incidence of MD in HVT vaccinated chickens overall but the relationship did not quite achieve statistical significance (Figure 8.5).

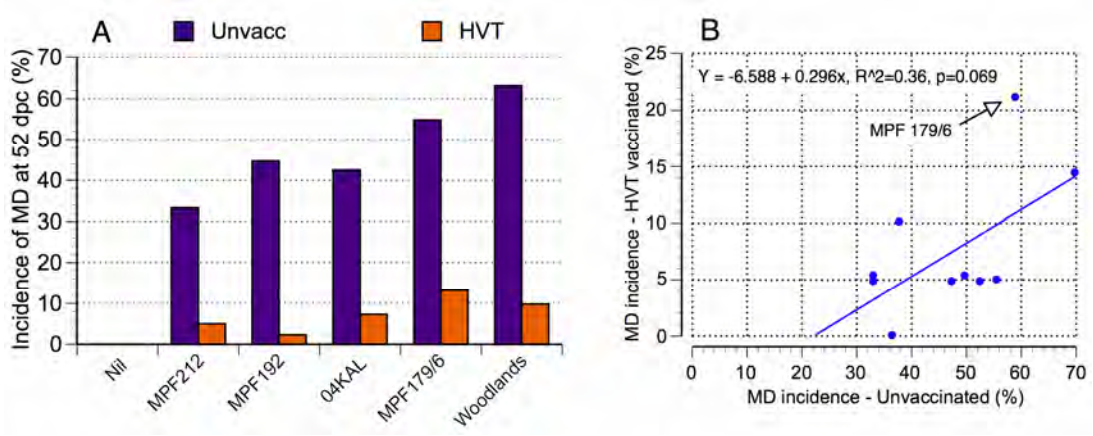


Figure 8.5. A. Incidence of MD at 52 dpc by challenge and vaccination treatments. B. Association between the incidence of MD in unvaccinated and HVT-vaccinated chickens.

Relative immune organ weights at 13 dpc

At day 13 pc 209 chickens were removed from the isolators for organ weight analysis. There were no significant effects of breed on the relative weight of the bursa, thymus and spleen (relative weight = organ weight/bodyweight x 100) so data by challenge treatment and vaccination are shown in Figure 8.6.

Relative thymic weight was markedly affected by operator (p<0.0001) with no other significant effects although there was a trend towards higher thymic weights in HVT-vaccinated compared with Unvaccinated chickens (0.399 v 0.375 % respectively. P=0.088) (Figure 8.6A).

Relative bursal weight was affected by vaccination (p=0.001) but not challenge treatment (p=0.95) or breed (p=0.57), and there was no significant interaction between these effects. Overall HVT-vaccinated chickens had higher relative bursal weights than unvaccinated chickens (0.197 v 0.175 % respectively) (Figure 8.6B).

Relative splenic weight was affected by vaccination (p=0.0001) and challenge treatment (p=0.0001) with significant interaction between these effects (p=0.0006) (Figure 8.6C). HVT-vaccinated chickens had lower relative splenic weights than unvaccinated chickens (0.108 v 0.139 % respectively). Nil

challenged chickens had lower relative splenic weights (0.094%) than those challenged with MPF179/6 (0.137%), 04KAL (0.135%), Woodlands 1 (0.132%) and MPF192 (0.128%) but not MPF212 (0.116%). The marked interaction between the effects of vaccination and challenge treatment was because splenic weight was increased by vaccination in the sham-challenged treatment, but decreased in the treatments challenged with MDV1 (Figure 8.6C).

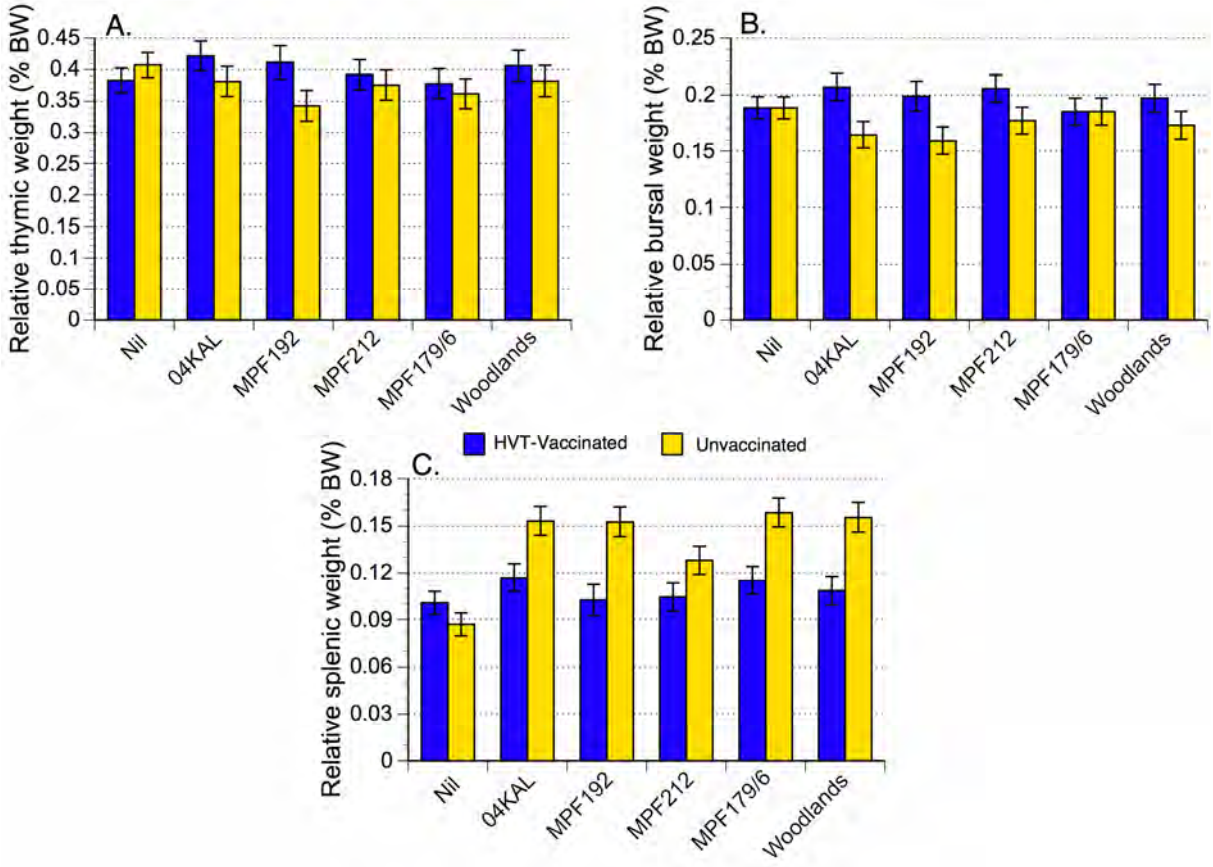


Figure 8.6. Relative immune organ weights (LSM±SEM) at 13 dpc by challenge and vaccination treatment. The legend is the same for each figure. Means are of 14-16 chickens for MDV1 challenge treatments and 24 chickens for sham-challenge treatments.

Relative immune organ weights at 52 dpc

At day 52 pc, 411 surviving chickens were sacrificed and spleen and bursa of Fabricius were removed and relative organ weights calculated as for 13 dpc. Data by challenge treatment and vaccination are shown in Figure 8.7. Males and birds with tumours in the relevant organ were excluded.

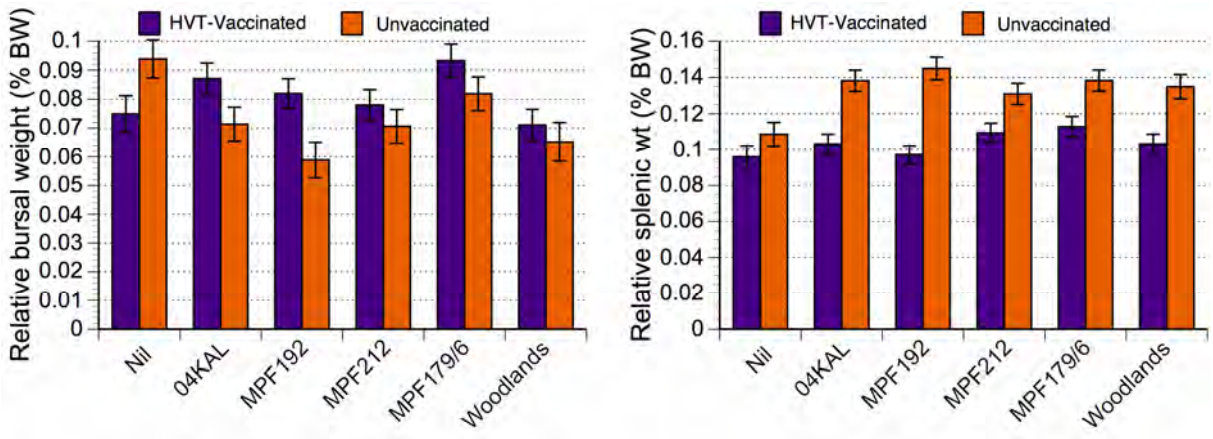


Figure 8.7. Relative immune organ weights and atrophy scores (LSM±SEM) at day 52 pc by challenge and vaccination treatment. Means are of 25-40 chickens per treatment combination. Relative splenic weight data is back-transformed from the log scale.

Relative bursal weight was affected by vaccination (p=0.031), challenge treatment (p=0.005) and chicken breed (p=0.0002), with significant interaction between the effects of vaccination and challenge treatment (p=0.021). HVT-vaccinated chickens had higher relative bursal weights than unvaccinated chickens (0.081 v 0.074 %). Ross chickens also had had higher relative bursal weights than Cobb chickens (0.084 v 0.071 %). Chickens challenged with MPF179/6 had significantly higher relative bursal weights (0.088%) than those challenged with Woodlands 1 (0.068%) and MPF192 (0.070%) with the other treatments intermediate. The interaction between the effects of vaccination and challenge treatment was because relative bursal weights were increased by vaccination in the MDV1 challenged treatments, but the reverse was true in sham challenge treatments (Figure 8.7A).

The $\log_{10}$ of relative splenic weight (LogRSW) was affected by vaccination (p<0.0001) and challenge treatment (p=0.014). HVT-vaccinated chickens had lower LogRSW than unvaccinated chickens (-0.985 v -0.879). Sham-challenged chickens had significantly lower LogRSW (-0.991) than those challenged with MPF179/6 (-0.903) with the other challenge viruses ranked between these and not differing significantly from either (Figure 8.7B).

Immune organ atrophy scores at 13 and 52 dpc

Thymic and bursal atrophy scores (0= no atrophy, 3 = extreme atrophy) by vaccination and challenge treatment at 13 and 52 dpc are presented in Figure 8.8. Scores were much higher at 52 dpc than they were at 13 dpc.

At 13 dpc thymic atrophy score was significantly influenced by challenge treatment (P<0.0001), vaccination (P=0.009) and operator (p=0.008), but not breed (P=0.07) with no significant interactions between these effects. Overall, HVT-vaccinated chickens had a lower mean thymic atrophy score than unvaccinated chickens (0.24% v 0.40 % respectively). The Nil challenge treatment had significantly lower mean thymic atrophy scores (0.07%) than those challenged with MPF179/6 (0.55%), 04KAL (0.41%) and MPF192 (0.38%), but not Woodlands 1 (0.28%) and MPF212 (0.24%) (Figure 8.8A). The effect of breed approached significance (P=0.07) with higher scores in Ross (0.38%) than Cobb (0.26%) chickens.

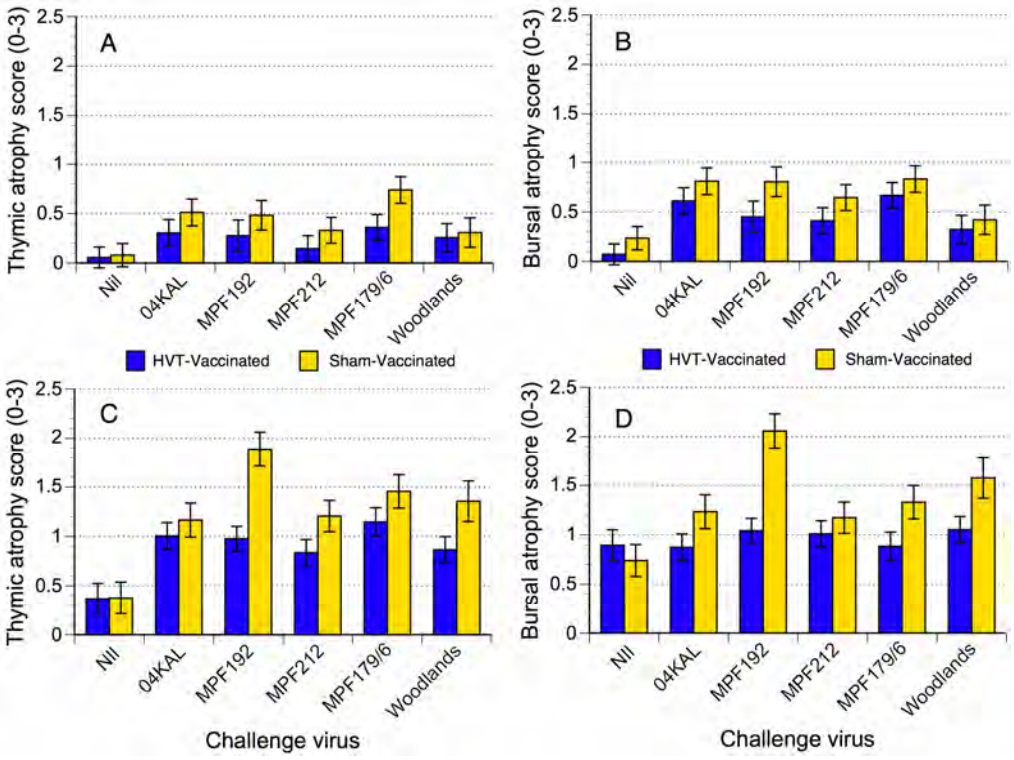


Figure 8.8. Least squares means (±SEM) for thymic and bursal atrophy score (0 = nil, 3 = severe) at 13 (A,B) and 52 (C,D) dpc by challenge and vaccination treatment.

At 13 dpc bursal atrophy score was significantly influenced by challenge treatment (P<0.0001), Breed (P=0.042) and vaccination (P=0.003) but not operator (P=0.79) with no significant interactions between these effects. Overall, HVT-vaccinated chickens had a lower mean bursal atrophy score than unvaccinated chickens (0.39 v 0.60 % respectively). The Nil challenge treatment had significantly lower mean bursal atrophy scores (0.17%) than those challenged with MPF179/6 (0.69%), 04KAL (0.69%) and MPF192 (0.56%) but not MPF212 (0.50%) and Woodlands 1 (0.37%) (Figure 8.8B). The effect of breed was significant (P=0.04) with higher scores in Ross (0.57%) than Cobb (0.42%) chickens. However the breed effect was evident only for 2 viruses, MPF179/6 and MPF212 with no trace of a breed effect for the other challenge groups.

At 52 dpc thymic atrophy score was significantly influenced by challenge treatment (P<0.0001), breed (P<0.0001), vaccination (P<0.0001) and operator (p=0.004), with significant interaction between the effects of challenge treatment and breed (P=0.0003). Overall, HVT-vaccinated chickens had a lower mean thymic atrophy score than unvaccinated chickens (0.84% v 1.25% respectively). The Nil challenge treatment had significantly lower mean thymic atrophy scores (0.04%) than all other challenge groups; MPF192 (1.35%), MPF179/6 (1.31%), Woodlands 1 (1.10%) 04KAL (1.07%) and MPF212 (1.03%) (Figure 8.8C). The overall effect of breed was highly significant (P<0.0001) with higher scores in Cobb (1.27%) than Ross (0.81%) chickens. The significant interaction between the effects of breed and challenge treatment (P=0.0003) was because for most viruses (04KAL, MPF179/6, MPF192 and Woodlands 1) TA score was higher in Cobb than Ross birds. However the reverse was true for MPF212, and for nil challenged chickens the two treatments were the same.

At 52 dpc bursal atrophy score was significantly influenced by challenge treatment (P=0.0002), breed (P=0.01), vaccination (P<0.0001) and operator (P<0.0001), with significant interaction between the effects of challenge treatment and breed (P=0.001), and between challenge virus and vaccination (P=0.008). Overall, HVT-vaccinated chickens had a lower mean bursal atrophy score than unvaccinated chickens (0.96% v 1.35% respectively). The Nil challenge treatment had significantly lower mean thymic atrophy scores (0.82%) than MPF192 (1.55%) and Woodlands1 (1.31%), but not MPF179/6 (1.11%), MPF212 (1.09%) and 04KAL (1.05%) (Figure 8.8D). The effect of breed was significant (P=0.01) with higher scores in Cobb (1.27%) than Ross (1.04%) chickens. The significant interaction between the effects of breed and challenge treatment (P=0.001) was because for most viruses (04KAL, MPF179/6, MPF192 and Woodlands 1) the BA score was higher in Cobb than Ross birds. However, as for TA, the reverse was true for MPF212, and for nil challenged chickens there was a slight trend towards higher scores in Cobb birds. The significant interaction between the effects of vaccination and challenge treatment (P=0.008) is shown in Figure 8.8D with no effect of vaccination in Nil challenged chickens, but progressively larger differentials with the challenge viruses MPF212, 04KAL, MPF179/6, Woodlands1, and MPF192.

MDV1 content of spleen at 13 dpc

None of the 16 unvaccinated nil challenged chickens whose spleens were tested for MDV1 were positive and so all nil challenged chickens are removed from the remainder of the analysis. Overall a high percentage of chickens challenged with MDV1 (74.5%) were positive for MDV1 at 13 dpc (Table 8.5). This proportion was significantly affected by challenge virus (P=0.016) and vaccination (P=0.0007) but not breed (P=0.62) with significant interaction between the effects of challenge and vaccination (P=0.05).

Table 8.5. Proportion of chickens in different treatment groups positive for MDV1 by qPCR at 13 dpc.

Challenge	Number of chickens					% Positive for MDV1				
	Total	Cobb	Ross	Vacc	Unvacc	Total	Cobb	Ross	Vacc	Unvacc
04KAL	32	16	16	16	16	75.0[ab]	68.8	75.0	81.3	68.8
MPF179/6	32	16	16	16	16	84.4[a]	81.3	87.5	68.8	100.0
MPF192	33	17	16	15	18	72.7[ab]	76.5	68.8	53.3	88.9
MPF212	32	16	16	16	16	56.3[b]	50.0	56.3	43.8	68.8
Woodlands	32	16	16	16	16	84.4[a]	87.5	75.0	75.0	93.8
Total	161	81	80	79	82	74.5	72.8	72.5	64.6	84.1

Chickens challenged with MPF179/6 and Woodlands 1 had significantly overall percentages of chickens positive to MDV1 than those challenged with MPF212 (P<0.01), with the other two viruses

being intermediate (Table 8.5). Fewer vaccinated chickens (64.6%) than unvaccinated chickens (84.1%) were positive for MDV1 (P=0.0007). The interaction between the effects of challenge and vaccination was because for 04KAL a higher proportion of vaccinated than unvaccinated chickens was positive for MDV1 whereas the reverse was true for the other challenge viruses.

Overall the log_{10} MDV1 load in spleen observed was considerably lower than that observed at an equivalent time in earlier pathotyping experiments using a challenge dose of 500pfu (Chapters 3 and 4). MDV1 Load was significantly affected by challenge virus (P<0.0001) and vaccination (P<0.0001) but not breed (P=0.67) with no hint of interaction between these effects (P>0.5) (Figure 8.9A). Chickens vaccinated with HVT had significantly lower log_{10} MDV1 VCN in spleen cells (1.01±0.14) than unvaccinated chickens (2.76±0.14). Chickens challenged with MPF179/6 (2.55±0.22) and Woodlands1 (2.35±0.22) had significantly higher MDV1 loads in spleen than those challenged with MPF212 (0.99±0.22) with O4KAL (0.18±0.22) and MPF192 (1.71±0.22) being intermediate. There was a very strong association between the mean MDV1 load in spleen in chicken treatment groups and the mean %MD observed in each group (24 Breed x Vacc x Chall groups) (Figure 8.9B).

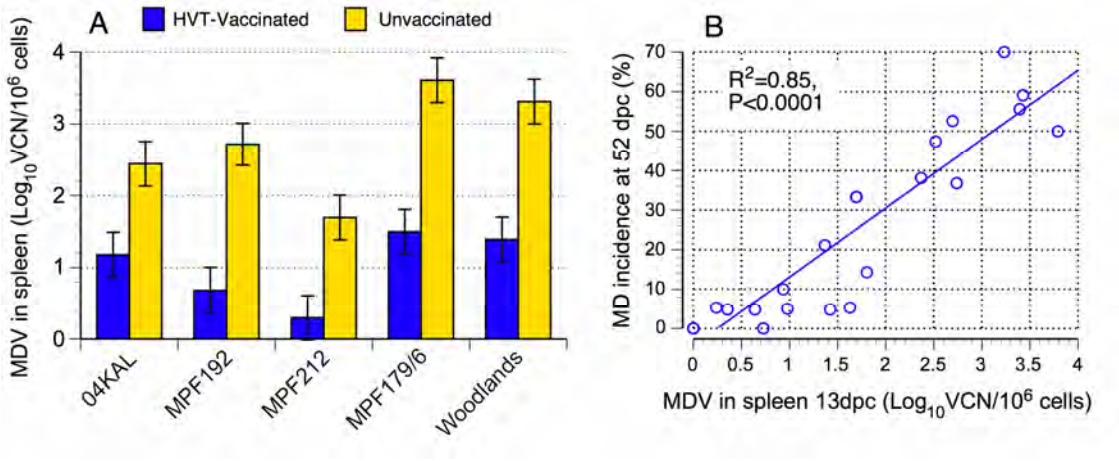


Figure 8.9. A. Least squares means (±SEM) for MDV1 load in spleen (Log_{10}VCN (0 = nil, 3 = severe) at 13 (A,B) and 52 (C,D) dpc by challenge and vaccination treatment.

