



Field application of a Rispens-specific qPCR test

Final Project Report

A report for the Australian Egg Corporation Limited

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Foreword

This project was conducted to fully test and validate the molecular diagnostic tests used to differentiate between wild type and Rispens CVI988 serotype 1 Marek's disease virus (MDV) developed under AECL Project 08/17 University of New England (UNE) for field application by industry. These tests differentiate clearly between Australian wild type MDV1 and Rispens CVI988 but will not differentiate some overseas strains of wild-type MDV1.

The project aimed to use these tests to investigate vaccination responses to Rispens CVI988 experimentally and in the field to develop effective field measurements of vaccine take. It also aimed to investigate the spread of Rispens between chickens and the effect of wild-type MDV challenge at different times post vaccination on the level of protection provided by Rispens vaccination.

We currently have adequate control of Marek's disease in Australia, largely based on the use of the Rispens vaccine in breeders and layers, and high titre cell-associated Herpesvirus of Turkeys (HVT) in broilers. However MDV has shown a marked propensity to change in virulence to overcome the effects of vaccination and we should not be complacent about current control. Use of routine vaccine take testing, coupled with other routine tests such as wild-type MDV1 levels in dust will enable industry to closely monitor vaccine performance and quickly detect breakdowns in protection. It will also help maintain ongoing MDV capability.

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

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Program Manager – R&D
Australian Egg Corporation Limited

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The Australian Egg Corporation Limited provided the funds which supported this project.

About the Authors

Professor Stephen Walkden-Brown [BVSc (Qld) 1981, PhD (Qld) 1992] is a Professor in Animal Health and Production in the Animal Science Group of the School of Environmental and Rural Sciences at the University of New England (UNE). He is an active researcher in several areas including, in poultry, the epidemiology and control of Marek's disease, and inactivation of poultry pathogens in litter. Prof. Walkden-Brown has previously had poultry projects funded by RIRDC/AECL (UNE 83-J), the Australian Poultry CRC (03-17, 06-15, 09-34 and 2.2.3) and the Australian Research Council. In recognition of his work on Marek's disease Prof. Walkden-Brown was invited to spend 10 months on sabbatical working at the World Organisation for Animal Health (OIE) international reference laboratory for Marek's disease, the BBSRC Animal Health Institute, Compton, UK with Dr Venogopal Nair's research group. He was awarded an Underwood fellowship to do this between November 2007 and September 2008. Prof Walkden-Brown has over 250 research publications of which more than 80 are original full scientific papers. He has supervised or is supervising 26 PhD, 6 Masters and 19 honours students.

Dr. Katrin Renz [BSc and MSc (Hohenheim, Gemany) PhD, UNE 2008) studied for her BSc and MSc in Animal Science majoring in microbiology at Hohenheim University in Germany. She then completed a PhD degree at UNE in October 2008 with the thesis title 'In vivo and in vitro characterisation of Australian MDVs' under the supervision of Drs Walkden-Brown and Cheetham. Dr. Renz worked as a part time research fellow on the project funds and was responsible for the day to day running of the project in consultation with the principal investigator. The aim of this project was to take the molecular diagnostic tests to differentiate between wild type and Rispens CVI988 serotype 1 Marek's disease virus developed under AECL Project 08/17 UNE and fully test and validate them for field application by industry. Dr. Renz previously was also successful in obtaining two small grants from the Poultry CRC in 2009, project 09-04 on "Screening for bacteriophages of selected poultry pathogens" and 09-26 on "Isolation and titration of selected avian pathogens in cell culture". In 2013, Dr. Renz has successfully obtained a major research project funded by RIRDC Poultry Meat, entitled "Loop-mediated isothermal amplification (LAMP) tests to detect poultry pathogens" as a principal researcher.

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Abbreviations

ab	Antibody
AE	Avian encephalomyelitis
Ag	Antigen
AEC	Animal Ethics Committee
AECL	Australian Egg Corporation Ltd.
AOV	Analysis of variance
BBSRC	Biotechnology and Biological Sciences Research Council
Bursa	Bursa of Fabricius
CEF	Chicken embryo fibroblasts
cm	Centimeter
CO ₂	Carbon dioxide
CPE	Cytopathic effect (in cell culture)
C _t	Critical threshold value
CV	Coefficient of variation
°C	Degree Celsius
DNA	Deoxyribonucleic acid
dNTP	generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP
DPC/dpc	Days post-challenge
DPI/dpi	Days post-infection
DPV/dpv	Days post-vaccination
DXB	DX Binding
DXF	DX Final Wash
DXL	DX Liquid Digest
DXW	DX Wash
EDS	Egg Drop Syndrome
ELISA	Enzyme Linked Immuno Sorbent Assay
FP	Fowl Pox
g	Gram/ gravity
h/hr	Hour
HEPA	High Efficiency Particulate air
HVT	Herpesvirus of Turkeys. Also known as Meleagrid herpesvirus 1 (MeHV-1) and Marek's disease virus serotype 3 (MDV3).
IA/ia	Intra-abdominal
IB	Infectious Brochitis
IgG	Anti-Chicken IgY
IP/ip	Intra-peritoneal
kPa	Kilo Pascal
kVA	Kilo Volt-Ampere
Ig	Immunoglobulin
L	Litre
LSM	Least square means
Ltd	Limited
m	Meter
M	Molar
mab	maternal antibody
MD	Marek's disease
MDV	Marek's disease virus
MDV1	Marek's disease virus serotype 1. Also known as Gallid herpesvirus 2 (GaHV-2).
MDV2	Marek's disease virus serotype 2. Also known as Gallid herpesvirus type 3 (GaHV-3).

Mg	Milligram
min	Minute
mm	Millimeter
mM	Millimolar
ml	Millilitre
ng	Nanogram
ND	Newcastle disease
nm	Nanometer
NSW	New South Wales
OIE	Office International des Epizooties. In May 2003 the Office became the World Organisation for Animal Health but kept its historical acronym OIE. The OIE is the intergovernmental organisation responsible for improving animal health worldwide.
OPD	o-phenylenediamine
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween 20
pc	Post challenge
PC2	Physical contamination level 2 laboratory
PCR	Polymerase chain reaction (conventional, end point form)
Pfu/PFU	Plaque forming units
PI	Protective index. (%MD in Sham-vaccinated chickens – %MD in HVT-vaccinated chickens) ÷ (%MD in Sham-vaccinated chickens) x 100
Pty	Unlimited proprietary company
pv	Post vaccination
qPCR	Quantitative real-time polymerase chain reaction (PCR)
QC	Quality control
Qld	Queensland
REML	Restricted maximum likelihood
RIRDC	Rural Industries Research and Development Corporation
rpm	Revolutions per minute
s	Second
sc	subcutaneous
SEM	Standard error of the mean
SPF	Specific pathogen free
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
UK	United Kingdom
UNE	The University of New England
USA	The United States of America
UV	Ultraviolet
VCI	Vaccination-challenge interval
VCN	Viral copy number
Vic	Victoria
vvMDV	Very virulent MDV. A pathotype of MDV1, 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.
VR	Virulence rank (100 – PI)
wk	Week

Executive Summary

The two general aims of the project were to fully test and validate the molecular diagnostic tests that are used to differentiate between wild type (generally pathogenic) serotype 1 Marek's disease virus (MDV) and Rispens CVI988 serotype 1 vaccine (developed under AECL Project 08/17 UNE) for field application by industry. Secondly, the project aimed to use the test to investigate the kinetics of viral replication and persistence within the host, and shedding pattern, of the Rispens CVI988 virus (Rispens) alone and in combination with wild type virus. The project commenced in August 2011 and finished on 30th September 2013.

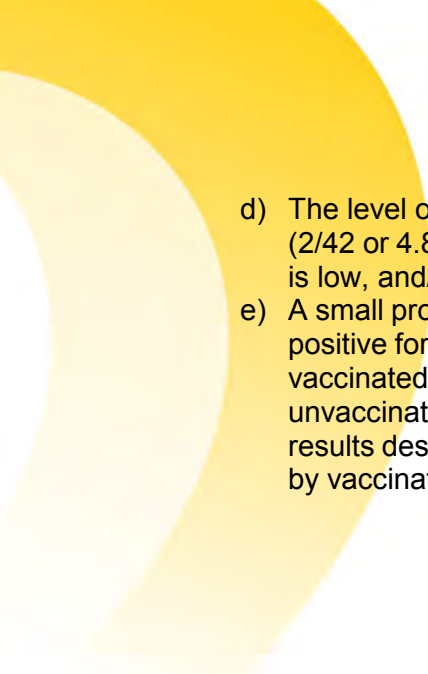
An initial experiment at University of New England (UNE) examined replication, shedding and transmission of Rispens CVI988 in chickens vaccinated with the three commercially available Rispens/CVI988 vaccines available in Australia (Milestone 2).

A second complex isolator experiment examined the protection provided by Rispens vaccination of ISA Brown chickens against challenge with very virulent MDV (vvMDV) at five different vaccination-challenge intervals (VCI) including challenge before vaccination (VCI of -10, -5, 0, 5 and 10 days respectively). This study also investigated the comparative replication rates and shedding of Rispens and vvMDV in these groups and others where birds were administered only one of the viruses (Milestone 3).

To evaluate the field application of the quantitative real-time polymerase chain reaction (qPCR) tests field experiments took place on three commercial layer farms in the Tamworth and Port Macquarie area (Milestones 1 and 4). The experiments monitored the vaccination responses and presence of wild-type MDV over the lifetime of commercial layers. A collective dust sample was taken in each shed. In addition feather and serum samples were collected at each visit from fifteen randomly selected chickens per age group. The samples were subject to either the qPCR test or an Enzyme Linked Immuno Sorbent Assay (ELISA) in order to develop practical indicators of vaccination success, compare sensitivity of the qPCR test with serology and test for pathogenic MDV1 infection in vaccinated flocks. It is acknowledged that the limited geographic range of the field testing may not encompass the full spectrum of wild-type MDV strains or challenge levels.

The project objectives were achieved successfully and some major findings of this work were:

- a) That currently commercially available Rispens CVI988 vaccine viruses in Australia are shed in significant amounts into the environment from vaccinated chickens and transmit successfully to unvaccinated in-contact chickens;
- b) The Rispens vaccine provided no significant protection when challenge preceded vaccination, with protective indices (PI) of -4% and 21% for VCI of -5 and -10 respectively. On the other hand it provided PI of 60%, 85% and 100% at VCI of 0, 5 and 10 respectively. The study also revealed that vvMDV load in peripheral blood lymphocytes (PBL) or feather tips at 14 and 21 days post infection (as determined by qPCR) were accurate early predictors of MD incidence at 56 days post challenge. The load of Rispens virus in PBL or feathers at the same times post vaccination did not offer similar predictive power;
- c) The Rispens CVI988 vaccine is consistently found in feather tips and dust samples from vaccinated commercial chickens up to 91 weeks of age (as determined by qPCR). MDV1 antibodies are present in serum samples (as determined by ELISA) in chickens up to 91 weeks of age. The antibody titre in young birds up to 4 weeks of age fluctuates around levels of 2.5-3 log₁₀ MDV antibody titre. In birds from 18-20 weeks of age up to 91 weeks, the antibody titres remain relatively stable at a higher level of 3.5-4 log₁₀ MDV antibody titre. These data indicate that infection with the Rispens vaccine virus is persistent, and that sampling to detect virus at any time from 3 weeks post vaccination is a good indicator of vaccine take;

- 
- d) The level of wild-type MDV found in dust samples from vaccinated chickens was low (2/42 or 4.8%), indicating that co-infection with wild type MDV in vaccinated chickens is low, and/or mostly below the detection threshold; and
 - e) A small proportion of MDV-positive dust samples from commercial broiler farms were positive for the Rispens virus (7/100 or 7%). Because chicks on these farms were not vaccinated with Rispens these results indicate possible “escape” of Rispens to unvaccinated farms. This would be consistent with the shedding and transmission results described at a) above which show that the Rispens virus is shed at high levels by vaccinated birds and transmits freely to unvaccinated birds.

Overall Conclusions

Based on the results of the project experiments we can conclude the following:

- a) Currently available Rispens CVI988 vaccine viruses are shed consistently in large amounts in feather dander between days 7 and 56 post infection;
- b) These vaccinal viruses transmit effectively to in-contact chickens;
- c) Australian MDV isolate 02LAR (vvMDV pathotype) has a higher replication rate than Rispens in peripheral blood lymphocytes (PBL), feather tips and dust, and that this is true in single and co-infected chickens. The differences are greatest in PBL and feathers, and least in dust;
- d) In co-infections 02LAR and Rispens have broadly similar actions on each other with prior infection with one virus leading to suppression of replication of the subsequent virus. However the effect of Rispens on vvMDV load was greater than the reciprocal effect and again the effect was greatest in PBL and feathers, and least in dust;
- e) Patterns of viral load in feather cells more closely resembled those in PBL than those in dust suggesting that they are measuring virus of at least partially different origin;
- f) Early measures of MDV in PBL, feathers and to a lesser extent dust, are good predictors of subsequent MD status in challenge experiments, and thus good predictors of protection. Measures of Rispens virus are less reliable predictors;
- g) Chickens vaccinated with Rispens remain infected for life and maintain high antibody titres;
- h) Measurement of Rispens in PBL, feathers or dust using qPCR are likely to be good indicators of vaccination success. Measurements in PBL from 1 week, feather tips from 2 weeks and dust samples from 3 weeks post vaccination would be suitable. Measurement in PBL is most accurate and most closely associated with subsequent antibody titre but is the most difficult and expensive measurements. Measurement in dust is the easiest and most economical because of the non-invasive collection method, and the small number of samples required, relative to samples from individual chickens;
- i) The level of co-infection with wild type MDV serotype 1 in vaccinated chickens in the field is low, and/or mostly below the detection threshold; and
- j) There is some evidence of “escape” of Rispens vaccine virus to unvaccinated farms, consistent with its ability to shed and transmit between chickens very effectively. This suggests that it may form part of a pool of circulating MD viruses with airborne or fomite-mediated transmission between farms.

This study expands our understanding of the interaction between pathogenic and vaccinal viruses following vaccination with imperfect vaccines and together with the companion paper (below) has implications for selection of appropriate samples to test for vaccination success.

Islam, T., Stephen, W., Walkden-Brown, S.W., Katrin, G., Renz, K.G., Fakhru Islam, A.F.M., and Ralapanawe, S. (2013): Replication kinetics and shedding of very virulent Marek's Disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. Veterinary Microbiology, (submitted).

1. General material and methods

1.1 Source of Marek's disease viruses

Rispens CVI988 viruses

The Rispens/CVI988 vaccines were sourced from the respective manufacturer and detail of each vaccine is given in **Table 1-1: Details of Rispens/CVI988 vaccines used**. Exact passage levels in cell culture for each vaccine were not made available by the manufacturers.

Table 1-1: Details of Rispens/CVI988 vaccines used

Vaccine company	Commerical name	Batch no.	Expiry	Doses	Manufacturer's specified dose*
Intervet Australia Pty Ltd	Nobilis® Rismavac	3957G-026	Feb 2013	1000	≥1000 TCID ₅₀
Fort Dodge Australia	Poulvac® CVI Vaccine	1696100	16 th Sept 2005	1000	≥1000 pfu
Bioproperties Pty Ltd	Vaxsafe® RIS	RIS 6111	Nov 2009	1000	≥4000 pfu

*Doses based on manufacturer's most recent re-titration of vaccine

At the same time of vaccination, the vaccine material used for the chickens was inoculated onto chicken embryo fibroblast (CEF) cell cultures to calculate the exact plaque forming units (pfu) for each of the three vaccines that were injected to the live birds. The titration was done on six well plates with confluent CEF seeded 24 hours prior. A serial dilution from 10⁻¹ to 10⁻⁴ from the original vaccination material was prepared and 200µl of each dilution added to duplicate wells of CEF. The cultures were incubated at 38.5 °C and 5% carbon dioxide (CO₂) for 3-5 days until plaques became visible. Plaques were counted under an inverted microscope at the dilution that gave the easiest distinction between plaques, where a guide of 10-60 plaques per well was appropriate. The titre was calculated by using the following equation:

$$\text{Counts} \times 5 \times \text{dilution factor} = \text{titre (pfu/ml)}$$

Result:

Intervet 5262 pfu/ml
Ford Dodge 676250 pfu/ml
Bioproperties 357500 pfu/ml.

Pathogenic MDV1 virus

The pathogenic MDV1 isolate 02LAR was sourced from cell-cultured stocks that had previously been grown and passaged on CEF at UNE. The media used was M199 containing 10% fetal calf serum and antibiotics/antimycotics (Invitrogen, Australia). The CEF cultures were incubated at 38.5°C and 5% CO₂ for 3-5 days until plaques became visible and titrated as described above prior to storage in liquid nitrogen (liqN) until used. Details of the challenge virus used are provided in **Table 1-2**.

Table 1-2: Details of the pathogenic MDV1 isolate 02LAR

Name	Batch number	Dose (pfu)	Source	Origin	Year of isolation
02LAR	18/11/2009	400	University of New England	Unvaccinated broilers, VIC, Australia	2002

1.2 Participating farms

The study involved 3 layer farms on geographically different areas in New South Wales (NSW) (Figure 1-1):

- Glendon farm, Tamworth
- Mid Coast Eggs farm, Port Macquarie
- Bowlers Lane farm, Tamworth.

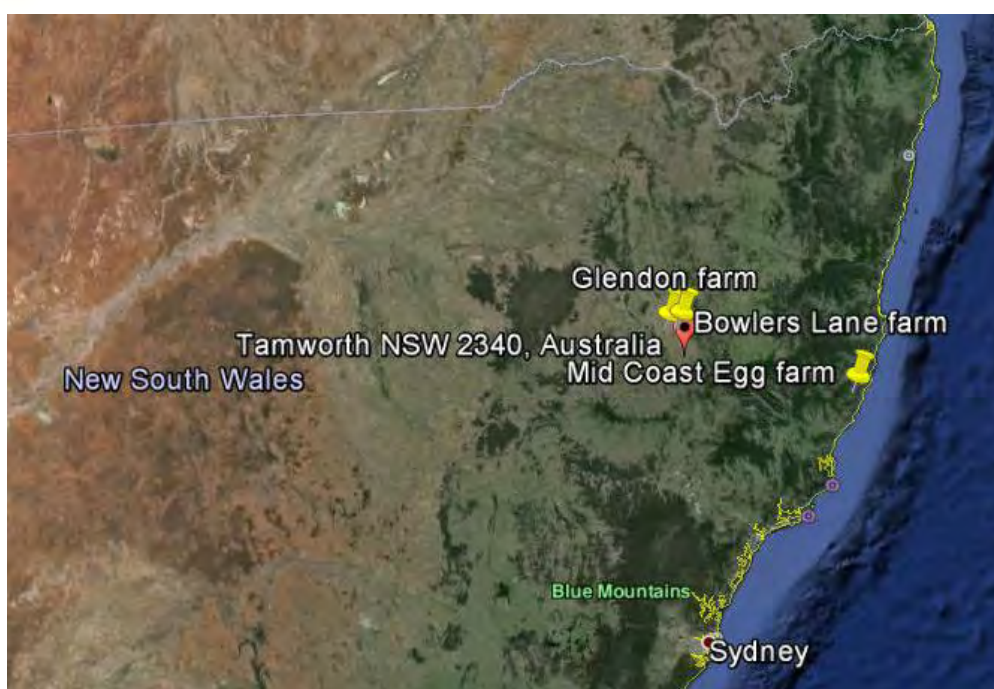


Figure 1-1: Location of participating farms

1.3 Experimental Chickens

Day-old specific pathogen free (SPF) white leghorn chickens (Australian SPF Services Pty Ltd, Melbourne, Australia) were used in the Rispens/CVI988 shedding and transmission experiment. For the vaccination-challenge interval experiment, day-old unvaccinated commercial female ISA Brown chickens from parent stock vaccinated with Rispens CVI988 (Bioproperties, Ringwood VIC) were used.

The layers on the farms participating in the field study were:

- Commercial Hy-Line Brown (Bowlers Lane, Tamworth)
- Commercial ISA Brown (Bowlers Lane, Tamworth and Port Macquarie)
- English Leghorn (Bowlers Lane, Tamworth).

All commercial layers in the field study came from parent stocks that had been vaccinated with Rispens CVI988 (Bioproperties Pty Ltd Australia).

1.4 Vaccination

Upon arrival at UNE, experimental chickens (SPF or commercial layer) were vaccinated subcutaneously (sc) under the loose skin on the dorsal aspect of the neck just below the head, using recommended doses of vaccine and diluent (Bioproperties Pty Ltd, Australia; Fort Dodge Australia and Intervet Australia Pty Ltd). Vaccines were thawed at 36°C in a water bath and used within 30 minutes of thawing as they are cell associated and viability is lost with loss of cell integrity. Disposable sterile 1ml syringes and 21G needles were used throughout. In the vaccine shedding and transmission experiment, vaccination was performed at hatch (day 0) and in the vaccination-challenge interval experiment, vaccination was performed sc at various days following experimental design. Three commercially available Rispens vaccines used in the vaccine shedding and transmission experiment were obtained from Bioproperties Pty Ltd Australia, Fort Dodge Australia and Intervet Australia Pty Ltd (**Table 1-1: Details of Rispens/CV1988 vaccines used**). In the vaccination-challenge interval experiment, only the Rispens vaccine from Bioproperties Pty Ltd Australia was used.

1.5 Challenge with pathogenic MDV1

In the vaccination-challenge interval experimental chickens were challenged with the very virulent MDV1 isolate 02LAR at a dose of 400pfu per chicken in 0.2ml via the sc route. Before use the virus was thawed at 37°C in a water bath and diluted with M199 media containing 10% fetal calf serum and antibiotics/antimycotics (Invitrogen, Australia) and used within 30 minutes of thawing. Disposable sterile 1ml syringes and 21G needles were used.

1.6 Animal management

The chickens used in all experiments were maintained and treated according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 2004, the NSW Animal Research Act 1985 and the NSW Animal Research Regulation 2005, and the experiments were approved by the UNE Animal Ethics Committee (AEC).

1.7 Euthanasia of chickens

Euthanasia was performed by AEC approved personnel following the method described by Zander *et al.* (1997). At the time of euthanasia the chicken was held in a fixed position by one hand. The thumb and the index finger of the other hand gripped the base of the skull and the middle and ring fingers were held under the beak. Cervical dislocation was completed by the rapid extension of the arm holding the head with a concurrent dorsal flexion of the head.

1.8 Marek's disease lesion detection and scoring from birds

Standard post-mortem examination was carried out for all dead and euthanized chickens throughout the experiment. Carcasses were wet in warm water containing water and detergent then 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. The bursa of Fabricius was examined and scored for atrophy as for the thymus. Tumorous enlargement of the spleen was 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.

1.9 Sample collection from chickens

Weekly blood samples for separation of lymphocytes and subsequent PCR were collected from the brachial vein by needle pricking using a sterile 23G needle followed by aspiration of blood from the resultant drop using a sterile plastic Pasteur pipette into a 1.5ml microfuge tube that was pre-loaded with 150µl of 3% sodium citrate. The sample was mixed briefly and chilled until further processing.

At the termination of the experiments at UNE, 3ml blood was collected from the brachial vein into 4ml vacutainers containing Z Serum clot activator (VACUETTE, Greiner Bio-one GmbH, Austria) for ELISA tests. Samples were centrifuged at 1450 x g for 15 minutes at 4°C and serum transferred to a 1.5ml microfuge tube and stored at -20°C until further processing. For the field studies, approximately 1ml blood from fifteen (15) randomly selected chickens per age group on each farm was collected into a 1.5ml microfuge tube as described above for ELISA tests.

Weekly feather samples from each individual bird were collected inside the respective isolators into a 1.5ml microfuge tube. Feathers were sampled initially by plucking from the wing (3-5 small wing feathers on days 7 and 14) then from the axillary tract (3-5 feathers for all subsequent sampling days) and stored until further processing. For the field studies, feathers from fifteen (15) randomly selected chickens per age group on each farm were collected as described above and stored at -20°C until further processing.

In the first experiment, dust samples were collected directly into a sterile 1.5ml microfuge tube from each room from a 40cm x 20cm galvanised steel settle plate and stored at -20°C until further processing. After collection each time the plate was thoroughly cleaned and disinfected with Virkon S so that the dust sample was representative of the entire week preceding the measurement.

In the second experiment, dust samples were collected from the dust deposits at the 90° bends in the exhaust air outlet of each isolator. To do this, the exhaust air outlet valve had to be closed briefly. Dander was scraped into a sterile 1.5ml microfuge tube using a disposable wooden spatula. After each collection, the valve was thoroughly cleaned and disinfected so that the next collection represented the past 7 days of dander accumulation.

For the field studies, a cumulative dust sample was collected from each shed from any suitable surface, usually the top of the cages, feed hoppers etc., into a 1.5ml microfuge tube and stored at -20°C until further processing.

1.10 Climate controlled rooms

The chickens of the vaccine shedding and transmission experiment were reared in four climate controlled rooms. The climate controlled rooms measured 3.6m × 4m and were supplied with temperature controlled filtered air. In each room, the chickens were placed in floor pens which were approximately 2.5m × 2.5 m. Pine wood shavings at a depth of 5-10cm were used as bedding material (**Figure 1-2**). Room temperature settings started at 35°C at day -2 and reduced by 2°C per day until 25°C was reached.

The chickens were offered feed (chicken starter and grower, Ridley Agricultural Products, Tamworth) and town water ad libitum throughout the experiment. Lighting was initially 24 hour light (days 1-2) followed by 12 hour light/12 hour dark controlled by an automatic timer. Birds were inspected twice daily for general well being.



Figure 1-2: Set up of the floor pens used in the vaccine shedding/transmission experiment. For the first few days, ice cude trays were used as additional water source

1.11 Isolator unit

Twenty-four purpose (24) built soft body chicken isolation units kept in the UNE isolator facility (Building W33) were used for the vaccination challenge interval experiment (**Figure 1-3**). The isolator facility is under constant negative pressure and all outgoing air is High Efficiency Particulate 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 a 49% open area. This is critical to enable housing of chickens from day-old to adult without faecal accumulation on the floor. Isolators are positive-pressure and soft-bodied with disposable plastic linings, gauntlets and gloves, disposed of after every experiment.



Figure 1-3: Interior of PC2 animal facility containing twenty four (24) isolators, also showing main air inlet duct which carries HEPA filtered, heated air to each isolator

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 airflow can be regulated manually to allow adjustment for isolator pressures. There are 12-23 airchanges/hour per isolator unit depending on fan settings. Isolators are individually fitted with heat lamps under separate thermostatic control. The entire feed supply for each experiment was loaded into a large feed hopper for each isolator and sealed for the duration of the experiment. Four nipple drinkers were provided in each isolator connected with a low pressure water supply. The entire facility was on an automated power backup via a 13 KVA generator.

Chickens were offered feed (commercial layer starter then grower, Ridley Agricultural Products, Tamworth) and water ad libitum throughout the experiment. Isolator temperatures were set at 34°C for the first two days and then decreased by 1°C every second day until a temperature of 22°C was reached. Lighting was initially 24 hour light (days 1-2) followed by 12 hour light/12 hour dark lighting set with an automatic timer.

Physical cleaning was performed in each room and isolator units including all appliances immediately after every experiment. All appliances and materials used to build isolators were physically cleaned with detergent followed by high-pressure steam cleaning. A second cleaning was carried out with detergent and complete treatment with a virucide (0.5-1% Virkon RS, Antec International Ltd, England, UK). The experimental rooms including isolation units were fumigated twice with formaldehyde before the start of the experiment. All materials passed into isolators was placed into the access box, sprayed with Virkon S and left for 20 minutes before being introduced into the isolator. Staff changed into protective clothing and footwear on entering the facility, and wore disposable hair nets while in it.

1.12 General laboratory Procedures

1.12.1 Separation of peripheral blood lymphocytes (PBL)

Approximately 300 µl of citrated blood sample was transferred slowly onto 300 µl of Ficoll Paque™ PREMIUM (Amersham Biosciences, Sweden) in a 1.5ml microfuge tube and centrifuged at 900 x g for 20 minutes at approximately 8°C. Lymphocytes were then carefully aspirated from the Ficoll paque interface and transferred to another 1.5ml microfuge tube containing 500 µl phosphate buffered saline (PBS). The samples were then centrifuged at 3500x g for 5 minutes at approximately 8°C. The supernatant was removed using a sterile pipette and the PBL pellet was stored at -20°C until further processing.

1.12.2 DNA extraction from PBL

DNA was extracted from PBL using the automated DNA X-tractor Gene and associated buffers and solutions (Corbett Robotics, Australia). Prior to loading the samples onto the robot, the PBL pellet was resuspended in 100 µl PBS and then diluted 1:5 in PBS. The diluted samples were loaded manually into the 96 well lysis block, 200 µl per well. The lysis block was transferred to the X-tractor gene.

The extraction protocol was as follows:

- 100 µl of DX Liquid Digest (DXL) with 10% DX digest enzyme was added per well to the lysis plate, mixed and incubated for 20 minutes
- 400 µl of DX Binding (DXB) with DX binding additive was added per well to the lysis plate and mixed and incubated for 5 minutes
- 600 µl from each well of the lysate was transferred from the lysis plate to the capture plate and vacuumed at 30kPa for 3 minutes
- 200 µl of DX Binding (DXB) with DX binding additive was added per well to the capture plate and again vacuumed at 30kPa for 3 minutes
- 600 µl of DX Wash (DXW) per well was loaded into the capture plate and vacuumed at 25kPa for 1 minute
- This step was repeated and 600 µl of DX Final Wash (DXF) per well loaded to the capture plate and vacuumed at 35 kPa for 5 minutes to dry the plate
- The carriage was moved to the elution chamber and 150 µl of elution buffer (E) per well was loaded to the capture plate, incubated for 5 minutes and again vacuumed at 30kPa for one and half minutes. At last the elution plate was moved from the robot and stored at -20°C.

1.12.3 DNA extraction from feather tips, dust and spleen

Prior to DNA extraction of feather samples, approximately 1cm of the proximal shaft (the feather tip) was finely chopped to approximately 3mm lengths using a sterile scalpel blade and transferred into a sterile 1.5ml microfuge tube.

DNA was extracted from 2-3 feather tips or 5mg dust or 10 (±1) mg of spleen respectively using the DNeasy Blood and Tissue Kit (Qiagen, Australia) for Experiment 1 and the ISOLATE Genomic DNA Mini Kit (Bioline, Australia) for Experiment 2 and the feather and dust samples from the field studies. Both kits were tested and revealed that there was no difference in either extracted DNA quantity or quality obtained. The DNA was extracted according to the manufacturer's instructions.

All extracted DNA was quantified using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA). The absorbance ratio of the sample at 260nm and 280nm was assessed as a measurement of DNA quality. All DNA was stored at -20°C until further analysis.

1.12.4 Quantitative real-time Polymerase Chain Reaction (qPCR)

All extracted DNA samples of the first experiment were subject to both the MDV serotype specific TaqMan® qPCR assays as developed in the AECL/RIRDC project UNE08-17 and generic TaqMan® qPCR assays as described previously by Islam *et al.* (2004).

The respective TaqMan® real-time qPCR assay was performed using a RotorGene 3000 real-time PCR instrument (Corbett Research, Sydney, Australia). The qPCR cycling parameters consisted of: 50°C for two minutes, 95°C for 2 minutes, followed by 40-45 cycles consisting of denaturation at 94°C for 15 seconds and annealing/ extension at 60°C for 45 seconds for MDV1 generic assay and 60 seconds for the Rispens specific and pathogenic assays. Each reaction tube contained 0.3 µM of each primer, 0.2 µM of the probe, 12.5 µL of Platinum® Quantitative PCR System-UDG (Invitrogen Australia Pty Ltd), 5 µL of DNA template (25 ng of DNA) in a total reaction volume of 25 µL. A Corbett CAS1200 liquid handling instrument (Corbett Research, Sydney, Australia) was used to prepare the reaction tubes for all qPCR assays.

A standard curve for each primer set was generated in each assay and used to derive the copy number of target sequences in unknown samples. For each assay run, individual standard curves were generated using 10-fold dilutions of MDV1 standards of known concentration of MDV1 DNA. All samples were randomised across assays for the first experiment and for the second experiment individual bird samples were done in one assay to minimise individual assay effects, a single reaction per sample was used for PBL DNA samples and duplicate reactions for feather, dust and spleen DNA samples. Standards and quality controls (QCs) were also assayed in duplicate for all assays. Details of Standards and QCs are given in **Table 1-3**. Samples that did not amplify or amplified with a C_t value below the lowest standard were determined negative.

Table 1-3: Details of standards and quality controls (QCs) used in the qPCR assays

qPCR assay	Standards and QCs	Origin	Dilution factor	Concentration (ng/ul)
Generix MDV1/ Pathogenic MDV1	MDV1 26.1	Pooled spleen DNA of SPF chickens infected with MDV1 (MPF57)	0	12.5
			100	0.125
			100	0.00125
			10	0.000125
	QC1	Pooled spleen DNA of SPF chickens infected with MDV1 (MPF57)	0	50
Rispens specific MDV1	Risp 1	DNA extracted from Marek's disease vaccine Rispens CVI988® vaccine (Bioproperties).	0	0.132
			100	0.00132
			10	0.000132
			10	0.000013
	QC TZ1	Spleen DNA 2 SPF chickens vaccinated with Marek's disease vaccine Rispens CVI988® vaccine (Intervet and Bioproperties respectively).	0	75.5
	QC TZ2	As above	25	2.5

1.12.5 Enzyme Linked Immuno Sorbent Assay (ELISA)

Sera samples were diluted in 1:20 with PBST (Phosphate Buffered Saline with 0.05% Tween 20) containing 1% skim milk powder and stored at 4°C until ready. ELISA plates were coated with Marek's antigen (Ag) 1:100 dilution (using 100 µl of Marek's concentrated Ag in 10mls of carbonate buffer per plate) and 100 µl diluted Ag was added per well to the plate. The antigen was prepared from the vaccine Rispens CVI988 vaccine (Bioproperties Pty Ltd). The preparation of antigen is described below.

