Field studies on the detection, persistence and spread of the Rispens CVI988 vaccine virus and the extent of co-infection with Marek's disease virus

S Ralapanawe*[a b], KG Renz[a], SK Burgess[a] and SW Walkden-Brown[a]

Objective: Use specific real-time quantitative polymerase chain reaction (qPCR) tests to determine: a) vaccination success determined by viral load of Rispens CVI988 (Rispens) in feathers or dust b) persistence of Rispens infection in vaccinated layer chickens; c) extent of co-infection with wild-type MDV (MDV) in vaccinated layers; and d) presence of Rispens virus in unvaccinated broiler flocks.

Methods: Feather and dust samples for qPCR to detect MDV and Rispens, and serum samples to detect anti-MDV antibody using ELISA were collected from birds aged three days to 91 weeks from three layer farms. DNA extracted from MDV-positive dust samples from 100 broiler flocks, was tested for the presence of Rispens using the qPCR.

Results: Overall 66% and 93% of feather and dust samples respectively from Rispens-vaccinated layers were Rispens–positive. Viral load in these samples varied between farms during early life reaching readily detectable levels at 2-3 weeks of age. Vaccinated chickens maintained high Rispens load in feathers and dust and MDV antibody levels until 91 weeks of age. MDV infection was detected in 6.7% of feather samples from vaccinated chickens. Rispens virus was detected in 7% of samples from unvaccinated broiler flocks.

Conclusion: Vaccine take can be measured effectively by Rispens-specific qPCR of feathers or dust from around three weeks post vaccination. Infection with Rispens is persistent with lifelong shedding and serological response. Detectable infection rate of vaccinated chickens with MDV is low and there is preliminary evidence of escape of Rispens virus to unvaccinated flocks.

[a] Animal Science, School of Environmental and Rural Science, University of New England, Armidale, NSW, Australia 2351 [b] Veterinary Research Institute, B365, Peradeniya, Sri Lanka 20400; sralapan@myune.edu.au

Key words Rispens vaccine; Marek's Disease Virus; imperfect vaccine; Viral load; Feather; Dust; Serology; Diagnostics

Abbreviations avian encephalomyelitis, AE; egg drop syndrome, EDS; enzyme linked immunosorbent assay, ELISA; fowl pox, FP; *Gallid herpesvirus 2*, GaHV-2; *Gallid herpesvirus 3*, GaHV-3; Herpesvirus of Turkeys, HVT; infectious bronchitis ,IB; infectious laryngotracheitis, ILT; Marek's Disease Virus, MDV; Marek's Disease, MD; *Meleagrid Herpesvirus 1*, MeHV-1; New Castle disease, ND; qPCR, quantitative real-time PCR; Rispens CVI988 vaccine, Rispens; viral copy numbers, VCN

Introduction

Marek's Disease (MD) was first described by Jozef Marek [1] and is an economically important poultry disease throughout the world. The MD virus (MDV) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Mardivirus*. The genus *Mardivirus* consists of five species of viruses, including *Gallid herpesvirus 2* (GaHV-2), *Gallid herpesvirus 3* (GaHV-3), *Meleagrid Herpesvirus 1* (MeHV-1). The early classification into MDV's into 3 serotypes which were known as serotypes 1, 2 and 3 (HVT or herpesvirus of turkeys) were dependent on variation in antigenic determinants[2, 3] which correspond to the different species. The MDV strains of serotype 1 belong to GaHV-2 species; serotype 2 to GaHV-3 and serotype 3 to MeHV-1. Serotype 1 MDV (GaHV-2) are pathogenic and cause tumours in chickens while serotype 2 (GaHV-3) from chickens[4] and serotype 3 (MeHV-1) from turkeys[5] are non-oncogenic.

MD is the first ever viral disease causing cancer to be successfully controlled by vaccination. [6]. The vaccine that currently offers the highest level of protection against MD in long lived layer and breeder chickens is the Rispens CVI988 vaccine.[7] Rispens CVI988 is an attenuated vaccine strain of a serotype 1 MDV first isolated in the Netherlands[8] and found to be protective in both laboratory and field trials.[9] Rispens has since proven to offer superior protection against clinical MD and is administered worldwide particularly to breeder and layer chickens.

Australia imported Rispens vaccine master seeds from France in 1997, in order to control a major outbreak of MD between 1993 to 1997 which caused considerable economic loss.[10] The introduction of this vaccine and the earlier introduction in 1996 of automated *in ovo* vaccination of broiler chickens with cell-associated HVT vaccine brought the MD outbreak under control and the disease has remained well controlled by these measures to the present. The original Rispens isolate transmitted successfully between chickens[9] and we have recently demonstrated that current commercial vaccine strains of Rispens in Australia are shed in high amounts from vaccinated chickens, commencing as early as 7 days and also transmit readily to unvaccinated chickens.[11] This raises the prospect of the Rispens virus "escaping" from vaccinated flocks and establishing itself amongst the population of free living MDVs.

Although the Rispens vaccine provides superior protection against MD, in common with other MD vaccines it does not prevent infection with wild type MD virus [9]. Such vaccines are known as imperfect vaccines [12] allowing both vaccinal and wild-type virus to replicate in the host, potentially driving MDV towards higher virulence.[13] The potential of co-infection of MDV has plagued measurement of Rispens vaccination efficacy as both Rispens and MDV are GaHV-2 (serotype 1) viruses, requiring tests that can differentiate between them. With the recent development of qPCR tests which achieve this task[14, 15] it has been possible to perform experimental studies involving co-infection with both viruses.[15, 16] This paper presents data from field studies using these tests with the main objectives being to: a) evaluate use of specific qPCR of feather or dust samples as measures of vaccine efficacy; b) investigate the long term kinetics of the virus and host antibody response in layer chickens; c) determine the extent of MDV co-infections in Rispens-vaccinated layers under field conditions and d) determine whether the Rispens vaccine virus has escaped into non-vaccinated broiler flocks.