Serology for CAV at 51 dpc

At 51 dpc 5 chickens from each isolator challenged with MDV1 material were bled and plasma samples sent to RMIT for serology for CAV. All samples were negative except for 2/5 samples in isolator 4 (04KAL, Cobb) and 5/5 samples in isolator 14 (04KAL, Ross). These two isolators were the only ones challenged with 04KAL and this result provides clear evidence of CAV contamination of the 04KAL infective material, despite it being negative for CAV by qPCR at RMIT.

Association between variables and prediction of MD incidence

The degree of association between the incidence of MD and other MD-associated variables such as relative immune organ weights is presented in Table 8.6.

The very strong overall association between MDV1 load in spleen at 13 dpc and the percentage of chickens with MD (Fig 8.9B) varied with the effects of breed and vaccination and challenge virus (Figure 8.10). When the effects of breed, vaccination and challenge virus on the incidence of MD at 52 dpc (%MD) were fitted in a model with MDV1 load in spleen at 13 dpc fitted as a covariate, the latter accounted for 70% of the total variation in MD% (P<0.001) with breed accounting for 15% (P=0.03), challenge virus accounting for 12% (P=0.53) and vaccination accounting for 3% (P=0.30). However when the 4 Nil challenge treatments were removed, the proportions of variation explained by the same effects were 14% (P=0.17) 14% (P=0.72) 40% (P=0.03) and 32% (P=0.05) respectively. These results indicate that the association between MDV1 load in spleen at 13 dpc and MD% is most powerfully modulated by the breed of chicken with Ross birds having a lower incidence of MD for a given MDV1 load in spleen at 13 dpc.

Table 8.6. Correlation matrix for MD-associated variables measured at days 13 and 52 pc. Correlation is based on 24 points representing each Challenge x Vaccine x Breed combination. With df=22 significant values of r are 0.404, 0.515 and 0.559 for P<0.05, P<0.01 and P<0.001 respectively (2 tailed test). Values of r over 0.85 (P<0.0000001) are bolded for convenience.

Variable	MD% d52	MDV1 d13	RTW d13	RBW d13	RSW d13	TA d13	BA d13	CAS d13	BA d52	TA d52	RSW d52	RBW d52	CAS d52
MD% d52	1	**0.92**	-0.54	-0.58	0.89	0.57	0.59	0.63	0.68	0.62	**0.92**	-0.48	0.68
MDV1d13	**0.92**	1	-0.44	-0.47	**0.90**	0.70	0.68	0.75	0.59	0.58	**0.86**	-0.34	0.61
RTWd13	-0.54	-0.44	1	0.72	-0.57	-0.52	-0.38	-0.48	-0.42	-0.40	-0.50	0.42	-0.43
RBWd13	-0.58	-0.47	0.72	1	-0.56	-0.41	-0.60	-0.56	-0.41	-0.39	-0.52	0.37	-0.42
RSWd13	**0.89**	**0.90**	-0.57	-0.56	1	0.72	0.70	0.77	0.73	0.70	**0.86**	-0.54	0.74
TAd13	0.57	0.70	-0.52	-0.41	0.72	1	0.71	**0.91**	0.26	0.28	0.52	-0.05	0.28
BAd13	0.59	0.68	-0.38	-0.60	0.70	0.71	1	**0.94**	0.30	0.45	0.57	-0.04	0.40
CASd13	0.63	0.75	-0.48	-0.56	0.77	**0.91**	**0.94**	1	0.31	0.40	0.59	-0.05	0.37
BAd52	0.68	0.59	-0.42	-0.41	0.73	0.26	0.30	0.31	1	0.83	0.72	-0.80	**0.95**
TAd52	0.62	0.58	-0.40	-0.39	0.70	0.28	0.45	0.40	0.83	1	0.58	-0.67	**0.96**
RSWd52	**0.92**	**0.86**	-0.50	-0.52	**0.86**	0.52	0.57	0.59	0.72	0.58	1	-0.40	0.67
RBWd52	-0.48	-0.34	0.42	0.37	-0.54	-0.05	-0.04	-0.05	-0.80	-0.67	-0.40	1	-0.76
CASd52	0.68	0.61	-0.43	-0.42	0.74	0.28	0.40	0.37	**0.95**	**0.96**	0.67	-0.76	1

When the nil challenge groups were included there was a significant association between the $\log_{10}$challenge dose in pfu and the incidence of MD at 52 dpc (P=0.03)

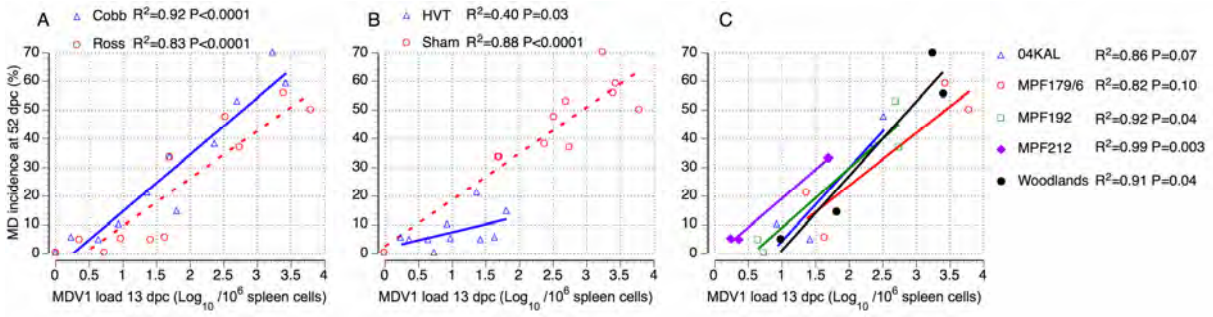


Figure 8.10. Association between MDV1 load in spleen at 13 dpc and the incidence of MD at 52 dpc (%) by breed of chicken (A), vaccination (B) and challenge virus (C).

Brief discussion and conclusions

The experiment was successfully implemented. All challenge viruses induced significant levels of MDV1, despite using much lower doses (in terms of pfu) than have been used in the past. Unchallenged isolators remained free of MDV1. This vindicates the use of challenge dose based on multiples of BID_{50}. Vaccination with HVT also induced significant protection against MDV1 challenge with all of the challenge viruses.

With regards the original stated hypotheses:

1. The broiler strains Ross and Cobb will not differ in susceptibility to the 5 MDV1 isolates whether unvaccinated or vaccinated with HVT.

This hypothesis is rejected. The experiment clearly showed that Cobb birds were more susceptible to MD. This was highly significant with 62/226 (27.4%) Cobb chickens developing MD lesions compared to 47/231 (20.3%) of Ross chickens. Of the 10 virus x vaccination combinations a higher proportion of Cobb than Ross chickens exhibited MD in 9. The exception was for 04KAL in unvaccinated chickens. Interestingly the experiment clearly showed that the effect of breed was not on early viral replication with no difference in MDV1 load in spleen at 13 dpc. However there was a different slope for the two breeds in the association between MDV1 load at 13 dpc and MD incidence

at 52 dpc with Ross birds exhibiting a slower rate of increase in MD incidence as MD load increased (Fig 8.10). This suggests that the resistance in the Cobb chicken is anti-tumour, rather than an anti-viral in nature. The improved FCR in Ross chickens seen in the experiment may be in part due to MD resistance as there was an overall adverse effect of MDV1 challenge on FCR.

2. HVT will only provide partial protection against MD.

This hypothesis is accepted although HVT generally provided a high level of protection against MDV1 challenge (range in PI of 64.3-100%). In the previous broiler experiment (MD05-C-PT1, Chapter3) in Cobb chickens PI was 52%, 48% and 81% for 02LAR, FT158 and MPF57 respectively. Only one virus was common between these experiments, MPF179/6 is a later passage of MPF57. Interestingly in the present experiment MPF179/6 (challenge dose 200pfu) had a PI of 64.3% based on induction of MD in 59.1% of unvaccinated chickens. MPF57 in the previous broiler experiment (challenge dose of 500pfu) had a PI of 81% based on induction of MD in 96.4% of unvaccinated chickens.

3. The MDV1 isolates used will vary significantly in their ability to induce MD in unvaccinated and HVT-vaccinated chickens.

This hypothesis is accepted. Using a common challenge dose of 6 x BID_{50} The isolate Woodlands 1 induced significantly more MD than MPF212. The combination of the two older reference viruses (Woodlands 1 and MPF 179/6=MPF 57 B2) induced more MD than the 3 newer isolates in combination. However as the dose of MPF179/6 was not known in BID_{50} and was substantially higher in pfu terms indicating that dose effects may have come into play here. We have previously shown that a 4-fold increase in MDV1 challenge dose resulted in significantly higher incidence of MD and MD associated mortality in commercial broiler chickens (Islam *et al.* 2007).

4. Pathotype rankings will not differ between chicken genotypes.

This hypothesis must be rejected although there was comparatively little range in the PI index observed for the different viruses. While there was broad agreement between genotypes in ranking for induction of MD in unvaccinated and HVT vaccinated chickens (Figure 8.11A) there were significant discrepancies in ranking for PI (Figure 8.11B). For example MPF212 had the second highest ranking for PI in Cobb birds, but the lowest in Ross birds.

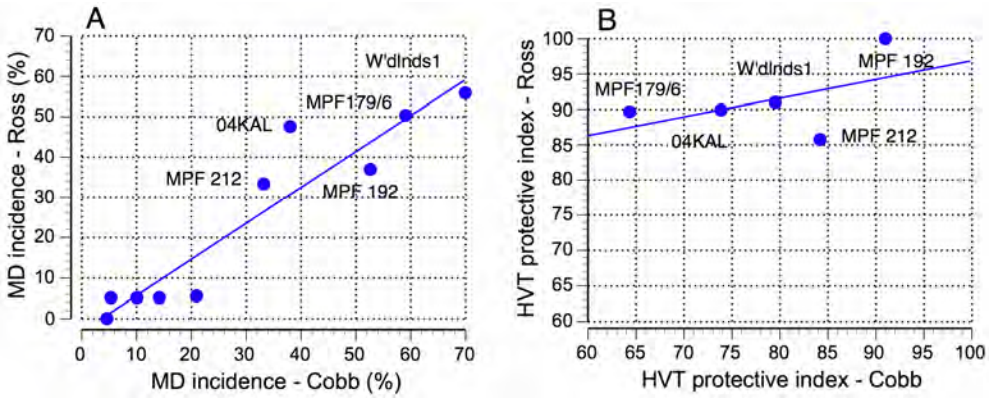


Figure 8.11. A) Breed comparison for incidence of MD in groups of unvaccinated and HVT-vaccinated chickens and B) HVT protective index. The low numbers in A) are for HVT-vaccinated chickens, the high numbers for unvaccinated chickens.

5. The order of rankings for pathogenicity will not differ between unvaccinated and HVT-vaccinated chickens (This has not been so in some earlier experiments).

This hypothesis is broadly accepted. There was non-significant trend (p=0.07) for a positive relationship between MD incidence in vaccinated and unvaccinated chickens within challenge virus and breed (Figure 8.12).

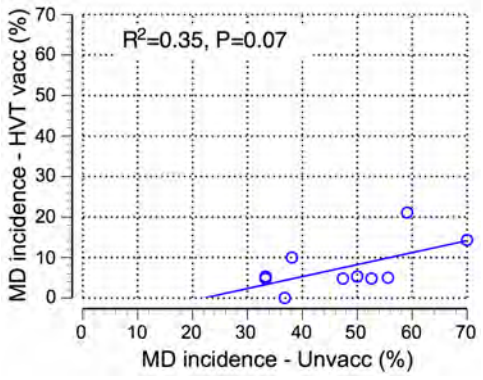


Figure 8.12. Association between of MD incidence in unvaccinated and HVT-vaccinated chickens grouped within breed.

6. Thymus atrophy scores, relative bursal weights and relative splenic weights at day 13 pc will be good predictors of pathogenicity.

This hypothesis is only partially accepted. Relative spleen weight at day 13 pc was very strongly positively associated with the final incidence of MD in the same treatment group (r=0.89 p<0.0001). Thymus and bursal weights or scores had negative and much weaker associations (r= 0.54-0.63). Once again the evidence is that use of a simple scoring system for thymic and bursal atrophy is as efficient as dissection and weighing of immune organs, for obtaining meaningful mean values for groups of chickens as indicators of immunosuppression.

7. MDV1 load in spleen at day 13 will be good predictor of pathogenicity.

This hypothesis was strongly supported with a strong positive overall association between MDV1 load in spleen at 13 dpc and the incidence of MD at 52 dpc (R^2=0.85 P<0.0001). This has been observed repeatedly in our pathotyping experiments. However while the nature of the association between the two variables was similar for the different challenge viruses, it was significantly influenced by both breed of chicken, and by vaccination. Thus models including these effects have an overall higher R^2 (Up to 0.94) than those based on prediction based on MDV1 load in spleen alone.

Other points of interest:

It was interesting to note that while Ross birds were lighter at hatch and at 13 dpc, this difference had disappeared by 52 dpc with superior late growth rates associated with superior performance in terms of FCR, mortality and resistance to MD.

There was a significant effect of bird density in the isolators on final weight. The magnitude of this effect overall was between 55-65g per extra bird in isolator within a bird density range of 10-21 chickens per isolator. This effect would tend to counteract the adverse effect of MD on performance as MD induces mortality and reduces bird density in the isolators. Therefore bird density should be included in any analysis of bird performance, and least squares means (ie. adjusted means) be reported rather than arithmetic means.

Six BID_{50} was able to induce MD in significant numbers of unvaccinated chickens (33-70%). The percentage of chickens infected initially as determined by qPCR of 13 dpc spleens revealed that an even higher proportion chickens was successfully infected with MDV1 (84% of unvaccinated chickens and 65% of vaccinated chickens). Unfortunately the issue of dose is clouded by the following:

- In the present experiment isolates with high challenge doses in pfu terms tended to induce more MD. MPF179/6 and Woodlands 1 had the highest pfu values and induced the most MD. However they are also both highly pathogenic viruses from earlier studies so it is not clear that this is a dose effect rather than a pathogenicity effect.
- We do not have a direct comparison between the 6xBID50 used in this experiment and a high pfu dose for the same isolate in another experiment. The overall percentage of MD induced in this experiment in unvaccinated chickens challenged with MDV1 (47.7%) was lower than in the last broiler experiment using challenge doses of 500pfu/chicken (85.7%) suggesting that 6xBID50 is inferior to 500pfu at inducing a high level of MD.

Acknowledgements.

Thanks to Baiada Poultry Pty Ltd for supplying the chickens and Bartter's Enterprises (Liam Morrisroe) for supplying the feed.

Reference

Islam AFMF, Walkden-Brown SW, Groves PJ, Underwood GJ (2007) Effects of HVT vaccine dose, Marek's disease virus (MDV) challenge dose and vaccination to challenge interval on protection against MDV challenge in broiler chickens. *Australian Veterinary Journal*, In press.

Chapter 9: Experiment 8. MD04-C2-DUST

Effect of temperature on MDV content and infectivity of poultry dust

Start: 20/10/04 Completion: 20/05/06 AEC: UNE AEC04/005
UNE AEC06/061

Introduction

The enveloped infectious virus, produced in the feather follicle epithelium, is vital in the natural transmission of Marek's disease virus (MDV). It is shed from the host in feather dander. Persistence of MDV viability in poultry house dust is of major importance in the transmission of the disease. There are a number of factors that impact on the survival, and therefore the transmission, of MDV. However heat inactivation appears to be the main reason for the loss of MDV infectivity.

This experiment examined the effect of temperature on the viability of the virus over time in the chicken dander, the natural medium of MDV transmission. A wide range of temperature was studied form -80^{o}C to +37^{o}C up to two years of storage. The hypothesis tested in this experiment was that the infectivity of MDV1 in chicken dander will decrease over time in higher temperature but not at lower temperatures, around and below 0ºC.

Materials and methods

A series of experiments was conducted to determine the infectivity of MDV1 in dust stored for various duration, starting from day 14 in weeks interval up to day 56 and then at days 90, 180, 356 and 735. Each trial used 70 Isa-Brown off-sex cockerels in a completely randomized design having seven treatment groups. The treatment groups were: uninfected control, storage temperatures of -80ºC, -20ºC, 4ºC, 10ºC, 26ºC and 37ºC. Ten chickens were used for each treatment groups.

The chickens were obtained from a flock vaccinated with serotype-1 MDV and were therefore presumably positive for MDV1 maternal antibody. Chickens were inoculated (from 14 to 180 days storage trials) on the day of hatch (day 0) and spleen samples were collected from individual birds at 7 day post infection (dpi) for quantification of MDV1. During later trials (360 and 725 days) chicken were inoculated with the dust MDV1 at day 5 and spleen samples were collected 7 dpi.

A preliminary test was conducted to test the procedures. Spleen samples were collected at 7 and 10 dpi following humane killing of the chickens. There was no significant difference in infectivity was observed at 7 and 10 dpi. Therefore, the spleen samples were collected at 7 dpi. Early termination of experiment was also to minimise the risk of later transmission of the virus.

The experiments were conducted in the climate control rooms of the UNE Animal House. Chickens were reared in multi-deck brooders. Layer starter feed and water was given *ad libitum*.

Infective feather dander was collected from a MDV1 challenge experiment (MD04-A6ISO) in which unvaccinated commercial broiler chickens were inoculated with MDV1 (strain MPF 57). The experiment (MD04-A6ISO) produced gross MD lesions in 77% of unvaccinated chickens and the dust was collected between 42 and 49 days post challenge. Dust was weighed into 2 mg aliquots and stored at various temperatures.

Intra-tracheal inoculation of was performed either by an 18g blunt needle and 3ml syringe. The dust (2mg) was placed in the hub of the needle and inoculated (insufflated) via the intra-tracheal route. The whole insufflation exercise for the first six trials was carried out in a fume hood with a thorough cleaning in between treatments. For the later two trials the process was carried out in a separate climate control room of the Animal House.

MDV1 was quantified from DNA extracted from 10 mg of spleen tissues using real-time PCR described in earlier chapters. The spleen samples of the trials for days 14 to 90 were assayed together

by Mrs. Peta Blake (Poultry CRC scholar) and all other samples were assayed at the end of the trials by Mr. Paul Reynolds.

Statistical analysis: Analysis of variance was used to test the effect of storage period and temperature and their interaction on Log_{10} MDV1 load (viral copy number or VCN per 10^6 spleen cells). Significance difference between means was determined using Tukey's HSD test. The difference in the incidence of MDV1 infection (MDV1 positive or negative by PCR) in chicks was tested by logistic regression.

Results

There was a significant effect of day (duration of storage) ($P<0.003$) and temperature (temperature of storage) ($P<0.001$) on the log_{10} MDV1 load (VCN per 10^6 spleen cells) with no significant interaction between them ($P=0.16$). In general the MDV1 load was decreasing with increasing temperature (Fig. 9.1A). The significant effect of day was due to highly variable MDV1 content in various time points, particularly due to higher VCN at day 725. Analysis excluding day 725 revealed no significant effect of day ($P=0.25$).

Logistic analysis revealed a significant effect of day ($P<0.001$) and temperature ($P<0.001$) on the incidence of MDV1 infection with a significant interaction ($P<0.001$) between them. The MD incidence was highly variable at various days in different temperatures (Fig. 9.1B). However, the incidence was much higher in the lower temperatures (-80°C, -20°C and 4°C) then higher temperatures (10°C, 26°C and 37°C).

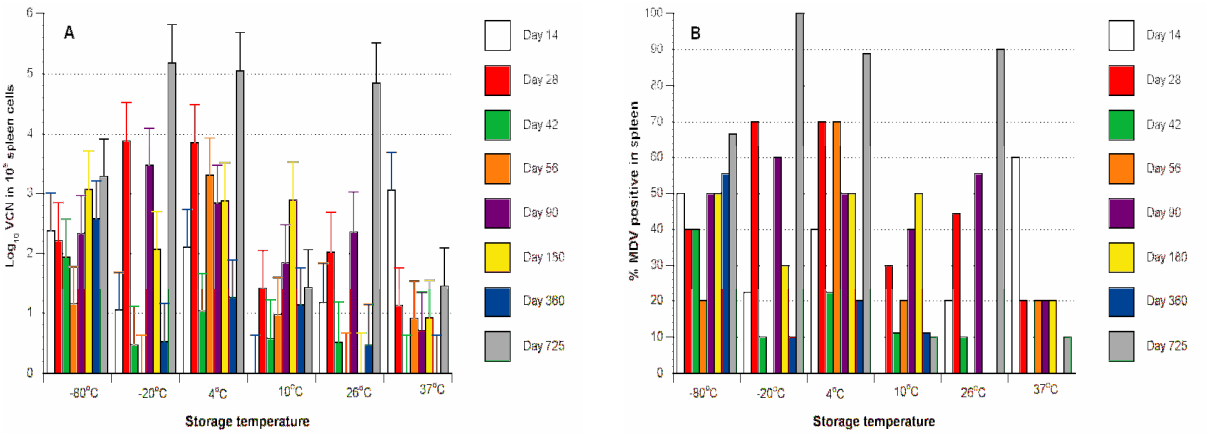


Fig 9. 1. A. MDV1 load (Log_{10} VCN) in spleen of chickens infected with dander containing MDV1, which were stored at various temperatures up to 725 days. B. Incidence of MDV1 infection in chickens challenged with dander containing MDV1 stored at various temperatures from day 14 to day 725.

