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

By

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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

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Dedication

I would like to dedicate this thesis to

my beloved father, Dieter Renz and my mother, Gundula Renz.

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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):

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Conference abstracts (refereed):

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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).

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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 isolatres 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 *Eco*RI-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