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Three vaccine trials on Marek's disease

A report for the Rural Industries Research and Development Corporation

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FOREWORD

Losses due to Marek's disease (MD) in the early nineties were excessive despite the use of vaccination. Because of the success of the Rispens strain, a serotype 1 vaccine, the RIRDC funded a project (Project No RMIT-12E) which commenced in 1994 to develop an Australian serotype 1 vaccine. At the time it seemed unlikely a serotype 1 vaccine would be introduced to the country.

This report covers three trials conducted to assess the newly developed candidate RMIT vaccine.

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Abbreviations

ANOVA	analysis of variance
FFU	focus forming unit
HVT	herpes virus of turkeys
LSD	least significant difference test
MD	Marek’s disease
MDV	Marek’s disease virus
MEM	Eagle's Minimal Essential Medium
MV	Maravac
PD_{50}	50% protective dose
PFU	plaque forming unit
RIR	Rhode Island Red (chickens)
RMIT	Royal Melbourne Institute of Technology
SE	standard error of the mean
SPF	specific-pathogen-free
$TCID_{50}$	50% tissue culture infective dose
TMC	The Marek's Company
US	United States
VIAS	Victorian Institute of Animal Science

Executive Summary

This report describes three trials which are part of and follow the development of a live attenuated serotype 1 Marek's disease virus (MDV) vaccine from a highly virulent Australian strain, the Woodlands No. 1 strain.

Clone 60/2, passage 78, of the attenuated virus was evaluated in a large-scale safety and protection test as part of this project.

These tests confirmed that the 60/2 clone was both safe and efficacious. No gross tumours were observed in any of the vaccinated birds, although some mild immune organ depletion was evident in a safety test. Mild immunosuppression and Marek's disease (MD) lesions are a deficiency of serotype 1 MD vaccines.

The 50% Protective dose of the candidate vaccine was calculated to be 97.7 PFU/dose, however there is difficulty in obtaining a meaningful comparison between vaccines because of many test variables.

The large-scale comparison of the 60/2 clone with other vaccines revealed high levels of protection, although, the Rispens vaccine appeared to perform marginally better. Further studies need to be undertaken in commercial birds to test the role of factors found under field conditions that may take a part in vaccine efficacy.

1. Introduction

In the development of the RMIT serotype 1 vaccine against Marek's disease (MD), a series of chicken trials have been conducted. The initial selection of a suitable candidate was conducted at VIAS, Attwood in 1995 (Morrow, C. J., 1995). Several of these were tested in other experiments for their relative protection to each other and comparison with commercially available vaccines. From these studies the 60/2 clone was selected due to its minimal pathogenicity and its high protective value. In order to overcome the residual pathogenicity observed in this clone, further attenuation was instituted by continuation of passage in cell culture.

Preparations of clone 60/2 at several different passage numbers were then assessed for safety (pathogenicity) and protection in a chicken experiment conducted at the Victorian Institute of Animal Science (VIAS). From these results the 78^{th} passage was selected for further testing in the following trials; a large-scale safety trial, determination of its 50% Protective dose (PD_{50}) and its efficacy compared with commercially available vaccines.

2. Objectives

The research aims of this project were:

1. To determine the safety of the RMIT candidate serotype 1 vaccine at various doses.

2. To determine the 50% Protective dose (PD_{50}) of the RMIT candidate serotype 1 vaccine.

3. To compare the efficacy of the RMIT candidate serotype 1 vaccine to existing local and imported MD vaccines in Australia.

3. Safety test

This experiment was conducted to assess the safety of the 60/2 clone at passage 78 in a large-scale test in order to give results with greater statistical significance.

Day-old mixed sex Specific Pathogen-Free (SPF) chickens (CSIRO) were assigned to three groups of around 50 - 100 (see Table 1). Birds were vaccinated subcutaneously in the back of the neck with 0.2 mL of the appropriate dose (Table 1) using MEM maintenance medium as the diluent. The negative control group received diluent alone.

All birds were housed together on the floor of rooms fitted with HEPA filters to inlet and outlet air flows at VIAS, Attwood. An additional 10 birds were housed separately in a bubble isolator at the University of Melbourne to act as true negative controls, in the unlikely event of contact spread of virus from vaccinated birds.

