

***In vitro* and *in vivo* characterization of selected
Australian isolates of Marek's disease virus**

By

Katrin Renz

BSc, MSc (Agricultural Sciences, University of Hohenheim, Germany)

A thesis submitted for the degree of

Doctor of Philosophy

in the Centre of Animal Health and Welfare, School of Environmental
and Rural Sciences, the University of New England.

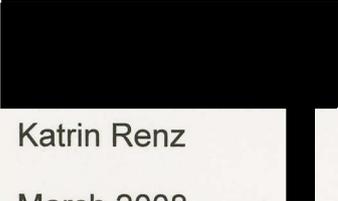


March 2008

Declaration of originality

I hereby certify that this work is the original work of the author except where acknowledged in the text. My involvement in Experiment MD05-C-PT3 was as a co-investigator to Prof. Stephen Walkden-Brown and these data were included in this thesis with his permission. Any help I have received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

The substance of this thesis has not been submitted for any degree, either in full or in part and it is not currently being submitted for any other degree or qualification at this or any other University.



Katrin Renz

March 2008

Dedication

I would like to dedicate this thesis to
my beloved father, Dieter Renz and my mother, Gundula Renz.

Acknowledgements

This research was financially supported by the Rural Industries Research and Development Corporation (RIRDC) project UNE-83J which is greatly appreciated. Furthermore, I have received both a UNERA and IPRS postgraduate scholarship from the University of New England for which I am grateful.

The journey towards a PhD degree can be treacherous but also exciting and pleasurable. I have many to thank for helping me through the hard times and sharing the good times throughout my graduate work. First and foremost, I would like to thank my supervisor, Prof. Stephen Walkden-Brown, for his supervision, support and overall guidance throughout my PhD candidature. His excitement and enthusiasm for any research is unsurpassed and this has incited me along until the end of my thesis and beyond. I would also like to thank my co-supervisor Dr Brian Cheetham for his greatly appreciated advice and invaluable support during the molecular work. His patience and knowledge has helped me to cope with the challenges of molecular Biology.

Special thanks are also extended to Dr Fakhrul Islam (UNE), Dr Zhanhai Gao (UNE), for their encouragement and helpful discussions.

For their technical assistance throughout my thesis, I would like to thank Susan Burgess (UNE), Paul Reynolds (UNE), Megan Sutherland (UNE), Jenny Druitt (UNE), Suresh Kumar (UNE), Kapil Chousalkar (UNE), Gary Taylor (UNE).

Last, but not least, I want to thank all my friends, especially Michael Zender, Judith Reiser, Dr Frank Klempau and the Kontner family for their undying moral support and inspiration. They always managed to make me smile during the hard times of my PhD candidature. And although being thousands of kilometres apart, both my family and friends always kept as close contact as possible with me and gave me all the help and support they could.

Finally, I would like to extend my special tribute to my parents for their incredible efforts, support and generosity to allow me to undertake the endeavour of a PhD degree. Very special thanks also to my sisters, Verena and Anne Renz, who both gave me continuous support and encouragement throughout the journey.

Publications

Materials from this thesis have been published in the following journals, conference papers and abstracts:

Refereed journal papers:

1. **Renz, K.G.**, Islam, A., Cheetham, B.F. and Walkden-Brown, S.W. (2006): Absolute quantification using real-time polymerase chain reaction of Marek's disease virus serotype 2 in field dust samples, feather tips and spleens. *J Virol Methods*. **135**, 186-91.
2. **Renz, K.G.**, Cheetham, B.F. and Walkden-Brown, S.W. (2008): Sequence analysis of the Marek's EcoRI-Q-encoded (meq) gene of five Australian isolates of Marek's disease virus serotype 1 (MDV1) and association with virulence. *Vet. Microbiol.* (under revision for re-submission).

Long conference papers (refereed):

3. **Renz, K.G.**, Cheetham, B.F., Islam, A. and Walkden-Brown SW (2006). Absolute quantification of Marek's disease virus serotype 2 using real-time polymerase chain reaction and its application to field dust samples. *Proceedings of the Australian Poultry Science Symposium* **18**, 153-156.
4. **Renz, K.G.** and Walkden-Brown SW (2007): Environmental enrichment strategies for improved welfare of experimental layer chickens housed in isolators. *Proceedings of the Australian Poultry Science Symposium* **19**, 41-44.

Short conference papers (refereed):

5. Walkden-Brown, S.W., Cooke, J., Islam, A., **Renz, K.G.**, Hussain, Z., Islam, A.M.F.M., Tannock, G. and Groves, P. (2007): Pathotyping of Australian isolates of Marek's disease virus. *Proceedings of the Australian Veterinary Poultry Association*, Sydney, pp 32-37.

Conference abstracts (refereed):

6. **Renz, K.G.**, Cheetham, B.F. and Walkden-Brown, S.W. (2008): Association between Marek's EcoRI-Q-encoded (*meq*) gene sequence and virulence in Australian and international isolates of Marek's disease virus (Abstract). In '*Proceedings of the 8th International Marek's Disease Symposium: 6-10th July, Townsville, Australia*'. Townsville p. (submitted).
7. **Renz, K.G.** and Walkden-Brown, S.W. (2008): Longitudinal quantification of MDV serotype 1 and HVT in feather tips and relationship to clinical Marek's disease in commercial layer chickens. In '*Proceedings of the 8th International Marek's Disease Symposium: 6-10th July, Townsville, Australia*'. Townsville p. (submitted).
8. **Renz, K.G.** and Walkden-Brown, S.W. (2008): Virulence of MDV challenge strain and vaccination influence the shedding profile of MDV in feather dander. In '*Proceedings of the 8th International Marek's Disease Symposium: 6-10th July, Townsville, Australia*'. Townsville p. (submitted).
9. Walkden-Brown, S.W., Cooke, J., Islam, A., **Renz, K.G.**, Hussain, Z., Islam, A.M.F.M., Tannock, G. and Groves, P. (2008): Pathotyping of Australian isolates of MDV. In '*Proceedings of the 8th International Marek's Disease Symposium: 6-10th July, Townsville, Australia*'. Townsville p. (submitted).