The covered plates were then incubated at 4°C for at least 16 hours. After coating, the plates were washed twice with PBST. After the second wash, the plate was inverted onto a stack of paper towels to remove any excess liquid. To block the plates, 100 µl/well of 1% skimmed milk in PBST was added per well. The plates were incubated in a humidified chamber at room temperature for 1 hour. Prior to loading 100µl of the diluted samples and standard per well to each plate, the plates were washed with PBST as described above, and incubated in humidified chamber at 37°C for 1 hour. The plates were then washed again as described above and 100 µl of Anti-Chicken IgY (IgG) whole molecule peroxidase conjugated (Sigma Cat no: A9046) 1:5000 dilution with PBST was added per well to all wells. Again the plates were covered and incubated at 37°C for 1 hour. The contents of the plates were again flicked out and washed 3 times with PBST. After the third wash plates were inverted and banged onto clean paper towel to remove any excess liquid. Finally, 100 µl of o-phenylenediamine (OPD) substrate (Sigma Chemicals, USA) was added per well to all wells. The plates were covered with aluminum foil and incubated at room temperature for 10 minutes. Adding 50

µl/well of 1M sulphuric acid (H₂SO₄) to plates stopped the reaction and the plates were read at 492 nm using a plate reader.

Preparation of antigen

One vial of Bioproperties Vaxsafe RIS vaccine containing live Rispens CVI988 virus was thawed by placing in warm water. Then dilution media was added to make it up in 5mls and mixed well. After that it was spun down for 10 minutes at 2000 x g at 4°C and the pellet was retained after pouring off supernatant. The pellet was then frozen at –20°C until frozen and then thawed, and this freeze/thawing step was repeated at least four times. After the last freeze/thawing, 5mls PBS was added and the pellet broken up and dispersed. Then it was sonicated for at least 1 minute at approximately 12 amps. Finally the sample was centrifuged for 10 minutes, 2500 x g at 4°C, then the supernatant was collected and stored at –20°C until use.

Validation of assays

A standard curve was generated in each assay. All samples were tested in duplicate. Samples individual birds samples were included on the same plate to minimise between-plate assay effects, but treatment effects were stratified across plates. The sensitivity of the assays for MDV1 was determined by running two-fold serial dilutions of the standards with known titres. Standards and quality control were also assayed in duplicate for all assays. Details of Standards are given in **Table 1-4**. Standards were made up from pooled sera from breeder broiler chickens vaccinated against MDV with Rispens vaccine. The titre cutoff value was determined from known negative control chickens sera and titres adjusted by subtraction of the highest of these values. The intra assay (plate) coefficient of variation (CV) was calculated from duplicates of each sample and inter assay CV calculated from negative control sample included in each assay run. The mean intra assay CV was 8.45% and the mean inter assay CV was 30%.

Table 1-4: Details of standards used in ELISA assay

Standards	Dilutions	Titre units
S 01	1:16	512
S 02	1:32	256
S 03	1:64	128
S 04	1:128	64
S 05	1:256	32
S 06	1:512	16
S 07	1:1024	8
S 08	1:2048	4
S 09	1:4096	2
S10	1:8192	1

2. Experiment 1: Viral kinetics, shedding profile and transmission of Rispens/CVI988 in maternal antibody-free chickens

This experiment aimed to investigate the kinetics and spread of three commercially available Rispens vaccines in vaccinated and in-contact specific pathogen free (SPF) chickens in a controlled environment in order to identify appropriate tissues (blood/ peripheral blood lymphocytes (PBL), feather tip, dust) and timing of sampling to use to monitor vaccine take by quantitative real-time polymerase chain reaction (qPCR). Sensitivity of the qPCR test was also compared with seroconversion in a non-serotype specific MDV Enzyme Linked Immuno Sorbent Assay (ELISA).

A particular focus of the study was the occurrence of the Rispens vaccine in in-contact chickens and the kinetical differences between the vaccines of three different vaccine suppliers. At the moment the literature is unclear about the extent, if any of transmission of Rispens, although there is significant shedding of virus and the original vaccine virus spread effectively (Rispens, 1972a).

The specific hypotheses under test were:

1. CVI988 will be shed in dander and shedding will be detected at the first measurement at day 7 post-vaccination (dpv)
2. CVI988 will transmit readily to in-contact chickens
3. Replication kinetics in PBL, feather tips, viral load in dust, final viral load in spleen and final antibody titre against MDV will not differ significantly between the three commercial vaccines
4. Replication rate and shedding of Rispens/CVI988 will be lower than published values for wild-type MDV
5. Anti-MDV antibody titre at day 56 will be predicted by early measurement of viral load in PBL, feather or dust.

PhD candidate Tanzila Islam contributed significantly to this experiment. The results of this experiment have been published:

Islam, T., Renz, K.G., Ralapanawe, S., and Walkden-Brown, S.W. (2013) Viral kinetics, shedding profile and transmission of serotype 1 Marek's disease vaccine Rispens/CVI988 in maternal antibody-free chickens. *Avian Diseases* 57, 454–463.

2.1 Overview of method

2.1.1 Experimental design

The experiment had a 3 x 2 factorial design with an external negative control treatment and duration of 56 days. The factors in the factorial design were:

- Three commercially available cell associated Rispens CVI988 vaccines: Vaccine A: Vaxsafe RIS® (Bioproperties Pty Ltd), Vaccine B: PoulvacCVI Vaccine® (Fort Dodge Australia), Vaccine C: Nobilis Rismavac® (Intervet Australia Pty)
- Two modes of infection: vaccination at day old and unvaccinated in-contact.

Seventy individually identified SPF white leghorn chickens (Australian SPF Services Pty Ltd, Melbourne, Australia) were used in the experiment providing 10 birds per treatment

combination and 10 birds as negative controls. The experiment was terminated at 56 days of age.

The experiment was conducted in four identical positive pressure rooms supplied with temperature controlled filtered air. One room was allocated to each of the three vaccines under test with an additional room for the negative controls.

All birds were provided commercial feed and water *ad libitum* for the duration of the experiment. The chickens were reared in 1.5m x 1.5m pens on pine shavings. The experiment was approved by the UNE Animal Ethics Committee (AEC No. UNE 09/091).

2.1.2 Vaccination

Three commercial Rispens CVI988 vaccines were used in the experiment. All three vaccines were diluted according to manufacturer's instructions using diluents provided by each manufacturer. Vaccination was performed manually in a volume of 0.2ml/bird subcutaneously (sc) at hatch (day 0). Negative control chickens were mock vaccinated with diluent only. The details of the vaccines and doses are given in **Table 1-1**.

2.1.3 Sample collection and DNA extraction

Blood and feather tip samples were collected from the vaccinated and control birds weekly starting from 7 days of age up to day 56. The same tissues were collected from in-contact birds weekly starting from 21 days of age up to day 56. Blood samples were collected into 1.5ml microfuge tubes pre-loaded with 150 µl of 3% sodium citrate prior to separation of PBL using Ficoll Paque™ PREMIUM (Amersham Biosciences, Sweden). Feathers were sampled initially by plucking from the wing (3-5 small wing feathers on days 7 and 14) then from the axillary tract (3-5 feathers) for all subsequent sampling days and stored at -20°C until further processing. Weekly dust samples were collected from each room commencing at day 7, using settle plates and stored at -20°C until further processing. Individual sera were collected at day 56 and stored at -20°C until further processing. Spleens were collected at day 56 from 5 randomly selected birds per treatment combination and stored at -20°C for further processing.

DNA was extracted from PBL using the automated X-tractor Gene including reagents (Corbett Robotics, Australia) according to the manufacturer's instructions (section 1.12.2

DNA extraction from PBL). DNA from feather tips was extracted from 6 birds per treatment at days 7, 14, 21, 28, 42 and 56 using the ISOLATE genomic DNA mini kit (Bioline, Australia) following the instructions given by the manufacturer (section 1.12.3 DNA extraction from feather tips, dust and spleen). DNA from spleen and dust was extracted using the DNeasy® blood and tissue kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's instructions. All extracted DNA samples were stored at -20°C until further analysis.

Prior to quantitative real-time PCR (qPCR), extracted DNA was quantified by spectrophotometric analysis using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA), and diluted to a concentration of 5 ng/µl for use in the qPCR assays.

2.1.4 qPCR and ELISA

For determination of viral load in PBL, feather tip, dust and spleen samples, a TaqMan® serotype-1 MDV specific real-time qPCR assays as published by Islam *et al.* (2004; section 1.12.4 Quantitative real-time Polymerase Chain Reaction (qPCR)) were performed using a Rotor Gene 3000 real-time PCR instrument (Corbett Research, Australia).

For determination of MDV-specific antibody titre in serum samples, an indirect ELISA (section 1.12.5 Enzyme Linked Immuno Sorbent Assay (ELISA)) adapted from that of Zelnik *et al.* (2004) was used. All samples were randomized prior to either analysis by qPCR or ELISA.

2.1.5 Statistical analysis

MDV copy number and antibody titre data were log transformed [$\log_{10}(y+1)$] to meet the assumptions of the analysis of variance (AOV). The data were analysed by AOV fitting the effects of Vaccine, Type of infection, Day (when required) and their interactions. When the analysis included in-contact chickens, data from days 7 and 14 were excluded, as samples were not collected from this treatment on these days.

For repeated measures (PBL, feather, dust) a mixed REML model was used with animal or room fitted as a random effect. The rate of which birds become infected was determined by detection of MDV in PBL and in feather and treatment effects were investigated using survival analysis (Kaplan-Meier product-limit method).

Association between variables was tested initially by multivariate pair wise correlation analysis followed by linear regression analysis of specific associations. Associations were explored between MDV load in PBL and feathers at various sampling times, and between these variables and day 56 MDV load in spleen and day 56 anti MDV antibody titre. For these, individual animal data were used.

For associations between these variables and MDV load in dust weekly room means were used. In all cases negative controls were excluded. Analyses were performed with JMP 10 (SAS Institute, NC, USA). Least squares means and standard errors of means are presented for continuous variables. A significant level of $P < 0.05$ is used throughout.

2.2 Results

2.2.1 Vaccination success and room biosecurity

At day 7, 28 out of 30 (93%) vaccinated chickens were positive for MDV1 in PBL. Of the remaining 2 chickens, 1 chicken tested positive at day 14 and the other chicken tested positive at day 28. All negative control chickens were negative for MDV in PBL, feather, spleen and dust at all measurement times indicating no transfer of infection between rooms.

2.2.2 Infection rate in vaccinated and in-contact chickens

At day 7, 28 out of 30 (93%) vaccinated birds were positive for MDV1 in PBL with the cumulative percentage of infected chickens rising to 100% by day 28 (). For the in-contact birds, 38% were positive at day 21 (first sampling), with a cumulative percentage of 96% positive by day 56. These data demonstrate clear transmission of Rispens/CVI988 virus to in-contact chickens with a lag phase of 21 days for vaccine A and vaccine B, 18 days for vaccine C. Whereas shows the cumulative proportion of birds becoming positive to MDV in PBL,

Figure 2-1 shows the actual proportion of positive chickens at any given week. In vaccinated chickens the 93% positive rate at day 7, decreased to 68% at day 14, rose steadily to 83% positive at day 42 before decreasing sharply to 33% positive at day 56. Amongst the in-contact chickens, 40% were positive at the first day of sampling (day 21) rising to 92% positive at day 42, before decreasing to 58% positive at day 56.

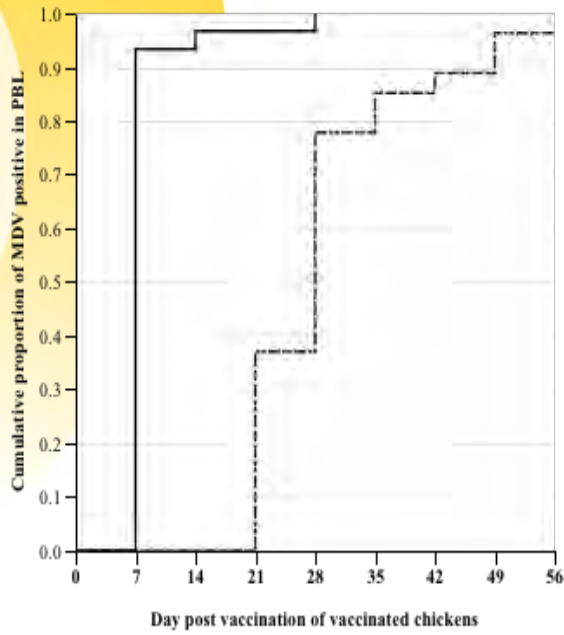


Figure 2-4: Cumulative proportion of vaccinated and in-contact chickens becoming MDV positive over time, as determined by qPCR of PBL for MDV1 commencing at days 7 (vaccinated) and 21 (in-contact) respectively (curves differ significantly, $P < 0.001$)

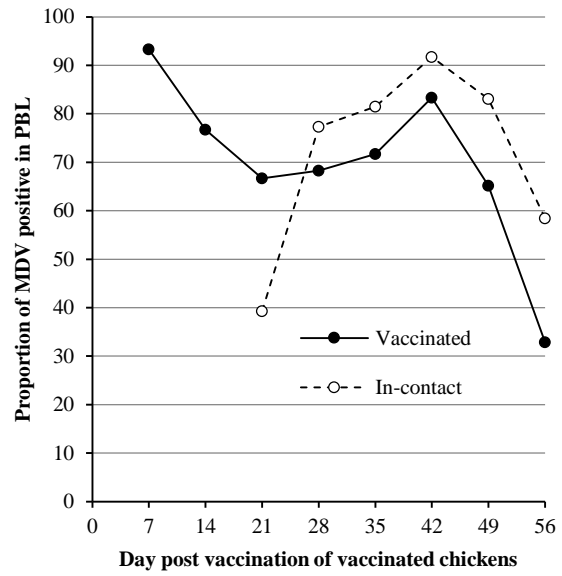


Figure 2-3: Proportion of vaccinated and in-contact chickens MDV positive in any given week as determined by qPCR of PBL for MDV1 commencing at days 7 (vaccinated) and 21 (in-contact) respectively. Asterisks indicate significant differences within time periods

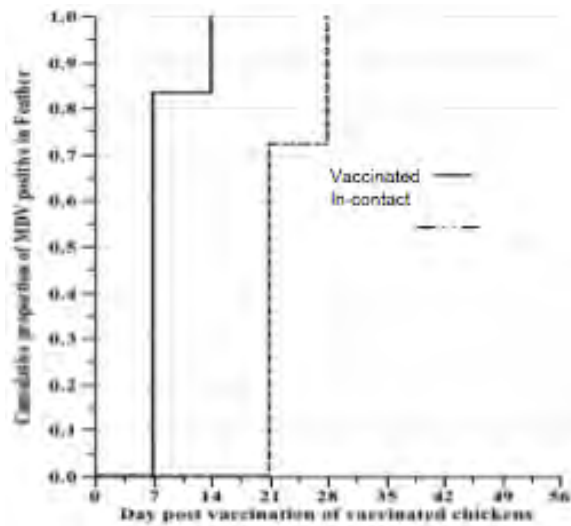


Figure 2-1: Cumulative proportion of vaccinated and in-contact chickens becoming MDV positive over time, as determined by qPCR of feather tips for MDV1 commencing at 7 dpv (vaccinated) and 21 dpv (in-contact) respectively (curves differ significantly, $P < 0.001$)

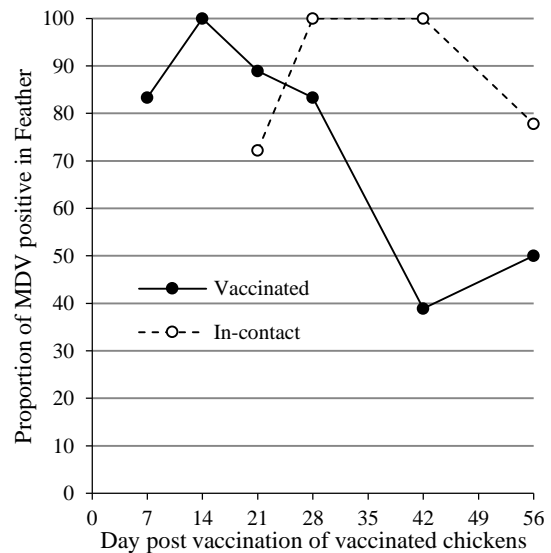


Figure 2-2: Proportion of vaccinated and in-contact chickens MDV positive in any given week as determined by qPCR of feather tips for MDV1 commencing at days 7 dpv (vaccinated) and 21 dpv (in-contact) respectively. Asterisks indicate significant differences within time periods

At day 7, 83% of vaccinated birds were positive for MDV in feather tips with the cumulative percentage of infected chickens rising to 100% by day 14 (

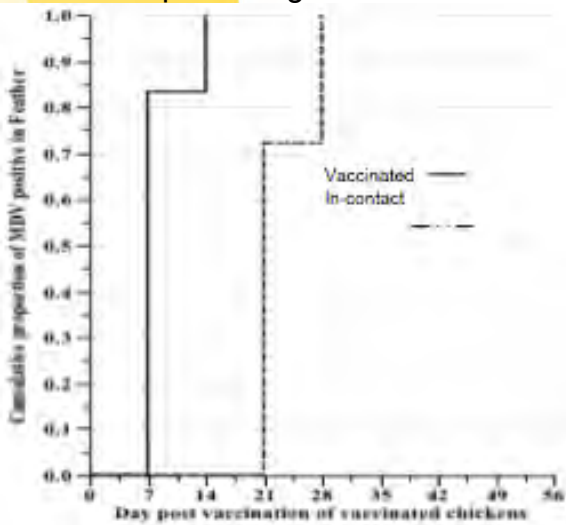


Figure 2-1). For the in-contact birds, 72% were positive at day 21 (first sampling), with a cumulative percentage of 100% positive by day 28. These data demonstrate clear transmission of Rispens/CVI988 virus to in-contact chickens with a lag phase of 14 days for vaccine A and vaccine B and 18 days for vaccine C. Whereas

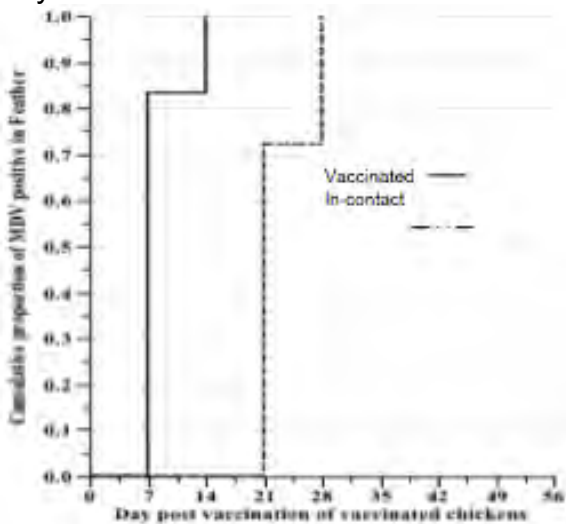
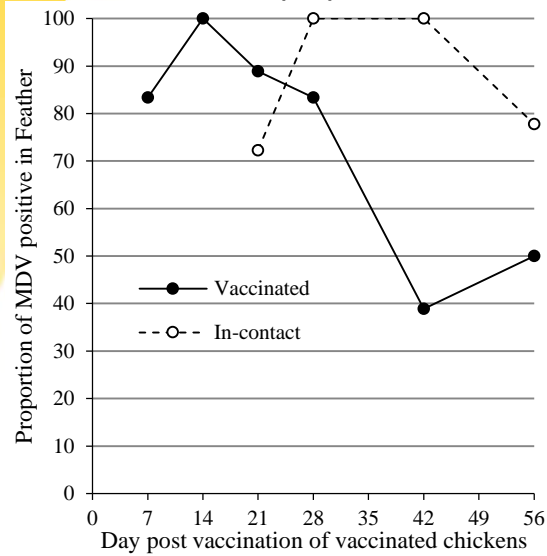


Figure 2-1 shows the cumulative proportion of birds becoming positive to MDV in



feather tips,

Figure 2-1 shows the actual proportion of positive chickens at any given week. In vaccinated chickens the 83% positive rate at day 7, increased to 100% at day 14, decreased gradually to 83% at day 28 before decreasing sharply to 39% at day 42 before ending at 50% at day 56. Amongst the in-contact chickens, 72% were positive at the first day of sampling (day 21) rising to 100% positive at days 28 and 42 before decreasing to 78% at day 56.

Using PBL to measure infection rate, Vaccine A showed a lower transmission rate

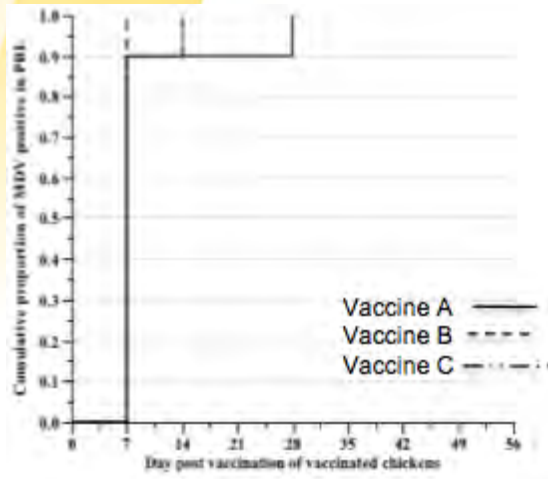


Figure 2-6: Cumulative proportion of in-contact chickens becoming MDV positive over time as determined by qPCR of PBL for MDV1 commencing 21 by vaccine (failure curves differ significantly, $P=0.0176$)

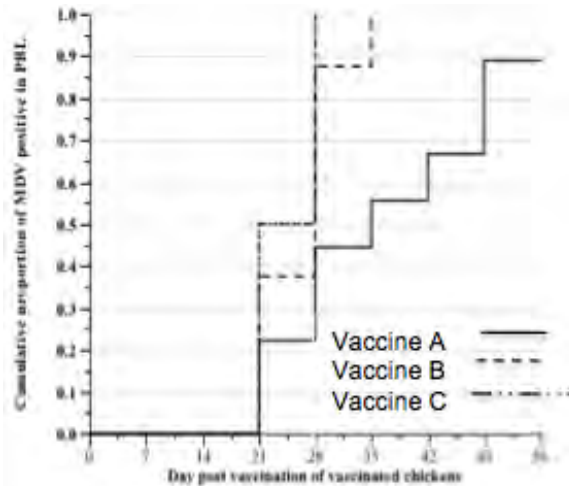


Figure 2-5: Cumulative proportion of vaccinated chickens becoming MDV positive over time as determined by qPCR of PBL for MDV1 commencing at days 7 by vaccine (no significant difference, $P=0.4788$)

than the other two vaccines (

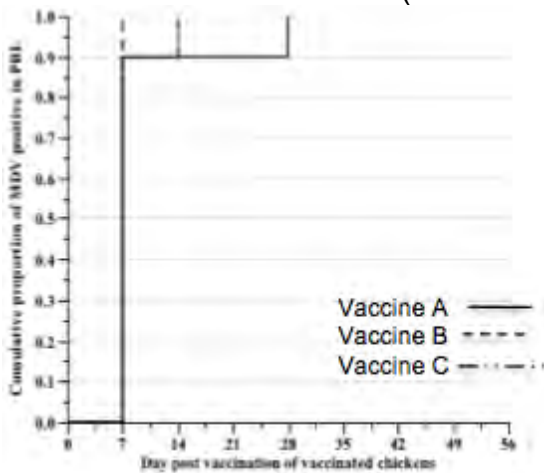
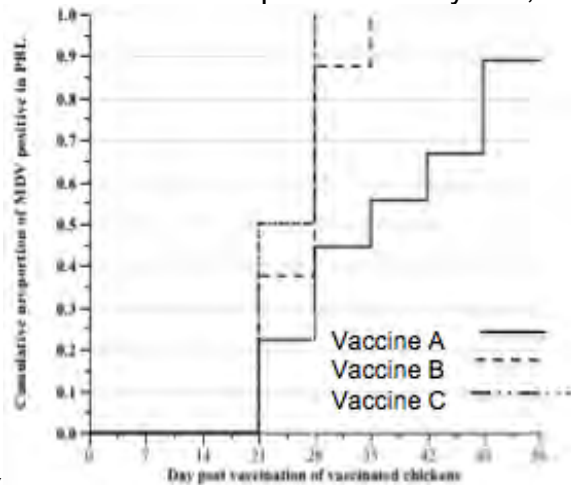


Figure 2-5). All vaccinated birds were 100% positive at days 28, 7 and 14 for vaccines



A, B and C respectively (

Figure 2-6). The in-contact birds from the vaccine A group failed to become 100% positive in PBL, those from the vaccine B and C groups became 100% positive in PBL at days 35 and 28 respectively.

Using feather tips to measure infection rate, vaccinated birds were 100% positive at for vaccines A, B and at day 14 for vaccine C (

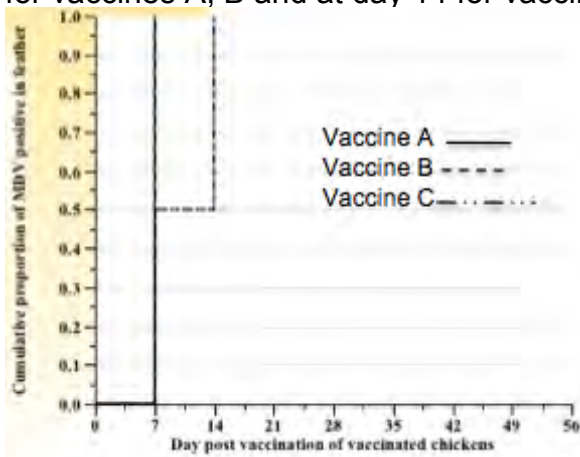


Figure 2-7). The in-contact birds in the vaccine A group became 100% positive at day 21 and from the vaccine B and C groups became 100% positive at day 28 (

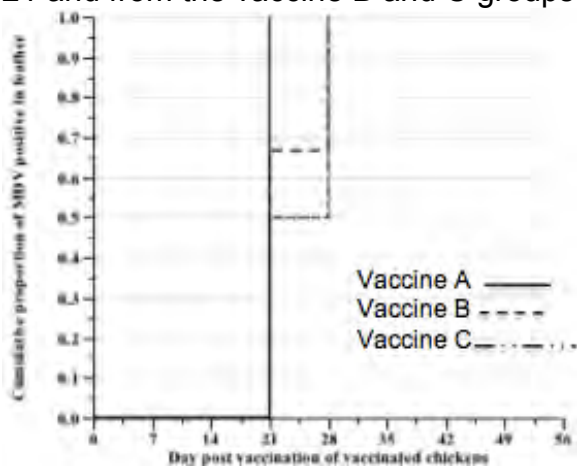




Figure 2-7).

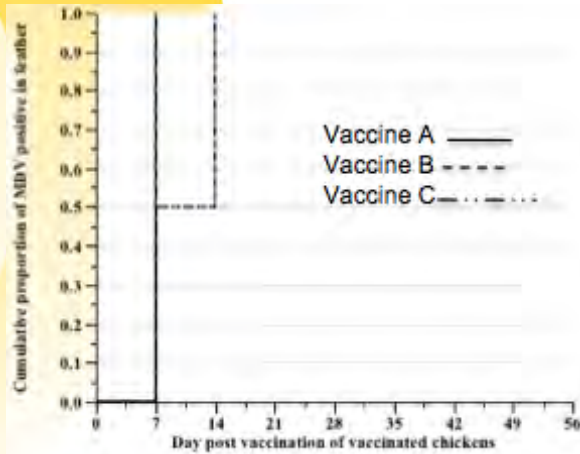


Figure 2-7: Cumulative proportion of vaccinated chickens becoming MDV positive over time as determined by qPCR of feather for MDV1 commencing at days 7 by vaccine (failure curves differ significantly, $P=0.0334$)

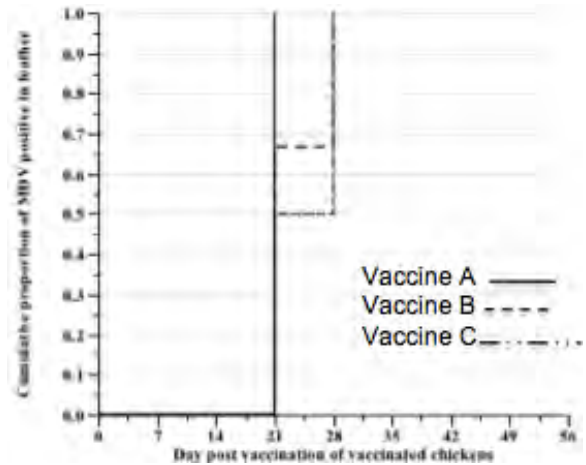


Figure 2-8: Cumulative proportion of in-contact chickens becoming MDV positive over time as determined by qPCR of feather for MDV1 commencing at day 21 by vaccine (no significant difference, $P=0.1603$)

2.2.3 MDV Load in PBL

There was a significant effect of mode of infection ($P=0.0069$) and vaccine ($P=0.0002$) with no significant interaction between these effects (**Figure 2-9**). There was also a significant effect of day ($P<0.0001$) but the interaction between day and vaccine was not significant ($P=0.300$). However the interaction between mode of infection and day was significant ($P=0.051$). Overall, Log_{10} ($y+1$) viral load in PBL (VCN/106 cells) was significantly ($P=0.0069$) lower in vaccinated (2.11 ± 0.15) than in-contact chickens (2.74 ± 0.16). Vaccine C had a significantly higher overall viral load (3.02 ± 0.18) than vaccine A (1.85 ± 0.19) and vaccine B (2.38 ± 0.21) (**Figure 2-9**).

In vaccinated chickens, the highest mean Log_{10} ($y+1$) MDV load in PBL was at day 7 (3.55 ± 0.19), decreasing until day 21 after which it stabilized at until day 42 before decreasing sharply until the termination of the experiment at 56 dpv. In the in-contact chickens, MDV load in PBL increased from first sampling at day 21 (1.67 ± 0.31) to day 28 (3.28 ± 0.30). Between days 28 and 49, the overall MDV load in PBL plateaued at around $3.29 (\pm 0.31)$ then decreased to $2.08 (\pm 0.31)$

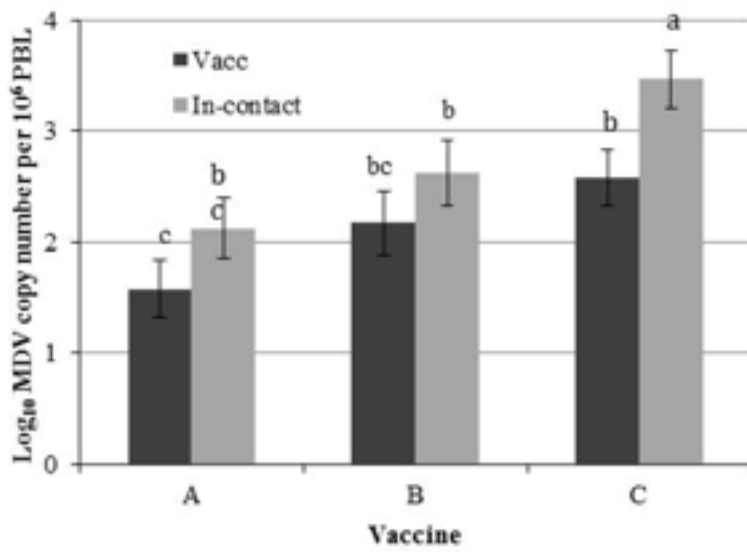


Figure 2-9: Overall effect of vaccine on mean (LSM±SEM) viral copy number per 10⁶ PBL of vaccinated and in-contact chickens (P=0.6960). All sampling times are included

MDV copy number per 10⁶ PBL at day 56 (

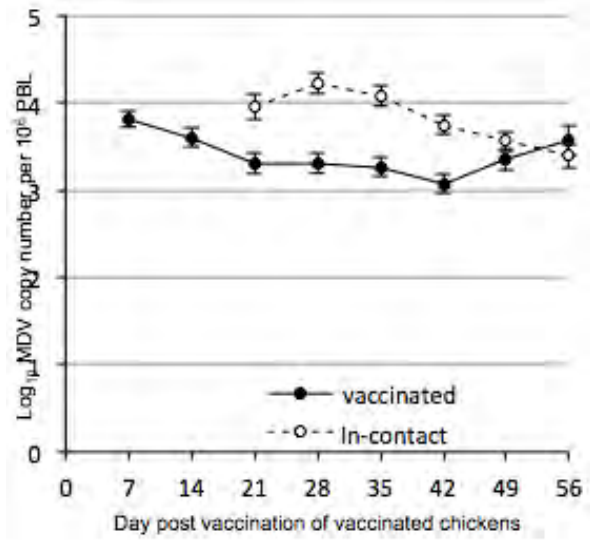


Figure 2-10: Mean (LSM±SEM) viral copy number per 10⁶ PBL of MDV-positive vaccinated and in-contact chickens over time (Effect of mode of infection P<0.0001)

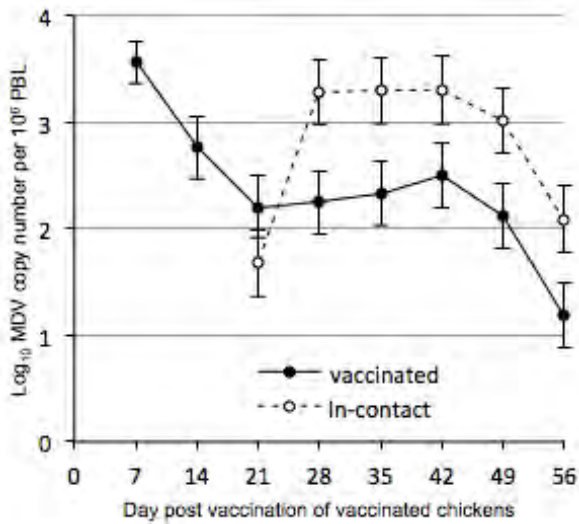
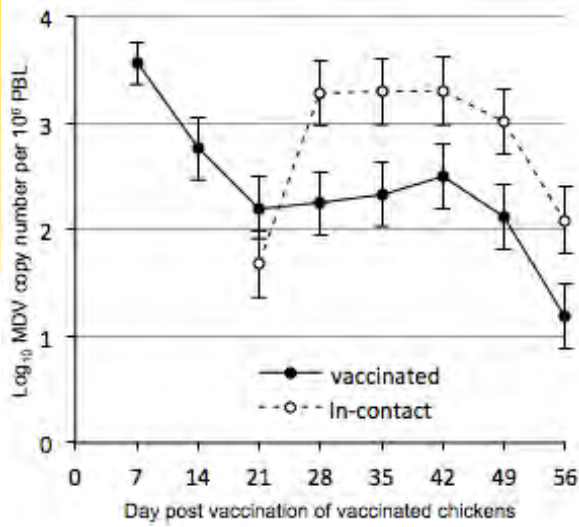


Figure 2-10). The declines in MDV load over time were due mainly to the decline in the proportion of chickens positive at any one sampling point. This is clear from



comparing

Figure 2-10 with

Figure 2-1 showing that the shape of the viral load curve is very similar to that of the proportion of positive for MDV. Analysis of viral load in only samples that were positive for MDV revealed a very similar pattern

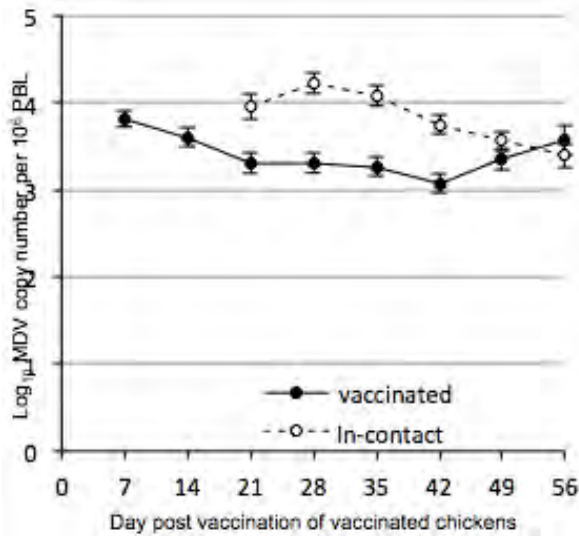


Figure 2-10 but with a reduced amplitude of change due to higher minimum values.