Vaccinated birds were maintained and observed for 10 weeks for any signs of MD. Any birds that died or required euthanasia were examined for gross and histological lesions. Ten weeks after vaccination, birds were killed and examined for gross lesions and assigned a thymus score. Measurements were made of individual bursa and body weights. Thymus scores were graded from 0 - 3 where a score of 3 was normal and one of 0 indicated total atrophy. Ten birds per group were examined histologically, together with 5 of the 10 negative control birds housed in the isolator. Tissues examined histologically included brachial, sciatic and caeliac nerves, left gonad, spleen, kidney, liver, proventriculus, bursa and thymus. Birds that died during the experiment were examined for gross and histological lesions.

3.1 Results and Discussion

Table 1. Large scale safety test results of the 60/2 clone at passage 78.

Group	Total birds		Histology positive	% MD =	Dermatitis
(dose)	at start	at completion*			
Negative control					
- mixed in room	53	43	0	0	0
- in isolator	10	10	0	0	0
2,000 PFU	95	69	2	2.9	3
40,000 PFU	99	87	2	2.3	5

* The total of birds at the *completion* of the experiment does not include those that were removed due to loss of wing tags or death due to causes other than MD.

= Calculated from birds which had died during the experiment and exhibited histological evidence of MD. Expressed as a percentage of the total number of birds at completion (see above*). No gross lesions were seen in birds that died during the experiment or those autopsied at completion.

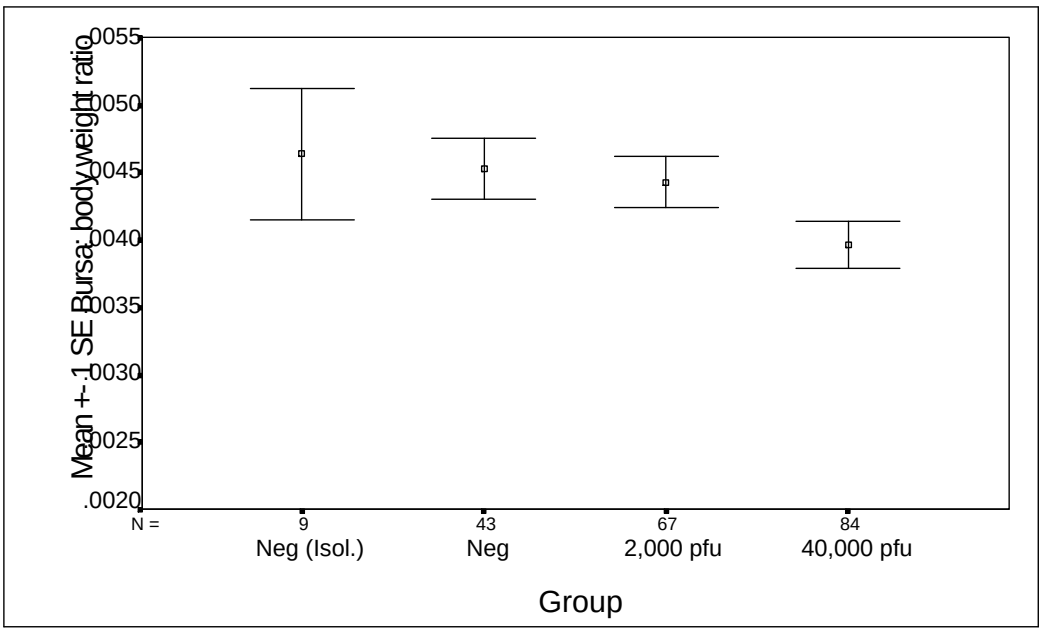
Table 1 shows histological evidence of MD in only 4 birds which were removed or died during the experiment. These lesions were consistent with a mild form of MD (mild to moderate lymphocyte infiltration of organs/nerves). Two of these chickens had been vaccinated with 2,000 PFU and two with 40,000 PFU.

No gross lesions were observed throughout the trial but 8 of 156 (5%) of vaccinated birds exhibited signs of dermatitis which had also been observed in a previous small-scale trial to assess attenuation of the 60/2 clone after additional passage in cell culture. Birds exhibiting dermatitis showed bursal and thymic atrophy, but the remaining vaccinated chickens were healthy and showed no gross signs of immune organ depletion. This was confirmed for bursal depletion when the bursa: body weight ratios were examined and no significant differences between the vaccinated groups and the negative controls were found (Figure 1); although not statistically significant, vaccinated groups showed slightly lower ratios.