Materials and methods

Experimental design

In order to meet the objectives, feather, blood and dust samples were collected at regular intervals from 3 commercial layer farms as summarised in Table 1. In addition, DNA samples extracted from dust samples from commercial broiler farms from around Australia were analysed for the presence of Rispens virus using the Rispens-specific qPCR test. The samples chosen were those previously shown to be positive for MDV using a generic MDV qPCR test [17] and they are summarised in Table 2.

Table 1. Overview of farms and sampling for the layer studies. All birds were vaccinated with the Rispens vaccine at hatch.

Farm	Location in NSW	Chicken population	Layer strain	Rispens vaccine	No of sampling visits	Date of first sampling	Ages sampled (weeks)
A	Tamworth	27,000	Hy-Line Brown	VaxSafe RIS®[a]	5	15/06/12	1, 2, 3, 4, 23, 33, 41, 51, 61, 71 and 78
B	Port Macquarie	16,000	ISA Brown	VaxSafe RIS®[a]	1	09/01/13	50, 57, 65, 72, 83, and 91
C	Tamworth	30,000	ISA Brown	VaxSafe RIS®[a]	3	13/02/13	0.43, 2.5, 3.5, 5.5, 6.5, 7.5, 8, 8.5, 12, 13,
C	Tamworth	3000	English Leghorn	VaxSafe RIS®[a]	3	13/02/13	2.5, 7.5, 8.5

[a]Bioproperties, Ringwood VIC 3134

Table 2. Overview of farms and dust samples for the meat chicken (broiler) studies.

State	No. of farms	Chicken strain	Age at sampling (days)	Rispens vaccination status	Number of dust samples
SA	15	Cobb, Cobb free-range	32-49	Unvaccinated	41
NSW	19	Cobb, Ross	21-54	Unvaccinated	49
QLD	2	Cobb, Ross	35-42	Unvaccinated	2
WA	1	Cobb, Ross	40-42	Unvaccinated	2
VIC	3	Cobb	42-49	Unvaccinated	6

Layer farm details Farm A had Hy-Line Brown chickens in 4-5 age groups about 19 weeks apart in age. There was one group of young pullets on the floor and four age groups of layers in two caged layer sheds (two age groups per shed). These sheds were mechanically ventilated. Replacement pullets were reared to 15 weeks of age in four barns separated from the older birds by approx. 0.5 km. Chicks were vaccinated at hatch with Rispens CVI988 (Bioproperties, Ringwood, Vic). Other vaccinations were for infectious bronchitis (IB) at day old and at 10 weeks fowl pox (FP) by wing stab, egg drop syndrome (EDS) and Newcastle disease (ND) by injection and avian encephalomyelitis (AE) by eye drop. There were 5 samplings, with ages 1, 2 and 3 respectively covered in the first 3 visits, ages 4, 23, 41, 61 and 78 weeks on the fourth visit and ages 33, 51 and 71 on final visit.

Farm B housed ISA Brown chickens purchased as started pullets around 15 weeks of age, run as layers to 65 weeks, then moulted and taken through a second lay to 90-92 weeks of age. Due to recent new shed construction, the farm had a skewed aged distribution with birds on the farm being 50, 57, 65, 72, 83 and 91 weeks of age at the time of the sampling. The 50-week-old group were in the new climate controlled shed with the other groups in the older conventional shed with open sided ventilation. There were 9000 birds in each from the older groups and 7000 birds from the youngest age group. The birds on this farm were vaccinated against MD at hatch with Rispens CVI988 (Bioproperties, Ringwood, Vic) and also vaccinated against IB, infectious laryngotracheitis (ILT), FP, AE, ND and EDS.

Farm C housed pre-lay pullets, mostly ISA Brown with one group of English Leghorns. At the time of the first sampling there were 5 age groups of 5600-6000 birds per age group in single age sheds. There were three sampling visits with age groups sampled on the first visit being 3 days and 2.5, 8, 12 and 13 weeks of age. The English leghorns were 2.5 weeks old and there were 3000 birds in one shed together with the same aged ISA Browns. At the 3rd visit, the age groups sampled were 3.5, 6.5 and 8.5 weeks old. Chickens from the oldest age group at the first visit had already been transferred to another layer farm, thus were not available for the last sampling. The birds were reared on the floor and were vaccinated against MD (Rispens CVI988, Bioproperties, Ringwood, Vic) at hatch and also against FP, ILT, IB, Coryza, ND and coccidiosis at various times.

Sampling procedures Blood and feather samples were collected from 15 individual birds per age group. From young chicks (to two weeks of age) large wing feathers were sampled while all subsequent samples were from the axillary tract along the side of the thorax. Dust samples were collected from available surfaces in the shed. Serum was separated from the blood samples and all samples stored at -20˚C until further processing.

Laboratory procedures

1. ELISA Antibody directed against MDV was detected in sera using a Marek's disease specific indirect ELISA adapted from Zelnik et al.,[18] as described below. This ELISA does not distinguish between antibody directed at the Rispens or other serotype 1 MDVs.

