Efficacy of Rispens CVI988 Vaccine against Challenge with Marek's Disease Viruses of Varying Virulence, Effects on Viral Kinetics and Field Application of a Rispens Specific qPCR Test

Sithara Ralapanawe

B.V.Sc. (Hons.), University of Peradeniya, Sri Lanka

M.Sc. (Applied Microbiology), University of Kelaniya, Sri Lanka

A thesis submitted for the degree of Doctor of Philosophy of

The University of New England, Armidale, Australia

August 2015

School of Environmental and Rural Science

Faculty of Arts and Science

une
University of
New England

Declaration

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree or qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged.

Ms. Sithara Ralapanawe

August 2015

I would like to dedicate this thesis to my beloved

late father, Chandra Saranath Bandara Ralapanawe, and

my mother, Palika Ralapanawe.

Acknowledgements

I would like to thank my principal supervisor, Prof. Stephen Walkden-Brown, for his supervision and overall guidance throughout my PhD candidature. His vast knowledge and enthusiasm about Marek's disease and statistics will continue to inspire me well beyond this thesis. I am grateful to him for his encouragement, constructive criticism, detailed corrections and suggestions for modifications throughout my candidature and in finalizing this thesis.

I would like to express my gratitude to my co-supervisor Dr. Katrin Renz for sharing her knowledge on molecular diagnostics and valuable advice on the laboratory techniques and procedures. I also extend my thanks for her assistance with laboratory work and sampling in the field experiment.

I appreciate the assistance and guidance of Dr. Fakhrul Islam in the isolator experiment and in the thesis as a whole.

This thesis would not be possible without the hard work of all the technical staff in the Animal Science Department of School of Environmental and Rural Science, UNE: Susan Burgess, Michael Raue, Mark Taylor, Grahame Chaffey, Andrew Blakely and Garry Taylor who all helped me with my experiments. I extend my thanks to Susan Burgess for designing the Marek's disease enzyme-linked immunosorbent assay and for showing me new methods for analyses whenever I needed them. I would also like to extend my gratitude to Joanne Porter for her kind help with the administrative tasks.

I am deeply grateful to the University of New England for accepting me as a PhD candidate. Financial support from the Australian Egg Corporation Limited (AECL) and the internal student grant from the University of New England, which supported my experimental work, are also greatly appreciated. I also would like to extend my appreciation to the Department of Education and Training of the Australian Government for awarding me the Endeavour Postgraduate Award which enabled me to pursue my studies in Australia. My sincere gratitude also goes to the case managers of the Endeavour award for being extremely supportive and caring throughout the degree program. I also acknowledge the travel grant from the Organizing Committee of the 10th International symposium of Marek's Disease and avian which enabled me to travel to USA to present my work.

I also want to thank my postgraduate colleagues Robin Achari and Brendan Sharpe for helping me with field sampling. My gratitude also to my dear friends all over the world specially Rahfiya, Dharma, Hong, Khadijah, Michelle, Christine, Elfira, Shalanee, Merani, Aruni, Udani and Kanchana for their moral support and for lifting my spirits whenever I was down. I am also thankful to the Kanagaratnam family for helping me to settle down in the cold weather of Armidale.

I would also like to thank the Director General, Department of the Animal Production and Health and the Director, Veterinary research institute in Sri Lanka for granting me study leave to continue my graduate studies in Australia. I am also grateful to Dr. Gnana Gunawardena, Dr. Hemal Kothalawla, Dr. Thilliapalam Sivakumar and Dr. Samantha Iddamadeniya at the Veterinary Research Institute in Sri Lanka for their invaluable support.

I would like to especially thank my late father for teaching me the importance of education and raising me to be a hardworking scientist. Being a senior lecturer himself, he showed me by example how to reach goals while having a young family. His inspiration kept me afloat through difficult times in this doctoral degree. My heartfelt gratitude also goes to my mother, Palika, and my two beautiful sisters, Dilru and Hiruni, for their encouragement throughout.

Last but not least I would like to express my gratitude to my husband Tharanga Chandrasena for being my pillar of strength. Also thank you to my little daughter, Omendree, and son, Ovindu, for bearing with me and having infinite patience.