Discussion and conclusion

The experiment showed that MDV1 could retain its infectivity in chicken dander a substantial period of time (more than two years) even at comparatively high temperatures (37°C). However at this temperature there is clear evidence of infectivity declining over time.

However, the whole experiment produced some inconsistent results, probably because of the method of infection used. Insufflation of dust into the trachea of day-old chickens is a difficult process and it requires training and expertise. A likely contributor to low and variable infectivity during the early stages of the experiment was failure of infection through intra-tracheal insufflation. After the first 3 samples, the insufflation method was optimised with infection of chickens at 5 days of age instead of day old and this appeared to induce more reliable infections.

The high incidence of MDV1 infection and very high MDV1 load in spleen at day 725 is a surprising finding and is not one for which we have a ready explanation. Although we used Isa-Brown cockerels for all experiments, we did not measure maternal antibody levels and assumed them to be similar between batches of chicks (all from vaccinated parents). Variations in maternal antibody level and possibly improvements in insufflation technique may have contributed to the high values at day 725.

This study leads us to conclude that:

- MDV1 can retain infectivity up to two years even if stored at 37^{o}C, although infectivity is reduced over time at this temperature. However, infectivity was not reduced over this time period if dust is stored at temperatures below 4^{o}C.
- Dust insufflation process is slow and difficult method of challenging large numbers of chickens, particularly when they are very young (eg. Day old). It is highly dependant on operator skill.
- Ideally chickens should be infected at 5 days of age or older.

Chapter 10: Epidemiological survey of MDV incidence and risk factors.

Introduction

The objectives of this strategy of the project were to:

- Estimate the incidence of MDV1 in dust in broiler sheds under commercial conditions
- Evaluate any associations between MDV1 incidence and broiler performance parameters
- Estimate the flock prevalence of CAV and investigate whether CAV presence modifies effects of MDV incidence rates on broiler performance
- Provide field data to verify & improve the epidemiological model being produced

Methodology

Eighty farms across the main broiler producing states were intended to provide data and samples for the survey. Sample size was selected in a formally random stratified manner. The numbers of farms in each state and from each broiler company were selected with regard to representing the relative size of each operation in each area. Major broiler producing areas in each state were defined.

Procedures for obtaining data were as follows:

- Farm questionnaire which would collect information on:
    - Proximity to other farms
    - Biosecurity practices
    - Hygiene
    - HVT vaccination
    - Shed/ventilation design
    - Bird density
    - Breed
    - Brooding factors
    - Bird performance (mortality, growth, FCR)

The questionnaire retrieved information on a farm and individual shed level, making analysis necessary on these two levels. A copy of the questionnaire is included in Appendix 1. The completed questionnaires were returned to University of New England with the samples described below. Once received by UNE, each farm was allocated a survey identification code and a copy of the questionnaire forwarded to Zootechny Pty Ltd for computer data entry.

The following samples were collected from each farm by the co-operators.

- Dust samples (settle plates for 1 week) collected at days 21, 28, 35, 42 and final in each shed.
    - Last sample will be tested by qPCR for MDV1: if positive, earlier samples screened until first negative
- Blood and spleen from 5 birds in 2 sheds / farm at 42 days
    - CAV serology (samples sent to RMIT University for ELISA assay)
    - MDV1 & 3 qPCR on spleen cells (samples sent to University of New England)

Farms were selected for entry in the following manner:

The survey period (12 months) was divided into four quarters being defined as:

- Spring: 1 October to 31 December 2004
- Summer: 1 January to 31 March 2005
- Autumn: 1 April to 30 June 2005
- Winter: 1 July to 30 September 2005

Twenty farms were targeted in each quarter, stratified according to farm numbers in a state and within each cooperating company. Individual farms were selected using randomly selected hatch dates. All possible hatch dates for each quarter were assigned a computer-generated random number and sorted in either ascending or descending order (by coin toss). The operations (companies and region) for each quarter were also sorted by random number and the dates and site thus aligned and allocated. Farms were thus selected by random hatch date (or closest date to the selected date if the operation did not hatch chicks on the specified day).

For each farm selected, up to 4 sheds on each farm would enter the survey (if farms had 4 or less sheds, all sheds were selected, if more than 4 sheds, shed selection was left to the co-operator's discretion).

Survey Analysis

The analysis objectives of the data set were to:

1. identify putative risk factors for flocks (either individual sheds or whole farms) being positive for Marek's Disease Virus type 1 (MDV1) in shed dust
2. evaluate the effect on bird health and performance of having MDV1 detected in dust from the shed or farm
3. identify putative risk factors for flocks having positive serology to Chicken Anaemia Virus (CAV) at 42 days of age
4. evaluate the effects on bird health and performance for flocks having positive CAV serology.

As can be gleaned from the questionnaire, the majority of the data were in the form of dichotomous independent variables (these could be assigned values of 0 or 1).

Questionnaire and sample result data were entered into a relational database (MS Access ™) on a farm and an individual shed basis. Once completed the information was downloaded in MS Excel spreadsheets and then into spreadsheets for a statistical data package (STATISTICA™, Statsoft, 2003) for analysis. The analysis approach followed that suggested by Hosmer and Lemeshow (2000) using forward stepwise logistic regression. The goal of developing a statistical model was to select those variables that result in a best model within the scientific context of the situation. Hosmer and Lemeshow (2000) provide a paradigm that, with thoughtful application, "yields the best possible model within the constraints of the data". All data fields were firstly characterised as to their statistical distributions and data type (i.e. categorical, ordinal or continuous). Distributions of continuous variables were graphed and compared to a normal distribution using Kilmogorov-Smirnov, Lillefors and Shapiro-Wilks analyses (Statsoft, 2001). Output graphs of these are shown for farm level variables in Appendix A and for shed level variables in Appendices B and C.

Analysis Process and Results

Risk factors for Marek's Disease Virus in Dust

1. At Farm Level

Descriptive Statistics

A total of 72 farms contributed to the survey over the 12 month period. Table 10.1 reports the distribution of the number of farms over various variables of the survey. Farms were spread in relation to poultry populations in each region of each state and across each cooperating company according to relative size. Within this survey 62.5% of farms carried Cobb birds, 30.6% grew Ross birds and about 7% had a mixture of these breeds. The number of sheds on each farm varied from 3 to 60 (mean of 6.7 sheds per farm). Only 36% of the surveyed farms were vaccinated against Marek's Disease using HVT vaccine. 38% of farms (of those that provided the information) had no other poultry farm within 2 km of their property.

Table 10.1 Sample Distribution: Number of Farms in categories

		Total= 72			
Variable	**Values**	**No. Flocks**	**Variable**	**Values**	**No. Flocks**
STATE	VIC	19	NUMBER OF SHEDS	3	4
	WA	4	ON EACH FARM	4	26
	NSW	27		5	18
	TAS	4		6	5
	SA	8		7	1
	QLD	10		8	9
				10	3
REGION	SE Qld	10		12	1
	Tamworth	4		24	1
	Hunter Valley	4		28	1
	Mangrove Mountain	10		60	1
	Sydney	3			
	Griffith	4	HVT VACCINATED?	NO	46
	Mornington Peninsula	10		YES	26
	Bendigo	2			
	Geelong	5	NUMBER OF OTHER	0	7
	SA	8	POULTRY FARMS	1	22
	WA	4	WITHIN 2 KM	2	8
	Tasmania	4		3	10
				4	7
COMPANY	Company A	20		5	2
	Company B	20		6	1
	Company C	15		8	1
	Company D	5		10	1
	Company E	3		11	1
	Company F	3		No Information	12
	Company G	4			
	Company H	2			
			TYPE OF NEAREST	Broiler	61
BREED	COBB	45	POULTRY FARM	Layer	5
	ROSS	22		Breeder	2
	MIXED	5		No Information	4

Table 10.2 shows the frequency of farms using biosecurity practices recorded from the questionnaire.

A number of these practices were common to most farms (exclusion of visitors, full cleanout with detergent), making any assessment of variation in risk from these factors difficult to lack of variation.

Disinfectant types used in cleanout varied markedly by product names so these were grouped according to their active ingredients. Glutaraldehyde based products were the most frequently used throughout the survey with halogen types (iodine or chlorine based) being next most common. About 71% of surveyed farms used fumigation as a cleanout practice.

There was considerable variation in source of drinking water used for the birds.

Table 10.2. Sample distribution and Biosecurity Practices across Farms

Variable	Values	No. Flocks	Variable	Values	No. Flocks
Records visitors	NO	24	Excludes Visitors	NO	6
	YES	48		YES	66
Disinfectant type used in cleanout	Nil	1	Cleanout practice	FULL with detergent	58
	Glutaraldehyde	30		Full no deterg	7
	Halogen	16		Partial	3
	Peroxide	2		No Information	4
	Formalin	5			
	Quaternary	3	Fumigation used	NO	51
	No Information	15		YES	21
Retain litter on farm	NO	60	Provision of clothing for visitors	NO	35
	YES	12		YES	37
Water source	Town	32	Hygiene of vehicles entering farm	NO	50
	Bore	21		YES	22
	Surface	12			
	Other	3			
	Bore/Town	1	Water sanitised	NO	30
	No Information	3		YES	42

Distributions of farm frequency with regard to continuous variables are shown in Appendix A. These were assessed for fit to a normal distribution using all of the Kolmogorov-Smirnov, Lilliefors and Shapiro-Wilks W tests for normality. If any of these tests returned a probability that the difference from normality was less than 5% due to chance, the data was considered to be non-normal in distribution. According to this criterion, raw Feed Conversion Ratios (FCR), FCR corrected to an average live weight of 2.45 kg, average processing age for the batch and final cumulative % mortality had normal distributions. Distributions for Final Live weight and Final Age (last group collected fro slaughter), average mortality from 0 to 14 days of age, turnaround time (period flock empty of birds prior to this placement) and proximity to the nearest poultry farm differed significantly from normality.

Descriptive Epidemiology

Exactly 50% of farms had at least 1 shed positive for MDV1 in dust sampled at the end of their batch. However of the farms with positive dust results, only 22% returned positive results in all sheds tested on that farm and only 47% had at least 3 out of 4 sheds with positive results.

Table 10.3 shows the prevalence of farms with positive MDV presence in dust at the end of the batch across categorical variables described in the survey.

Table 10.3. Descriptive Statistics - Distribution of MDV1 Dust status Farms over categories

Variable	Parameter value	MDV1 in dust result		%	*P* *
		Negative	Positive	Positive	
* P value from Pearson's Chi-square or Fisher's Exact test (two tailed)			Fisher's exact test used if a cell value <5		
STATE	VIC	10	9	47.4	
	WA	4	0	0.0	
	NSW	12	15	55.6	
	TAS	3	1	25.0	
	SA	2	6	75.0	
	QLD	5	5	50.0	
	Totals	36	36	50.0	*0.19*
COMPANY	LaIonica	1	1	50.0	
	Company A	11	9	45.0	
	Company B	10	10	50.0	
	Company D	2	2	50.0	
	Company C	8	7	46.7	
	Company E	1	3	75.0	
	Company G	1	3	75.0	
	Company H	1	1	50.0	
	Totals	35	36	50.7	*0.94*
REGION	Mornington Peninsula	5	5	50.0	
	Geelong	2	3	60.0	
	Bendigo	1	1	50.0	
	Hunter Valley	2	2	50.0	
	Tamworth	3	1	25.0	
	Mangrove Mountain	5	5	50.0	
	Sydney	1	2	66.7	
	Griffith	0	4	100.0	
	SEQld	5	5	50.0	
	SA	2	6	75.0	
	Tasmania	3	1	25.0	
	WA	4	0	0.0	
	Totals	33	35	51.5	*0.33*
QUARTER	Oct - Dec 04	13	5	27.8	
	Jan - Mar 05	7	13	65.0	
	Apr - Jun 05	5	13	72.2	
	Jul - Sep 05	11	5	31.3	
	Totals	36	36	50.0	*0.011*
SEASON	Winter - Spring	24	10	29.4	
	Summer - Autumn	12	26	68.4	*0.001*
HVT VACCINATED	NO	23	23	50.0	
	YES	13	13	50.0	
	Totals	36	36	50.0	*1.00*
HVT STATUS IN DUST AT 21 DAYS	NEGATIVE	19	19	50.0	
	POSITIVE	17	17	50.0	
	Totals	36	36	50.0	*1.00*
OTHER POULTRY FARMS WITHIN 2 km	NO	5	2	28.6	
	YES	22	31	58.5	
	Totals	27	33	55.0	*0.23*
NUMBER OF SHEDS ON FARM	5 OR LESS	26	22	45.8	
	MORE THAN 5	8	14	63.6	
	Totals	34	36	51.4	*0.17*

Table 10.3 (cont)

Variable	Parameter value	MDV1 in dust result		%	*P* *
		Negative	Positive	Positive	
BREED	COBB	22	23	51.1	
	ROSS	12	10	45.5	
	MIX	2	3	60.0	
	Totals	36	36	50.0	*0.82*
OLD LITTER RETAINED ON FARM	NO	33	27	45.0	
	YES	3	9	75.0	
	Totals	36	36	50.0	*0.11*
CLEANOUT TYPE	FULL with detergent	28	30	51.7	
	Full no deterg	5	2	28.6	
	Partial	1	2	66.7	
	Totals	34	34	50.0	*0.43*
DISINFECTANT TYPE	GLUTARALDEHYDE	15	15	50.0	
	HALOGEN	7	9	56.3	
	NIL	1	0	0.0	
	PEROXIDE	1	1	50.0	
	FORMALIN	4	1	20.0	
	QUATERNARY AMMONIUM	1	2	66.7	
	Totals	29	28	49.1	*0.64*
FUMIGATED	NO	24	27	52.9	
	YES	12	9	42.9	
	Totals	36	36	50.0	*0.44*
DAYS TURNAROUND BETWEEN THIS AND PREVIOUS BATCH	1	0	1	100.0	
	4	1	2	66.7	
	5	1	1	50.0	
	6	0	4	100.0	
	7	6	3	33.3	
	8	2	1	33.3	
	9	3	2	40.0	
	10	4	5	55.6	
	11	5	5	50.0	
	12	4	0	0.0	
	13	1	2	66.7	
	14	5	3	37.5	
	16	1	0	0.0	
	17	0	2	100.0	
	21	0	1	100.0	
	30	0	1	100.0	
	Totals	33	33	50.0	*0.64*
KEEPS RECORD OF VISITORS	NO	11	13	54.2	
	YES	25	23	47.9	
	Totals	36	36	50.0	*0.62*
PROVIDES CLOTHING FOR VISITORS	NO	14	21	60.0	
	YES	22	15	40.5	
	Totals	36	36	50.0	*0.099*

Table 10.3 (cont)

Variable	Parameter value	MDV1 in dust result		%	*P* *
		Negative	Positive	Positive	
DISINFECTION OF VEHICLES	NO	23	27	54.0	
	YES	13	9	40.9	
	Totals	36	36	50.0	*0.31*
WATER SOURCE	TOWN or BORE	32	28	46.7	
	SURFACE	4	8	66.7	
	Totals	36	36	50.0	*0.34*
WATER SANITISED	NO	15	15	50.0	
	YES	21	21	50.0	
	Totals	36	36	50.0	*1.00*
CAV SEROLOGY	NEGATIVE	13	5	27.8	
	POSITIVE	23	30	56.6	
	Totals	36	35	49.3	*0.04*
PERFORMANCE POOL RESULT	Farm in top half of pool	17	14	45.2	
	Farm in bottom half of pool	10	17	63.0	
	Totals	27	31	53.4	*0.18*

Probability values (*P*) shown in Table 10.3 reflect contingency Table 10.analysis for differences due to chance between observed and expected values by Pearson's χ^2 test or Fisher's Exact test if a cell value was lees than 5. This gives a guide to putative associations between the variable's status and the risk of the farm having a positive presence of MDV1 in the final dust samples.

Variable Selection for further Analyses

The associations shown in Table 10.3 suggest some factors may be associated with the likelihood of a farm having dust positive for MDV1. Many of these factors however may interact or confound each other. It is also possible that even if individual variables not to show strong confounding but may contribute to considerable confounding when taken collectively (Hosmer and Lemeshow, 2000).

Variables which have zero cells are particularly troublesome. The cross-tabulation Table 10.for State in Table 10.3 shows a zero cell for positive dust for Western Australia, and from a limited number of cooperating farms. The inclusion of WA data creates severe distortion of the multiple variable analyses. The geographic and industry differences in WA make it difficult to simply collapse their data into another category (e.g. combining it with South Australia or Tasmania is not biologically valid). Given its small contribution to the overall survey the best strategy for handling this is to eliminate WA data from further analysis. Tasmania also has only 1 positive result from a very small number of observations and the industry and geography in this state is also much varied from the other states. Tasmania similarly was also excluded from further analyses. Table 10.4 shows the analysis for state and MDV1 status in dust with these states removed.

Table 10.4. State comparison of Farms and MDV1 status in dust (WA and Tasmania removed)

Variable	Parameter value	MDV1 in dust result		% Positive	*P* *
		Negative	Positive		
STATE	VIC	10	9	47.4	
	NSW	12	15	55.6	
	SA	2	6	75.0	
	QLD	5	5	50.0	
	Totals	29	35	54.7	*0.61*

A criterion for selection of variables for inclusion in a statistical model of risk factors for a farm being MDV1 positive in dust was adopted along the lines recommended by Hosmer and Lemeshow (2000). The number of variables to be considered needs to be minimised so that the resultant model will be more numerically stable. Variables for further analysis were chosen if their univariate P value (Table

10.3) was les than 0.25. A higher than usual P value is chosen as a more rigorous value (e.g. P=0.05) may fail to identify variables that are important in a survey such as this (Hosmer &Lemeshow, 2000; Dohoo, 1993). Variables selected or rejected for analysis are listed in Table 10.5. The variable "QUARTER" referred to the 3 month period of replication throughout the survey. Clearly quarters 2 and 3 showed significantly higher frequency of MDV1 positive farms. This was collapsed into the variable "SEASON" as this grouping provided a better description of the variation.

Table 10.5. Selection of Farm Level variables for Multivariate analysis

Variables Selected		**Variables rejected**	
Putative Risk Factor	P	Non-Associated Factor	P
Season (Summer-Autumn)	0.001	State	0.61
Another poultry farm within 2 km	0.23	Region within state	0.33
Number of sheds on farm (>5)	0.17	Company of ownership	0.94
Retention of litter from previous batch on farm	0.11	Turnaround time between previous batch and this batch	0.64
Provision of clothing for visitors (No)	0.099	Cleanout procedure	0.43
Positive serology to CAV at 42 days	0.04	Disinfectant type used	0.64
		Fumigation of farm used	0.44
		Recording of visitors	0.62
		Provision for disinfection of vehicles	0.31
		Water source	0.34
		Water sanitation	1.00
		HVT vaccination	1.00
		Dust positive for HVT at 21 days	1.00
		Breed	0.87

There appeared to be no association of the risk of being positive in dust for MDV1 at end of batch with geographic location, company of ownership or breed of broiler chicken used. Vaccination with HVT (and as detected by dust qPCR at 21 days on farm) had no effect on the risk of being positive for MDV1 at end of batch. It is known that vaccination does not prevent infection with or shedding of wild MDV type 1 so this finding was to be expected. We were left with six putative risk factors (Table 10.5) that required analysis, controlling for the presence of each factor and for possible interactions between these factors. Forward Stepwise Multiple Logistic Regression was then applied to the variables selected (Statsoft, 2001), using P = 0.25 to enter and P = 0.30 to remove variables during the analysis. Table 10.6 shows the full output from the multiple analysis.

Table 10.6. Full output from Multiple Logistic Regression for farm Level Factors

Variable	Estimate	Standard Error	**Ω***	**95% Confidence Limits of Ω**		Wald stat	p
				Lower	Upper		
Intercept	0.344499	0.713527				0.233	0.629
RetainLitter	0.508645	0.492354	**1.66**	0.63	4.37	1.067	0.302
FarmCloth	-0.741327	0.389988	**0.48**	0.22	1.02	3.613	0.057
SEASON	1.316950	0.439717	**3.73**	1.58	8.84	8.970	0.003
5+sheds	1.105299	0.485945	**3.02**	1.17	7.83	5.174	0.023
farmsin2km	1.093035	0.587909	**2.98**	0.94	9.44	3.457	0.063
CAV POS	0.234222	0.422186	**1.26**	0.55	2.89	0.308	0.579
Scale	1.000000	0.000000					

*Odds Ratio for risk the farm of being positive for MDV1 in dust at end of batch (=$e^{Estimate}$)

Table 10.7 shows the stepwise analysis procedure *STATISTICA*™ output.