Thymus scores (Figure 2) for both vaccine doses were slightly lower than the negative control and this was statistically significant. These results indicate that although there was no sign of serious immune organ depletion, some depletion of these organs was evident.

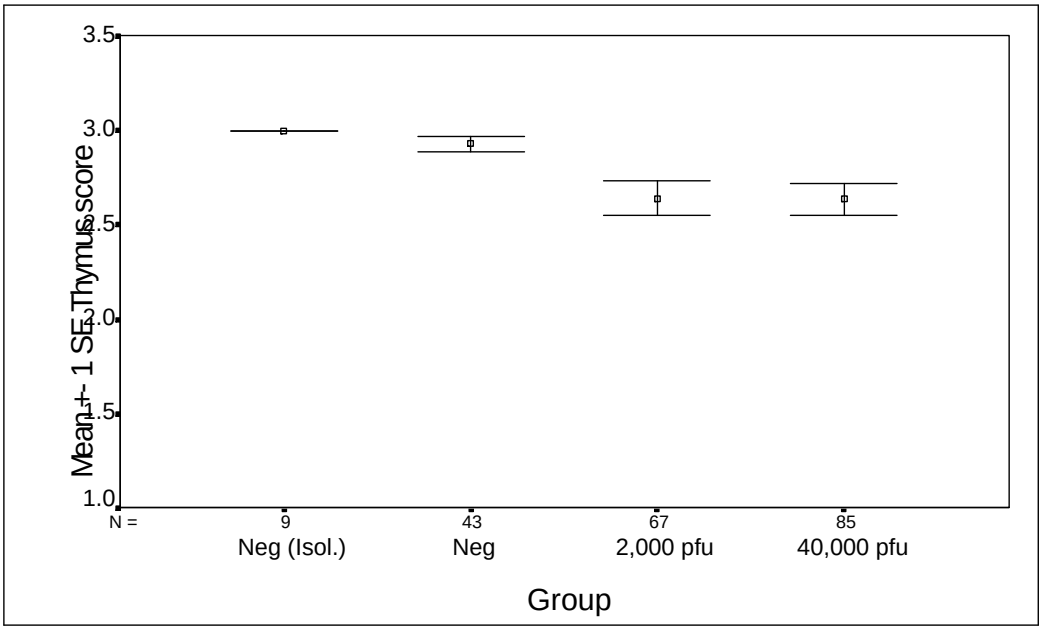
Evidence of MD lesions caused by vaccine strains of MDV or HVT has been described by several authors. The original Rispens (CVI-988) strain (Section 5), generally considered to be safe and of low pathogenicity, was shown by Pol *et al.* (1986) to cause paralysis and neuritis in 88% of the highly MD-susceptible strain of Rhode Island Red (RIR) chickens. Von Bülow (1977) also demonstrated pathogenicity of the CVI-988 strain for RIR chickens with classical symptoms of MD in 28.5% of birds when inoculated with a high dose (6,640 - 12,000 PFU). In addition, Pol *et al.* (1986) demonstrated paralysis in 2 and endoneural inflammation in 3 of 36 RIR chickens tested using the US strain of HVT, FC126 (Section 5.). Another serotype 1 vaccine, the Md11/75C/R2 strain, caused lower body and bursa weights

and resulted in up to 28% gross lesions (Witter *et al.*, 1987). Despite these findings, many of these vaccines are in common use throughout the world. The pathogenicity which is observed in highly MD-susceptible lines, such as the RIR and the CSIRO SPF chickens used in this experiment, is not evident when used in commercial breeds of chicken which are usually less MD-susceptible and may posses some protective maternal antibody against early MDV challenge.



Analysis of variance (ANOVA) results:
Sex effects were significant (p 0.000)
Group effects were not significant (p 0.067) with no significant differences ($p<0.05$) between groups by the least significant difference (LSD) test.