Table of contents

Declaration of originality	ii
Dedication	iii
Acknowledgements	iv
Publications	v
Table of contents	vii
List of Tables	xvii
List of Figures	xix
Abstract	xxv
List of abbreviations	xxix
General introduction	1
Chapter 1: Review of literature	4
1.1 Introduction	4
1.2 Brief history of Marek’s disease	6
1.3. MD situation in Australia	7
1.4 Economic significance	9
1.5 Aetiology of Marek’s disease	10
1.5.1 Classification of MDVs	10

1.5.2 Morphology	13
1.5.3 Genome organisation.....	14
1.5.4 MDV genes and gene products.....	15
1.5.4.1 MDV1-specific genes and gene products.....	17
1.5.4.2 MDV2 genes	19
1.5.4.3 HVT genes	19
1.5.5 Other Herpesviruses related to MDV	20
1.6 Pathobiology and epidemiology.....	21
1.6.1 Pathogenesis	21
1.6.1.1 Entry to the host and infection.....	22
1.6.1.2 Phase 1: early cytolitic phase.....	23
1.6.1.3 Phase 2: Latency	24
1.6.1.4 Phase 3: late cytolitic phase.....	26
1.6.1.5 Fully productive infection in the feather-follicle epithelium (FFE)	26
1.6.1.6 Phase 4: Transformation	27
1.6.1.7 Immunosuppressive effects of MDV infection	28
1.6.1.8 Other consequences of MDV infection.....	30
1.6.2 Factors affecting pathogenesis	30
1.6.2.1 Virus serotype and pathotype.....	30
1.6.2.2 Host genotype	31
1.6.2.3 Host immunity	33
1.6.3 Co-infection with other pathogens.....	34
1.6.4 Transmission.....	35
1.7 Control of MD	36
1.7.1 Vaccination against MD	36
1.7.2 Hygiene and biosecurity.....	38
1.8 Diagnosis of MD and characterization of MDV.....	38
1.8.1 Diagnosis of MD in vivo	38

1.8.2 Pathotyping of MDV isolates	40
1.8.3 Diagnosis of MD in vitro	42
1.8.3.1 Virus isolation and identification	42
1.8.4 Molecular methods for MDV diagnosis	44
1.8.4.1 Polymerase chain reaction (PCR)	44
1.8.4.2 Real – time PCR	45
1.8.4.3 PCR methods for detection of MDV	47
1.8.4.4 Advantages and disadvantages of molecular diagnostic methods.....	48
1.8.4.5 Genomic analysis of MDV	49
1.8.4.6 Cloning vectors for sequencing purposes	50
1.8.4.7 Summary of the application and potential of MDV genome analysis	52
1.9 Conclusions	52

**Chapter 2: Absolute quantification of MDV2 genome copy
number in dust samples, spleens and feather tips using real-
time PCR..... 54**

2.1 Introduction	54
2.2 Materials and Methods	55
2.2.1 Development of plasmid standard.....	55
2.2.2 Development of plasmid constructs	56
2.2.3 Processing plasmid DNA for standard curve determination	57
2.2.4 Quantitative real-time PCR (qPCR)	57
2.2.4.1 Primer/probe sets and qPCR protocol.....	57
2.2.4.2 Validation of assays	58
2.2.4.3 Conversion of previous MDV2 standard	58
2.2.5 MDV samples.....	58

2.3 Results	59
2.3.1 Development of plasmid constructs	59
2.3.2 Sensitivity	59
2.3.3 Reproducibility	60
2.3.4 Conversion of previous MDV2 standards.....	62
2.3.5 Absolute quantification of MDV2 in field dust, spleen and feather tip.	63
2.4 Discussion	65
2.5 Conclusion	66
Chapter 3: Association between Marek’s EcoRI-Q-encoded (<i>meq</i>) gene sequence and virulence in Australian and international isolates of Marek’s disease virus	67
3.1 Introduction	67
3.2 Materials and Methods	69
3.2.1 Sample material for DNA extraction.....	69
3.2.2 PCR amplification of <i>meq</i> gene.....	71
3.2.3 Cloning of PCR fragments	71
3.2.4 DNA sequencing and analysis	72
3.2.5 Statistical analysis.....	73
3.3 Results	73
3.3.1 <i>Meq</i> gene sequencing and alignment of Australian isolates	73
3.3.2 Comparison of Australian <i>meq</i> sequences with <i>meq</i> of reference isolates Md5 and Rispens/ CVI988	76
3.3.3. Association between proline content and repeats and virulence.....	80