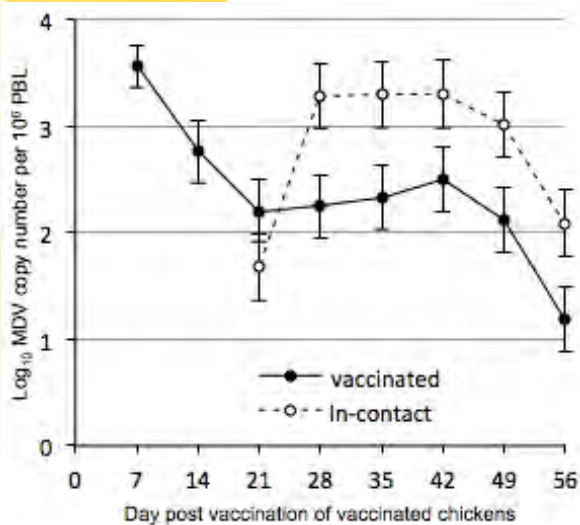


Figure 2-11: Mean (LSM±SEM) viral copy number per 10⁶ PBL of all vaccinated and in-contact chickens over time (Effect of mode of infection P=0.0514)

2.2.4 MDV load in feather tips

There was a significant effect of mode of infection ($P < 0.0001$) with no significant effect of vaccine ($P = 0.79$) but there was significant interaction between these effects ($P = 0.02$; **Figure 2-12**). There was also a significant effect of day ($P < 0.0001$) but the interaction between day and vaccine was not significant ($P = 0.07$). However the interaction between mode of infection and day was significant ($P < 0.0001$). Overall Log₁₀ ($y+1$) viral load in feather (VCN/ 10^6 cells) was significantly ($P < 0.0001$) lower in vaccinated (2.45 ± 0.18) than in-contact chickens (3.56 ± 0.18) (**Figure 2-12**).

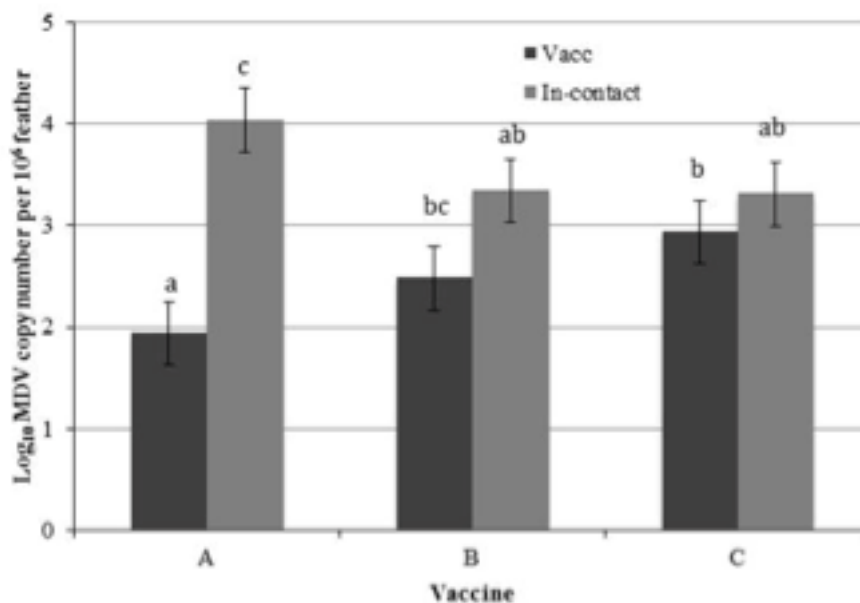
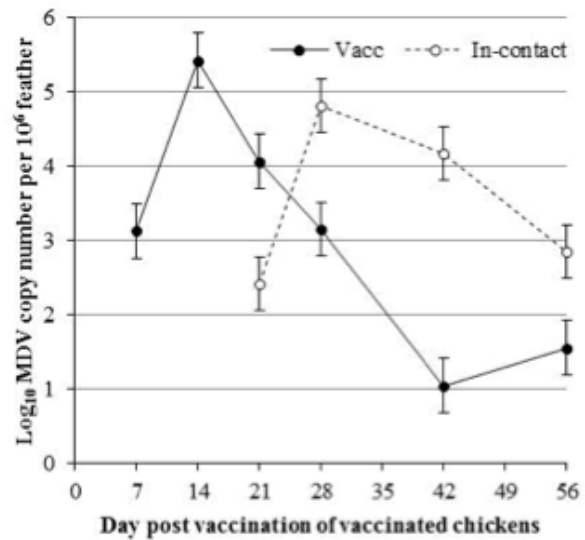


Figure 2-12: Overall effect of vaccine on mean (LSM±SEM) viral copy number per 10⁶ feather tips of vaccinated and in-contact chickens ($P = 0.0191$)

In vaccinated chickens, the highest mean Log_{10} (y+1) MDV load in feather tips was at day 14 (5.41 ± 0.36), decreasing until day 42 after which it increased slightly by the



termination of the experiment at 56 dpv (

Figure 2-13). In the in-contact chickens, MDV load in feather tips increased from first sampling day 21 (2.41 ± 0.31) to day 28 (4.81 ± 0.36). After that it decreased gradually to (2.84 ± 0.36) MDV copy number per 10^6 feather tips at day 56 (

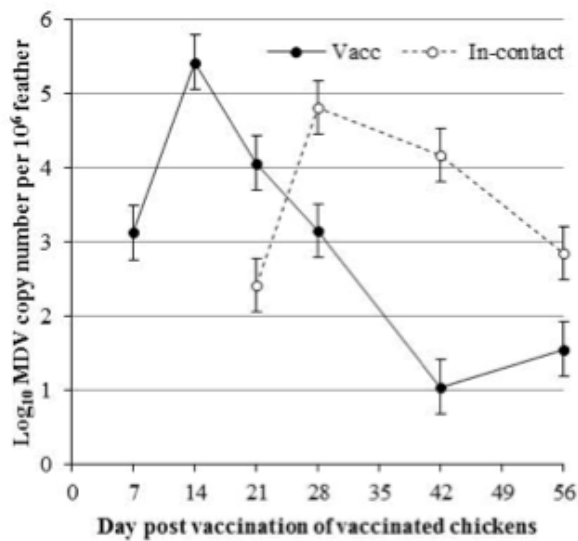


Figure 2-13).

The declines in MDV load over time were due mainly to the decline in the proportion of chickens positive at any one sampling point. This is clear from comparing

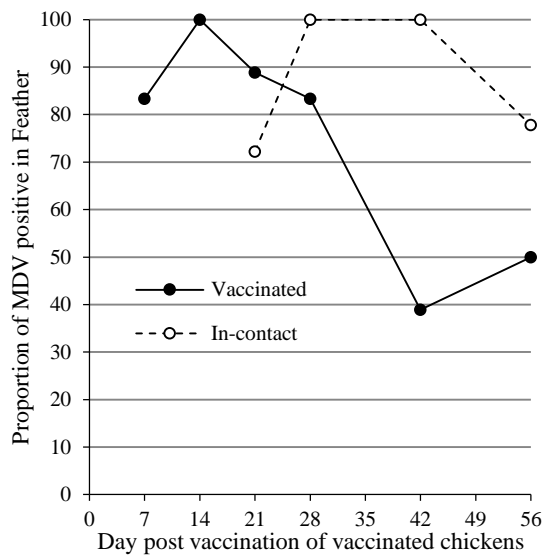
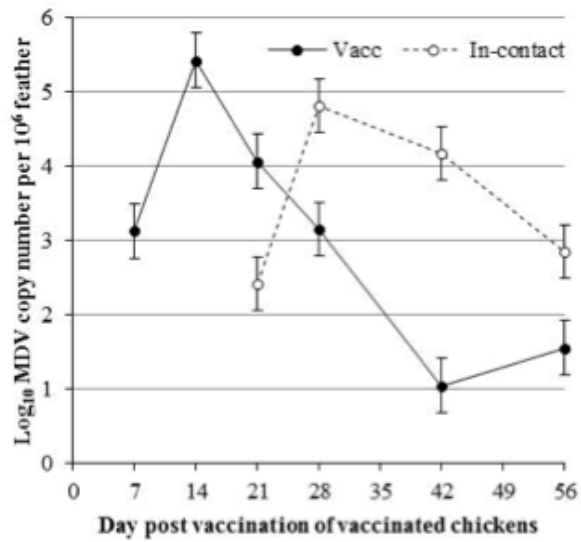


Figure 2-13 with

Figure 2-1 showing that the shape of the viral load curve is very similar to that of the proportion of positive chickens in any given week. Analysis of viral load in only

samples that were positive for MDV revealed a very similar pattern (

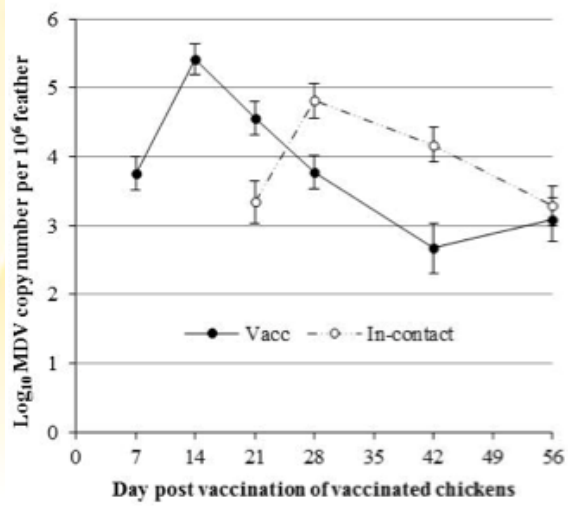


Figure 2-13) but with a reduced amplitude of change due to higher minimum values.

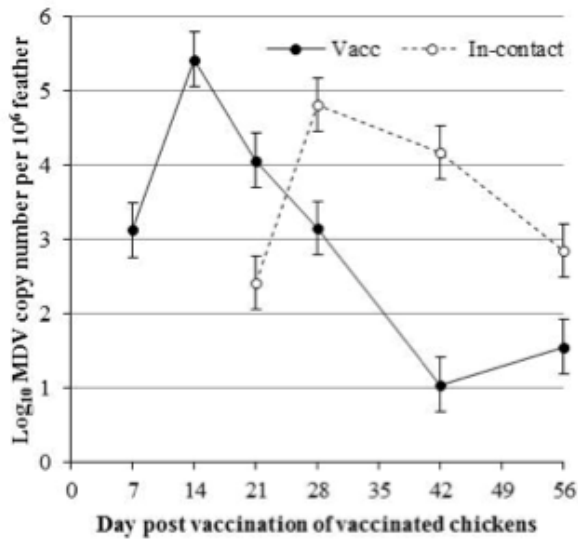


Figure 2-14: Mean (LSM±SEM) viral copy number per 10⁶ feather of vaccinated and in-contact chickens over time (Effect of mode of infection P<0.0001)

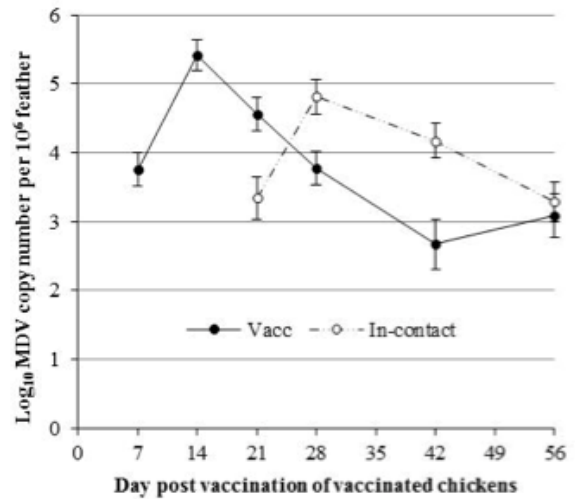


Figure 2-13: Mean (LSM±SEM) viral copy number per 10⁶ feather of MDV-positive vaccinated and in-contact chickens over time (Effect of mode of infection P<0.0001)

2.6.5 MDV Load in spleen cells

There was no significant effect of type of infection (P=0.17) or vaccine (P=0.09) on the MDV copy number in spleen at day 56 with no significant interaction between these effects (P=0.17) (Figure 2-15). The overall Log₁₀ (y+1) MDV load in spleen of vaccinated chickens (1.93 ± 0.37) was not significantly different from that of in-contact chickens (2.72 ± 0.42).

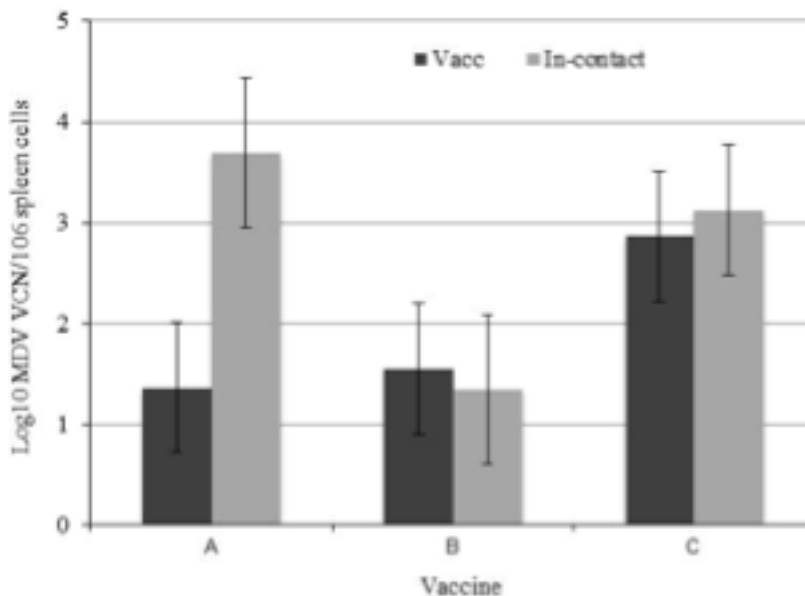


Figure 2-15: Interaction plot showing mean (LSM±SEM) viral copy number per 10⁶ spleen cells of chickens vaccinated with vaccines A, B and C and in-contact chickens at day 56 post vaccination (Interaction P=0.17)

2.2.6 MDV load in dust

There was a significant effect of vaccine ($P=0.045$) and day ($P<0.0001$). Dust samples from all the three vaccine groups tested positive at day 7 between 2-3 \log_{10} VCN/mg dust and then increased up to day 21 (4.2-5.2 \log_{10} VCN/mg dust) after which the MDV load plateaued or increased slightly (4.4-5.7 \log_{10} VCN/mg dust) in all three vaccines until day 56 (Figure 2-16). Vaccine C had a significantly higher viral load in dust ($5.09\pm 0.14 \log_{10}$ VCN/mg dust) than vaccine A ($4.57\pm 0.14 \log_{10}$ VCN/mg dust) ($P=0.017$; Figure 2-16).

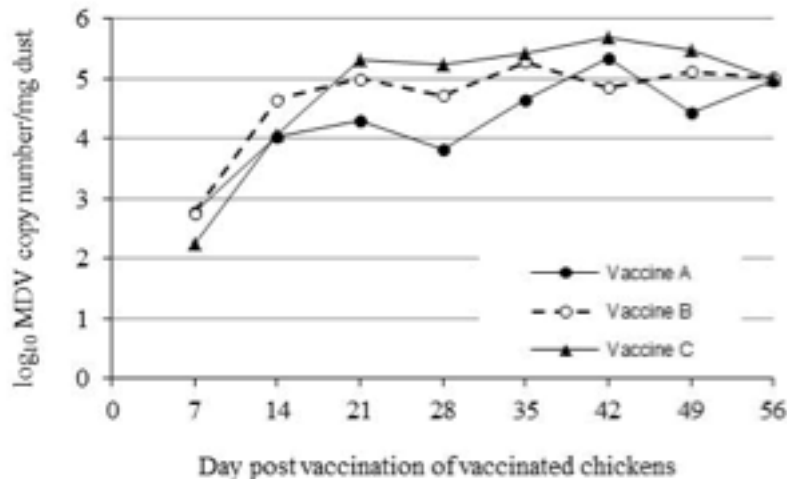


Figure 2-16: Overall effect of vaccine on the mean \log_{10} viral copy number per mg dust over time (LSM \pm SEM). Vaccinated and in-contact chickens combined as they were in the same room

2.2.7 MDV-specific serology

There was no significant effect of mode of infection ($P=0.36$) or vaccine ($P=0.19$) on the \log_{10} MDV antibody titre at day 56 with no significant interaction between these effects ($P=0.71$) (Figure 2-17).

Overall, 50/53 (94%) birds were positive with 25/27 (93%) positive from the vaccinated groups and 25/26 (96%) positive from the in-contact groups at day 56.

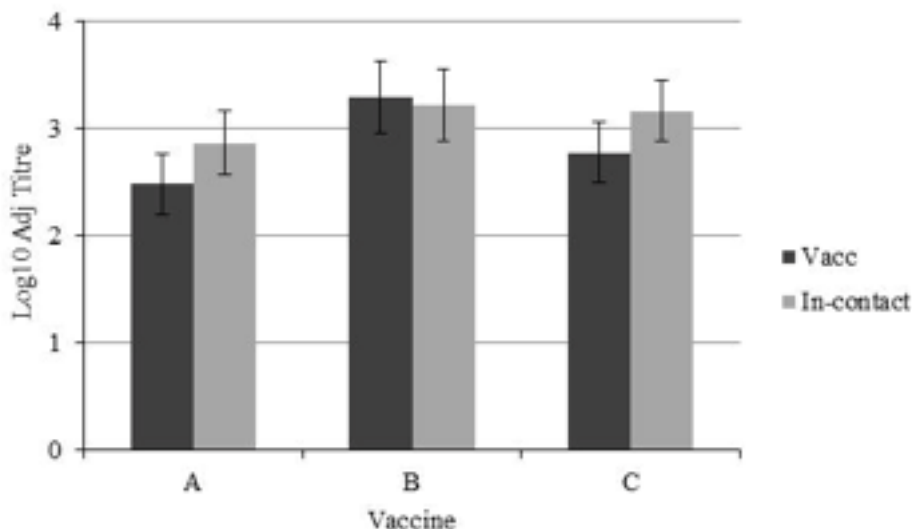


Figure 2-17: Mean (LSM \pm SEM) serum Log₁₀ MDV antibody titre of vaccinated and in-contact chickens grouped by three vaccines at day 56 post vaccination. There are no significant differences

2.2.8 Association between variables and prediction of anti MDV titre

Individual sample data

There was a significant positive overall linear association between \log_{10} MDV load in PBL and feathers overall ($n=180$, $R^2=0.15$ $P<0.0001$). The slope of the linear fit was very similar for vaccinated and in-contact chickens and the association was highly significant in both cases ($P<0.001$). The association was also positive at all sampling times, being statistically significant at days 14, 28, 42, 56. The maximal R^2 value of 0.66 was observed at day 14.

At day 56 MDV load in spleen had a positive and significant relationship with MDV load in both PBL ($R^2=0.34$, $P=0.0002$) and feather tips ($R^2=0.36$ $P=0.008$).

Anti-MDV1 titre at day 56 also had a significant positive linear association with day 56 MDV load in spleen ($R^2=0.31$ $P=0.0005$) and PBL ($R^2=0.10$ $P=0.009$), but not feather tips ($R^2=0.003$ $P=0.73$). MDV load in feather tip at earlier sampling dates had no significant predictive value for Anti-MDV1 titre at day 56 (R^2 range 0.0004- 0.15; P range 0.11-0.90). On the other hand, MDV load in PBL was a significant predictor at days 7 ($R^2=0.18$ $P=0.027$), 28 ($R^2=0.11$ $P=0.017$), 35 ($R^2=0.14$ $P=0.006$), 42 ($R^2=0.12$ $P=0.013$) and 49 ($R^2=0.08$ $P=0.05$).

Associations with weekly dust samples (room means)

MDV load in weekly dust samples was not significantly associated with MDV load in either PBL ($P=0.37$) or feather tip ($P=0.61$) with a weak negative association in both cases. Analysis within individual weeks (3 data points only) revealed a significant positive association between MDV1 in dust and PBL on days 28, 35, 49 and a significant negative association between dust and feathers on day 42. MDV load in weekly dust samples were not significant predictors of day 56 anti MDV titre, or day 56 MDV load in spleen apart from day 42 dust samples which were significantly and positively associated with the latter ($R^2 =0.99$, $P=0.018$).

2.7 Discussion

The study has demonstrated that all three Rispens vaccine viruses commercially available in Australia shed in dust as early as day 7 post-vaccination. The experiment also revealed that the shed virus transmits very effectively to in-contact chickens. The MDV detection rate in PBL peaked soon after infection and declined rapidly between 42 and 56 days later. Similarly, the MDV detection rate in feather tips also peaked soon after infection but declined gradually over the experimental period until day 42 then increased slightly until day 56. However, these declines are not matched by a decline in the amount of shed virus, which was maintained at high levels during this period.

With regards to the first hypothesis this study demonstrated that current vaccinal strains of CVI988 are shed readily from the vaccinated chickens into the environment in large amounts (10^5 - 10^6 VCN/ mg dust), and are detected readily in dust samples from day 7 onwards. The concentration of CVI988 in dust increased up to 42 and then plateaued until 56. Given the increase in dander production that occurs with increasing age, total virus shed per bird would have increased steadily with age during this study. Renz (2008) reported that dander production in layer birds increased from 5.2mg at 12 days of age to 55mg per chicken at 61 days of age, considerably less than the values reported for broiler chickens (Islam *et al.*, 2007). A previous study showed that the dust content in the air increased with the age of broiler chickens (Islam *et al.*, 2008) and at 5 days of age a broiler chicken can potentially inhale up to 50L of air per day rising to more than 300L per day at 15 days of age (Fedde *et al.*, 1998). With the increasing shedding rate of Rispens in the environment and the increasing air intake of the chickens over time, it is clear that chickens will inhale increasing amounts of Rispens virus with increasing age.

With regards to the second hypothesis, this study has clearly demonstrated efficient transmission of the vaccine virus to in-contact birds. When Rispens/CVI988 was first detected and tested for its properties, Rispens *et al.* (1972b) reported that at a passage level of 35 the virus spread directly to contact chickens as determined by virus isolation and antibody levels. In that study, 35 unvaccinated chickens were placed in contact with 190 vaccinated chickens under isolated conditions to monitor the contact transmission of the vaccinal virus. The birds were kept over a 2-year period. Antibodies were found in all vaccinated and contact birds. Antibody levels were high throughout the observation period and they did not differ significantly from those found after a natural MDV infection. To confirm the spreading capacity of CVI988, Rispens *et al.* (1972b) performed an additional experiment. In this experiment the virus could be reisolated from buffy coat cells from 2/5 contact chickens at 4 weeks. At week 5, virus was reisolated from buffy coat (1/5), feather tips (3/5) and serology results were positive for all 5 in-contact birds (Rispens *et al.*, 1972a). However, a plaque purified clone of CVI 988 named CVI 988/C with passage level of 65 (4) showed only limited transmission to in-contact chickens (0/8 by virus isolation and 4/10 by serology) (Witter *et al.* 1987) and CVI988 with an initial passage level of 42 showed inefficient transmission to in-contact chickens (Witter *et al.* 1995). In the latter report low levels of transmission were possibly due to limited transmission in isolators given the rapid rate of removal of airborne dander. These results suggest that the ability of CVI988 to transmit is negatively associated with passage level in cell culture. The passage level of current commercial vaccine strains is confidential, but given that CVI988 seed was made available to vaccine companies at passage level 33 (van Iddekinge *et al.* 1999) it is a reasonable inference that currently used vaccine strains have a passage level in the range of 35-45 and this is a possible explanation for the improved transmission in vivo compared with CVI988/C.

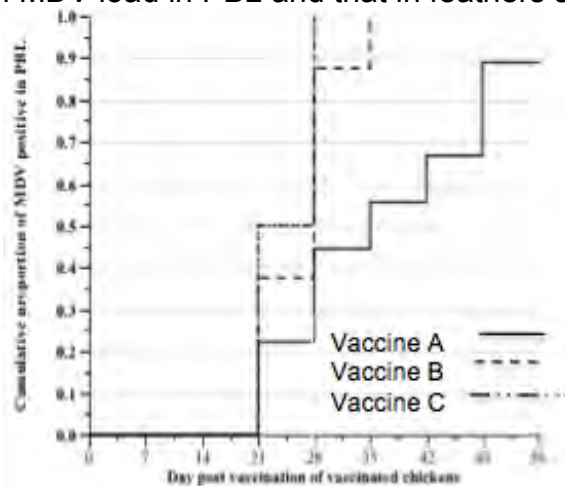
The third hypothesis was that the replication kinetics in PBL, feather tips, viral load in dust, final viral load in spleen and final antibody titre against MDV would not differ significantly between the three commercial vaccines used. Some data, such as those for MDV load in feather tips, antibody titre and MDV load in spleen at day 56 showed no differences and thus supported the hypothesis. However, there were clear differences between vaccines in the overall MDV load in PBL and dust and the rate of transmission of MDV from vaccinated to in-contact birds as determined by MDV presence in PBL.

The fourth hypothesis was that replication rates and shedding of Rispens CVI988 would be lower than published values for wild-type MDV due to the effects of attenuation. A previous study has shown that the most common MD vaccine used in broilers, Herpesvirus of Turkeys (HVT) is shed in dander at lower rates than MDV1, but that apathogenic MDV2 can shed at higher rates than MDV1 (Islam *et al.* 2007) so the association between virulence/attenuation and rate of virus shedding is not clear. The same authors found little difference in the shedding rate of three isolates of MDV1 differing little in virulence, while Renz (2008), working with MDV1 isolates with a wider virulence range, did find a positive association between virulence and rate of shedding of MDV. This is supported by the findings of the present experiment in which the mean vaccine virus load in PBL never exceeded 10^4 VCN/ 10^6 PBL while values of 10^5 - 10^6 VCN/ 10^6 PBL have been reported for pathogenic MDV up to 35 dpi using the same methods (Islam *et al.* 2006). The mean vaccine viral load in feather tips also never exceeded 10^6 VCN/ 10^6 feather tip cells while values of 10^8 VCN/ 10^6 feather tips have been reported for pathogenic MDV at 56 dpi using same method (Islam *et al.* 2006). In addition, MDV load in PBL continued to increase up to day 35 with virulent MDV (Islam *et al.* 2006), while with the CVI988 vaccine, values peaked early (days 7-14) then declined in both the present study and that of Baigent *et al.* (2005). With regards to virus shedding, vaccinal MDV values in dander in the present study were approximately $2 \log_{10}$ lower than reported values of 10^6 - 10^7 VCN/mg of dander for virulent wild type virus (Islam *et al.* 2007). These findings support the fourth hypothesis.

The early decline in viral load in PBL and feather tips appears to be a characteristic of current strains of CVI988. Baigent *et al.* (2005) reported the presence of the virus in both PBL and

feather tips at 4 dpv with numbers increasing to a peak at 14 dpv before gradually decreasing until the last day of their experiment at 28 dpv. Haq *et al.* (2012) also reported the same pattern in feather tips. The pattern was somewhat different in the present experiment with a peak viral load in PBL at day 7, declining by about 1 log to day 21 with a further 1.5 log₁₀ decline between days 42 and 56, the viral load in feather tips peaked at day 14 and then declined 1 log₁₀ every week until day 42 then increased slightly by until day 56. This appears to differ from wild-type MDV in which load of MDV in PBL continues to increase, at least to day 35 post infection (Islam *et al.* 2006). In our study the decline in load in PBL and feather tips was due largely to birds reverting to a negative PCR result for MDV in PBL and feather tips over time. Given the continuing high levels of shedding of virus at a group level, it appears that these birds are not eliminating infection but that infection is falling below the detection threshold. This may be due to virus entering latency, perhaps in response to the host immune response. The lack of a correlated decline in shedding of MDV in dander supports our notion that the replicative cycle of MDV and the pathogenic cycle are to some degree independent from each other. So from the above discussion it can be said that PBL sampling at day 7 post vaccination and feather sampling at day 14 post vaccination would be the best time to sample chicks to measure success of vaccination.

With regard to association between the measured variables there was a strong association between MDV load in PBL and that in feathers supporting the similar



pattern observed in

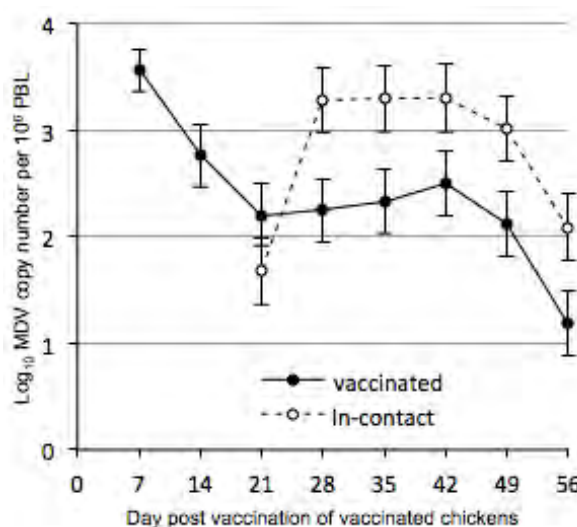


Figure 2-6 and

Figure 2-10. Both of these at day 56 were also significantly associated with MDV load in spleen. On the other hand, there was no overall association between MDV load in

weekly dust samples and that in PBL or feathers suggestive of a weaker relationship, as can be observed in the different patterns seen in

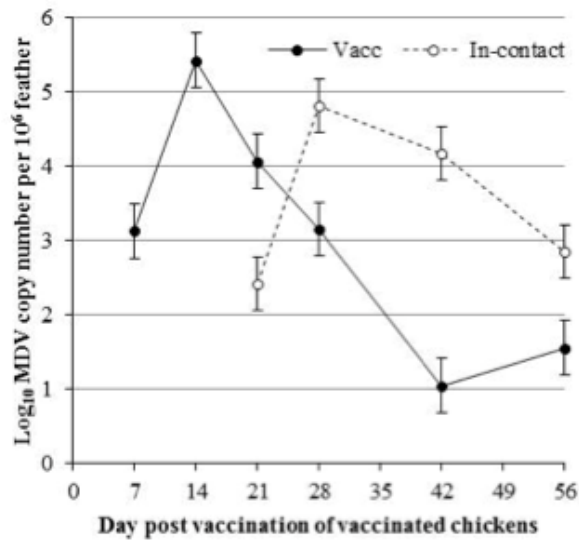
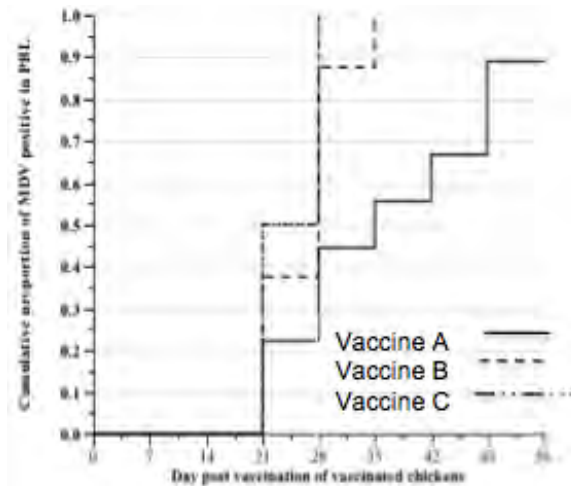


Figure 2-13 where dust samples show no decline in values towards the end of the



experiment unlike those in PBL

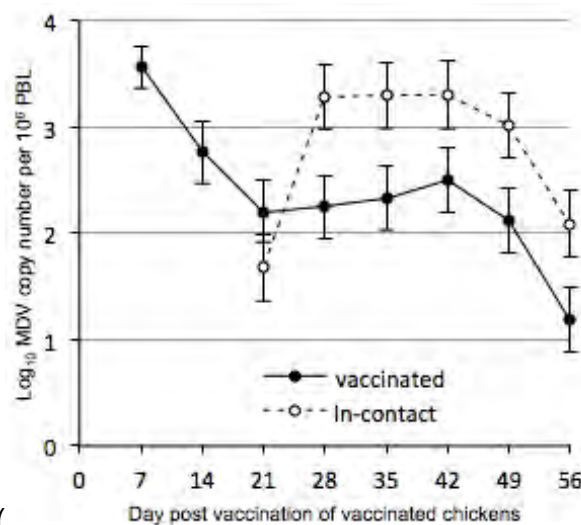
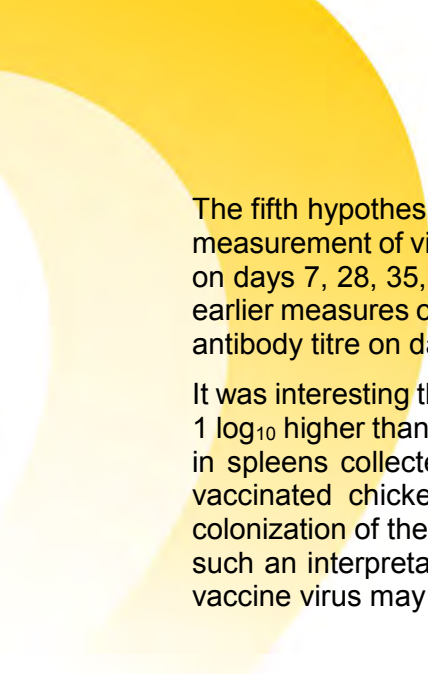


Figure 2-6 and feathers (

Figure 2-10. This suggests that the extracted DNA from feather tips may be more representative of leucocytes in the feather pulp, than of dander.

