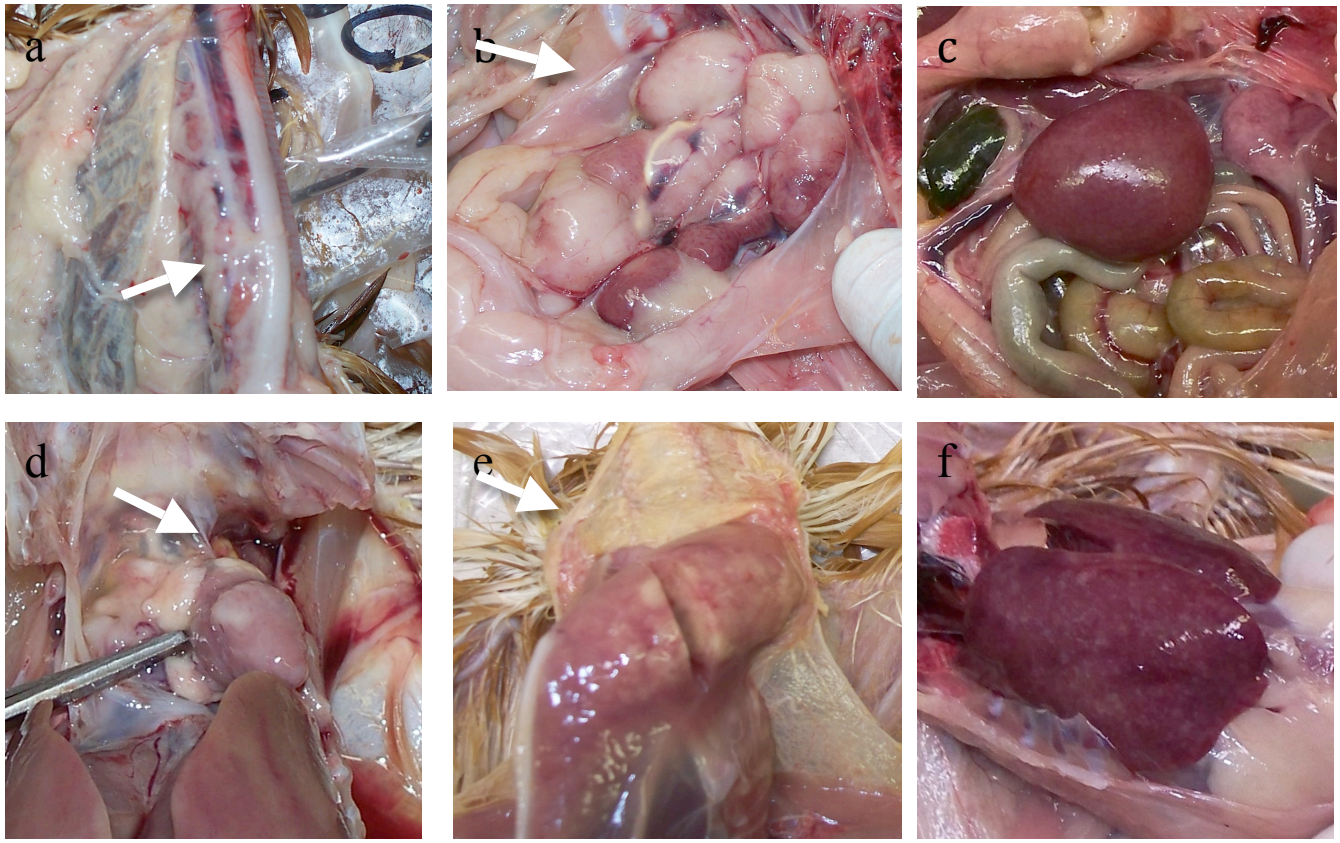

Figure 2.3 Examples of MD gross lesions observed during *post mortem*, a) thymic atrophy, b) lymphoma in ovary and kidney, c) enlarged spleen, d) lymphoma in heart, e) lymphoma in breast muscle, f) lymphoma in liver.

