Mareks disease studies in Australia

A report for the Rural Industries Research and Development Corporation

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Foreword

Marek's disease (MD) continues to cause major economic losses in broilers, broiler breeders and layer birds in all poultry producing countries of the world. In Australia, control of MD has been achieved with locally produced vaccines, namely HVT (herpes virus of turkeys; serotype 3) and Maravac (serotype 2). Unlike other countries, Australia had not used a serotype 1 vaccine until 1995.

World wide it has been observed that prolonged use of a particular vaccine or a particular vaccine combination eventually results in the emergence of virus strains which are not well controlled by vaccination. These strains are referred to as very virulent MDV (vvMDV).

The emergence of vvMDVs in Australia has coincided with the introduction and widespread used of imported breeds of birds. These imported strains seem to be less responsive to vaccination than Australian developed breeds.

The Australian poultry industry became extremely concerned about the apparent failure of Australian vaccines in the early 1990s and designed a project to develop an Australian serotype 1 vaccine from a local virulent strain of Marek's disease virus (MDV), at a time when it seemed unlikely a serotype 1 vaccine would be imported into the country.

The Woodlands No. 1 strain was a vaccine candidate for its pathogenic qualities observed in the field. A successful vaccine was developed and showed good levels of protection compatible with other commercially available vaccines that have since entered the Australian chicken industry.

Since the commencement of this project, the industry has successfully lobbied government to allow the importation of overseas type 1 vaccines. This report also describes the laboratory trials which were undertaken to assess the efficacy of one of these imported vaccines, the efficacy of currently used Australian vaccines and that of the Woodlands No. 1 developmental Australian vaccine.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report, an addition to RIRDC's diverse range of over 1000 research publications, forms part of our Chicken Meat and Egg R & D programs, which aim to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images, and to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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All other work reported was conducted by RMIT University and was wholly or partially funded by the RIRDC. Some of the studies reported are taken from theses submitted by T. L. Cipriani, D. B. De Laney and D. Jaikumar in part-fulfilment of requirements towards the degrees B. App. Sc. (Hons), PhD and M. App. Sc.

Abbreviations

ANOVA	analysis of variance
ARI	Animal Research Institute
ca	cell associated
CAV	chicken anaemia virus
Cefs	chicken embryo fibroblast cells
Ceks	chicken embryo kidney cells
CFM	cell freezing medium
LSD	least significant difference
MD	Marek's disease
MDV	Marek's disease virus
MPF	Marek's problem flock
NATA	National association of testing authorities
PFU	plaque forming unit
PI	protective index
QDPI	Queensland Department of Primary Industries
RT	room temperature
sc	subcutaneous
SE	standard error of the mean
SOPs	standard operating procedures
SPF	specific-pathogen-free
$TCID_{50}$	50% tissue culture infective dose
TMC	The Marek's company
VIAS	Victorian Institute of Animal Science

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Executive Summary

During the early 1990s, Australian poultry producers began to experience increasing difficulties in controlling Marek's disease. In light of this, the RIRDC decided to fund a series of contract trials to evaluate, under controlled conditions, the efficacy of local, commercially available vaccines against a more recent field strain of Marek's disease virus (MDV). As there were no serotype 1 vaccines available in Australia at the time, the RIRDC also supported the development of one at RMIT.

At the outset of this project, the likelihood of importing the serotype 1 Rispens (CR6) vaccine seemed remote due to quarantine restrictions. However, soon after approval was given for the importation of the seed of the CR6 vaccine and therefore comparative trials were conducted on both vaccines.

This report describes the successful development of a live attenuated serotype 1 MDV vaccine from a highly virulent Australian strain, the Woodlands No. 1 strain, its evaluation in the vaccine trials, and numerous laboratory studies on Australian strains of MDV.

Development of a serotype 1 vaccine

Attenuation of the Woodlands No. 1 strain was achieved by serial cell culture passage, firstly in chicken embryo kidney (CEK) and then in chicken embryo fibroblast (CEF) which are more convenient for use in vaccine manufacture. Clones of the virus were prepared and tested at passages 32, 40, 60, 80 and 99. Attenuation of the virus was observed at passage 60 in a chicken pathogenicity test, which was confirmed in later pathogenicity experiments on Clone No. 2 from the same passage (Clone 60/2).

In a protection study using a virulent challenge virus, clones prepared from passages 80 and 99 were tested in parallel with a clone at passage 40 and Clone 60/2. Clone 60/2 provided the best protection and immunogenicity in the higher passage clones was greatly reduced. The 40^{th} passage clone retained some virulence and offered no protection against virulent challenge. Tests on other clones at the 60^{th} passage (clones 60/5 and 60/7) indicated lower protection rates than were achieved using the 60/2 clone. Further testing of the 60/2 clone in both commercial (Cobb and Isa Brown lines) and SPF birds revealed good levels of protection when compared with Australian commercial vaccines that included the original Rispens European strain CVI-988 and the CR6 derivative. The Steggles MD-1 experimental vaccine performed poorly in SPF birds (PI=23), although, it did not appear to have the same level of residual virulence as the RMIT clone 60/2 vaccine. The Marek's Company (TMC) Rispens (original/European strain) provided significantly better protection than the same vaccine produced by Solvay, when used at the manufacturer's recommended dose in both lines of commercial birds. When the Rispens vaccines were combined with either HVT and/or Maravac vaccines, there were fewer differences between the two manufacturers.

Further passage of the Clone 60/2 in cell culture was carried out to determine if additional attenuation could be achieved without the loss of immunogenicity. Results indicated that passage 78 of the 60/2 clone should be used for further evaluation in large-scale safety and protection tests. The studies confirmed that Clone 60/2 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 (some bursal and thymic atrophy present, but differences were not statistically significant). Mild immunosuppression and MD lesions is a deficiency of serotype 1 MD vaccines.

In addition to the development of a MD vaccine, two cell culture-prepared MDV challenge viruses were produced from the Woodlands No. 1 and MPF 57 strains. The development of these standardised challenge viruses was essential for the evaluation of MD vaccines. Previously, lymphocyte preparations of these viruses were used which were difficult to standardise in terms of the effective dose received by each. Passage of the highly virulent MPF 57 challenge strain in cell culture

effectively removed a chicken anaemia virus contaminant that has been present in the original lymphocyte isolate.

Australian serotype 1 vaccine experimental challenge studies

The 50% protective dose of clone 60/2 was calculated to be 97.7 PFU/dose, although, there was 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 in SPF birds revealed high levels of protection, although the Rispens vaccine showed superior performance. Vaccine combinations which include the TMC HVT provided superior protection to that of the Steggles HVT vaccine.

In commercial birds, presumably because of the presence of maternal antibodies to other vaccines, Clone 60/2 (hereafter referred to as the RMIT vaccine) provided poor protection. When used in combination with TMC HVT, it was relatively efficacious. However, again the Rispens vaccine was superior when it, too, was used in combination with TMC HVT(Protective Index (PI) = 97), but still only marginally better than the RMIT combination. The growth characteristics of the Rispens CVI-988 and RMIT 60/2 vaccine strain in CEFs were similar.

The introduction of the Rispens/CR6 vaccine to the Australian chicken industry has lessened the need for an Australian serotype 1 MD vaccine. Nevertheless, the RMIT vaccine may be useful if further change occurs in the evolution of MDVs. Since the RMIT vaccine virus is derived from a recent Australian isolate, it may share more antigens with recent strains. The Rispens vaccine was derived from a mildly virulent serotype 1 MDV isolated in the 1970s in The Netherlands and, as such, may not possess antigens common to recent Australian strains.

Experimental challenge studies of commercially available vaccines in Australia

Existing Australian serotype 2 and 3 vaccines in commercial birds induced PIs too low to be protective in the field due partly to the presence of maternal antibody. However, HVT cell-associated vaccine was superior to the cell-free vaccine.

The trivalent vaccine, HVT + Maravac + CR6 gave the best PI in a trial on commercial birds of known maternal antibody status. The newly imported CR6, alone, produced very poor protection, with a PI in chickens with HVT maternal antibody not statistically different from that of chickens with CR6 maternal antibody. Indications from this trial suggest that the influence of maternal antibody was not due to the vaccine used in the parental flock.

Laboratory Studies

Development of a MDV serotype 1 specific DIG-labelled probe for detection of MDV by dot-blot hybridization

Rapid tests for the detection of MDV are required to ensure that chicken flocks are protected by vaccination and to assist in containment measures to prevent the spread of infection. Currently, MDV is detected in peripheral blood or tissue samples by 1) virus isolation in cell culture, which is time consuming and relatively insensitive, 2) serology, which is nonspecific for individual serotypes or 3) the polymerase chain reaction (PCR), which is relatively expensive.

To overcome these problems, a probe labelled with digoxigenin (DIG) was developed for the detection of MDV 1 by dot-blot hybridization. Dot-blot hybridization is a detection technique based on the hybridization of a labelled nucleic acid probe homologous to a target region, which has been fixed to a membrane. Detection is then based on the use of autoradiography for radiolabelled probes or for nonisotypic labelling systems, the addition of a substrate to produce a fluorescent or colour signal. The test is easy to perform and rapid, providing a result within 24 hr compared with up to 14 days by virus isolation. It is also inexpensive and allows large numbers of samples to be screened on a single blot. The test also allows the detection of virus in samples that were collected during the latent stage of infection.

The probe was labelled by the incorporation of DIG in a PCR reaction product that consisted of the amplified 132 bp repeat located within the inverted repeat long region of the MDV 1 genome. The sensitivity and specificity of virus isolation and dot-blot hybridization were compared with PCR, which was used as the reference procedure. Highest sensitivity rates were achieved by dot-blot hybridization using the 132 bp PCR probe, compared with virus isolation and identification by immunoperoxidase or immunofluorescence. Despite their much lower sensitivity, higher specificity was obtained by both culture detection methods than for the dot-blot hybridization. These results indicate that non-specific binding of the probe to areas other than the target region which may give rise to false-positive results. Another possibility is that false-negative results obtained by PCR affected the specificity of results obtained by dot-blot hybridization. Although PCR is currently recognized as the most sensitive technique available for the detection of MDV, several factors were found to affect its performance as a sensitive test. These included the presence of inhibitory factors in specimens, occasional difficulties in the detection of positive samples under the UV transilluminator and contamination of reagents. These findings cast doubt on the value of PCR for use as a reference procedure.

Collection and characterisation of MDV field isolates

In order to achieve a representative collection of MDV strains, approximately 300 blood samples were collected from flocks in different parts of the country that had been experiencing losses due to MD. These blood samples were processed so they could be screened for the presence of the serotype 1 MDV. MDV 1 samples were stored in liquid nitrogen for future reference. It was proposed that polymorphisms (differences in their DNA sequence) between these serotype 1 strains could be studied by molecular and biological procedures. Characterisation of the virus in this way would allow trends in the evolution of MDV to be studied.

Characterisation of MDV after adaptation to Vero continuous cell-line

Production of MD vaccines has been limited to primary chicken and duck embryo fibroblast (CEF and DEF) cultures. These have a limited life span and cannot be readily stored in liquid nitrogen. Moreover, the need to prepare CEF and DEF cells on a regular basis from 10-11 day-old embryos derived from a flock that must be tested continuously for the presence of avian pathogens adds to the cost of vaccine production. A continuous cell line that would support MDV replication could have significant advantages for the rapid large-scale preparation of MD vaccines. For this reason, serotype 1 MDV and HVT have been adapted to the Vero continuous cell line (Jaikumar *et al*., 2001). Both adapted viruses were characterised by immunological and molecular techniques.

HVT grew more rapidly and produced more extensive CPE and higher virus yields than the serotype 1 virus. The growth pattern of HVT was consistent with the amount and distribution of serotype-specific antigen in infected cells as detected by immunofluorescence. The Vero cell line appeared to be more susceptible to HVT than to serotype 1 viruses.

When the genome of adapted serotype 1 viruses was examined, an expansion of the 132 bp DR sequence indicated that the infected cell line contained serotype 1 MDV DNA. The presence of intact DNA with a size of approximately 180 kb in both serotype 1- and HVT-infected Vero cells after isolation and characterisation indicated that whole copies of both types of DNA were present and provided further evidence for adaptation to growth of the serotype 1 virus. This is the first report of the growth of either virus in a continuous line.

Titres of both viruses were lower than could be obtained in CEF cell culture.

Efficacy of γ-inulin as an adjuvant for live caHVT vaccination

Commercial broiler chickens were used to assess the possible role of γ-inulin as an adjuvant to improve the efficiency of HVT vaccination against MD. Chickens were administered a cell-associated HVT (caHVT) vaccine with or without γ-inulin using three vaccination procedures: i) Inovoject®, an automated egg injection system that delivers the vaccine into the embryo, ii) by hand *in ovo* or iii) subcutaneously (sc) at day old. All birds were challenged with a virulent MDV1 challenge virus at 3 days of age. Effective vaccination by HVT was assessed according to the development of viraemia at day 17. The route of vaccination had a significant effect on HVT viraemia ($P < 0.0005$).

These studies indicated that sex ($P<0.001$) and vaccine dose ($P<0.005$), but not route of vaccination ($P=0.34$) and γ-inulin, had significant effects on the live weights of chickens. MD was present in all treatment groups but its incidence in groups treated with γ-inulin was not significantly different from non-treated groups ($p<0.05$). Differences in the percentages MD in groups administered γ-inulin using the Inovoject® method or sc at day 1 were also non-significant ($p<0.05$). No adverse effects due to γ-inulin were noted in any group. The incidence of MD was significantly affected by the vaccine dose ($P<0.001$), the sex of the chickens ($P<0.001$) and the method of vaccine administration ($P<0.005$). In the absence of γ-inulin, the Inovoject® route of vaccination provided protection which did not differ significantly whether administered on the 18.8^{th} or 17.8^{th} day of incubation. Protection also did not differ than that provided by hand *inovo* vaccination.