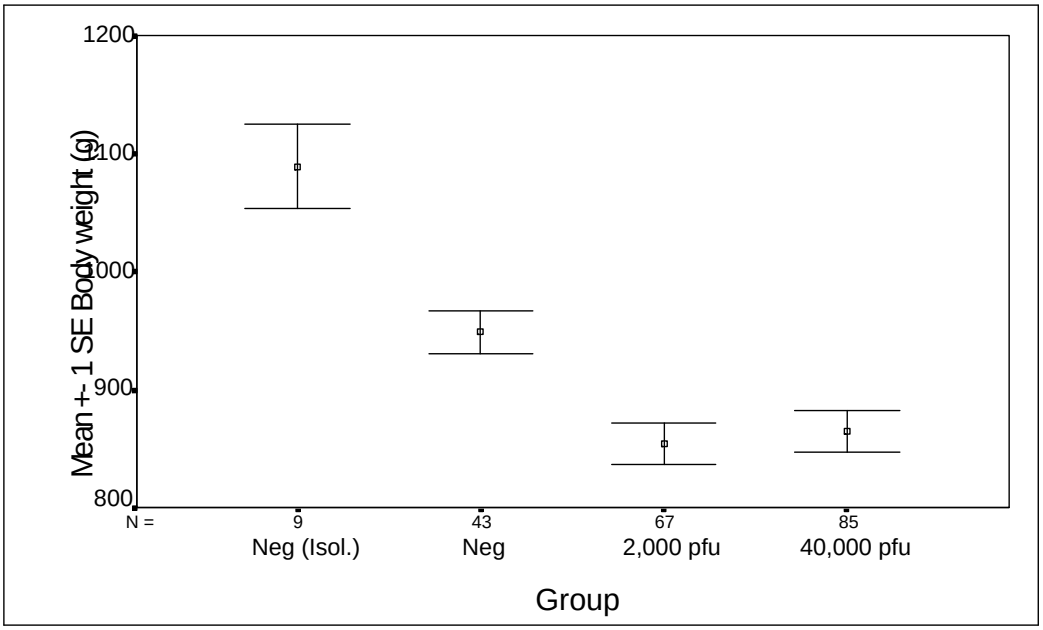
Figure 1. Bursa: body weight ratio (mean $\pm$ SE) for large scale safety test



Analysis of variance (ANOVA) results:
Sex effects were significant (p 0.017)
Group effects were significant (p 0.034)

	40,000 pfu	2,000 pfu	Neg	Neg (Isol.)
40,000 pfu				
2,000 pfu				
Neg	*	*		
Neg (Isol.)				

Figure 2. Thymus score (mean ± SE) for large scale safety test



Analysis of variance (ANOVA) results:
Sex effects were significant (p 0.000)
Group effects were significant (p 0.000)

	2,000 pfu	40,000 pfu	Neg (isol)	Neg
2,000 pfu				
40,000 pfu				
Neg (isol)	*	*		
Neg	*	*	*	

(*) Indicates significant differences ($p<0.05$) between groups by the least significant difference (LSD) test.

Figure 3. Body weight (mean $\pm$ SE) for large scale safety test

4. Determination of 50% Protective Dose

Thirty-eight day-old SPF chickens of mixed sex (CSIRO) were assigned to each of six groups. Day-old chickens from each group were inoculated subcutaneously in the back of the neck with the RMIT vaccine (clone 60/2 at passage 78) at 1000, 200, 40, 8, and 1.6 PFU/ 0.2 mL dose. The control group was inoculated with diluent alone.

Birds were housed together on the floor of rooms fitted with HEPA filters to inlet and outlet air flows at VIAS, Attwood. Nine days after vaccination, all groups were challenged with a cell culture-grown MPF 57 challenge virus intra-peritoneally at the standard dose (50 PFU/ 0.2 mL). Birds were maintained for 10 weeks after challenge and any that died or required euthanasia were examined for gross and histological MD lesions.

Ten weeks after challenge, birds were killed and examined for gross lesions and assigned a thymus score and measurements of bursa and body weights were taken. Birds were considered protected if there was no evidence of MD at autopsy. MD was confirmed for all birds that died during the experiment, except for those that died before challenge due to other causes. These birds and those that lost wing tags or could otherwise not be properly identified were not included in the PD_{50} calculation.

4.1. Results and Discussion

Table 2. MD observed at different doses of the 60/2 clone at passage 78

Dose (PFU/ bird dose)	MD	% MD	PI (%)
1000	11/32	34.4	59.3
200	15/33	45.5	46.2
40	23/35	65.7	22.3
8	27/34	79.4	6.1
1.6	25/30	83.3	1.5
Challenge only (positive control)	33/39	84.6	NA

Table 3. Calculation of the 50% Protective Dose for clone 60/2 at passage 78, by the method of Reed & Muench (1938).