Table 10.7. Forward Stepwise Multivariate Logistic Regression Analysis. Farm Level Putative Risk factors for MDV1 positive dust at end of batch

	Effect	Degr. Of Freedom	Wald statistic	Wald P=	Score stat.	Score P=	Variable status
Step 1	RetainLitter	1			1.325946	0.249528	Out
	FarmCloth	1			3.711080	0.054052	Out
	SEASON	1			7.360988	0.006665	Entered
	5+sheds	1			3.687480	0.054822	Out
	farmsin2km	1			2.735025	0.098170	Out
	CAV POS	1			2.513508	0.112874	Out
Step 2	SEASON	1	6.970886	0.008285			In
	FarmCloth	1			4.296796	0.038184	Out
	RetainLitter	1			2.025548	0.154673	Out
	5+sheds	1			5.080968	0.024190	Entered
	farmsin2km	1			3.649963	0.056070	Out
	CAV POS	1			3.149977	0.075928	Out
Step 3	SEASON	1	7.931232	0.004859			In
	5+sheds	1	4.701596	0.030135			In
	RetainLitter	1			2.094208	0.147858	Out
	FarmCloth	1			5.106773	0.023833	Out
	farmsin2km	1			5.252958	0.021909	Entered
	CAV POS	1			1.525068	0.216854	Out
Step 4	SEASON	1	8.815853	0.002986			In
	5+sheds	1	5.682886	0.017131			In
	farmsin2km	1	4.410193	0.035725			In
	FarmCloth	1			4.728586	0.029665	Entered
	RetainLitter	1			2.171881	0.140554	Out
	CAV POS	1			0.812138	0.367489	Out
Step 5	SEASON	1	8.888991	0.002869			In
	5+sheds	1	6.078701	0.013682			In
	farmsin2km	1	3.725600	0.053584			In
	FarmCloth	1	4.266690	0.038867			In
	RetainLitter	1			1.452096	0.228192	Entered
	CAV POS	1			0.670374	0.412921	Out
Step 6	SEASON	1	8.971615	0.002742			In
	5+sheds	1	5.898999	0.015149			In
	farmsin2km	1	3.778615	0.051912			In
	FarmCloth	1	3.681470	0.055020			In
	RetainLitter	1	1.406014	0.235719			In
	CAV POS	1			0.309882	0.577753	Out

Attempts at evaluating the effects of interactions between these variables yielded unstable results due to numerous empty cells. There was a possibly significant interaction between Season and farm proximity but the numbers of farms falling into several of the combined categories was small. Table 10.8 shows the final analysis for risk factors at farm level for positive dust results for MDV1 following stepwise analysis. Five variables were retained in the model. Effects of CAV serology fell out of the model after controlling for the presence of other factors.

Table 10.8. Risk factors for positive MDV1 dust at end of batch farms

Variable	Estimate	Standard Error	**Ω***	95% Confidence Limits of Ω		Wald stat	p
				Lower	Upper		
Intercept	0.344499	0.713527				0.233	0.629
Farm Clothing provided	-0.741327	0.389988	**0.48**	0.22	1.02	3.613	0.057
SEASON (Summer-Autumn)	1.316950	0.439717	**3.73**	1.58	8.84	8.970	0.003
>5 sheds on farm	1.105299	0.485945	**3.02**	1.17	7.83	5.174	0.023
Other farms within 2km	1.093035	0.587909	**2.98**	0.94	9.44	3.457	0.063

*Odds Ratio for risk the farm of being positive for MDV1 in dust at end of batch ($=e^{Estimate}$)

After adjusting for the presence of other factors, the analysis identified several putative risk factors for establishment of type 1 MDV on farms (as demonstrated by presence of detectable virus in shed dust at end of the batch). Placing birds in the Summer – Autumn period (January to June) increases the risk of being MDV1 positive 3.73 times over the Winter – Spring period. Larger farms (more than 5 sheds) are 3 times more at risk of being MDV1 positive and farms with other poultry farms within 2 km of them are also about 3 times more at risk of being MDV1 positive than more isolated farms.

Provision of clothing for visitors on farm appears to have a protective effect (approximately halving the risk of being MDV1 positive).

The effect of being CAV positive on also being MDV1 positive was not significant after controlling for the other factors' presence.

2. At Shed Level

Descriptive Statistics

Across the 72 farms discussed above, a total of 288 sheds were enrolled in the survey. Descriptive statistics for some variables from the sheds data set are graphed in Appendix B. As can be seen, most of the data for variables describing number of pick-up entries made for each shed, final age at which the shed was processed, time form first pick-up to final pick-up for slaughter, final live weight at slaughter, average age at which birds were picked up, average live weight for the birds from each shed over the entire pick-up and late mortality (raw or standardised) differed significantly from a normal distribution. Analyses of these variables therefore require either non-parametric methods or the variable needed to be rescaled into a categorical data set.

Table 10.9 reports the distribution of sheds numbers across several variables where information was provided in the survey. Totals for each variable vary due to missing data in some fields from some farms.

Donor flock representation was evenly spread between younger and older flocks. The majority of sheds was of the tunnel ventilation style and most used gas-fired space heaters for brooding purposes. Only 44% of sheds were vaccinated with HVT and of these 82.6% were vaccinated using the *in ovo* route. The available vaccine brands were relatively evenly represented across the survey.

Only 2 sheds on each farm were sampled for CAV serology at 42 days. Of these, only 62.4% gave any positive results and only 20.8% had all 5 samples positive at 42 days

Eighty-five sheds (29.5%) had positive dust results for the presence of MDV1 at the end of their batch.

Table 10.9. Sample Distribution: Number of sheds in categories

Variable	Value	No. FLOCKS	% FLOCKS
STATE	VIC	70	25.4
	NSW	104	37.7
	SA	31	11.2
	QLD	39	14.1
	WA and TAS	32	11.6
COMPANY	C	58	20.1
	A	81	28.1
	B	80	27.8
	OTHER	69	24.0
BREED	COBB	175	60.8
	ROSS	76	26.4
	MIX	16	5.6
AGE OF DONOR FLOCK	<= 43 WEEKS	118	41.0
	> 43 WEEKS	106	36.8
SEXING OF FLOCK	AS HATCHED	138	47.9
	SEXED	122	42.4
VENTILATION STYLE	TUNNEL	193	67.0
	OTHER	75	26.0
BROODER TYPE	SPACEHEATER	253	87.8
	HOVER TYPE	19	6.6
OTHER POULTRY FARM WITHIN 2KM	NO	60	20.8
	YES	216	75.0
LITTER TYPE	SHAVINGS OR SAWDUST	178	69.5
	OTHER	66	27.8
HVT VACCINATED?	NO	161	55.9
	YES	127	44.1
HVT VACCINATION ROUTE	NIL	167	58.0
	DAY OLD	39	13.5
	IN OVO	82	28.5
HVT VACCINE BRAND	INTERVET	35	12.2
	BIOPROPERTIES	30	10.4
	FORT DODGE	41	14.2
DUST STATUS FOR MDV1	NEGATIVE	191	66.3
	POSITIVE	85	29.5
CAV POSITIVE SEROLOGY AT 42 DAYS*	NO	36	35.6
	YES	63	62.4
No. birds/5 positive for CAV serology per shed	0	36	16.3
	1	11	11.1
	2	6	5.9
	3	10	10.1
	4	15	14.9
	5	21	20.8

*5 birds in only 2 sheds on each farm sampled for CAV at 42 days

CAV serology was only conducted on 2 sheds per farm and hence the data set is not inclusive for this parameter across all sheds. CAV effects therefore are only meaningfully assessed at farm level (see "Risk factors for positive Chicken Anaemia Virus" below).

Descriptive Epidemiology

Table 10.10 shows the univariate association of variables at the shed level with being positive for MDV1 by qPCR on dust at the end of the broiler batch. As with the farm level analysis, the states of Western Australia and Tasmania were removed as they had 0/19 and 1/13 positive sheds for MDV1 respectively.

Table 10.10. Univariate Analysis: Distribution of MDV1 status of sheds across variables

Variable	Value	No. Sheds qPCR for MDV1 result		% positive	Measure of association* P=
		MDV1 negative	MDV1 positive		
STATE	VIC	48	22	31.4	
	NSW	65	39	37.5	
	SA	19	12	38.7	
	QLD	28	11	28.2	0.65
COMPANY	A	36	24	40.0	
	B	42	19	31.1	
	C	40	15	27.3	
	OTHER	42	26	38.2	0.42
BREED	COBB	106	46	30.3	
	ROSS	32	27	45.8	
	MIX	8	8	50.0	0.05
DONOR FLOCK AGE	<= 43 WEEKS	67	30	30.9	
	>43 WEEKS	56	33	37.1	0.38
FLOCK SEXED	AS HATCHED	67	34	33.7	
	SEXED	72	47	39.5	0.37
BROODER TYPE	SPACE HEATER	135	78	36.6	
	HOVER TYPE	15	4	21.1	0.17
SEASON	WINTER-SPRING	95	17	15.2	
	SUMMER-AUTUMN	65	67	50.8	0.0000
VENTILATION STYLE	TUNNEL	111	67	37.6	
	OTHER	38	12	24.0	0.07
LITTER TYPE	SHAVINGS / SAWDUST	118	49	29.3	
	OTHER (straw, paper)	32	33	50.8	0.002
POULTRY FARM	NO	42	11	20.8	
WITHIN 2 KM	YES	113	67	37.2	0.026
HVT VACCINATED	NO	80	46	36.5	
	YES	80	38	32.2	0.48
DUST AT 21 DAYS	NO	80	48	37.5	
POSITIVE FOR HVT	YES	78	35	31.0	0.29
HVT TYPE	INTERVET	18	12	40.0	
	BIOPROPERTIES	22	7	24.1	
	FORT DODGE	26	10	27.8	0.38
HVT ROUTE OF	NIL	82	50	37.9	
ADMINISTRATION	DAY OLD S/C	20	15	42.9	
	IN OVO	58	19	24.7	0.08
FINAL AGE OF	<47 DAYS	23	18	43.9	
BATCH (Quartiles)	47 - 51 DAYS	31	18	36.7	
	51- 55 DAYS	45	17	27.4	
	>=55 DAYS	44	28	38.9	0.34
AVERAGE AGE OF	< 41.2 DAYS	22	12	35.3	
BATCH (Quartiles)	41.2 - 43.9 DAYS	40	16	28.6	
	43.9 - 46.0 DAYS	37	21	36.2	
	>46 DAYS	38	22	36.7	0.78
AVERAGE LIVE WEIGHT	<2295 gm	32	16	33.3	
OF BATCH (Quartiles)	2295-2555 gm	39	16	29.1	
	2555-2764 gm	40	13	24.5	
	>2764 gm	29	28	49.1	0.037

*Probability association due to chance by Chi-square analysis or Fisher's exact test if a cell value <5.

The effects of season and proximity to other farms again show out as highly significant in the risk of being positive in dust for MDV1 at the end of the batch. Other factors which were identified as showing statistical association with risk of being MDV1 positive at the individual shed level included breed, ventilation and brooding styles and litter type. At this level of analysis it seems that the Cobb breed was at lower risk of being MDV1 positive than Ross or mixed flocks , tunnel ventilated sheds

were at higher risk and the use of wood shavings or sawdust decreased the risk compared to straw or other litter materials.

Although the average age sheds were grown to and the final age of pick up for slaughter are not closely associated with risk of being MDV1 positive, batches grown to a higher average live weight seem more likely to be MDV1 positive at the end of the batch. Geographic location (state), company of ownership, age of donor flock and sexing of the flock appeared to have no association with the risk of being MDV1 positive. While HVT vaccination, type of vaccine used and whether HVT was detectable in dust in the shed at 21 days do not show significant associations with detection of MDV1 in dust at the end of the batch, the route of vaccination does appear to have had an effect.

Results from other studies (Islam *et al*. 2005b) using quantitative PCR (qPCR) tests in birds vaccinated with HVT have shown HVT virus to be detectable in dust. Table 10.11 shows the results of qPCR analysis for HVT virus on dust samples from sheds at 21 days of age in relation to the recorded history of HVT vaccination. Out of 126 sheds described as HVT vaccinated in the survey, HVT virus was not detected in dust from 25 sheds at 21 days. This may indicate either a limit in the sensitivity of the test in detection of HVT in vaccinated birds, a mistake in the vaccination history of the flocks or failure of sufficient "take" of the vaccine in an apparently vaccinated flock that would allow detection of the virus by this test.

Of more concern was the detection of HVT virus in 17 flocks that were designated as not being vaccinated with HVT. False positives are unlikely with this test (Islam *et al*., 2005a) and hence this would indicate mistakes in the history of the sheds or errors by the hatchery involved (e.g. placing a group of vaccinated birds in an incorrect destination shed).

Table 10.11. Association of presence of HVT in dust at 21 days with HVT vaccination status

	DUST qPCR result at 21 days		
HVT vaccinated	HVT negative	HVT positive	Totals
NO	141	17	158
YES	25	101	126
Totals	166	118	284

Variable Selection for further Analyses

As for the farm level analysis, variables were selected for further study based on their univariate level of association with detection of MDV1 in dust at the end of the batch (Table 10.10). These were fit into a forward stepwise multiple logistic regression model, as described for the farm level variables above, to control for the presence of each factor in combination with the others of interest. This gave a subset of 8 variables. The output from the stepwise analysis is shown in Table 10.12. Interactions between these variables were assessed and were attempted to be fitted into the model. However no interactions managed to achieve a significant improvement in the model or they failed to be analysable due to instability in the resulting data.

The stepwise analysis with the selected factors is instructive. Each step of the process enters one variable (selected on its univariate strength of association) and then compares the model with this variable and without other variables already entered. In this way, the effect of considering each variable in the presence of others is controlled. Season was the most significant effect and enters at step 1. The breed effect enters at step 2 but is removed at step 6 after the effect of route of vaccination has entered the model.

Table 10.12. Forward Stepwise Logistic Regression of selected shed variables against risk of detectable MDV1 in dust at end of batch.

Step	Effect	Degr. Of freedom	Wald statistic	Wald P=	Score statistic	Score P=	Variable status
Step 1	Other farm within 2km	1			2.24713	0.133862	Out
	Tunnel ventilation	1			7.37681	0.006607	Out
	Shavings or sawdust litter	1			6.88368	0.008699	Out
	Space heater brooding	1			1.35766	0.243943	Out
	Cobb breed	1			8.84363	0.002941	Out
	Avg L Weight quartile	3			6.30378	0.097731	Out
	Summer-Autumn	1			24.88261	0.000001	Entered
	HVT vaccination route	2			7.29712	0.026029	Out
Step 2	Summer-Autumn	1	23.04751	0.000002			In
	Tunnel ventilation	1			7.65824	0.005651	Out
	Shavings or sawdust litter	1			8.49143	0.003568	Out
	Space heater brooding	1			2.44324	0.118032	Out
	Cobb breed	1			9.11748	0.002532	Entered
	Avg L Weight quartile	3			7.03131	0.070906	Out
	Other farm within 2km	1			2.55748	0.109773	Out
	HVT vaccination route	2			7.14915	0.028027	Out
Step 3	Summer-Autumn	1	23.09434	0.000002			In
	Cobb breed	1	8.80345	0.003007			In
	Shavings or sawdust litter	1			7.11719	0.007635	Entered
	Space heater brooding	1			2.92659	0.087131	Out
	Tunnel ventilation	1			5.15585	0.023168	Out
	Avg L Weight quartile	3			6.13827	0.105073	Out
	Other farm within 2km	1			1.83478	0.175563	Out
	HVT vaccination route	2			3.34969	0.187338	Out
Step 4	Summer-Autumn	1	23.94822	0.000001			In
	Cobb breed	1	7.51497	0.006119			In
	Shavings or sawdust litter	1	6.88222	0.008706			In
	Space heater brooding	1			5.06388	0.024429	Entered
	Tunnel ventilation	1			3.58474	0.058312	Out
	Avg L Weight quartile	3			3.67966	0.298197	Out
	Other farm within 2km	1			1.14553	0.284487	Out
	HVT vaccination route	2			2.85763	0.239592	Out
Step 5	Summer-Autumn	1	24.95300	0.000001			In
	Cobb breed	1	8.04258	0.004569			In
	Shavings or sawdust litter	1	8.92021	0.002820			In
	Space heater brooding	1	4.67024	0.030690			In
	Tunnel ventilation	1			3.89737	0.048362	Entered
	AVGLWTQ	3			7.35011	0.061537	Out
	Other farm within 2km	1			2.27600	0.131390	Out
	HVT vaccination route	2			3.82458	0.147742	Out
Step 6	Summer-Autumn	1	25.23260	0.000001			In
	Cobb breed	1	5.90650	0.015085			In
	Shavings or sawdust litter	1	6.86427	0.008794			In
	Space heater brooding	1	4.99293	0.025451			In
	Tunnel ventilation	1	3.73169	0.053389			In
	Avg L Weight quartile	3			8.05153	0.044959	Out
	Other farm within 2km	1			1.23127	0.267161	Out
	HVT vaccination route	2			6.75406	0.034149	Entered

Table 10.12 (cont).

Step	Effect	Degr. Of freedom	Wald statistic	Wald P=	Score statistic	Score P=	Variable status
Step 7	Summer-Autumn	1	25.79802	0.000000			In
	Cobb breed	1	1.50518	0.219875			Removed
	Shavings or sawdust litter	1	5.52074	0.018792			In
	Space heater brooding	1	7.05443	0.007907			In
	Tunnel ventilation	1	6.27751	0.012228			In
	HVT vaccination route	2	6.50498	0.038678			In
	Other farm within 2km	1			0.87857	0.348595	Out
	Avg L Weight quartile	3			7.96395	0.046763	Entered
Step 8	Summer-Autumn	1	26.00765	0.000000			In
	Avg L Weight quartile	3	6.91398	0.074691			In
	Shavings or sawdust litter	1	4.06397	0.043807			In
	Space heater brooding	1	10.11241	0.001473			In
	Tunnel ventilation	1	8.73407	0.003123			In
	HVT vaccination route	2	8.28169	0.015909			In
	Other farm within 2km	1			0.60405	0.437036	Out
	Cobb breed	1			2.21260	0.136888	Out

The final stepwise analysis showing odds ratios for each identified risk factor is shown in Table 10.13. This has left us with 6 putative risk factors at the shed level, being season, brooder type, ventilation type, litter type used, average live weight quartile and route of vaccination with HVT. The odds ratio (**Ω**) describes the magnitude and direction of the putative association between the risk factor and the dependent outcome (being MDV1 positive in dust). An odds ratio greater than unity ascribes the factor as contributing to the risk of the outcome while a factor with an odds ratio less than unity indicates a protective effect against the outcome. An odds ratio of 1.00 means that the two factors are independent (no association). The 95% confidence interval for an odds ratio should not include unity (or should only just do so) for the association to be considered meaningful (Dohoo, 1993).

The analysis suggests that risk factors for sheds being MDV1 positive in dust at the end of the batch are: being placed in summer-autumn, using space heating for brooding and using tunnel ventilation style. Potentially protective factors include using wood shavings or sawdust litter (as opposed to straw or rice hulls), only keeping birds to an average live weight below 2764 gm and using the *in ovo* route for HVT vaccination. However, as can be seen from Table 10.13, the 95% confidence intervals for several variables overlap unity considerably and must therefore be assessed with caution. These factors are average live weight quartile and route of HVT vaccination.

We are thus left with seasonal, brooding ventilation factors and litter type as contributing significantly to the risk of being MDV1 positive in dust at the shed level.

The effects of ventilation style and brooder type may show co-linearity. Within the data set the majority of tunnel ventilated sheds use space heating and there are only 4 sheds which have neither tunnel ventilation nor space heating and none of these was MDV1 positive. Combining an interaction term for ventilation style and brooder type and using this instead of these factors as individual variables improves the model fit only minimally.

Table 10.13. Risk factors for positive MDV1 dust at shed level

Variable	Level of effect \2	Estimate	Standard error	Ω \1	95% Confidence Interval of Ω		Wald statistic	P=
					Lower	Upper		
Intercept		-2.231	0.539				17.110	*0.000*
Season	Summer-Autumn	1.090	0.214	2.97	1.96	4.52	25.993	*0.000*
Brooder type	Space heater	1.257	0.382	3.51	1.66	7.43	10.821	*0.001*
Ventilation style	Tunnel	0.796	0.322	2.22	1.18	4.17	6.105	*0.013*
Litter type	Shavings or sawdust	-0.428	0.238	0.65	0.41	1.04	3.222	*0.073*
Avg Live Weight Quartile 1 \3	<2295 gm	-0.425	0.398	0.65	0.30	1.43	1.142	*0.285*
Avg Live Weight Quartile 2 \3	2295 - 2555 gm	0.048	0.338	1.05	0.54	2.03	0.020	*0.887*
Avg Live Weight Quartile 3 \3	2555 - 2764 gm	-0.503	0.379	0.60	0.29	1.27	1.762	*0.184*
HVT vaccination route \4	NIL	0.340	0.305	1.41	0.77	2.55	1.245	*0.264*
HVT vaccination route \4	Day Old s/c	0.435	0.366	1.55	0.75	3.17	1.416	*0.234*

\1 Ω=Odds ratio for risk of having detectable MDV1 in dust at end of batch for the given variable (=$e^{estimate}$)
\2 Where variables are polychotomous, odds ratio compares against the reference level of the variable
\3 Reference level is Quartile 4 (>2764 gm)
\4 Reference level is *in ovo* vaccination route

Table 10.14 shows the mean Log_{10} MDV1 virus copy number per mg of dust for samples that were positive by qPCR. There did not appear to be a seasonal difference in viral load in dust samples. As viral copy data was not distributed normally, nonparametric tests were used to compare means (Mann-Whitney U test where there were only 2 independent levels within the variable or Kruskal-Wallis ANOVA where there were multiple levels). Vaccination by the *in ovo* route was significantly associated with lower MDV1 load in dust. There was a breed effect observed where the Ross bird flocks had significantly higher viral loads in dust but almost none of the Ross flocks in the survey were vaccinated. This breed effect is confounded in the company results as well (as Company A uses only Ross stock). Sexed flocks also had higher MDV1 dust loads compared with "as hatched" flocks. Farms with another farm within 2 km also tended to have higher dust MDV1 loads.

Of great interest is the difference in MDV1 dust load with flocks using shavings or sawdust litter compared to other litter types (rice hulls or straw). The reason for this is unclear but we could speculate that the litter type may have some effect on the dust characteristics which may have a direct effect on the ability of the qPCR to detect the virus. Possibly, wood source litter types may create more litter-derived dust than either straw or rice hulls and this could have a dilution effect on the dander in shed dust and hence on the qPCR assay.

Dust viral loads were higher in flocks slaughtered out at less than 51 days (which may also reflect the breed effect to some extent).