Table of Contents

List of Tables

Table of Figures

List of Abbreviations

aa	Amino acid
ADOL	Avian Disease Oncology Laboratory
AE	Avian encephalomyelitis
AEC	Animal ethics committee
AGPT	Agar gel immunoprecipitation test
ANOVA	Analysis of variance
B cells	B lymphocytes
BAC	Bacterial artificial chromosomes
bp	Base pairs
Bursa	Bursa of fabricius
CEF	Chicken embryo fibroblasts
CKC	Chicken kidney cells
CMI	Cell mediated immunity
Ct	Cycle threshold
CV	Coefficient of variance
DEF	Duck embryo fibroblasts
DNA	Deoxy ribonucleic acid
dpc	Days post challenge
dpi	Days post infection
dpv	Days post vaccination
EDS	Egg drop syndrome
ELISA	Enzyme linked immunosorbent assay
EMS	Early mortality syndrome
FFE	Feather follicular epithelium
FP	Fowl pox
G	Gauge
GaHV-2	Gallid herpesvirus 2
GaHV-3	Gallid herpesvirus 3
HEPA	High efficiency particulate air
HP	Highly protective
hr	Hours
HVT	Herpesvirus of turkeys
IB	Infectious bronchitis
IF	Immunofluorescence test
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILT	Infectious laryngotracheitis

IRL	Internal repeat long
IRS	Internal repeat short
Kbp	Kilobase pairs
LAMP	Loop mediated isothermal amplification
LAT	Latency-associated transcripts
LL	Lymphoid leucosis
LP	Low protective
m	Mild
mab	Maternal antibody
MAMA	Mismatch mutation assay
MATSA	Marek’s tumour associated surface antigen
MD	Marek's disease
MDV	Marek's disease virus
MDV-1	MDV serotype 1
MDV-2	MDV serotype 2
MDV-3	MDV serotype 3
MeHV-1	Meleagrid herpesvirus 1
MHC	Major histocompatibility complex
mRNA	Messenger RNA
ND	Newcastle disease
NO	Nitric oxide
P	Proline
PBL	Peripheral blood leucocytes
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PC2	Physical containment level 2
PCR	Polymerase chain reaction
PFU	Plaque forming units
pp38	Phosphoprotein of 38kDA
QC	Quality control
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
REV	Reticuloendothelial virus
Rispens	Rispens CVI988
RNA	Ribonycleic acid
Rnase	Ribonuclease
rpm	Revolutions per minute
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
T cells	T lymphocytes
TP	Transient paralysis

TRL	Terminal repeat long
TRS	Terminal repeat short
UL	Unique long sequence
UNE	University of New England
US	Unique short sequence
USDA	United States Department of Agriculture
v	Virulent
VCI	Vaccination challenge interval
VCN	Viral copy numbers
VN	Virus neutralization
VR	Virulence rank
vv	Very virulent
vv+	Very virulent plus

List of Publications

Peer reviewed journal articles

1. **Ralapanawe, S.,** Walkden-Brown, S. W., Islam, A. F. M. F., & Renz, K. G. (2016). Effects of Rispens vaccination followed by challenge with Marek's disease viruses of differing virulence on the replication kinetics and shedding of the vaccine and challenge viruses. *Veterinary Microbiology, 183*, 21-29.

2. **Ralapanawe, S.**, Walkden-Brown, S. W., Renz, K. G., & Islam, A. F. M. F. (2016). Protection provided by Rispens CVI988 vaccine against Marek's disease virus isolates of different pathotype and early prediction of vaccine take and MD outcome. *Avian Pathology, Accepted on 2nd Aug 2015, In Press*.

3. **Ralapanawe, S.**, Walkden-Brown, S. W., Renz, K. G., & Burgess, S. (2016). Protection provided by Rispens CVI988 vaccine against Marek's disease virus isolates of different pathotype and early prediction of vaccine take and MD outcome. *Australian Veterinary Journal, Accepted on 6th Nov 2015*.

Other reviewed journal articles (not reported in this thesis)

1. Islam, T., Walkden-Brown, S. W., Renz, K. G., Islam, A. F. M. F., & **Ralapanawe, S**. (2014). 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, 173*(3-4), 208-223.

2. Islam, T., Renz, K. G., Walkden-Brown, S. W., & **Ralapanawe, S.** (2013a). Viral kinetics, shedding profile, and transmission of serotype 1 Marek's disease vaccine Rispens/CVI988 in maternal antibody-free chickens. *Avian Diseases, 57*(2s1), 454-463.

3. Islam, T., Walkden Brown, S. W., Renz, K. G., Islam, A. F. M. F., & **Ralapanawe, S.** (2013b). 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.