Dose (PFU/ bird dose)		MD	Numbers protected		Cumulative numbers protected	
whole No.	as Log		+	-	+ (↑)	- (↓)
1000	10^{3}	11/32	21	11	63	11
200	$10^{2.3}$	15/33	18	15	42	26
40	$10^{1.6}$	23/35	12	23	24	49
8	$10^{0.90}$	27/34	7	27	12	76
1.6	$10^{0.20}$	25/30	5	25	5	101

$$\frac{42 - 26}{(42 - 26) + (49 - 24)} = \frac{16}{41} = 0.39$$

Therefore the $PD_{50} = 10^{1.6 + 0.39} = 10^{1.99} = 97.7$ PFU/ dose

The 50% Protective Dose (PD_{50}) is defined as the particular concentration of vaccine virus that induces protection in 50% of vaccinates. It is used to set an effective vaccinating dose and vaccine manufacturers will set different standards anywhere from <10 - 100 x PD_{50}. There are many test variables in the determination of the PD_{50} and these include the challenge virus strain and dose, the genetic susceptibility and sex of the chickens and environmental factors. As one might expected, a study by de Boer *et al.* (1986) demonstrated that PD_{50} determinations for a given vaccine varied depending upon the challenge virus, however the ranking for various vaccines would also change depending upon the challenge virus used. For example, with the vvMDV Tun challenge strain, the Rispens (CVI-988) clone C derivative at passage 65 (CVI-988, CEF_{65} clone C) gave a PD_{50} of 5.2 and the HVT FC126 vaccine 60.8, however with a vvMDV Md5 challenge, PD_{50}'s of 19.9 and 7.6 respectively were obtained. The study revealed the same phenomenon for other vaccines, therefore demonstrating the complex nature of PD_{50} determinations and the difficulty in obtaining meaningful comparisons between vaccines, even when variables such as the challenge strain are constant.

5. Comparison of the RMIT vaccine with commercial vaccines

This experiment was conducted to compare the efficacy of the RMIT vaccine with other commercial vaccines in large numbers of birds. Fifty-two female day-old SPF chickens (CSIRO) were assigned to each of eight groups. Day-old birds were vaccinated subcutaneously in the back of the neck with 0.2 mL of the appropriate vaccine and dose (Table 4) using MEM Maintenance medium as the diluent. Mixed vaccines were combined as a single 0.2 mL dose; the control groups were inoculated with diluent alone.

Birds were housed together on the floor of rooms fitted with HEPA filters to inlet and outlet air flows at VIAS, Attwood. Nine days after vaccination all groups, except for the contact control (negative) group, were challenged with the cell culture-grown MPF 57 challenge virus (De Laney *et al*. 1998, Morrow *et al*. 1997) intra-peritoneally at the standard dose of 50 PFU/ 0.2 mL.

Birds were maintained for 10 weeks after challenge and any that died or required euthanasia were examined for gross and histological MD lesions. Ten weeks after challenge, birds were killed and examined for gross lesions and assigned a thymus score; measurements of bursa and body weights were then taken. Five birds per group were also examined histologically. Gross and histological examination was used to confirm MD for birds that died during the experiment. Birds that lost wing tags or could otherwise not be properly identified were not included in the protection calculations.

Table 4. Vaccine doses used in the commercial vaccine comparative study

Vaccine		**Batch**	**Dose**	
Full title	**abbreviation**		**Manufacturer**	**Estimated RMIT equivalent**
RMIT (Woodlands 60/2 pass 78)	RMIT	2/6/97	4,000 PFU[a]	4,000 PFU[a]
The Marek's Company Rispens	Rispens	M7101	4,000 PFU[a]	4,000 PFU[a]
The Marek's Company HVT	TMC HVT	H7301	8,000 PFU[a]	8,000 PFU[a]
Steggles HVT	Stegg. HVT	FC9741A	1318 $TCID_{50}$	910 PFU[b]
Cyanamid Websters Maravac	MV	62200	343 FFU[c]	323 FFU[d]

a Titre determined by RMIT plaque assay method and vaccines diluted to the minimum required dose as shown.
b Equivalent titre determined by assuming 1 $TCID_{50}$ = 0.69 PFU (Luria *et al*., 1978). This relationship has been confirmed by parallel testing of both quantal and plaque assays.
c Titre determined by manufacturer. (Minimum recommended dose for Maravac is 250 FFU).
d Based on RMIT agarose overlay technique.