A large, abstract yellow graphic consisting of overlapping curved shapes is located in the top-left corner of the page.

The fifth hypothesis was that anti MDV antibody titre on day 56 would be predicted by early measurement of viral load in PBL, feather or dust. This was certainly true for viral load in PBL on days 7, 28, 35, 42 and 49 as well as being significantly associated on day 56. However, earlier measures of MDV load in feathers and dust were not significant predictors of anti MDV antibody titre on day 56. Thus the hypothesis is supported for PBL but not feathers or dust.

It was interesting that the viral load in PBL and feather tips of in-contact chickens were nearly 1 log₁₀ higher than that of vaccinated birds at an equivalent point post infection and viral load in spleens collected at day 56 were also 1 log₁₀ higher in contact chickens compared to vaccinated chickens. This suggests that natural infection may result in more effective colonization of the host although potential differences in dose and duration of infection make such an interpretation speculative. Additionally the single passage through the host by the vaccine virus may influence its infective capacity.

3. Experiment 2: Replication kinetics and shedding of very virulent Marek's Disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections

This experiment (milestone 3, part 1) was designed in order to determine the effects of widely divergent vaccination to challenge intervals, including challenge prior to vaccination, on the replication kinetics of Rispens and vvMDV in PBL, feather tips and dust samples.

The specific hypotheses under test were:

1. Viral load of pathogenic MDV in PBL, feather cells and dust will be higher than that of the Rispens virus
2. Vaccination with Rispens vaccine virus will significantly reduce the load of pathogenic MDV in PBL, feather cells and dust if administered before challenge and this effect will be greater the longer the vaccination to challenge interval
3. Challenge with pathogenic MDV1 will not influence the load of Rispens vaccine virus in PBL, feather and dust
4. The pattern of viral load of over time will differ between pathogenic MDV and Rispens in PBL and feathers but not dust and this will not be affected by co-infection.

PhD candidate Tanzila Islam contributed significantly to this experiment. The results of this experiment have been published:

Islam, T., Stephen, W., Walkden-Brown, S.W., Katrin, G., Renz, K.G., Fakhrul Islam, A.F.M., and Ralapanawe, S. (2013): Replication kinetics and shedding of very virulent Marek's Disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. *Veterinary Microbiology*, (submitted).

3.1 Overview of method

3.1.1 Experimental design

The experiment involved 600 female ISA Brown chickens of a single age group in an incomplete 2 x 2 x 3 x 3 factorial design replicated twice at the isolator level using a total of 24 isolators. Two isolators contained unvaccinated and unchallenged controls injected with relevant diluent only.

The factors and levels in the design were:

- Challenge virus: Two levels; unchallenged (diluent only) or challenged with vvMDV isolate 02LAR @ 400pfu/bird
- Vaccine virus: Two levels, unvaccinated (diluent only) or vaccinated with Rispens CVI988 (Bioproperties) @ 3200pfu/bird
- Challenge day: Three levels i.e. challenged at days 0, 5 or 10 of age
- Vaccination day: Three levels i.e. vaccinated at days 0, 5 or 10 of age.

Based on challenge and vaccination days there were five different vaccination to challenge intervals (VCI) i.e. -10, -5, 0, 5 and 10 days. Twelve treatment combinations were selected from the complete factorial design (**Table 3-1**) with each treatment replicated in two isolators.

Vaccination and challenge was performed at UNE as outlined in the general materials and methods section.

Table 3-1: Combination of treatments showing treatment abbreviations and vaccination challenge interval and the treatments are included in each of the statistical analyses (X indicates inclusion)

No.	Treatment	Day of vaccination	Day of challenge	VCI	Analysis 1	Analysis 2	Analysis 3
1	MDV d0	Unvacc.	0	n/a	X		
2	MDV d5	Unvacc.	5	n/a	X		
3	MDV d10	Unvacc.	10	n/a	X		
4	RIS d0	0	Unchall.	n/a		X	
5	RIS d5	5	Unchall.	n/a		X	
6	RIS d10	10	Unchall.	n/a		X	
7	VCI -10	10	0	-10		X	X
8	VCI -5	5	0	-5		X	X
9	VCI 0	0	0	0	X	X	X
10	VCI 5	0	5	5	X		X
11	VCI 10	0	10	10	X		X
12	Neg cont.	Unvacc.	Unchall.	n/a			

MDV: Challenged with MDV isolate 02LAR; RIS: Vaccinated with Rispens CVI988; Unvacc.: Unvaccinated; Unchall.: Unchallenged; n/a: not applicable.

3.1.2 Experimental chickens and management

The experimental chickens were newly hatched female ISA Brown commercial layer chickens. The chickens were unvaccinated but came from a Rispens CVI988-vaccinated parent flock so would have had maternal antibody directed against MDV1. There were 25 chickens placed in each isolator initially giving 50 chicks per treatment combination.

Chickens were fed *ad libitum* on commercial layer starter then grower diets (Ridley Agricultural Products, Tamworth, Australia). After two days of 24 hour light, chicks were then exposed to a 12L:12D lighting cycle in positive pressure isolators containing autoclaved rice hulls in scratch trays and hanging twine for environmental enrichment. Temperature settings were reduced from 35°C to 21°C at a rate of 1°C every second day. The experiment was approved by the University of New England Animal Ethics Committee (AEC No. UNE 10/057).

3.1.3 Sample collection, DNA extraction and qPCR

Blood samples, feathers and isolator dust were collected longitudinally from five individually wing tagged birds per isolator weekly throughout the experiment commencing at 7 days post infection (dpi). Where the vaccine and challenge virus had different dpi, chicks were sampled separately for each virus so they could be compared at the same dpi. These samples were used to quantify viral copy numbers of both viruses in peripheral blood lymphocytes (PBL), feather tips and dust respectively.

For blood and feather samples, five birds from each isolator were tagged at the beginning of the experiment and blood and feather samples collected from them. Blood samples were collected into 1.5ml microfuge tubes pre-loaded with 150 µl of 3% sodium citrate prior to separation of PBL using Ficoll Paque™ 195 PREMIUM (Amersham Biosciences, Sweden). Feathers were sampled at 7 and 14 dpi from the wing and subsequently from the axillary tract (3-5 feathers per sampling). However, only feather samples from 7, 14 and 21 dpi were analysed for MDV load by qPCR. Weekly dust samples were collected from the exhaust duct of each isolator. All samples were stored at -20°C until further processing.

DNA was extracted from PBL using the automated X-tractor Gene including reagents (Corbett Robotics, Australia) according to the manufacturer's instructions. DNA from feather tips (proximal end) was extracted from 6 birds per treatment at days 7, 14, 21, 28, 42 and 56 using the ISOLATE genomic DNA mini kit (Bioline, Australia) following the instructions given by the manufacturer. Approximately 5mg of dust was extracted using the same kit according to the manufacturer instructions. All extracted DNA samples were stored at -20°C until further analysis.

Prior to quantitative real-time PCR (qPCR), extracted DNA was quantified by spectrophotometric analysis using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies Wilmington, USA), and diluted to a concentration of 5 ng µl⁻¹ for use in the qPCR assays.

For determination of viral load in PBL, feather tip and dust samples, TaqMan® serotype-1 MDV real-time qPCR assays specific for either pathogenic MDV1 or Rispens were performed using a Rotor Gene 3000 real-time PCR instrument (Corbett Research, Australia).

3.1.4 Statistical analysis

Analyses were performed using JMP10 (SAS Institute Inc. 2010). Three different analyses were performed on different data sets within the experiment to test the various hypotheses. For each analysis a mixed restricted maximum likelihood (REML) model was used with individual chicken or isolator fitted as a random effect. This accounts for the repeated measures taken on individual chickens (PBL, feathers) or isolators (dust).

Analysis 1 investigated the effects of challenge day and vaccination status on pathogenic MDV load in chickens challenged with pathogenic MDV on days 0, 5 or 10 of age, with or without vaccination with Rispens on day 0 (full factorial with 6 treatments). Effects fitted included chicken (or isolator) as a random effect, challenge day, vaccination status (vaccinated or not at day 0), dpi and interactions between these effects.

Analysis 2 investigated the effects of vaccination day and challenge status on Rispens virus load in chickens vaccinated with Rispens on days 0, 5 or 10 of age, with or without challenge with pathogenic MDV on day 0 (full factorial with 6 treatments). Effects fitted included chicken (or isolator) as a random effect, vaccination day, challenge status (challenged or not at day 0), dpi and interactions between these effects.

Analysis 3 investigated the effects of VCI on the viral load in chickens infected with both Rispens and pathogenic MDV virus at VCI of -10, -5, 0, 5 and 10 days (non factorial, 5 treatments). Effects fitted included chicken (or isolator) as a random effect, VCI, virus type (Rispens or pathogenic), dpi and interactions between these effects.

MDV load in PBL, feather and dust were log transformed [$\text{Log}_{10}(y + 1)$] prior to analysis. Each analysis was generally performed twice for each tissue in which MDV was quantified. The first analysis included all samples, including those negative for the virus, while the second analysis included positive samples only. However, day 7 samples were excluded from second analysis of dust in Analysis 1 and Analysis 3. Differences between different levels within a significant main effect were tested using Student's t test. Associations were explored between MDV load in PBL, feathers and dust at various sampling times. Association between VCI and MDV load in different tissues were tested by pairwise correlation and linear

regression analysis of specific associations. Least squares means (LSM) and standard errors of the mean (SEM) are presented for continuous variables. A significance level of $P < 0.05$ is used throughout.

3.2 Results

3.2.1 Application of treatments

All vaccination and challenge treatments were successfully applied and maintained, as determined by MDV1 differential real-time qPCR. All the control chickens were negative for Rispens and pathogenic MDV1 in PBL, feather and dust. Challenged birds from each challenge day were positive for pathogenic MDV1 and vaccinated birds from each vaccination day were positive for Rispens.

In PBL the percentage of samples positive for pathogenic MDV was 68% overall and ranged from 14-98% depending on treatment. For Rispens only 45% of samples were positive overall and the range was 20-59% depending on treatment (**Figure 3-1a**).

In feather cells the proportion of samples positive for pathogenic MDV was 62% overall and ranged from 33-90%. For Rispens it was 54% overall and the range was 10-83% (**Figure 3-1b**). In dust the proportion of samples positive for pathogenic MDV was 78% overall and ranged from 68-94%. For Rispens it was 89% overall and the range was 68-100% (**Figure 3-1c**).

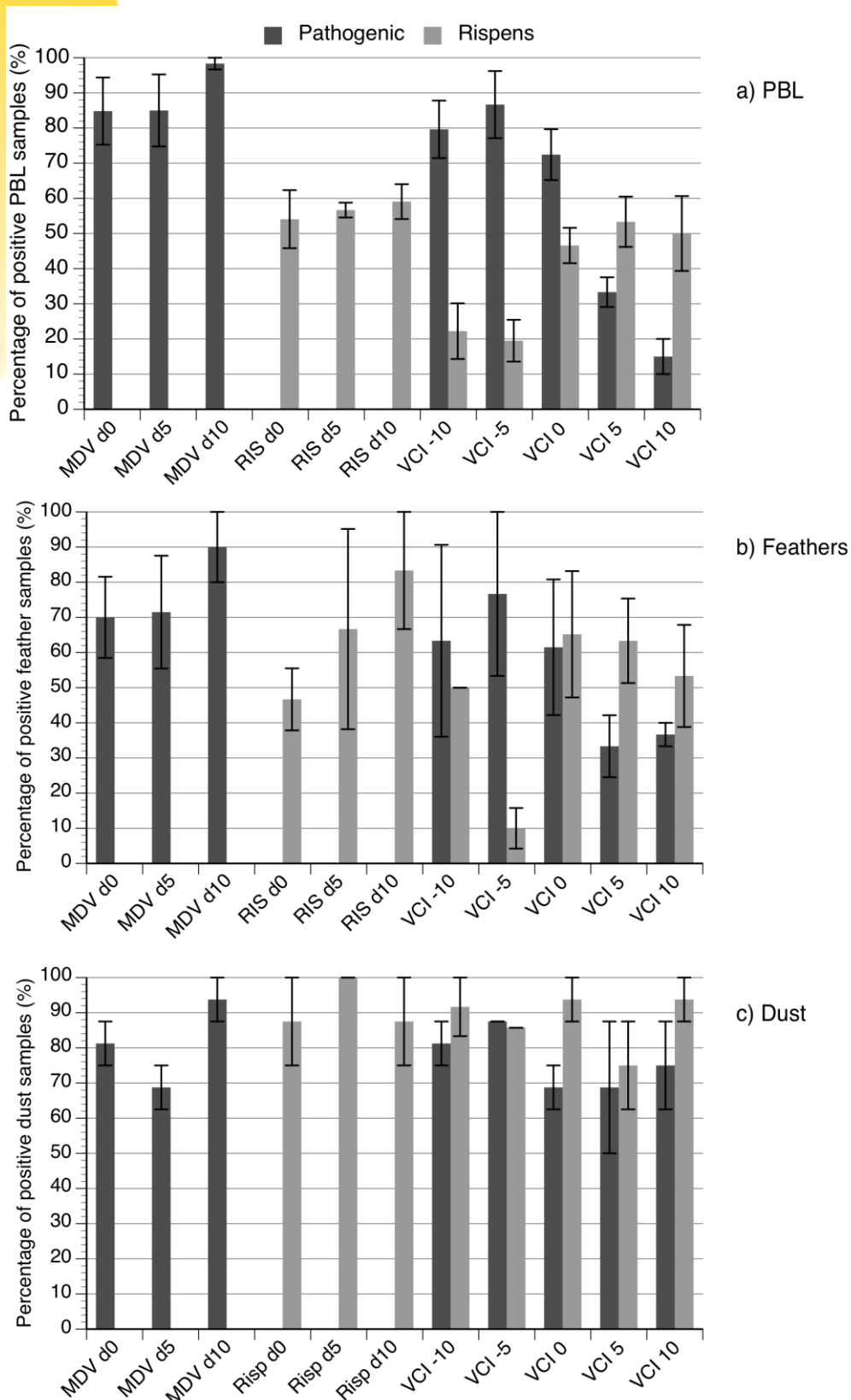


Figure 3-1: Percentage of chickens positive for pathogenic MDV and Rispens virus in each treatment in a) PBL, b) feather cells and c) dust as determined by qPCR

3.2.2 Viral load in PBL

Analysis 1 (Pathogenic MDV)

With samples from all chickens included there were significant effects of vaccination status ($P < 0.0001$), challenge day ($P = 0.02$) and dpc ($P < 0.0001$) on overall pathogenic MDV viral load in PBL in all birds with no significant 3-way interaction between them ($P = 0.2$). However the interaction between vaccination status and challenge day was significant ($P < 0.0001$; **Figure 3-2a**); as was the interaction between vaccination status and dpc ($P = 0.005$; **Figure 3-3a**).

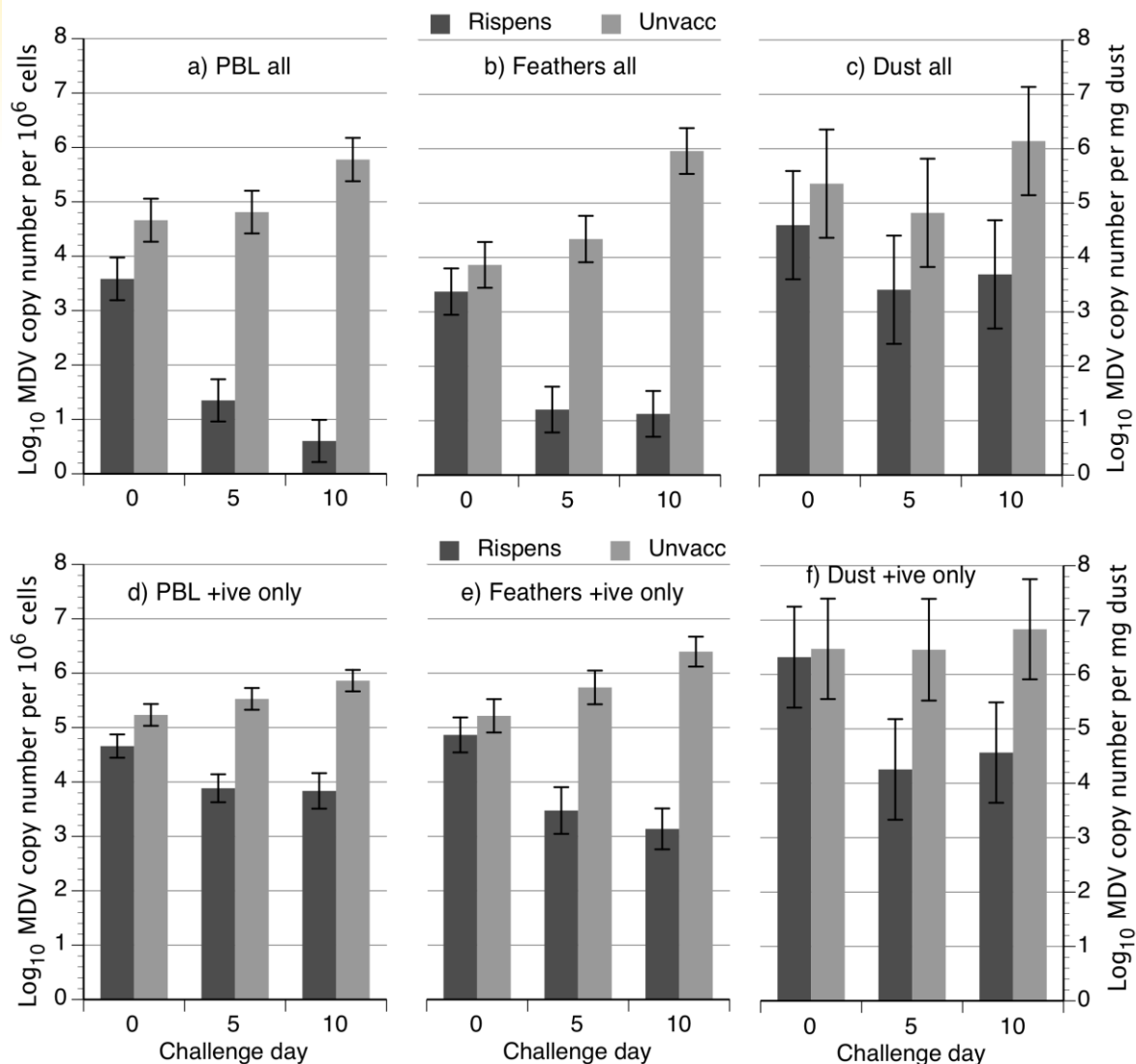


Figure 3-2: Analysis 1. Mean (LSM±SEM) Log₁₀ viral copy number of pathogenic MDV in PBL (a), feather cells (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of vaccination status (Rispens or unvaccinated) and challenge day (0, 5 or 10). Chickens were vaccinated or not at day 0 (day of hatch) and challenged with vvMDV 02LAR on days 0, 5 or 10

Overall, pathogenic MDV viral load in PBL [$\text{Log}_{10} (y + 1) \text{VCN}/10^6 \text{ cells}$] was significantly ($P < 0.0001$) higher (5.08 ± 0.23) in unvaccinated chickens than vaccinated chickens (1.85 ± 0.22) with this due to significant effects for challenge days 5 and 10 but not 0 (**Figure 3-2a**). Moreover the pathogenic MDV viral load was significantly higher in unvaccinated chickens than vaccinated chickens at every sampling time from 7 to 56 dpc (**Figure 3-3a**). In unvaccinated chickens, the pathogenic MDV load increased rapidly from 7 dpc (3.01 ± 0.32) to 42 dpc (6.06 ± 0.34) then plateaued until 56 dpc (6.08 ± 0.38). On the other hand in vaccinated chickens, the pathogenic MDV load increased gradually from 7 dpc (0.73 ± 0.32) to 21 dpc (2.19 ± 0.32) then plateaued until 42 dpc (2.33 ± 0.32), after which it decreased slightly to 56 dpc (1.97 ± 0.33).

Analysis of positive samples only revealed significant effects of vaccination status ($P < 0.0001$) and dpc ($P < 0.0001$) but not challenge day ($P = 0.5$) on overall pathogenic MDV viral load in PBL.

The interaction between vaccination status and challenge day 277 was significant ($P = 0.007$; **Figure 3-2d**); with no significant interaction between vaccination status and dpc ($P = 0.3$; **Figure 3-3d**).

Overall, pathogenic MDV viral load in PBL [$\text{Log}_{10} (y + 1) \text{VCN}/10^6 \text{ cells}$] was significantly ($P < 0.0001$) higher (5.54 ± 0.12) in MDV positive samples from unvaccinated chickens than vaccinated chickens (4.13 ± 0.16) with the difference again due to chickens challenged at days 5 and 10 but not 0 (**Figure 3-2d**). Pathogenic MDV load in MDV positive samples was significantly higher in samples from unvaccinated chickens than vaccinated chickens at all dpc (**Figure 3-3d**). In MDV-positive samples from unvaccinated chickens, the pathogenic MDV load increased gradually from 7 dpc (4.73 ± 0.18) to 42 dpc (5.98 ± 0.15) then plateaued until 56 dpc (5.99 ± 0.16). In MDV positive samples from vaccinated chickens, the pathogenic MDV load showed a broadly similar pattern increasing gradually from 7 dpc (3.29 ± 0.31) to 28 dpc (4.47 ± 0.21) then plateauing until 42 dpc (4.52 ± 0.20), after which it decreased slightly to 56 dpc (4.40 ± 0.24).

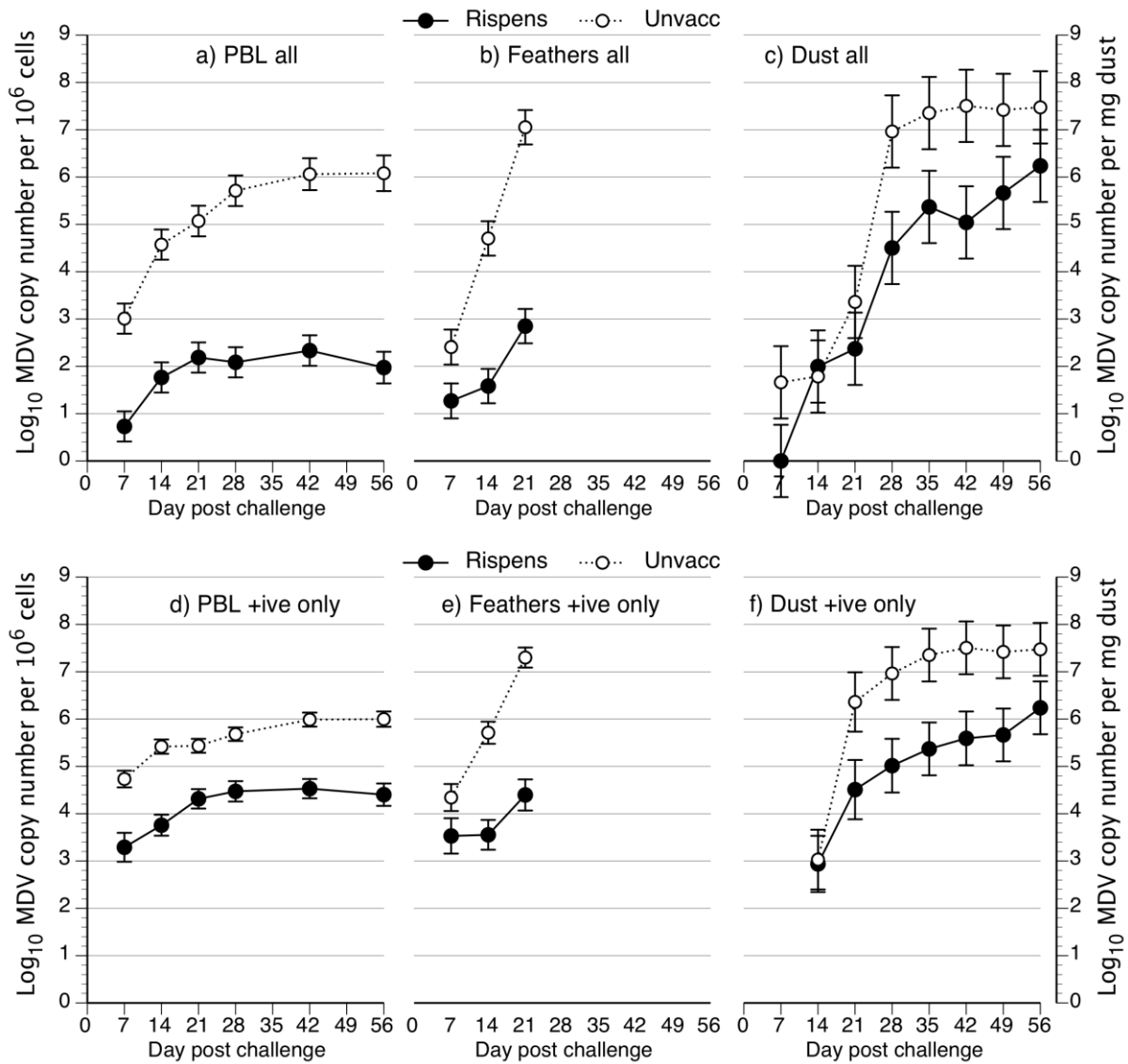


Figure 3-3: Analysis 1. Mean (LSM±SEM) Log₁₀ viral copy number of pathogenic MDV in PBL (a), feather tips (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of vaccination status (Rispens or unchallenged) and day post challenge. Chickens were either vaccinated or not at day 0 (day of hatch) and challenged with vvMDV 02LAR on days 0, 5 or 10

Analysis 2 (Rispens virus)

With samples from all chickens included there was a significant effect of challenge status ($P = 0.0009$), on overall Rispens MDV viral load in PBL, but not vaccination day ($P = 0.7$), dpv ($P = 0.2$) or 3-way interaction between them ($P = 0.5$). Neither the interaction between challenge status and vaccination day ($P = 0.3$; **Figure 3-4a**) nor that between challenge status and dpv ($P = 0.9$; **Figure 3-5a**) were significant.

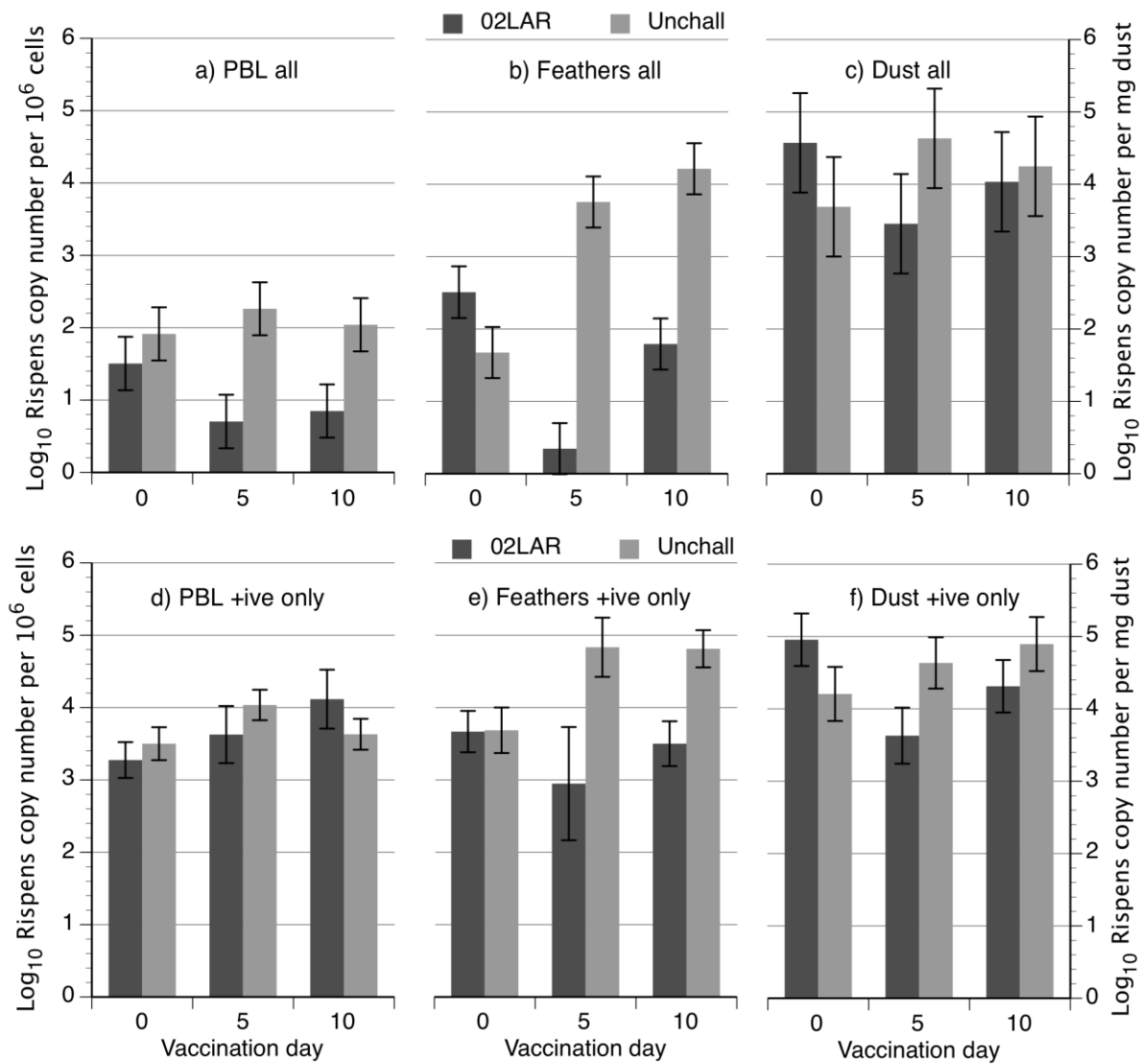


Figure 3-4: Analysis 2. Mean (LSM±SEM) Log₁₀ viral copy number of Rispens MDV in PBL (a), feather cells (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of challenge status (02LAR or unchallenged) and vaccination day (0, 5 or 10). Chickens were challenged or not at day 0 (day of hatch) and vaccinated with Rispens on days 0, 5 or 10

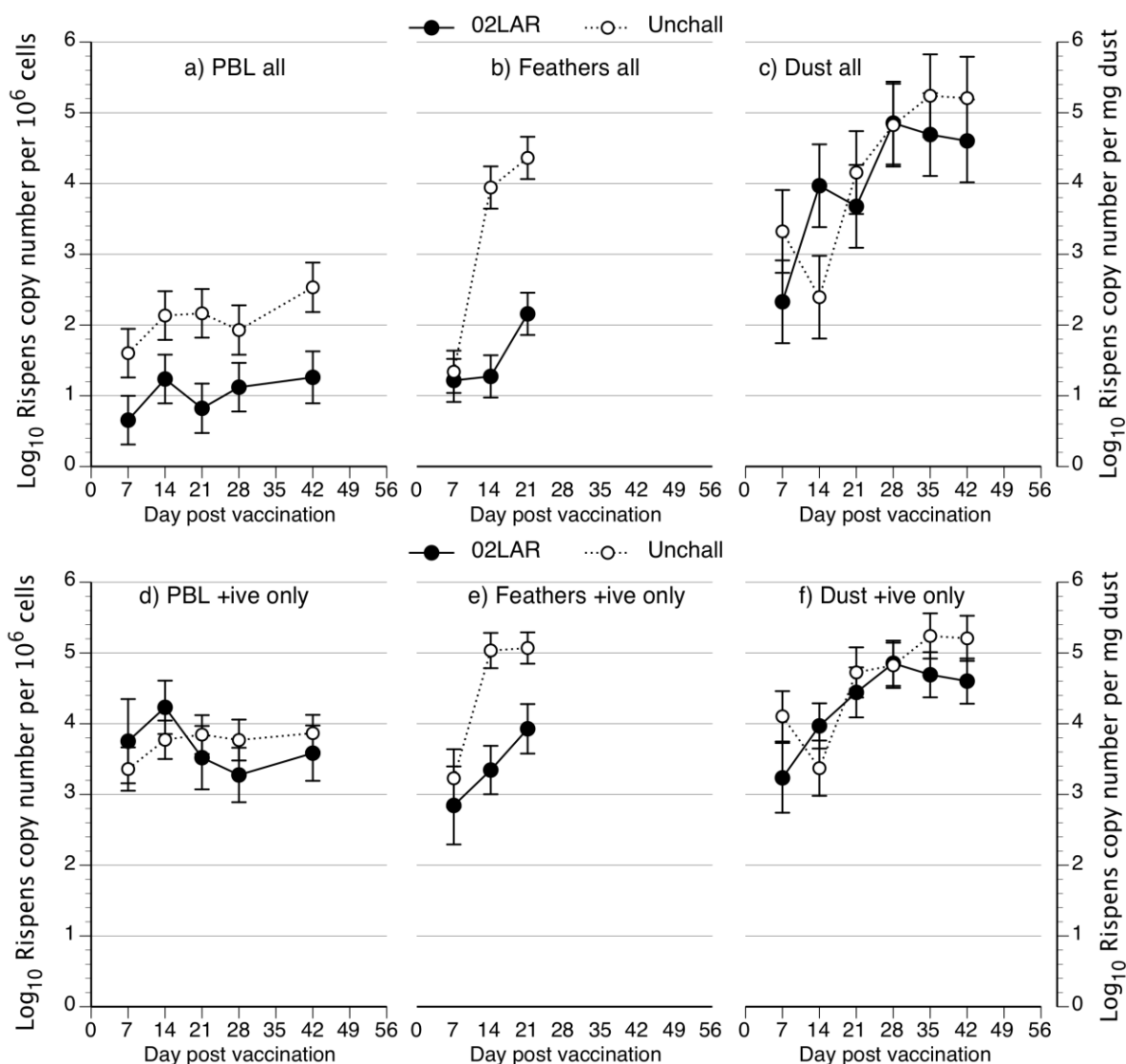


Figure 3-5: Analysis 2. Mean (LSM±SEM) Log₁₀ viral copy number of Rispens MDV in PBL (a), feather tips (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of challenge status (02LAR or unchallenged) and day post vaccination. Chickens were challenged or not at day 0 (day of hatch) and vaccinated with Rispens on days 0, 5 or 10

Overall, Rispens MDV viral load in PBL [Log₁₀ (y+1) VCN/10⁶ cells] was significantly ($P = 0.0009$) higher (2.07 ± 0.21) in unchallenged chickens than challenged chickens (1.01 ± 0.21) due mainly to significant effects in chickens vaccinated on days 5 and 10 (**Figure 3-4a**). In unchallenged chickens, the Rispens MDV load increased from 7 dpv (1.6 ± 0.34) to 14 dpv (2.13 ± 0.34) then plateaued until 28 dpv (1.93 ± 0.35), before increasing slightly until 42 dpv (2.53 ± 0.35). Challenged chickens showed a broadly similar response increasing irregularly from 7 dpv (0.65 ± 0.34) to 42 dpv (1.26 ± 0.36).

