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

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August 2015

School of Environmental and Rural Science
Faculty of Arts and Science
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.

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<tbody>
<tr>
<td>aa</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ADOL</td>
<td>Avian Disease Oncology Laboratory</td>
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<tr>
<td>AE</td>
<td>Avian encephalomyelitis</td>
</tr>
<tr>
<td>AEC</td>
<td>Animal ethics committee</td>
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<tr>
<td>AGPT</td>
<td>Agar gel immunoprecipitation test</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>B cells</td>
<td>B lymphocytes</td>
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<td>BAC</td>
<td>Bacterial artificial chromosomes</td>
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<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>Bursa</td>
<td>Bursa of fabricius</td>
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<td>CEF</td>
<td>Chicken embryo fibroblasts</td>
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<td>CKC</td>
<td>Chicken kidney cells</td>
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<td>CMI</td>
<td>Cell mediated immunity</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<td>CV</td>
<td>Coefficient of variance</td>
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<td>DEF</td>
<td>Duck embryo fibroblasts</td>
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<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<tr>
<td>dpc</td>
<td>Days post challenge</td>
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<td>dpi</td>
<td>Days post infection</td>
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<td>EDS</td>
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<td>ELISA</td>
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<tr>
<td>HEPA</td>
<td>High efficiency particulate air</td>
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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  Ribonucleic acid
Rnase  Ribonuclease
rpm  Revolutions per minute
SNP  Single nucleotide polymorphism
SPF  Specific pathogen free
T cells  T lymphocytes
TP  Transient paralysis
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<td>TRS</td>
<td>Terminal repeat short</td>
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<td>UL</td>
<td>Unique long sequence</td>
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<td>US</td>
<td>Unique short sequence</td>
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<td>v</td>
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<td>vv+</td>
<td>Very virulent plus</td>
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List of Publications

Peer reviewed journal articles


Other reviewed journal articles (not reported in this thesis)


Peer reviewed long conference publications


Short non-peer reviewed conference publications


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.