Preparation of antigen for Marek's disease ELISA Rispens CVI988 vaccine (Bioproperties Vaxsafe RIS®) was used to prepare the ELISA antigen. A vaccine vial was thawed, diluted in 2.5 ml of vaccine diluent, centrifuged at 748 xg for 5 minutes at 4 ˚C and the supernatant discarded. The remaining pellet was frozen at -20 ˚C and subjected to four freeze-thaw cycles. After the last thawing the pellet was broken up and dispersed in PBS using a sonicator (MSE Soniprep 150) for 2 minutes at 12 amperes. The homogenized antigen was centrifuged at 4 ˚C for at 1455 x g for 10 minutes and the supernatant retained. The concentration of the antigen was determined by a spectrophotometer (M7 Bio-Rad SmartSpec^{TM} 3000) using bovine albumin serum standards (Sigma, A-3803) and Bradford reagent (Sigma-Aldrich). Antigen was stored in aliquots at -20 ˚C until required. The optimum antigen concentration to coat the plates was determined by serial dilution of the antigen against serial dilutions of known positive samples and conjugate.

ELISA procedure The test serum samples were diluted 1:100 with PBST (0.5ml/litre Tween 20 added for 1litre of PBS). The ELISA plates (Immulon ® 2 flat bottom microtitre plates, Cat. No. 011-010-3455) were coated with Marek's antigen (1: 100 dilution, diluted with carbonate buffer 0.05M, pH 9.6). 100 µl of the diluted antigen was added to each well and incubated at 4 ˚C for 16 hours followed by washing twice with PBST. 100 µl of PBST containing 1% skim milk was added to each well to block the plates. The plates were covered and left for 1 hour at room temperature. The contents were removed by inverting the plates and 100 µl of the diluted samples, standards, negative control samples and blanks (PBST+1% skim milk) added, followed by incubation for one hour at 37 ˚C. Positive control sera were from experiments in which specific pathogen free (SPF) chickens were challenged with MDV. Negative control sera were from unchallenged SPF chickens. After incubation the plates were washed twice with PBST followed by addition of 100 µl of rabbit anti-chicken antibody (2^{nd} antibody) conjugated with horseradish peroxidase enzyme (Sigma cat no. A9046, diluted 1:5000 with PBST). The plate was covered and incubated for 1 hour at 37 ˚C. The plates were then washed with PBST three times and 100 µl of substrate (34 mg of o-Phenylenediamine and hydrogen peroxide 30% w/v [Univar/Chem supply] with 100ml of citrate phosphate buffer [pH 5.0]) added to all wells. Plates were covered with aluminium foil and incubated for 10 minutes at room temperature. The chemical reaction was stopped by addition of 50 µl of 98% sulphuric acid per each well. The plate was mixed for 5 seconds and read by microplate reader at 490 nm (Bio-Rad, Benchmark), and the optical density values were obtained, averaged over duplicate samples. The antibody titre was derived from the optical density values of the standards of known dilution in the standard curve. The standard curve consisted of 10 standards in duplicate comprising a 2-fold serial dilution. A cut off value of 500 was used to differentiate positive from negative samples.

2. Quantitative Polymerase Chain Reaction (qPCR) The DNA was extracted from the feather calamus (referred to as feather tip), which connects the shaft of the feather to the skin. Prior to DNA extraction, feather tips from 3-5 feathers from each sample were cut 2-3mm from its proximal end using a sterile scalpel blade for each sample. These feather tips were transferred into a new labelled microfuge tube. The processed feather samples underwent DNA extraction using a Genomic DNA Mini Kit (Bioline, Australia) followed by Rispens-specific qPCR to determine absolute viral genome copy numbers as described by Renz et al.[14] Furthermore, 120 selected feather DNA samples were subjected to wild-type MDV-specific qPCR as described by Renz et al.[14] All dust samples were subjected to DNA extraction using a Genomic DNA Mini Kit (Bioline, Australia) and analysis by qPCR for Rispens and pathogenic MDV viruses. Before qPCR analysis, DNA of all samples was quantified using spectrophotometry using a Nanodrop® ND-1000 UV-Vis spectrophotometer (NanoDrop® Technologies, Wilmington, USA). The DNA samples were diluted to a constant concentration of 5 ng μl^{-1} before use in the qPCR assay.

Extracted DNA from dust samples from unvaccinated commercial meat chicken flocks in Australia which were positive for MDV from previous analyses was tested for presence of Rispens using the Rispens-specific qPCR. These were positive for a generic MDV serotype 1 qPCR that does not differentiate between Rispens and MDV. The selected samples therefore represent a non-random sample biased towards those potentially containing the Rispens virus.

Statistical analysis Data were analysed using JMP11 statistical software (SAS Institute Inc. 2014). The ELISA antibody titres were cube root transformed and viral loads determined by qPCR were log transformed [Log_{10} (y + 50)] to better approach a normal distribution of

variances. To ascertain patterns of antibody concentration and viral loads on different farms at different chicken ages, smoothed spline curves were fitted (λ = 1000 or 5000). Association between measured variables was assessed by correlation and linear regression analysis. Sensitivity of the Rispens qPCR tests for feathers and dust were assessed relative to serological results for the same individual bird in the case of feathers and on a shed basis in the case of dust. For the latter, any positive serological result within the shed resulted in a positive shed serological result. Only data after 3 weeks of age were included in the calculations.

Results

Early assessment of vaccine take

The percentage of samples positive for anti-MDV antibody in serum was 100% at 3 days of age declining to 50% at 4 weeks of age (Fig. 1A). Thereafter, it increased to 100% at eight weeks of age remaining relatively constant to 12 weeks of age. Anti-MDV antibody titre during early life differed markedly between farms A and C (Fig. 2A). On farm C it was high initially, decreased somewhat to week four then increased to 12 weeks. On farm A, it remained at low levels around the detection level for the first four weeks of life during which measurements were made.

