Pathotyping of Australian isolates of Marek's disease virus

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Marek's disease has changed significantly since it was first described, with marked increases in the virulence of Marek's disease virus (MDV) evident in some countries both prior to, and following, the introduction of vaccination in 1970 . Increases in virulence have been associated with sequential vaccine failure, and a MDV pathotyping scheme based on the protective index provided by HVT and HVT/MDV2 bivalent vaccines was developed at the USDA Avian Diseases and Oncology Laboratory (ADOL) and later adapted to enable easier international comparison of isolates . The original method involves administration of a fixed challenge of MDV to a defined susceptible genotype of chicken (line 15x7 cross) with maternal antibody against MDV. Viruses are classified under this as shown in Table 1.

Table 1. USDA-ADOL classification of MDV pathotype (Witter, 1997).

Classification	Description
mMDV (mild)	Induces mainly paralysis and nerve lesions with little or no mortality in pathotyping experiments. Vaccination with HVT confers good protection. The predominant pathotype in "classical" MD. Classification based on significantly lower pathogenicity than JM/102/W.
vMDV (virulent)	Causes low levels of mortality by day 56pc, but induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT vaccination confers good protection. The reference US strain is JM/102/W and classification is based on lack of significant difference from JM/102/W in HVT-vaccinated chickens.
vvMDV (very virulent)	Causes moderate levels of mortality by day 56 post challenge (pc) and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT vaccination is only partially protective but HVT/MDV2 vaccines provide a high level of protection. The reference US strain is MD5 and classification is based on lack of significant difference from MD5 in HVT/SB1-vaccinated chickens.
vv+MDV (very virulent plus)	Causes high levels of mortality by day 56pc and induces lymphomas and nerve lesions in a high proportion of susceptible unvaccinated chickens. HVT and HVT/MDV2 are only partially protective. Classification based on significantly higher pathogenicity than MD5 in HVT/SB1-vaccinated chickens.

Whether there has been an equivalent evolution of virulence in Australian MDVs is equivocal. Some practices thought to influence evolution of virulence such as vaccination of broilers against MD and reuse of litter have been practiced to a lesser extent in Australia over the years than some of the more intensive poultry rearing regions overseas. Nevertheless, Australian field isolates of MDV have been classified as vvMDV on the basis of very poor protection provided by HVT in maternal antibody negative (mab-) SPF chickens as early as 1985 and also in the early 1990s . However the methods used in these experiments differed from the "Gold

Standard" USDA-ADOL pathotyping test. The work reported in this paper was designed to investigate the pathogenicity of Australian MDVs in SPF and commercial chickens using methods more closely aligned with the USDA-ADOL system, and to also look for early markers of pathogenicity, that may remove the need for full length challenge experiments (49-56 days post-challenge).

The results of 3 experiments are summarised and discussed. The key features of each experiment are shown in Table 2. MDV isolates were obtained from a variety of Australian sources and materials between 1992 and 2004 and grown to titres of $>10^4$ pfu/ml in chick kidney cell culture following limited passage (usually <10) (Table 3). Infective material was free of other avian pathogens including chicken infectious anaemia virus. All pathotyping experiments were carried out in positive pressure isolators with each treatment combination replicated in two isolators. Some chickens from each treatment were removed from the isolators at days 7 (Expt 2) and 13-14 (all Expts) post challenge (pc) for determination of bodyweight, relative immune organ weights and MDV load in spleen determined by real time-PCR . Chickens that died or were euthanised during the course of the experiment were examined for gross MD lesions, as were all survivors at day 56 pc when the experiments were terminated. The total incidence of MD lesions over the course of the experiment (%MD) was calculated as the percentage of birds "at risk" of exhibiting MD lesions, in which lesions are present. This was defined as the population of chickens alive at the time the first gross MD lesion was detected. The vaccinal protective index (PI) was calculated as: (%MD in Sham-vaccinated chickens – %MD in HVT-vaccinated chickens) ÷ (%MD in Sham-vaccinated chickens) x 100 . In the USDA-ADOL system isolates are ranked in virulence on the basis of Virulence Rank (100-PI) . Data were analysed using appropriate analyses in JMP 6.0 .

