Replication kinetics, shedding, transmission and protective efficacy of Rispens/CVI988 vaccine virus in single and combined infections with very virulent Marek's disease virus

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A thesis submitted for the degree of Doctor of Philosophy

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July 2013

Declaration

I hereby certify that this work is the original work of the author except where acknowledged in the text. 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.

Tanzila Islam

Dedication

I would like to dedicate this thesis to my beloved

father, Md. Nurul Islam and

mother, Mahfuza Islam

Acknowledgements

I would like to pay my deepest gratitude to Almighty Allah who has given me opportunity and strength to pursue my PhD study.

This research was financially supported by the Australian Egg Corporation Limited (AECL) and University of New England, which is greatly appreciated.

I would like to thank my principal supervisor, Prof. Stephen Walkden-Brown, for his supervision, inspiration, 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 like to specially acknowledge the support and input he gave me during writing and finalizing the thesis.

I would like to express my gratitude to my co- supervisors Dr. Katrin Renz and Dr. Fakhrul Islam for their greatly appreciated advice and invaluable support during the molecular work and analysis.

Special thanks are also extended to Dr Katrin Renz, Sithara Ralapanawe (UNE), for doing the lab work (preparation of DNA and qPCR) for all of my feather samples.

I am deeply grateful to School of Environmental and Rural Science (UNE) specially Frank Leayr and Shirley Fraser for understanding my situation and supporting me financially.

For their technical assistance throughout my thesis, I would like to thank Susan Burgess (UNE), Michel Raue (UNE), Sara Bowers (UNE), Andrew Blakely (UNE), Grahame Chaffey (UNE) and Garry Taylor (UNE).

I also want to thank all my friends, especially Reza Barekatain, Fatima Tuzzohora, Tahmina Islam, A/Prof Habib Zafarullah, Izaz Raisul and Belal 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.

Lastly, I would like to thank my family for all their love and encouragement. Thanks to my parents who raised me with a love of science and supported me in all my pursuits. Thanks to my sisters, Tahrima and Tasmiyah Islam for their sincere encouragement and inspiration throughout my research work and lifting me uphill this phase of life. I owe everything to them. I wish to extend my special tribute to my loving, supportive, encouraging, and patient husband Mohammad Forhad Hossain whose faithful support during every single moment of this PhD is so appreciated. And most of all, my precious daughter, Fatiha Binte Fuad, who encourages me to finish the final stage of PhD by giving lovely smile always.

List of Publications from this thesis

Journal Articles

1. **Islam, T**., Renz, K. G., Walkden-Brown, S. W., & Ralapanawe, S. (2013). Viral Kinetics, Shedding Profile and Transmission of Serotype 1 Marek's Disease Vaccine Rispens/CVI988 in Maternal Antibody-negative Chickens. *Avian Diseases, 57*, 454-463.
2. **Islam, T**., Walkden-Brown, S. W., Renz, K. G., Islam, A. F. M. F., & Ralapanawe, S. (2013). Vaccination-challenge interval markedly influences protection provided by Rispens/CVI988 vaccine against very virulent Marek's disease virus challenge *Avian Pathology*, 42(6), 516-526.
3. **Islam, T**., Walkden-Brown, S. W., Renz, K. G., Islam, A. F. M. F., & 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*, Re-submitted.

Conference Presentations

1. **Islam, T**., Renz, K. G., Walkden-Brown, S. W., & Ralapanawe, S. (2011). Viral load, shedding rate and lateral transmission of Marek's disease vaccinal virus (Rispens/CVI988) in SPF chickens. Proceedings of the Australian Poultry Science Symposium, Sydney, 22, 239-242.
2. **Islam, T.**, Walkden-Brown, S. W., Renz, K. G., Islam, A. F. M. F., & Ralapanawe, S. (2013). Effect of widely divergent vaccination-challenge intervals on protection provided by Rispens/CVI988 vaccine against very virulent Marek's disease virus challenge in ISA Brown chickens. Proceedings of the Australian Poultry Science Symposium, Sydney, 24, 243-246.
3. **Islam T**, Renz K. G., Islam, A. F. M. F., Walkden-Brown S.W. (2012) Effect of wide variation in the timing of vaccination relative to challenge on protection against MDV provided by Rispens CVI988 vaccine.Proceedings of the 9th International Meeting on Marek's Disease and Avian Herpesviruses Berlin, 24-28 June 2012, 39.

4. **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. Proceedings of the 9th International Meeting on Marek's Disease and Avian Herpesviruses Berlin, 24-28 June 2012, 50.