3.3.4 Phylogenetic tree alignment.....	81
3.4 Discussion.....	83
3.4.1 Sequence variation in the meq gene of Australian and international isolates of MDV1.....	83
3.4.2 Correlation between sequence variation and virulence.....	84
3.4.3 Phylogenetic tree alignment.....	86
 Chapter 4: Pathotyping of recent Australian isolates of Marek's disease virus (MDV) in commercial layer chickens.....	 88
4.1 Introduction	88
4.2 Materials and methods	92
4.2.1 Experimental design	92
4.2.2 Experimental time course	92
4.2.3 UNE isolator facility.....	93
4.2.4 Experimental chickens and their management	94
4.2.5 Vaccination	95
4.2.6 Challenge with MDV1	95
4.2.7 Post mortem procedure	97
4.2.8 DNA extraction.....	98
4.2.9 Determination of MDV genome copy numbers by quantitative real-time PCR (qPCR)	98
4.2.10 Statistical analysis.....	99
4.3 Results.....	100
4.3.1 Confirmation of successful application of treatments	101
4.3.2 Mortality/ Survival.....	101
4.3.3 MD incidence, protective index and virulence rank	103

4.3.4 Anatomical distribution, number and severity of MD lesions	106
4.3.5 Body weight, relative immune organ weights and MDV1 load in spleen at 14 dpc and 56 dpc	108
4.3.5.1 Bodyweight.....	108
4.3.5.2 Bursa of Fabricius	109
4.3.5.3 Thymus	110
4.3.5.4 Spleen	112
4.3.5.5 MDV1 load in spleen at 14 dpc	113
4.3.6 Prediction of MD incidence	114
4.3.6.1 MDV load in spleen	114
4.3.6.2 Relative immune organ weight	115
4.3.7 Association between incidence of MD lesions in unvaccinated and vaccinated chickens.....	116
4.4 Discussion.....	117
Chapter 5: Longitudinal quantification of Marek’s disease virus serotype 1 (MDV1) and Herpesvirus of Turkeys (HVT) in feather tips and their relationship to clinical Marek’s disease in commercial layer chickens	122
5.1 Introduction	122
5.2 Materials and methods	124
5.2.1 Experimental design	124
5.2.2 Experimental chickens	125
5.2.3 Scoring of MD lesions	126
5.2.4 Feather sample processing for qPCR.....	126
5.2.5 Statistical analysis.....	127
5.3 Results	128

5.3.1 Application of treatments	128
5.3.2 Mortality/ Survival.....	129
5.3.3 Incidence of MD and protective index.....	130
5.3.4 Effect of vaccination and challenge virus on MDSS.....	131
5.3.5 MDV1 load in feather tips.....	132
5.3.5.1 Descriptive overview: Individual chicken profiles grouped by MDSS	132
5.3.5.2 Descriptive overview: Mean MDV1 and HVT load in individual unvaccinated and HVT vaccinated chickens by MDSS	134
5.3.5.3 Effect of challenge virus, vaccination and days post challenge	135
5.3.5.4 Effect of MDSS and days post challenge	137
5.3.6 MDV1 load in spleens at 14 dpc	138
5.3.7 MDV1 load in isolator exhaust dander	139
5.3.8 HVT load in feather tips	139
5.3.8.1 Effect of challenge virus and days post vaccination	139
5.3.8.2 Effect of MDSS and days post vaccination.....	141
5.3.8.3 Association between MDV1 and HVT load in vaccinated chickens	142
5.3.9 Associations between MDV1 load in feather, dust and spleen and MD outcome on a group (isolator) mean basis.....	143
5.4. Discussion.....	145
Chapter 6: Shedding profile of three pathogenic isolates and two vaccinal strains (HVT and SB-1) of Marek's disease virus in dander from commercial layer chickens	151
6.1 Introduction	151
6.2 Materials and methods	153

6.2.1 Experimental design	153
6.2.2 Experimental chickens	154
6.2.3 Collection and processing of dander for qPCR analysis	154
6.2.4 Estimation of daily dander production per chicken.....	155
6.2.5 Statistical analysis.....	155
6.3 Results	156
6.3.1 MDV1 viral load in dander.....	156
6.3.2 HVT load in dander	158
6.3.3 MDV2 load in dander	159
6.3.4 Viral load of all three serotypes in dander from chickens vaccinated with bivalent vaccine and challenged with MDV1	160
6.3.5 Daily dander production per chicken and daily shedding rates of MDV	161
6.3.6 Prediction of MD incidence	163
6.3.6.1 Association between MDV load in isolator exhaust dust and the subsequent incidence of MD	163
6.3.6.2 Association between HVT and MDV2 load in isolator exhaust dust and the subsequent incidence of MD.....	164
6.4 Discussion.....	165
Chapter 7: Evaluation of two environmental enrichment strategies for improved welfare of layer chickens in pathotyping experiments	174
7.1. Introduction.....	174
7.2. Materials and Methods	175
7.2.1 Experimental design	175

7.2.2 Measurements	176
7.2.2.1 Feather pecking	176
7.2.2.2 Feather coverage	176
7.2.2.3 Feather condition score.....	177
7.2.2.4 Skin injury score.....	177
7.2.3 Statistical analysis.....	177
7.3. Results	177
7.3.1 Incidence of feather pecking	177
7.3.2 Feather coverage.....	178
7.3.3 Skin injury and feather condition score	179
7.4. Discussion.....	180
Chapter 8: General discussion and conclusions	183
8.1 Molecular methods for absolute quantification of MDV2 using real-time PCR.....	183
8.2 Association between sequence variation in the <i>meq</i> gene and virulence of Australian and international isolates of MDV1 (Chapter 3)	186
8.3 Pathotyping Australian isolates of MDV1 in commercial layer chickens (Chapter 4).....	188
8.4 Evaluation of various tissues/ sample materials for MD prediction and routine MD monitoring (Chapters 4, 5 and 6)	191
8.5. Kinetics of MDV1 and HVT load in feather tips and dust (Chapters 5 and 6)	195
8.6 Behavioural aspects of chickens kept under experimental conditions (Chapter 7).....	196
8.7 Applications of findings	197
8.8 Future work	199