Analysis of positive samples only revealed no significant effects of challenge status ($P = 0.8$), vaccination day ($P = 0.2$) and dpv ($P = 0.7$) on overall Rispens MDV viral load in PBL, with no significant interaction between the effects of challenge status and vaccination day ($P = 0.4$; **Figure 3.4d**) or challenge status and dpv ($P = 0.5$; **Figure 3-5d**).

Analysis 3 (Effect of VCI on both viruses)

With samples from all chickens included there were significant effects of VCI ($P < 0.0001$), dpi ($P < 0.0001$) and virus ($P < 0.0001$) on overall viral load in PBL in all birds. The interaction between VCI and virus was significant ($P < 0.0001$; **Figure 3-6a**); as was the interaction between dpi and virus ($P = 0.01$; Figure 3.7a). Overall, viral load in PBL [$\text{Log}_{10} (y + 1)$ VCN/ 10^6 cells] of pathogenic MDV (2.94 ± 0.11) was significantly higher ($P < 0.0001$) than that of the Rispens virus (1.31 ± 0.11). Viral load of pathogenic MDV in PBL was significantly higher at VCI -10, -5 and 0, but not 5 or 10 (**Figure 3-6a**). It was also significantly higher ($P < 0.05$) than that of Rispens at all times dpi except at 7 dpi (**Figure 3-7a**).

Analysis of positive (positive to either virus) samples only revealed significant effects of VCI ($P = 0.002$), dpi ($P = 0.01$) and virus ($P < 0.0001$) on overall viral load in PBL. The interaction between VCI and virus was significant ($P = 0.0008$; **Figure 3-6d**); as was the interaction between dpi and virus ($P = 0.02$; Figure 3.7d). Overall viral load in PBL [$\text{Log}_{10} (y + 1)$ VCN/ 10^6 cells] of pathogenic MDV was significantly ($P < 0.0001$) higher (4.69 ± 0.11) than that of Rispens virus (3.46 ± 0.14). It was significantly ($P < 0.05$) higher at all VCI except in VCI 5 and VCI 10 (**Figure 3-6d**). Moreover, Rispens viral load was significantly lower ($P = 0.02$) than pathogenic viral load at all times dpi except at 7 and 14 dpi when the difference was not significant (**Figure 3-7d**).

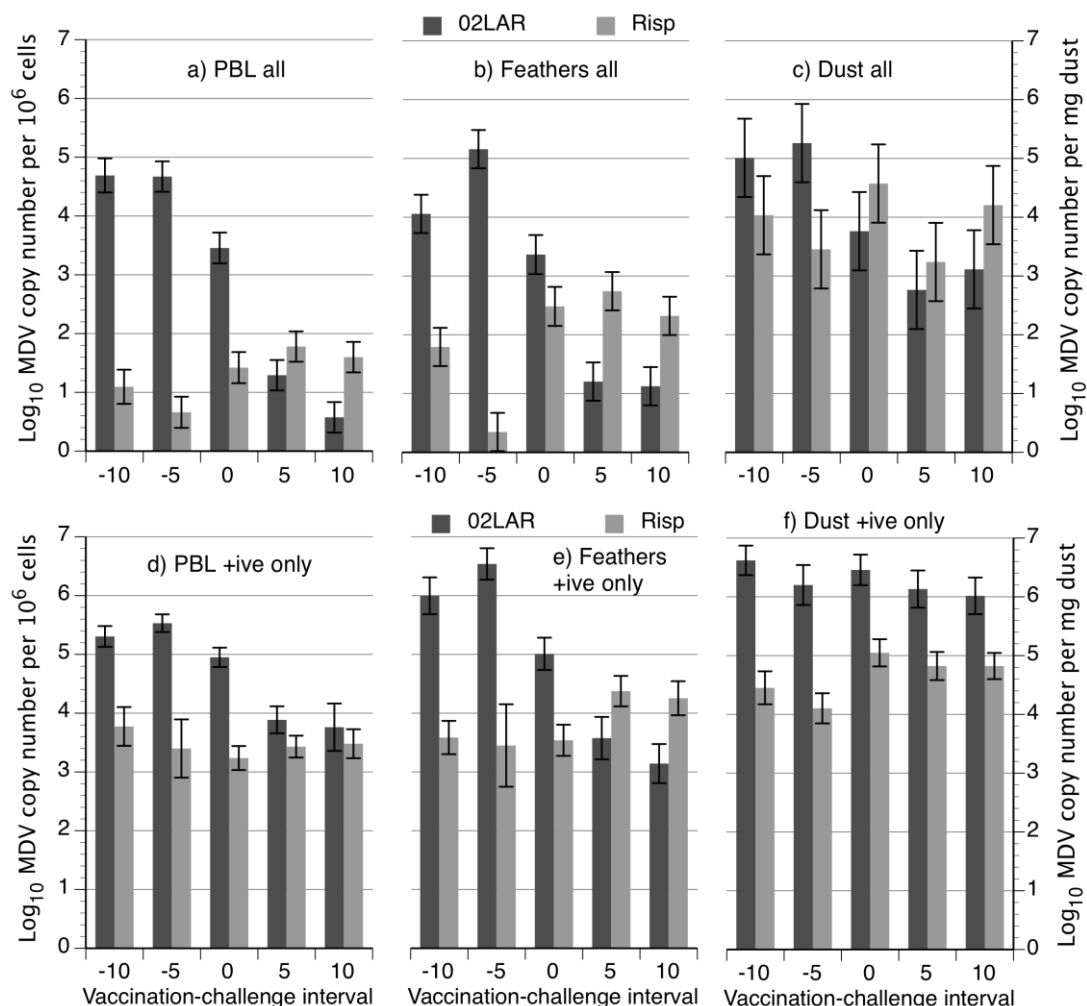


Figure 3-6: Analysis 3. Mean (LSM \pm SEM) Log_{10} viral copy number in PBL (a), feather cells (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of virus (02LAR or Rispens) and vaccination challenge interval (-10, -5, 0, 5 or 10)

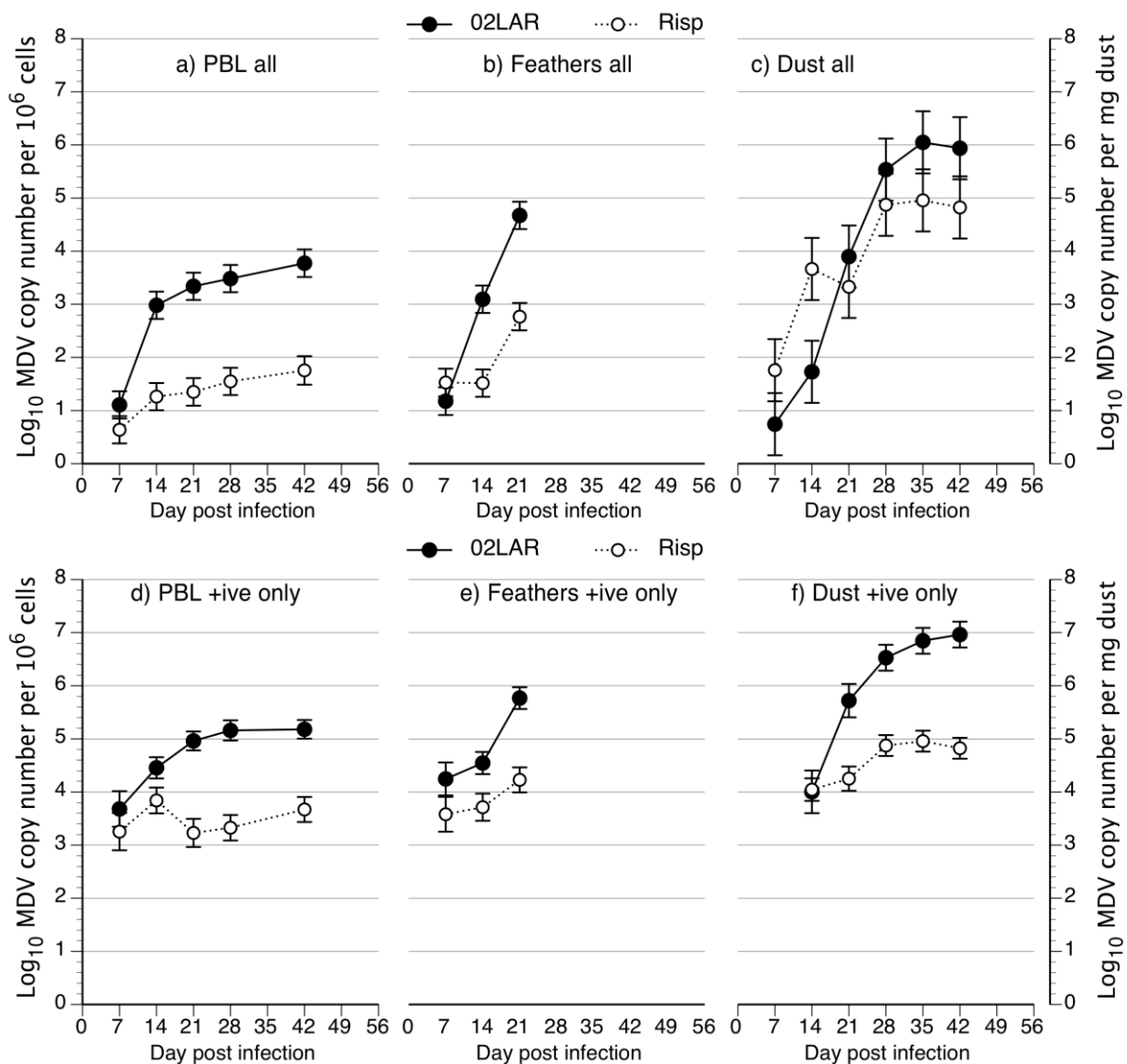


Figure 3-7: Analysis 3. Mean (LSM±SEM) Log₁₀ viral copy number in PBL (a), feather cells (b) and dust (c) in all samples, and in the same sample types (d-e) in positive samples, showing interaction between the effects of virus (02LAR or Rispens) and day post infection

3.2.3 Viral load in feather cells

Analysis 1 (Pathogenic MDV)

With all samples included there were significant effects of vaccination status ($P < 0.0001$) and dpc ($P < 0.0001$) but not challenge day ($P = 0.09$) on overall pathogenic MDV viral load in feather cells with significant three-way interaction between them ($P = 0.003$). The interaction between vaccination status and challenge day was also significant ($P < 0.0001$; **Figure 3-2b**) as was the interaction between vaccination status and dpc ($P < 0.0001$; **Figure 3-3b**). Overall, pathogenic MDV viral load in feather cells [$\text{Log}_{10} (y + 1) \text{VCN}/10^6 \text{ cells}$] was significantly ($P < 0.0001$) higher (4.72 ± 0.24) in unvaccinated chickens than vaccinated chickens (1.90 ± 0.24) with this effect due to significant differences at challenge days 5 and 10 (**Figure 3-2b**).

Pathogenic MDV viral load was significantly higher in unvaccinated than vaccinated chickens at each of the time periods assessed (7, 14 and 21 dpc) (**Figure 3-3b**). In unvaccinated chickens, the pathogenic MDV load increased rapidly and linearly from 7 dpc (2.41 ± 0.37) to 21 dpc (7.05 ± 0.36). In vaccinated chickens, the rate of increase over time was much less with pathogenic MDV load increasing gradually between 7 dpc (1.26 ± 0.36) and 21 dpc (2.85 ± 0.36) (**Figure 3-3b**).

Analysis of positive samples only revealed significant effects of vaccination status ($P < 0.0001$) and dpc ($P < 0.0001$) but not challenge day ($P = 0.4$) on pathogenic MDV viral load in feather cells. The interaction between vaccination status and challenge day was significant ($P = 0.0002$; **Figure 3-2e**); with significant interaction also between vaccination status and dpc ($P = 0.002$; **Figure 3-3e**).

Overall, pathogenic MDV viral load in feather cells [$\text{Log}_{10} (y + 1)$ VCN/ 10^6 cells] was significantly ($P < 0.0001$) higher (5.78 ± 0.17) in positive samples from unvaccinated chickens than vaccinated chickens (3.82 ± 0.21) with this effect due to significant differences at challenge days 5 and 10, but not 0 (**Figure 3-2e**). Pathogenic MDV load was significantly higher in positive samples from unvaccinated chickens than those from vaccinated chickens at 14 and 21, but not 7 dpc (**Figure 3-3e**).

In unvaccinated chickens, the pathogenic MDV load increased linearly from 7 dpc (4.34 ± 0.29) to 21 dpc (7.29 ± 0.21). On the other hand in vaccinated chickens, the pathogenic MDV load was low at 7 dpc and 14 dpc (approx. 3.5) then increased to 21 dpc (4.39 ± 0.33).

Analysis 2 (Rispens virus)

With all samples included there were significant effects of challenge status ($P < 0.0001$), vaccination day ($P = 0.1$) and dpv ($P < 0.0001$) on overall Rispens MDV viral load in feather cells with significant three-way interaction between these ($P < 0.0001$). The interaction between challenge status and vaccination day was significant ($P < 0.0001$; **Figure 3-4b**); as was the interaction between challenge status and dpv ($P < 0.0001$; **Figure 3-5b**).

Overall, Rispens MDV viral load in feather cells [$\text{Log}_{10} (y + 1)$ VCN/ 10^6 cells] was significantly ($P = 0.0009$) higher (3.21 ± 0.20) in unchallenged chickens than challenged chickens (1.54 ± 0.20). Rispens MDV viral load was significantly higher in unchallenged chickens than challenged chickens at challenge days 5 and 10, but not 0 (**Figure 3.4b**). In unchallenged chickens, the Rispens MDV load increased rapidly from 7 dpv (1.33 ± 0.29) to 14 dpv (3.94 ± 0.29) then more slowly to 21 dpv (4.36 ± 0.29). The pattern in challenged chickens was quite different with low values at 7 and 14 dpv (1.2 - 1.3) before increasing as 21 dpv to 2.16 ± 0.29 (**Figure 3-5b**).

Analysis of only positive samples revealed significant effects of challenge status ($P = 0.001$) and dpv ($P = 0.004$) but vaccination day ($P = 0.2$) on overall Rispens load in feather cells. The interaction between challenge status and vaccination day was significant ($P = 0.04$; **Figure 3-4e**); but that between challenge status and dpv was not ($P = 0.1$; **Figure 3-5e**). Overall, Rispens load in feather cells [$\text{Log}_{10} (y + 1)$ VCN/ 10^6 cells] was significantly ($P = 0.001$) higher (4.44 ± 0.19) in unchallenged chickens than challenged chickens (3.37 ± 0.29) again due to significant differences on challenge days 5 and 10 but not 0 (**Figure 3-4e**). In unchallenged chickens, the Rispens MDV load increased rapidly from 7 dpv (3.22 ± 0.41) to 14 dpv (5.03 ± 0.24) then plateaued until 21 dpv (5.06 ± 0.22). On the other hand in challenged chickens, the Rispens MDV load increased steadily from 7 dpv (2.84 ± 0.55) to 21 dpv (3.92 ± 0.35) (**Figure 3-5e**).

Analysis 3 (Effect of VCI on both viruses)

With all samples included there was significant effect of VCI ($P = 0.0003$), dpi ($P < 0.0001$) and virus ($P = 0.0001$) on overall viral load in feather cells with significant three-way interaction between them ($P < 0.0001$). The interaction between VCI and virus was also

significant ($P < 0.0001$; Figure 3.6b); as was the interaction between dpi and virus ($P < 0.0001$; **Figure 3-7b**). Overall, viral load in feather cells [$\text{Log}_{10} (y + 1) \text{VCN}/10^6 \text{ cells}$] was significantly ($P = 0.0001$) higher for pathogenic (2.98 ± 0.15) than Rispens virus (1.94 ± 0.15) but there was marked interaction with VCI such that load of pathogenic MDV was significantly higher than that of Rispens for VCI -10 and VCI -5 but the reverse was true for VCI 5 and 10, with no difference between the two for VCI 0 (**Figure 3-6b**). Viral load of pathogenic MDV was significantly higher ($P < 0.0001$) than that of Rispens at 14 and 21, but not 7 dpi (**Figure 3-7b**).

Analysis of only positive samples revealed significant effects of VCI ($P = 0.001$), dpi ($P < 0.0001$) and virus ($P < 0.0001$) on overall viral load in feather cells in positive to MDV only birds. The interaction between VCI and virus was significant ($P < 0.0001$; **Figure 3-6e**); but not the interaction between dpi and virus ($P = 0.1$; **Figure 3-7e**). Overall, viral load in feather cells [$\text{Log}_{10} (y + 1) \text{VCN}/10^6 \text{ cells}$] was significantly ($P < 0.0001$) higher for pathogenic MDV (4.85 ± 0.14) than Rispens (3.84 ± 0.18) but this effect was only observed for VCI 5 and VCI 10, not VCI 0 (**Figure 3-6e**).

3.2.4 Viral load in dust

Analysis 1 (Pathogenic MDV)

With all samples included there were significant effects of dpc ($P < 0.0001$), but not vaccination status ($P = 0.1$), challenge day ($P = 0.6$) or three-way interaction between them ($P = 0.1$) on pathogenic MDV viral load in dust in all birds. Neither the interaction between vaccination status and challenge day ($P = 0.7$; **Figure 3-2c**) nor that between vaccination status and dpc were significant ($P = 0.2$; **Figure 3-3c**).

Pathogenic MDV viral load in dust [$\text{Log}_{10} (y + 1) \text{VCN}/\text{mg}$] was numerically higher (5.44 ± 0.57) in unvaccinated chickens than vaccinated chickens (3.89 ± 0.57) but the difference did not achieve statistical significance ($P = 0.01$), possibly due to the much smaller number of dust samples analysed, relative to those for PBL and feathers. In unvaccinated chickens, the pathogenic MDV load increased rapidly from 7 dpc (1.66 ± 0.76) to 28 dpc (6.96 ± 0.76) then increased further slightly at 35 dpc before plateauing until 56 dpc (7.47 ± 0.76). In vaccinated chickens all samples were negative at 7dpc but the pathogenic MDV load increased rapidly from 14 dpc (1.99 ± 0.76) to 35 dpc (5.36 ± 0.76) before increased slowly until 56 dpc (6.24 ± 0.76) 1.23 logs lower than for pathogenic MDV (**Figure 3-3c**).

Analysis of only positive samples revealed significant overall effects of dpc ($P < 0.0001$) but not vaccination status ($P = 0.08$), challenge day ($P = 0.6$) on pathogenic MDV viral load in dust and interaction between vaccination status and challenge day ($P = 0.5$; **Figure 3-2f**). On the other hand there was significant interaction between the effects of vaccination status and dpc ($P = 0.01$; **Figure 3-3f**). Pathogenic MDV viral load in dust [$\text{Log}_{10} (y + 1) \text{VCN}/\text{mg}$] was numerically higher (6.58 ± 0.53) in unvaccinated chickens than vaccinated chickens (5.04 ± 0.53). In unvaccinated chickens, pathogenic MDV load increased from low levels at 14 dpi (3.03 ± 0.63) rapidly to 35 dpc (7.35 ± 0.55), before plateauing at around 7.5 (± 0.55) until 56 dpc. On the other hand in vaccinated chickens, the pathogenic MDV load increased from 14 dpc (2.93 ± 0.59) to 56 dpc (6.23 ± 0.55) with the bulk of the increase occurring between days 14 and 21 (**Figure 3-3f**).

Analysis 2 (Rispens)

With all samples included there was a significant effect of dpv ($P < 0.0001$) on overall Rispens MDV viral load in dust, but not challenge status ($P = 0.7$), vaccination day ($P = 0.9$) or three-way interaction between them ($P = 0.9$). Two-way interactions between challenge status and vaccination day ($P = 0.3$; **Figure 3-4c**) and challenge status and dpv ($P = 0.1$; **Figure 3-5c**) were also not significant. In unchallenged chickens, the $\text{Log}_{10} (y + 1)$ Rispens MDV load decreased between 7 dpv (3.32 ± 0.59) and 14 dpv (2.39 ± 0.59), then increased gradually to 35 dpv (5.23 ± 0.59) before plateauing until 42 dpv. In challenged chickens, the Rispens

MDV load increased irregularly from 7 dpv (2.32 ± 0.59) to 28 dpv (4.85 ± 0.59) then plateaued or fell slightly until 42 dpv (**Figure 3-5c**).

Analysis of only positive samples revealed a significant effect of dpv ($P < 0.0001$) on Rispens MDV viral load in dust with no significant effects of challenge status ($P = 0.4$) or vaccination day ($P = 0.4$) or two-way interactions between challenge status and vaccination day ($P = 0.1$; **Figure 3-4f**) and challenge status and dpv ($P = 0.2$; **Figure 3.5f**). In unchallenged chickens, the Rispens MDV load [$\text{Log}_{10}(y + 1)$ VCN/mg] decreased between 7 dpv (4.10 ± 0.36) and 14 dpv (3.37 ± 0.39), before increasing gradually to 35 dpv (5.24 ± 0.32) and plateauing until 42 dpv. On the other hand in challenged chickens, the Rispens MDV load increased gradually from 7 dpv (3.23 ± 0.49) to 28 dpv (4.85 ± 0.32) then decreased slightly until 42 dpv (4.60 ± 0.32 ; **Figure 3-5f**).

Analysis 3 (Effect of VCI on both viruses)

With all samples included there was a significant overall effect of dpi ($P < 0.0001$) but not VCI ($P = 0.4$) or virus ($P = 0.8$) on viral load with no significant three-way interaction between them ($P = 0.7$). However the interaction between VCI and virus was significant ($P = 0.02$; **Figure 3-6c**); as was the interaction between dpi and virus ($P = 0.04$; **Figure 3-7c**). Pathogenic MDV load did not differ significantly with VCI other than VCI -5 for which pathogenic MD load was significantly higher (5.26 ± 0.67) than that of Rispens (3.45 ± 0.67 ; **Figure 3-6c**). Pathogenic MDV load increased sharply between 7 dpi (0.74 ± 0.58) and 35 dpi (6.05 ± 0.58), then plateaued until 42 dpi. On the other hand, the Rispens MDV load increased irregularly from 7 dpi (1.75 ± 0.58) to 28 dpi (4.87 ± 0.58) then plateaued until 42 dpi, more than a log lower than for pathogenic MDV (**Figure 3-7c**).

3.2.5 Association between variables

Analysis 1 (Pathogenic MDV)

The pathogenic MDV loads in feather tips at 14 and 21 dpc were significantly ($P < 0.0001$ to $P = 0.007$) and positively associated with pathogenic MDV viral load PBL at all dpc ($R^2 = 0.35$ to 0.75). At 7 dpc MDV load in feather tips was significantly ($P = 0.0007$ to $P = 0.04$) and positively associated with pathogenic MDV viral load at 7, 14, 21 and 28 dpc PBL ($R^2 = 0.26$ to 0.43).

Pathogenic MDV load in dust samples from 28, 35, 42, 49 and 56 dpc were significantly ($P < 0.0001$ to $P = 0.03$) and positively associated with MDV load in PBL at 14, 21, 28, 42 and 56 dpc ($R^2 = 0.63$ to 0.95). They were also significantly ($P = 0.01$ to $P = 0.04$) and positively associated with MDV load in feather tips at 21 dpc ($R^2 = 0.59$ to 0.69).

Analysis 2 (Rispens)

The Rispens load in feather tips at 7 dpv had a significant ($P = 0.005$) positive association with load in PBL at 28 dpv ($R^2 = 0.36$) while load in feathers at 14 dpv was significantly ($P = 0.01$ to $P = 0.03$) and positively associated with load in PBL at 7 and 21 dpv ($R^2 = 0.27$ to 0.32). Rispens load in feathers at 21 dpv feather cells had significant ($P = 0.002$ to $P = 0.03$) and positive association with load in PBL at 7, 14 and 21 dpv ($R^2 = 0.28$ to 0.39).

The Rispens load in PBL at 7 dpv was significantly ($P = 0.001$ to $P = 0.01$) and positively associated with load in dust at 35 and 42 dpv ($R^2 = 0.70$ to 0.81) while that in PBL at 14 dpv was significantly ($P = 0.05$) and positively associated with load in dust at 21 dpv dust ($R^2 = 0.57$) but significantly ($P = 0.04$) and negatively associated with load in dust at 56 dpv ($R^2 = 0.71$). Rispens load in PBL at 21 dpv was significantly ($P = 0.02$ to $P = 0.03$) and positively associated with that in dust at 35 and 42 dpv ($R^2 = 0.63$ to 0.65). Load in PBL at 28 dpv was significantly ($P = 0.04$) and positively associated with load in dust at 49 dpv ($R^2 = 0.63$) while load in PBL at 42 dpv was significantly ($P = 0.03$ to $P = 0.04$) and positively associated with load in dust at 7 and 42 dpv ($R^2 = 0.61$ to 0.63).

Rispens load in feather tips at 14 dpv was significantly ($P = 0.007$) and positively associated with load in dust at 35 dpv dust ($R^2 = 0.72$) while load in feathers at 21 dpv feather cells was significantly ($P = 0.02$ to $P = 0.04$) and positively associated with load in dust at 42 and 49 dpv ($R^2 = 0.60$ to 0.72).

Analysis 3 (Effect of VCI on both viruses)

There was negative association between VCI and overall pathogenic MDV load in PBL ($n = 5$, $R^2 = 0.87$, $P = 0.02$), feather tips ($n=5$, $R^2 = 0.76$, $P = 0.05$) and dust ($n = 5$, $R^2 = 0.55$, $P = 0.15$) (Figure 3-8, 1st row). On the other hand there was a positive association between VCI and Rispens MDV load in PBL ($n = 5$, $R^2 = 0.79$, $P = 0.04$), feather cells ($n = 5$, $R^2 = 0.33$, $P = 0.31$) and dust ($n = 5$, $R^2 = 0.11$, $P = 0.59$) (Figure 3-8, 2nd row).

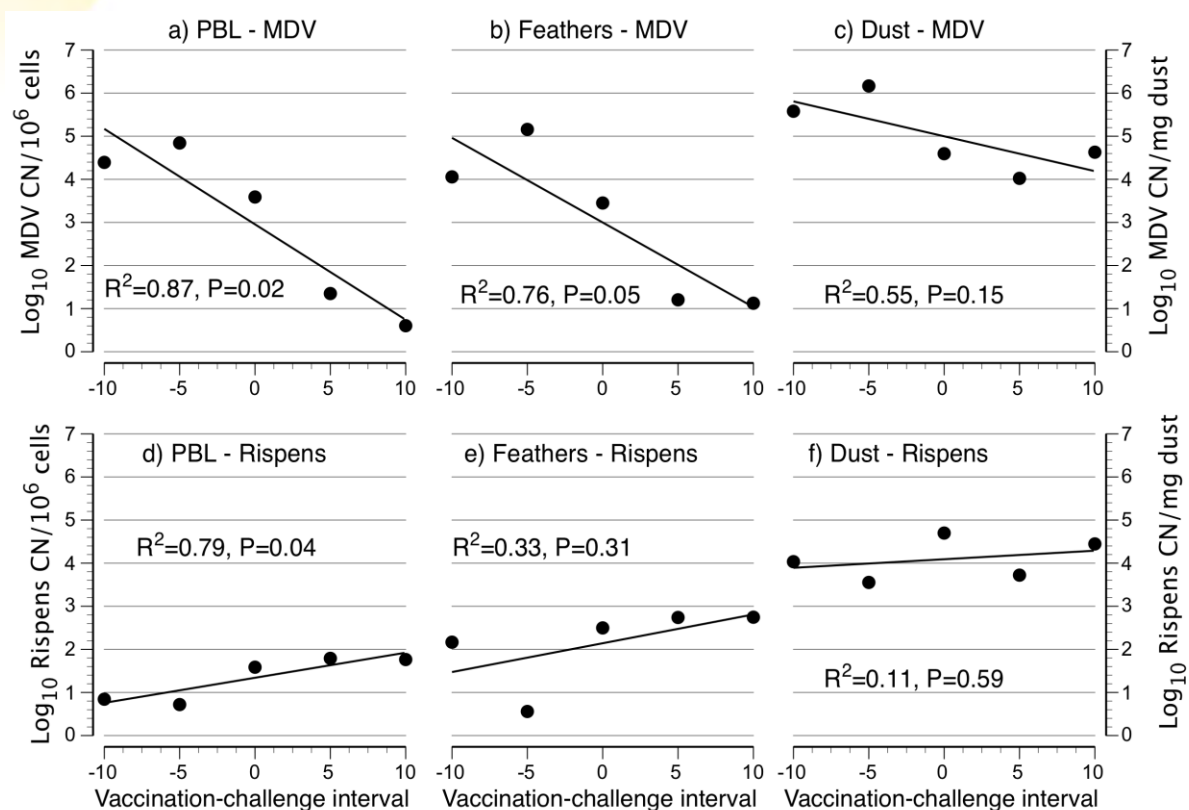


Figure 3-8: Association between VCI and pathogenic MDV (1st row) or Rispens (2nd row) load in PBL (left), Feather tips (middle) and dust (right). Each point is the mean of data from two isolators for each VCI value and the line is a linear regression curve

3.6 Discussion

This study examined the competitive kinetics and shedding profiles of very virulent MDV1 (02LAR) and vaccinal MDV1 (Rispens/CVI988) in co-infected chickens and the effect of VCI on this. It revealed that Rispens vaccination greatly reduced the pathogenic MDV viral load in PBL, feather cells and dust, but only if vaccination preceded challenge. Similarly challenge with pathogenic MDV significantly reduced the Rispens viral load in PBL and feather cells but not dust if challenge preceded vaccination. This resulted in pathogenic MDV load having a significant negative association with VCI and Rispens load having a significant positive association with VCI.

The first hypothesis that viral load of pathogenic MDV in PBL, feather cells and dust will be higher than that of the Rispens virus in co-infected birds was supported by the data found in

analysis 3 but the level of difference was affected by sample type, VCI and whether all samples or only positive samples were measured. In PBL the viral load of pathogenic MDV was greater than that of Rispens only when challenge preceded or was coincident with vaccination, being approximately 3.5, 4 and 2 logs higher at VCI -10, VCI -5 and VCI 0 respectively. Similarly in feather cells it was approximately 2 and 4.5 logs higher at VCI -10 and VCI -5. However in dust pathogenic MDV load was only significantly higher than Rispens by just under 2 logs at in VCI -5. For the latter variable analysis of positive samples only led to a much clearer and consistent advantage in favour of pathogenic MDV.