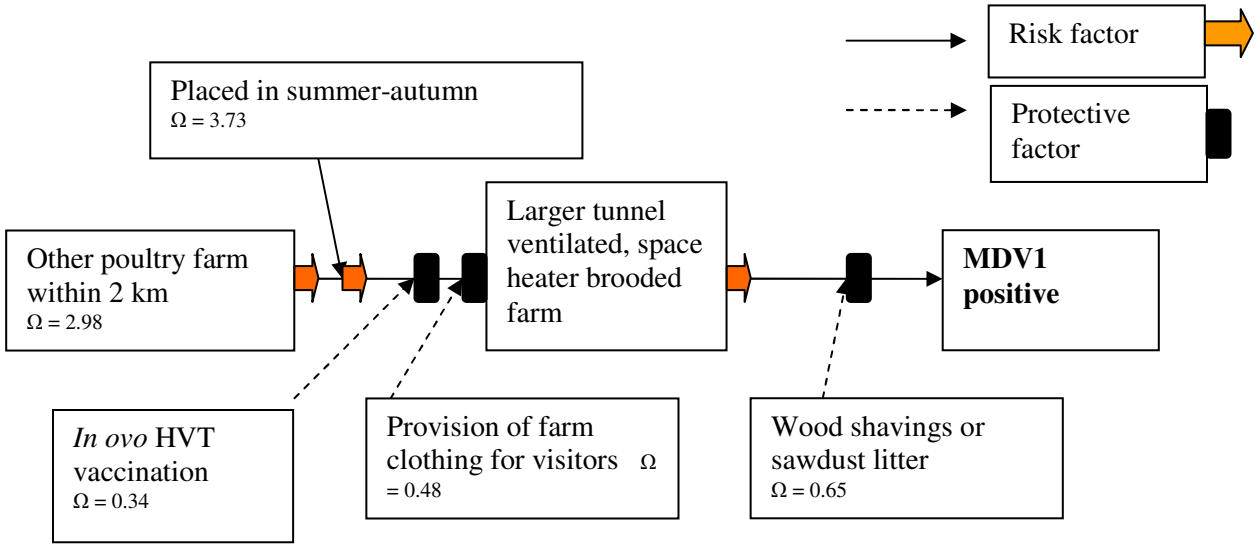
Table 10.14. Breakdown of Mean Log_{10} virus count in dust positive samples

Variable	Value	MDV1 titre Mean Log10 virus copies /mg dust \1	Significance comparison Mann-Whitney U test P=	Kruskal-Wallis ANOVA P=
SEASON	WINTER-SPRING	3.94		
	SUMMER-AUTUMN	3.46	0.08	
BREED	COBB	3.16		
	ROSS	4.28	0.052	
COMPANY	A	4.53 a		
	B	3.09 b		
	C	3.31 ab		
	OTHER	2.56 b		0.004
SEXED	AS HATCHED	3.22		
	SEXED	4.01	0.008	
DONOR FLOCK AGE GROUP	<= 43 WEEKS	3.59		
	> 43 WEEKS	3.85	0.41	
In ovo HVT VACCINATION	NIL	3.89 a		
	YES	2.89 b	0.005	
VENTILATION SYSTEM	TUNNEL	3.68		
	OTHER	3.21	0.64	
LITTER TYPE	SHAVINGS / SAWDUST	3.02 a		
	OTHER	4.51 b	0.000001	
OTHER POULTRY FARM WITHIN 2 KM	NO	2.97		
	YES	3.79	0.054	
Average AGE Quartile	<41.2 days	3.37		
	41.2 - 43.9 days	3.16		
	44 - 46 days	4.00		
	> 46 days	3.67		0.68
FINAL AGE Quartile	<=47 days	4.00 ab		
	47 - 51 days	4.60 b		
	> 51 - 55 days	3.11 ab		
	> 55 days	3.01 a		0.01
Average LIVE WEIGHT Quartile	< 2295 gm	3.34 ab		
	2295 - 2555 gm	2.80 b		
	>2555 - 2764 gm	3.38 ab		
	>=2764 gm	4.25 a		0.03

\1 a,b - means within the same variable group with different postscripts differ significantly (P<0.05)

1. Path Model for Risk of MDV1 in dust

The farm and individual shed analyses leave us with several putative risk factors for the presence of detectable Marek's Disease Virus (serotype 1) in dust collected over the last week of the batch of broilers. The following simple path model for risk of exposure to MDV1 as evidenced by qPCR-detectable presence of virus in end-of-batch dust samples is proposed.



Effects of MDV1 Status on Flock Performance and Health

Performance parameters are measurable at two levels within the survey. Bird growth rates and mortalities are recorded at shed level while Feed Conversion Ratios (FCR) and Position in the Grower Pool are calculated only on a farm basis. There is much variation in these factors between companies (management practices, sexing or non-sexed flocks, nutritional philosophy, market requirements for bird size, etc) and breeds used (breeds differ in growth profile by age, potential FCR, mortality, disease susceptibility, etc). Making a standardised comparison across these variations is a challenge. Several parameters needed to be standardised to account for these inherent differences.

Any analysis needed to be controlled for breed, as this is a major variable. FCR will vary according to average and final weight targets and needs to be adjusted to provide a comparable outcome. The usual industry practice is to adjust raw FCR for a farm to an average live weight (usually 2.45kg at the survey time), although this varied by company. However, all companies use a change of 0.03 FCR units for every 100 gm change in live weight. This assumes a linear relationship of the two parameters which is not correct and the correction becomes less accurate as the difference between actual weight and target weight becomes larger.

Table 10.15 is a triangular matrix of correlation coefficients of performance characteristics of the broiler farms within the survey. Although average live weight has a significant correlation with FCR, the association of FCR with average age of the batch is stronger (r=0.27 compared to r= 0.59 respectively) and the latter is highly significantly correlated (P<0.001).

Table 10.15. Correlation coefficients of performance parameters

Parameter	FCR	Average AGE	Average Final Weight	Average Live weight	Average Final Age
FCR	1.00				
Average AGE	**0.59**	1.00			
Average Final Weight	0.33	0.54	1.00		
Average Live weight	0.27	0.69	0.68	1.00	
Average Final Age	0.57	0.63	0.81	0.41	1.00

All correlations are significant at P<0.05

"Average Age" is calculated as an average of the product actual ages of each pick-up and the number of birds picked up at each age (weighted average). "Average Live Weight" is similarly a weighted average of the average live weights of birds picked up at each shed entry.

Figure 1 is a regression of raw FCR against Average Age of the flock in days. These two parameters show a significant correlation and suggest a moderately strong linear relationship.

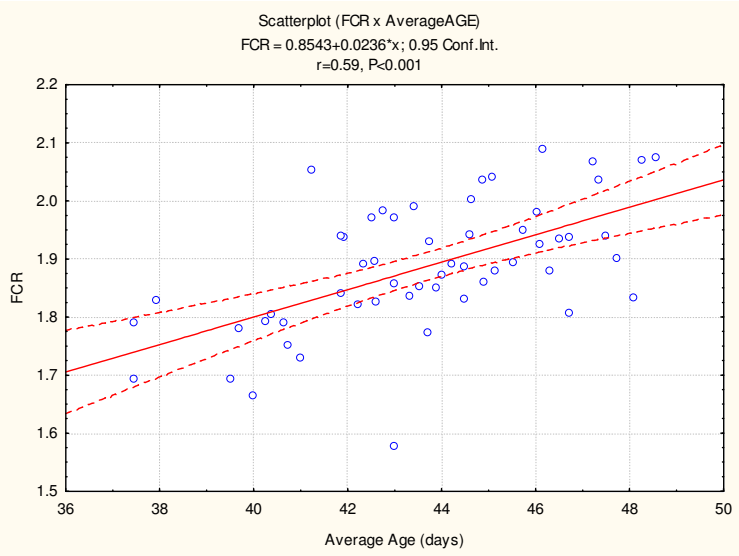


Figure 10.1. Linear Regression of raw FCR and Average Age of slaughter

Both raw FCR and Average Age data were normally distributed (see Appendix A, figures A1 and A3). Standardising FCR to average age would appear more meaningful than the industry practice of standardising to a set average live weight within the context of this survey. Final Age was also moderately correlated with FCR but this variable was not normally distributed (see Appendix A, figure A4), hence standardising to Average Age appears to be a better selection.

Using the regression equation for the relationship between FCR and average age (see Figure 1), an adjustment for FCR to the average age for the survey population (43.522 days) could be calculated as follows:

Adjusted FCR = 0.0236*(43.522-average age for flock) + FCR for flock.

Table 10.A6 (Appendix A) shows that the adjusted FCR values are normally distributed. Table 10.16 shows a summary of adjusted FCR across some farm-level factors.

Table 10.16. Adjusted FCR for average age across various farm level variables

VARIABLE	VALUE	Interaction 1	Interaction 2	Adjusted FCR	P= *
STATE	VIC			1.897	
	NSW			1.907	
	SA			1.841	
	QLD			1.829	0.13
BREED	COBB			1.892	
	ROSS			1.858	0.22
Breed x MDV status	COBB	MDV1 negative		1.893	
	COBB	MDV1Positive		1.890	
	ROSS	MDV1 negative		1.912	
	ROSS	MDV1Positive		1.822	0.19
Breed x vaccination status	COBB	Not vaccinated		1.868	
	COBB	HVT vaccinated		1.908	
	ROSS	Not vaccinated		1.858	
	ROSS	HVT vaccinated		-	0.24
Breed x MDV status x Vaccination status	COBB	MDV1 negative	Not vaccinated	1.878	
	COBB	MDV1 negative	HVT vaccinated	1.900	
	COBB	MDV1Positive	Not vaccinated	1.861	
	COBB	MDV1Positive	HVT vaccinated	1.920	
	ROSS	MDV1 negative	Not vaccinated	1.912	
	ROSS	MDV1 negative	HVT vaccinated	-	
	ROSS	MDV1Positive	Not vaccinated	1.822	
	ROSS	MDV1Positive	HVT vaccinated	-	0.25

*Compared using Analysis of Variance

There were no significant differences between adjusted FCR results across location, breed, breed by MDV1 status or vaccination status interactions. There was considerable variation between companies in adjusted FCR (data not shown for confidentiality reasons). Vaccination status was affected by company and breed. No Ross flocks within the survey were vaccinated with HVT. Attempts to control the data for these interactions lead to too small sample sizes for meaningful comparisons. Nevertheless, no differences in adjusted FCR could be demonstrated between farms that were found to be positive in dust for MDV1 and those that were negative.

Another parameter needing standardisation was late mortality. The industry measures mortality thus:

Mortality = (No. birds dying) / (No. birds placed)*100 %

This is a "Risk Rate". This ignores the dynamic nature of the end of flock life where there may be substantial numbers removed for slaughter at various times. Correct assessment requires knowing the numbers of birds **at risk** at a particular time. Table 10.17 reveals the variation in population dynamics during the pick-up period for slaughter in each shed. There was much variation in these factors throughout the surveyed sheds. Assessment of the rate of bird loss requires the use of an accurate denominator of the numbers of birds in the shed over time.

Table 10.17. Variation in population dynamics during pick-up for slaughter

Activity	Mean	Minimum	Maximum
End of batch age (days)	50.7	35	60
No. shed entries for slaughter	3.6	1	8
Days after first pick up to depopulation	15.7	0	25

The survey provided data in several areas to assist in evaluating the change in shed populations during the final period:

- Number of birds placed
- Crude Mortality to 35 days
- Number birds collected at first pick up
- Number of birds at final pick up
- Total Final (crude) mortality
- Age at first and last pick up

Meaningful assessment of bird losses requires adjustment of the denominator for birds withdrawn for slaughter, thus:

Mortality risk rate = (deaths in period) / (average population at risk in time period)

$$\text{i.e. Mortality risk rate} = \frac{\text{(birds lost 35 days to final age)}}{\text{(No. alive day 35 – half withdrawals)}}$$

This figure can then be used to calculate a comparable late mortality rate over a standardised time period as pick-up period varied markedly. In this case we selected the average time birds were kept past 35 days as the standardised period (15 days - approximately the average for the survey group). This adjustment can be made using the following formula (Martin *et al.,* 1988):

$$\textbf{Standardised Late Mortality Risk Rate} = 1-(1-\text{actual Risk rate})^{15/\text{(actual days)}}$$

Distribution of the standardised late mortality estimates from this transformation is shown in Appendix B, Figure B8 (and Appendix C, Figure C5 for states considered in the analyses above). This parameter is not normally distributed, being heavily skewed to the right.

These transformations provide us with standardised parameters for better comparison of effects across factor categories.

Summaries of the relative performance of flocks which differed in status with respect to having MDV1 detected in dust from the sheds at the end of the batch are shown in Table 10.19.

Distributions of Average Age and Average Live Weight (weighted average of live weights over the pick up period) were not normally distributed (see Appendix B, Figures B5 and B6). The final age of pick up and the birds' final live weights (age and weight of last birds picked up for slaughter) varied considerably across the survey and were also not normally distributed (Appendix B, Figures B2 and B4). The lack of normally distributed data for these parameters means that the statistical assumptions for parametric analysis (e.g. Analysis of Variance or Student's t-test) are not met. To allow for assessment of effects of MDV1 status in dust and of HVT vaccination on these performance parameters, the parameters were divided into quartiles and contingency tables were used (χ^2 or Fisher's exact test) to assess comparisons. Table 10.18 shows the quartile boundaries used for each parameter.

Table 10.18. Quartile boundaries for non-normally distributed performance parameters

Parameter	Unit	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Final Age	Days	<47	47 - 51	51 - 55	>55
Final Live weight	Gm	<2960	2960-3128	3128-3470	>3470
Average Age to 2.45kg	Days	<41.2	41.2 – 43.9	43.9 – 46	>46
Average Live Weight	Gm	<2295	2295 - 2555	2555 - 2764	>2764

Distribution of numbers of flocks and statistical comparisons across the performance parameter quartiles with regard to MDV1 in dust status are shown in Table 10.19 and for HVT vaccination status in Table 10.20.

Table 10.19. Flock MDV1 status in dust across performance parameter quartiles

Performance Parameter	Quartile	MDV1 negative	MDV1 positive	Total	% flocks positive for MDV1	P (from χ^2 analysis)
Final Live weight	1	42	22	64	34.38	
	2	30	15	45	33.33	
	3	57	26	83	31.33	
	4	44	19	63	30.16	0.95
	Totals	173	82	255	32.16	
Final Age	1	32	19	51	37.25	
	2	46	18	64	28.13	
	3	47	17	64	26.56	
	4	44	28	72	38.89	0.32
	Totals	169	82	251	32.67	
Average Live Weight	1	41	17	58	29.31	
	2	44	16	60	26.67	
	3	48	14	62	22.58	
	4	34	28	62	45.16	0.04
	Totals	167	75	242	30.99	
Average Age	1	41	14	55	25.45	
	2	43	16	59	27.12	
	3	38	21	59	35.59	
	4	42	22	64	34.38	0.54
	Totals	164	73	237	30.80	

The distribution of MDV1 positive flocks across quartiles of Final Live Weight, Final Age and Average Age at processing was fairly even, however there was a significantly higher proportion of flocks with positive MDV1 dust results in the highest quartile for Average Live Weight. As this was not associated with higher age, being MDV1 positive in dust was thus associated with a higher growth rate. This was surprising.

Table 10.20. HVT vaccination status of flocks across performance parameter quartiles

Performance Parameter	Quartile	Not HVT vaccinated	HVT vaccinated	Total	% HVT vaccinated	P (from $\chi 2$ analysis)
Final Live Weight	1	54	17	71	23.94	
	2	26	20	46	43.48	
	3	41	43	84	51.19	
	4	20	44	64	68.75	<0.00001
	Totals	141	124	265	46.79	
Final Age	1	48	10	58	17.24	
	2	43	21	64	32.81	
	3	28	38	66	57.58	
	4	17	57	74	77.03	<0.00001
	Totals	136	126	262	48.09	
Average Live Weight	1	42	20	62	32.26	
	2	24	38	62	61.29	
	3	24	39	63	61.90	
	4	39	23	62	37.10	0.0004
	Totals	129	120	249	48.19	
Average Age	1	45	15	60	25.00	
	2	29	33	62	53.23	
	3	29	30	59	50.85	
	4	27	38	65	58.46	0.0009
	Totals	130	116	246	47.15	

The distribution of flocks which were HVT vaccinated was highly significantly associated with all growth rate parameters, being more prevalent in flocks taken to older ages and higher live weights (both final and average for the flock).

Appendix D shows the distributions of flocks by mortality rates over ages 0 – 14 days, 0 – 35 days, 0 – Final Age and Standardised Late Mortality (as calculated above). As can be seen, these do not follow normal distributions and require nonparametric tests to compare averages across different groupings.

Table 10.21 shows the average mortality rates over the defined periods for flocks with regard to their MDV1 status in dust at end of batch and Table 10.22 shows the same data according to HVT vaccination status of the flocks.

Table 10.21. % Mortality rates by period according to MDV1 status in dust at end of batch

Mortality period	MDV1 status in dust at end of batch				Mann-Whitney U test P=
	No. negative	% mortality in negative flocks	No. positive	% mortality in positive flocks	
0 – 14 days	173	1.58	79	1.75	0.97
0 – 35 days	170	3.02	78	3.43	0.07
>35 days (standardised to 15 days)	125	2.72	74	2.87	0.93
0 days - Final	166	4.77	81	5.30	0.21

Table 10.22. % Mortality rates by period according to HVT vaccination status

Mortality period	HVT vaccination status of flock				Mann-Whitney U test P=
	No. non vaccinated	% mortality in non vaccinated flocks	No. vaccinated	% mortality in vaccinated flocks	
0 – 14 days	141	1.56	123	1.68	0.93
0 – 35 days	137	3.07	122	3.13	0.64
>35 days (standardised to 15 days)	92	2.60	114	3.02	0.10
0 days - Final	135	4.49 a	122	5.28 b	0.004

There was no significant difference between raw mortality rates for different MDV1 status across flocks, although the slightly higher level over 0-35 days in positive flocks approached significance. Flocks which were vaccinated with HVT had significantly higher total mortality than unvaccinated flocks and this difference appears to be due to late mortality (after 35 days). This would appear as rather anomalous as the point of vaccinating is to improve bird performance. It was difficult to find if there was any confounding occurring in the data to produce this result. The only factor found which resulted in an interaction across vaccination status and late mortality was route of vaccination.

Table 10.23. Standardised late mortality and vaccination route

HVT vaccination route:	Non-vaccinated	Day old vaccination (sub-cutaneous)	*In ovo* vaccination	Kruskal-Wallis ANOVA P=
Standardised Late Mortality % (15 days)	2.60 [a]	4.06 [b]	2.62 [a]	0.0114

[a,b] means with different superscripts differ significantly (P<0.05)

As can be seen from Table 10.23, the increased mortality observed from vaccinated flocks in Table 10.22 was due to much higher rates in day old (sub-cutaneously) vaccinated birds only. There were only 2 companies which used this method in the survey and they used it exclusively. This lack of variation in vaccination route in these companies does not allow us to differentiate the cause of the observed mortality difference. This may not have been due to the vaccination *per se*, but could have been due to any number of management factors peculiar to those operations.

Interactions between HVT vaccination and MDV1 status are shown in Table 10.24.

Table 10.24. Interactions of MDV1 status across HVT vaccination practices

HVT vaccination route	MDV1 status in dust	No. flocks	Standardised late mortality % (15 days)	Mann-Whitney U test P=
Non-vaccinated	Negative	54	2.54	0.77
	Positive	42	2.51	
Day old (s/c)	Negative	17	3.37	0.06
	Positive	14	4.93	
In ovo	Negative	54	2.70	0.13
	Positive	18	2.10	

In day old vaccinated chickens, late mortality was higher in MDV1 positive flocks than in MDV1 negative flocks, while mortality for any flock vaccinated day old exceeded that for *in ovo* vaccinated or non-vaccinated flocks.

Test result Interactions and Agreement

Tests of agreement and association between the qPCR assays were carried out.

Table 10.25 is a contingency Table 10.comparing MDV1 qPCR results from dust samples and spleen samples collected from the same sheds. Results shown are drawn from only sheds where spleens were collected (2 sheds per farm only).

Table 10.25. Comparison of spleen and dust results for qPCR for MDV1

Dust qPCR result	**Spleen qPCR for MDV1 result**		Totals
	Negative	Positive	
Negative	81	16	97
Positive	26	16	42
Totals	107	32	139
KAPPA (K)	**0.23**	P= 0.09	

The level of agreement between these two tests is only fair (K >0.21 but <0.40, Petrie & Watson, 1999). Dust collected over the last week of the flock's life detected more positive sheds than did spleens from 5 birds per shed at 42 days (30% versus 23%). The dust test did not detect virus in 16 sheds that had positive spleen results at day 42.

We have shown that being HVT positive in dust at 21 days was associated with lower MDV1 viral copies in dust at end of batch (Table 10.14). It was of interest to see if the amount of HVT virus found in dust at 21 days had any correlation with the later amounts of MDV1 found in dust. Log_{10} viral copy numbers for MDV1 in dust at end of batch and Log_{10} HVT viral copies in dust at 21 days are not normally distributed. Analysing for sheds that were found positive for both viruses, the Spearman Rank correlation coefficient (r_s) here was 0.134, indicating a poor correlation between these levels.

Risk Factors for positive Chicken Anaemia Virus Serology

At the outset, it was assumed that, owing to the strong survivability of CAV in the environment, resistance to disinfectants and potential for vertical transmission that if the virus was present within a shed or flock that spread throughout the birds would be virtually complete by late in the flock's life span. Within the resources of the project, and expecting that a high proportion of birds would be expected to be sero-positive to CAV by 42 days, it was decided to only sample 5 birds from each of 2 sheds from each farm within the survey.

As can be seen from Table 10.9, only about 21% of sheds tested had 100% of birds sampled with positive CAV serology. Furthermore, less than 50% had 3 or more birds out of 5 positive and 11% had only 1 positive sample. The difficulty this creates is that, with many sheds with only a low proportion of birds positive from such a small sample size, it is impossible to confidently declare a shed with 0 out 5 positive results to actually be "negative" for CAV. A detailed analysis was not pursued owing to this outcome. Descriptive results for associations of positive CAV serology at 42 days are provided below but these must be assessed with the above limitations strongly in mind.