9. References	200
----------------------------	------------

List of Tables

- Table 1.1 Current taxonomy and attributes for MDV.
- Table 1.2: Serotypes and pathotypes of MDV (modified from Witter, 1998).
- Table 2.1: Primers and probes used for quantitative real-time PCR assay and primers used in standard PCR for amplification of fragments of *DNApol* gene for cloning into vector.
- Table 2.2: qPCR analysis of 30 field dust samples (each from a different broiler farm), grouped by the pattern of results for all three MDV serotypes. Data are taken from Islam (2006) and Renz, K.G. (unpublished data) and MDV1 and HVT were assayed as described by Islam et al., 2006a. (N.A. = not available, VCN= viral copy number).
- Table 2.3: qPCR analysis of 6 feather tip samples and 10 spleen samples. (VCN= viral copy number).
- Table 3.1: Details of the Australian MDV isolates used, the incidence of gross MD lesions (%MD) in unvaccinated chickens by 56 dpc, the protective index (%PI) provided by HVT and the putative pathotype based on the classification of Witter (1997) in the pathotyping experiment (Walkden-Brown et al., 2007a).
- Table 3.2: Sequences of *meq*-gene primers and pGEM® T-easy universal primers used for PCR amplification and sequencing.
- Table 3.3: GenBank® accession numbers of the *meq* gene sequences and the proline content of the derived amino acid sequences of the Meq protein used in this study.
- Table 3.4: Amino acid substitutions in the 5 Australian and the international isolates of the Meq protein.
- Table 4.1: Details of the challenge isolates used in this experiment.
- Table 4.2: Summary of number of chicken spleen samples positive for MDV1 and HVT at 14 dpc.
- Table 4.3: Summary of MD mortality between 6 dpc and 56 dpc.
- Table 4.4: Summary of MD incidence, protective index (PI) of vaccines, and virulence rank of the 3 challenge isolates of MDV1.
- Table 4.5: Anatomical distribution of MD lesions in chickens showing gross lesions.

- Table 5.1: MD severity score (MDSS). Individual chickens were scored for MD severity from 0 to 2 on the following scale (Islam. et al., 2006b).
- Table 5.2: Feather tip samples of the treatment combinations which were analysed for MDV1 and HVT by qPCR, grouped by MDSS.
- Table 5.3: Success of vaccination and challenge treatments determined by qPCR analysis of feather tips at 7 and 14 dpc.
- Table 5.4: Mortality in the experiment grouped by vaccination and challenge treatment.
- Table 5.5: MD incidence (% with gross MD lesions) and protective index.
- Table 5.6: Number of birds in the MDSS categories grouped by vaccination and challenge treatment.
- Table 5.7: Number and percentage of samples with positive/ negative HVT qPCR results, grouped by challenge virus.
- Table 5.8: Matrix of coefficients of correlation amongst isolator mean values for MD incidence at 56 dpc (%MD) and correlates of spleen at 14 dpc.
- Table 6.1: Comparison of bodyweight (BW), metabolic BW (BW^{0.75}) and daily dust production between layers and broilers.
- Table 8.1: Comparison of various sample materials with regards the value of their MDV1 load as a predictive tool for MD status.

List of Figures

- Figure 1.1 Genome organisation of MDV (adapted from Nair and Kung, 2004).
- Figure 1.2 Schematic drawing depicting the different stages in the cycle of MD.
- Figure 1.3 Past and projected evolution of Marek's disease virus towards greater virulence in the USA.
- Figure 1.4 The change of the clinical picture of MD over the years (Osterrieder et al., 2006).
- Figure 1.5 Diagram showing the released reporter fluorophore (y axis) plotted as a function of the amplification cycle number (x axis) for a serial tenfold dilution in duplicates of plasmid copies (10^5 - 10^1), from left to right (Renz et al., 2006).
- Figure 1.6 Typical chromatogram showing a section of a sequenced DNA sample.
- Figure 1.7 pGEM® – T Easy plasmid vector circle map.
- Figure 2.1: MDV2 qPCR assay. The released reporter fluorophore (y axis) is plotted as a function of the amplification cycle number (x axis). A serial tenfold dilution in duplicates of pKR-DNApol plasmid copies is shown (10^5 - 10^1), from left to right). The lowest dilution of 100 plasmid copies did not amplify.
- Figure 2.2: Standard curve generated from one of the three qPCR MDV2 assays with the pKR-DNApol plasmid [$y = -3.337 \log(x) + 40.532$, with $R^2 = 0.9994$]. The dotted lines represent the 95% confidence intervals of the line.
- Figure 2.3: Plots of three standard curves generated with pKR-DNApol plasmid in three separate MDV2 assays. The slopes and intercepts did not differ significantly ($p < 0.05$).
- Figure 2.4: Parallelism of standard curves generated from MDV2 positive reference standard and pKR-DNApol plasmid standard. All data points display means of three separate assays.
- Figure 2.5: Linear regression plot of reference MDV2 concentration and pKR-DNApol plasmid number per reaction ($y = 1.0812 \log(x) + 2.6$, $R^2 = 0.9988$).
- Figure 3.1: Overview of the structure of *meq*.