The percentage of feather samples positive for the Rispens virus increased erratically to a peak of 93 % at 8 weeks fluctuating between 40 and 95% (Fig. 1B). As with antibody titre, there were differences in Rispens viral load young chickens between farms A and C (Fig. 2B). On farm C viral load was high and sustained over the first 12 weeks whereas on farm A viral load was low but increasing during the 0-4 week sampling period.

The percentage of dust samples positive for the Rispens virus was 100% throughout the first 12 weeks apart from 50% at 2 weeks of age (Fig. 1C). Rispens viral load in dust during the first few weeks of life again varied markedly between farms A and C, being high but declining on farm C while low initially but rising sharply on farm A during the 0-4 week sampling period (Fig. 2C).

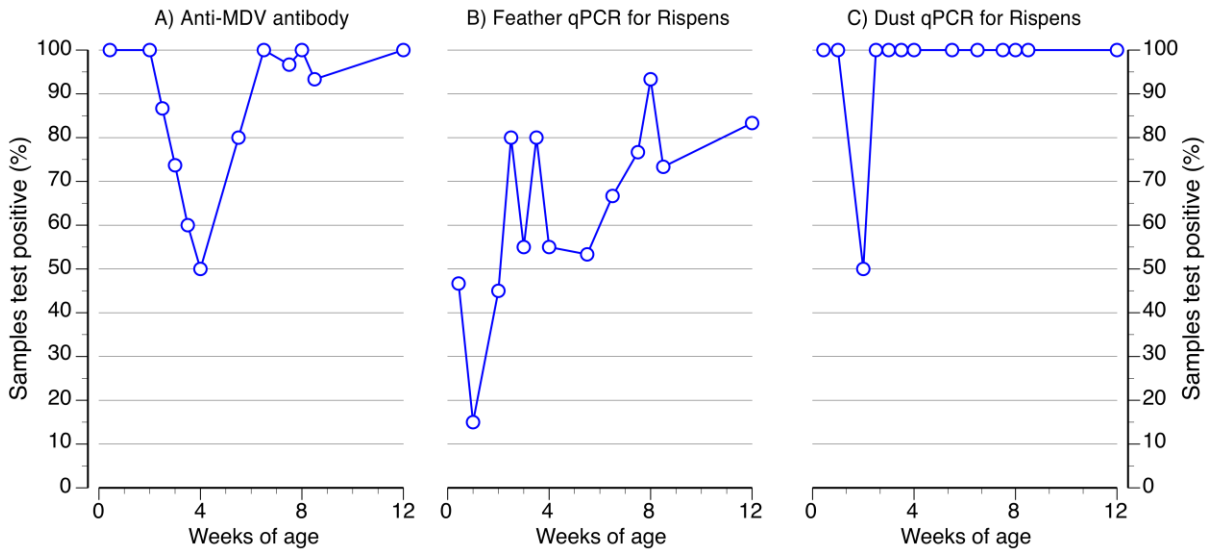

Figure 1 Percentage of serum samples positive for anti-MDV antibody (A), feather samples qPCR positive for the Rispens virus (B) and dust samples qPCR positive for the Rispens virus (C) in layer chickens vaccinated with Rispens CVI988 vaccine up to 12 weeks of age. All farms combined.

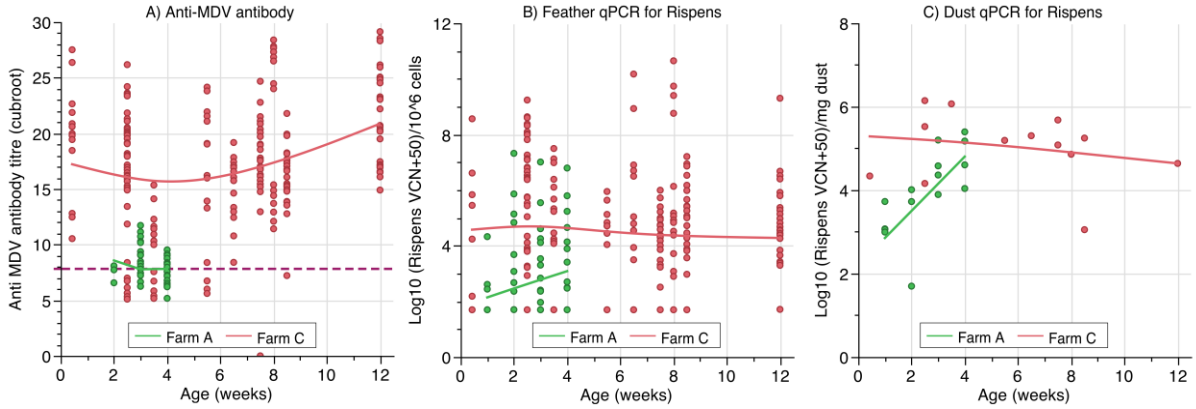

Figure 2 Serum anti-MDV titre (cube root transformed) (A), Rispens viral load (log transformed) in feathers (B), and dust (C) in layer chickens vaccinated with Rispens CVI988 vaccine up to 12 weeks of age on two farms. Each point represents a single sample and curves are smoothed spline curves ($\lambda = 1000$). The dotted line in (A) represents the positive cut off value.