5.1. Results and Discussion

Table 5 shows that the highest rate of protection (97.6%) was obtained for the Rispens vaccine when used alone, which was significantly greater than the figure obtained for the RMIT vaccine when used alone (81.0%). However, protection induced by either vaccine when used in combination was not significantly different from each other or from a Maravac + TMC HVT combination. By contrast the Maravac and TMC HVT, when used in combination, provided significantly better protection than the Maravac + Steggles HVT combination. These results suggest that vaccine combinations which include the TMC HVT provide superior protection to that of the Steggles HVT vaccine.

The relatively poor performance of the Steggles HVT vaccine may have been due to its significantly lower titre compared with TMC HVT (910 compared with 8,000 PFU; Table 4). The validity of the challenge using cell culture-grown MPF 57 challenge virus is apparent from the 92% incidence of MD in the positive controls, confirming the results obtained in earlier experiments (Section 6.).

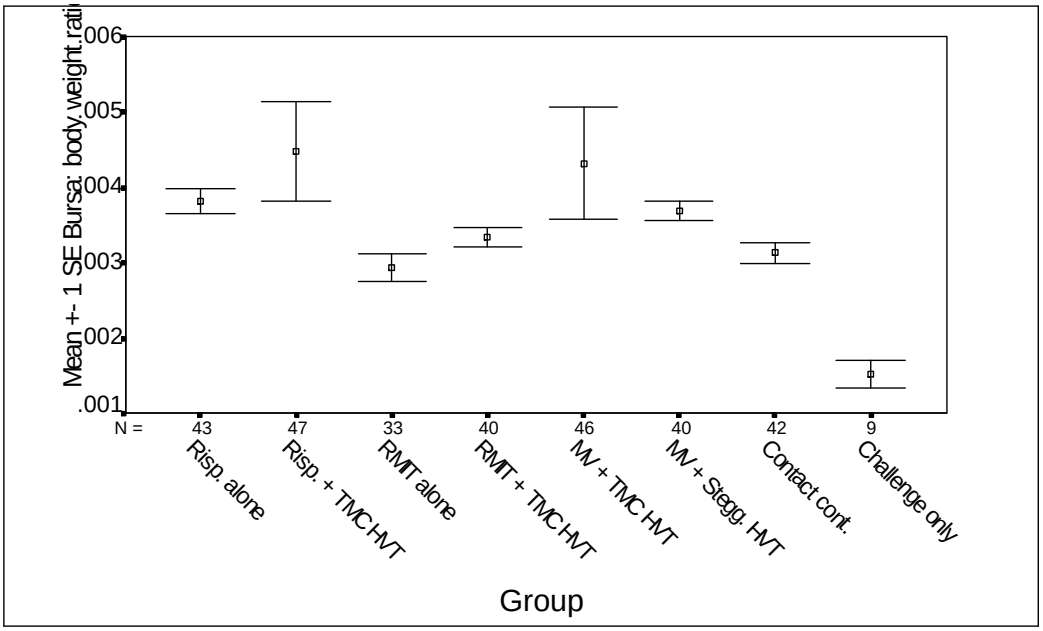
Figure 4 shows the mean bursa: body weight ratios for each group. These results also show the effectiveness of the cell culture-grown MPF 57 challenge, with the challenge only (positive control) group having the lowest bursa: body weight ratio. As expected from the experimental design, the contact control group also experienced a decrease in bursa: body weight ratio which is not significantly different from the group inoculated directly with challenge virus and demonstrates the efficiency of transmission of the challenge virus by contact. Unlike other vaccine groups, the two vaccine groups which received the RMIT vaccine (RMIT alone and RMIT + TMC HVT) were not significantly different from the directly inoculated challenge group, suggesting that the RMIT vaccine either does not protect birds from the immunodepressive effects of the MD challenge as effectively as the other vaccines, or may have contributed to the immunodepression caused by the challenge virus (see Section 3.1.).

The thymus scores in Figure 5 indicate that all vaccine groups were significantly greater from the directly inoculated challenge group. However, the score for the group that received the RMIT vaccine alone was significantly lower than for other vaccine groups and reflects the results obtained for bursa: body weight ratios.

Table 5. Protection results for large scale comparison of RMIT and commercial vaccines in chickens challenged with MPF 57.