These findings are consistent with the general observation that virulent viruses tend to have higher replication rates in the host than attenuated or mild strains. In the current experiment this difference was clearly much greater in lymphoid tissues than dust, suggesting that attenuation or low pathogenicity affects affinity for, or replication in, lymphoid tissues far more than it does the shedding of virus in dander. As lymphocytes are the primary target cell in the pathogenesis of Marek's disease, this is perhaps not surprising. What is more surprising is the greater magnitude of the difference between vvMDV and Rispens load in feather cells compared to dust. In feather cells over the measurement period (7 - 21 dpc) the higher viral load in pathogenic MDV than Rispens was as marked than it was for PBL, something not observed for dust samples (**Figure 3-7**, a,b,c). This suggests that measurements of MDV load in feathers and dust are measuring MDV in different cell populations or with a different efficiency, despite the high correlation between measurements of MDV in dust and feather tips reported elsewhere (Baigent *et al.* 2013). When only positive samples were analysed (**Figure 3-7**, d,e,f) the differences between sample types were greatly reduced, suggesting that there may be a greater false negative rate in dust samples than the other samples, leading to greater variation and less power to detect differences between virus strains.

The second hypothesis was that vaccination with Rispens would significantly reduce the load of pathogenic MDV in PBL, feather cells and dust if administered before challenge and that this effect would be greater the longer the vaccination to challenge interval. The hypothesis was strongly supported as vaccination with Rispens significantly reduced the vvMDV load in PBL, feather cells and dust and the effects increased with day of challenge post vaccination (Analysis 1). Vaccination at hatch with Rispens led to significant overall reductions in pathogenic MDV loads in PBL (3-4 logs) and feather cells (3-5 logs) in chickens challenged at 5 and 10 dpv but not those challenged at the same time as vaccination (**Figure 3-2**, a, b). This reduction was due to both a reduction in the number of chickens positive for MDV (**Figure 3-1a**) and a reduction in the viral load in positive samples (**Figure 3-2**, d, e). Differences between vaccinated and unvaccinated chickens were evident in PBL and feather samples from the first sampling at 7 dpi and generally increased with time post challenge (**Figure 3-3**, a,b,d,e).

In dust samples the results were somewhat different, with the effects of vaccination becoming significant later, at days 21 to 28 dpv (**Figure 3-3**, e, f). This resulted in no significant difference in overall MDV viral load between vaccinated and unvaccinated chickens in all dust samples (**Figure 3-2c**) but in positive samples significant reductions in vvMDV load (> 2 logs) were observed in samples from chickens challenged 5 and 10 days post vaccination (**Figure 3-2f**). Haq *et al.* (2012) also reported a reduction in MDV load in feather cells in SPF chickens vaccinated in ovo with Rispens vaccine and challenged at day 5 of age. However, in that study the reduction in MDV due to vaccination was delayed (first observed at 14 dpc) and much smaller (1- 1.5 logs) than that observed in the present study. Baigent *et al.* (2011) vaccinated SPF chickens with pCVI988 (prepared from a BAC clone of CVI988) at 1 day of age and challenged them with wild type MDV strain RB-1B at 15 days of age. Vaccination significantly slowed RB-1B replication and reduced its load in spleen, kidney, liver and feather cells from 3 to 20 dpc but not at 56 dpc. At 20 dpc differences in spleen of approximately 4 logs and in feathers of approximately 6 logs were of an even greater magnitude than observed in the current experiment.

These findings are consistent with studies showing that other MD vaccines also reduce MDV load. Islam *et al.* (2006b) vaccinated commercial broiler chickens at hatch with HVT and challenged at 2 days post vaccination with MPF57 revealed that HVT vaccination reduced MDV load in PBL at 28 and 35 dpc, whereas in our data showed that Rispens vaccination reduced MDV load in PBL from day 7 until 56 dpc. Islam and Walkden-Brown (2007) showed that in commercial broiler chickens both HVT and a bivalent vaccine combining HVT and MDV2 significantly reduced MDV1 shedding in dust between 14 and 28 dpc and challenge with MDV1 enhanced both HVT and MDV2 replication and subsequent shedding in dust. Walkden-Brown *et al.* (2013) reported from the same study that these vaccines reduced MDV viral loads at 7 and 14 dpc, but not 56 dpc in spleen. Islam *et al.* (2008) showed that vaccination with HVT significantly reduced pathogenic MDV load in dust and the reduction was greatest for treatments with VCI of 4 and 7 rather than 2 days.

It is interesting that in the present study the inhibitory effects of vaccination on MDV were far greater for PBL and feathers than for dust, in which the differences became clear up to 4 weeks later and were of a smaller magnitude (**Figure 3-3**, a, b, c). This has been observed in other studies. For example Walkden-Brown *et al.* (2013) reported large reductions of MDV load in spleen cells at various times post challenge in chickens vaccinated with HVT or bivalent HVT/MDV2 but in the same study, there were comparatively minor reductions in the shedding of MDV in dander induced by vaccination (Islam and Walkden-Brown 2007). This is suggestive of a differential action of vaccination on replication in lymphoid tissues central to disease pathogenesis relative to that in epithelial tissues more involved in reproductive success of MDV. However, early and large reductions in MDV load due to vaccination are detected in feather tips which runs counter to this, and raises the issue of the relationship between measures of MDV in feather tips (including a heterogeneous cell population in feather pulp) and those in dust. In the only study directly examining this issue, a close association was reported (Baigent *et al.* 2013).

Our third hypothesis that challenge with pathogenic MDV1 will not influence the load of Rispens vaccine virus in PBL, feather and dust was not supported. Just as vaccination prior to challenge will reduce the load of challenge virus, it was observed that challenge prior to vaccination also reduces the load of vaccinal virus in PBL and feather cells (**Analysis 2**, **Figure 3-4** and **Figure 3-5**). This is reflected in a significant positive association between VCI and Rispens load, but a negative association between VCI and vvMDV load (**Figure 3-8**). Challenge with pathogenic MDV 5 and 10 days prior to vaccination reduced Rispens viral load in PBL by approximately 2 and 1.5 logs respectively and in feather cells by approximately 3.5 and 2.5 logs respectively. However there were no significant effects in dust (**Figure 3-4**, c, f). Significant suppression occurred from 7 dpv in PBL and from 14 dpv in feather cells (**Figure 3-5**, a, b). Baigent *et al.* (2011) and Haq *et al.* (2012) found that challenge with pathogenic MDV1 had no significant effect on the level of BAC-cloned CVI988 in spleen, liver, kidney and feather tips and CVI988 in feather cells respectively, probably because, unlike the present experiment the effect of challenge prior to vaccination was not tested in those experiments.

In combination these results show that if MDV challenge occurs after vaccination there is little effect on Rispens load, but that if it occurs prior to vaccination, challenge will depress the replication rate of the Rispens virus. This may be mediated by development of an adaptive immune response to MDV following challenge. The lack of an effect of post-vaccination challenge on Rispens virus load contrasts markedly with effects on HVT and MDV2 viral load in dander which are markedly increased following challenge (Islam and Walkden-Brown 2007). The reasons for this difference are not clear.

Our fourth hypothesis was that pattern of viral load of over time will differ between pathogenic MDV and Rispens in PBL and feathers but not dust and this will not be affected by co-infection. Previously it has been shown that pattern of Rispens viral load over time differed from that of pathogenic MDV in PBL and feather cells but not in dust (Baigent *et al.* 2005b and Islam *et al.* 2013a). The hypothesis is largely supported by the results. For PBL clear

differences in the profiles of the two viruses in chickens infected with each virus on its own were evident (**Figure 3-3a** and **Figure 3-5a**) with pathogenic MDV increasing more uniformly and to a far greater extent over time, than Rispens. Co-infection with the two viruses did not greatly alter this basic difference (**Figure 3-7a**). A considerable part of the difference in overall profiles was due to a lower proportion of positive samples for Rispens, so that when only positive samples were considered, the differences in load between the two viruses were much reduced (**Figure 3-3d**, **Figure 3-5d** and **Figure 3-7d**). For feather cells clear differences in the profiles of the two viruses in chickens infected with each virus on its own were evident (**Figure 3-3b** and **Figure 3-5b**) with pathogenic MDV increasing more sharply and to a far greater extent from day 7 to 21, than Rispens. Co-infection with the two viruses did not greatly alter this basic difference (**Figure 3-7b**). Again a considerable part of the difference in overall profiles was due to a lower proportion of positive samples for Rispens, so that when only positive samples were considered, the differences in load between the two viruses were much reduced (**Figure 3-3e**, **Figure 3-5e** and **Figure 3-7e**). For dust, no differences in the profiles of the two viruses in chickens infected with each virus on its own were evident (**Figure 3-3c** and **Figure 3-5c**) though pathogenic MDV increased to a far greater extent over time than Rispens. Co-infection with the two viruses did not alter this basic difference (**Figure 3-7c**). Moreover when only positive samples were considered, the differences in load between the two viruses showed similar results (**Figure 3-3f**, **Figure 3-5f** and **Figure 3-7f**).

It has been shown that both HVT and a bivalent vaccine combining HVT and MDV2 significantly suppressed pathogenic MDV1 shedding in dust between 14 and 28 dpc (Islam and Walkden- Brown 2007). Islam *et al.* (2006b) shows that pathogenic MDV1 load was unaffected by vaccination with HVT until 28 dpc, after that chickens having significantly lower pathogenic MDV1 loads. This differs from the pattern seen in PBL and dust in the present experiment where Rispens vaccination had great suppression effect at 7 to 56 dpc in PBL and 28-56 dpc in dust though the pattern was different.

As Rispens virus transmits effectively from vaccinated to contact chickens (Islam *et al.* 2013a) and there was no significant effect pathogenic MDV challenge post vaccination on Rispens shedding, it is possible that the Rispens virus may establish itself in chicken populations. However the consistently lower shedding rate of the Rispens virus relative to vvMDV as shown in this experiment suggests that it may not be competitive in free competition with wild-type virus.

4. Experiment 3: Influence of the vaccination-challenge interval on the protection provided by Rispens CVI988 vaccine against very virulent Marek's disease virus challenge

Using the same experimental chickens and design, this experiment (milestone 3, part 2) also investigated the effects of the widely divergent vaccination-challenge interval (VCI) on the level of vaccinal protection provided by the Rispens vaccine. This is the first investigation of the effect of VCI on protection against challenge in birds vaccinated with Rispens CVI 988 and the correlation of early vaccinal and pathogenic MDV load with the subsequent incidence of MD.

This work will contribute to improved understanding of vaccinal protection provided by Rispens CVI 988, and also assist with determination of vaccination and future MD status of vaccinated flocks using q-PCR measurement of MDV load in PBL, dust or feathers.

This chapter reports the vaccinal protection and MD prediction aspects of the study and tests the following hypotheses:

1. Vaccination with Rispens CVI988 5 or 10 days after MDV challenge will not provide significant protection
2. Vaccination 5 or 10 days prior to, or simultaneous with, challenge will provide significant protection and the level of protection will be positively correlated with VCI
3. Early measures (up to 21 days) of pathogenic and vaccinal MDV load in PBL, feathers and dust will be good predictors of subsequent MDV status.

PhD candidate Tanzila Islam contributed significantly to this experiment. The results of this experiment have been published:

Islam, T, Walkden-Brown, SW, Renz, KG, Fakhrul Islam, AFM, Ralapanawe, S (2013c) Vaccination-challenge interval markedly influences protection provided by Rispens CVI988 vaccine against very virulent Marek's disease virus challenge. *Avian Pathology* 1-35.

4.1 Overview of method

4.1.1 Experimental design

The experimental design as outlined in Experiment 2 also applied to this aspect of the same experiment. The experimental chickens, treatment allocations and sampling times were the same as Experiment 2. Blood, feather and dust samples were processed either for real-time PCR analysis or ELISA as described under General Material and Methods section.

4.1.2 MD diagnosis and lesion scoring

Diagnosis of MD was based on gross pathology observed on post mortem examination of all dead and euthanised chickens. 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. The bursa of Fabricius was examined and scored for atrophy as for the thymus. Tumorous enlargement of the spleen and bursa of Fabricius were recorded as gross MD lesions. Chickens without visible MD tumours were treated as negative. Histopathological confirmation was not carried out. All MD lesions were scored 1-3 subjectively for severity on an ascending scale based on the size and extent of the lesion.

4.1.3 Vaccinal protection index (PI)

The protective index (PI) provided by the Rispens vaccine against challenge with 02LAR at various VCI was calculated as: $(\%MD \text{ in unvaccinated chickens} - \%MD \text{ in Rispens-vaccinated chickens}) \div (\%MD \text{ in unvaccinated chickens}) \times 100$ where %MD is the percentage of birds “at risk” of exhibiting MD lesions, in which lesions are present. This was taken as the population of chickens alive at the time the first gross MD lymphoma was detected. The chickens that died or were euthanised before the first MD case at 31 dpc were excluded from the calculation. This includes a sub-sample of 140 birds removed at 14 dpc for organ weight analysis, which is not reported here.

4.1.4 Statistical analysis

Analyses were performed using JMP10 (SAS Institute Inc. 2010). Two separate analyses were performed:

- Analysis 1 included only the treatments with an effective VCI and tested the effect of VCI
- Analysis 2 included all treatments and tested the effect of treatment as shown in **Table 3-1**.

Where the chicken was the experimental unit measured, categorical data such as mortality (died/survived), presence of MD (positive/negative), were analyzed using the Pearson Chi-square statistic test in the case of 2-way tables. Mortality data were also analyzed using survival analysis (Kaplan-Meier method). Vaccinal protective index (PI) was calculated for each isolator and treatment effects tested using analysis of variance after fitting linear models for analyses 1 and 2. MDV load in PBL, feather and dust were log transformed [$\text{Log}_{10}(y+1)$] prior to analysis. MD titre data were also analysed after log transformation. Treatment effects were tested using analysis of variance after fitting linear models for analyses 1 and 2. Differences between different levels within a significant main effect were tested using Student's t test. Association between MD incidence and MDV load in different tissues were tested by pairwise correlation and linear regression analysis of specific associations. Two separate analyses were done; one excluding unchallenged treatments and the other excluding unchallenged treatments and unvaccinated treatments. Least squares means (LSM) and standard errors of the mean (SEM) are presented for continuous variables. A significance level of $P < 0.05$ is used throughout.

4.2 Results

4.2.1 Application of treatments

All vaccination and challenge treatments were successfully applied and maintained, as determined by MDV1 differential real-time qPCR. All the control chickens were negative for Rispens and pathogenic MDV1 in PBL, feather and dust at all sampling days. Challenged birds were positive for pathogenic MDV1 and vaccinated birds were positive for Rispens.

4.2.2 Mortality/Survival

The first MD tumours were detected at 31 dpc. In total 17.7% birds died in the experiment of which 13.8% died with gross MD lymphomas (**Table 4-1**). All the negative controls chickens had no mortality with MD. Of the 18 chickens that died or were euthanased without gross MD

tumours, nine were non-starters/dehydrated, five had yolk sac infection and four were accidentally injured or killed (crushed behind feeder).

Analysis 1 revealed significant effects of VCI ($P < 0.0001$) for both mortality and mortality with MD lesions. Significantly more birds died in the VCI -5 treatment (55%) than in all other treatments. This was also the case for mortality with MD lesions (52.5%) (Table 4-1).

Analysis 2 showed that overall treatment also had a significant effect ($P < 0.0001$) on both mortality and mortality with MD lesions. Unvaccinated birds challenged at different days showed higher mortality (27.5%, 22.5% and 37.5% after challenge at days 0, 5 and 10 respectively) than unchallenged birds vaccinated at different days (7.7%, 0% and 0% for day 0, 5 and 10 respectively) (Table 4-1). Mortality with MD lesions showed similar trends (Table 4-1).

Survival analysis showed a significant effect of VCI ($P < 0.0001$; Figure 4-1). There was no significant effect of day of challenge in the challenge only treatments ($P = 0.38$; Figure 4-1, middle panel) whereas there was a significant effect of day of vaccination in vaccination only treatments ($P = 0.0001$; Figure 4-1, bottom panel) due mainly due to birds that died from miscellaneous causes other than MD.

Table 4-1: Effect of vaccination with Rispens CVI988 vaccine and challenge with vvMDV isolate 02LAR in various time combinations on total mortality, mortality with MD, incidence of MD to 56 dpc and protection index by VCI and treatment in commercial ISA Brown chickens. Mortality is for eligible chickens from 2 dpc; MD incidence from 31 dpc when the first MD case occurred

Treatment	Vacc*	Chall	Total mortality		Mortality with MD lesions (%)		MD incidence (%)		PI (%)
			No.	%	No.	%	No.	%	
MDV d0	Unvacc	02LAR	11/40 ^{ab}	27.5	8/40 ^a	20	21/37 ^a	56.8	
MDV d5	Unvacc	02LAR	9/40 ^{abd}	22.5	8/40 ^a	20	17/39 ^a	43.6	
MDV d10	Unvacc	02LAR	15/40 ^{ac}	37.5	11/40 ^a	27.5	22/38 ^a	57.9	
RIS d0	Risp	Unchall	3/39 ^d	7.7	0/39 ^c	0	0/39 ^c	0	
RIS d5	Risp	Unchall	0/50 ^c	0	0/50 ^c	0	0/50 ^c	0	
RIS d10	Risp	Unchall	0/48 ^c	0	0/48 ^c	0	0/49 ^c	0	
Neg cont	Unvacc	Unchall	2/20 ^{bd}	10	0/20 ^c	0	0/18 ^c	0	
VCI -10	Risp	02LAR	6/40 ^{bd}	15	6/40 ^{ab}	15	18/40 ^a	45	20.9 ^c
VCI -5	Risp	02LAR	22/40 ^a	55	21/40 ^d	52.5	23/39 ^a	59	-3.6 ^c
VCI 0	Risp	02LAR	10/40 ^a	25	8/40 ^a	20	9/40 ^b	23.1	60.4 ^b
VCI 5	Risp	02LAR	1/30 ^d	3.3	1/30 ^{bc}	3.3	2/30 ^{bc}	6.7	84.8 ^{ab}
VCI 10	Risp	02LAR	2/30 ^d	6.7	0/30 ^c	0	0/28 ^c	0	100 ^a
TOTAL			81/457	17.7	63/457	13.8			

*Vacc.: Vaccination; Unvacc.: Unvaccinated; Risp: Rispens; Chall: Challenge; Unchall: Unchallenged; PI: Protective index

^{abc} Values within columns not sharing a common letter in the superscript differ significantly ($P < 0.05$)

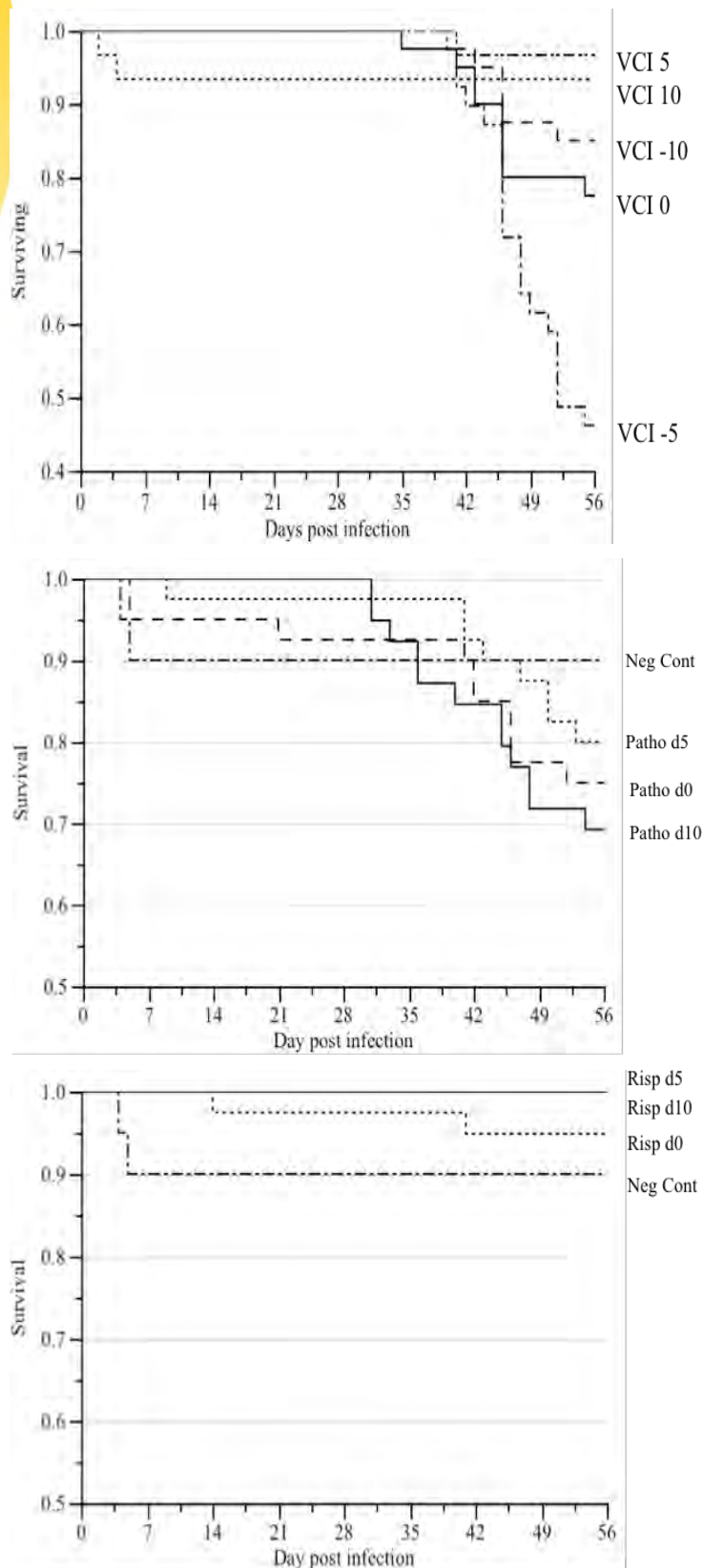


Figure 4-1 Survival patterns of commercial ISA Brown layer chickens vaccinated with Rispens CVI988 vaccine and/or challenged with MDV isolate 02LAR showing the effects of vaccination-challenge intervals (VCI) ranging from -10 to 10 (top; $P < 0.0001$), challenge with 02LAR in unvaccinated chickens (middle; $P=0.38$) and vaccination with Rispens CVI988 in unchallenged chickens (bottom; $P<0.0001$)

4.2.3 Incidence of MD and vaccinal protective index

The combined incidence of gross MD lesions in chickens that died with MD or had MD lesions on post mortem after euthanasia at 56 dpc is summarized by treatment and VCI in **Table 4-1**. Overall, there was a significant effect of VCI on MD incidence ($P = 0.001$) with a higher incidence in treatments VCI -10 (59%) and -5 (45%) than VCI 0 (23.1%), VCI 5 (6.7%) and VCI 10 (0%). VCI had a significant effect on PI ($P = 0.002$) with VCI 0, 5 and 10 showed significantly higher protective indices (60.4%, 84.8% and 100% respectively) than VCI -5 (-3.6%) and -10 (20.9%) (**Table 4-1**). Not surprisingly treatment in Analysis 2 also had an overall significant effect on MD incidence ($P < 0.0001$). The incidence ranged from no MD in unchallenged birds through to a mean of 52.6% in challenged but unvaccinated birds (**Table 4-1**).

On an individual isolator basis there was a significant ($P = 0.0008$) negative linear association between VCI and MD incidence ($R^2 = 0.77$; **Figure 4-2a**) and a significant ($P = 0.0009$) positive linear association between VCI and PI ($R^2 = 0.77$; **Figure 4-2b**).

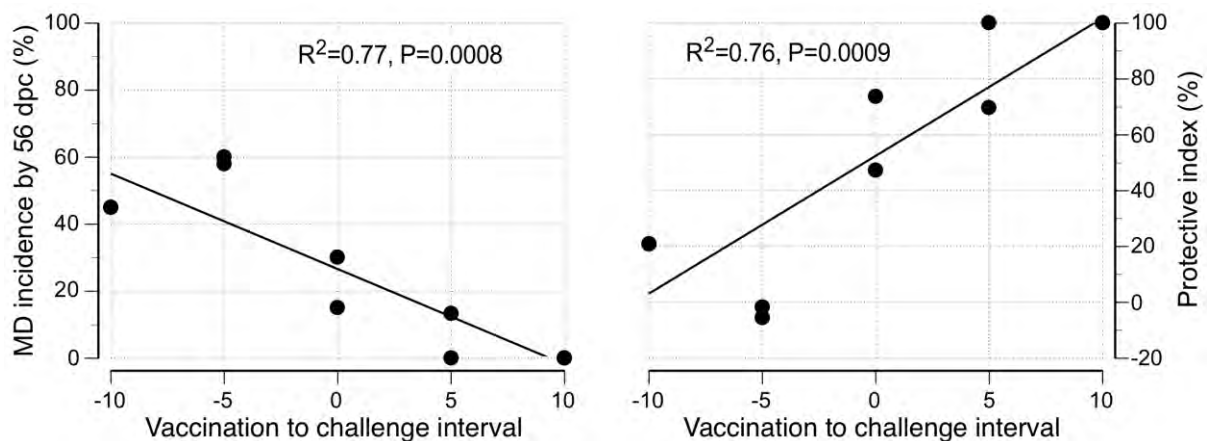


Figure 4-2: Association between vaccination challenge interval and (a) MD incidence, (b) Protection Index. Each point represents an individual isolator

4.2.4 MDV-specific serology

Analysis 1 revealed that there was a significant effect of VCI ($P = 0.006$; **Figure 4-3**) and dpc ($P < 0.0001$) on the \log_{10} anti MDV IgY titre with no significant interaction between these effects ($P = 0.1$). VCI 10 showed significantly higher IgY titre (2.05 ± 0.21) than all other VCI except VCI 5 where the titre level was non-statistically lower than VCI 10.

Analysis 2 showed that there was a significant effect of treatment ($P < 0.0001$) and days post challenge or vaccination ($P < 0.0001$) on the \log_{10} MDV IgY titre with significant interaction between these effects ($P = 0.0006$; **Figure 4-4**). There were no significant differences between Rispens only treatments with vaccine administered at different ages. The Rispens only treatments also showed significantly higher IgY titre than pathogenic only treatments at each of the equivalent times of infection.

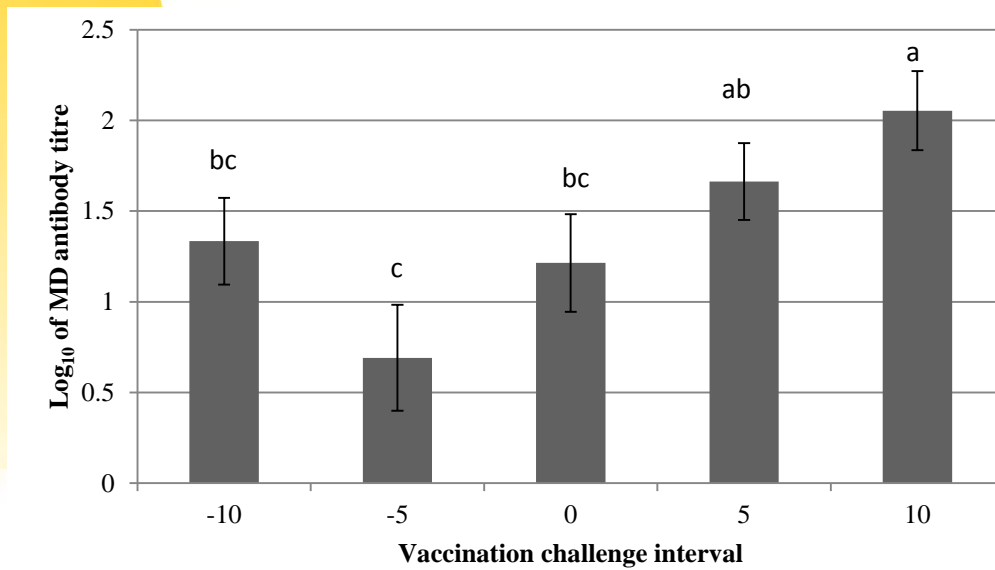


Figure 4-3: Mean (LSM±SEM) serum Log₁₀ of MD antibody titre for the different VCI treatments measured at 21 and 56 dpc. The effect of VCI was significant (P = 0.006). ^{abc}Means columns not sharing a common letter differ significantly (P<0.05)

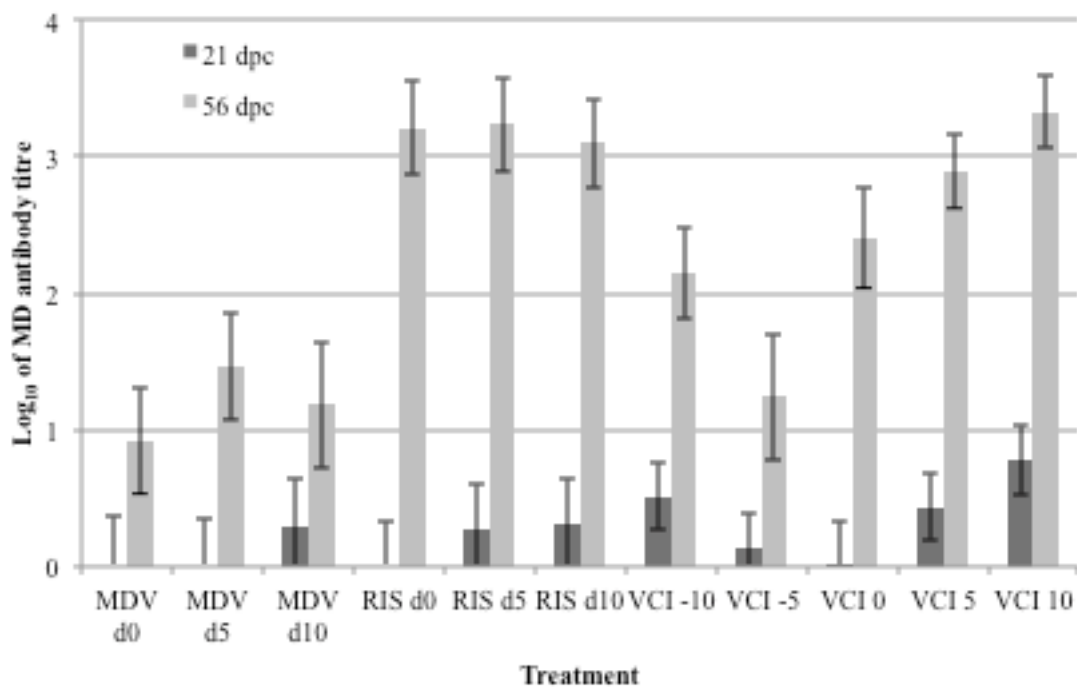


Figure 4-4: Mean (LSM±SEM) serum Log₁₀ of MD antibody titre for all treatments measured at 21 and 56 dpc for all treatments involving MDV challenge or 21 and 56 dpv in the RIS treatments. The effect of treatment was significant (P = 0.0006)

4.2.5 Relationship between Marek's disease incidence at 56 dpc and MDV load in PBL, feathers, dust, immune organ weights and MD serology

In the analysis where unchallenged isolators are excluded, Pathogenic MDV load in PBL on 14 ($R^2 = 0.69$; $P < 0.0001$) and 21 ($R^2 = 0.69$; $P < 0.0001$) dpc were significantly positively associated with MD incidence at 56 dpc, but there was no significant relationship at 7 dpc ($R^2 = 0.20$; $P = 0.09$) (Figure 4-5, 1st row). On the other hand Rispens load in PBL of chickens on 7, 14 and 21 days post vaccination (dpv) had no significant relationship with MD incidence by 56 dpc with a trend towards a negative association (Table 4-2).