Persistence of infection and immune response

Over the full spectrum of ages up to 91 weeks, 426/463 (92%) serum samples were positive for anti-MDV antibodies. After week 5, 80% or more of samples were positive and all samples between the ages of 12 and 83 weeks were positive (Figure 3A). On farm A antibody titres increased from low levels in the first 4 weeks of life to high levels at week 20 and beyond. The older birds on Farm C maintained high titres between weeks 50 and 91. The antibody titres of Farm A tended to be lower than those of Farm B where ages overlapped.

Of 498 feather samples sampled up to 91 weeks of age, 330 (66%) were positive for the Rispens virus by qPCR. The percentage of positive samples increased erratically to a maximum of 93 % at 8 weeks fluctuating between 53 and 80% thereafter (Fig. 3B). In older birds, as with antibody titre, there were slight differences in Rispens viral load between farms A and B (Fig. 4B). Viral loads in birds 50-90 weeks of age were as high or higher as those seen at earlier ages, indicating persistent infection. The mean viral load in positive samples was 10^5 viral copy numbers (VCN) per 10^6 feather cells with little variation over time. Feather sample sensitivity relative to the MDV ELISA result for the same chicken aged over 3 weeks was 71% (252 positive matches for 356 positive ELISA samples).

Of 42 dust samples subjected to Rispens assay 39 (93%) were positive for the Rispens virus (Fig. 3C). Older birds on farm A exhibited a slightly declining trend in Rispens load while farm B displayed an increasing trend (Fig. 4C). Between weeks 50 and 91 viral load varied between 1 and 25 x 10^3 with an overall mean of 15.8 x 10^3 per mg of dust. Dust sample

sensitivity relative to the MDV ELISA result from the 15 chickens in the shed the dust was sampled from for birds was 96%. Only samples from birds older than 3 weeks were included.

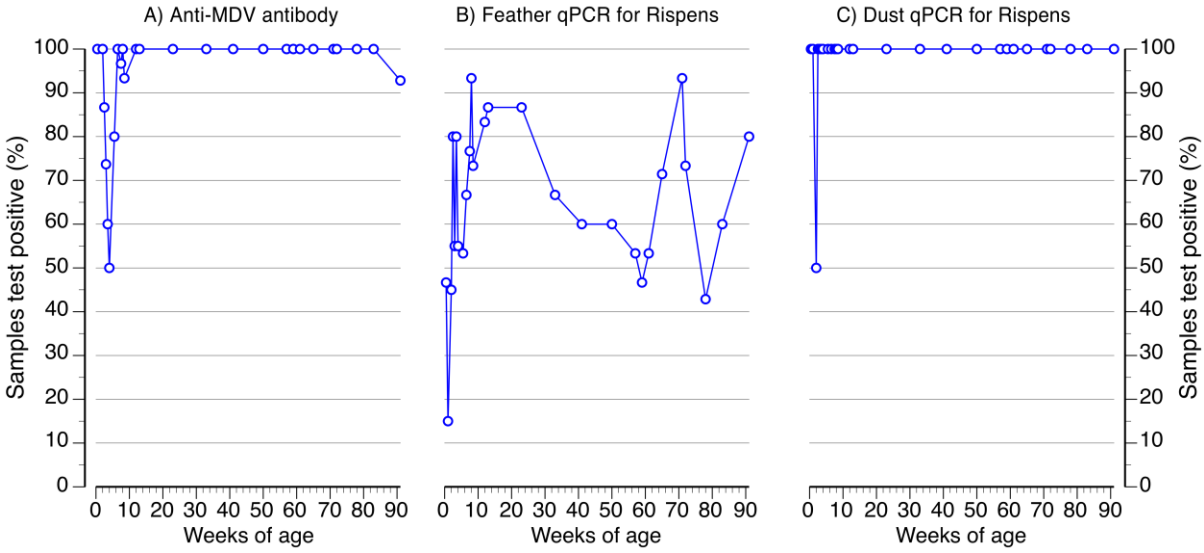

Figure 3 Percentage of serum samples positive for anti-MDV antibody (A), feather samples qPCR positive for the Rispens virus (B) and dust samples qPCR positive for the Rispens virus (C) in layer chickens vaccinated with Rispens CVI988 vaccine up to 91 weeks of age. All farms combined.

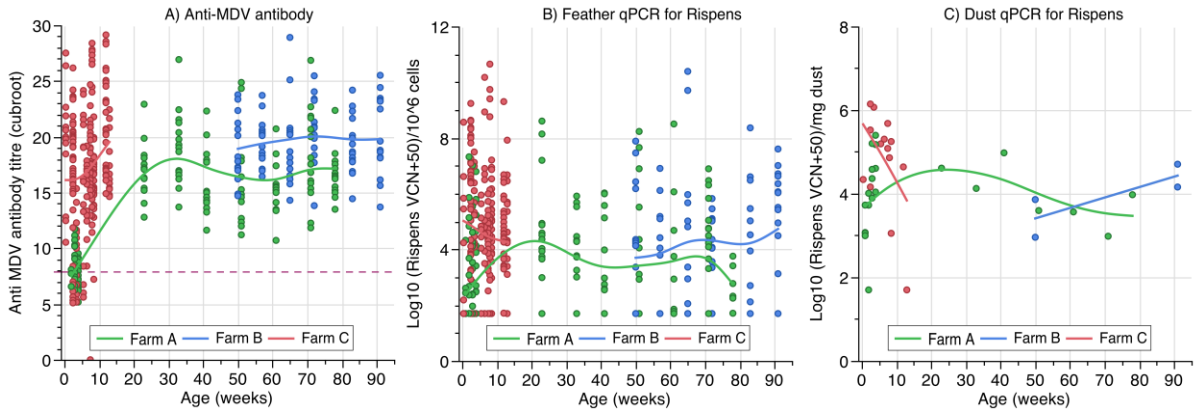

Figure 4 Serum anti-MDV titre (cube root transformed) (A), Rispens viral load (log transformed) in feathers (B), and dust (C) in layer chickens vaccinated with Rispens CVI988 vaccine up to 91weeks of age. Each point represents a single sample and curves are smoothed spline curves ($\lambda = 5000$). The dotted line in (A) represents the positive cut off value.