- Figure 3.2: Deduced amino acid sequences of the Meq protein for each of the isolates. Identical amino acids are denoted by an asterisk, dashes indicate missing amino acids and shading indicates regions of heterozygosity.
- Figure 3.3: Section of analysed DNA of the *meq* gene showing an insertion of 177 bases in all Australian isolates which is similar to the 180 bp insertion in the *meq* of CVI988 except for a deletion of the three initial bases in the Australian isolates (marked by dashes) and a one nucleotide position in the Australian isolates (shaded).
- Figure 3.4: Section of analysed DNA of the *meq* gene showing differences compared to Md5 and CVI988.
- Figure 3.5: Association between proline content and pathotype. Mean (\pm SEM) number of PPPP repeats (left panel) and overall proline content (% , right panel) grouped by pathotype for MDV isolates used in this study.
- Figure 3.6: Phylogram of the five Australian Meq protein sequences, the two reference sequences and a range of other Meq sequences from GenBank® with their putative classification into pathotypes.
- Figure 4.1: Survival patterns from 5 dpc showing the effects of Challenge (Left, $P < 0.0001$) and Vaccination treatments (Right, $P < 0.0001$).
- Figure 4.2: Classification of the three challenge viruses into pathotypes based on their mean VR according to the system suggested by Witter (1997).
- Figure 4.3: Interaction plot showing the differential effect of vaccination on the MD lesion severity score induced by the different challenge viruses.
- Figure 4.4: Interaction plot showing the effects of MDV challenge and vaccination on chicken bodyweight (LSM \pm SEM) at 14 dpc (left panel) and 56 dpc (right panel).
- Figure 4.5: Interaction plot showing the effects of MDV challenge and vaccination on relative bursal weight (LSM \pm SEM) at 14 dpc (left panel), and 56 dpc (right panel).
- Figure 4.6: Interaction plot showing the effects of MDV challenge and vaccination on relative thymic weight (LSM \pm SEM) at 14 dpc (left panel), and 56 dpc (right panel).
- Figure 4.7: Association between mean relative thymic weight and mean number of lobes dissected by each operator at 14 dpc (left panel), and association between mean relative thymic weight and mean thymic atrophy score (0-3) at 14 dpc (right panel).

- Figure 4.8: Interaction plot showing the effects of MDV challenge and vaccination on relative splenic weight (LSM±SEM) at 14 dpc (left panel), and 56 dpc (right panel).
- Figure 4.9: Load of MDV1 (LSM±SEM) in spleen of chickens at 14 dpc, by vaccination treatment and challenge virus.
- Figure 4.10: Association between MDV1 load in spleen at 14 dpc and the incidence of MD by day 56pc (% birds with gross MD lesions).
- Figure 4.11: Association between relative thymic weight (% of bodyweight) at 14 and 56 dpc (left panels), thymic atrophy score (right panel) and the incidence of MD by 56 dpc (% birds with gross MD lesions).
- Figure 4.12: Association between relative bursal weight (% of bodyweight) at 14 and 56 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions).
- Figure 4.13: Association between relative splenic weight (% of bodyweight) at 14 and 56 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions).
- Figure 4.14: Association between the incidence of MD lesions (MD%) in unvaccinated chickens or chickens vaccinated with HVT or Bivalent vaccine (HVT/SB-1).
- Figure 5.1: Chicken survival by challenge virus (left panel) and vaccination (right panel). Treatments not sharing a common letter in the superscript differ significantly ($P<0.05$).
- Figure 5.2: Individual profiles of MDV1 load in feather tips over time, grouped by MDSS score. Left panels represent unvaccinated chickens, right panels HVT vaccinated chickens. (2a, d): Chickens with no MD lesions (MDSS 0). (2b, e): Chickens with MD lesions at 56 dpc after euthanasia (MDSS 1). (2c, f): Chickens dying with MD lesions before 56 dpc (MDSS 2).
- Figure 5.3: Mean MDV1 load in feathers of individual chickens (\log_{10} scale) in ascending order, by vaccination treatment (Left panel HVT vaccinated, Right panel unvaccinated) and MDSS.
- Figure 5.4: Mean MDV1 and HVT load in feathers (\log_{10} scale) and MDSS of individual chickens sorted by HVT load. There was a strong trend towards higher HVT load in FT in chickens not exhibiting any gross MD lesions (MDSS 0).
- Figure 5.5: Main effect of days post challenge. Least squared means of MDV1 virus load in feather tips (\pm SEM, \log_{10} scale). Means not sharing a common letter differ significantly using Tukey's HSD ($P<0.05$).

- Figure 5.6: (Left panel) Interaction plot of MDV1 virus load in feather tips (LSM±SEM, log₁₀ scale) over time for the two challenge viruses. The interaction between challenge virus and days post challenge was highly significant (P<0.0001). (Right panel) Interaction plot of MDV1 virus load in feather tips (LSM±SEM, log₁₀ scale) over time for unvaccinated and HVT vaccinated chickens. The effect of vaccine was not significant (P=0.54). Asterisks mark times when treatments differ using Tukey's HSD (P<0.05).
- Figure 5.7: Mean (LSM ±SEM) MDV1/ 10⁶ cells in feather tips of chickens grouped by MDSS over time (log₁₀ scale). The effect of days post challenge and MDSS was highly significant (P<0.001). The interaction between MDSS and days post challenge was not significant (P=0.32).
- Figure 5.8: Mean MDV1/ 10⁶ spleen cells at 14 dpc (LSM ±SEM, log₁₀ scale) grouped by challenge virus in unvaccinated and HVT vaccinated chickens. Means not sharing a common letter differ significantly using Tukey's HSD (P<0.05).
- Figure 5.9: Mean MDV1 load in dust (LSM±SEM, log₁₀ scale) over time, grouped by challenge isolate (left panel) and vaccination treatment (right panel). Asterisks mark times when treatments differ using Tukey's HSD (P<0.05).
- Figure 5.10: Plot of mean HVT virus load in feather tips (±SE, log₁₀ scale) over time. The solid line includes all chickens (many zero values) while the dashed line includes only chickens positive for HVT.
- Figure 5.11: Proportion of chickens grouped by MDSS score that were positive for HVT at each time point.
- Figure 5.12: (Left panel) Association between MDSS and mean log₁₀ HVT load/ 10⁶ cells in feathers of individual chickens. Each data point represents the overall mean HVT value for one individual chicken. (Right panel) Relationship between MDV1 and HVT copy number at various days post vaccination. Each data point represents one chicken at a specific sampling time, all sampling times are included. The line is a linear regression.
- Figure 6.1: MDV1 load in dander (LSM±SEM, log₁₀ scale) over time by vaccine treatment (Left panel) or challenge treatment (right panel). The effects of vaccine, challenge and day post challenge were significant (P<0.0001).
- Figure 6.2: Interaction plot of challenge virus and vaccine. The asterisk marks treatments which were significantly different using Tukey's HSD test (P<0.05).