Marek's disease vaccine assay facility

The need for a vaccine assay facility

Different vaccine viruses of a particular serotype are likely to provide different levels of protection when vaccinated with the same dose which means that the particular immunising dose set by manufacturers for different viruses is likely to vary. With an ever-increasing number of vaccines available to the poultry industry, it was imperative that an independent and reliable assay facility be available to evaluate their effectiveness in relation to one another. In response to this, RMIT University set up such a facility in consultation with vaccine manufacturers and industry representatives. The facility has been largely used by only one manufacturer (90%), despite its availability to all Australian manufacturers.

A reference virus preparation was run in parallel with assays on each vaccine batch in order to monitor the relative sensitivity of each assay. A nominal cell count and virus titre, with maximum and minimum limits, were set for two reference preparations during the establishment phase of the assay facility. The assay result was required to fall within these limits (mean ± 2 standard deviations for the cell count and mean ± 1 standard deviation for virus titre) or the assay was considered invalid and had to be repeated. A moving average was calculated for the reference titre, which was a better indicator of assay trends rather than individual data. The nominal cell count and virus titre were revised for each reference virus throughout the period of the project. Revised values indicated the stability of each reference preparation for both MDV 1 and HVT.

The Virology Laboratory at RMIT University was granted accreditation in June 2003 under sections 8.02 Tests on Veterinary Pharmaceutical and Biological Products, and 0.20 Potency of Immunological Products with the National Association of Testing Authorities (NATA), as a reference vaccine assay facility. Standard operating procedures (SOPs) and methods were completed and incorporated into the existing quality manual of the laboratory.

Optimisation of methods for standardising MDV vaccines

The use of chilled diluent in administering vaccines was shown to decrease vaccine titre by about 25%. Fluctuations in titre have been observed recently in the industry and, hence, were further investigated. Assays were set up in the laboratory to simulate this practice. Reconstituting vaccine with diluent held at 4°C resulted in a highly significant loss of 37% in vaccine titre (Student's *t*-test; $P < 0.05$) opposed to reconstituting in diluent held at RT. A loss in titre was also evident when the vaccine and diluent mixture was held for two hours. Therefore, it is critical to maintain diluent at RT and to administer MDV vaccines promptly to maintain their potency.

The critical role of the operator was demonstrated during the course of the project when there were changes of operator performing the vaccine assays. Changes in virus titre were observed due to the introduction of small variations in the standard method. These discrepancies were corrected and the subsequent differences between vaccine titre of parallel assays were minimal and statistically non-significant.

MDV tests and assays available at RMIT University

RMIT University now offers a national independent vaccine assay facility for MD vaccines, several diagnostic tests for isolation and detection of serotypes 1, 2 and 3 MDVs and the maintenance of a serotype 1 challenge virus for use in vaccine efficacy studies. These services are available to all vaccine manufacturers, researchers and members of the chicken industry.

1. Introduction

Marek's Disease (MD) was first described in 1907 as a peripheral polyneuritis affecting chickens (Marek, 1907). This original description was termed Marek's disease (Biggs, 1961) to differentiate it from other, often similar, lymphoproliferative diseases. MD is characterised by a mononuclear infiltration of one or more of the peripheral nerves, gonad, iris, various viscera, muscle or skin (Calnek & Witter, 1997) and is caused by a cell-associated herpesvirus, Marek's disease virus (MDV). In its classical form, the most common clinical sign is partial or complete paralysis of the legs and wings. Nerve function is affected giving rise to lameness and drooping of wings. Enlarged feather follicles or occasionally tumours may be felt on the feathered parts of the body (Purchase, 1985). In the acute form, birds are often clearly depressed and some may die without showing earlier signs (Payne, 1995).

MDV serotypes are classified into three groups, with the prototype virus designated as serotype 1. Two nononcogenic herpesviruses also exist and share antigenic determinants with MDV as detected by immunological assays (von Bulow & Biggs, 1975a, b). The nonpathogenic chicken isolates are designated as serotype 2 and those isolated from turkeys (HVTs), are designated as serotype 3 viruses. Serotype 2 and 3 viruses, along with attenuated variants of serotype 1, are used as vaccines to protect chickens against tumour induction following exposure to virulent MD (Witter, 1998).

MD occurs in poultry producing countries worldwide and prior to the use of vaccines, it constituted a serious economic threat to the industry. Mortality rates in flocks of 10-15% were common and losses of up to 80% have been recorded (Payne *et al*., 1976).

As reporting systems vary between countries it is difficult to determine the true incidence of MD. However, chickens that are raised in areas where poultry is prevalent have experienced some loss. Losses are especially high in areas where broiler raising is intensive. These areas continue to have the greatest risk even with vaccination (Calnek & Witter, 1997).

Since the introduction of vaccines, the incidence of MD has been greatly reduced; the initial protection rate was estimated to be at about 97% under field conditions (Witter, 1991). Despite the reduction in such losses, a high level of concern persists as the virus is now breaking through many formerly successful vaccine programs (vaccine breaks); the virus, it seems, is increasing in virulence and as such reducing the efficacy of existing vaccines.

1.1 Vaccination against Marek's disease

1.1.1 Attenuated serotype 1 MDV vaccines

Attenuated virulent serotype 1 vaccines

The first effective vaccine against MD was developed by Churchill *et al.,* (1969a,b) following the discovery that the oncogenic HPRS-16 MDV isolate could be attenuated by passage in cell culture and that the attenuated virus protected chickens against MD. The attenuated HPRS-16 vaccine was used extensively in Europe following its development and was considered effective, but has now been superseded by other vaccines. Reversion to virulence and spread have not been reported.

Attenuated mildly virulent serotype 1 vaccines

Rispens *et al*., (1972 a,b) described a vaccine derived from strain CVI-988, a mildly virulent serotype 1 MDV isolate which was further attenuated by 20 cell culture passages. This vaccine has been used extensively in The Netherlands where it was developed and, more recently in the USA and Australia for protection against MDV strains of very high virulence. The vaccine has been shown to be mildly

virulent for highly susceptible lines of chickens and maintains its ability to spread by contact (Rispens *et al*., 1972a; von Bulow, 1977).

Attenuated serotype 1 vaccines derived from highly virulent strains

Witter (1982) described an attenuated very virulent strain, Md11/75C, which was considered effective against challenge with very virulent MDVs. However, it was readily neutralized *in vivo* and was relatively ineffective in chickens with maternal antibodies (Witter & Lee, 1984). The vaccine virus was also shown to be partially virulent after backpassage in chickens.

1.1.2 Serotype 2 MDV vaccines

The protective efficacy of serotype 2 viruses was first observed by Zander *et al*., (1972) in vaccination experiments which were later found to contain the serotype 2 strain HN-1. The SB-1 strain (Schat & Calnek, 1978) is used extensively in the USA and has been shown to be non-oncogenic since no proliferative lesions have been observed. Jackson *et al*., (1977) used a naturally non-oncogenic MDV-19 strain as a vaccine. This Australian isolate is marketed as Maravac® (Fort Dodge Australia Pty Ltd.) and was used extensively throughout Australia until recently.

1.1.3 Serotype 3 MDV vaccines

The Herpesvirus of turkeys (HVT; Serotype 3 viruses) are non pathogenic for turkeys and chickens, and were first identified in turkey kidney cell cultures by Kawamura and co-workers in 1969. Since their discovery and commercial propagation, serotype 3 viruses, and in particular, the FC126 strain (Witter *et al*., 1970) have been widely used throughout the world, including Australia, as a cell-associated form of vaccine against MD. A cell-free form of vaccine also exists but is less protective than its cell-associated counterpart (Witter, 2001).

1.1.4 Polyvalent vaccines

Better protection from MD was found when combinations of serotypes were used together in a vaccine than either serotype alone. Termed 'Protective synergism', it is unique to MD and is strongly serotype specific. It was demonstrated between the SB-1 strain (serotype 2) and FC126 strain (HVT) (Schat *et al* 1982; Witter, 1982), which stimulated the development of the first commercial vaccine based on this phenomena between serotype 2 and 3 viruses (Schat *et al*., 1982, Witter, 1982). Synergism is especially evident between serotypes 2 and 3 but seems to be less pronounced with other combinations.

With the recent introduction of serotype 1 vaccines, trivalent combinations of all three serotypes have been used. Trivalent vaccines FC126 + 301/B + CVI988/C (serotypes 1, 2 and 3 respectively) and FC126 +SB-1 + CVI988/C were introduced in 1990 and are currently available in the USA, recommended only for high risk flocks. Trivalent vaccines are not available in Australia but one combination was trialed within this report.

1.1.5 Recombinant vaccines

There have been numerous attempts to develop recombinant vaccines against MD using HVT (Ross *et al*., 1993), fowlpox (Heine *et al*., 1997; Nazerian *et al*., 1996) and Newcastle disease (Morgan *et al*., 1992,; Sonoda *et al*., 1996; Reddy *et al*., 1996) viruses as vectors but none have proved as efficacious as the standard HVT vaccine. An experimental recombinant fowlpox vaccine expressing glycoprotein

genes from the Woodlands virus was developed by Cyanamid Websters Ptd. Ltd. and was evaluated with an HVT vaccine in this report.

1.2 Marek's disease in Australia

In the early 1970's, the success of vaccination against MD became apparent in other countries but Australian quarantine restrictions prevented the importation of any such vaccine. This necessitated the development of a local vaccine. In 1974, field trials began using an isolate of herpesvirus of turkeys (HVT) designated HVT NSW 1/70 (Jackson *et al*., 1974). The vaccine was shown to be safe and gave an overall protection of 85.2%, which compared favourably with results reported in other countries. Seed material of NSW 1/70 was initially released by Arthur Webster Pty. Ltd. and was also distributed between various companies and the University of Sydney, resulting in the manufacture of Australia's first MD vaccine. Vaccination with this strain was very effective and controlled MD in Australia until about 1980.

Despite the availability of serotype 1 and 2 vaccines in other countries, HVT was the most commonly used vaccine throughout the world until the late 1970's when MD outbreaks of increasing severity of lesions were reported in vaccinated chickens (Eidson *et al*., 1978; Witter, 1983). Viruses isolated at this time, such as Md5 (Witter, 1983) and RB1B (Schat *et al*., 1982), were classified as very virulent (vvMDV). This prompted the use of serotype 2 & 3 bivalent vaccines (Witter, 1982; Calnek *et al*., 1983).

MD continued to be ubiquitous in Australia, resulting in breaks in vaccination programs during the 1980's. Vaccination breaks were reported in Victoria with losses between 2-25% of pullets and breeder chickens (Nicholls, 1984; Reece *et al*., 1986). In Eastern Australia, excessive MD losses occurred, despite good management practices (McKimm-Breschkin *et al*., 1990). Reports of up to 50% mortality in Australian-bred parent meat birds (Jackson, 1996) established that vv MDV strains had emerged in Australia during this time. Consequently, an Australian cell-associated serotype 2 vaccine Maravac (Jackson *et al.,* 1977) which was licensed for sale in 1977, was used alternatively with HVT or in combination.

In the early 1990's, despite the use of Maravac, polyvalent vaccines and increased doses of HVT, an increase in the prevalence of MD was once again observed. Clearly, there were more serious and continuous outbreaks. In Eastern Australia, flocks vaccinated with HVT only, were succumbing to MD with 25% mortality (De Laney *et al*., 1995) and chickens vaccinated with both cell-associated serotype 2 and HVT vaccines displayed similar mortalities (Zerbes *et al*., 1994). During this time, imported breeds of chickens became available in Australia. The influx of imported pullets coincided with increases in MD mortality to over 50%, which was never seen in Australia (Jackson, 1996). Field and breed comparison trials demonstrated that imported strains were responding poorly to Australian-derived MD vaccines. The incidence of MD in imported breeds appeared to be higher than in existing strains of birds.

In view of this situation, and the emergence of strains of increased virulence, changes in vaccination strategies included the vaccination of broiler chickens (previously not routine practice in Australia; Groves, 1995).

A meeting was convened by Websters and the AVPA in 1993 where the industry reported to the government and vaccine manufacturers the full extent of MD in Australia. Websters proposed that a serotype 1 vaccine be imported from overseas. This option was unpopular with the majority of the poultry industry and instead, the industry decided to increase its funding of MD research, particularly at providing evidence that a serotype 1 vaccine would give better protection than existing Australian vaccines (Jackson, 1995).

1.3 Why vaccine breaks occur

The occurrence of excessive MD losses in a vaccinated flock are termed vaccine breaks. Vaccine breaks are often blamed on highly virulent pathotypes, although inefficient control practices and vaccination management can also result in excess losses.

1.3.1 Challenge strain

Virulence or oncogenicity is usually associated with serotype 1 MDVs. Large variation in the pathogenic potential of different strains within the serotype has led to their classification into various pathotypes mild (m), virulent (v), very virulent (vv) or very virulent plus (vv+). Field strains are classified into one of the four pathotypes according to their relative pathogenicity for chickens receiving different vaccines (Witter 1997). This classification is somewhat arbitrary and has also been based on factors such as the clinical signs observed (i.e. acute, classical or neural signs) and in comparison with that produced by well-characterised strains.

The emergence of these pathotypes, first noted in 1907, is thought to represent a continuous evolution of MDV towards greater virulence. Up to about 1950, MD was a classic disease induced by viruses of the m MDV pathotype. A more virulent form of MD associated with viruses of the vMDV pathotype then became dominant during the 1960s. A major pathotype shift from vMDV to vv MDV occurred in the late 1970's. This was probably a response to the first use of HVT vaccines and led to the use of combined vaccines (serotypes 2 and 3).

In the early 1990's, the incidence of MD suddenly increased in all countries although the vaccines were all the same as those introduced in the 1970s. This was the emergence of even more virulent forms of MDV(vv+). These strains could not be controlled by the use of combined vaccines and led to the development and use of newer and better serotype 1 vaccines.

The emergence of increasingly virulent viral strains, coupled with an apparent reduction of vaccine efficacy during the past 30 years, has provoked justifiable concern. This suggests that vaccination, itself, does not provide complete control and is not the ultimate solution for MD (Calnek & Witter, 1997). Strict biosecurity and management measures to reduce early exposure and the presence of genetic resistance are essential components of a successful vaccination program.