Extent of co-infections with MDV

Of 120 randomly selected feather DNA samples from Rispens-vaccinated layers only eight (6.7%) were positive for wild type MDV (Table 3). Of the 120 samples only 60 were positive for the Rispens virus and 3 (5%) of these were positive for both viruses. Only farms A and B had samples positive for wild type MDV and the viral load varied from 0.2 to 3.98 x 10^3 copies per 10^6 feather cells. The age of MDV-positive birds ranged from 4 to 83 weeks with no particular association between viral load and age.

Of the 42 dust samples from Rispens-vaccinated layers only two (4.8%) were positive for pathogenic MDV. The two samples were also positive for Rispens virus with approximately 4 logs higher load of Rispens than MDV.

Table 3: The proportion of positive samples by the farm, sample type and the age (weeks). Dashes signify that no sample was tested or collected for that age group, often because birds were in a multi-age shed.

Farm	Sample	Test result	Age (weeks)											Overall result
A			**1**	**2**	**3**	**4**	**23**	**33**	**41**	**51**	**61**	**71**	**78**	
	Feather	Rispens qPCR	3/20	9/20	11/20	11/20	13/15	10/15	9/15	8/15	8/15	14/15	6/14	102/184
		MDV qPCR	1/1	-	-	-	0/7	0/10	1/11	1/4	2/3	0/4	1/6	6/46
	Dust	Rispens qPCR	4/4	2/4	4/4	4/4	1/1	1/1	1/1	1/1	1/1	1/1	1/1	21/23
		MDV qPCR	0/4	0/4	0/4	0/4	-	0/1	0/1	0/2	0/1	0/1	0/1	0/23
	Sera	MDV ELISA	-	1/4	14/20	10/20	15/15	15/15	15/15	15/15	15/15	15/15	15/15	130/149
B			**50**	**57**	**65**	**72**	**83**	**91**						
	Feather	Rispens qPCR	9/15	7/15	10/14	11/15	9/15	12/15						58/89
		MDV qPCR	1/12	0/4	0/4	0/6	1/4	0/4						2/34
	Dust	Rispens	2/2	-	-	-	-	2/2						4/4
		MDV	0/2	-	-	-	-	0/2						0/4
	Sera	MDV ELISA	15/15	15/15	14/14	15/15	15/15	15/15						89/89
C			**0.43**	**2.5**	**3.5**	**5.5**	**6.5**	**7.5**	**8**	**8.5**	**12**	**13**		
	Feather	Rispens qPCR	7/15	36/45	12/15	8/15	10/15	23/30	14/15	22/30	25/30	13/15		170/225
		MDV qPCR	-	-	-	0/8	0/8	0/5	0/4	0/3	0/7	0/5		0/40
	Dust	Rispens qPCR	1/1	3/3	1/1	1/1	1/1	2/2	1/1	2/2	2/2	0/1		14/15
		MDV qPCR	0/1	1/3	1/1	0/1	0/1	0/2	0/1	0/2	0/2	0/1		2/15
	Sera	MDV ELISA	15/15	39/45	9/15	12/15	15/15	29/30	15/15	28/30	30/30	15/15		207/225

Rispens virus escape into non-vaccinated flocks

Of the 100 dust samples from broiler flocks not vaccinated with Rispens but positive for MDV in a generic MDV-1 qPCR test, 7 were found to be positive for the Rispens virus. Details of these samples are provided in Table 4. Comparison of the viral loads detected by the Rispens-specific and generic MDV-1 qPCR tests for these samples are shown in Figure 5.

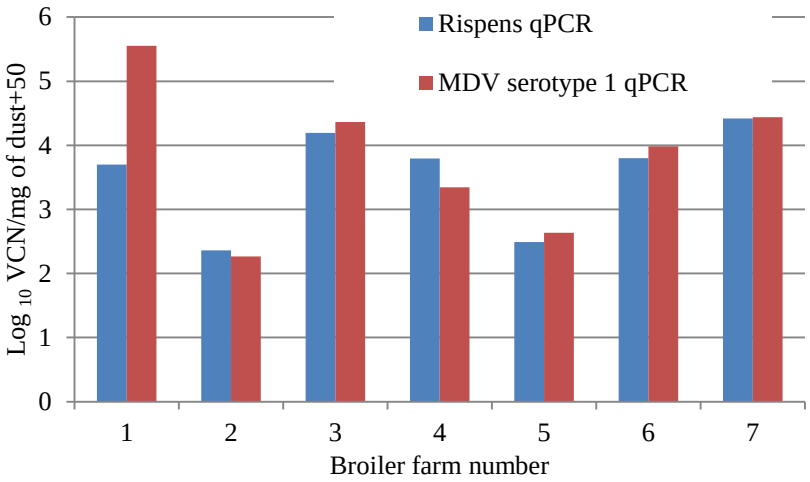

Figure 5: Comparison of viral copy number detected in dust samples from seven commercial broiler farms using the Rispens specific or generic MDV serotype 1 qPCR test. Chickens on these farms were not vaccinated with Rispens.