Peer reviewed long conference publications

1. **Ralapanawe, S.**, Walkden-Brown, S. W., Islam, A. F. M. F., & Renz, K. G. (2015). Kinetics of Rispens CVI988 vaccine virus in single and mixed infections with very virulent Marek's disease virus in ISA Brown chickens *Proc. Aust. Poult. Sci. Symp.* **26**, 183-186.

2. **Ralapanawe, S.,** Walkden-Brown, SW, Islam, AFMF, Renz, KG (2013) Protection provided by Rispens CVI988 vaccine against very virulent Marek's disease virus challenge in ISA Brown chickens *Proc. Aust. Poult. Sci. Symp.* **24**, 268-271.

Short non-peer reviewed conference publications

1. **Ralapanawe, S.**, Islam, A. F. M. F., Renz, K., Walkden-Brown, S. (2014) *Protection provided by CVI988 vaccine against two virulent Australian Marek's disease virus isolates.* In '10th International Symposium on Marek's Disease and Avian Herpesviruses. East Lansing, Michigan, USA', 20-23 July 2014. (Ed. H Cheng) pp. 61. (Michigan State University and USDA-ARS Avian Disease and Oncology Laboratory
2. **Ralapanawe, S.**, Renz, K., Walkden-Brown, S. (2014) *Field studies on the Rispens CVI988 vaccine virus in layer and unvaccinated broiler chickens.* In '10th International Symposium on Marek's Disease and Avian Herpesviruses. East Lansing, Michigan, USA', 20-23 July 2014. (Ed. H Cheng) pp. 89. (Michigan State University and USDA-ARS Avian Disease and Oncology Laboratory

Abstract

Marek's disease (MD) is an economically important poultry disease, which is successfully controlled by imperfect vaccines. The imperfect vaccines for MD, herpesvirus of turkeys (HVT) and HVT/Gallid herpesvirus 3 (GaHV-3) (bivalent) are likely to have contributed to the observed increase in virulence which has led to sequential failure of these vaccines in some parts of the world. The Gallid herpesvirus 2 (GaHV-2 ,MDV-1) Rispens CVI988 vaccine, first developed in 1972, has not been affected by this failure and is considered to be the gold standard Marek's disease vaccine, being widely used worldwide to vaccinate long lived layers and breeders. Two experiments were designed to investigate this vaccine and its efficacy in Australia. An experiment in isolators investigated the protection provided by Rispens vaccine against Australian pathogenic GaHV-2 isolates of varying virulence (virulent, vMDV and very virulent vvMDV), and the kinetics of viral genome copy number of Rispens and the pathogenic MDV isolates in single and mixed infections. In the second experiment, a Rispens virus specific qPCR test was used to measure the vaccine take in invasive and non-invasive samples and the long-term viral kinetics of the Rispens virus in the field. Co-infection levels of Rispens and pathogenic GaHV-2 in the field and the possibility of establishment of Rispens virus in unvaccinated broiler flocks were also examined. Experiment one used 236 commercial ISA Brown chickens having maternal antibody directed against Rispens vaccine in 12 isolators. Chicks were vaccinated or not vaccinated with Rispens vaccine at hatch and challenged with vMDV isolate MPF57, vvMDV isolate FT158 at 5 days of age or left unchallenged. Each of the six treatment combinations was replicated in two positive pressure isolators. The protective index provided by Rispens vaccine did not vary with GaHV-2 challenge pathotype being 66% and 61% for MPF57 and FT158, respectively. Pathogenic viral loads in PBL, feather cells and dust up to 21 dpc were good early predictors for subsequent MD incidence. The early Rispens viral loads of PBL, feather, dust and spleen samples were, however more useful measures of the vaccine take than subsequent MD incidence. Investigation of the kinetics of the three viruses provided evidence that Rispens vaccination reduced the viral load of MPF57 more than FT158, thus providing an environment that favours the higher virulence isolate as has been shown for HVT, the other major MD vaccine. Patterns of, and treatment effects on, viral load in PBL and feathers were broadly similar, but differed markedly from those of virus shed in dust, so the former measurements cannot be used to predict the latter. In the field study 498 feather

and 42 dust samples were collected from three different farms at a wide range of age groups of chickens. By analysing these samples using Rispens virus -specific qPCR, we found that feather and dust samples from chickens between 2 and 3 weeks of age provided good early indicators of vaccine take. Co-infection of vaccinated chickens with pathogenic GaHV-2 was found in only 7% of 120 randomly selected feather DNA samples and in 5% in dust samples. Preliminary evidence of spread of the Rispens vaccine virus to unvaccinated broiler flocks was detected with 7/100 GaHV-2 positive dust DNA samples from unvaccinated broiler farms found to be positive for the Rispens viral genome.