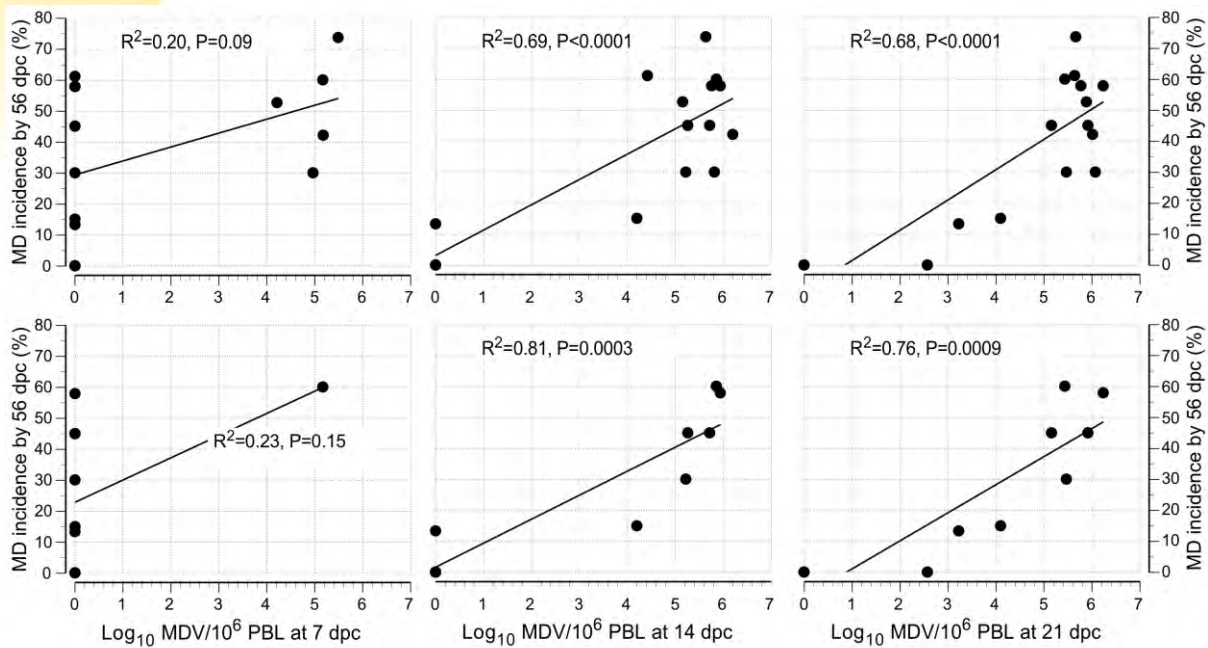


Figure 4-5: Association between MD incidence and pathogenic MDV load in PBL at various times post infection. Each data point represents the mean Log₁₀ MDV copy number in PBL of five chickens from each isolator and the corresponding MD incidence of that group up to 56 dpc. 1st row: Unchallenged isolators excluded; 2nd row: Unchallenged and unvaccinated treatments excluded

Table 4-2: Pairwise correlation of MD incidence (MD%) at d56 with viral load in various tissues, immune organ weight and MD antibody titre of different challenge days. Correlation is between least squares mean values for each isolator. Analysis includes 16 isolators excluding only treatments not challenged with pathogenic MDV

Variable	Virus	Day	Correlation with MD 56 dpc	N	Lower 95%	Upper 95%	P value
PBL	MDV1	7	0.4425	16	-0.0681	0.7694	0.0861
		14	0.8305	16	0.569	0.9394	<0001*
		21	0.8264	16	0.5603	0.9379	<0001*
	Risp	7	-0.1696	10	-0.7221	0.515	0.6394
		14	-0.0668	10	-0.6683	0.5875	0.8545
		21	-0.5693	10	-0.8826	0.094	0.0858

Feather	MDV1	7	-0.5193	16	-0.8072	-0.0318	0.0392*
		14	0.5118	16	0.0215	0.8036	0.0427*
		21	0.8386	16	0.5868	0.9425	<0001*
	Risp	7	-0.5559	10	-0.8782	0.1135	0.0952
		14	-0.1181	10	-0.6959	0.5527	0.7453
		21	-0.4052	10	-0.8245	0.3012	0.2453
Dust	MDV1	7	0.0882	16	-0.4262	0.5594	0.7455
		14	-0.0915	16	-0.5617	0.4234	0.7361
		21	0.3208	16	-0.208	0.7045	0.2258
	Risp	7	-0.0988	10	-0.6858	0.566	0.7859
		14	-0.083	10	-0.6772	0.5768	0.8197
		21	0.3316	10	-0.3767	0.7952	0.3493
Relative splenic wt (%)		14	-0.4476	16	-0.772	0.0619	0.0821
		56	0.4678	16	-0.0364	0.7821	0.0677
Relative bursal wt (%)		14	0.093	16	-0.4221	0.5628	0.7318
		56	-0.2485	16	-0.6626	0.2819	0.3534
Log ₁₀ anti MDV titre		14	0.338	16	-0.1894	0.7141	0.2004
		56	-0.004	15	-0.5152	0.5093	0.9887

Pathogenic MDV load in feather tips on 14 ($R^2 = 0.26$; $P = 0.04$) and 21 ($R^2 = 0.70$ $P < 0.0001$) dpc were found significantly positively associated with MD incidence at 56 dpc, but there was a significant negative relationship at 7 dpc ($R^2 = 0.27$; $P = 0.04$) (**Figure 4-6**, 1st row). On the other hand Rispens load in feather tips of chickens at 7, 14 and 21 dpv had no significant association with MD incidence to 56 dpc with a trend towards a negative association (**Table 4-2**).

There were no significant associations between pathogenic MDV load in isolator exhaust dust at 7, 14 and 21 dpc and MD incidence to 56 dpc (**Table 4-2**). There was also no significant relationship between Rispens load in dust and MD incidence to 56 dpc with a trend towards a negative association at 7 and 14 dpv and positive association at 21 dpv.

There were also no significant relationships found between MD incidence up to 56 dpc and splenic or bursal weights at 14 and 56 dpc as well as MD antibody titre at 21 and 56 dpc (**Table 4-2**). In the analysis where unchallenged isolators and unvaccinated isolators were excluded, MD incidence to 56 dpc was found to be significantly positively associated with pathogenic MDV load at 14 ($R^2 = 0.81$; $P = 0.0003$) and 21 ($R^2 = 0.76$; $P = 0.0009$) dpc but not at 7 dpc ($R^2 = 0.23$; $P = 0.15$) (**Figure 4-5**, 2nd row).

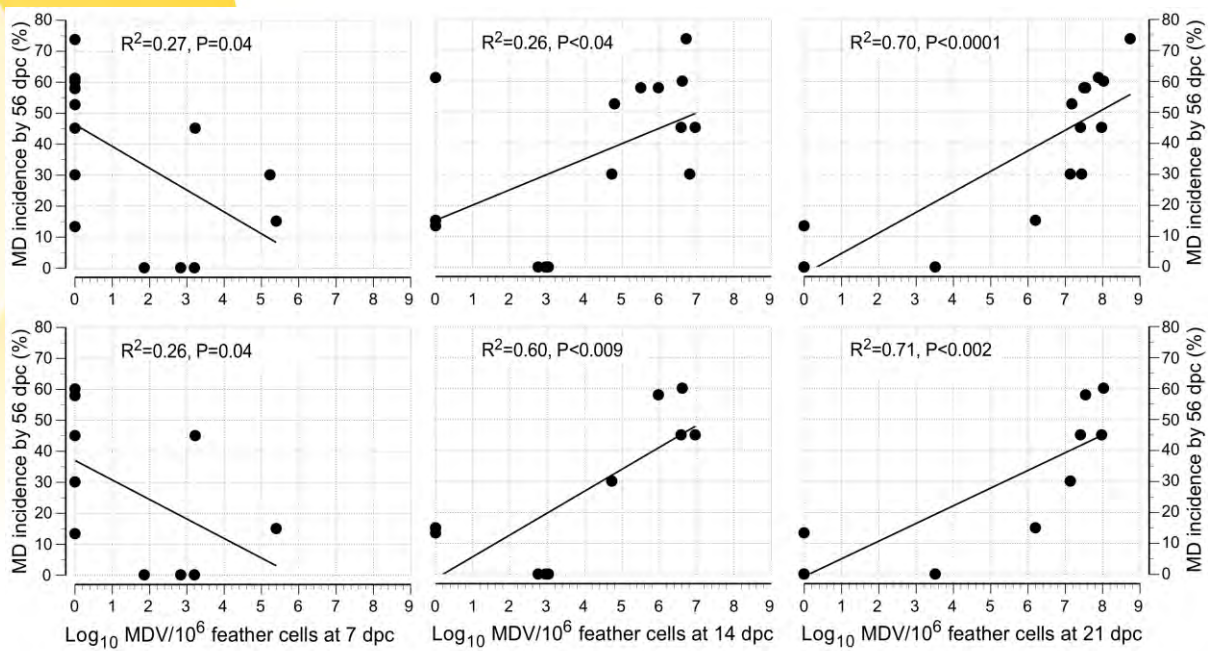


Figure 4-6: Association between MD incidence and pathogenic MDV load in feather cells at various times post infection. Each data point represents the mean Log_{10} MDV copy number in feather cells of five chickens from each isolator and the corresponding MD incidence of that group up to 56 dpc at the end of the experiment. 1st row: Unchallenged isolators excluded; 2nd row: Unchallenged and unvaccinated treatments excluded

On the other hand it was not associated with Rispens load in PBL of chickens at 7, 14 and 21 dpv with a trend towards a negative association (

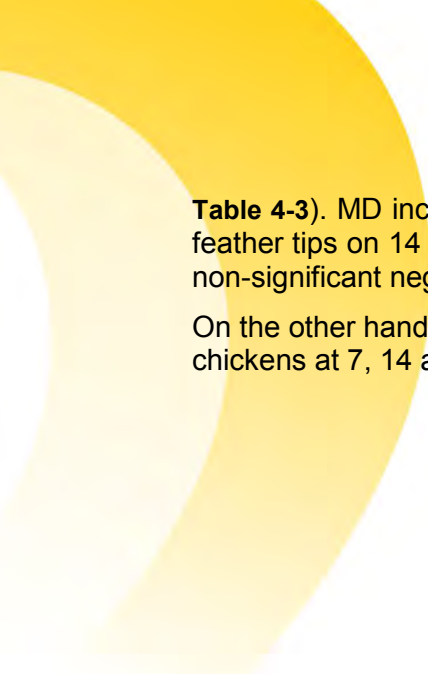


Table 4-3). MD incidence at 56 dpc was positively associated with pathogenic MDV load in feather tips on 14 ($R^2 = 0.60$; $P = 0.009$) and 21 ($R^2 = 0.71$; $P = 0.002$) dpc but there was a non-significant negative relationship at 7 dpc ($R^2 = 0.26$; $P = 0.13$) (**Figure 4-6**, 2nd row).

On the other hand it was not significantly associated with Rispens load in feather cells of chickens at 7, 14 and 21 dpv with a trend towards a negative association (

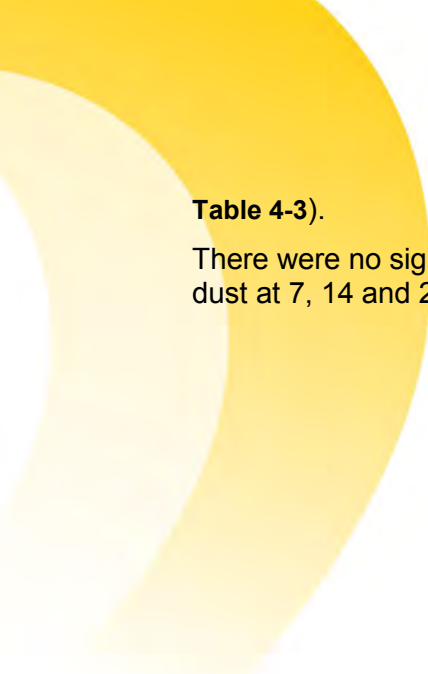


Table 4-3).

There were no significant association between pathogenic MDV loads in isolator exhaust dust at 7, 14 and 21 dpc with MD incidence up to 56 dpc (

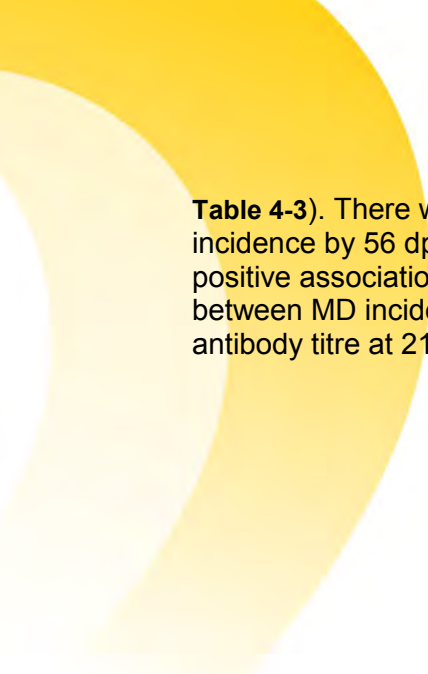


Table 4-3). There was also no significant association between Rispens load in dust and MD incidence by 56 dpv with a trend towards negative associations at 7 and 14 dpv and a positive association at 21 dpv. In this analysis there were also no significant associations between MD incidence to 56 dpc and splenic and bursal weights at 14 and 56 dpc, or MD antibody titre at 21 and 56 dpc (



Table 4-3).

Table 4-3: Pairwise correlation of MD incidence (MD%) at d56 with viral load in various tissues, immune organ weight and MD antibody titre of different challenge days. Correlation is between least squares mean values for each isolator. Analysis includes 10 isolators in VCI treatments involving infection with both the Rispens CVI988 vaccine virus and vvMDV isolate 02LAR

Variable	Virus	Day	Correlation with MD to 56 dpc	N	Lower 95%	Upper 95%	P value
PBL	MDV1	7	0.4871	10	-0.2056	0.8546	0.1533
		14	0.9055	10	0.6418	0.9777	0.0003*
		21	0.8739	10	0.543	0.9699	0.0009*
	Risp	7	-0.1696	10	-0.7221	0.515	0.6394
		14	-0.0668	10	-0.6683	0.5875	0.8545
		21	-0.5693	10	-0.8826	0.094	0.0858
Feather	MDV1	7	-0.5083	10	-0.8621	0.1784	0.1355
		14	0.7717	10	0.2763	0.9431	0.0089*
		21	0.8401	10	0.4467	0.9613	0.0023*
	Risp	7	-0.5334	10	-0.8706	0.1449	0.1124
		14	-0.4576	10	-0.8441	0.2416	0.1835
		21	-0.5979	10	-0.8918	0.0508	0.0679
Dust	MDV1	7	0.2616	10	-0.4406	0.7652	0.4653
		14	-0.2331	10	-0.7523	0.4647	0.5169
		21	0.4817	10	-0.6858	0.566	0.7859
	Risp	7	-0.0988	10	-0.6858	0.566	0.7859
		14	-0.083	10	-0.6772	0.5768	0.8197
		21	0.3316	10	-0.3767	0.7952	0.3493
Relative splenic wt (%)		14	-0.4447	10	-0.8393	0.2568	0.1978
		56	-0.0447	10	-0.6559	0.6018	0.9023
Relative bursal wt (%)		14	0.3693	10	-0.3392	0.8105	0.2937
		56	0.4575	10	-0.2417	0.844	0.1837
Log ₁₀ anti MDV titre		14	0.358	10	-0.3507	0.8059	0.3098
		56	-0.1154	10	-0.6945	0.5546	0.751

4.3 Discussion

This study has demonstrated that VCI has a significant effect on the protective efficacy of the Rispens/CVI988 vaccine with full protection observed only when vaccination occurred 10 days before challenge, and no significant protection occurring if challenge precedes vaccination by 5 or 10 days. Pathogenic MDV viral load in PBL and feather tips at 14 and 21, but not 7, dpc were good predictors of subsequent MD incidence, whereas the Rispens CVI988 viral load in PBL and feather tips were not.

The first hypothesis was supported as vaccination with Rispens, 5 or 10 days after challenge with vvMDV provided no significant protection against MD. The second hypothesis was also supported, as there was significant protection against MD when challenge occurred concurrently with vaccination or at days 5 or 10 following it. The respective PI values were 60.4%, 84.8% and 100% with a highly significant positive linear association between PI and VCI on an individual isolator basis (Figure 4-2). These data suggest that maximal protection is obtained by day 10 following vaccination. This is supported by the findings of Baigent *et al.* (2007) of uniformly high PI (> 90%) in anti-MDV maternal antibody-negative chickens vaccinated with Rispens CVI988 at 1 day of age and challenged with MDV at 14, 21 and 28 days post vaccination. A positive association between VCI and PI has also been reported in other studies.

Islam *et al.* (2007) reported that vaccination of maternal anti MDV antibody-positive broilers with a range of HVT doses induced higher protection against MDV challenge at a VCI of 5 (mean PI of 79%) than a VCI of 2 (mean PI of 15%). Similarly Islam *et al.* (2008) reported PI of HVT vaccination against MDV challenge in maternal antibody-positive broilers of 48, 69 and 77% for VCI of 2, 4 and 7 days respectively. In a second experiment they reported PI of 66, 33, 53, 76 and 76% for VCI of 0, 2, 4, 7 and 10 days respectively, concluding that for HVT, no improvement in protection is obtained beyond a VCI of 7 days. Interestingly the latter experiment also showed significant protection when the vaccine and challenge virus were co-administered, an observation also seen in the present experiment. In all studies using maternal antibody-positive chickens those with longer VCI are challenged later, and thus have lower levels of maternal antibody directed against MDV at challenge time than those with shorter or negative VCI.

It is established that the presence of maternal antibody directed against MDV slows the pathogenesis of MD (Chubb *et al.* 1969), presumably by slowing the MDV replication rate. It is therefore possible that part of the observed effect of VCI is mediated by a reduced maternal antibody (passive immunity) inhibition of challenge virus, in addition to the enhanced active immunity expected when the immune response has time to develop. The significantly higher mortality rate with MD in chickens with VCI of -5 relative to those with a VCI of -10 was unexpected. Chicks in both cases were challenged with MDV at day 0. Possibly vaccination at day 5 rather than 10 of age resulted in greater maternal antibody inhibition of the homologous vaccine virus. Alternatively vaccination during the early cytolitic phase of pathogenesis at 5 dpc facilitates MD pathogenesis relative to vaccination at 10 dpc during the latent phase of pathogenesis. It should be noted that many factors in addition to VCI affect the efficiency of vaccinal protection against MD. These include chicken genotype, maternal antibody status, vaccine virus batch and dose and the level and route of challenge, which may interact to alter the optimum VCI. The 10-day optimum VCI observed in this experiment is therefore likely to be indicative, rather than applicable to all circumstances.

The protection index (PI) provided by the Rispens vaccine in the experiment is numerically greater than that provided by HVT or HVT/MDV2 against the same challenge virus in the same chicken strain some years ago. Renz (2008) reported PI of 27.2 and 63.1% for HVT and HVT/SB1 vaccines administered to ISA Brown chicks at hatch, followed by challenge with 500 pfu of 02LAR at day 5 (VCI=5). This compares with a value of 85% for VCI 5 in the present experiment. This supports field experience that the Rispens CVI988 vaccine is one of the most effective vaccines against MDV currently available.

With regards the third hypothesis, pathogenic MDV viral copy number (VCN) in PBL and feather, but not dust, measured at 14 and 21 dpc provided very good prediction of subsequent MD incidence. However, the relationship was much weaker at 7 dpc PBL ($R^2 = 0.23$) and at 7 dpc feather cells ($R^2 = 0.26$). On the other hand, Rispens load in PBL at 7 and 14 and 21 dpv and in feather cells at 7 and 14 and 21 dpv showed mostly weak non-significant negative associations with subsequent MD incidence (PBL, $R^2 = 0.06 - 0.56$; Feather cells, $R^2 = 0.06 - 0.11$). Other studies have shown that the load of MDV in lymphocytes or splenocytes in the first few weeks after challenge are good predictors of subsequent MD

status (Gimeno *et al.* 2008; Islam, *et al.* 2008; Islam *et al.* 2007; Islam, *et al.* 2006; and Walkden-Brown, *et al.* 2013) but that the load of HVT is not (Gimeno, *et al.* 2008 and Islam, *et al.* 2006). Our results are consistent with these earlier findings in chickens vaccinated HVT and or MDV2 vaccines. In the case of isolator exhaust dust no significant association were found between either pathogenic MDV1 or Rispens virus on subsequent MD incidence. This contrasts with the findings of Walkden-Brown *et al.* (2013) who showed that MDV load in dust at 14 and 21 dpc were powerful early predictors of subsequent MD status in commercial broiler chickens.

There were also no significant relationships found between relative immune organ weights or MD IgY titre at 21 and 56 dpc and MD incidence to 56 dpc (**Table 4-3**). However, Renz (2008) showed significant association between subsequent MD status at 56 dpc and relative bursal weight at 14 and 56 dpc but no significant association with splenic weight. Previous studies showed that in commercial broiler chickens the association between relative immune organ weights and subsequent MD status are very strong (Renz *et al.* 2012).

From the above discussion it can be concluded that the viral load of pathogenic MDV in PBL and in feather tips at 14 and 21 dpc is the best predictor of subsequent MD incidence. This finding supports that of Islam *et al.* (2006) that quantitation of MDV in PBL of broiler chickens (Renz *et al.* 2012) during the early stages of infection has predictive value for subsequent MD incidence. Other studies have shown that MDV load in spleen is an excellent predictor of future MD in SPF, commercial broiler and layer chickens (Islam *et al.* 2007; Renz 2008; Renz *et al.* 2012; and Walkden-Brown *et al.* 2013) suggesting that MDV load in circulating PBL or splenocytes may be equally good early predictors of subsequent MD incidence.

Anti-MDV antibody titres in chickens injected with the Rispens vaccine only on days 0, 5 and 10 were significantly higher than those of chickens challenged with pathogenic MDV only on the same days (**Figure 4-4**). This may be due to the higher dose of Rispens vaccine used compared to 02LAR (3200 pfu v 400 pfu), or possibly the Rispens virus is more immunogenic.

We also found that the 56 dpi antibody titres were significantly higher than the 21 dpi titres in all treatments (**Figure 4-4**), indicating an active response to infection and the likely initial inhibitory effect of maternal antibody directed against MDV. There was also a broadly positive relationship between antibody titre and VCI (**Figure 4-3**) and thus with protective index. Interestingly administration of pathogenic MDV in addition to the Rispens vaccine virus generally reduced antibody titres, except with a VCI of 10 (**Figure 4-4**). This suggests that pathogenic MDV interferes with the immune response to Rispens vaccination. Given the well-documented immunosuppressive effects of pathogenic MDV infection due to lytic infection of B and T lymphocytes this is not surprising, and may also explain the lower antibody titres induced by pathogenic MDV alone, compared with vaccinal Rispens virus alone.

Measuring the level of specific antibody is generally an effective way to determine vaccination success. However the antibody response to pathogenic and vaccinal MDV cannot be differentiated by standard immunological tests. Moreover, in maternal antibody positive commercial chickens interpretation of early titres are confounded by passive maternal antibody and active responses to vaccination are very low or non detectable at 21 dpv as we observed in the present experiment (**Figure 4-4**). On the other hand, differential qPCR can detect the viral load of different viruses from day 7 and provides a good prediction of future MD status at days 14 and 21. Thus tests for vaccinal success based on qPCR quantification of vaccinal or pathogenic MDV offer significant advantages. Unfortunately, in the present experiment no significant association between pathogenic or vaccinal MDV in isolator exhaust dust and future MD status was observed. The reasons for this are not clear given the good associations reported previously (Walkden-Brown *et al.* 2013). It is of concern that in the present experiment, many dust samples from isolators known to have MDV active in them were negative for MDV. This is suggestive of failure of MDV detection (false negatives) in dust using the methods used in this experiment.

The survival rate of unvaccinated but challenged chickens to 56 dpc was comparatively high, being 72.5%, 77.5% and 62.5% for chickens challenged with pathogenic virus at day 0, day 5 and day 10 respectively. It is also interesting to note that there was none of the early paralysis and mortality observed between 9-15 dpc with the same challenge virus in SPF chickens (Renz *et al.* 2012). The higher survival rate and absence of early paralysis and mortality in the present experiment is most likely due to the protective effect of maternal antibody directed against MDV which is well known to inhibit or delay the pathogenesis of MD (Chubb *et al.* 1969). Although not directly measured, such maternal antibody would have been present in chickens in the current experiment as they came from parents vaccinated against MD. Similarly high survival rates and an absence of early mortality have been reported in unvaccinated commercial broiler chickens challenged with 02LAR (Walkden-Brown *et al.* 2013). These chickens would have been maternal antibody positive as they were hatched from parents vaccinated against MD.

In summary, we can conclude that a) the Rispens CVI988 vaccine provides increasing levels of protection as VCI increased from 0 to 10 days with 100% protection observed at a VCI of 10 days; b) It provides no significant protection if chickens are vaccinated 5 or 10 days after challenge with vvMDV, c) qPCR quantitation of pathogenic MDV1 in PBL or feathers at 14-21 dpc are highly predictive of MD incidence at 56 dpc whereas quantitation of the Rispens vaccine virus provided little predictive value.

5. Field trials to monitor vaccination responses and presence of wild-type Marek's disease virus

The overarching aim of this field study was to develop effective field measurements of Rispens vaccine take (Milestones 1 and 4). Another aim of this study was to define the long-term viral kinetics of infection with Rispens CVI988 under commercial conditions, beyond eight weeks post vaccination. Use of routine vaccine take testing, coupled with other routine tests such as wild-type MDV1 levels in dust will enable industry to closely monitor vaccine performance and quickly detect breakdowns in protection. The data of this study is the first of its kind, providing detailed information on the longterm kinetics of the Rispens vaccine under field conditions.

The specific hypotheses under test were:

1. Analysis of either feather or dust samples from vaccinated flocks at around 3 weeks post vaccination will be the best timeframe for determination of Rispens vaccination success
2. Vaccinated chicken will remain infected with Rispens throughout their lifespan
3. The qPCR Rispens test on feathers or dust will have similar sensitivity to MDV serology, but superior specificity as it will differentiate vaccinal from wild-type MDV1
4. CVI988 will be found in some unvaccinated surrounding flocks indicating that it has established itself in the poultry population.

It was hoped that the field trial could involve chickens vaccinated with all 3 commercial Rispens vaccines, but it was only possible to investigate farms vaccinated with the Bioproperties vaccine. In retrospect this was probably a good thing, as it allows a clearer analysis of the effects of age, without confounding by vaccine type.

5.1 Overview of method

5.1.1 Experimental Design

The study involved 3 layer farms on geographically different areas (**Figure 1-1**). On each farm, the following sampling procedure was performed:

- 15 randomly selected birds per age group were selected and 3-5 feathers from the axillary tract as well as approx. 500ul blood sampled from the wing vein as described in the General material and methods
- Shed dust samples were collected from each shed on the farm as described in detail in the General material and methods.

Farm 1: Glendon, Tamworth

The genotype of the birds used at Glendon was Hy-Line Brown. The younger birds were housed in separate barns from the older birds from 0-15 weeks of age - marked as 'B' in **Figure 5-1**. The first sampling was carried out when the birds were one week old and continued until they were 4 weeks old. The sampling schedule for this farm is given in **Table 5-1**.



Figure 5-1: Aerial view of Glendon farm Tamworth. A-Cage system for older birds; B-barn type system for younger birds 0-15 weeks

Table 5-1: Sampling schedule at Glendon farm, Tamworth

Date	Experiment day	Young chick sampling	Older birds sampling
08/06/12	0	Chicks placed in 4 sheds	
15/06/12	7	1 dust and 3-4 birds for each shed for blood and feathers	
22-06-12	14	As above	
29-06-12	21	As above	
06-07-12	28	As above	
06-07-12	28	As above	Sample 4 older ages. 15 blood and feather sampled from each age group

After the birds reach 15 weeks old they were transferred to the battery cage systems (**Figure 5-2**, right panel), shown on the farm map as “B”. On the 28th experimental day we sampled four older age groups - 23, 41, 61 and 78 weeks of age. On the 97th experimental day there were only 3 batches of birds remaining that were 33, 59 and 71 weeks old respectively.

In general this farm has at least 5 age groups at any given time, on young group on the floor and 4 age groups in the two caged layer sheds (2 age groups per shed) with a re-stocking interval between batches of chickens of 19 weeks. The Rispens CVI988 vaccine used in this farm was sourced from Bioproperties. The number of birds per age group was approximately 27,000. The birds were also vaccinated for Infectious Bronchitis (IB) at day old, and at 10 weeks Fowl Pox (FP) by wing stab, Egg Drop Syndrome (EDS) and Newcastle disease (ND) by injection and avian encephalomyelitis (AE) by eye drop.



Figure 5-2: (left panel) Brooding system for chicks from age 0-4 weeks. (right panel) cage system for chickens from 15 weeks of age onwards.

Farm 2: Mid Coast Eggs farm, Port Macquarie

This farm housed ISA Brown chickens purchased pullets around 15 weeks of age, run as layers to 65 weeks, then moulted and taken through a second lay to 90-92 weeks of age. Due to investment in a new controlled environment shed and the need to populate the new shed the farm had a skewed aged structure with birds on the farm being between 50 to 91 weeks of age, in 2 sheds (see aerial view of the sheds in **Figure 5-3**). One shed is tunnel ventilated and climate controlled, the other one is conventional shed with side blinds. There were 6 age groups at the time of sampling, these being 50, 57, 65, 72, 83 and 91 weeks of age. The next batch of pullets was to be purchased shortly after the visit, to replace the 91 week old flock.

There was one sampling day for this farm (**Table 5-2**).

Table 5-2: Sampling schedule at Mid Coast Eggs farm, Port Macquarie

Date	Sampling task
09/01/13	15 blood and feather samples from each age group

The youngest age group (50 weeks) were housed in the old style conventional cage system, which is shown in **Figure 5-4**. It is the narrow shed beside the larger tunnel ventilated shed in the aerial view.

The birds on this farm were vaccinated for Marek's with the Bioproperties Rispens vaccine. The birds were also vaccinated against IB, ILT, FP, AE, ND and EDS. There were 9000 birds in each of the five older groups and 7000 birds in the youngest age group.



Figure 5-3: Aerial view of Mid coast eggs farm, Port Macquarie



Figure 5-4: Conventional shed and cage system with blinds to control ventilation

Farm 3: Bowlers Lane farm, Tamworth

The chickens on Bowler's lane farm were ISA Browns and one age group of English Leghorns. The farm rears layer pullets up to point of lay when the chickens are transferred to layer farms off site (**Figure 5-5**). At the time of the first sampling there were 5 age groups that had 5600-6000 birds per age group in single age sheds. The age groups sampled on the first visit were 0.43, 2.5, 8, 12 and 13 weeks old. The English leghorns were 2.5 weeks old (**Figure 5-6**) and there were 3000 birds in one shed together with same aged ISA Browns. At the last visit for sampling, the age groups sampled were 3.5, 6.5 and 8.5 weeks old. Chickens from the oldest age group at the first visit had already been transferred to another layer farm, thus were not available for the last samping visit.

The sampling schedule for this farm is given below (Table 5-3).

Table 5-3: Sampling schedule at Bowlers Lane farm, Tamworth

Date	Sampling of young chick and older birds
13/02/13	15 blood and feather samples from each age group and 1 dust sample from each shed
20/03/13	15 blood and feather samples from each age group and 1 dust sample from each shed
27/03/13	15 blood and feather samples from each age group and 1 dust sample from each shed

The birds were reared on deep litter and were vaccinated with FP, ILT, IB, Coryza, ND, and Eimeria® vaccines. The vaccine used for Marek's disease was the Rispens vaccine from Bioproperties.



Figure 5-5: Aerial view of Bowler's Lane farm, Tamworth



Figure 5-6: Floor pen, English Leghorns at Bowler's Lane

5.1.2 Sample processing for qPCR and ELISA

Blood, feather and dust samples were processed either for real-time PCR analysis or ELISA as described in the General material and methods. A detailed sample analysis table is given below (Table 5-4).

Table 5-4: Summary of sample types and qPCR tests performed by farm of origin (n)

Farm	Sample type	MDV qPCR assay samples (n)		ELISA samples (n)
		Rispens	Pathogenic	MDV generic
Glendon	Feather	184	46	
	Dust	23	23	
	Serum			149
Bowlers Lane	Feather	225	40	
	Dust	15	15	
	Serum			225
Mid Coast Egg	Feather	89	34	
	Dust	4	4	
	Serum			89

5.1.3 Statistical analysis

The results were analysed using JMP 10 statistical software (SAS Institute Inc., Cary NC, USA). A statistical significance level of $P < 0.05$ is used throughout unless stated otherwise. For ELISA test results, a cubic root model was used to analyse the anti-MDV anti- antibody titre by farm and strain with age as the effect. Likewise feather qPCR results were analysed using Rispens viral load in feather as the y variable, by farm and strain and age as the effect. The dust samples were analysed by farm as virus type as the effect. The percentage of positive results were analysed by Chi-square test.

5.2 Results

5.2.1 Anti MDV antibody titre

Antibodies against MDV were detectable in chicken sera up to the age of 91 weeks by ELISA. The MDV IgY titre (cube root transformed) at the Bowler's Lane farm in young chickens (day old up to 17 weeks of age) ranged from 14 to 22. The MDV IgY titre (cube root) of 3.5 week old ISA Browns' at Bowler's Lane was significantly lower compared to any of the other age groups. The MDV IgY titre (cube root) at the Glendon farm in young chickens (one to two weeks old) ranged from 7.6 to 8.6 before increasing to a peak of 19 at 33 weeks of age, and plateauing around 16-18 thereafter (Figure 5-7). At the Mid Coast Eggs farm, only older chickens (between 50 to 91 weeks of age) were sampled, and the MDV IgY titre (cube root) titre in these birds ranged from 18 to 20.

There were significant effects due to the effect of age ($P < 0.0001$) on the MDV IgY titre (cube root) titre, particularly in the younger birds both at Bowlers Lane (English Leghorns and ISABrowns) as well as at Glendon (Hy-Line Browns) (Figure 5-7). There was no significant effect of age at Mid Coast Eggs farm ($P = 0.64$).

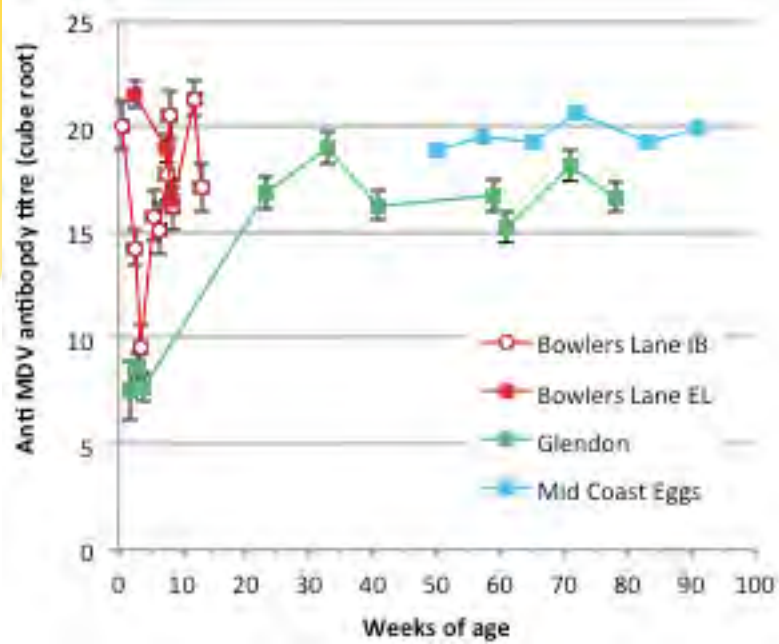


Figure 5-7: Mean (LSM±SEM) concentration of anti MDV antibody titre (cubic root transformed) in chickens vaccinated with Rispens CVI988 vaccine across all 3 farms of up to 91 week old chickens

A detailed profile of the MDV IgY titre (cube root) for the young birds from the Bowler's Lane and Glendon farms is shown in **Figure 5-8**. The MDV IgY titre (cube root) within the first five weeks of age on both farms showed a wide range of fluctuation ranging from 8 to 21, rising and stabilizing thereafter between 15 – 21 MDV IgY titres (cube root).