1.3.2 Ineffective vaccination and early exposure to virulent field strains of MDV

One of the most common causes of MD breaks is ineffective vaccination. Vaccines that are not stored and diluted according to the manufacturers' instructions may be lower in titre and may be unable to induce a protective immune response.

Protective immunity is not usually acquired until 5-8 days after administration of MD vaccines (Okazaki and Burmester, 1971). Minimal biosecurity, intensive stocking practices, use of multi-aged sites and poor hygiene, all contribute to early infection in immunologically immature chickens. The failure to prevent early exposure is perhaps the most important single cause of vaccine failures (Calnek & Witter, 1997).

1.3.3 Maternal antibody

Maternal antibodies can be present in chickens which are derived from parent hens naturally exposed to field strains of MDV and/or vaccinated with MD vaccine viruses. Maternal antibodies can interfere with the multiplications of vaccine virus in the chicken and hence reduce the effectiveness of vaccination. However, interference can be overcome by administering serotype 2 vaccines to one generation and serotype 3 to the next. Most breeders in Australia used this program routinely until around 1993 (Groves 1997).

1.3.4 Immunodepression

Immunity may fail to develop in chickens that have become immunodepressed due to the stress of poor management and other immunosuppressive viruses such as chicken infectious anaemia virus (Otaki *et al*. 1988, Yuasa & Imai, 1988) and infectious bursal disease (Sharma, 1984). Stresses occurring in young chickens include beak trimming, poor quality feed, water deprivation and heat stress.

1.3.5 Genetic Resistance

Genetic resistance to MD has been well documented and is associated with the major histocompatibility complex (MHC) or *B*-locus (Hansen *et al*., 1967; Biggs *et al*., 1968; Cole, 1968). Since successful vaccination programs have been in place, the selection of MD resistant birds has been used as an adjunct to vaccination, rather than as the primary means of disease control. In recent years with the advent of increasingly virulent strains of MDV and the realisation that existing vaccines are becoming increasingly less effective, there has been renewed interest in the selection and understanding of the mechanisms of genetic resistance to MD.

1.4 Need for a serotype 1 vaccine in Australia

During the early 1990s in Australia, the need for a serotype 1 MD vaccine was apparent due to the frequent isolation of increasingly virulent strains of MDV. Poultry farms were experiencing excessive losses due to MD, despite the use of vaccination with serotype 2 and 3 vaccines available at that time. A similar situation in the USA resulted in the introduction of the serotype 1, Rispens CVI988 strain. This strain was derived from a virus of unclassified pathogenicity (probably mild) that was initially isolated in The Netherlands (Rispens *et al.,*1972a,b). The original CVI988 strain has been used successfully in Europe and other countries since the early 1970s (Maas *et al*., 1974) but has only been available in the United States since 1994. The use of a serotype 1 vaccine in the USA appeared to be the solution to many of the problems being experienced with the newly emergent virulent strains. In Australia however, the likelihood of importing the Rispens vaccine appeared at the time remote and the industry supported the development of a serotype 1 vaccine from an Australian MDV strain.

2. Objectives

The overall aim of the project was to develop a serotype 1 Marek's disease (MD) vaccine in Australia and to evaluate its performance in comparison with existing Australian MD vaccines. The outcomes were to assist the industry in its decision to pursue a new local vaccine or to facilitate the selection of an imported vaccine. Accordingly, a programme was developed with the following objectives:

1. To develop a serotype 1 MD vaccine from a highly virulent Australian strain of MDV by attenuation in cell culture.

2. To test the efficacy and safety of any vaccine developed.

3. To compare any vaccine developed with that of currently available MD vaccines in Australia.

3. Development of an Australian MD Serotype 1 vaccine

3.1 Attenuation of the Woodlands No. 1 strain: preparation and selection of clones

3.1.1 Attenuation of MDVs by serial passage in cell culture

Attenuation of virulent viruses by serial passage in cell culture has been used in the development of MD, human and other veterinary viral vaccines. Soon after the first isolation of MDV in cell culture, a loss in pathogenicity was reported for chickens infected with a strain of MDV which had been passaged in cell culture (Churchill *et al*., 1969a; Nazerian, 1970). The HPRS-16 strain of MDV was reported to show a gradual increase in the extent of CPE developed after passage. By the 60th passage, plaques produced were larger and developed faster than those obtained from lower passage material. A loss of pathogenicity for chickens by the virus occurred after passage 33 (Churchill *et al*., 1969a). An attenuated strain derived from this isolate, HPRS-16/Att, was the first vaccine used against MD (Churchill *et al*., 1969b) but was soon largely replaced by HVT. Nazerian (1970) reported a reduction in the recovery of virus from chickens after inoculation with higher passages of the HPRS-16 strain; virus could be recovered from 50% of chickens inoculated with HPRS-16 at passage 100, compared with 100% at passage 23.

Although the mechanism of attenuation is still unclear, several theories have been proposed. Payne *et al*. (1976) and later Witter & Offenbecker (1979) hypothesised that attenuated viruses may be unable to infect lymphocytes. Support for this came from Schat (1985) who was also able to demonstrate the absence of a cytolytic infection which is characteristic of the early pathogenesis of oncogenic viruses. It is not known whether attenuation causes a block in viral replication within the lymphocyte or whether it prevents efficient transfer of the virus to the target cell. However, the effect is clearly selective as it does not influence the replication of virus in cell cultures. Another suggestion is that the loss of restriction enzyme fragments of viral DNA observed after attenuation could be associated with the loss of oncogenicity (Hirai *et al*., 1981, 1984; Tanaka *et al*., 1984), however it is unclear if the absence of certain DNA sequences in attenuated viruses is related to the failure of the virus to transform or to infect lymphocytes (Schat, 1985).

3.1.2 Attenuation of the MDV Woodlands No. 1 strain

The Woodlands No. 1 strain was originally isolated from a vaccinated flock of 15 week old chicken meat breeder line at a farm where mortality due to Marek's disease was occurring of up to 3% per week. The initial isolation was made by Zerbes *et al*. (1994) at The University of Newcastle, NSW. Subsequent passage in cell culture forms the basis of the project.

Initial isolation was achieved by collecting heparinised blood from the original flock and inoculation into day-old specific-pathogen-free (SPF) chickens, which were housed with uninoculated contact birds. After 7 weeks, the contact birds were bled and the lymphocytes from the blood pooled and stored in liquid nitrogen. The procedure was repeated twice in day-old SPF chickens to amplify any virus present. Isolated lymphocytes were then inoculated into chicken embryo kidney (CEK) cell cultures which were passaged weekly. Passage in CEKs resulted in a substantial increase in virus titre and in the extent of CPE produced. The virus was then passaged into mixed cultures containing CEK and increasing proportions of chicken embryo fibroblast (CEF) cells and finally into cultures consisting entirely of CEF cells. At various passage levels, clones were prepared for evaluation as vaccine candidates (see Section 3.1.3.).

3.1.3 Preparation and selection of clones

Cloning by limit dilution

Eight clones per passage were prepared at passages 32, 40, 60, 80, 100 and 120. Limiting dilutions were performed three times in an attempt to obtain pure lines of infectious virus; three additional passages were then carried out in order to expand stocks of each clone for storage in liquid nitrogen. The passage numbers above refer to the final cell culture passage which was stored and used for any subsequent testing. Limiting dilutions were carried out by inoculating 24-well plates with 10-fold dilutions of a particular passage. Briefly, infected CEF cultures were trypsinised and suspended into MEM Growth Medium and 10-fold dilutions were prepared in the same medium. One hundred microlitres per dilution were inoculated to each of 12 - 24 wells and, after 6 - 7 days, each well was examined for plaques. Wells demonstrating a single plaque were selected for further cloning. Cells in the well were released by trypsinising and 10-fold dilutions in MEM Growth Medium were prepared and inoculated onto fresh CEF cultures in 24-well plates, using 4 wells per dilution. Again, wells showing a single plaque were released and the procedure repeated. Each clone was then passaged three more times to expand the stock for storage in liquid nitrogen. Frozen clone stocks were then tested for infectivity using the quantal assay (50% tissue culture infective dose, $TCID_{50}$; Reed & Muench, 1938; see Table 3.1) and cell viability.

Identification of cloned virus by IFA and PCR

Virus present in each clone was confirmed as serotype 1 MDV by an indirect immunoflurorescence antibody (IFA) test. A polymerase chain reaction (PCR) assay was carried out on clones from passages 32, 40, 60 and 80 in an attempt to differentiate between pathogenic and non-pathogenic clones of serotype 1 viruses, according to the presence of an expanded 132 bp region within the IR_L region of the MDV genome. Pathogenic serotype 1 MDVs possess 1 - 3 copies of the 132 bp sequences, whereas attenuated and non-pathogenic viruses may contain up to 30 copies. All of those clones that showed evidence of attenuation by PCR and several that did not were selected for tests of pathogenicity in chickens (see Table 3.1). Clones at the 80^{th} passage were not available at the time of pathogenicity testing.

Table 3.1. *Titre and MDV PCR results for clones derived from the Woodlands No.1 strain*

Passage/ Clone No.	$TCID_{50}$/mL	Evidence of attenuation by PCR[a]	Passage/ Clone No.	$TCID_{50}$/mL	Evidence of attenuation by PCR[a]
32/1 *	$10^{3.98}$	Yes	60/1	$10^{5.31}$	No
32/2	$10^{3.20}$	No	60/2 *	$10^{5.22}$	No
32/3	$10^{4.10}$	No	60/3	$10^{5.45}$	No
32/4	$10^{4.10}$	NA	60/4	$10^{5.45}$	No
32/5	$10^{3.75}$	No	60/5 *	$10^{5.35}$	No
32/6 *	$10^{3.98}$	No	60/6	$10^{5.00}$	No
32/7 *	$10^{4.68}$	Yes	60/7 *	$10^{4.89}$	Yes
32/8	$10^{4.35}$	No	60/8	$10^{5.00}$	No
40/1	$10^{4.75}$	No	80/1	$10^{5.90}$	Yes
40/2 *	$10^{5.00}$	No	80/2	$10^{5.55}$	No
40/3	$10^{4.75}$	No	80/3	$10^{5.90}$	No
40/4	$10^{4.75}$	No	80/4	NA	NA
40/5 *	$10^{4.96}$	No	80/5	$10^{5.50}$	No
40/6	$10^{4.75}$	No	80/6	$10^{5.91}$	No
40/7	$10^{5.00}$	No	80/7	$10^{5.90}$	No
40/8	$10^{4.00}$	No	80/8	$10^{5.45}$	Yes

***** Clones selected for pathogenicity testing in chickens.

a Clones showing obvious attenuation in MDV PCR, as indicated by:
- an increase in the number of bands and/or increase in intensity of higher repeat bands
- presence of a high MW smear

NA Not attempted

3.1.4. Tests of pathogenicity in chickens

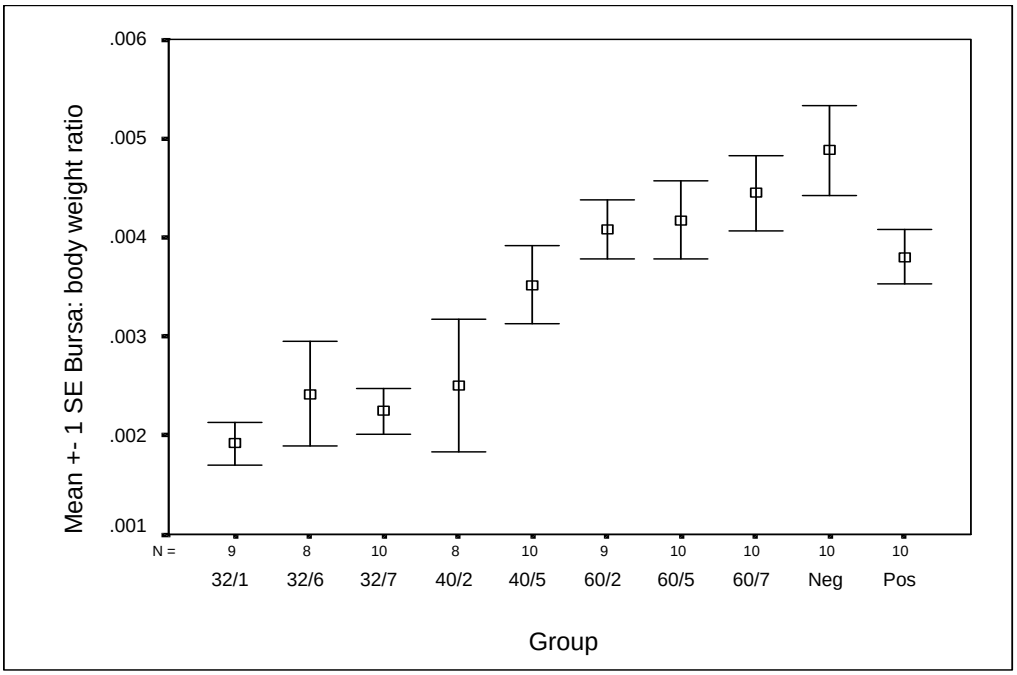
Mixed sex day-old SPF chickens (obtained from Steggles Vaccine Laboratory, Blackhill, NSW) were assigned to ten groups of ten birds. Day-old birds in each group were inoculated intra-peritoneally with 0.1 mL of the appropriate clone or control preparation (see below). The clones chosen are indicated in Table 3.1 (asterisk) and were diluted to the equivalent of 1000 PFU/ dose [assuming 1 $TCID_{50}$ = 0.69 PFU (Luria *et al*., 1978)] in Cell Freezing Media (CFM). Titres were determined by the quantal assay because many of the clones, especially those derived from lower passages, did not produce plaques that could be detected by a plaque technique. Passage 80 clones were not available at the time of pathogenicity testing in birds. Positive control birds were inoculated with a suspension of Woodlands No.1-infected lymphocytes (see Section 3.1.6. for a discussion on this preparation). Standardisation in subsequent experiments was achieved with the use of cell culture-grown challenge viruses (see Chapter 3.2). Negative controls were inoculated with CFM alone. Each group was housed in a separate bubble isolator (Dennett & Bagust, 1979) at the Victorian Institute of Animal Science (VIAS), Attwood, VIC. Seven days after inoculation, 5 day-old SPF birds per group were added to each group as contact controls. Any birds that died or required euthanasia during the time of observation were examined for gross and histological lesions. Six weeks after inoculation, birds were killed and examined for gross lesions and measurement of bursa and body weights. Samples of both sciatic nerves, both brachial nerves, the caeliac ganglion and the left gonad were taken for histology. Any gross lesions were also sampled. Tissues were scored according to the criteria listed in Table 3.2.