Table 2. Summary of the main features of experiments 1-3.

Variable	**Expt 1**	**Expt 2**	**Expt 3**
Full experiment code	MD04-R-PT2	MD05-C-PT1	MD05-C-PT3
Chicken type	SPF (SPAFAS Australia - bird Ex CSIRO HWL)	Cobb (broiler)	IsaBrown (Layer)
Sex	Mixed	Female	Female
Initial bird number	482	648	540
Parents vaccinated against MDV	No	Yes (Rispens CVI988)	Yes (Rispens CVI988)
Vaccination treatments[1] (day 0, sc)	HVT, Sham	HVT & Bivalent (HVT/MDV2), Sham	HVT & Bivalent (HVT/MDV2), sham
Vaccine dose (pfu)	8,000	8,000 (alone or combined)	8,000 (alone or combined)
Challenge MDV treatments[2] (day 5, ip)	Sham, MPF57, 02LAR, FT158, Woodlands1, 04CRE, MPF132/5	Sham, MPF57, 02LAR, FT158	Sham, MPF57, 02LAR, 04CRE
Challenge dose (pfu)	500	500	500
Final day (days post challenge)	56	56	56

[1]Vaccine strains were HVT strain FC126 and MDV2 strain SB1.

[2]Australian isolates of MDV 1992 - 2004. Grown and titrated on chicken kidney cell culture.

Table 3. Details of the MDV isolates used (sorted by date of origin).

Name of the virus	Origin	Year of origin	Bird strain	Vaccination history	Batch number
Woodlands-1	SE Qld	1992	Broiler breeder	Bivalent (serotype 2 & 3)	310804 P14
MPF57	NSW	1994	Layer	unknown	140904 P6 (Expt 1) B1[1] (179/6) 200904 P7 (Expts 2&3).
MPF132/5	NSW	2001	Broiler	unknown	050904 P5
FT158	Northern NSW	2002	Broiler breeder	Rispens CVI988	260904 P7
02LAR (179/3)	Victoria	2002	Broiler	Unvaccinated	120904 P6
04CRE (179/2)	Sydney	2004	Layer pullets 6 wo	Rispens CVI988	260904 P8

MPF 57 B1: One passage through chickens before re-isolation in CK's.

Challenge with MDV generally induced a high incidence of MD lesions in sham-vaccinated chickens (53-96%) with vaccination providing variable levels of protection (HVT: 31-100%; Bivalent: 58-82%) (Table 4). The bivalent vaccine provided similar levels of protection as HVT in Expt 2 (broilers), but superior protection in Expt 3 (layers, $P<0.05$). In SPF chickens without mab against MDV the more virulent MDV isolates also induced an early mortality/paralysis syndrome between days 11-15pc consistent with lesions reported for highly pathogenic MDV in the USA . Male chickens were significantly more susceptible to this phenomenon than females, the reverse of the situation for later mortality associated with lymphomas, for which females were significantly more susceptible. The early mortality syndrome was not observed in commercial chickens protected with mab. Using the criteria in Table 1 the viruses fall into three broad groups:

- Lower virulence (approximating vMDV). MPF132/5. This virus induced relatively low early mortality, had mild suppressive effects on thymus and bursa at day 13pc and while there was a high incidence of MD tumours in sham-vaccinated mab negative chicks (72%) HVT-vaccination provided a high level of protection against all of these effects.

- Higher virulence (approximating vvMDV). 02LAR, FT158, Woodlands1. These viruses caused early mortality syndrome with some neurological signs and had marked suppressive effects on thymus and bursa at day 13pc in mab negative chicks. However HVT provided good protection against these effects. On the other hand, protection against gross MD tumours by day 56pc was limited in both mab + and mab- chickens, in the range 35-59%.

- Intermediate/Difficult to classify. 04CRE and MPF57. 04CRE showed many hallmarks of high pathogenicity but induced a comparatively low level of MD in unvaccinated chickens. MPF57, the main reference virus used, gave markedly different results depending on the genotype of chicken used and their mab status. While it uniformly induced high levels of MD in unvaccinated chickens (69-96%) vaccination with HVT and Bivalent vaccine produced highly variable levels of protection. For example HVT provided protective indices of 100%, 85% and 35% in SPAFAS, Cobb and IsaBrown chickens respectively.