Table 4: Details of dust DNA samples positive for generic and Rispens assays

Sample submission date	State	Farm	Chicken Strain	Chicken Age	MD vaccination status
15/02/2012	NSW	1	Unsexed Barn Ross	49	Unvaccinated
01/05/2012	SA	2	Unsexed Barn Cobb	40	Unvaccinated
08/08/2012	NSW	3	Unsexed FR Cobb	N/A	Unvaccinated
19/09/2012	SA	4	Cobb free-range	32	Unvaccinated
11/10/2012	SA	5	Ross (Cobb)	39	Unvaccinated
21/05/2013	VIC	6	Unsexed Barn Ross	49	Unvaccinated
23/01/2013	NSW	7	Unsexed Barn Ross	34	Unvaccinated

Association between variables

There was a significant positive association between Rispens viral load in feathers and ELISA antibody titres (Figure 6 A, $y=0.088x + 2.56$, $P < 0.001$, $R^2 = 0.045$,).

The association was also significant within each individual farm (Figure 6 B, Farm A: $P = 0.036$, $R^2 = 0.03$; Farm B: $P = 0.035$, $R^2 = 0.05$ and Farm C: $P = 0.016$, $R^2 = 0.026$;).

In addition there was a significant positive association between Rispens viral load in feathers and Rispens viral load in dust (Figure 6 C, $y=0.31x + 3.1$, $P < 0.0001$, $R^2 = 0.05$).

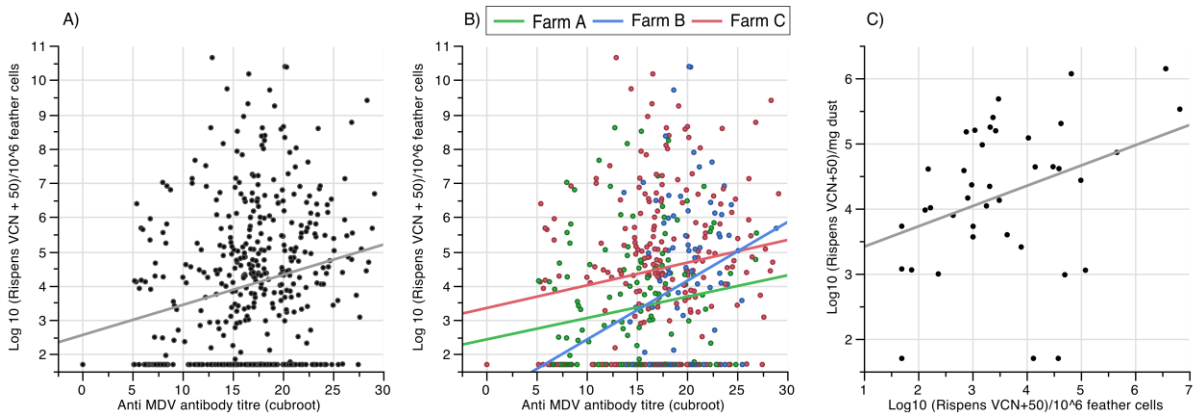

Figure 6 Association between anti-MDV titre and Rispens viral load (log transformed) in feathers in individual chickens on all farms A) and in individual chickens within farms B). Association between Rispens viral load in feathers (mean of 15 samples) and in dust (single sample) within shed and age category is shown in C). Lines are linear regressions and details are available in the text.

Discussion

The first objective was to determine if and when Rispens vaccination success could be measured effectively by Rispens-specific qPCR analysis of either feather or dust samples. There was a high percentage of positive feather samples (80%) and a high mean Rispens viral load of 2.5×10^5 VCN per 10^6 feather cells by 2.5 weeks of age. These results are consistent with studies carried out in isolators or other experimental conditions in which all feather samples from Rispens vaccinated birds were positive at 14 days post vaccination. [11, 19, 20] The Rispens viral load in dust was highest at 3.5 weeks at approx. 10^6 VCN/mg of dust. This is higher than observed in an experiment carried out in housed ISA Brown chickens where the load of Rispens in dust 3.5 weeks post vaccination was approx. 3.2×10^4 VCN/mg.[16] Thus, based on the results, we can conclude that feather samples collected from 2.5 weeks and dust samples from 3.5 weeks of age onwards would be effective measures the vaccine "take". Rispens viral loads both in dust and feather increased with age from one to four weeks on Farm A but not C, although viral loads on farm C were 1-2 logs higher in both sample types. The observed large differences between farms in Rispens viral load are not readily explained with the information available to us, but indicate that significant differences are possible in vaccination outcome, despite ostensible use of the same vaccine and age at vaccination.

In the present experiment significant viral loads in dust were observed in very young chickens prior to the onset of shedding of virus in significant quantities as ascertained in experimental studies.[11,16] This is likely due to contamination of the dust samples with material from the previous batch of chickens. In this study dust was simply escaped from surfaces in the shed to evaluate this simple test, but in retrospect, the use of settle plates may have been preferable. A reliable molecular dust test for early detection should be based on samples free from direct contamination from earlier batches, either by using settle plates, or by sampling from an identified shed surface cleaned thoroughly prior to chick placement.