Table 3.2 *Criteria for scoring tissue according to lymphocyte infiltration*

Score	Features
1	No infiltration with lymphoid cells
2	Slight infiltration
3	Moderate infiltration
4	Extensive infiltration with loss of tissue architecture
5	Gross lesion

Mean tissue scores for the five nerves from each chicken were taken and the mean of these calculated for the entire group. The mean visceral organ score (taken from histological examination of the left gonad) and the mean bursa: body weight ratio for each group were also calculated. Viraemia was assessed in each bird from blood collected at the completion of the experiment. Lymphocyte preparations were inoculated into cell cultures and individual viraemic titres were expressed as $TCID_{50}$/mL of blood.

3.1.5. Results

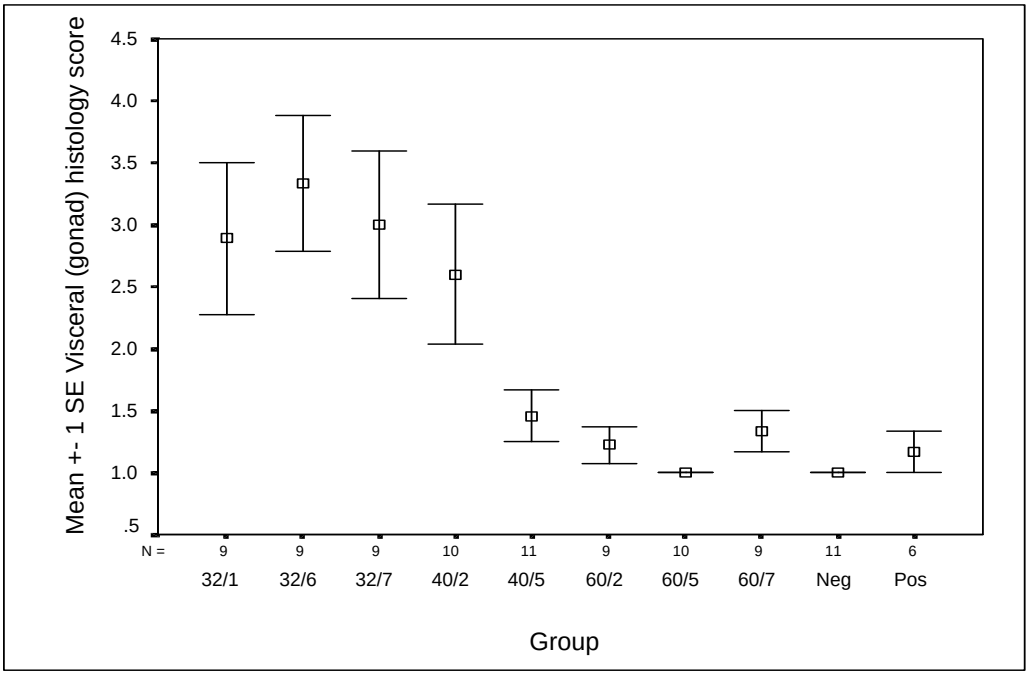


Analysis of variance (ANOVA) results:
Sex effects were not significant (p 0.466)
Group effects were significant (p 0.000)

	32/2	32/7	32/6	40/2	40/5	Pos	60/2	60/5	60/7	Neg
32/2										
32/7										
32/6										
40/2										
40/5	*	*								
Pos	*	*	*	*						
60/2	*	*	*	*						
60/5	*	*	*	*						
60/7	*	*	*	*						
Neg	*	*	*	*	*	*				

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

Figure 3.1 *Bursa: body weight ratios (mean $\pm$ SE) of chickens infected with Woodlands No.1 clones in the pathogenicity test.*

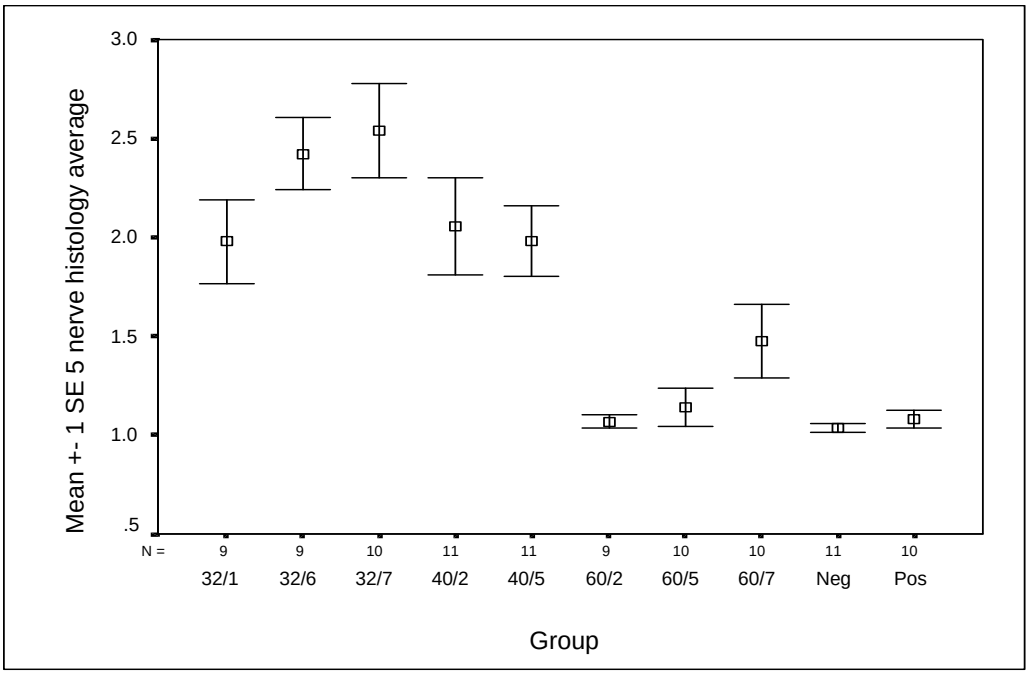


Analysis of variance (ANOVA) results:
Sex effects were significant (p 0.009)
Group effects were significant (p 0.00)

	60/5	Neg	Pos	60/2	60/7	40/5	40/2	32/2	32/7	32/6
60/5										
Neg										
Pos										
60/2										
60/7										
40/5										
40/2	*	*	*	*	*	*				
32/2	*	*	*	*	*	*				
32/7	*	*	*	*	*	*				
32/6	*	*	*	*	*	*				

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

Figure 3.2 Visceral (gonad) scores (mean $\pm$ SE) of chickens infected with Woodlands No.1 clones in the pathogenicity test.

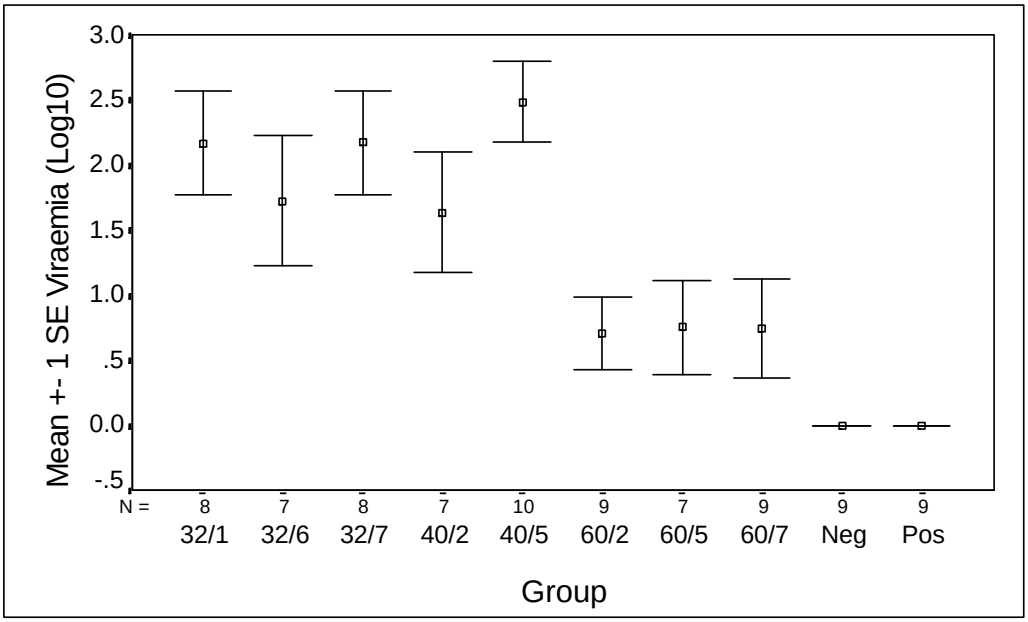


Analysis of variance (ANOVA) results:
Sex effects were not significant (p 0.233)
Group effects were significant (p 0.00)

	Neg	60/2	Pos	60/5	60/7	32/2	40/5	40/2	32/6	32/7
Neg										
60/2										
Pos										
60/5										
60/7										
32/2	*	*	*	*	*					
40/5	*	*	*	*	*					
40/2	*	*	*	*	*					
32/6	*	*	*	*	*					
32/7	*	*	*	*	*	*	*	*		

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

Figure 3.3 *Nerve scores (5 nerve mean/bird; group mean $\pm$ SE) of chickens infected with Woodlands No.1 clones in the pathogenicity test.*



1.6 Log_{10} $TCID_{50}$/mL of blood is the limit of test detection. Mean values include all assay results where a negative test is recorded as 0.

Analysis of variance (ANOVA) results:
Sex effects were not significant (p 0.738)
Group effects were significant (p 0.00)

	Neg	Pos	60/5	60/2	60/7	40/2	32/6	32/1	32/7	40/5
Pos										
Neg										
60/5										
60/2										
60/7										
40/2	*	*								
32/6	*	*	*	*	*					
32/1	*	*	*	*	*					
32/7	*	*	*	*	*					
40/5	*	*	*	*	*					

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

Figure 3.4 *Viraemic titre (mean Log_{10} $TCID_{50}$/mL of blood ± SE) of chickens infected with Woodlands No.1 clones in the pathogenicity test.*

Table 3.3 *Mean viraemic titre and percent of birds viraemic for different clones of the Woodlands No.1 strain.*

Group	32/1	32/6	32/7	40/2	40/5	60/2	60/5	60/7	Neg	Pos
Mean viraemic titre * (Log_{10} $TCID_{50}$ /mL blood)	2.17	1.72	2.17	1.64	2.48	0.71	0.75	0.75	0.0	0.0
Percentage of birds viraemic	88	71	88	100	100	44	43	33	0	0

* Where 1.6 Log $TCID_{50}$ /mL blood is the limit of detection. Mean values include all assay results where a negative test was recorded as 0.

3.1.6. Discussion

According to all measures of pathogenicity except bursa: body weight ratios (Figures 3.1 - 3.3), the positive control group was not significantly different from the negative control group indicating that the positive control did not produce adequate MD pathology. However clones at lower passages were significantly more virulent from both the higher passage clones and the negative control and, therefore, serve as an internal positive control for the test. The poor positive control result highlights problems arising from the use of unstandardised lymphocyte preparations as challenge viruses. The batch of Woodlands No.1-infected lymphocytes used in this experiment had not previously been tested for pathogenicity, although earlier passage material had been shown to be pathogenic for chickens. Virus used in this experiment could not be readily detected in cell culture. This problem was overcome in subsequent experiments by the use of standardised cell culture-grown challenge viruses (see Chapter 3.2).

ANOVA results confirm that group effects were significant for all measures of pathogenicity and that sex effects were only significant for the gonad (visceral) scores which one would expect due to the high number of lesions seen in the ovary.

Clones at higher passages tended to be less virulent by bursa: body weight ratios and tissue scores (Figures 3.1 - 3.3), with clones at the 60^{th} passage not significantly different from the negative control. Individual clones at the same passage were variable in their virulence. Virulence, however, did not correlate with results obtained by PCR (Table 3.1). Low passage clones, such as 32/1 and 32/7, showed an increase in the number of repeat 132 bp bands by PCR, suggesting some degree of attenuation. However none of the 40 passage clones and only the 60/7 clone of the passage 60 clones showed such an increase (Table 3.1). This is not surprising as virulence is probably a multi-genetic phenomenon (see Section 3.1.1.). Despite these findings for individual clones, PCR results for uncloned material at different passages indicates a trend to higher 132bp repeat sequences with higher passage. Uncloned material probably contains a mixed virus population with a higher proportion of viruses showing evidence of genomic expansion at higher passage levels.

All clones retained the ability to infect chickens, but measurements of viraemia (Table 3.3) showed that, with increased passage, the mean viraemic titre and the proportion of birds with viraemia decreases. These observations have previously been reported for virulent US strains of MDV after cell culture passage and is thought to be caused by the decreased efficiency of the virus to replicate *in vivo*. This problem has been overcome by backpassage of the virus through chickens, resulting in virus that can replicate efficiently *in vivo* (Witter & Lee, 1984; Witter, 1991a). However, in the present studies the lower efficiency of *in vivo* replication of the 60/2 clone derived from the Woodlands No.1 strain

did not appear to adversely affect its ability to effectively protect the chicken against virulent MDV challenge (see Sections 3.3, 3.4 and Chapter 4).