Overall, 64% of flocks had some level of positive serology detected within 5 samples at 42 days.

Table 10.26 shows breakdowns of means of continuous variables for sheds with zero positive samples out of 5 for CAV serological titres compared to sheds with 1-5 positive results / 5 samples. As these variables do not follow a normal distribution (see Appendix B), nonparametric tests were used for comparisons. Mean results were compared using the Mann-Whitney U test (Petrie and Watson, 1999).

Table 10.26. Comparisons of mean results of continuous data variables across flocks with differing CAV serology. * Compared using Mann-Whitney U test (Statsoft, 2001)

FACTOR (units)	**CAV SEROLOGY**				***P*=** *
	0 / 5		**>=1 / 5**		
	n	**Mean**	**n**	**Mean**	
Bird No. (number)	40	30008	75	31269	***0.49***
Placement density (birds/m^2)	37	19.7	63	18.7	***0.005***
Average density days 35 to final(birds / m^2)	35	12.8	57	11.3	***0.08***
Donor flock age (weeks)	32	40.4	68	42.7	***0.24***
Donor flock CAV titre	3	5476	6	5574	***0.79***
Donor flock IBD titre	7	6327	4	7768	***0.06***
Mortality from 0 to 14 days (%)	44	1.77	74	1.61	***0.19***
Mortality from 0 to 35 days (%)	44	3.17	72	3.06	***0.48***
Standardised late mortality (birds/1000 at risk from 35 days over 15 days)	36	19.1	66	27.1	***0.027***
Total batch Mortality (%)	43	4.44	72	4.71	***0.28***
No. entries to shed for pick-up	42	3.5	75	3.6	***0.63***
Days to pick up (days post 35 days)	41	14.2	75	16.3	***0.03***
Average flock Age (days)	41	43.4	72	43.8	***0.62***
Average Live Weight of batch (gm)	38	2499	76	2566	***0.26***
Final Age of batch (days)	44	49.17	76	51.3	***0.01***
Final Live Weight (gm)	44	2991	76	3200	***0.02***
FCR corrected to 2.45kg live weight	40	1.81	64	1.84	***0.24***
LogMDV1 in dust at end of batch (virus copies/mg dust)	10	3.45	27	3.83	***0.71***

Table 10.27 shows a cross-tabulation of numbers of flocks within categories which had either 0 or ≥1 positive serological results from 5 samples for CAV. Probability values shown were calculated using Chi-square contingency Table 10.analysis or Fisher's exact test if a cell's value was <5.

Table 10.27. Categorical variables compared between flocks with 0/5 positive CAV serology and those with some positive results.

Variable	Value	No. Flocks <45% positive for CAV serology	No. Flocks >45% positive for CAV serology	Total flocks	% flocks >45% positive	*P*= *
STATE	NSW	12	35	47	74.47	
	QLD	8	8	16	50.00	
	SA	6	10	16	62.50	
	VIC	14	20	34	58.82	
	WA & TAS	6	9	15	60.00	
	Totals	46	82	128	64.06	*0.39*
COMPANY	OTHER	5	22	27	81.48	
	A	18	19	37	51.35	
	B	10	26	36	72.22	
	C	13	15	28	53.57	
	Totals	46	82	128	64.06	*0.03*
REGION	SEQld	8	8	16	50.00	
	Tamworth	4	4	8	50.00	
	Hunter Valley	2	4	6	66.67	
	Griffith	0	8	8	100.00	
	Mangrove Mtn	0	15	15	100.00	
	Sydney	6	0	6	0.00	
	Mornington Peninsula	3	17	20	85.00	
	Bendigo	2	2	4	50.00	
	Geelong	5	1	6	16.67	
	South Australia	6	10	16	62.50	
	Tasmania	3	5	8	62.50	
	Western Australia	3	4	7	57.14	
	Totals	42	78	120	65.00	*0.0002*
BREED	COBB	23	56	79	70.89	
	ROSS	14	19	33	57.58	
	MIX	5	2	7	28.57	
	Totals	42	77	119	64.71	*0.048*
SEXED	AS HATCHED	17	41	58	70.69	
	SEXED	23	35	58	60.34	
	Totals	40	76	116	65.52	*0.24*
LITTER TYPE	SHAVINGS / SAWDUST	31	57	88	64.77	
	OTHER	11	21	32	65.63	
	Totals	42	78	120	65.00	*0.93*
BROODER TYPE	SPACE HEATER	41	71	112	63.39	
	HOVER TYPE	3	7	10	70.00	
	Totals	44	78	122	63.93	*0.68*
DONOR FLOCK AGE	<=43 WEEKS	22	38	60	63.33	
	>43 WEEKS	10	30	40	75.00	
	Totals	32	68	100	68.00	*0.22*
VENTILATION STYLE	TUNNEL	26	59	85	69.41	
	OTHER	18	17	35	48.57	
	Totals	44	76	120	63.33	*0.03*

Table 10.27. Cont

Variable	Value	No. Flocks <45% positive for CAV serology	No. Flocks >45% positive for CAV serology	Total flocks	% flocks >45% positive	*P*= *
HANDWASH AT SHED ENTRY	NO	30	56	86	65.12	
	YES	16	26	42	61.90	
	Totals	46	82	128	64.06	*0.72*
HVT VACCINATED	NO	30	44	74	59.46	
	YES	16	38	54	70.37	
	Totals	46	82	128	64.06	*0.20*
OTHER FARM WITHIN 2 KM	NO	14	10	24	41.67	
	YES	32	69	101	68.32	
	Totals	46	79	125	63.20	*0.015*
SEASON	WINTER-SPRING	21	40	61	65.57	
	SUMMER-AUTUMN	25	42	67	62.69	
	Totals	46	82	128	64.06	*0.73*

* Probability difference due to chance, Chi-square analysis.

Within the limitations of sample size discussed above, flocks were more likely to have positive CAV serology at 42 days if they:

- Were placed and maintained at lower stocking density
- Were Cobb breed
- Were grown in a tunnel ventilated shed
- Had another poultry farm within 2 km

Donor flock serological history was requested in the survey but few respondents were able to supply this information. From minimal data there was no evidence that difference in CAV serological titre in the birds' parents had any effect on risk of being serologically positive at 42 days. Higher IBDV titres in parents may have been associated with a higher risk of having positive serology at 42 days however.

CAV positive flocks also tended to have had longer pick-up periods for slaughter and were generally kept to slightly higher live weights and ages.

As opposed to risks for being MDV1 positive, there was no seasonal effect noticeable with the risk of being CAV positive, nor did litter type or brooding system have an impact.

Flocks which had at least 1 positive serological result from 5 samples for CAV at 42 days had a significantly higher late mortality (standardised over 15 days post 35 days of age) than those flocks which had no positive results.

There were some indications of differences between regions and companies with regard to CAV serology. The regional differences really only reflected low rates of positives in two regions with only 6 sheds each represented in the survey (Sydney and Geelong). This also contributes to the perceived differences between companies as company locations are geographically dependent. The significance of this regional effect is questionable. The interactions of company and location are considered in more detail below.

Table 10.28 shows a breakdown of state and company distributions of flocks with 0/5 positive samples for CAV serology (i.e. statistically $\leq$ 45% of birds likely to be positive) compared with flocks that had 1 or more positive samples /5 (>45% likely to be positive).

There was no significant difference between proportion of flocks positive in each state but there were significant differences (P=0.036, Kruskal-Wallis ANOVA) between companies. Company comparisons showed that Company B had 72% flocks with at least 1 positive serum /5 while Company

A and Company C averaged only 52% and 54% respectively in this category. Amongst "Other" companies, basically each company was present only in one state and results varied in the geographic location as well here. Basically, if we regard the comparison as valid (ignoring the problem with sample size), this may indicate that there may be some intra-company practices which alter the risk of being "positive" serologically for CAV at 42 days. Any assumptions here need to be guarded.

Table 10.28. Comparison of CAV serology results by State and Broiler Company

STATE	COMPANY	No. Flocks <45% positive for CAV serology	No. Flocks >45% positive for CAV serology	Total flocks	% flocks >45% positive
NSW	OTHER	0	11	11	100.00
	A	2	12	14	85.71
	B	0	8	8	100.00
	C	10	4	14	28.57
NSW Total		12	35	47	74.47
QLD	OTHER	0	4	4	100.00
	A	5	3	8	37.50
	B	3	1	4	25.00
Qld Total		8	8	16	50.00
SA	B	4	4	8	50.00
	C	2	6	8	75.00
SA Total		6	10	16	62.50
VIC	OTHER	5	7	12	58.33
	A	8	0	8	0.00
	B	0	8	8	100.00
	C	1	5	6	83.33
Vic Total		14	20	34	58.82
WA&TAS	A	3	4	7	57.14
	B	3	5	8	62.50
WA & Tas Total		6	9	15	60.00
Grand total		46	82	128	64.06

Table 10.29 shows a cross tabulation of performance parameters and results of CAV serology.

Table 10.29. Performance and CAV serology results

Performance Parameter	CAV serology results (5 birds per shed) at 42 days		Mann-Whitney U test
	Negative	Positive	P=
Adjusted FCR	1.835	1.899	0.24
Average Age (days(=)	43.44	43.77	0.27
Average Live Weight (gm)	2499	2566	0.26
Final Live Weight (gm)	2991	3200	0.008
Mortality % days 0-14	1.77	1.60	0.19
Mortality % days 0-35	3.16	3.00	0.48
Late mortality (standardised)	1.95	2.71	0.03
Mortality % 0 days – final	4.40	4.87	0.28

The estimate of CAV positivity generated by the data suggests possible significant associations of CAV positive serology at 42 days with slightly higher late mortality (after 35 days). Flocks with positive CAV serology were processed with higher final live weights within this survey.

Discussion of Results

Objective:

Estimate the incidence of MDV in dust in broiler sheds under commercial conditions

Exactly 50% of farms had at least 1 shed positive for MDV1 in dust sampled at the end of their batch. However of the farms with positive dust results, only 22% returned positive results in all sheds tested on that farm and only 47% had at least 3 out of 4 sheds with positive results. This outcome was somewhat surprising in comparison to the presumed ubiquitous existence of Marek's Disease. This finding indicates that the exposure to MDV is not ubiquitous as was the prior conception, and that variation and inconsistent incidence findings with this disease in the literature (e.g. Calnek & Witter, 1997) may be due to variation in exposure to virus, not just due to variation in virulence of virus strains in different flocks. Even spread of the virus between sheds on the same property may be inconsistent.

There were no differences demonstrable in **incidence** of MDV1 in final dust samples due to geography (state or region), company of ownership/integration, HVT vaccination, HVT vaccine brand used, choice of disinfectant class, use of fumigation or turnaround time.

There were several risk factors identified that were significantly associated with differing MDV1 status in dust. These were:

Season: Birds being placed in summer-autumn (January through June) had 3-4 times the risk of being MDV1 positive in dust compared to birds placed in winter-spring. This was unexpected and segments of the industry have assumed that late spring-summer were higher risk periods and had used strategic vaccination to meet this belief. Data quoted from USA (Calnek & Witter, 1997) showed a consistently higher rate of clinical MD cases in broilers in early winter (based on abattoir condemnation records). This is not inconsistent with our findings, as birds placed in autumn would be processed in early winter, when signs could be detected.

Farm size: Larger farms (>5 sheds) were about 3 times more at risk of being MDV1 positive in dust at end of batch in at least one shed.

Proximity to other farms: Having another chicken farm within 2 km of the premises increases risk of being MDV1 positive in dust at end of batch three fold. This is consistent with findings with a small scale survey of layer farms previously conducted (Groves, 1993). Bearing in mind the method of spread of this virus, this finding makes biological sense.

Ventilation & brooding style: Tunnel ventilated sheds with space heater brooding had an increased risk of being MDV1 positive. Reasons for this are unclear but may be associated with farm size (newer tunnel farms tend to be larger). Forced ventilation may increase the risk of drawing airborne infected dust particles into the shed.

Retention of litter from previous batches: Effect of having retained litter on site was associated with higher risk of MDV1 positivity although the confidence levels for the odds ratio extended over unity. Used litter could obviously harbour MDV but would be less likely to result in infected aerosol particles than would live birds in the vicinity.

Some potentially protective factors were also identified. These were:

Provision of clothing on farm for visitors: Most frequent visitors to broiler farms are company service personnel, veterinarians and pick up crews, all of which have frequent and close contact with other poultry and are a potential source of infection. Providing clean clothing and footwear for these visitors could obviously assist in decreasing the chance of viral introduction, especially when considering a virus like MDV, with long survival times in dust which could be easily carried on clothing.

Use of wood based litter material: This approximately halved the risk of being MDV1 positive (Ω = 0.65) compared to other type materials (rice hulls, straw, paper). Possible reasons are not immediately clear. We could speculate that the litter type may have some effect on the dust characteristics which may have a direct effect on the ability of the qPCR to detect the virus. Alternatively, wood source litter types may create more litter-derived dust than either straw or rice hulls and this could have a dilution effect on the dander in shed dust and hence on the qPCR assay.

Differences in the amount of viral copies found in dust were observed to be associated with several factors. MDV1 copy numbers in dust were modified by the factors shown in Table 10.30 (summarised from Table 10.14).

Table 10.30. Factors associated with quantity of MDV1 in dust at end of batch

Factors associated with higher MDV1 copy numbers in dust	Factors associated with lower MDV1 copy numbers in dust
Breed (Ross)	Company (smaller integrations)
Sexed flocks	*In ovo* HVT vaccination
Proximity to other farms (<2km)	Litter type (wood based)
Higher average live weight	Older final age of processing

Ross birds tended to have higher MDV1 loads in dust but then very few of the Ross flocks in the survey were vaccinated. Use of *in ovo* HVT vaccine, although not affecting presence of the virus, was associated with lower recovery rates of MDV1 in dust. Proximity to other poultry also increased the likelihood of higher dust viral levels. Wood based litter material was associated strongly with lower viral load.

Somewhat surprisingly, flocks kept to an older age tended to have lower viral loads in dust. This may have been associated with more of these older flocks being HVT vaccinated (see below). Also interestingly, flocks which achieved a higher average live weight (weighted average of the weights picked up over time) had increased viral copies in dust. This, coupled with the higher levels seen in birds processed younger, may indicate that **more rapidly growing birds may shed more MDV**.

Evaluate any associations between MDV incidence and broiler performance parameters

We introduce two new methods of standardising measurement and assessment of bird performance. These were:

1. Adjustment of FCR to average flock age rather than a standard live weight. These parameters showed a stronger correlation than the usual industry method.
2. Standardisation method for assessment of late mortality, which is severely complicated by population dynamics involved with multiple shed entries for pick up for slaughter over a variable time.

We were unable to show any differences in performance in the areas of Final Live Weight, Final Age, Average Age or mortality rates with the presence of detectable MDV1 in dust at end of batch. Average Live Weights for flocks which were positive for MDV1 in dust were actually higher than negative flocks. There was a trend however for positive flocks to fall into the lower half of the performance pool (Table 10.3).

Further, it was not possible to demonstrate beneficial effects from HVT vaccination on raw analysis, whether flocks were positive for MDV1 or not. One confounding effect in this respect was found to be route of vaccination. Flocks in this survey that were vaccinated at day old by sub-cutaneous injection, had higher late mortality and poorer performance. The reason for this was not easily discernable and may not be related to the vaccination itself as this outcome as not separable from the overall effect of the operations using this technique (lack of variation within the cohort).

Interactions within and between flocks' MDV1 in dust status and HVT vaccination status with performance outcomes were complex within the survey data. There were no significant differences

within adjusted FCR and MDV or HVT status (Table 10.16). However FCR is a complex parameter, determined by breed, flock age and growth rates among a host of other contributory factors. Historically, when Marek's Disease caused major problems in the mid 1990's, HVT vaccination resulted in marked improvements in FCR (commonly improvements as high as 0.15 to 0.20 were observed). However as vaccination proceeded over time, and studies conducted where farms were vaccinated every second batch, this observable difference disappeared. This was due to an improvement in the non-vaccinated flocks, not deterioration in the vaccinated group. This was assumed at the time to relate to an overall decline in the challenge exposure of the entire population, batch to batch. With widespread HVT vaccination of broilers in many areas and the improved control of MD in layers and breeders following the introduction of Rispens MDV vaccine, this situation would seem to have continued.

Estimate the flock prevalence of CAV and investigate whether CAV presence modifies effects of MDV incidence rates on broiler performance

The small sample size chosen for CAV serology limited the conclusions possible for this objective.

Only 21% of sheds tested had 100% of birds sampled with positive CAV serology. Furthermore, less than 50% had 3 or more birds out of 5 positive and 11% had only 1 positive sample. This was quite unexpected. The difficulty this creates is that, with many sheds with only a low proportion of birds positive from such a small sample size, it is impossible to confidently declare a shed with 0 out 5 positive results to actually be "negative" for CAV. Statistically, failing to find a positive in 5 samples in a typical broiler flock would only provide 95% confidence that the positive proportion was only something less than 45% (Martin *et al*., 1988). Descriptive results for associations of positive CAV serology at 42 days are provided below but these must be assessed with the above limitations strongly in mind. We have not devoted a major analysis to this factor as a result of this shortcoming. Overall, 64% of flocks had some level of positive serology detected within 5 samples at 42 days.

Within the limitations of sample size discussed above, flocks were more likely to have positive CAV serology at 42 days if they:

- Were placed and maintained at lower stocking density
- Were Cobb breed
- Were grown in a tunnel ventilated shed
- Had another poultry farm within 2 km

Implications

Information generated by the survey will assist the industry to better target HVT vaccination, especially with regard to strategic use of the vaccine by season. This will enable marked cost savings in the reduced need to vaccinate al year round. Continued use of the dust qPCR will enable a longer term grasp of the epidemiological changes that may occur with a highly adaptive virus like MDV.

We now have evidence that MDV is not necessarily as ubiquitous as formerly believed.

Several of the identified risk factors for MDV1 exposure and establishment in as flock are not amenable to easy control by the management (season, proximity to other farms, existing farm size and shed construction) but some factors may allow manipulation to decrease risk. Provision of on-farm clothing and avoiding retention of used litter may significantly lower risk rates.

Recommendations

Further work is necessary to understand the effects of wood based litter materials and higher growth rates on the detectability of MDV1 in shed dust. For the commercialisation of the qPCR the effect of the litter material on dust and the ability to isolate the virus will need to be determined more fully. It

will also be important to confirm the observation of more positive flocks and higher levels of MDV in dust from faster growing birds, as this could bias the meaningfulness of the qPCR on dust outcome in the field.

More investigation should be carried out to determine other underlying factors that lead to the observed poorer performance of day old vaccinated birds, within the operations using this method.

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Glossary

MDV1 – sero-type 1 Marek's Disease Virus
CAV – Chicken Anaemia Virus
HVT – Herpes Virus of Turkeys

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Chapter 11

Development of a deterministic epidemiological model of MD in broiler chickens

Introduction

This work was carried out by Dr Zhanhai Gao, a post-doctoral fellow at UNE and member of the project team. It was developed in consultation with other project members, especially Drs Walkden-Brown, Groves and Islam, with inputs also from Dr Greg Underwood and Dr Evan Sergeant of Aus Vet Animal Health Services. The work was initiated under ARC Linkage project LP0211607 and expanded during this project.

Our original intention was to initially develop an effective model of the spread and consequences of MDV infection within a shed of broiler chickens, given certain starting conditions. A sophisticated model achieving this was successfully developed and is submitted with this final report. We had hoped for widespread testing by industry personnel, but found it difficult to get industry veterinarians to devote time to do this, so it has had limited feedback from industry.

The subsequent intention was to extend the model to include spread of MDV between sheds on a farm, and between farms in a district. This was to be based on the findings of the Field Epidemiological study (Chapter 10). This was not achieved due to Dr Gao's contract finishing at UNE prior to the full details from the field study becoming available. Thus we in the project now have

a) A detailed epidemiological model of MD in a shed of chickens. This is available in MATLAB, in which it was originally programmed and in Excel, into which it was re-programmed subsequently to improve its availability to likely end users.

b) A set of epidemiological equations and relationships describing factors influencing MDV presence in the field, based on the field epidemiological study (Chapter 10).

While not formally linked into a single model, the two sets of information greatly enhance our understanding of MD epidemiology in Australia.

In the absence of a formal report on the model from Dr Gao the instructions for operating the model and his 3 conference papers on the subject are in the Appendices, and the full model is included with the Final report. A final journal paper describing the model was drafted some time ago, but still requires work.

Brief description of the model

The introductory page of the model is shown in Figure 11.1. The user inputs a number of variables influencing MDV spread and expression of MD. These are shown in Table 11.1. The model is then run, and a solution derived for each day of the batch and chickens move through the different stages of the pathogenesis of MD at various speeds and the MD virus spreads from chicken to chicken. The output variables are presented graphically on a second sheet, over time and at the end of the batch or at any given day during the batch in windows on the main screen. The output variables are also shown in Table 11.1.