- Figure 6.3: HVT load in dander (LSM±SEM, log₁₀ scale) over time by vaccine treatment (Left panel) or challenge treatment (right panel). The effects of challenge (P=0.59) and vaccination (P=0.95) were not significant.
- Figure 6.4: MDV2 load in dander (LSM±SEM, log₁₀ scale) over time by challenge treatment. Chickens were vaccinated with bivalent vaccine at hatch. Each data point is the mean of two isolators in each challenge treatment. The effect of challenge was not significant (P=0.64).
- Figure 6.5: (Left Panel) Interaction between the effects of MDV serotype and dpc on viral load in dander from chickens vaccinated with bivalent vaccine and challenged with MDV1 (LSM±SEM) (n=6 isolators). (Right panel) Interaction between the effects of MDV serotype and challenge MDV on viral load in isolator exhaust dust from the same chickens (LSM±SEM) (n=6 isolators).
- Figure 6.6: Mean 24 h dust production per chicken over time in randomly selected isolators. Each data point represents the mean of six isolators.
- Figure 6.7: (Left panel) Mean 24 h MDV1 shed per chicken over time by vaccination status (LSM±SEM). Each data point represents the mean of six isolators. (Right panel) Mean 24 h MDV2/HVT shed per chicken over time (LSM±SEM). For MDV2, each data point represents the mean of two isolators, for HVT, each data point represents the mean of three isolators.
- Figure 6.8: Association between MDV1 load in isolator exhaust dust at 7, 14 and 21 dpc and the incidence of MD by 56 dpc (% birds with gross MD lesions) in chickens challenged with MDV1 (unchallenged isolators excluded). Each point represents one isolator. The lines are linear regression curves.
- Figure 6.9: Association between MDV2 load in isolator exhaust dust at 33, 40, 47 (upper panel), 54 and 61 dpv (lower panel) and the incidence of MD by 56 dpc (61 dpv, % birds with gross MD lesions) in chickens challenged with MDV1 (unchallenged isolators excluded). Each point represents one isolator. The lines are linear regression curves.
- Figure 7.1: Mean incidence of feather pecking by treatment (left panel) and by treatment and week of the experiment (right panel).
- Figure 7.2: Mean feather coverage score by treatment (left panel) and by treatment and chicken age (right panel).
- Figure 7.3: Mean skin injury score by treatment (left panel) and by chicken age (right panel).

Figure 8.1: Association between MD incidence (%MD) induced in Cobb broilers and ISABROWN layers either unvaccinated, or vaccinated with HVT or bivalent vaccine (graph adapted with permission of Prof. S. W. Walkden-Brown).

Abstract

Chapter 1 reviews the literature on Marek's disease relevant to the work carried out in this thesis. Methods for TaqMan[®] quantitative real-time PCR (qPCR) assays to detect the three serotypes of Marek's disease virus (MDV) are already available, and absolute quantification has been developed for MDV serotype 1 and serotype 3. The development of a method for absolute quantification of Marek's disease virus serotype 2 (MDV2) is described in Chapter 2. Using plasmid DNA, the lower detection limit of the MDV2 assay was determined to be 10 copies of the viral genome. Three independent assay runs showed highly reproducible C_t values and calculated copy numbers, with mean intra- and inter-assay coefficients of variation of less than 3 % for C_t and 21.5 % for calculated copy number. Absolute quantification of MDV2 was performed successfully on dust samples collected from poultry farms across Australia, material from infectious spleens and feather tips from chickens vaccinated with an avirulent strain of MDV2. Thus, it is now possible to use qPCR assays for absolute quantification of all three serotypes of MDV in a sample.

The sequencing of the *meq* gene of 5 Australian isolates of MDV1 isolated between 1992 and 2004 which had been pathotyped in an experiment using unvaccinated and HVT-vaccinated specific pathogen free (SPF) chickens is reported in Chapter 3. The sequencing results were compared with a range of MDV1 *meq* sequences published in GenBank[®] and associations with virulence examined. Compared to the USA strain Md5, there was a 177 bp insertion as well as distinct point mutations in all of the five isolates. There was no clear association between ascribed pathotype and *meq* sequence amongst Australian isolates, but amino acid alignment revealed that the number of repeats of sequences of 4 prolines in the *meq* gene (overall range 2-8) was strongly associated with pathotype across all strains with the most pathogenic strains having the fewest such repeats. All of the Australian isolates had 5 such repeats placing them in the moderately virulent range. The number of PPPP repeats was more closely associated with pathotype than the overall percentage of P in the Meq protein. A phylogenetic tree based on published *meq* sequences from a

range of American, Asian and European isolates placed the Australian isolates as a separate group within a clade of attenuated and lower virulence isolates containing the 177-180 bp insertion. Our results suggest that the insertion is not an indicator of marked attenuation or low pathogenicity in its own right, as the Australian isolates all contained the insertion but induced MD in 53-94 % of challenged chickens. Rather, the number of PPPP repeats, independent of the presence of the insertion is a better indicator of pathogenicity. In general the insertion will increase the number of such repeats, leading to its association with reduced virulence.