None of the clones tested showed evidence of virus spread to contact birds (introduced to each group 7 days after inoculation) by any of the criteria used. Overall, these results suggest that the ability of the virus to be spread by contact is lost after passage in cell culture, which confirms the findings of Witter (1991a) for strain Md11/75C/R2. The inability of a vaccine to spread by contact is a desirable property for a vaccine virus, as it decreases the chance of the reversion to virulence after passage through chickens (see Section 3.4.3.). The Rispens (CVI-988) serotype 1 vaccine strain has been shown to spread by contact (von Bülow, 1977).

Lesions were scored according to the criteria outlined in Table 3.2 and the mean corrected scores (mean negative group score is subtracted from the mean group score) were used to classify virulence based on the criteria of Biggs and Milne (1972); (see Table 3.4). Using this system to compare both visceral (gonad; Figure 3.2) and nerve scores (Figure 3.3) for the various clones, all clones at the 60th passage were considered apathogenic. Clones at passages 40 and 32, according to the same criteria, were acutely pathogenic (both 40 and 32 passage level clones obtain corrected visceral scores of >1.5 [except the 40/5 clone] and corrected nerve scores of >1.0).

Table 3.4 *Pathogenicity classification according to peripheral nerve and visceral organ scores (Biggs & Milne 1972).*

Category of Pathogenicity	**Peripheral nerves**	**Visceral organs (Gonads)**
Apathogenic	<1.0	<1.0
Classical	>1.0	<1.5
Acute	0 - 5	>1.5

From the results described above, clone 2 at the 60th passage (60/2) was considered to be sufficiently attenuated and the best candidate for further vaccine evaluation. In the following chapters the 60/2 clone and other selected clones are evaluated for their capacity to induce protection against challenge viruses.

3.2 The development of two Marek's disease challenge viruses

3.2.1 Introduction

Marek's disease vaccines have been evaluated by challenging immunised chickens with virulent strains of serotype 1 viruses and observing for deaths, morbidity and typical lesions over a predetermined period, generally from 6 to 10 weeks (Biggs & Milne, 1972; Witter, 1991a). Birds that die during the period and those that survive are subjected to post-mortem examination and assessed for the presence of tumours, nerve and other MD-specific lesions. Many MD challenge viruses consist of preparations of lymphocytes from birds experimentally infected with a virulent strain of Marek's disease virus (MDV), however this procedure is both time consuming and expensive. It requires the use of chickens and appropriate holding facilities and introduces significant risks from spread of the disease to other susceptible birds. Virus from lymphocyte preparations is often undetectable by cell culture techniques but is still highly infectious for chickens. Lymphocyte preparations may also contain adventitious viral contaminants, such as the chicken anaemia virus (CAV) that can act synergistically with MDV in causing disease. Many of these disadvantages can be overcome using challenge viruses prepared and assayed in cell culture (Okazaki *et al.*, 1971; Payne & Rennie, 1973; Witter, 1982). However, passage in cell culture has been shown to be associated with a reduction of virulence (Churchill *et al*., 1969a; Konobe *et al*., 1979; Witter, 1991a) and the extent of this reduction varies according to experimental conditions. Churchill *et al*. (1969a) described a loss of pathogenicity for chickens for the HPRS-16 strain after passage 33 and a report by Morgan *et al.* (1996) suggested that some attenuation occurs after as few as 18 passages.

The development of two cell culture-grown challenge viruses and their evaluation are described in the following section. The two highly virulent Australian strains of MDV were passaged a limited number of times in chicken embryo kidney (CEK) and chicken embryo fibroblast (CEF) cultures prepared from specific-pathogen-free (SPF) embryos and shown to retain their virulence. Preparations of each strain could be readily stored as seed-lots and assayed by standard techniques.

3.2.2 Preparation of challenge viruses in cell culture

Viruses

Two Marek's disease virus strains, the Woodlands No.1 strain (Zerbes *et al*., 1994) and strain MPF 57 (De Laney *et al.*, 1998), were isolated from flocks in Eastern Australia that had been vaccinated with serotype 2 and 3 vaccines and were experiencing high MD mortalities. Each strain was first isolated in CEK cultures from lymphocyte preparations of field blood samples. The isolates were passaged an additional 4 - 6 times in CEK cultures and then 3 times in mixed CEK and CEF tissue cultures containing increasing proportions of CEF cells. Each strain was finally passaged 3 - 4 times in CEF cultures, suspended in CFM and stored. The Woodlands No. 1 strain was tested in chickens at passage 14 and strain MPF 57 at passage 12.

The Woodlands No.1 and MPF 57 lymphocyte challenge control preparations were obtained from clinical material that had undergone 3 - 5 bird-to-bird passages of the original lymphocyte material. The lymphocyte preparation used in this experiment contained 6.8 and 1.5 x 10^6 cells/ 0.2 mL dose for the Woodlands No.1 and MPF 57 strains, respectively, with viabilities of 37 and 47%. The infectious titre of strain MPF 57 in CEK cultures was 0.26 $TCID_{50}$/dose; virus could not be detected by cell culture techniques for the Woodlands No.1 preparation.

Neither strain has been fully pathotype classified according to the system described by Witter (1997), as it has not been possible to classify the Australian stains by comparison with the prototype US strains used in this system of classification (see Discussion Section 3.2.4.).

Identification of viral antigens in cell culture

An immunoperoxidase test, incorporating monoclonal antibodies (Mabs) H19, Y5 or L78, specific for serotypes 1 - 3, respectively (Lee *et al*., 1983), was used to confirm the presence of MDV serotype 1-specific antigens of the challenge strains.

PCR studies on cell culture and lymphocyte challenge virus preparations

The PCR test for MDV was used in an attempt to differentiate between pathogenic and non-pathogenic serotype 1 MDVs, according to the presence of an expanded 132 bp region within the IR_L region of the MDV genome. There was no difference in the number of 132 bp repeats observed between the lymphocyte and the low-passage tissue culture preparations of either challenge virus strain, suggesting that minimal change in the IR_L region had taken place as a result of tissue culture passage. The low number of repeat bands observed in these challenge preparations is in contrast to the higher number of bands seen with further passages of the Woodlands No.1 strain.

3.2.3 Dose response testing of cell culture-grown challenge viruses in chickens

Experimental design

Eighteen 8-day-old mixed-sex SPF chickens (CSIRO) with a known susceptibility of 80 - 90% to the Woodlands No. 1 strain were inoculated intra-peritoneally with cell culture preparations containing $10^{1.16}$, $10^{2.16}$ or $10^{3.16}$ $TCID_{50}$ of the Woodlands No.1 strain or $10^{0.66}$, $10^{1.66}$ or $10^{2.66.}$ $TCID_{50}$ of strain MPF 57 [an estimated equivalent of 10^1, 10^2 or 10^3 plaque forming units (PFU) of the Woodlands No.1 strain or $10^{0.5}$, $10^{1.5}$ or $10^{2.5}$ of strain MPF 57; assuming 1 $TCID_{50}$ = 0.69 PFU (Luria *et al*., 1978)]. Control groups consisted of birds inoculated with diluent alone or lymphocyte-grown preparations. Birds were housed in flexible-film isolators fitted with HEPA filters to inlet and outlet air flows (Dennett & Bagust, 1979).

Seven-day-old SPF contact birds were introduced to the isolators 2 days after inoculation and removed 5 weeks later. Inoculated birds were maintained for 10 weeks post-inoculation, with 5 birds per group being removed after 4 weeks for the evaluation of viraemia. Birds removed during the experiment were bled for the assessment of viraemia and examined for gross lesions and measurement of bursa and body weights. Tissues from birds removed at the completion of the experiment were examined histologically. Birds that died or were euthanased during the experiment were examined for gross and histological lesions. Lesions were graded according to the criteria set out in Table 3.2. Tissues examined histologically included brachial, sciatic and splanchnic nerves, left gonad, spleen, liver, heart, lung, bursa, intestine and proventriculus.

Samples of serum and lymphocytes were collected at the completion of the experiment and tested for the evidence of CAV by serology and PCR techniques.

Results

All doses of cell culture-grown virus for both strains produced death or gross tumours in 60 - 100% of birds inoculated (Table 3.5.). A bimodal pattern of deaths occurred for birds inoculated with either strain, with the first occurring 2 weeks after inoculation and the second around 6 weeks. There was an even distribution of deaths between peaks in each group, except for those inoculated with MPF 57 lymphocytes where most deaths occurred within the first peak at 2 weeks, a well recognised characteristic of very virulent MDVs (Witter, 1983). The MPF 57 lymphocyte inoculum was subsequently shown by PCR to be contaminated with CAV and inoculated birds were seropositive for

CAV, which may account for the increased virulence and the increased number of early deaths in this group. CAV could not be detected in the Woodlands No.1 lymphocyte inoculum or in either strain after growth in cell culture. The latter finding is not unexpected as CAV cannot be cultured in CEK or CEF cultures (S.C. Cunningham, PhD thesis, Royal Melbourne Institute of Technology, 1997).

MDV viraemia was detected in inoculated birds of all groups, with the exception of the group infected with the Woodlands No.1 lymphocyte-grown virus where viraemia was only detected in the contact birds (Table 3.6).

Gross lesions/tumours observed were predominantly ovarian but also included a significant number of liver tumours and tumours in other organs, including the kidney, heart, lung, testes, nerve and spleen. Results of histological examination revealed slight to severe (scores 2 - 5) lymphocyte infiltration in the nerves and other organs of birds in each group. Similar lesions and histopathology have been described in detail for the original isolate of the Woodlands No. 1 strain by Zerbes *et al.* (1994).

Viraemia was detected in many of the contact birds (Table 3.6) which, together with lower bursa: body weight ratios and the demonstration of liver and ovarian tumours, was evidence of transmission from inoculated birds, proving that both of the cell culture-grown challenge viruses retained their ability to spread by contact.

The bursa: body weight ratios of infected birds measured at the completion of the experiment (Figure 3.5) shows that lower ratios were obtained for increasing doses of cell culture-grown virus. Ratios for the highest doses of each cell culture-grown virus were comparable with those of unstandardised lymphocyte preparations of the same strain. Such trends were not apparent for the bursa: body weight ratios measured for birds removed at 4 weeks where, for each strain, the ratio for higher doses was actually higher than for lower doses. This may reflect an early inflammatory response which precedes the later cytolytic response to infection (Calnek & Witter, 1997).

Table 3.5. *Death, tumours and histology results for birds inoculated with cell culture-grown and lymphocyte preparations of the Woodlands No.1 and MPF 57 challenge viruses.*

Challenge virus	**Dose**[a]	**Death or gross tumours**[b]		**Histology**[bc] **(≥3 score)**	
		total	**positive**	**total**	**positive**
MPF 57:					
cell culture	0.66	10	6	10	8
	1.66	10	9	10	9
	2.66	10	7	10	8
lymphocytes	0.26	10	10	10	10
Woodlands No.1:					
cell culture	1.16	11	8	11	10
	2.16	10	8	10	8
	3.16	10	10	10	10
lymphocytes	ND	9	8	9	8
Uninfected	0	10	0	10	0

a Log_{10} $TCID_{50}$ per bird.

b Includes birds that died or were euthanased during the experiment and those examined at its completion. Birds removed at four weeks were not included.

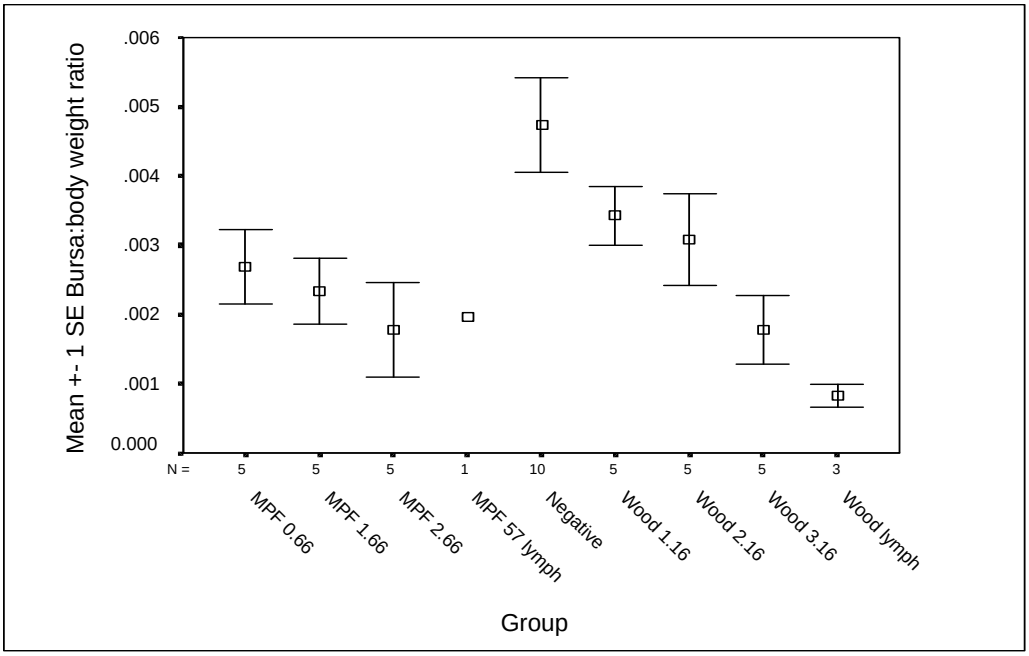
c Includes birds with a histology score of ≥3 in any tissue.

ND Not detected in CEK cultures.