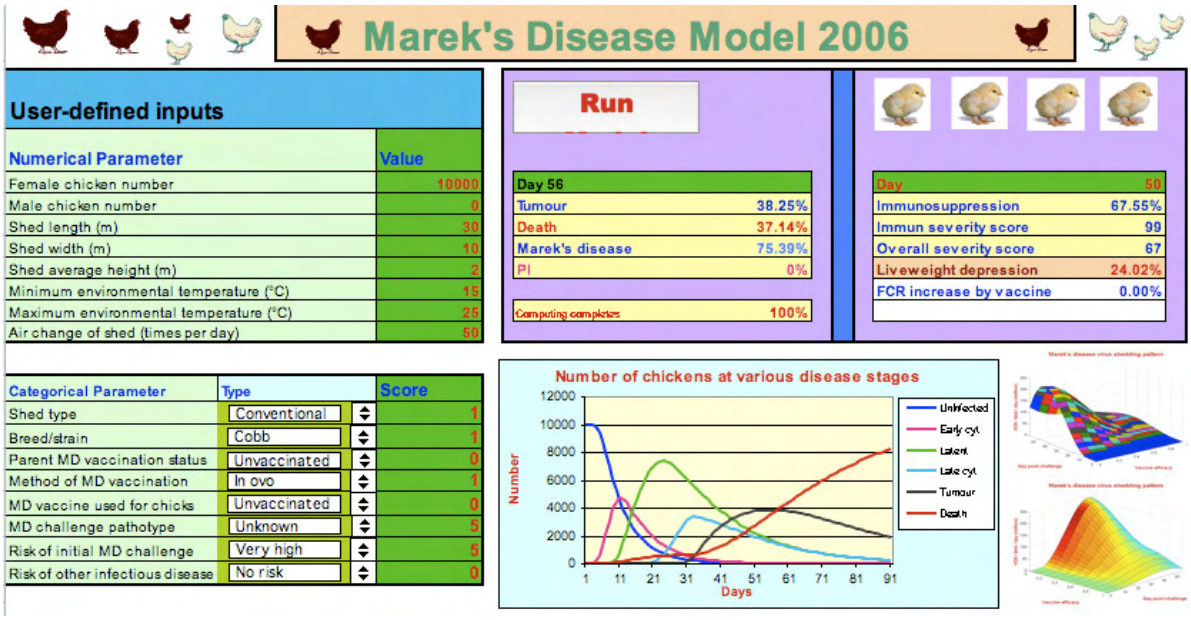

Figure 1. Opening Screen of the spreadsheet version of the MD epidemiological model.

Table 1. Input and output variables of the MD epidemiological model

Model input variables	Type	Model outputs (daily)
1. Number of chickens by sex	Enter numeric data	Cumulative chickens at different stages of pathogenesis (uninfected, early cytolytic, latent, late cytolytic, tumour, dead)
2. Shed dimensions to enable calculation of air volume	Enter numeric data	Daily new cases of chickens at different stages of pathogenesis
3. Min and Max shed temperatures	Enter numeric data	Rate of MDV shedding per chicken per day
4. Number of daily air changes in the shed	Enter numeric data	Cumulative tumour and death cases by vaccination status
5. Shed type	Pull down menu	Reduction in tumour and death cases induced by vaccination
6. Chicken strain	Pull down menu	Proportion of chickens immunosuppressed
7. Parent MD vaccination status	Pull down menu	Severity of immunosuppression
8. Method of MD vaccination	Pull down menu	Flock liveweight depression
9. MD vaccine used for broilers	Pull down menu	Flock FCR depression
10. Likely pathotype of MDV challenge	Pull down menu	
11. Likely risk of MDV challenge	Pull down menu	
12. Risk of other disease challenge	Pull down menu	

General Discussion of Results

A more detailed discussion of the results of each particular experiment or study can be found at the end of each experimental chapter. What follows here is a broad discussion of the results in light of the milestones set.

Milestone 6.1.1a: Isolation of field strains of MDV, adaptation to cell culture and elimination of contaminants

Despite the improvements in methodology introduced in 2004 the yield of high titre isolates for use in pathotyping experiments remained disappointing. While a significant proportion of isolates grew in cell culture and exhibited CPE, titres were generally low. Apart from one run of cell cultures in 2004 during which 6 isolates grew to a titre of 10^4 pfu/ml or higher, no other cell culture run during the experiment yielded titres of this magnitude. This is despite multiple efforts using fresh, highly infective spleen material from SPF chickens at UNE, and in many cases the very same MDV isolates that had grown to high titre in 2004. In the USA titres of 10^6 pfu/ml are commonly achieved (Schat 2004 pers comm.). This issue led to significant disruption of the pathotyping program at UNE, although it also resulted in some very interesting and novel work being carried out on alternative routes of infection, alternative infective materials, and alternative methods of titration of infectivity of infective materials.

While infective materials with titres below 10^4 pfu/ml are clearly infective as shown in Chapter 7, their use for formal pathotyping experiments is limited. For example, the standard USDA ADOL pathotyping protocol requires 17 birds in each of 3 vaccination treatments, replicated twice, to be challenged with 500pfu of MDV. This is a requirement of 102 birds x 500pfu = 51,000 pfu of infective material (with no allowance for wastage). At an infective titre of 100pfu/ml, day old chicks weighing 40g would need to be challenged with 5ml of material, and a total of 510ml of material would be required. To test the same batch of an isolate in several experiments (eg broiler, layer, SPF) as proposed in this project, would thus require between 1 and 2 litres of infective material. A typical cell culture run to passage 6 produces 20-30ml of material (ie the volume of a single batch), so clearly for cell culture material to be useful for formal pathotyping experiments, titres of 10^4 and greater are required. The isolates achieving this level of growth in culture during the project were MPF57, Woodlands 1, 02LAR, FT158, MPF132/5 and 04CRE, short of the 12 new isolates originally aimed for, and a low return on the 755 total submissions or 453 submissions positive for MDV1 on qPCR.

The reasons for the failure of Australian MDV isolates to consistently grow to adequate titre in this project are unknown, but they do not include the quality of the original infective material. Fresh infective spleen material from SPF or other chickens deliberately infected with MDV is the recommended starting material for MDV isolation, was the starting material for most of the successful high-titre isolates in 2004, and was a common material used in the period 2004-2006. Rather, the lack of growth appears to be a property of the culture system used, or our Australian strains of MDV. The fact that isolates which grew to high titre in one cell culture run often failed to grow similarly after back-passage in chickens and re-isolation in cell culture, or after a culture was seeded with the same original seed material suggests that failure to grow to significant titre is not a characteristic of the viral strain. Thus most evidence suggests that it is some feature of the cell culture system being used which is resulting in failure of reliable growth to high titre.

The immunoperoxidase staining with H19 appears to provide satisfactory confirmation that the MDV1 producing the CPE in cell cultures is a pathogenic MDV1 rather than the vaccinal strain CVI988. Material showing CPE at RMIT almost invariably induced clinical MD in chickens at UNE. Growth in cell culture followed by PCR for CAV to screen for CAV infection also appeared to provide good protection against cultures infected with CAV. However there was one clear exception to this. In Chapter 8 (Expt MD06-C-PT4) it was clear that the material for isolate 04KAL was infected with CAV, despite these precautions.

Milestone 6.1.1b: Preliminary characterisation of MD isolates *in vivo*

This original aim of this work was to confirm the infectivity and freedom from contamination of infective material from RMIT to be used in pathotyping experiments. In the end it also served a means of screening significant amounts of infective material for MDV infectivity, and for growing virus in SPF chickens prior to sending spleens back to RMIT for re-isolation on cell culture.

This milestone was more than met with major screening experiments (Chapters 2 and 5) screening 7 and 11 MDV isolates respectively, more than double the 8 targeted isolates. In Chapter 2, infection of SPF chickens via the respiratory route with infective dust from the field successfully induced MDV for 4 of 7 dusts without transmission of any other avian pathogens (0/7) as determined by serology for 18 avian pathogens. This single experiment yielded most of the isolates which eventually grew to high titre at RMIT (MPF57, 02LAR, 04CRE, MPF132/5). In experiment 5 all 11 sets of infective material from RMIT induced MDV. However serology for diseases other than MDV was not conducted as we were unsure of the extent to which the different isolates would grow subsequently and did not want to waste resources on isolates which might not grow to reasonable titre subsequently. This proved to be a sound decision as none of the 11 isolates grew to high titre following the experiment. However 5 were used in Chapter 8 (MD06-C-PT4) and of these one isolate (04KAL) was found to be contaminated with CAV despite growth on CK cell culture which does not support CAV, and a negative CAV PCR test on the cell culture material used for infection.

Although the infective dose was not controlled in these experiments they provided valuable information about the infectivity of various materials and the pathogenicity of the MDV involved, allowing confident selection of isolates for later use in pathotyping experiments. An additional large screening experiment (MD05-R-PT3) was carried out on RIRDC project UNE-83J. In that experiment infective material from 19 isolates was used, with 14 inducing tumours.

This work has also shown than MDV can be successfully amplified in chickens from a variety of challenge materials including dust/dander (intra-tracheal or simple exposure), stored whole blood, splenocytes and infective cell culture material.

Milestone 6.1.1c: Characterisation of recent MDV isolates in commercial strains of broiler and layer chickens

The intent of this work was to test 6 new isolates of MDV in 2 strains of commercial broiler chickens and 2 strains of commercial layer chickens. Unlike the pathotyping work done in SPF chickens (RIRDC Project UNE 83-J) these experiments would include bivalent vaccine (HVT/SB1) in addition to HVT vaccine, as per the USDA AOL gold standard.

In the end the availability of challenge virus meant that complete experiments, each testing 3 MDV isolates, were achieved for Cobb broilers (Chapter 3) and IsaBrown layers (Chapter 4). Following this there was an intense period of virus screening experiments at UNE (Chapter 5, RIRDC Expt MD05-R-PT3) in an attempt to produce fresh highly infective spleen material to grow virus at RMIT. As it became clear that this might not occur, more experiments were conducted at UNE to determine whether the infectivity of some of the known infective material could be titrated in chickens. This would enabling the next round of pathotyping experiments to use dose rates of MDV infective material determined, not by CPE in cell culture, but multiples of bird infective dose 50 (BID_{50}) infectivity in chickens. This work (Chapters 6 and 7) demonstrated the dose responsiveness of infectivity, and showed that BID_{50} could be calculated readily from short, inexpensive experiments using MDV load in spleen at 10-12 days of age (determined by qPCR) as the measure of infection. This work enabled a final pathotyping experiment to be carried out (Chapter 8) in which the pathogenicity of several isolates was compared in both Ross and Cobb chickens, using an infective dose rate of 6 x $BID_{50.}$ Time and resources did not permit the final layer experiment to be conducted, but on reflection, the value of experiments 6-8 is probably greater than that of the single missed layer experiment, because it demonstrated a clear alternative method for titration of viral infectivity based upon use of SPF chickens rather than cell culture and determination of MDV by qPCR as the end point, rather than plaque counts or $TCID_{50}$. The complete dependence of the pathotyping work on the availability of challenge virus was made clear in the project milestones.

Many of the key findings of the pathotyping work are summarised by (Walkden-Brown *et al.* 2007) and include:

- Bivalent vaccine provided significantly improved protection against MD in IsaBrown layer, but not Cobb broiler chickens (Chapters 3 and 4).
- In commercial chickens containing maternal antibody, Australian strains of MDV do not induce early paralysis and mortality syndromes, in marked contrast to the situation in SPF chickens in which they do (Chapters 3, 4 and 5, and RIRDC Expt MD05-R-PT3). In SPF chickens (eg Chapter 5) the syndrome reported by (Walkden-Brown *et al.* 2006) was again observed but it was absent in the other experiments with chickens having maternal antibody. The syndrome is an early paralysis/mortality syndrome between days 9 and 20 after MDV challenge, usually with a peak in the first half of this period. 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. 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 has been observed only in the most pathogenic strains, and has a significantly higher prevalence in males than females.
- On an international scale, our viruses rank in the v to vv category of the USDA AOL system (Witter 1997; Witter *et al.* 2005) with no evidence of vv+ pathotypes. HVT continues to provide significant protection in the face of challenge with 500pfu, with bivalent vaccine offering improved control in some chicken genotypes. Thus, on the evolution of virulence diagram presented by (Witter 1998) Australia appears to be at an earlier stage than the USA. This is consistent with the idea that blanket vaccination with an imperfect vaccine is a driver of increased virulence (Gandon *et al.* 2001) as broiler chickens in Australia have only had widespread vaccination against MD in the last decade, whereas in the USA vaccination has been widespread since the 1970s. With the limited number of Australian isolates formally pathotyped, there was no trend towards increased virulence over time. Indeed MPF23, isolated in the mid 1980's and putatively classified as vv by (McKimm-Breschkin *et al.* 1990) exhibited the hallmarks of one of the more virulent MDVs in one of our experiments (Chapter 5).
- Chicken genotype and maternal antibody status has a marked effect on the apparent virulence of MDVs and can change the virulence rank of individual virues and their rankings against each other markedly. The most extreme example was MPF57. The protective index provided by HVT against MPF57 challenge from the same single batch was 100%, 84.6% and 34.9% in SPF (RIRDC experiment MD05-R-PT2), Cobb broiler (Chapter 3) and IsaBrown layer chickens (Chapter 4) respectively.
- The two broiler strains Cobb and Ross, differ significantly in susceptibility to MD with the Cobb strain being more susceptible (Chapter 8).
- Overall there was a poor relationship between the incidence and severity of MD induced in unvaccinated chickens (virulence), and that induced in vaccinated chickens with the same virus (vaccine resistance). This suggests that the two may be separate traits. Current pathotyping methods are based on vaccinal protection and are therefore measures of vaccine resistance rather than true virulence.
- There was a very strong overall relationship between MDV viral load in spleen at a variety of ages (from d7 onwards) and the percentage of chickens with gross MD tumours. This suggests that MDV replication rate in lymphoid tissue is a good marker of virulence.

- Similarly there was a good association between MDV load in dander shed from challenged birds and the incidence of MD tumours in IsaBrown layers (Chapter 4). However the effect was not as clear in Cobb broilers (Chapter 5).

Milestone 6.1.2a: Validation of novel measurements to assist in pathotyping MDV strains

This was a particularly gratifying and rewarding aspect of the project. The work on this project and the other related projects has shown repeatedly that the MDV load in spleen at all ages, but particularly from 1-4 weeks of age is a very strong predictor of the subsequent incidence of MDV in a group of chickens (eg. Chapters 3, 4, 8). Because this trait can be measured very early (1-2 weeks) it offers the potential to screen MDV isolates for pathogenicity in chickens even prior to chickens becoming infective, removing the requirement for isolators. MDV load in spleen was a good predictor of MD incidence in SPF chickens (Walkden-Brown *et al.* 2006) as well as the commercial chickens in the current experiment indicating that the presence of maternal antibody does not reduce its efficacy. The close association between MDV load in spleen and MDV incidence in populations of chickens is consistent with our observation within individual chickens that MDV load in circulating lymphocytes at days 14-21 is a good predictor of subsequent MD status (Islam *et al.* 2006).

For groups of chickens, measuring the amount of MDV in dander or dust is an attractive non-invasive option. In the project experiments these proved a less sensitive predictor of subsequent MD status than MDV load in spleen both in Cobb broiler (Chapter 3) and IsaBrown layers (Chapter 4) although they had considerable predictive power in the latter. Measurement of MDV load in dust over time in these experiments revealed some very interesting information including:

- Even effective vaccination has a comparatively minor effect on the shedding rate of MDV1 from chickens halving it in Cobb broiler chickens (Chapter 5), and reducing it by up to 10 fold in IsaBrown layers (Chapter 4). Thus vaccinated chickens infected with MDV1 can continue to shed very large amounts of MDV1 into the environment (10^6-$10^{7.5}$ VCN/mg dander after 21 days post challenge)
- Challenge with MDV1 greatly increases the rate of shedding of vaccinal HVT and MDV2 (Chapter 3).

The relative weights of immune organs such as the Bursa of Fabricius, the thymus and the spleen are other potential markers of MD infection. In mab-negative SPF chickens, the level of atrophy of the thymus and bursa, and enlargement of the spleen at early ages are good markers of subsequent MD status (Walkden-Brown *et al.* 2006), but their predictive value is lower in mab positive commercial chickens, with the mab providing significant protection against immunosuppression as well as the early paralysis/mortality syndrome. This is consistent with observations in other studies in commercial broilers (Islam *et al.* 2002). While the bursa and spleen are relatively easy organs to remove and weigh, removal of the thymus is time consuming and open to error. The project demonstrated that simple scoring of thymic and bursal atrophy on a scale 0-3 was almost as effective as dissection and weighing of the organs in predicting MD outcomes for groups of chickens.

In terms of alternative pathotyping methodology the project provides strong support for alternative methods, particularly in earlier screening experiments. The potential alternative methods, most of which have been tested and found effective during the project include:

a) Use of titrated infective material other than cell culture adapted MDV. MDV grows more effectively and rapidly in chickens than in cell culture and significant amounts of virus can be recovered from chickens in 2-4 weeks post inoculation, prior to the onset of clinical MD. Splenocytes can be harvested, and their infectivity titrated in SPF chickens with an endpoint of BID_{50} calculated 10 days post challenge by qPCR of spleen (Chapters 6 and 7). Some birds could be left to grow out to 28d and sera could be tested for the presence of contaminating pathogens. Use of chickens vaccinated against MDV would be best for this purpose. Advantages include:

- No artificial barrier to MDV growth. All MDVs can be grown to high titre in chickens, not the 1-2% currently obtained from cell culture. It is low risk and low cost relative to cell culture.
- Speed. From a submitted infective sample to a titrated batch of infective splenocytes could take as less than one month, compared with the minimum of 6 weeks currently taken to grow virus in cell culture out to 6 passages and longer if higher passage levels are used.
- More sensitive test of contaminants. A chick inoculation test with serology for the key avian pathogens is a very sensitive test of contaminants in the infective material. Rather than being a specific extra step, this is part of the bulking up of the virus so does not add significant extra cost.
- Wider range of starting materials can be used. Infective dander is the easiest and most stable form in which to keep MDV. It is also comparatively easy to collect from an infective situation and to test for MDV1 content. It does not require special transportation features and can be sent in the regular mail. It is comparatively easy to infect chickens with dust and we have shown that this route of infection tends not to transmit other avian pathogens. Cell cultures cannot be inoculated with infective dust.
- Potentially cheaper and may use fewer chickens. To titrate a single virus as in Chapter 7 requires on lot of 30 chickens kept to 10-12 days of age. These do not need to be SPF chickens, but can be male layers which would be discarded anyway. Six cell culture runs using CK cells requires the use of fresh 10-day old SPF chickens for each of the 6 passages (approximately weekly). Specialised cell culture facilities are not required, however isolator facilities would be necessary for production of infective material free of contaminants. They would not be required for the actual titration or the pathotyping experiments as both can be done prior to transmission of MDV between birds.

This should not be though of as a "low tech" return to pre-cell culture days. This methodology is only possibly because modern highly sensitive and accurate molecular methods enable early, rapid, precise and relatively inexpensive measurement of infective status of chickens prior to them becoming infective. Why should we continue to define the infectivity of MDVs in an artificial *in vitro* situation when we can do it *in vivo*? It should be noted here that not all quantitative real-time PCR tests are equivalent. Sensitivities can vary by a thousandfold or more depending on assay design and it is important that a sensitive, accurate and repeatable assay is used for the viral measurements.

b) Use of a short 7-14 day experiments to screen MDVs for pathogenicity, and vaccines for vaccinal efficacy. Several possible scenarios can be envisaged viz:

- Use of SPF chickens. For these, the key endpoint would be MDV load in spleen at day 7-14, but may include thymic and bursal atrophy and splenomegaly. The presence of early mortality/paralysis could also be an important measure. Reducing the length of the experiment to day 10 would mean it could potentially be done outside isolators as the chickens would not be infective. On ethical grounds a 10-day experiment would also be more desirable as expression of nearly all of the early mortality/paralysis syndrome would be avoided.
- Use of commercial chickens. For these, the key endpoint would be MDV load in spleen. Immune organ weights and atrophy scores are unlikely to add much and current isolates do not induce the early mortality/paralysis syndrome in these chicks.

Of course such experiments do not measure oncogenicity directly, and it is likely that they would need to be calibrated from time to time with full-length pathotyping experiments.

Milestone 6.2.1: Field epidemiology study

This study, the first large-scale epidemiological study on MDV in Australia, was successfully implemented (Chapter 10). The reader is referred to the comprehensive discussion of this study at the

end of Chapter 10. It has confirmed earlier observations that MDV is not ubiquitous on Australian broiler farms with only 50% of farms having dust samples positive for MDV. Presence of MDV was strongly seasonal with a summer-autumn dominance. Larger farms, and those within 2km of another farm were at increased risk as were tunnel ventilated sheds and those re-using litter. Provision of protective clothing was protective, as was use of wood shavings for litter. Surprisingly, vaccination with HVT did not influence the incidence of MDV, perhaps confirming the limited effect of vaccination on shedding levels of MDV1 in Chapter 3. The majority of shed contained chickens which were serologically positive for CAV although it was surprising that in the majority of them, less than 100% of chickens were positive.

Milestone 6.2.2a: Enabling research into MDV to provide inputs into a simulation model.

As discussed above a method for successfully titrating the infectivity of MDV-infected dust was developed in Chapter 6. This enables direct comparisons to be made of viral load in dust, and infectivity of that dust in terms of BID_{50}. The method is limited by the range of dust doses that can be used, and for highly infective dusts, dilution with non-infective dust may be required.

A long term experiment (Chapter 9) investigated the effect of storage temperature on the infectivity of MDV infective dust over a period of 2 years. Dust stored at all temperatures (-80˚C, -20˚C, 4˚C, 16˚C and 37˚C) remained infective over this period, as determined by MDV1 presence in spleen 7 days post infection intra-tracheally. Infectivity was reduced, but not eliminated at the higher temperatures, relative to the lower ones. This data confirms the longevity of MDV in the environment under a wide range of temperature conditions, and supports the notion of using infective dust as a storage medium for MDV.

Milestone 6.2.2b: Modelling of MDV epidemiology

As discussed in Chapter 11 this milestone was met with the production of a fully featured model of MDV infection, spread and consequences in a single shed of chickens. However industry validation of the model was not achieved, nor was it widely used as a teaching tool. The original aim of expanding the model to model the spread of MDV from shed to shed and from farm to farm was not achieved, with our understanding of the latter best captured by the statistical models in Chapter 10.