Two recent Australian isolates of MDV1, 02LAR and 04CRE, which showed a range in pathogenicity in a previous experiment using specific pathogen free (SPF) chickens, were evaluated for pathogenicity in commercial layer chickens against the reference isolate MPF57 (Chapter 4). An adaptation of the USDA ADOL pathotyping method was used which involved measuring MD incidence in unvaccinated chickens and those vaccinated with HVT and HVT/MDV2 bivalent vaccines and determining vaccinal protective index. There was clear differentiation of the 3 challenge viruses with 02LAR inducing significantly more MD (57.5 %) than MPF57 (45.8 %) which in turn induced significantly more MD than 04CRE (30.8 %). There was also clear differentiation of HVT and Bivalent vaccines, with HVT providing a mean protective index of 43.8 % and the Bivalent vaccine 67.2 %. Compared to a similar recent study in commercial Cobb broiler chickens, the results of the layer study indicated that the genetic background of the host plays a vital role in the expression of MD in both vaccinated and unvaccinated chickens and can significantly affect virulence ranks.

Serotype-specific quantitative real-time PCR (qPCR) assays were used to investigate the shedding profile of all three MDV serotypes in feather tips (FT) of individual layer chickens (Chapter 5) and isolator exhaust dust from the pathotyping experiment reported in chapter 4 (Chapter 6). Feather dust production by layer chickens and subsequent shedding of virus in the dust/environment were also determined and results compared to the related earlier study in broiler chickens. All three serotypes were detected from 7 days post challenge (dpc) onwards in both feather tips and dust and continued to be shed throughout the experimental period until 56 dpc.

In the feather tips (Chapter 5), there was no effect of both vaccination ($P=0.54$) on MDV1 load but there was a marked effect of challenge isolate which was dependant on the time after challenge. The more virulent isolate (02LAR) had significantly higher MDV load in FT up to 21 dpc than the less virulent MPF57, but by day 35 the situation was reversed with 02LAR having very much lower MDV1 load, which continued for two weeks thereafter. This effect was linked with a marked reduction in MDV1 load in FT observed after 21 dpc in chickens that went on to develop MD tumours by 56 dpc had significantly lower load of MDV1 and HVT in feather tips ($P<0.05$). This is a novel and unexpected finding and the underlying mechanisms behind it are unknown. The lower level of MDV1 in FT of chickens going on to develop MD helps explain the lack of an effect of vaccination on MDV1 in FT as the expected effect of vaccination of reducing MDV1 load would have been negated by the lower MDV values in unvaccinated chickens, most of which went on to develop MD. There was no relationship between MDV1 and HVT load in feather tips of individual chickens, either overall ($R^2=0.0088$, $P=0.18$), or at any individual time point. A significant positive association between MDV1 load in feathers and subsequent MD on a whole isolator basis was only present at 14 dpc. This study has shown that despite the fact that feather tips can be easily and regularly sampled, and are easy to store and transport, their value as a prediction tool for subsequent MD outcome, is confounded by differential effects on MDV load at different times. In this regard, MDV load in other tissues such as spleen, peripheral lymphocytes or even dander which have been shown to be closely related to MD outcome and are therefore likely to be superior to feathers as robust predictors of future MD status.

In the MDV dust study (Chapter 6), the effect of vaccination with HVT and bivalent vaccine was clearly demarcated from 14 dpc. HVT significantly reduced MDV1 load relative to unvaccinated chickens by 4.7 % overall and the bivalent vaccine reducing it significantly further, by 9.8 %. The MDV1 load in dander also varied significantly in proportion to virulence, with the most virulent virus (02LAR) inducing the highest levels of shedding. Differences in shedding between challenge viruses were greatest at day 14 but were sustained throughout the experiment. This is the first report of increased shedding of MDV with increasing virulence, an important consideration in the modelling of evolution in virulence of

MDVs. Unlike MDV1, HVT and MDV2 load in dander decreased after 19-26 days post vaccination with a subsequent increase again around 54 days post vaccination. There were strong relationships seen between the amount of MDV1 in dust and subsequent MD outcome at 14-49 dpc, but a poor relationship was evident at 7 dpc, which is likely because of a sparing effect of maternal antibodies. Measurement of MDV1 load in dust in groups of birds in the first few weeks prior to challenge appeared to have excellent potential to predict MD outcomes and replace full pathotyping experiments. These measurements can be made prior to the onset of clinical MD, offering significant ethical advantages. This study is the most powerful demonstration to date of the utility of qPCR assays in poultry dust which integrates information from a number of birds in a single sample.

Chapter 7 reports a behavioural study which aimed to reduce feather pecking in layer chickens kept in isolators for research purposes by inclusion of bunches of string and/or a sand box in a 49 day experiment in non beak trimmed SPF white leghorn chickens. Inclusion of string from day 0 significantly reduced the incidence of feather pecking and skin injury and significantly improved ($P < 0.001$) feather coverage and feather condition score. Inclusion of a sand box from day 14 onwards did not significantly affect these variables although there was a trend for improvement in each case, particularly when combined with the presence of string. The inclusion of a sand box at day 0 warrants further investigation. This shows that simple environmental enrichment within isolators can lead to a significant reduction in feather pecking behaviour and now routinely include string in our experiments.