Table 3.6. *Viraemia results for birds inoculated with cell culture-grown and lymphocyte preparations of the Woodlands No.1 and MPF 57 challenge viruses.*

Challenge virus	**Dose**[a]	**Viraemia**		
		Inoculated birds at:		**contact**
		4 weeks	**10 weeks**	**birds**
MPF 57:				
tissue culture	0.66	3/6	0/5	1/7
	1.66	3/7	1/5	3/8
	2.66	2/6	1/4	0/7
lymphocytes	0.26	3/4	0/1	2/8
Woodlands No.1:				
tissue culture	1.16	3/7	1/5	1/5
	2.16	4/8	2/4	1/6
	3.16	4/8	2/5	0/5
lymphocytes	ND	0/4	0/3	2/4
Uninfected	0	0/4	0/10	0/6

a Log_{10} $TCID_{50}$ per bird.
ND Not detected in CEK cultures



Analysis of variance (ANOVA) results:
Sex effects were not significant (p 0.336)
Group effects were significant (p 0.08)

	W Lym	W 3.16	M 2.66	M Lym	M 1.66	M 0.66	W 2.16	W 1.16	Neg
W Lym									
W 3.16									
M 2.66									
M Lym									
M 1.66									
M 0.66									
W 2.16	*								
W 1.16	*								
Neg	*	*	*		*	*			

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

Figure 3.5. *Bursa: body weight ratio (mean $\pm$ SE) for lymphocyte preparations and different cell culture-grown doses of the Woodlands No.1 and MPF 57 challenge viruses 10 weeks after inoculation.*

3.2.4 Discussion

These results suggest that two very virulent Australian strains, the Woodlands No.1 strain and strain MPF 57, can be grown to workable titres after relatively few passages and yet retain their virulence for chickens. Both strains produced around 80% deaths or gross lesions when tested over a 100-fold range of inoculum doses (Table 3.5.), which is in conformity with the recommended figure of at least 70% (Anonymous, 1995). Virulence for the two cell culture-grown viruses was also reflected in the lower bursa: body weight ratios (Figure 3.5) compared with the uninfected (negative) control birds. There was no significant difference in the ratios between any of the MPF 57 cell culture or lymphocyte groups, nor between the Woodlands No.1 cell culture groups, although the Woodlands No.1 lymphocyte group was significantly different from the two lowest cell culture groups ($10^{1.16}$ and $10^{2.16}$ $TCID_{50}$). The highest doses for each strain ($10^{2.66}$ $TCID_{50}$ for strain MPF 57; $10^{3.16}$ for the Woodlands No.1 strain) gave bursa: body weight ratios that were approximately 50% of the negative control birds. Similar ratios were obtained by McKimm-Breschkin *et al*. (1990) for 13 highly virulent Australian strains. Both cell culture and lymphocyte preparations of each strain could be transmitted by contact (Table 3.6), although there was no evidence for a dose-dependent response. Contact transmission for the Woodlands No.1 strain appears to be lost after passage 32 (see Chapter 3.1).

Further evidence for the retention of virulence in the two cell culture-grown viruses was the low number of 132bp repeats in the IR_L region of the genome when tested by PCR. For each strain, both the cell culture-grown and lymphocyte preparations demonstrated the same number of 132bp repeats. Similar numbers of repeats have been reported for very virulent US and Israeli strains of MDV (Silva, 1992; Becker *et al*., 1992), although their significance for pathogenicity is not well understood. However, the extent of attenuation following passage in cell culture may be unpredictable and Morgan *et al.* (1996) described the isolation of four equally passaged clones from a parent strain, two of which were indistinguishable from the parent strain while a third could not be transmitted horizontally and a fourth did so with reduced efficiency.

It is concluded from this chapter that the degree of attenuation following limited passage of the two Australian strains of MDV, Woodlands No.1 and MPF 57, is minimal. Both strains at doses of 50 PFU/ bird and 100 PFU/ bird, respectively, have been used as standard challenge viruses in Australia and have given reproducible results in a number of breeds in tests carried out in different regions of Australia (unpublished observations; see Sections 3.3, 3.4, 4.1). The strains have been maintained as a seed-lot in liquid nitrogen and passaged no more than twice to produce a working stock at the required passage level. Further cloning of the preparations was not undertaken because of the potential for further attenuation following an additional 3 - 4 tissue culture passages. Titres of $10^{4.5}$ and $10^{5.5}$ $TCID_{50}$ /mL, respectively, can readily be achieved for strain MPF 57 and Woodlands No.1 strain. Data from Table 3.5 and Figure 3.5 suggest that strain MPF 57 was more virulent over the range of doses tested than the Woodlands No.1 strain. Virulence may also be related in some way to the extent of its capacity to grow in cell culture, which is a well recognised property of field isolates and is reflected in these results. After passage of the MPF 57 strain in cell culture, the CAV contaminant appeared to be removed and could not be detected by serology or PCR in birds inoculated with the cell culture-grown virus. The MPF 57 cell culture-grown virus can now be used in MD challenge experiments without the added immunosuppressive effects of the CAV contaminant.

As indicated earlier (Section 3.2.2), pathotype classification of Australian strains by the system of Witter (1997) has not been possible because the strains used for comparison in this system are not available in Australia. Under experimental conditions, chickens were not adequately protected from challenge with MPF 57 or the Woodlands No.1 strain after vaccination with bivalent (serotype 2 + 3) vaccines (see Sections 3.3 and 4.1). This suggests that under the particular set of conditions used, these viruses can be considered to be vvMDVs. However, these results are highly variable depending upon several factors such as the vaccine used, chicken line and experimental conditions. For example, Table 3.6 shows the variation in protection given by two different bivalent vaccines when using the

same MPF 57 cell culture-grown challenge virus in SPF chickens. Birds vaccinated with the bivalent Maravac + Steggles HVT vaccine achieved only 57.0 % protection whereas those vaccinated with Maravac and TMC HVT achieved 84.5 %. The significantly higher dose and the different HVT strain of the TMC vaccine may account for these differences. Such variations and the unavailability of international reference challenge strains (such as Md5) in Australia render comparisons according to the classifications system described by Witter (1997) very difficult. One of the key criteria used to distinguish between vv and vv+ MDV strains is to compare the protection afforded against the Md5 challenge virus in vaccinated chickens. Without the standard international strains for comparison, differences in genetic resistance between chicken lines and the presence of maternal antibody and other factors are difficult to control. In addition to these difficulties, the application of uniform criteria for gross necroscopy has been identified by Witter (1997) as a cause of highly variable results between research groups.

3.3 Clone protection studies and selection of the 60/2 clone

3.3.1 Evaluation of Clones in Challenge Experiments

The protection induced by clones prepared from a range of passage levels was evaluated in a challenge experiment. Pathogenicity tests had previously shown that by passage 60, the Woodlands No.1 isolate had become sufficiently attenuated to be considered safe for use as a vaccine (Chapter 3.1). However, it was still uncertain as to whether clones prepared from the 60th passage or from further passages were able to protect chickens from virulent challenge.

In the following experiment, single clones from passages 40, 60, 80 and 99 were selected for use in a challenge experiment. The 40/5 and the 60/2 clones were chosen because of their low pathogenicity (Chapter 3.1) while those at passages 80 and 99, which had not been tested for pathogenicity, were chosen randomly. The experiment was carried out at the Animal Research Institute (ARI), QLD by Dr. Peter Young and included groups vaccinated with commercial cell-free and cell-associated HVT (serotype 3), Maravac (serotype 2) and CR6 (serotype 1) vaccines and a fowlpox recombinant containing the MDV gB gene . Twenty to forty commercial female Isa Brown birds per group were used; female birds were used because of their greater susceptibility to MD. Another group of 20 mixed sex SPF chickens were inoculated with CR6 and cell-free HVT vaccines, the latter serving as a maternal antibody-free control group. Vaccines were administered to day-old chickens at the recommended dose (see Table 3.7). Birds were challenged at day 9 with a lymphocyte preparation of the Woodlands No.1 strain and any that died or were culled during the experiment were examined for gross pathology and the presence of visible tumours. Birds were maintained for 10 weeks after challenge, and were culled if they appeared severely depressed or were unable to rise or walk before that time. All surviving birds were then euthanased and necropsied for MD lesions.

Table 3.7 *Protection evaluation of RMIT clones at several passage levels by virulent challenge with Woodlands No.1 MDV.*

Vaccine/ treatment	**Chicken line**					
	Isa Brown			**SPF**		
	Birds with lesions	**% lesions**	**% protection**	**Birds with lesions**	**% lesions**	**% protection**
RMIT 40/5	36/40	$_{90}$[a]	0			
RMIT 60/2	26/40	$_{65}$[b]	24			
RMIT 80/1	16/20	$_{80}$[a]	6			
RMIT 99/2	33/40	$_{83}$[a]	3			
CR6	23/40	$_{58}$[b]	32	2/17	$_{12}$[a]	87
cf HVT	21/40	$_{53}$[b]	38	3/17	$_{18}$[a]	82
ca HVT + MV	20/39	$_{51}$[b]	39			
FP-MDV + HVT	28/40	$_{70}$[b]	18			
nonvaccinated + challenged (positive controls)	34/40	$_{85}$[a]		12/15	$_{80}$[b]	
nonvaccinated/ unchallenged (negative controls)	8/35	$_{23}$[c]		6/20	$_{30}$[a]	

a,b,c Different superscripts when the same column indicate significant differences ($p<0.05$).
CR6: Rispens vaccine, CR6 derivative (Cyanamid Websters Pty Ltd)(1000 PFU)
cf HVT: Cell-free HVT vaccine (Cyanamid Websters Pty Ltd)(250 FFU)
ca HVT: Cell-associated HVT vaccine (Steggles vaccine laboratory)(690 PFU)
MV: Maravac®, serotype 2 MDV vaccine (Cyanamid Websters Pty Ltd)(250 FFU)
FP-MDV: Recombinant fowl pox virus with MDV gB
All vaccines were administered at the manufacturers recommended dose.
RMIT clones: First number signifies passage number, second signifies clone number. All clones were administered at 1000 PFU/ dose.
% Protection calculation includes birds that died prior to the end of the experiment.

The results from Table 3.7 suggest that the best protection was obtained using 60th passage virus and that a loss in immunogenicity occurred at passages 80 and 99, as evidenced by much lower rates of protection. No protection was observed in birds inoculated with the 40th passage clone, due probably

to the residual virulence of the clone. Residual virulence for that clone may have been responsible for the increased incidence of lesions in the group (90%) compared with the positive controls (85%), although the difference was not significant. The good rates of protection obtained for the 60/2 clone, together with the pathogenicity results in Chapter 3.1, provided an indication of the need for further evaluation and development of this clone as a vaccine candidate.

It was clear from the incidence of MD lesions observed in the negative control groups of each bird line that adequate isolation between groups had not been achieved. Despite this, the experiment provided useful data and it seems unlikely that airborne transmission of virulent challenge virus to challenged birds due to inadequate separation of the groups would have affected the end point of the experiment.

3.3.2 Protection studies on the RMIT 60/2 clone in SPF birds and comparison with other vaccines in commercial birds.

The 60/2 clone was further evaluated in a challenge experiment by Dr. Peter Young at the ARI. Here, the protection by the 60/2 clone was compared with that induced by commercial vaccines and with a Steggles experimental type 1 MD vaccine. An additional group of birds vaccinated with the 60/2 clone were not challenged but were examined for lesions in a pathogenicity test. Pens were set up containing approximately 20 female birds of the Isa Brown line per group (one isolator per group) or SPF chickens (two isolators per group but the data was pooled). Vaccine was administered to day-old chickens at the recommended dose and, after 9 days, birds were challenged with lymphocyte preparations of the Woodlands No.1 MDV challenge virus. Ten weeks after challenge, birds were killed and examined for the presence of MD, as previously described in Section 3.3.1. The air supply had been modified to ensure that adequate isolation between groups was achieved.

Table 3.8 *Protection results for the RMIT 60/2 clone in SPF birds challenged with Woodlands No.1 MDV.*

Vaccine	**Challenge**	**Chicken line**					
		Isa Brown			**SPF**		
		Birds with lesions	**% lesions**	**% protection**	**Birds with lesions**	**% lesions**	**% protection**
RMIT 60/2*	-				0/40	0	NA
RMIT 60/2*	+				2/40	5	94[c]
CR6 (s.c)	+	8/20	40	60[b]	5/40	13	83[c]
CR6 (i.m)	+	9/20	47	53[b]			
CR6 (s.c) x 10	+	13/20	65	35[a]			
HVT	+	7/20	65	25[a]	6/40	15	81[c]
MV + HVT	+				3/40	8	90[c]
Steggles MD 1	-				0/40	0	NA
Steggles MD 1	+				24/40	60	23[a]
nonvaccinated	+	20/20	100	NA	31/40	78	NA[a]
nonvaccinated	-	0/25	0	NA	0/50	0	NA

a,b,c,d Different superscripts in the same column indicate significant differences ($p<0.05$).
CR6: Rispens vaccine, CR6 derivative (Cyanamid Websters Pty Ltd); s.c., subcutaneous; i.m., intramuscular.
HVT: Cell-associated HVT vaccine (Steggles vaccine laboratory)
MV: Maravac®, serotype 2 MDV vaccine (Cyanamid Websters Pty Ltd)
Steggles MD 1, serotype 1 Steggles Vaccine Laboratories experimental type 1 MD vaccine
All vaccines were administered at the manufacturers recommended dose
* The RMIT 60/2 clone used in this experiment was at passage 63 (those tested previously were at passage 60) and was administered at 1000 PFU/ dose.
% Protection calculation includes birds that died prior to the end of the experiment.

The results in Table 3.8 indicate that 94% protection was induced by clone 60/2, a figure that was superior to that achieved by any of the commercial vaccines tested ($p<0.05$ for all vaccines except the Maravac + HVT group). No lesions were observed for the 60/2 clone in the pathogenicity test, confirming earlier safety data for 60/2 clone (Section 3.1). The pathogenicity test in this experiment differed from that carried out in Section 3.1, in that an 11-week post-vaccination observation period was used. The experiment described in Section 3.1 was carried out according to the procedure of Biggs & Milne (1972) using a 6 week observation period. The 10-week observation period after challenge has been adopted by the Australian chicken industry as the standard observation period for challenge experiments (C.A.W. Jackson, personal communication).

The Steggles MD-1 experimental vaccine did not appear to have the same level of residual virulence as the RMIT clone 60/2 vaccine. However, this vaccine was used at an early stage of development and may have been over-attenuated as the protective index was 23 in SPF birds.

CR6 provided excellent protection in SPF birds (PI=83) however, the PI was reduced to 60% in Isa birds possessing maternal antibody. In birds with maternal antibody, delivery of the CR6 vaccine by the im route provided a similar level of protection to a sc injection. Using CR6 at a 10x dose did not appear to overcome the effects of maternal antibody. The level of protection achieved was actually lower than the standard dose given by the same route. Cleary there is no benefit in using a megadose of CR6.