With the limited number of isolates tested it is not possible to be definitive about trends in virulence over time but there was no evidence of any increase in virulence in the isolates from 2000-2004 (MPF132/5, 02LAR, FT158, 04CRE) compared to isolates from the early 1990's (MPF57, Woodlands1).

Table 4. Summary of the effects of challenge and vaccination on the incidence of gross MD lesions in experiments 1-3. Final bird numbers (at risk of MD) per treatment combination ranged from 16-42.

Variable	Expt	Challenge MDV						
		Sham	MPF57	02LAR	FT158	Woodlands1	04CRE	MPF132/5
%MD Sham vacc.	1	0	84.2^{ab}	94.4^{a}	84.2^{ab}	81^{ab}	52.9^{b}	72^{ab}
	2	0	96.4^{a}	70.4^{b}	89.7^{ab}	-	-	-
	3	0	69.4^{ab}	86.5^{a}	-	-	61.5^{b}	-
%MD HVT vacc	1	0	0^{d}	58.3^{a}	50^{ab}	47.8^{ab}	25^{abc}	12.5^{cd}
	2	0	14.8^{a}	31.0^{a}	34.6^{a}	-	-	-
	3	0	45.2^{a}	59.5^{a}	-	-	17.5^{b}	-
%MD HVT/SB1 vacc	2	0	17.2^{a}	27.6^{a}	37.9^{a}	-	-	-
	3	0	28.6^{ab}	31^{a}	-	-	13.2^{b}	
%PI (HVT)	1		100	38.2	41.2	41.0	52.7	82.6
	2		84.6	56.0	61.4	-	-	-
	3		34.9	31.2	-	-	71.5	-
%PI (HVT/SB1)	2		82.2	60.8	57.7	-	-	-
	3		58.8	64.2	-	-	78.5	-

^{ab}Means not sharing a common letter in the superscript within rows differ significantly (P<0.05)

With respect to early prediction of pathogenicity, these studies showed that in mab- SPF chickens from a relatively resistant line (CSIRO HWL - McKimm-Breschkin et al. 1990) MDV induced marked immunosuppression by day 13pc as evidenced by reduced relative thymic and bursal weights (expressed as a % of bodyweight). There were also strong correlations between immunosuppression at day 13pc and subsequent incidence of MD in that group. This is consistent with findings in the USA . The relationship was the opposite with relative spleen weight, with a positive association with subsequent MD incidence. However these associations were not as strong in commercial chickens with mab suggesting a marked protective effect of mab (Figure 1). In contrast, in all classes of chicken, there was a strong positive association between MDV content in spleen at day 14pc and subsequent incidence of MDV suggesting that this is a useful marker for virulence (Figure 2).

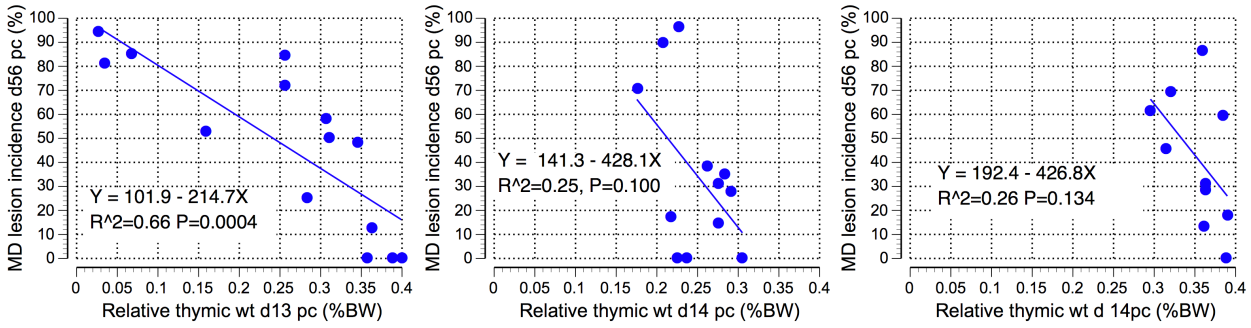

Figure 1. Association between relative thymic weight at day 13-14 pc and incidence of MDV to day 56 pc, in experiments 1-3 (left to right). Each point represents a treatment combination.