Our experience with this model has been that the greatest contribution is made to researchers, for whom the model is a means of determining what areas of knowledge are deficient in our understanding of the disease, and determining what research approaches will fill this deficit. However people outside of research appear to be highly sceptical about the outputs of mathematical models and are unlikely to accept their outputs unless there is extensive co-development and validation with end users.

The model and the work associated with it have provided important new insights into the epidemiology of MD. For example two commonly held ideas about MDV have been shown by our work to be demonstrably false – these are

a) MDV is a ubiquitous virus wherever chickens are kept intensively. We have shown in a number of detailed studies that we cannot detect MDV in some poultry producing areas under current management procedures.

b) Infection with MDV occurs early in life, soon after placement. In fact the rate of spread of MDV from chicken to chick is measurable, and takes considerable time. Typically it is not until 15-20 days after exposure to significant challenge that all chickens are infected.

Implications

The project has succeeded in demonstrating the use of quantitative real-time PCR as a tool to investigate and understand a key endemic poultry disease, Marek's disease. The work of this project could serve as a template for the use of such tools to improve understanding of the epidemiology and control of other endemic poultry diseases.

The major implications of the findings of this project are:

1. The current strains of MDV in Australia are in the v to vv pathotype category with little evidence of vv+ isolates. While HVT does not provide complete protection against these isolates in challenge studies with 500pfu of MDV, it mostly provides adequate control in the field. We appear to be behind the USA and Europe in the evolution of virulence in our MDVs. Limited reuse of litter and good biosecurity practices on Australian farms probably contribute to the adequate level of control provided at present. However, given the widespread use of vaccination of broilers against MDV since 1996, it can be anticipated that more virulent pathotypes of MDV will appear in the future.
2. A recently introduced bivalent vaccine (HVT/SB1) induced significantly improved protection against MD in IsaBrown layer, but not Cobb broiler chickens.
3. Chicken genotype significantly influences the outcome of pathotyping experiments, with clear challenge virus x chicken strain x vaccine interactions evident. Because of this pathotyping experiments should ideally be done therefore in the major industry genotypes. Ross broiler chickens are demonstrably more resistant to Marek's disease than Cobb birds.
4. Chicken maternal antibody status greatly influences the level of immunosuppression induced in chickens affected with MD and the expression of early neurological syndromes (days 9-15 post challenge). Maternal antibody offers strong protection against both. While this is clearly evident in the literature also, discussion and some publications on MD virulence and pathotype are confounded by claims in these areas which could easily be resolved by clearly stipulating the maternal antibody status of the birds being used and the consequences of this for the pathology observed.
5. Virulence (capacity to induce disease in the host) and vaccine resistance (capacity to overcome the protective effects of vaccination) appear to be separate traits and should be investigated as such. Current pathotyping protocols emphasise vaccine resistance.
6. Current procedures for isolation and growth of MDV in cell culture in Australia provide unacceptably low titres of virus. The reasons for this are not understood but need to be ascertained.
7. The project has researched alternative methods for the isolation, testing for contaminants, and titration of infectivity of MDV isolates using SPF chickens rather than cell culture. These methods have been shown to work and offer some significant advantages over the use of cell culture, including in the area of cost.
8. MDV load in spleen, and to a lesser extent, dander, measured in a sensitive, accurate qPCR assay has proved to be a very strong predictor of future MD incidence in groups of chickens from an early age. This offers considerable potential for alternative, cheaper and shorter experiments to rank MD isolates, or control methods, in terms of their efficacy.
9. Information generated by the field epidemiological survey will assist the industry to better target HVT vaccination, especially with regard to strategic use of the vaccine by season. This will enable marked cost savings in the reduced need to vaccinate al year round. Continued use of the dust qPCR will enable a longer term grasp of the epidemiological changes that may occur with a highly adaptive virus like MDV.
10. We now have evidence that MDV is not necessarily as ubiquitous as formerly believed.
11. Several of the identified risk factors for MDV1 exposure and establishment in as flock are not amenable to easy control by the management (season, proximity to other farms, existing farm size and shed construction) but some factors may allow manipulation to decrease risk. Provision of on-farm clothing and avoiding retention of used litter may significantly lower risk rates.

12. The project together with an earlier project has developed a sophisticated within shed model of MD epidemiology. This continues to be of utility to researchers and may form the basis of a more extended suite of models into the future.

Recommendations

The project has not directly produced products of commercial value. However it has greatly increased our knowledge of a key endemic disease and offers new methods of monitoring and controlling it.

The major recommendations arising from the works are as follows.

1. The reasons why most Australian isolates of MDV do not grow readily or reliably to high titre in cell culture should be determined in a project involving international collaboration with a laboratory that routinely grows isolates to high titre.
2. Consideration should be given to supporting the development of formal MDV challenge protocols using dose rates based on BID_{50} rather than pfu. Such protocols should allow the use of infective contaminant-free splenocytes in addition to infective contaminant-free cultured cells as the challenge material.
3. In addition to maintaining stocks of infective cells containing key isolates of MDV in liquid N2, consideration should be given to maintaining stocks of the same virus as infective dust stored at -20˚C at a separate institution. The dust material would be derived from chickens infected with the given isolate in isolators. This is a security measure to ensure the long-term availability of MDV stocks from different eras.
4. A broader range of current and past isolates of MDV (say 20) should be amplified and tested for contamination in SPF chickens, stored as infective splenocytes, titrated in chickens to determine BID_{50} and screened for pathogenicity based on a short pathotyping experiment of 10 days duration with MDV1 load in spleen as the end point. This will provide a set of stock infective materials for current MDV isolates, with preliminary ranking of virulence based on MDV load induced at day 14 of age. Ideally such isolates would be tested against international reference strains available from the USDA (Witter et al., 2005) or BACs of these isolates. This work would provide a more complete picture of Australia's standing with regard the virulence of its MDV strains – something we hoped to achieve in this project and the RIRDC project, but which was only partially achieved due to the non-availability of sufficient cell-culture adapted strains for testing.
5. Further work is necessary to understand the effects of wood based litter materials and higher growth rates on the detectability of MDV1 in shed dust. For the further commercialisation of the qPCR test for dust the effect of the litter material on dust and the ability to isolate the virus will need to be determined more fully. It will also be important to confirm the observation of more positive flocks and higher levels of MDV in dust from faster growing birds, as this could bias the meaningfulness of the qPCR on dust outcome in the field.
6. More investigation should be carried out to determine other underlying factors that lead to the observed poorer performance of day old vaccinated birds, within the operations using this method.

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Excellent technical support on this project was provided by Julie Cooke and Nadeene Clarke at RMIT, and Paul Reynolds and Sue Burgess at UNE. Paul prepared the isolator facility for all UNE isolator experiments and was associated with all facets of the experiments, including lab analysis. He also played a key role in coordinating the sending out of kits and receiving of samples for the epidemiological survey, the conducting of the qPCR assays and the collation of the data prior to it being sent to Dr Groves for analysis.

At UNE, Experiment 1 (Chapter 2) 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 Experiments 2 (Chapter 3) and 4 (Chapter 5). Dr Fakhrul Islam was a key collaborator participating in most experiments at the final post-mortems, and leading the work in Experiment 8

(Chapter 9) part of which also formed the B.Rur.Sci. Honours project work of Peta Blake, a CRC honours scholarship holder. Dr Zhanhai Gao was another key collaborator on the project, leading the MDV modeling work and developing the in-shed epidemiological model of MD.

At RMIT Julie Cooke was a key RMIT collaborator in the project and wrote Chapter 1 and parts of the General Materials and Methods. Brian Meehan was a valuable collaborator and also contributed to the RMIT sections of the report. Jianming Tan was involved in his own project at RMIT during this project, but made valuable contributions of time and ideas to this project as well. RIRDC project UNE-83J was also 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.

Appendices

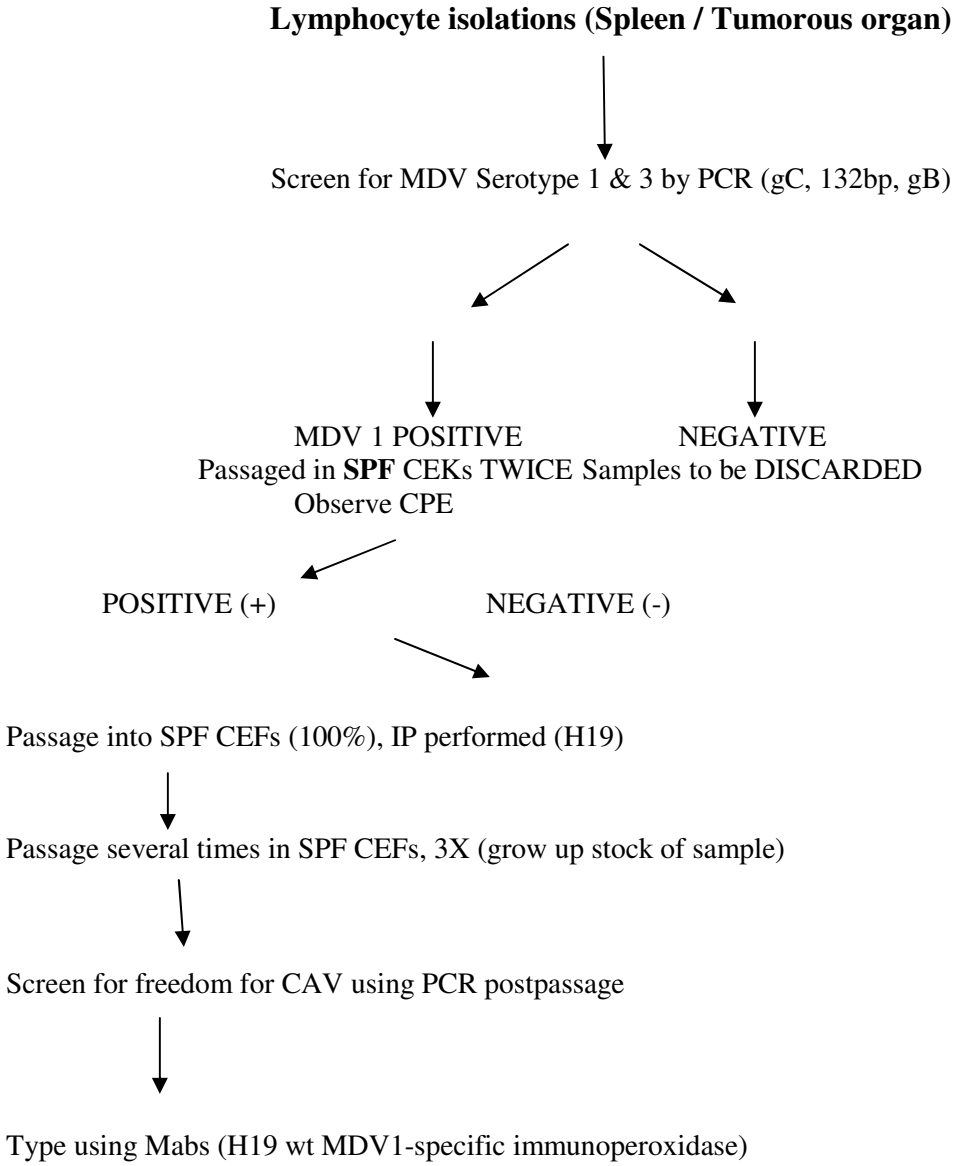
Appendix 1. Flow chart for characterisation of MDV field isolates (MSCM June 2003)

Appendix 2. Information regarding the collection of samples and flock history

Appendix 3. Flow chart for isolation of MDV from field samples (from 2004)

Appendix 4. MDV modelling papers by Dr Zhanai Gao.

APPENDIX 1: FLOW CHART FOR CHARACTERISATION OF MDV FIELD ISOLATES (MSCM June 2003)



APPENDIX 2: INFORMATION REGARDING THE COLLECTION OF SAMPLES AND FLOCK HISTORY

TO BE COMPLETED AND SENT WITH THE SAMPLES FOR TESTING

Spleen samples must be collected in an aseptic manner placed separately in cold sterile PBS (saline) solution and labelled with the bird number and the farm/shed name. Samples to be transported on a frozen ice brick. Samples must arrive at the laboratory for testing within 24 hours of the sample collection.

A quick email to advise us of the consignment note number and time the samples left is of great use.

Samples to be sent to:

RMIT UNIVERSITY
BUNDOORA WEST CAMPUS
BUILDING 223, LEVEL 1, RECEPTION
PLENTY RD.
BUNDOORA, VIC. 3083

RMIT Contact Details:

JULIE COOKE
PHONE: (03) 9925 7147
FAX: (03) 9925 7110
EMAIL: julie.cooke@rmit.edu.au

Senders contact details:

Name / Company:

Email address:

Phone number:

FLOCK HISTORY:

INFORMATION	
Vaccine status of flock: Vaccine type(s) Age at vaccination	
Site of flock	Company: State: Town: Farm: Shed:
Age of flock at time of blood collection	
Clinical signs (morbidity & mortality rates)	
Reason for testing (please ✓)	Isolation of virulent MDV 1 strains

OTHER COMMENTS:

(Shed Hygiene etc)

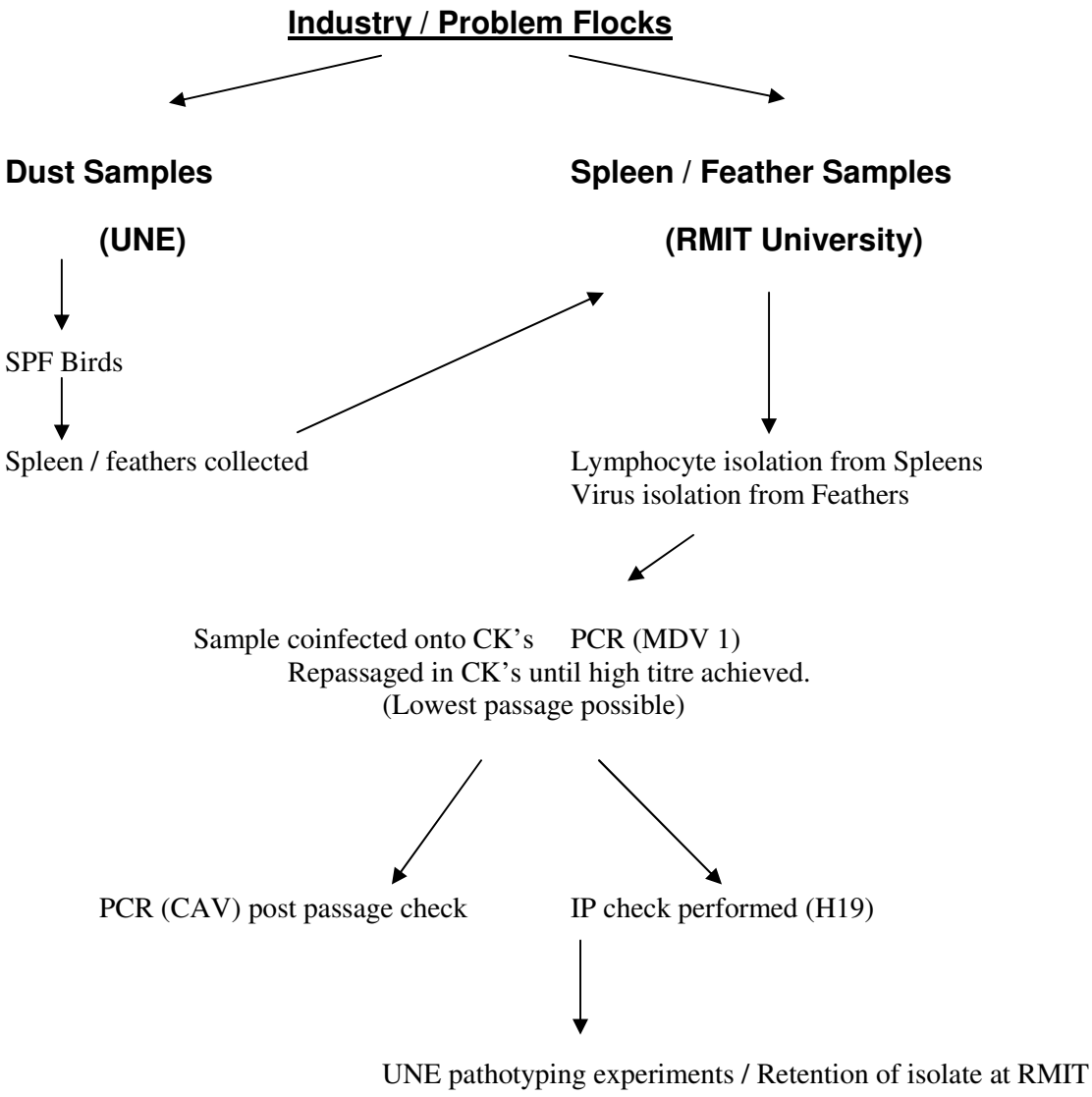
_____
_____
_____
_____

This information remains strictly confidential

THANKYOU FOR YOUR TIME AND INFORMATION

Julie Cooke
(Coordinator of Veterinary Virology – RMIT University, Melbourne)

APPENDIX 3: FLOW CHART FOR ISOLATION OF MDV FROM FIELD SAMPLES (From 2004)



Appendix 4. MDV modelling papers by Dr Zhanai Gao.

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Glossary

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)

References

References are provided at the end of each relevant section.

Plain English Compendium Summary

Project Title:	Improved diagnostics and disease surveillance through the application of molecular biotechnology
Project No.:	03-17
Researcher:	Associate Professor Stephen W Walkden-Brown
Organisation:	University of New England
Phone:	02-6773 5152
Fax:	02-6773 3922
Email:	swalkden@une.edu.au
Objectives	• Isolation of field strains of MDV, adaptation to cell culture and elimination of contaminants • Characterisation of recent MDV isolates in commercial strains of broiler and layer chickens (pathotyping/determination of virulence) • Validation of novel measurements to assist in pathotyping MDV strains (alternatives to full pathotyping experiments) • Epidemiological studies and modelling for Marek's disease and CAV infection.
Background	Developments in molecular technology now enable us to accurately measure the number of copies of the different serotypes of MDV in a wide range of samples including feather dander shed by chickens. This project uses Marek's disease as a case study for the application of such technology, specifically in the areas of pathotyping of MDVs in the light of marked increases in virulence and vaccine failure in other countries, and in the area of field monitoring and epidemiological studies based on analysis of MDV in poultry dust,
Research	At RMIT methods of isolation and growth of MDV in cell culture were optimised and 755 submitted infective samples processed. At UNE 8 major experiments were conducted to pathotype MDV strains in commercial chickens, to amplify MDV by back passage in chickens and to develop and test ways of growing and measuring MDV infectivity that do not require cell culture. Extensive measurements of viral load and other variables were made to test alternatives to full pathotyping. One experiment investigated the long term survival of MDV in dust at different temperatures. A major epidemiological study designed and analysed by Zootechny Pty Ltd involving 80 broiler farms was conducted. It involved intensive sampling for MDV1 in dust and spleen samples and serology for CAV coupled with an intensive questionnaire. At UNE ongoing improvements to an epidemiological model of MD infection in broilers was made in the light of industry feedback and new developments in understanding.
Outcomes	Despite considerable optimisation, isolation and growth of MDV in cell culture proved difficult with only 25% of the 755 submitted samples producing growth in cell culture and only 6 samples resulting in sufficient growth to produce virus for pathotyping experiments. In response to this, methods for the isolation, testing for contaminants, bulking up of virus, and measurement of infective dose that do not require adaptation of MDV to cell culture were developed and found to be effective. These methods use chickens rather than cultured cells, and

	are made possible by the early detection of viral load using real-time qPCR, prior to the onset of clinical disease. Five isolates of MDV were thoroughly pathotyped and shown not to include the most virulent pathotypes currently seen in the USA and elsewhere. Significant issues with current pathotyping protocols were identified, mainly relating to the effects of host genotype and maternal antibody status of the chicken strains involved, and differences between virulence (the ability to induce disease) and vaccine resistance (the ability to resist the effects of vaccination) which have not been recognised in the published literature. MDV load in spleen at an early age 1-3 weeks, proved a very accurate predictor of future MD incidence in groups of chickens in several experiments. This should enable very short screening experiments to be done to test for MDV virulence, or the efficacy of MD control methods such as vaccination. The epidemiological study showed that MDV is far from ubiquitous with no MDV detected on 50% of farms. Incidence was markedly seasonal and a range of risk factors and protective factors was identified. A within shed model of MDV was finalised and programmed in Excel to make it more accessible. The model is a valuable tool for researchers and forms a basis from which more comprehensive models could be based.
Implications	The reasons why Australian isolates of MDV are not readily growing to useful titres in cell culture need to be established. The project has provided alternatives to working with MDV that avoid the artificial bottleneck of adaptation to cell culture. These methods offer significant advantages on their own, or in combination with limited cell culture work. Adoption and standardisation of these methods is recommended. Due to chicken strain effects, pathotyping experiments are probably best done in the commercial strains of the day. Measurement of viral load in the first few weeks after challenge is a key measure, and may be the sole endpoint for large scale screening experiments. The terms virulence and vaccine resistance, each measured separately are probably more useful terms than the current "pathotype" definitions. The advances in methodology above would enable a rapid screening of say 20 current MDV isolates for virulence and vaccine resistance and it is recommended that this be done, preferably using one or more international strains as a benchmark. The findings of the epidemiological survey should be made widely available to industry, to assist with management of MDV.
Publications	Dr Walkden-Brown will be on Sabbatical in the UK from 21/9/07-20/9/08 and is expected to write a number of full journal papers on the work arising from this project. Current publications are: Blake PA (2004) Measurement of storage temperature and duration effects on the *in vivo* infectivity of Marek's Disease virus in feather dust. B. Rur. Sci. (Hons), University of New England.

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