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Abstract

The attenuated MDV-1 Rispens/CVI988 vaccine is widely used to vaccinate chickens worldwide and is the most effective current vaccine against MD. Two experiments were designed to investigate transmission of the Rispens/CVI988 vaccine virus (Rispens/CVI988) between chickens, the viral kinetics and shedding profile of Rispens/CVI988 and very virulent MDV (vvMDV, isolate 02LAR) in single and mixed infections and the effect of vaccination to challenge interval on the protection provided by Rispens/CVI988 against vvMDV challenge. Experiment 1 used 70 specific pathogen free (SPF) chickens in four climate controlled rooms. In three rooms, 10 chickens were vaccinated with one of the three commercial Rispens/CVI988 vaccines at day old and left in contact with 10 unvaccinated chickens. The fourth room held 10 unvaccinated control birds. As determined by MDV-specific qPCR of weekly room dust, PBL and feather samples the commercially available Rispens/CVI988 vaccine virus strains are shed in significant quantities and transmit effectively to in-contact chickens. Experiment 2 used 600 commercial ISA Brown layers of the same age (day old) in 24 isolators. Chickens were vaccinated with Rispens/CVI988 (3200 pfu) and/or challenged with the 02LAR (400 pfu) on days 0, 5, 10 post hatching providing vaccination to challenge intervals (VCI) of -10, -5, 0, 5, 10 days with the negative values indicating challenge prior to vaccination. As determined by MDV-specific qPCR able to differentiate between the viruses, vaccination with Rispens/CVI988 greatly reduced the viral load of vvMDV in PBL and feather cells but only if birds were vaccinated prior challenge. The effects on shedding of vvMDV in dust were smaller and occurred later. Similarly, pathogenic MDV-1 also significantly reduced the viral load of Rispens/CVI988 in PBL and feather cells if birds were challenged prior to vaccination. There was no significant effect on shedding in dust. Because of these effects VCI was significantly and negatively associated with vvMDV load and significantly and positively with Rispens/CVI988 load. The Rispens/CVI988 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 showed that vvMDV load in PBL or feather tips at 14 and 21 dpc were accurate early predictors of MD incidence at 56 dpc.

List of abbreviations

A	Adenine
aa	Amino acid
ARC	Australian Research Council
BAC	Bacterial artificial chromosome
B-cell	Bursa-derived cell
BHQ	Black hole quencher
BLAST	Basic local alignment search tool
bp	Base pairs
bZip	Basic leucine zipper
Bursa	Bursa of Fabricius
C	Cytosine
CAV	Chicken infectious anaemia virus
CEF	Chicken embryo fibroblasts
CKC	Chicken kidney cells
CPE	Cytopathic effect (in cell culture)
cDNA	Complimentary DNA
CNS	Central nervous system
C_t	Cycle threshold
CV	Coefficient of variance

CY5	Fluorescent reporter dye
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpc	Days post-challenge
dpi	Days post-infection
dpv	Days post-vaccination
FAM 5	Carboxyfluorescein (Fluorescent reporter dye)
FFE	Feather follicle epithelium
G	Guanine
GaHV-2	Gallid herpesvirus type 2
GaHV-3	Gallid herpesvirus type 3
gp	Glycoprotein
h	Hour
HEPA	High Efficiency Particulate air
HEX	Fluorescent reporter dye
HVT	Herpesvirus of Turkeys. Also known as Meleagrid herpesvirus 1(MeHV-1) and Marek's disease virus serotype 3 (MDV3).
IA/ia	Intra-abdominal
IP/ip	Intra-peritoneal

IBDV	Infectious bursal disease virus
ICP4	Intracellular protein 4
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
kDa	Kilo dalton
kB	Kilo base pairs
LAT	Latency-associated transcript
mab	Maternal antibody
MATSA	Marek's disease-associated tumour surface antigen
MD	Marek's disease
MDV	Marek's disease virus
MDV-1	Marek's disease virus serotype 1. Also known as Gallid herpesvirus 2 (GaHV-2).
MDV-2	Marek's disease virus serotype 2. Also known as Gallid herpesvirus type 3 (GaHV-3).
MeHV-1	Meleagrid herpesvirus type 1
MHC	Major histocompatibility complex
mMDV	Mild MDV. A pathotype under the USDA ADOL classification. MDV, which induces mainly paralysis and nerve lesions. HVT

	provides good protection.
mRNA	Messenger RNA
NK	Natural killer
NO	Nitric oxide
ORF	Open reading frame
PBL	Peripheral blood lymphocytes
PBS	Phosphate buffered saline
pc	Post challenge
PCR	Polymerase chain reaction (conventional, end point form)
pfu	Plaque forming units
PI	Protective index. (% MD in Sham-vaccinated chickens – % MD in HVT-vaccinated chickens) ÷ (% MD in Sham-vaccinated chickens) x 100
pp38	Phosphoprotein of 38 kDa
pv	Post vaccination
qPCR	Quantitative real-time PCR
REV	Reticuloendotheliosis virus
RNA	Ribonucleic acid
RNase	Ribonuclease
ROX	Fluorescent reporter dye
rpm	Revolutions per minute