Successful modification of the air supply ensured there was adequate isolation between groups, this was evidenced by the MD-free status of the negative control birds of both SPF and Isa groups (see Table 3.8).

3.3.3 Protection studies on other 60th passage clones

Other clones from the 60th passage were evaluated to determine the protection they induced in comparison with clone 60/2. Pens were set up containing approximately 20 birds per group for the RMIT clones, 15 per group for birds vaccinated with HVT and 30 for the unvaccinated/challenged controls. Duplicate groups were set up for each treatment using either female Cobb or Isa Brown birds. All chickens were first vaccinated within a day of hatching with infectious bronchitis virus vaccine (Cyanamid Webster, strain Vic-S) and MDV vaccines at the recommended dose. Birds were challenged at day 9 with lymphocyte preparations of MPF 57 and were maintained and assessed for the presence of MD at week 11, as described in Section 3.3.1.

Table 3.9 *Protection results for other 60th passage clones after challenge with MPF 57 MDV.*

Vaccine	Chicken line					
	Cobb			Isa Brown		
	Birds with lesions	% lesions	% protection	Birds with lesions	% lesions	% protection
RMIT 60/2	6/19	$_{32}$[a]	57	5/20	$_{25}$[a]	73
RMIT 60/5	14/20	$_{70}$[b]	6	18/20	$_{90}$[b,d]	3
RMIT 60/7	14/20	$_{70}$[b]	6	8/20	$_{40}$[a,c]	57
cf HVT	4/12	$_{33}$[a]	55	9/13	$_{69}$[b,d]	26
ca HVT	4/13	$_{31}$[a]	59	9/12	$_{75}$[b,d]	19
ca HVT + MV	13/17	$_{76}$[b]	0	13/20	$_{65}$[b,c]	30
Positive control (unvaccinated/ challenged)	23/31	$_{74}$[b]		27/29	$_{93}$[d]	

a,b,c,d Different superscripts in the same column indicate significant differences ($p<0.05$).
cf HVT: Cell-free HVT vaccine (Cyanamid Websters Pty Ltd)
ca HVT: Cell-associated HVT vaccine (Steggles vaccine laboratory)
MV: Maravac®, serotype 2 MDV vaccine (Cyanamid Websters Pty Ltd)
All vaccines were administered at the manufacturers recommended dose
RMIT clones: first number signifies passage number, second signifies clone number. All clones were administered at 1000 PFU/ dose.
% Protection calculation includes birds that died prior to the end of the experiment.

The results in Table 3.9 indicate that protection by the 60/2 clone was superior to that induced by the 60/5 and 60/7 clones in both commercial chicken lines tested. The 60/2 clone also induced significantly better rates of protection than commercial vaccines in the Isa Brown line and comparable protection in the Cobb line of birds. Accordingly, the 60/2 clone was selected for continued development.

An inconsistent result was obtained for commercial vaccines in Cobb birds when the cell-associated HVT alone was compared with cell-associated HVT + Maravac. Previous experience suggested that bivalent vaccines induce protection that is superior to, or at least equal, to that provided by cell-associated HVT alone. For reasons that are unclear, lower protection rates were observed in the Cobb, but not the Isa Brown birds, using the bivalent vaccine compared with cell-associated HVT alone (0% versus 59%). It is also notable that the MPF 57 challenge used in this experiment was a lymphocyte preparation, which was shown to be contaminated with CAV (Section 3.2). CAV can act synergistically with MDV to cause immunosuppression and disease (Jeurissen & de Boer, 1993). It is unclear whether the presence of CAV affected the results of this experiment, although it may have

resulted in the generally lower protection rates observed (Table 3.9). If the presence of CAV did result in lower protection, such a reduction would have been likely to occur uniformly between groups so that the rank order of group protection rates would have remained the same. However this assumption may not be justified if the extent of immunosuppression varied with different stains of MDV.

3.3.4. Further testing of the RMIT 60/2 clone

Comparison with CR6 at different doses

A bird trial was conducted to confirm previous results obtained for the 60/2 clone and to compare the protection induced by the RMIT and CR6 vaccines. Day-old chickens from Isa Brown and Cobb parents were vaccinated according to the schedule outlined in Table 3.10 and were challenged at 9 days with a cell culture-grown preparation of MPF 57 at 10 PFU/ bird (see Section 3.4). The Isa Brown parents had been previously vaccinated with CR6 and the Cobb parents with HVT. Plaque assays were performed on the RMIT and CR6 vaccines to determine the *actual dose* of vaccine each bird received at the time of vaccination. The assays were performed on vaccines after ampoules had been thawed and diluted for administration. The assay was carried out at Baiada Poultry Pty Ltd, Bringelly, NSW, by Dr. Edla Arzey. The assay results (*actual dose*) were compared to the *intended dose* (the manufacturer's stated dose) and are shown in Table 3.10. Birds were maintained and assessed for the presence of MD, as described in Section 3.3.1.

Table 3.10 *Comparison of the protection afforded by the RMIT 60/2 clone and the CR6 vaccine at two different doses after MPF 57 challenge.*

Vaccine/ treatment	Dose (PFU/ bird)		Chicken line					
			Cobb			Isa Brown		
	Intended	Actual	Birds with lesions	% lesions	% protection	Birds with lesions	% lesions	% protection
RMIT 60/2*	1000	925	21/36	$58^{a,b}$	16	11/40	$28^{a,b}$	55
RMIT 60/2*	2000	1850	24/39	62^{a}	11	4/40	10^{a}	84
CR6 (1^{st} batch)	2000	4650	7/20	35^{b}	49	8/22	36^{b}	41
CR6 (2^{nd} batch)	2000	5150	15/20	75^{a}	0	8/19	$42^{b,c}$	31
CR6 (2^{nd} batch)	1000	2575	11/18	$61^{a,b}$	12			
cf HVT	1 dose	ND	13/20	$65^{a,b}$	6	12/41	$29^{a,b}$	52
nonvaccinated + challenged			27/39	69^{a}		24/39	62^{c}	

a,b,c Different superscripts in the same column indicate significant differences ($p<0.05$).
CR6: Rispens vaccine, CR6 derivative (Cyanamid Websters Pty Ltd)
cf HVT: Cell-free HVT vaccine (Cyanamid Websters Pty Ltd) administered at the manufacturers recommended dose (250 FFU).
* The RMIT 60/2 clone used in this experiment was at passage 63 (those tested previously were at passage 60)
ND: Not done
% Protection calculation includes birds that died prior to the end of the experiment.

Although the results in Table 3.10 vary widely, the RMIT 60/2 clone appeared to produce better protection in the Isa Brown birds than CR6, despite having received a lower *actual* dose. In Cobb birds, the CR6 vaccine appears to have given better protection than the RMIT 60/2 clone, although an examination of the *actual* dose received indicates that the CR6 vaccine was administered at much higher doses. When the *actual* dose is approximately equivalent [1850 PFU of the RMIT 60/2 and 2575 PFU of the CR6 (2^{nd} batch)], similar rates of protection are obtained in the Isa Brown birds with observed protection rates of 11% and 12%, respectively.

The *actual dose* of the RMIT vaccine (determined at Baiada Poultry) was equivalent to the *intended dose* (determined at RMIT), which demonstrated good inter-laboratory agreement (see Table 3.10). However, the *actual dose* of the CR6 (determined at Baiada Poultry) was approximately twice the

intended dose (determined by the manufacturer) which indicates problems in inter-laboratory standardisation due to the use of different plaque assay methods and operators.

There are many other possible causes of variation between assays but a fundamental difference can be attributed to the use of a semi-solid agar or a liquid overlay. A semi-solid overlay prevents the formation of secondary plaques. Liquid overlays do not prevent the release of virus into the medium and may, therefore, result in the formation of secondary plaques by infecting other parts of the cell monolayer. Plaques produced in the semi-solid overlay method can usually be read macroscopically and the technique relies upon the formation of large plaques that can be readily seen. Strains that cause small plaques (usually low-passage serotype 1 strains, such as the challenge viruses described in Section 3.2, are often difficult to detect by this method. Other variations for both assays include the length of time plates are held before reading (which is especially important for liquid overlays, due to the formation of secondary plaques), the time of adsorption before the overlay is added, whether the inoculum is added to the medium, the type of medium used, whether primary or secondary CEF cultures are used and other variables, such as quality of the monolayers.

Comparison with Rispens (Original/ European strain)

This experiment was conducted to determine if there were differences in the protection offered by the Rispens vaccine produced by two manufacturers, The Marek's Company and Solvay, and to compare protection rates with the RMIT 60/2 clone. Both Rispens products were administered at different doses alone and in different combinations with Maravac and HVT (see Table 3.11). The RMIT 60/2 clone (now used at passage 78; see Section 3.4) was used at a nominal dose of 2,000 PFU and at a 5x dose (10,000 PFU; see Table 3.11). All vaccine titres were determined by the respective manufacturers. Birds were challenged at 9 days with a cell culture-grown preparation of MPF 57. The experiment was carried out according to the protocol outlined in Section 3.3.1.

Table 3.11 *Protection comparison of new serotype 1 vaccines at different doses and in combination with serotype 2 and/or 3 vaccines after MPF 57 challenge.*

Vaccine	Dose	x std dose	Isa Brown		Cobb		% Viraemia at 14 days
			% tumours	% protection	% tumours	% protection	
TMC Rispens	4,000 PFU	1	13.5^{b}	82.5	38.5^{c}	59.3	33.3
TMC Rispens	8,000 PFU	2			$13.5^{d,e}$	85.7	57.1
Solvay Rispens	1,000 $TCID_{50}$	1	68.2^{a}	11.8	63.9^{b}	32.5	16.7
Solvay Rispens	2,000 $TCID_{50}$	2			59.0^{b}	37.6	28.6
RMIT 60/2*	2,000	1	73.0^{a}	5.6	64.1^{b}	32.2	
RMIT 60/2*	10,000	5			$27.0^{c,d}$	71.5	
TMC HVT	8,000 PFU	1	22.9^{b}	70.4	18.9^{c}	80.0	100
TMC Rispens + TMC HVT		1			$12.5^{d,e}$	86.6	
TMC Rispens + Maravac		1			$22.2^{c,d,e}$	76.5	
TMC Rispens + HVT + Maravac		1			$12.5^{d,e}$	86.8	
Solvay Rispens + TMC HVT		1	15.6^{b}	79.8	$18.9^{c,d,e}$	80.0	
Solvay Rispens + Maravac		1			76.9^{b}	18.7	
Solvay Rispens + HVT + Maravac		1			8.6^{e}	90.9	
Nil	0	0	77.3^{a}		94.6^{a}		

a,b,c,d,e Different superscripts when the same column indicate significant differences ($p<0.05$).
* RMIT 60/2 clone at passage 78

For comparing vaccines it is clearly desirable to test at equivalent doses. As indicated in Section 3.3.4., standardisation of MD vaccines is difficult and assay results may vary considerably between testing laboratories. From an industry perspective, the need to test vaccines of different strains or from different manufacturers at equivalent doses is not important because the protective titre set by the manufacturer more accurately reflects vaccine use in the field. To clearly demonstrate which vaccine offers superior protection when used at comparable doses, it is important to determine vaccine titres by a common method. Because the experiment in Section 3.3.4 was conducted primarily to answer industry concerns, vaccine doses recommended and determined by the manufacturer were used (Table 3.11).

In Cobb birds, there was no significant difference in the protection offered by the RMIT vaccine compared with the TMC Rispens at the higher dose (10,000 and 8,000 PFU, respectively) whereas, at the lower dose (2,000 and 4,000 PFU, respectively), the TMC Rispens provided significantly better protection. However, at the lower dose, the TMC Rispens was used at twice the infectious titre. In Isa Brown birds, the TMC Rispens vaccine provided superior protection than either the RMIT or the Solvay Rispens vaccines.

From Table 3.11 it can be seen that the TMC Rispens product provided significantly better protection compared than the Solvay vaccine when used at the manufacturers recommended dose in both the Cobb and Isa Brown lines. In the Cobb birds, only the TMC Rispens, when used at the 2x dose, provided significantly better protection than HVT alone. When the Rispens vaccines were combined with either HVT and/or Maravac vaccines, there were fewer differences between the two manufacturers, due probably to the significant contribution of the HVT vaccine. The Maravac vaccine appears to contribute less to protection and, therefore, differences in protection provided by either Rispens vaccine is more apparent when used in combinations with Maravac. The lower protection of the Maravac vaccine may be due to the significantly lower titre used compared with the TMC HVT vaccine (250 and 8,000 PFU manufacturers minimum recommended dose, respectively). It may also account for the lower protection of the Solvay Rispens compared with the TMC Rispens vaccine (see Table 3.11).

3.3.5 Summary

The results from this series of experiments were variable and reflect the experimental variables used, including the use of different chicken lines, challenge viruses vaccine doses and testing facilities. When these differences in experimental design are taken into consideration, the RMIT 60/2 clone appears to be effective at protecting chickens from virulent challenge by two highly virulent strains of MDV and compares favourably with both Rispens vaccines (original European strain and CR6). In situations where the RMIT vaccine gave low protection, a similar situation was observed for other vaccines (see Table 3.9 and Table 3.10).

In addition to experimental variation, other factors which are more difficult to control, such as genetic variation between individual birds and maternal antibody status, could have also contributed to the variable results observed. High maternal antibody may have resulted in the need for higher vaccine dose to achieve adequate protection (Eidson *et al*., 1978), as seen in the commercial bird experiment (Table 3.11). Higher rates of protection were observed for SPF birds, which do not have maternal antibody, than for commercial Cobb or Isa Brown birds which usually possess maternal antibody from vaccination of the parent flock. Genetic differences may have also contributed to these differences. These experiments also were limited by the small number of birds per group that could be housed in a single isolator. In Section 4.1, large-scale testing of the 60/2 clone at the 78th passage was carried out to confirm the safety and efficacy of the 60/2 clone, compared with commercially available vaccines.