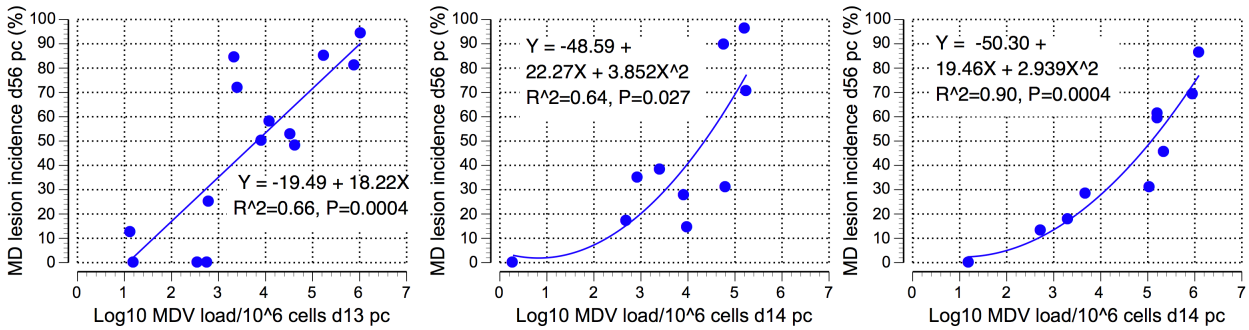

Figure 2. Association between MDV load in spleen cells (viral copy number/10^6 cells) at d13-14 pc and incidence of MDV to day 56 pc, in experiments 1-3 (left to right). Each point represents a treatment combination.

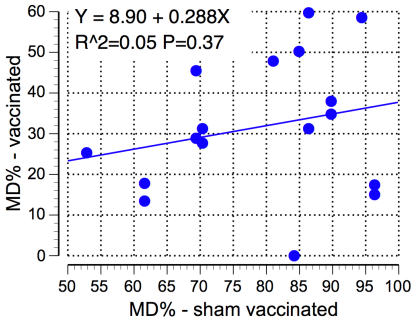

Figure 3. Association between the incidence of MD lesions (MD%) in sham-vaccinated chickens or chickens vaccinated with HVT or HVT/SB1 when challenged with the same MDV isolate.

Amongst the key findings to emerge from this work are:

a) A moderate range of MDV pathotypes is present in Australia but there is little evidence of vv+MDV. The vvMDV type isolates present are capable of causing significant early mortality and paralysis in the absence of gross lesions at days 11-15 post-challenge in mab- chickens. With limited numbers of MDV isolates examined there was no marked trend for increased virulence over the last decade.

b) Effective early markers (days 7-14pc) for subsequent MD incidence include thymic, bursal and splenic relative weights or a combination of these in mab- chickens. In both mab- chickens and mab+ chickens MDV load in spleen is a good marker.

c) The results raise significant issues about current approaches to pathotyping MDV.

- For some viruses (eg MPF57) the pathotype depended markedly on the chicken strain and maternal antibody status. This suggests that virulence is not solely a property of the virus, but rather the result of virus-host interaction as has been previously demonstrated for MDV . Given this, it would appear sensible to “pathotype” MDVs in current strains of commercial chickens to obtain relevant rankings.
- Overall there was a poor relationship between the incidence of MD induced in unvaccinated chickens (virulence?), and that induced in vaccinated chickens with the same virus (vaccine resistance?) (Figure 3). This suggests that the two may be separate traits. Current pathotyping methods are based on vaccinal protection and are therefore measures of vaccine resistance rather than true virulence.

d) Isolation and growth of field isolates of MDV to sufficient titre to use in the pathotyping experiments proved very difficult and a very small proportion of field submissions yielded

such an isolate. Of material that was positive for MDV on PCR, 23% produced cytopathic effects in culture but only 2.4% grew to sufficient titre for inclusion in pathotyping experiments of this type. Clearly alternative or complementary approaches to the growth and titration of infectivity of field MDVs are required.

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