RT-PCR	Reverse transcriptase PCR (conventional, end point form)
s	Second
SPF	Specific pathogen free
Syn	Synonym
T	Thymine
T-cell	Thymus - derived cell
UNE	The University of New England
USDA ADOL	United States Department of Agriculture Avian Diseases and Oncology Laboratory
VCI	Vaccination challenge interval
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/MDV-2 are only partially protective.
VN	Virus neutralising
VR	Virulence rank (100 – PI)
wk	Week

General Introduction

Marek's disease (MD) is a tumour causing lymphoproliferative disease of chickens caused by Gallid herpesvirus 2 or Marek's disease virus serotype 1 (MDV) which is a DNA virus belonging to the family *Herpesviridae*, sub-family *Alphaherpesvirinae* and genus *Mardivirus* (King, *et al.*, 2012). There are two other species under the genus Mardivirus: Gallid herpesvirus 3, avirulent chicken herpesvirus (MDV serotype 2); and Meleagrid herpesvirus 1, naturally occurring turkey herpesvirus (HVT) (MDV serotype 3).

The disease was first described by Josef Marek in 1907 causing paralysis in older chickens and it was described as polyneuritis (Marek, 1907). In 1926 it was revealed this disease also causes tumours in organs such as ovary, liver, kidneys, lungs, adrenals and muscle (Pappenheimer, *et al.*, 1926). In 1957 the disease appeared with neural involvement in 8 - 10 week old chickens with a mortality rate of 40 % in layers (Benton and Cover, 1957) and was described as acute leukosis (Biggs, *et al.*, 1965). In 1969 the disease was first successfully controlled by vaccinating chickens with live attenuated serotype 1 virus (Churchill, *et al.*, 1969b). In the 1970s HVT vaccine was introduced and found to be effective against virulent Marek's Disease Virus (vMDV) (Witter, 1983; Witter, *et al.*, 1970c). In the late 1970s new very virulent strains of MDV were isolated in the USA (vvMDV) associated with frequent HVT vaccination failure (Eidson, *et al.*, 1978b; Witter, 1983). To overcome this situation a new bivalent vaccine (HVT + MDV-2) was introduced (Calnek, *et al.*, 1983; Witter, 1981; Witter, 1982). Again in early 1990s major outbreaks occurred due to vaccine failure associated with ongoing increase in virulence. This led to assignment of a new pathotype category for MDV designated as very virulent plus MDV (vv+MDV) (Witter, 1996, 1997). This vv+MDV causes earlier and severe forms of MDV in younger chickens resulting in very high mortality to younger birds (Ficken, *et al.*, 1991; Spencer, *et al.*, 1992; Venugopal, *et al.*, 1996; Witter, 1996). An attenuated vaccine strain of serotype 1 named Rispens/CVI988 provided good protection against this vv+MDV (Rispens, *et al.*, 1972b; Rispens, *et al.*, 1972a; Witter, *et al.*, 1995) and is now widely used. This brief overview illustrates how Marek's disease virus has changed over the decades becoming more virulent over time, with increasing virulence inducing vaccine failure. Rispens/CVI988 is the most effective vaccine against vv+

pathotypes of MDV but if history is to repeat itself, evolution of virulence may also render this vaccine ineffective.

In Australia, the first vaccine was introduced in 1971. It was HVT. Due to maternal antibody interference with cell free HVT vaccine and contamination of HVT with REV a serotype 2 MD vaccine (Maravac) was introduced in 1978 (Jackson, 2000a, 2000b). Between 1992 and 1997, MD caused very severe losses in both the layer and broiler industries as conventional Australian vaccines and vaccination programs failed to control MD in the imported genotypes of chickens. The problem in layers and broiler breeders was brought under control by the importation of seed for the Rispens/CVI988 vaccine (attenuated MDV-1) in 1997 and MD remains well controlled in layers and breeders by this vaccine at present. Moreover, since 1996 imported strains of HVT (FC126, and NBSL S.AR) are now widely used as vaccines for broiler chickens in cell-associated form administered *in ovo* (Jackson, 2000a, 2000b). Very virulent MDV strains, against which HVT confers only partial protection first identified in Australia in 1985 (McKimm-Breschkin*, et al.*, 1990) and isolated from subsequent outbreaks of MD in vaccinated birds (De Laney*, et al.*, 1995; Zerbes*, et al.*, 1994). In consequent challenge experiments using titrated doses of a local isolate of MDV (MPF57), failure of HVT to provide complete protection has repeatedly been demonstrated (Islam*, et al.*, 2007a; Islam*, et al.*, 2006b; Islam*, et al.*, 2002). Formal pathotyping experiments have identified v and vv MDV pathotypes, but not the vv+ pathotypes reported overseas (Renz*, et al.*, 2012; Walkden-Brown*, et al.*, 2013b).