Group	MD			Group size	MD Total	Protective Index[a]
	Deaths	Tumours	Total		%	(PI) %
Rispens alone	1	0	1	45	2.2[a]	97.6
Rispens + TMC HVT	2	0	2	49	4.1[a,b]	95.5
RMIT alone	6	1	7	40	17.5[b]	81.0
RMIT + TMC HVT	3	0	3	43	7.0[a,b]	92.4
MV + TMC HVT	3	4	7	49	14.3[a,b]	84.5
MV + Stegg HVT	8	11	19	48	39.6[c]	57.0
Contacts (negative controls)	9	17	26	51	51.0[c]	44.6
Challenge only (positive controls)	40	6	46	50	92.0[d]	

a Protective Index (PI%) = $\frac{\%\text{ MD Positive control} - \%\text{ MD observed group}}{\%\text{ MD Positive control}}$

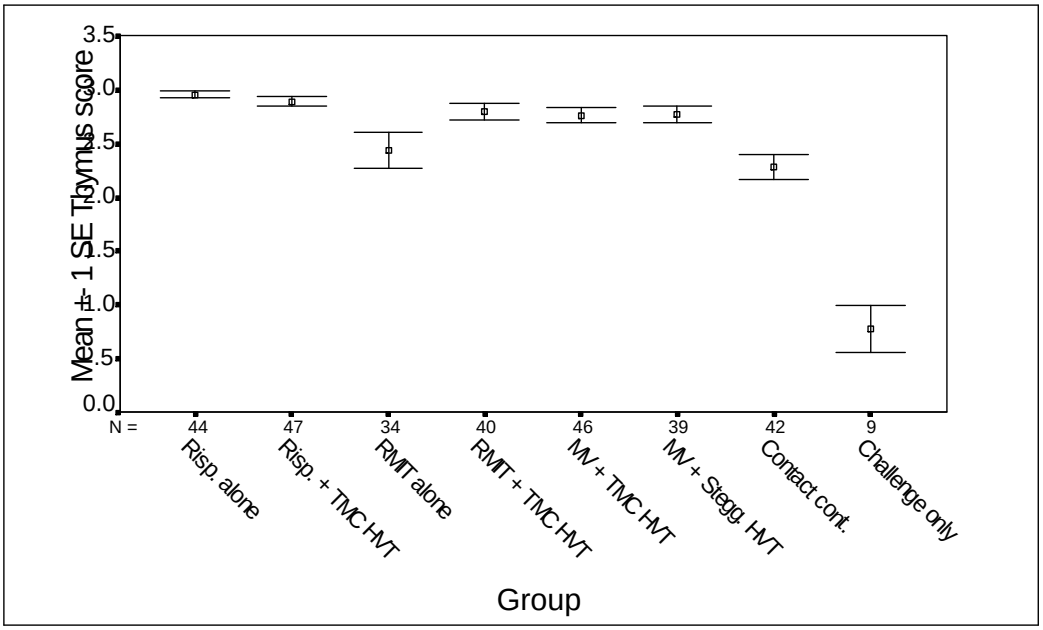


Analysis of variance (ANOVA) results:
Group effects were significant (p0.024)

	Challenge only	RMIT alone	Contact cont.	RMIT + TMC HVT	MV + Stegg. HVT	Risp. alone	MV + TMC HVT	Risp. + TMC HVT
Challenge only								
RMIT alone								
Contact cont.								
RMIT + TMC HVT								
MV + Stegg. HVT	*							
Risp. alone	*							
MV + TMC HVT	*	*	*					
Risp. + TMC HVT	*	*	*					

(*) Indicates significant differences ($p<0.05$) between groups by the least significant difference (LSD) test.

Figure 4. Bursa: body weight ratio (mean $\pm$ SE) for large scale comparison of RMIT and commercial vaccines