While feather sampling may provide an earlier and more individual measure of vaccine take it is potentially far more costly than dust testing as many individual chickens may need to be tested (or samples pooled), and unlike dust samples, feather samples require a cold-chain process to get to the lab and extensive preparation prior to DNA extraction. With the feather test there was a significant proportion of negative samples long after infection should have been established from the initial vaccination or by transmission of the virus between chicks following vaccination. It is likely that these negative samples reflect "false negatives" with regards the vaccination status of the chickens. The evidence supporting this is that the majority of the chickens with negative feather samples were serologically positive for MDV, suggestive of vaccination success. After excluding the data of first three weeks, the sensitivity of Rispens qPCR for feathers was 71% on individual bird basis and for dust 96% on shed basis.

Our second objective was to investigate the long-term kinetics of the virus and host antibody response in layer chickens. One of the major characteristics of herpesviruses is latency, with associated persistent life-long infection.[21] The preliminary studies carried out by Rispens et al.,[8] showed that the Rispens CVI988 virus (at passage 26 in duck embryo fibroblast culture) could be isolated from feathers up to two years of age following vaccination of day old birds reared in isolated facilities, even though the frequency of virus isolation varied between 30 to 70%. These authors also reported that the anti-MDV antibody level for the Rispens virus also remained undiminished throughout these two years and was similar to the levels after an actual MD infection. This is consistent with lifelong infection with maintenance of high antibody titre following infection with pathogenic MDV.[22] Indeed, more recent studies have shown that chickens co-infected with all 3 serotypes of MDV shed all serotypes in dust up to the end of the experiment at eight weeks of age, suggestive of concurrent persistent infection of all serotypes of MD.[23] Latent infection with MDVs is associated mainly with lymphocytes, with fully productive replication possible in the feather follicle epithelium concurrent with latent infection in lymphoid tissues.[24] Latency in epithelial cells is possible but remains to be proven.[24] The results of our study are consistent with this model of lifelong latent infection in lymphoid tissues but fully productive active infection in the feather follicular epithelium. The virus was readily detected in feathers and dust in the chickens aged between 50-91 weeks with no obvious decline in viral load with age. MDV antibody titre also remained at maximal levels during this period. These results indicate both active infection, at least in the feather follicle epithelium, and ongoing immune response. One issue, as noted earlier, is that 34% of chickens had feather samples negative for Rispens by qPCR while only

8% were negative for MDV antibody detected by ELISA. The lower detection level of the qPCR test may be due to chickens throwing off the infection, variations in the structure of feather samples eg. the ratio of pulp to keratinised shaft or the presence of Rispens virus below the threshold of detection. The lack of evidence of a systematic decline in Rispens viral load over time, the presence of an ongoing high level antibody response and the absence of an observed effect of feather quality on the outcome of qPCR test result are more suggestive of the latter possibility.

The third objective of this study was to determine the extent of MDV co-infections in Rispens-vaccinated layers in the field. Rispens being an imperfect vaccine[12] prevents clinical disease and tumours when infected with pathogenic MD however, does not prevent infection, replication and shedding of the pathogenic virus. We found that there was co-infection of Rispens and wild-type viruses in the field; however it was at a very low level, being 7% in feather samples and 5% in dust samples. Much higher levels of co-infection may be seen under conditions of experimental challenge following vaccination. In a recent study[16] involving MDV challenge 10 days after day-old vaccination with Rispens about 35% of feather samples and 75% of dust samples were positive for the pathogenic MDV in the post-challenge period. Our data suggests that the vaccinal protection against infection is high, possibly due to a long period of time between vaccination and challenge. The level of such challenge is also likely to be lower than under experimental conditions and via natural routes of infection, rather than the various forms of inoculation used in challenge studies. It is possible that the true infection level was much higher, but below our threshold of detection, but the results do indicate the vast bulk of MDV being shed in Rispens vaccinated layer flocks is of vaccinal origin.

Our fourth objective was to determine whether the Rispens virus has escaped into non-vaccinated flocks. As virtually all layer and breeder flocks are vaccinated with Rispens, this, of necessity, required detection of Rispens CVI988 in unvaccinated broiler flocks. A low level of presence (7%) was detected in MDV-positive dust DNA samples providing preliminary evidence that it is capable of circulating freely poultry flocks. Of the 7 positive samples, six appeared to be infected with Rispens only (Figure 5). To the best of our knowledge, none of these flocks was placed with surplus vaccinated breeder birds. This natural spread of the Rispens virus is consistent with previous studies showing that Rispens CVI988 virus is shed high amounts in feather dander and transmits readily between chickens.[9, 11] Apathogenic MDV viruses have been isolated from the field in early studies and found to have a protective effect against MD.[25] The Rispens virus has a lower replication and shedding rate than virulent MDV[16], but our findings and those of Jackson et al.,[25] suggest that less virulent MDVs with lower replication rates may continue to be present and circulate in the poultry population.

Conclusion

Based on these results we can conclude that Rispens-specific qPCR of feathers from around 2 weeks post vaccination or dust from 3 weeks post vaccination are effective measures of

vaccination success following Rispens vaccination. PCR-based methods offer the advantage over serological testing of specificity for the vaccine virus. Vaccination with the Rispens vaccine induces persistent infection with lifelong shedding of the virus and a sustained serological response. Detectable co-infection rate of vaccinated chickens with wild-type MDV is low suggesting that protection against infection provided by the vaccine is high in the field. Consistent with our understanding of the shedding and transmission of Rispens there is preliminary evidence of natural spread of the Rispens virus to unvaccinated flocks.

Acknowledgements

The work was funded by the Australian Egg Cooperation Limited (AECL) under project 1UN111. We thank the participating farmers for their cooperation and Mark Porter and Michael Raue for assistance with on-farm sampling. We also thank Mrs Sue Sharpe and Dr Peter Groves for their assistance with the broiler study.

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