While MDV vaccines have provided effective protection against Marek's disease they failed to prevent evolution of virulence of MDV. Indeed it is speculated that MDV vaccines have played an important role in the increase in virulence of MDV (Witter, 1996, 1997). This is because vaccination of MD is imperfect i.e. the vaccine prevents the disease but not the infection and both pathogenic virus and vaccinal virus replicate together in the chickens (Atkins*, et al.*, 2012; Gandon*, et al.*, 2001). In such a situation increases in virulence may be favoured, as the higher replication rate of the more virulent viruses is not counterbalanced by increased mortality rate and reduction in virus fitness due to termination of replication by host death. Thus, in vaccinated hosts more virulent MDVs are likely to have greater overall reproductive success than less virulent MDVs. Recent modelling studies based on measured shedding data in chickens co-infected with vaccinal and pathogenic virus have confirmed this situation and the hypothesis of vaccination-induced evolution in virulence, together with an

important role for marked reductions in the mean lifespan of chickens (Atkins, *et al.*, 2012). At this moment it is not clear whether MDV will continue to increase in virulence and overcome the protection provided by the Rispens/CVI988 vaccine. While the kinetics of co-infection of MDV with HVT and MDV-2 have been comparatively well documented (Atkins, *et al.*, 2011; Islam, *et al.*, 2006a; Islam, *et al.*, 2008; Renz, *et al.*, 2006; Walkden-Brown, *et al.*, 2013b) co-infection with serotype 1 vaccine is much more difficult to study due to difficulties in differentiation between vaccinal and pathogenic MDV-1. This difficulty has also led to practical issues in measuring vaccine take following serotype 1 vaccination, and in diagnostic differentiation between the two viruses. This issue of differentiation has recently been solved with the development of a fully quantitative real-time PCR test that differentiates Australian wild-type MDV from the Rispens/CVI988 vaccine (Renz, *et al.*, 2013).

As Rispens/CVI988 is the most effective vaccine against MD at present time we need to understand the nature of this virus and its interaction with pathogenic viruses in co-infected hosts. Baigent *et. al.* (2005b) reported the replication kinetics of the Rispens/CVI988 virus in PBL and feather tips up to 28 days post vaccination (dpv) and extending their findings in feather tips showed that presence of high levels of this virus in feather tips at 13 days post vaccination was strongly correlated with subsequent protection against MDV challenge (Baigent, *et al.*, 2007). Moreover, Rispens *et al.* (1972a) showed that at a passage level of 35 the virus spread readily from vaccinated to in-contact chickens (Rispens, *et al.*, 1972a). Subsequent publications report that both a plaque purified clone of CVI988 (988 C) at a passage level 65 and CVI988 at passage 42 showed very limited transmission between birds (Witter, 1987; Witter, *et al.*, 1995). Given currently used vaccine strains have likely passage level in the range of 40-45 it is important to determine the extent to which this virus is shed and transmits between birds, and whether it is able to circulate independently in chicken flocks. The main focus of my thesis is therefore on the detailed understanding of Rispens/CVI988 virus kinetics in single and mixed infections to gain insight into the likely effects of Rispens/CVI988 vaccination on evolution of virulence and to also determine the optimum sampling regimen to confirm Rispens/CVI988 vaccine take. This study aims to

- Investigate the kinetics, shedding and transmission of Rispens/CVI988 including the time of appearance and level of virus in PBL, feather dust and feathers.
- Investigate the protective efficacy followed by Rispens/CVI988 vaccine against very virulent MDV

- Investigate the effect of vaccination challenge interval on the protective efficacy of Rispens/CVI988 vaccine
- Investigate interaction between Rispens/CVI988 and pathogenic MDV in viral kinetic studies in co-infected chickens including shedding of virus in dust.
- Investigate the effect of vaccination to challenge and challenge to vaccination intervals on the kinetics and shedding of both pathogenic MDV and the Rispens/CVI988 vaccine virus.