The main implications of this study are; 1) The Rispens vaccine take can be measured in commercial layer flocks using qPCR testing of feathers from 14 days post vaccination (dpv) or dust from 21 dpv, 2) There is a low level of co-infection of Rispens virus with pathogenic GaHV-2 in commercial layer flocks, 3) There is a preliminary evidence for the Rispens virus has 'escaped' into the unvaccinated broiler chicken population and 4) Further evidence that unlike HVT and bivalent vaccines, the protective index provided by the Rispens vaccine is not influenced the pathotype of the challenge GaHV-2 virus, in this case between v and vv MDV.

General Introduction

Marek's disease (MD) is an economically important disease of poultry which is characterised mostly by lymphotropic infiltrations in visceral organs causing T-cell lymphomas as well as a range of pathological syndromes including classical and acute paralysis. MD is caused by a DNA virus *Gallid herpesvirus 2* (GaHV-2; conveniently referred to by serotype as MDV-1) belonging to the *Mardivirus* genus of the *Alphaherpesvirinae* subfamily of *Alphaherpesviridae*. There are four other species belonging to genus *Mardivirus* and the most important are *Gallid herpesvirus 3* (GaHV-3, MDV-2) and *Meleagrid herpesvirus 1* (MeHV-1, MDV-3). The other two species are *Anatid herpesvirus 1* and *Columbid herpesvirus 1*. GaHV-3 is a non-pathogenic species from chickens and MeHV-3 is a naturally occurring herpesvirus of turkeys (HVT) which is non-pathogenic in chickens.

MD can cause up to 60% mortality in commercial poultry and is usually successfully controlled by live viral vaccines. However, the MD vaccines are imperfect vaccines that do not provide sterile immunity. Therefore, the vaccines successfully reduce MD incidence, lymphomas, and mortality but not superinfection, replication, and shedding of pathogenic GaHV-2. Therefore, it is postulated that the MD vaccines themselves could be one of the causes of the evolution of virulence of GaHV-2 and emergence of new pathotypes (Atkins *et al.*, 2013; Read *et al.*, 2015). This had been previously reported by Witter *et al.* (1997) who classified the GaHV-2 isolates as mild (m), virulent (v), very virulent (vv) and very virulent plus (vv+) partly on the basis of their ability to induce MD in chickens vaccinated with HVT and HVT+ GaHV-3 bivalent vaccines.

The Rispens CVI988 vaccine (Rispens vaccine) is currently considered the "gold standard" of MD vaccination (Davison & Nair, 2005) and is an attenuated GAHV-2 vaccine. In general, Rispens vaccine offers significantly better protection against vv+MDV isolates than HVT and HVT+GaHV-3 (bivalent) vaccines (Witter *et al.*, 1995). However, previous pathotyping and protection experiments have shown that the protection provided by Rispens vaccine against GaHV-2 isolates is variable (Witter *et al.*, 2005; Zhang *et al.*, 2015). Furthermore, only limited studies have investigated the protection provided by Rispens vaccine against vMDV isolates as they are adequately protected against by HVT and bivalent vaccines. Moreover, most pathotyping and protection studies using Rispens vaccine have been conducted in maternal antibody (mab) –ve chickens (Buscaglia *et al.*, 2004; Gong *et al.*, 2014; Zhang *et*

al., 2015) or experimental strains of mab +ve chickens (Witter *et al.*, 1995; Witter *et al.*, 2005) which do not reflect the situation in the field. Only a few protection studies have been conducted in commercial mab +ve chickens using Rispens vaccine (Lee *et al.*, 2010; Lee *et al.*, 2013; Chang *et al.*, 2014; Islam *et al.*, 2013a). In this thesis I tested the protection provided by the Rispens vaccine against two Australian isolates of MDV differing in virulence in mab +ve commercial chickens of a major international genotype (ISA Brown) using industry-standard vaccination procedures.

Quantitative PCR (qPCR) methods have been developed to differentiate between GaHV-2, GaHV-3, and HVT, and this has enabled the development of methods to correlate viral loads with subsequent MD status in protection studies. As Rispens/CVI988 vaccine is an attenuated GaHV-2, qPCR methods that differentiate Rispens vaccine from pathogenic GaHV-2 have only become available more recently (Baigent *et al.*, 2011; Haq *et al.*, 2012; Renz *et al.*, 2013; Gimeno *et al.*, 2014).