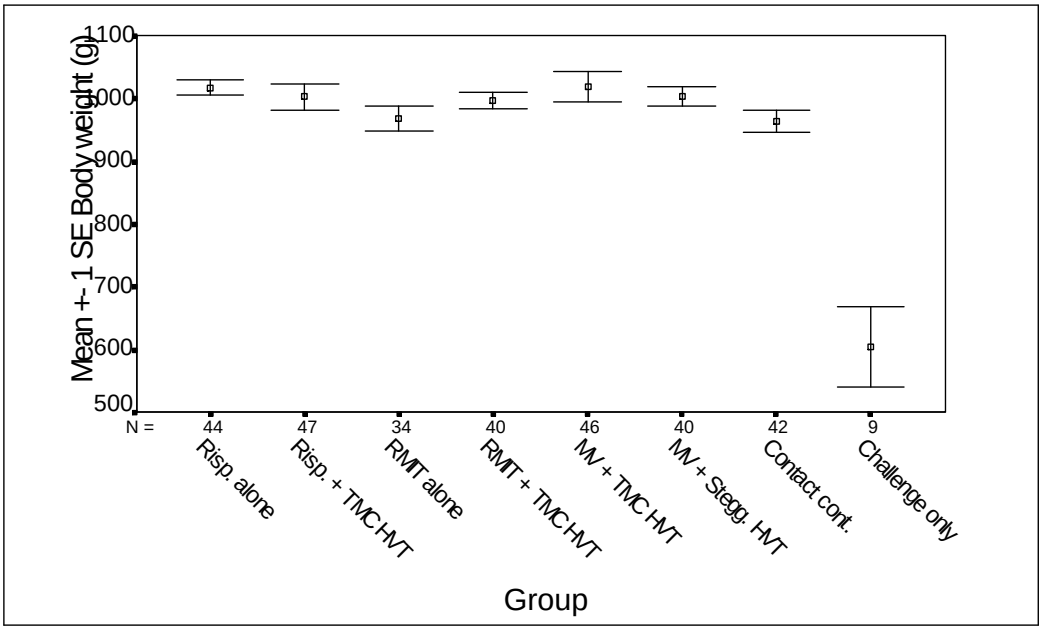


Analysis of variance (ANOVA) results:
Group effects were significant (p0.000)

	Challenge only	Contact cont.	RMIT alone	MV + TMC HVT	MV + Stegg. HVT	RMIT + TMC HVT	Risp. + TMC HVT	Risp. alone
Challenge only								
Contact cont.	*							
RMIT alone	*							
MV + TMC HVT	*	*	*					
MV + Stegg. HVT	*	*	*					
RMIT + TMC HVT	*	*	*					
Risp. + TMC HVT	*	*	*					
Risp. alone	*	*	*					

(*) Indicates significant differences ($p<0.05$) between groups by the least significant difference (LSD) test.

Figure 5. Thymus scores (mean $\pm$ SE) for large scale comparison of RMIT and commercial vaccines



Analysis of variance (ANOVA) results:
Group effects were significant (p0.000)

	Challenge only	Contact cont.	RMIT alone	RMIT + TMC HVT	Risp. + TMC HVT	MV + Stegg. HVT	Risp. alone	MV + TMC HVT
Challenge only								
Contact cont.	*							
RMIT alone	*							
RMIT + TMC HVT	*							
Risp. + TMC HVT	*							
MV + Stegg. HVT	*							
Risp. alone	*	*						
MV + TMC HVT	*	*						

(*) Indicates significant differences ($p<0.05$) between groups by the least significant difference (LSD) test.

Figure 6. Body weight (mean ± SE) for large scale comparison of RMIT and commercial vaccines

6. Conclusion

In previous experiments, it was shown that various passage levels of the 60/2 clone exhibited a dermatitis syndrome in 30 - 40% of vaccinated birds (group size 10). *Pseudomonas spp.* was identified from cultures of the lesions. In this larger safety study (Section 3.), the incidence of dermatitis was only 5% and only *Proteus spp.* could be isolated. Birds with dermatitis exhibited bursal and thymic atrophy whereas vaccinated birds without any signs of dermatitis (both high and regular doses of the RMIT vaccine) were healthy and showed no overt signs of immune organ depletion.

Figure 1 shows that bursa: body weight ratios were only moderately lower than the negative control birds and, from Figure 2, the thymus scores were approximately the same as that of the negative controls. This suggests that the few birds which acquired dermatitis may have developed immune organ depletion and were more susceptible to skin infection. However, the majority of birds did not show significant signs of immunodepression and did not develop dermatitis. No tumours were detected.

Although the Rispens vaccine appeared to perform marginally better than the RMIT vaccine (Section 5.), further studies need to be undertaken in commercial birds. Under field conditions other factors, such as the genetic characteristics of the chicken and maternal antibody status, circulating field strains and the environment, may play an important role in vaccine efficacy.

The RMIT vaccine may provide superior protection under Australian conditions as it has been derived from a recent very virulent Australian strain of MDV, unlike the Rispens strain that was derived from a strain isolated in The Netherlands over 20 years ago before the advent of field strains of increasing virulence.

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