2.1.9 Sample collection from chickens

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

Weekly feather samples from each individual bird were collected inside the respective isolators into a 1.5 ml Eppendorf 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 at -20 °C until further processing.

At the termination of the experiment, 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 g for 15 minutes at 4 °C temperature and serum aspirated and transferred to a 1.5 ml Eppendorf tube and stored them at -20 °C until further processing.

In the first experiment, dust samples were collected directly into a sterile 1.5 ml Eppendorf tube from each room from a large 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 samples were collected from the dust deposits at the 90º 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 1.5 ml Eppendorf 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.

Spleen samples were collected from all the dead and euthanized birds after dissection for enumeration of viral load by qPCR. The spleens were collected into a sterile 5 ml tube using forceps, weighed with the tube and then stored at -20 degrees until further processing.

2.2 General laboratory Procedures

2.2.1 Separation of peripheral blood lymphocytes (PBL)

Approximately 300 µl of citrated blood sample was transferred slowly onto 300 µl of Ficoll Paque^{TM} PREMIUM (Amersham Biosciences, Sweden) in a 1.5 ml Eppendorf tube and centrifuged at 900 g for 20 minutes at approximately 8 °C. Lymphocytes were then carefully aspirated from the Ficoll Paque interface and transferred to another 1.5 ml Eppendorf tube containing 500 µl PBS. The samples were then centrifuged at 3500 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.

2.2.2 DNA extraction from PBL

DNA was extracted from PBL using the automated DNA X-tractor Gene (Corbett Robotics, Australia). Prior to loading the samples on 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 30 kPa 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 30 kPa for 3 minutes
- 600 µl of DX wash (DXW) per well was loaded into the capture plate and vacuumed at 25 kPa 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 30 kPa for one and half minutes. At last the elution plate was moved from the machine and stored at -20 °C.

2.2.3 DNA extraction from feather tips, dust and spleen

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

DNA was extracted from 2 - 3 feather tips or 5mg dust or 10 (± 1) mg of spleen respectively using either the DNeasy Blood and Tissue kit (Qiagen, Australia,) for Experiment 1 and the ISOLATE Genomic DNA Mini Kit (Bioline, Australia,) for Experiment 2. Both kits were

tested and revealed that there was no difference in either extracted DNA quantity or quality and VCN obtained. The DNA was extracted according to the manufactuer's instructions.

The method for Qiagen kit was as follows:

5 mg of dust or 10 mg of spleen was weighed into a 1.5 ml microcentrifuge tube. 200 µl buffer X1 was added to each sample. To make up 10 ml of X1 buffer the following reagents and amounts were used:

100 mM Tris Cl pH 8	1 ml
0.5M EDTA pH 8	200 µl
1M NaCl	1 ml
10 % SDS	2 ml
1 M DTT	400 µl **Added just before use**
250 ug/ml Proteinase K	125 µl **Added just before use**
Milli Q water	5.275 ml

Next the tube with sample and buffer was incubated at 55 ºC in a water bath for 1 hr, mixing every 15 minutes. If there was a large amount of sediment present after incubation the sample was centrifuged at 8000 rpm (4722 g) for 15 seconds then supernatant was taken off and used this for the extraction.

Next 200 µl buffer AL and 200µl absolute ethanol was added to sample and mixed by vortexing. Then the mixture was pipetted into DNeasy spin column and centrifuged at 8000 rpm (4722 g) for 1 minute. Then the tube was discarded and the column was placed into a new collection tube. Next 500 µl of AW1 buffer was added and the spin column centrifuged at 8000 rpm (4722 g) for one minute. Again the tube was discarded and the column placed into another new collection tube. After that 500 µl of AW2 buffer was added to the tube and

centrifuged 4 minutes at 13000 rpm (12470 g). Then the column was placed into 1.5 ml microcentrifuge tube after discarding the collection tube.