Chapter 8 presents a general discussion of the thesis with conclusions about application of the findings and future work.

List of abbreviations

A	Adenine
aa	Amino acid
ab	Antibody
AEC	Animal Ethics Committee
ALV	Avian Leukosis Virus
ANOVA	Analysis of variance
AP-1	Activator protein 1, a transcription factor
ATF	Activation transcription factor
BAC	Bacterial artificial chromosome
B-cell	Bursa-derived cell
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
Bursa	Bursa of Fabricius
BW/BWT	Body weight
C	Cytosine
CAV	Chicken infectious anaemia virus
CD4+	A subpopulation of T cells that express the CD4 receptor. Also referred to as T helper cells.
CD8+	A subpopulation of T cells that express the CD8 receptor. Also known as cytotoxic T cells.
CEF	Chicken embryo fibroblasts
CEKC	Chicken embryo kidney cells
CKC	Chicken kidney cells
cm	Centimeter
CMI	Cell-mediated immunity
CPE	Cytopathic effect (in cell culture)
cDNA	Complimentary DNA
CNS	Central nervous system
CSIRO	Australian Commonwealth Scientific and Industrial Research Organisation
C_t	Critical threshold value
CV	Coefficient of variance
CXC	Chemokine
CY5	Fluorescent reporter dye
Da	Dalton
DEF	Duck embryo fibroblasts
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
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
ds	Double stranded
EARC	Ellipsoid-associated cell
EBV	Epstein Barr Virus
Exp	Exposure
FAM 5	carboxyfluorescein (Fluorescent reporter dye)
FFE	Feather follicle epithelium
Fos	An oncogene, identified in a mouse osteosarcoma, encoding a transcription factor
FRET	Fluorescence Resonance Energy Transfer
FT	Feather tip
G	Guanine
g	Gram
h	Hour
HEPA	High Efficiency Particulate air
HEX	Fluorescent reporter dye
HSV-1	Herpes Simplex Virus-1
HVT	Herpesvirus of Turkeys. Also known as Meleagrid herpesvirus 1 (MeHV-1) and Marek's disease virus serotype 3 (MDV3).
HWL	Hybrid white leghorn
IA/ia	Intra-abdominal
ICTV	International Committee on Taxonomy of Viruses
IP/ip	Intra-peritoneal
ICP	Intracellular protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	isopropyl- β -D-thiogalactopyranosidase
IR _L	Internal repeat long region of the MDV genome
IR _S	Internal repeat short region of the MDV genome
Jun	Retrovirus-associated DNA sequence (jun) originally isolated from the avian sarcoma virus 17 (asv 17). A proto-oncogene.
kDa	Kilo dalton
kb	Kilo base pairs
LORF	Long open reading frame
LSM	Least squares means
mab	maternal antibody
MATSA	Marek's disease-associated tumour surface antigen
mb	Mega base pairs
MCS	Multiple cloning site
MD	Marek's disease
MDSS	Marek's disease severity score
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).
MEM	Minimal essential medium
meq	Marek's <i>EcoRI</i> -Q encoded gene
MHC	Major histocompatibility complex

min	Minute
mM	Millimolar
mMDV	Mild MDV. A pathotype under the USDA ADOL classification. MDV, which induces mainly paralysis and nerve lesions. HVT provides good protection.
ml	Millilitre
mRNA	Messenger RNA
ng	Nanogram
NK	Natural killer
nm	Nanometer
NO	Nitric oxide
ORF	Open reading frame
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
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
pp	Phosphoprotein
pv	Post vaccination
qPCR	Quantitative real-time PCR
qRT-PCR	Quantitative reverse transcriptase real-time PCR
REML	Restricted maximum likelihood
REV	Reticuloendotheliosis virus
R _L	Repeat long region of the MDV genome
RMIT	The Royal Melbourne Institute of Technology (RMIT University).
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	Fluorescent reporter dye
rpm	Revolutions per minute
R _S	Repeat short region of the MDV genome
RT-PCR	Reverse transcriptase PCR (conventional, end point form)
s	Second
sc	subcutaneous
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SORF	Short open reading frame
SPF	Specific pathogen free
T	Thymine
T-cell	Thymus-derived cell
TR	Telomerase
TR _L	Terminal repeat long region of the MDV genome
TR _S	Terminal repeat short region of the MDV genome
µg	Microgram
µl	Microliter
UNE	The University of New England

U _L	Unique long region of the MDV genome
U _S	Unique short region of the MDV genome
USDA ADOL	United States Department of Agriculture Avian Diseases and Oncology Laboratory
VCN	Viral copy number
vIL	Viral interleukin
vMDV	Virulent MDV. A pathotype under the USDA ADOL classification. MDV, which causes low levels of mortality by day 56pc, but induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT provides good protection.
vvMDV	Very virulent MDV. A pathotype under the USDA ADOL classification. MDV, which causes moderate levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT is only partially protective but HVT/MDV2 vaccines provide a high level of protection.
vv+MDV	Very virulent plus MDV. A pathotype under the USDA ADOL classification. MDV, which causes high levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/MDV2 are only partially protective.
VN	Virus neutralising
VP	Virus protein
VR	Virulence rank (100 – PI)
vTR	Viral telomerase RNA subunit
VZV	Varicella Zoster Virus
wk	Week
wo	week old
WT-1	Wilm's tumour suppressor protein
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside
YAC	Yeast artificial chromosome