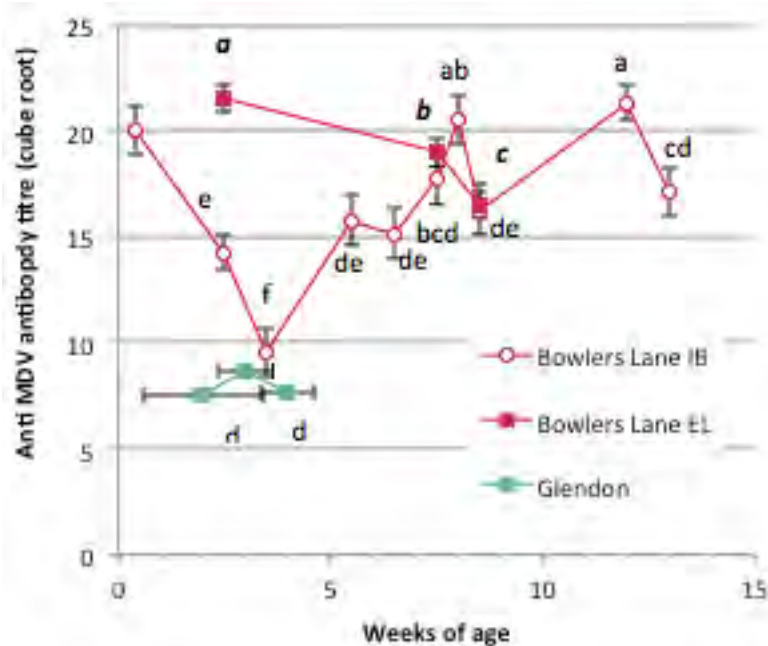


Figure 5-8: Mean (LSM±SEM) cubic root concentration of anti MDV antibody titre in chickens vaccinated with Rispens CVI988 vaccine up to 13 weeks of age in two farms. The levels which are not connected by the same letter are significantly different (P<0.05)

5.2.2 Rispens and MDV load in feather tips

The Rispens/CVI988 load in feather tips was detected throughout all age groups. Overall, 330/498 (66.27%) of the feather samples were positive by qPCR specific for Rispens across all ages whereas 168/498 (33.73%) samples were negative (Figure 5-9).

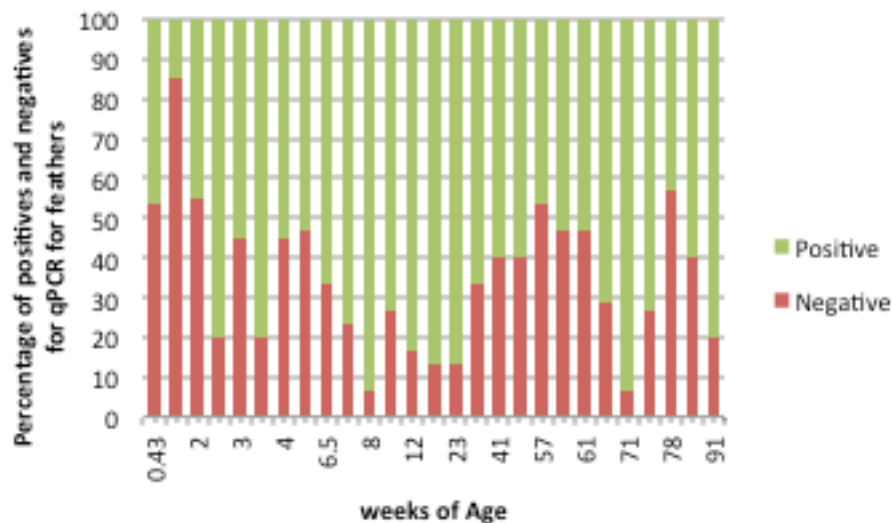


Figure 5-9: Contingency analysis of feather tip samples across all farms and age groups tested for presence of Rispens using the Rispens specific qPCR test

There was a significant effect of age on the mean feather viral load for Rispens for the English leghorns at Bowlers lane ($P=0.01$) as well as at Glendon farms ($P<0.0001$) whereas it was not significant for ISA Browns ($P= 0.37$) at Mid Coast Eggs farm ($P=0.38$) (Figure 5-10).

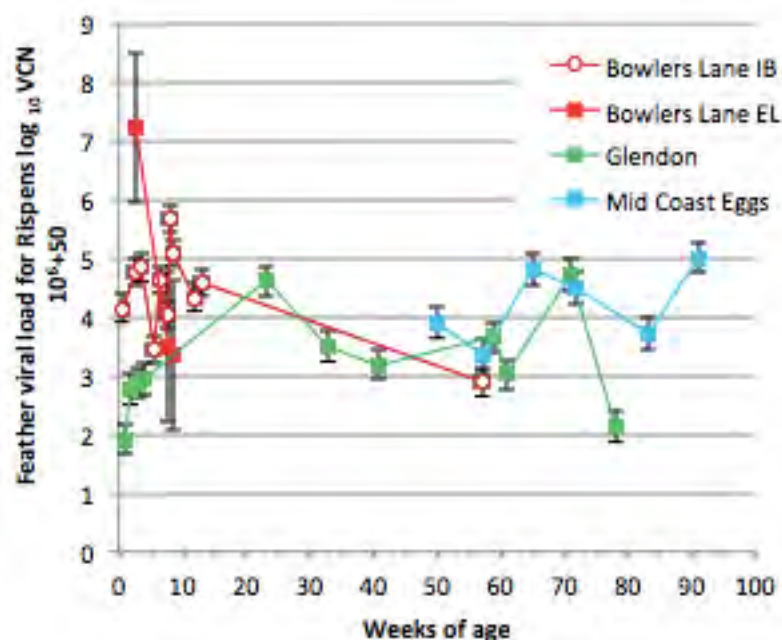


Figure 5-10: Mean log₁₀ (LSM±SEM) Rispens load per 10⁶ +50 cells in feathers from chickens vaccinated with Rispens CVI988 vaccine by farms up to 91 weeks of age

Up to 10 weeks of age, the Rispens load in feathers ranged from 1.9-7.2 mean log₁₀ (LSM±SEM) per 10⁶ +50 cells, whereas after 10 weeks of age, the Rispens load in feathers across all farms plateaued around 4.5-2.1 mean log₁₀ (LSM±SEM) per 10⁶ +50 cells (Figure 5-11). Over the longer term to 91 weeks of age, Rispens load varied around approximately 4 logs without a significant age trend (Figure 5-12).

5.2.3 Pathogenic MDV detection in feather tips

One hundred and twenty feather samples were selected randomly and were subjected to pathogenic MDV qPCR test. Only eight samples (6.67%) were positive for the pathogenic MDV.

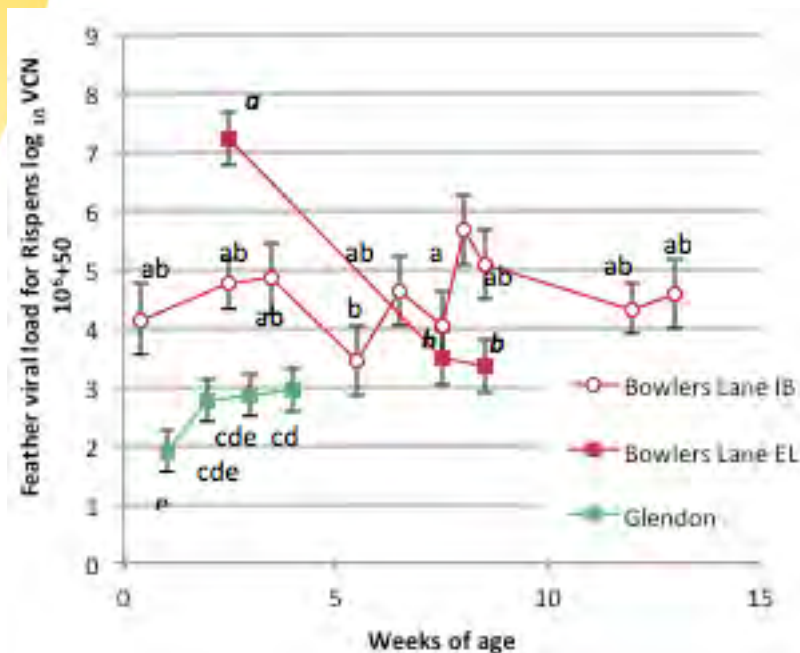


Figure 5-11: Mean log₁₀ (LSM±SEM) Rispens load per 10⁶ +50 cells in feathers from chickens vaccinated with Rispens CVI988 vaccine in two farms. The levels which are not connected by the same letter are significantly different (P<0.05)

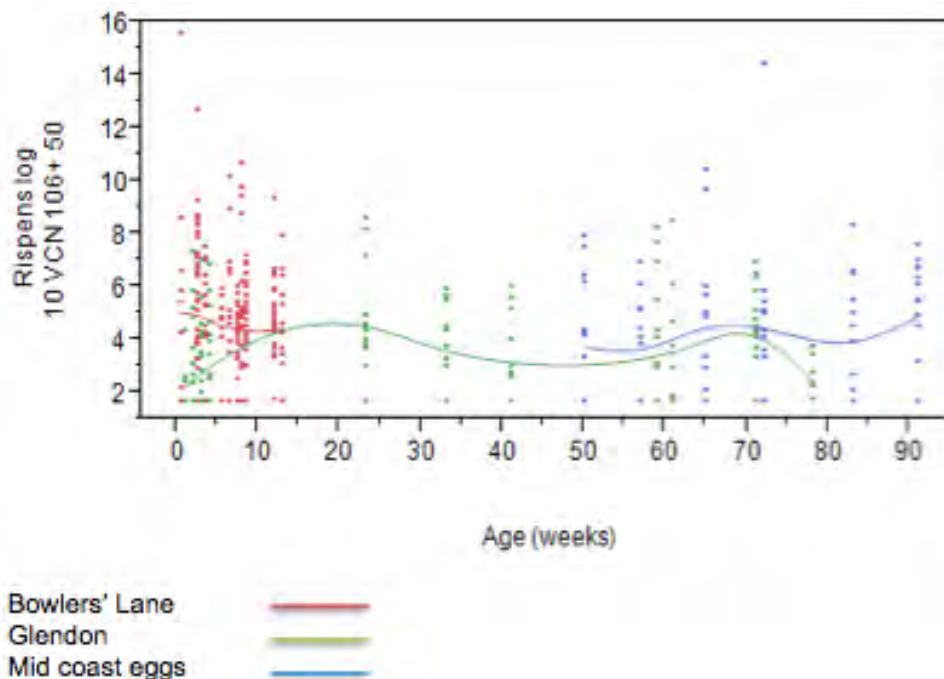


Figure 5-12: Smoothing spline fit $\lambda = 1000$ of feather viral load in chickens vaccinated with Rispens CVI988 vaccine by farm up to the age of 91 weeks

5.2.4 Detection of Rispens and wild-type virus in dust

A total of 42 dust samples from sheds across the three farms were collected. Overall, in the Rispens specific qPCR assay, 39/42 samples were positive (92.86%). In the pathogenic MDV specific qPCR assay, 2/42 samples positive (4.8%).

Figure 5-13 shows the Rispens MDV profile in shed dust over time in each participating farm.

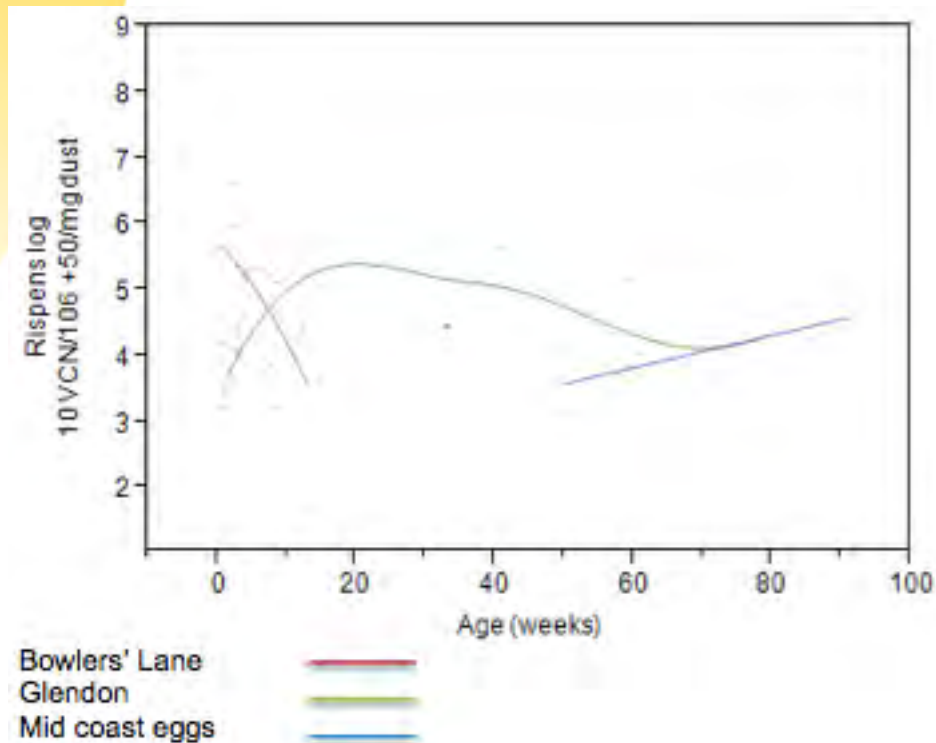


Figure 5-13: Smoothing spline fit $\lambda = 1000$ of mean viral load of Rispens virus in dust by chicken age and farm

5.2.5 Detection of Rispens in commercial dust samples

Dust samples from commercial broiler farms rearing chickens that had not been vaccinated with Rispens were analysed with the Rispens specific qPCR test. Out of the 100 DNA samples collected between early 2012 into 2013, seven samples (7%) were positive for Rispens. The same samples had been analysed with a generic MDV1 qPCR assay, **Figure 5-14** shows the results of the generic MDV1 assay alongside the Rispens specific qPCR assay results.

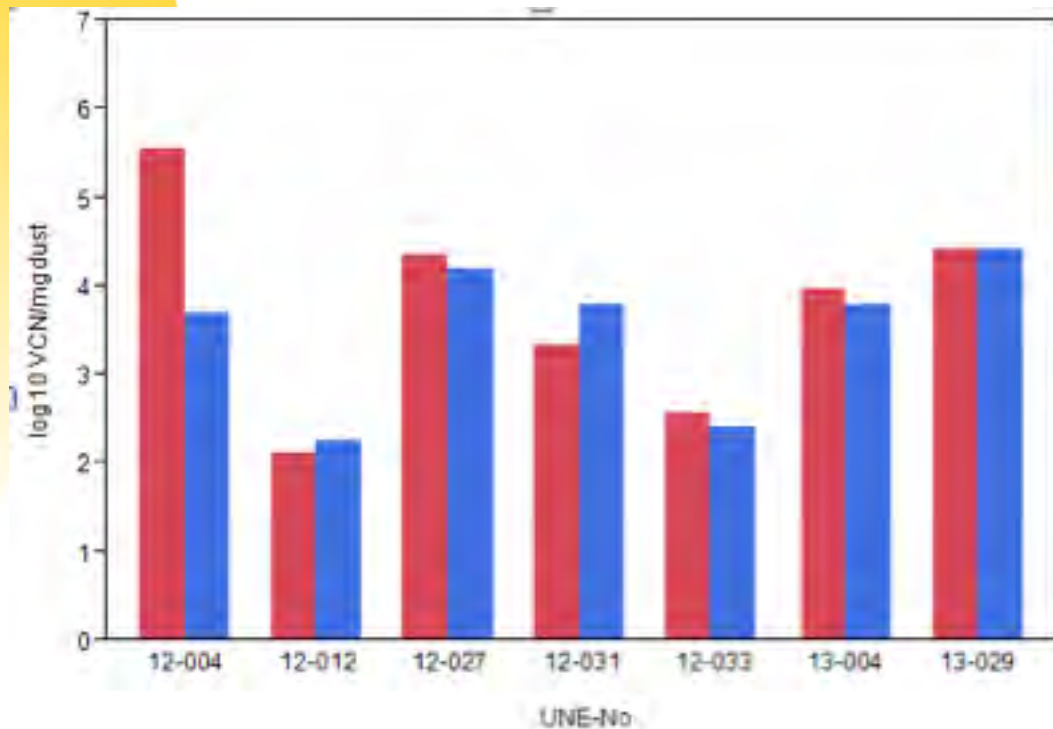


Figure 5-14: Bar chart showing seven commercial dust samples originated from unvaccinated broilers which were positive using the generic MDV qPCR test and positive using the Rispens specific qPCR test

5.2.6 Association between variables

Figure 5-15 shows scatterplots illustrating the level of association amongst key variables. There was a significant positive correlation between ELISA and dust Rispens assays ($r = 0.42$, $P = 0.02$). There was also a negative correlation between feather pathogenic and feather Rispens assays ($r = -0.52$, and $P = 0.01$).

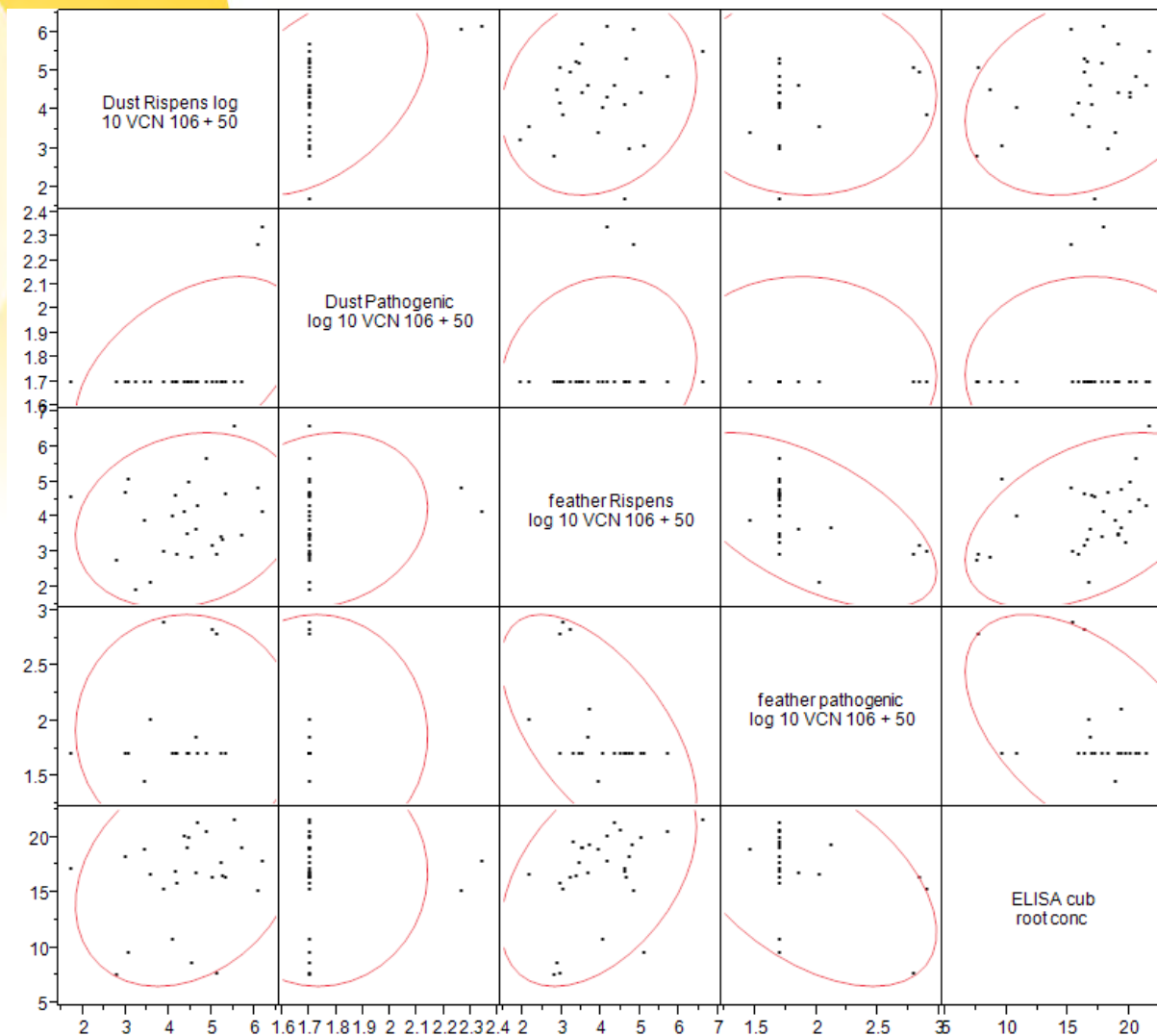


Figure 5-15: Association amongst key variables

5.3 Discussion

This study is the first of its kind, reporting detailed data for the Rispens CVI988 vaccine virus in various types of layer chickens up to 91 weeks of age under commercial conditions. The Rispens virus was detected successfully in various sample types using the Rispens specific qPCR assay developed under AECL project 07/18-UNE (Renz *et al.* 2013) and seroconversion to MDV in serum samples was determined using an adapted ELISA (Zelnik *et al.* 2004).

Unfortunately, all three participating farms in this study used the same supplier of the Rispens vaccine (Bioproperties Pty. Ltd.), so Rispens data from the other suppliers Fort Dodge and Intervet could not be obtained.

The data supports of this study supports hypothesis one; analysis of feather or dust samples from any time after 3 weeks post vaccination is a suitable timeframe to determine Rispens vaccination success. With regards to feather tip samples, the highest proportion of birds in this study was positive for Rispens between 1-2 weeks post vaccination (50-approx. 80%) and the highest Rispens load in feather tips was detected in 3 week old birds, with feather tips harbouring 3.3-7.2 \log_{10+50} VCN/ 106 cells. This is also in concordance with findings in Experiment 1 of this project where feathers from all vaccinated birds were positive for

Rispens by 14 days post vaccination. Also, previous experiments report peaking loads of Rispens in feather tips at 13-15 days post vaccination (Baigent *et al.* 2005; 2007), with a delayed peak if birds did receive only a fraction of the Rispens vaccine (Baigent *et al.*, 2007). This further supports the ideal timeframe for determination of vaccination success between 2-3 weeks post vaccination. Furthermore, the feather development between 2-3 weeks of age / post vaccination is ideal with regards to the consistence of the calamus of the feather (referred to as the feather tip in this report) which is the target for DNA extraction as it is reasonably soft and has not yet fully keratinized at this age.

Similarly, the Rispens load in dust samples was generally the highest from birds around 3 weeks post vaccination (4-5 \log_{10+50} VCN / mg dust). This is consistent with the findings of Rispens loads in dust in experiment 1 of this project where Rispens dust levels peaked between 2-3 weeks post vaccination at very similar levels (4-5 \log_{10} VCN / mg dust) as well as previous field studies investigating the presence of pathogenic MDV1 in broiler chickens (Walkden-Brown *et al.* 2013).

Hypothesis two, that vaccinated chicken will remain infected with Rispens throughout their lifespan, was supported. Between the ages of 78-91 weeks, 43-80% of all feather tip samples were positive by the Rispens specific qPCR test. At Glendon, the Rispens load in feather tips from 78 week old chickens was 2.1 \log_{10+50} VCN/ 10^6 cells and at Mid Coast Eggs, the oldest flock sampled at 91 weeks of age had a Rispens load in feathers of 5.0 \log_{10+50} VCN/ 10^6 cells. However, due to complete keratinization of the feather calamus at those ages, it is possible that the DNA extraction in some samples failed or was only partially successful despite the increase of the incubation time during the digestion step of the DNA extraction to counteract this fact. This might have resulted in some false negative results in the Rispens qPCR assay. Comparable data from other studies is hardly available which is mainly due to the fact that a differentiation between pathogenic and vaccinal MDV1 was not possible previously. Also, the main focus of previous studies were younger age groups of up to around 6 weeks, in order to determine vaccination success and association between early measurements with subsequent disease outcome (Islam *et al.* 2006, Renz *et al.* 2012 and Walkden-Brown *et al.* 2013).

With regards to hypothesis three, which is supported, the ELISA test was successful on all age groups and farms up to 91 weeks. The MDV IgY titre in older birds between 78-91 weeks remained at a high level between 16-20 at both Glendon and Mid Coast Egg farms respectively. Overall, the serological results showed a highly similar pattern compared to the Rispens qPCR results in feathers, especially in young birds up to 15 weeks of age (**Figure 5-7** vs **Figure 5-10**). The Rispens shedding pattern in shed dust showed a similar pattern compared to the serology results over time for Glendon and Mid Coast Eggs farms, but not for the Bowler's Lane farm. The Rispens load in shed dust on this farm decreased over time when analysed by the Rispens specific qPCR test, whereas the MDV IgY titre in serum samples first decreased until 3-4 weeks of age, before increasing again (**Figure 5-7**). A possible explanation is the detection of wild type virus detected on this property which was shown in Experiment 2 to suppress replication of the vaccine virus.

With regards to hypothesis four, that CVI988 will be found in some unvaccinated surrounding flocks indicating that it has established itself in the poultry population, seven out of 100 commercial broiler dust samples were positive for Rispens, thus supporting the hypothesis. Based on the findings of the first experiment of this project, that Rispens is successfully shed in high amounts in feather dander and transmits freely between chickens, it was thought possible that Rispens may be present in dust samples from unvaccinated broilers flocks especially when the broiler flocks were in reasonably close proximity to any layer/breeder farms. Data from similar studies is unfortunately unavailable, but it would be interesting to investigate in detail to which extent such 'escape' vaccinal virus spreads into unvaccinated flocks and to what extent it can offer protection against MDV infection in such flocks.

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7. Plain English Summary

Project Title	Field application of a Rispens-specific qPCR test.
AECL Project No.	1UN111
Researchers involved	Stephen W. Walkden-Brown, Katrin G. Renz
Organisations involved	Animal Science W49, School of Environmental and Rural Science, University of New England, Armidale, NSW 2351
Phone	(02) 6773 5152
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Email	swalkden@une.edu.au
Objectives	<ol style="list-style-type: none"> 1. To take the molecular diagnostic tests to differentiate between wild type and Rispens CVI988 serotype 1 Marek's disease virus developed under AECL Project 08/17 UNE and fully test and validate them for field application by industry as tests of vaccination success, and as a means of detecting wild type (pathogenic) MDV1 in Rispens-vaccinated flocks. 2. To use the test to investigate the kinetics of viral replication and persistence within the host, and shedding pattern, of the Rispens CVI988 virus alone and in combination with wild type virus. This will shed light on the natural epidemiology of the vaccine virus and the conditions under which protection following vaccination are optimised.
Background	<p>Vaccination against Marek's disease using live vaccines provides protection against clinical Marek's disease but not against co-infection with wild-type pathogenic MDVs, which continue to multiply in the host and be shed in feather dander at very high levels. Thus vaccinated chickens may harbour mixed populations of MDVs. While the Australian broiler industry has access to molecular tests to differentiate between vaccinal (HVT and MDV serotype 2) and pathogenic (MDV serotype 1) serotypes of MDV the layer and breeder industries do not as they rely more on an attenuated serotype 1 vaccine (Rispens CV1988) to provide long-term protection against MD.</p> <p>Our group developed the first reliable, quantitative tests that differentiate between Rispens and wild type MDV1 under AECL project 07/18-UNE. These tests clearly differentiate between Australian wild type MDV1 and Rispens CVI988. The sensitivity and specificity of the tests have been well characterised on the earlier project, but there is a need to conduct animal studies in the lab and in the field to determine useful end points for the test as a diagnostic tool for industry. It is also important to determine how the Rispens virus is influenced by co-infection with pathogenic MDV-1 and vice versa. Determining these is the purpose of this project.</p>

<p>Research</p>	<p>The project research comprised 3 experiments as follows:</p> <ol style="list-style-type: none"> 1. Rispens virus replication, shedding and transmission post vaccination in SPF chickens. To determine viral kinetics and shedding and transmission between birds. 2. Co-infection and protection in ISA Brown layers vaccinated with Rispens and/or challenged with pathogenic MDV-1 at different vaccination-challenge intervals (VCI). To determine the effect of VCI on level of vaccinal protection and on virus replication rates and shedding of both vaccinal and pathogenic virus. 3. Field studies on 3 farms to investigate Rispens virus presence in feathers and dust, and anti-MDV antibody titre in Rispens-vaccinated chickens from 1-91 weeks of age. To determine optimum times and tissue types for sampling for vaccination success. Additional work involved detecting wild-type MDV-1 in vaccinated layers and presence of Rispens in broiler shed dust samples, from populations not vaccinated with Rispens. To determine the efficacy of vaccination in blocking infection in the field, and to determine if the Rispens vaccine virus has “escaped” into unvaccinated flocks.
<p>Outcomes</p>	<ol style="list-style-type: none"> 1. Demonstration that current strains of the Rispens vaccine virus are shed in large amounts from vaccinated birds and spread effectively to in-contact unvaccinated birds. 2. Demonstration that the Rispens virus inhibits replication and shedding of pathogenic MDV, but only if vaccine administration is prior to or concurrent with challenge. The same is true for the effect of pathogenic virus on Rispens, i.e. Challenge prior to vaccination suppresses replication of the vaccine virus. 3. Probably due to the kinetics described above, vaccine is only protective to a significant extent when challenge is delayed 5-10 days post vaccination. There is a small protective effect when vaccine is administered concurrently with challenge, but none if challenge precedes vaccination by 5 or 10 days. 4. Rispens infection and antibody levels in vaccinated commercial hens persist lifelong and rates of detectable co-infection with pathogenic MDV are very low. 5. Rispens virus appears to have “escaped” into some unvaccinated flocks. 6. Sampling of feathers or dust from vaccinated chickens from 3 weeks of age is suitable for determination of vaccination success using Rispens-specific qPCR. Dust has significant advantages over feathers from sample number, collection, transportation and processing perspectives.
<p>Implications</p>	<p>The main deliverable is an effective field test for measuring vaccination success following vaccination with Rispens CVI988 and effective methods for detecting MDV breaks in vaccinated flocks. The tests are available on a commercial basis to industry through UNE.</p> <p>The other main deliverable is useful information on the spread and natural epidemiology of the Rispens MDV strain, and the efficacy of vaccination against challenge at different times post vaccination.</p> <p>Our understanding of the kinetics of virulent and non-virulent MDV1 in the same host is greatly enhanced by this project. Unlike the situation with serotype 2 and 3 vaccinates, challenge with pathogenic MDV reduces rather than increases the shedding of the Rispens vaccine virus. The higher replication and shedding rates of pathogenic MDV-1 compared to the Rispens vaccine, support the theory that vaccination favours natural selection of more virulent MDVs over attenuated or less virulent MDVs by allowing them to replicate at higher rates, but removing early death of the host as a factor limiting reproductive success.</p>

Key words	Marek's disease, vaccination, Rispens CVI988, qPCR
Publications	<p>Full Journal Papers (refereed)</p> <p>Islam, T, Walkden-Brown, S, Renz, K, Islam, A, Ralapanawe, S (2014) Replication kinetics of very virulent Marek's disease virus and vaccinal Rispens/CVI988 virus during single and mixed infections varying in order and interval between infections. <i>Veterinary Microbiology</i> (in press)</p> <p>Islam, T, Walkden-Brown, SW, Renz, KG, Fakhrul Islam, AFM, Ralapanawe, S (2013) Vaccination-challenge interval markedly influences protection provided by Rispens CVI988 vaccine against very virulent Marek's disease virus challenge. <i>Avian Pathology</i> 1-35.</p> <p>Islam, T, Renz, KG, Ralapanawe, S, Walkden-Brown, SW (2013) Viral kinetics, shedding profile and transmission of serotype 1 Marek's disease vaccine Rispens/CVI988 in maternal antibody-free chickens. <i>Avian Diseases</i> 57, 454–463.</p> <p>Refereed long conference papers</p> <p>Islam, T, Walkden-Brown, SW, Renz, KG, Islam, AMFM (2013) Effect of widely divergent vaccination-challenge intervals on protection provided Rispens CVI988 vaccine against very virulent Marek's disease virus challenge in Isa Brown chickens. <i>Proc. Aust. Poult. Sci. Symp.</i> 24, 243-246.</p> <p>Islam, T, Renz, KG, Islam, AMFM, Walkden-Brown, SW (2012) Effect of wide variation in the timing of vaccination relative to challenge on protection against MDV provided by Rispens CVI988 vaccine. <i>Proceedings of the 9th International Meeting on Marek's Disease and Avian Herpesviruses Berlin, 24-28 June 2012</i> 39.</p> <p>Islam, T, Renz, KG, Walkden-Brown, SW (2011) Viral load, shedding rate and lateral transmission of Marek's disease vaccinal virus (Rispens/CVI988) in SPF chickens. In 'Proceedings of the Australian Poultry Science Symposium Volume 22. Sydney'. (Ed. JR Roberts) Volume 22 pp. 239-242. (The Poultry Research Foundation University of Sydney:</p> <p>Short conference paper</p> <p>Islam, T, Renz, KG, Walkden-Brown, SW (2012) Viral load, shedding rate and lateral transmission of Marek's disease vaccinal virus (Rispens/CVI988) in SPF chickens. <i>Proceedings of the 9th International Meeting on Marek's Disease and Avian Herpesviruses Berlin, 24-28 June 2012</i> 50.</p>