Next the DNA was eluted with 100 µl of AE buffer and incubated at room temperature for 5 minutes. Finally the tube was centrifuged at 8000 rpm (4722G) for one minute and the supernatant was stored at – 20 °c after discarding the column.

The method for the Bioline kit was as follows:

5 mg of dust weighed into a 1.5 ml microcentrifuge tube or place 3 - 4 small sections (3 mm) of feather tips cut using a sterile scalpel blade into such a tube. Then 400 µl Lysis Buffer D and 25 µl Proteinase K added, mixed by vortexing, incubated at 50 ºC mixing every 15 minutes until the sample is completely lysed, usually 1 - 2 hours. If there is a large amount of sediment present after incubation the sample is centrifuged at 12000 rpm (10000 g) for 1 minute then supernatant taken off and saved.

Next 200 µl binding buffer D was added to sample and mixed by vortexing for 15 seconds. Then the mixture was pipetted into spin column D and centrifuged at 12000 rpm (10000 g) for two minutes. Then the tube was discarded and the column was placed into a new collection tube. Next 700 µl of wash buffer was added and the column centrifuged at 12000 rpm (10000 g) for one minute. The filtrate was discarded and the washing procedure was repeated. After this the column was centrifuged for two minutes at 13000 rpm (12470 g) to remove all traces of ethanol.

Next the column was placed into 1.5 ml microcentrifuge tube and the DNA eluted with 100 µl elution buffer at room temperature for one minute. Finally the tube was centrifuged at 8000 rpm (4722 g) for one minute and the supernatant containing the extracted DNA was stored at – 20 ºC.

2.2.4 DNA Quantification

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

2.2.5 Quantitative real-time Polymerase Chain Reaction (qPCR)

All extracted DNA samples of the first experiment were subject to MDV serotype specific TaqMan® qPCR assays or generic assays. The primer and probe target for MDV-1 was the meq gene as published by Islam *et al.* (2004) and the sequences are given in Table 2.1. All extracted DNA samples of the second experiment were subject to two TaqMan® qPCR assays one of which only detects Rispens/CVI988 MDV-1 while the complimentary test only detects wild type pathogenic MDV-1 (Renz*, et al.*, 2013). The target gene for both of these tests was the *meq* gene and primers and probes are are given in Table 2.1. MDV genome copy number was determined by absolute quantification as reported by Islam *et al*. (2006a) and Renz *et al.* (2013).

Table 2.1 Sequences of meq-gene primers and probe used for qPCR MDV-1 and differentiation between Rispens/CVI988 and pathogenic assays

Target gene	Primer/probe sequence
Meq (MDV serotype 1)	Probe: 5'-(FAM) CGTCTTACCGAGGATCCCGAACAGG (BHQ-1)-3' F primer: 5'-GGAGCCGGAGAGGCTTTATG-3' R primer: 5'-ATCTGGCCCGAATACAAGGAA-3'
Meq (Rispens serotype 1)	Probe: 5'-(FAM)-TGCAGAGGGCCAATGAACACCTACG (BHQ1)-3' Primer BCH402: 5'-TCGGAGAAGACGCAGGAA-3' Primer BCH403: 5'-GCTCATGACAAGCCAACTGTA-3'
Meq (Non-Rispens serotype 1)	Probe: 5'-(FAM)-TGCAGAGGGCCAATGAACACCTACG (BHQ1)-3' Primer BCH406: 5'- TCGGAGAAGACGCAGGTC-3' Primer BCH445: 5'-GTAAGCAGTCCAAGGGTCACC -3'

The respective TaqMan® real-time qPCR assay was performed using a RotorGene 3000 real-time PCR machine (Corbett Research, Sydney, Australia) (Figure 2.4). The qPCR cycling parameters consisted of: 50 °C for two minutes, 95 °C for two minutes, followed by 45 cycles consisting of denaturation at 94 °C for 15 seconds and annealing/extension at 60 °C for 45 seconds for MDV-1 generic assay and 60 seconds for the Rispens/CVI988 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. Corbett CAS1200 Robotic machine (Corbett Research, Sydney, Australia) was used to prepare the reaction tubes for all qPCR assays.