Viral loads of various samples have been studied to predict subsequent MD status with or without vaccination (Yunis *et al.*, 2004b; Islam *et al.*, 2006b; Gimeno *et al.*, 2008; Islam *et al.*, 2008; Dunn *et al.*, 2014). In this thesis, using the differential qPCR method described by Renz (2013), I explored genome copy numbers of both pathogenic GaHV-2 and Rispens vaccine viruses in peripheral blood leucocytes (PBL), feather, spleen and dust in mab +ve commercial ISA Brown birds used in the protection study referred to above. I also investigated the level of immunosuppression focussing mainly on lymphoid organ atrophy induced by the two Australian MDV pathotypes by assessing effects on relative bursal, thymic, and splenic weights.

The differential qPCR assays for Rispens vaccine and pathogenic GaHV-2 have enabled the study of viral kinetics of vaccinal and challenge MD viruses in the same host (Baigent *et al.*, 2011; Haq *et al.*, 2012; Baigent *et al.*, 2013; Islam *et al.*, 2014). All MDV vaccines are imperfect vaccines, and therefore allow superinfection, replication, and shedding of pathogenic MDV in the host (Eidson *et al.*, 1971; Rispens *et al.*, 1972a; Islam & Walkden-Brown, 2007). Witter (1998b) had postulated that the increased virulence of the field GaHV-2s may be caused by the vaccination itself. It has been postulated that imperfect or "leaky" vaccines may lead to evolution of more virulent pathogens (Gandon *et al.*, 2001; Atkins *et al.*, 2013) and this has been supported by recent modelling of experimental data (Atkins et al., 2013). However, only very recently it has been confirmed experimentally that the use of the

imperfect HVT vaccine does indeed favour more virulent GaHV-2 isolates (Read *et al.*, 2015). Several co-infection studies of HVT and bivalent vaccines with pathogenic MDV in the same host have been carried out. However, this thesis reports the first study that compares the viral kinetics of GaHV-2 pathotypes of differing virulence in chickens vaccinated or not with the Rispens vaccine. This will provide insight into whether the ongoing efficacy of the Rispens vaccine is due to unique features of the vaccine that do not favour more virulent MDVs, or whether it is similar to the other MD vaccines in favouring the more virulent isolates. In the latter case, there must be some other reason for the ongoing success of this vaccine, first introduced in the early 1970s.

Although important viral kinetic studies have been carried out with Rispens vaccine, which provide useful suggestions on what samples should be collected at what times to best monitor the vaccine take (Baigent *et al.*, 2011; Haq *et al.*, 2012; Baigent *et al.*, 2013; Islam *et al.*, 2014a), very few studies have been carried out in the field. This thesis reports a field study into measures of Rispens vaccine take in vaccinated layer hens of different ages. Furthermore, although the initial studies of Rispens vaccine using the 26th DEF passage of Rispens CVI988 concluded that Rispens vaccine infection is lifelong (Rispens *et al.*, 1972a), the existing current commercial vaccine has not been tested in the field for lifelong infection. The current co-infection level of Rispens vaccine with pathogenic GaHV-2 is also unknown in the field. A recent study has confirmed that current commercial Rispens vaccines will effectively transmit laterally from vaccinated to unvaccinated chickens (Islam *et al.*, 2014). Therefore, part of this study was also to investigate whether the Rispens vaccine actually has escaped and become established in unvaccinated chicken populations.

Therefore, the main aims of the work reported in this thesis are to:

1. Investigate the protection levels provided by the Rispens vaccine against GaHV-2 pathotypes of varying virulence (vMDV and vvMDV) and test early predictions of vaccinal protection;
2. Identify which invasive and/or non-invasive samples should be collected, and when, to best detect the vaccine take of Rispens vaccine in commercial layer populations;
3. Study the viral kinetics of Rispens vaccine virus and GaHV-2 pathotypes of varying virulence in single and co-infected hosts, including determining whether Rispens vaccination favours the replication of more virulent GaHV-2 over less virulent GaHV-2;

4. Determine the long-term viral kinetics of Rispens vaccine in commercial layer chicken populations; and
5. Determine whether there has been natural spread to, and establishment of, Rispens vaccine virus infections in unvaccinated broiler flocks.