Figure 2.4 RotorGene 3000 real-time PCR machine

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 serial dilutions of MDV-1 standards of known concentration of MDV-1 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 quality controls (QCs) are given in Table 2.2 Samples that did not amplify or amplified with a Ct value below the lowest standard were determined negative. The intra assay co-efficient of variation (CV) was calculated from duplicates of each sample and inter assay CV calculated from a quality control sample included in each assay run. These CVs were based on both calculated viral copy number (VCN) and Ct values. Inter- and intra- assay CVs of 20 or less are generally acceptable but value of 10 or less is ideal. This would apply to the CV values for ct which is the actual measure determined by the instrument which would broadly equate to the CVs of log VCN value. CVs for VCN on the normal scale will necessarily be much higher. PBL DNA was not assayed in duplicate so the intra assay CV cannot be calculated for these samples. The details are presented in Table 2.3.

Table 2.2 Details of standards and QCs used in the qPCR assays

qPCR assay	Standards and QCs	Dilution factor	Concentration	Origin
Generic MDV-1/Pathogenic MDV-1	MDV-1 26.1	0	12.5 ng/ul	Pooled spleen DNA of SPF chickens infected with MDV-1 (MPF57)
	MDV-1 26.2	100	.125 ng/ul	
	MDV-1 26.3	100	.00125 ng/ul	
	MDV-1 26.4	10	.000125 ng/ul	
	QC1	0	50 ng/ul	Pooled spleen DNA of SPF chickens infected with MDV-1 (MPF57)
	QC2	20	2.5 ng/ul	
Rispens specific MDV-1	Risp 1	0	0.132 ng/ul	DNA extracted from Marek's disease vaccine Rispens CVI988® vaccine (Bioproperties).
	Risp 2	100	.00132 ng/ul	
	Risp 3	10	.000132ng/ul	
	Risp 4	10	.000013 ng/ul	
	QC TZ1	0	75.5 ng/ul	Spleen DNA 2 SPF chickens vaccinated with Marek's disease vaccine Rispens CVI988® vaccine (Intervet and Bioproperties respectively).
	QC TZ2	25	2.5ng/ul	

Table 2.3 Overall mean intra and inter assay coefficients of variation (CV) based on mean Ct values and mean calculated viral copy number (VCN) of all samples.

Sample	Experiment 1 (Generic MDV-1 qPCR assay)				Experiment 2 (Pathogenic MDV-1 qPCR assay)				Experiment 2 (Rispens MDV-1 qPCR assay)			
	Intra Assay CV		Inter assay CV		Intra Assay CV		Inter assay Cv		Intra Assay CV		Inter assay Cv	
	VCN (%)	CT (%)	VCN (%)	CT (%)	VCN (%)	CT (%)	VCN (%)	CT (%)	VCN (%)	CT (%)	VCN (%)	CT (%)
PBL	NA	NA	12.8	1.0	NA	NA	47.4	3.6	NA	NA	86.7	4.3
Feather	21.2	0.8	38.2	2.7	24.3	1.9	66.3	6.7	35.2	1.6	142.3	6.6
Spleen	37.0	2.0	13.0	15.0	ND	ND	ND	ND	ND	ND	ND	ND
Dust	19.0	1.1	4.0	2.6	8.0	0.4	53.5	8.5	13.4	1.5	121.0	7.0

ND – Not done

2.2.6 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. As antigen we used Marek's disease vaccine Rispens CVI988® vaccine (Bioproperties). 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 100 µl 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 one 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 one hour. The contents of the plates were again flicked out and washed three 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 chemical company, 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 1 M H_2SO_4 to plates stopped the reaction and the plates were read at 492 ηm using a plate reader.

Preparation of antigen: One vial of Bioproperties Vaxsafe RIS vaccine (Live) was thawed by placing in warm water. Then dilution media was added to make it up in 5 mls and mixed well. After that it was centrifuged for 10 mins at 2500 rpm (748 g) at 4 °C and the pellet was retained after pouring off supernatant. The pellet was then frozen at -20 °C then thawed; this was repeated at least 4 times. After the last freeze/thawing, 5 mls PBS was added and the pellet broken up and dispersed before being was sonicated for at least 1 minute at approx 12 amps. Finally the sample was centrifuged for 10 mins 3000 rpm (1077 g) at 4 °C, the supernatant was collected and stored at -20 °C until use as antigen.

Validation of assays: A standard curve was generated in each assay. All samples from an individual bird were done in duplicate on one plate to minimise between-plate effects. The sensitivity of the assays for MDV-1 was determined by running 2-fold serial dilutions of the standards with known titres. Standards and quality control (QC) were also assayed in duplicate for all assays. Details of Standards are given in table 2.4. Standards were made up from pooled sera from breeder broiler chickens vaccinated against MDV with Rispens/CVI988 vaccine. The region of sensitive detection was the linear part of the standard curve showing in the figure 2.5. The titre cut off value was determined from known negative control sera and titres adjusted by subtraction of the highest value from the control chickens. The intra assay (plate) co-efficient 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 2.4 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
S 10	1:8192	1

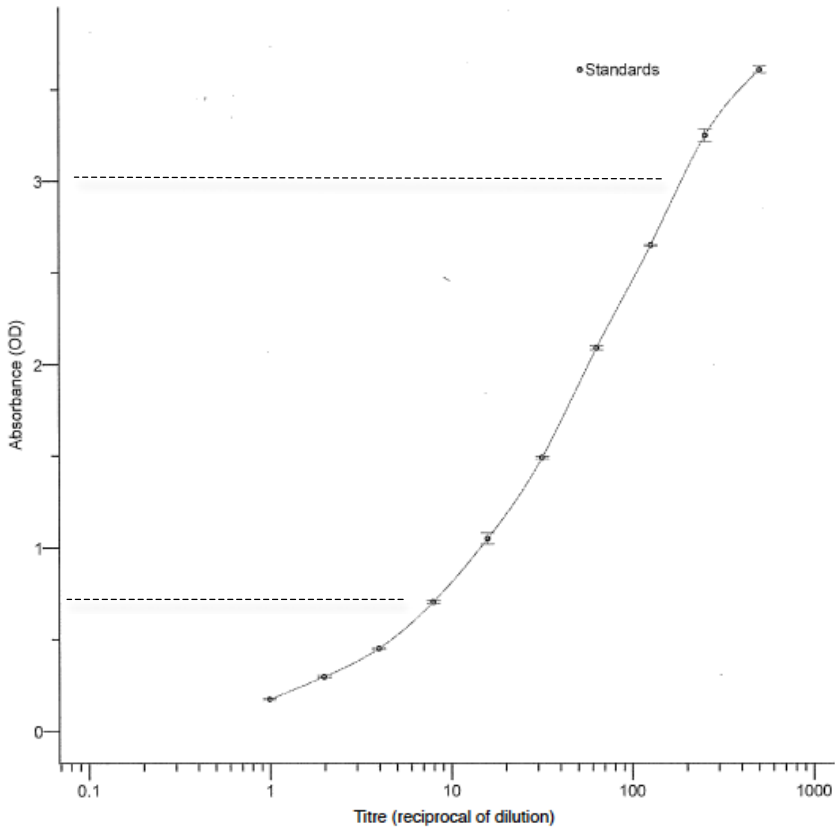

Figure 2.5 Standard curve of the ELISA assay. Dashed line showing the region of sensitive detection points