3.4 Further attenuation of the 60/2 clone and reversion to virulence safety tests

3.4.1 Introduction

Additional passage of the clone was carried out in order to assess if further attenuation could be achieved without the loss of immunogenicity. Tests for pathogenicity and protection in chickens were undertaken at passages intervals of 5 (see Table 3.12 & 3.13).

Packed cell volumes (PCV) were used as an additional parameter of MDV virulence. Spencer *et al*. (1996) showed that the PCV could be used to detect extravascular haemolytic anaemia caused by MD and concluded that the technique may be useful for measuring protection.

An effective vaccine must be safe and offer high levels of protection against virulent challenge viruses. There are several attributes of a live MD vaccine that are considered to contribute to its safety. As outlined in previous Chapters, the vaccine virus must not be pathogenic however, minimal levels of pathogenicity may be tolerable (see Section 4.1.1.). The inability of a vaccine virus to spread by contact is considered an advantage as passage of the virus through several chickens may lead to the virus reverting to virulence. This is particularly of concern for vaccines that have been derived from highly virulent strains. The capacity for a virus strain to revert to virulence was assessed in a *backpassage test*. This test involves the serial passage of a virus by chicken-to-chicken inoculation (see Section 3.4.3).

3.4.2. Further attenuation of the 60/2 clone by additional cell culture passage and evaluation in chickens

The 60/2 clone was passaged in CEFs three times per week in a similar manner to that described in Section 3.1. Each passage was stored in liquid nitrogen as stock material for evaluation in chickens.

Fifteen female day-old SPF chickens (CSIRO) were assigned to each of ten groups (Table 3.12). Two groups of day-old chickens per passage of the 60/2 clone were inoculated subcutaneously in the back of the neck with a 0.2 mL dose containing 1000 PFU. Two control groups were inoculated with diluent alone. After 10 days, birds from one of the two groups per passage and one control group were challenged with cell culture-grown Woodlands No.1 challenge virus intra-peritoneally at 100 PFU/ 0.2 mL dose. Birds were maintained for 10 weeks post-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 taken. The thymus score was graded from 0 - 3 where 3 was normal and 0 indicated total atrophy. Blood was taken at 5 weeks after vaccination and at completion of the experiment for measurements of viraemia and PCV. Five contact control birds (unvaccinated/unchallenged) per group were introduced 14 days after the commencement of the experiment to check for contact transmission of virus. These birds were removed and killed four weeks later and tested for viraemia, PCV, bursa and body weights and gross lesions.

Table 3.12 *Chicken experiment groups for additional passages of the 60/2 clone*

Group	Passage No. of 60/2 clone used for vaccination	Challenge*
Safety test		
78 alone	78	-
73 alone	73	-
68 alone	68	-
63 alone	63	-
Protection test		
78/C	78	+
73/C	73	+
68/C	68	+
63/C	63	+
Controls		
Challenged only (C)	-	+
Negative controls (Neg.)	-	-

* Woodlands No.1 cell culture-grown challenge virus, administered 10 days after vaccination at 100 PFU/ 0.2 mL bird dose.

Results and Discussion

The results for the experiment in which selected passage levels of the 60/2 clone were tested for both pathogenicity and protection after virulent challenge are shown in Table 3.13 and Figures 3.6 – 3.9. They suggest that the passage 78 material is the safest and provides the best protection, followed closely by 63 then passage 73 and 68. While many of these trends are not statistically significant, passage 78 is significantly better than passage 68 for the pathogenicity test component by body weight, thymus score and PCV. Amongst challenge (protection) groups, passage 78/C is significantly better than passage 63/C by both body weight and PCV. From the pathogenicity test component in Table 3.13, passage 63 and 78 both have the highest viraemia at 50% and an equal number of birds affected with dermatitis, however passage 78 showed no evidence of tumours and had the lowest number of overall deaths. When assessing the protective ability of these passages using a challenge, the passage 78/C material exhibited the least number of deaths, equally least number of tumours, and the highest viraemia.

Cultures taken from the dermatitis lesions grew a *Pseudomonas spp*. Interestingly, the dermatitis was only seen in the vaccinated groups which suggests that either a contaminant was present in the inoculum or the vaccine virus was causing immunodepression in these birds. In order to test for contamination, ampoules of the vaccine at each passage were cultured for bacterial growth by centrifuging the contents and inoculating the pellet onto horse blood agar (HBA) and chocolate agar plates at 35^0C in air and, HBA anaerobically for 2 days. No growth was observed. It therefore seems likely that if a *Pseudomonas* contaminant had been introduced from the vaccine, it was probably through the vaccine diluent as the passage 63 material had not previously demonstrated this problem

(Section 3.3) and contamination of cell culture stock could not be demonstrated for any of the passage material tested. Unfortunately, the diluent was unavailable for sterility testing.

The new cell culture-grown challenge virus (Section 3.2**)** was shown to be sufficiently virulent, causing 78% death or tumours in the unvaccinated challenge group. This is in conformity with the recommended figure of at least 70% (Anonymous, 1995) and that which has been found previously when using the cell culture challenge (Sections 3.2 & 3.3). The negative control birds remained uninfected by all parameters measured.

The newly tested measurements of PCV and thymus score accurately reflected other methods for assessing pathogenicity, such as the bursa: body weight ratio as previously outlined.

There was no evidence of vaccine virus transmission to contact birds by viraemia or other criteria tested, which confirms earlier observations (Sections 3.1 & 3.3) that the 60/2 clone (and other clones) have lost the ability to spread by contact.

Due to constraints in the number of birds that could be used in this experiment, a larger safety test and challenge study was conducted using the 78th passage of clone 60/2 (see Section 4.1). Passage 78 was chosen due to its generally better performance by all criteria. The large-scale testing also incorporated comparisons with other commercially used vaccines and a Protective Dose 50% test.

Table 3.13 *Chicken experiment results for additional passages of the 60/2 clone*

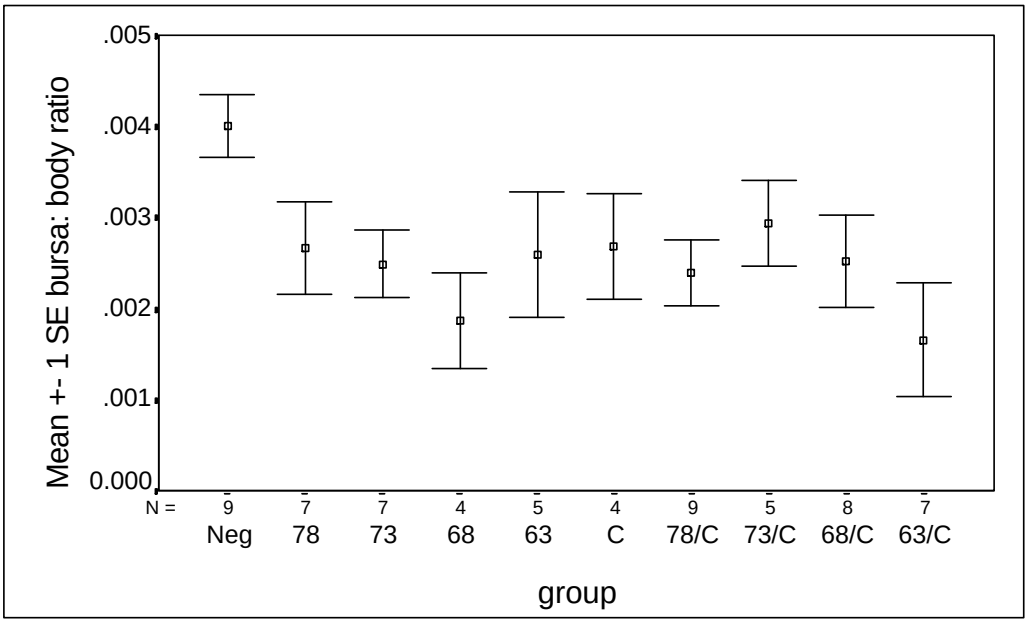
Group[a]	Tumours			Dermatitis[c]			Total Deaths[b]	Viraemia at 5
	Deaths	Survivors	Total	Deaths	Survivors	Total		weeks
78	0	0	0/11	1	2	3	3	2/4
73	0	1	1/10	2	2	4	2	0/5
68	0	1	1/10	4	0	4	6	0/4
63	1	0	1/10	3	0	3	6	2/4
78/C	1	0	1/10	0	2	2	1	2/5
73/C	1	0	1/10	3	0	3	5	1/5
68/C	1	0	1/12	2	2	4	4	1/3
63/C	1	1	2/12	3	3	6	5	0/3
Challenged only (C)	4	2	6/9	0	0	0	5	1/5
Negative control (Neg.)	0	0	0/10	0	0	0	0	0/3

a See Table 3.12

b Total deaths includes all deaths regardless of cause and may include causes other than tumours or dermatitis.

Most deaths were seen within the first 5 week period.

c Cultures of the dermatitis lesions grew *Pseudomonas spp*.

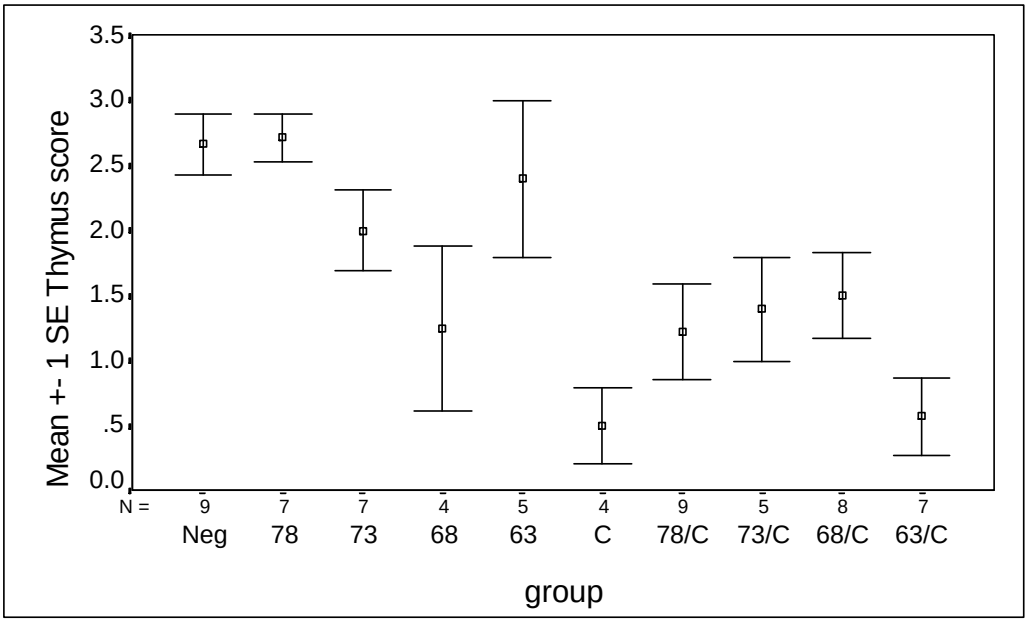


See Table 3.12 for group descriptions.
Analysis of variance (ANOVA) results:
Group effects were not significant (p 0.618)

	63/C	68	78/C	73	68/C	63	78	C	73/C	Neg
63/C										
68										
78/C										
73										
68/C										
63										
78										
C										
73/C										
Neg	*	*	*	*	*	*	*			

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

Figure 3.6 *Bursa: body weight ratio (mean ± SE) for additional passages of the 60/2 clone*

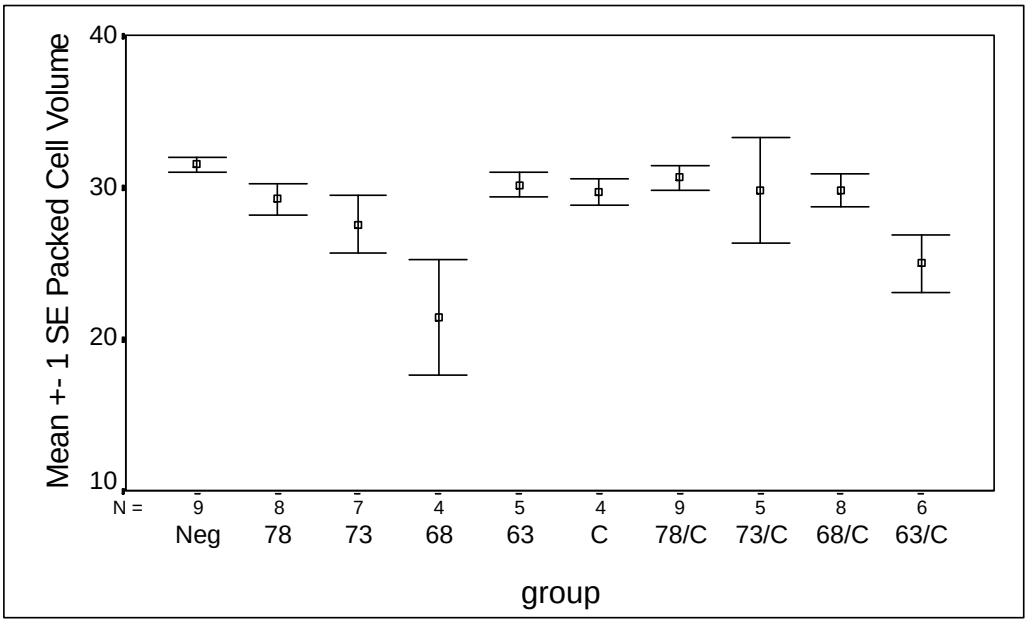


Analysis of variance (ANOVA) results:
Group effects were significant (p0.000)

	C	63/C	78/C	68	73/C	68/C	73	63	78	Neg
C										
63/C										
78/C										
68										
73/C										
68/C										
73	*	*	*							
63	*	*	*							
78	*	*	*	*	*	*				
Neg	*	*	*	*	*	*				

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

Figure 3.7 *Thymus scores (mean $\pm$ SE) for additional passages of the 60/2 clone*

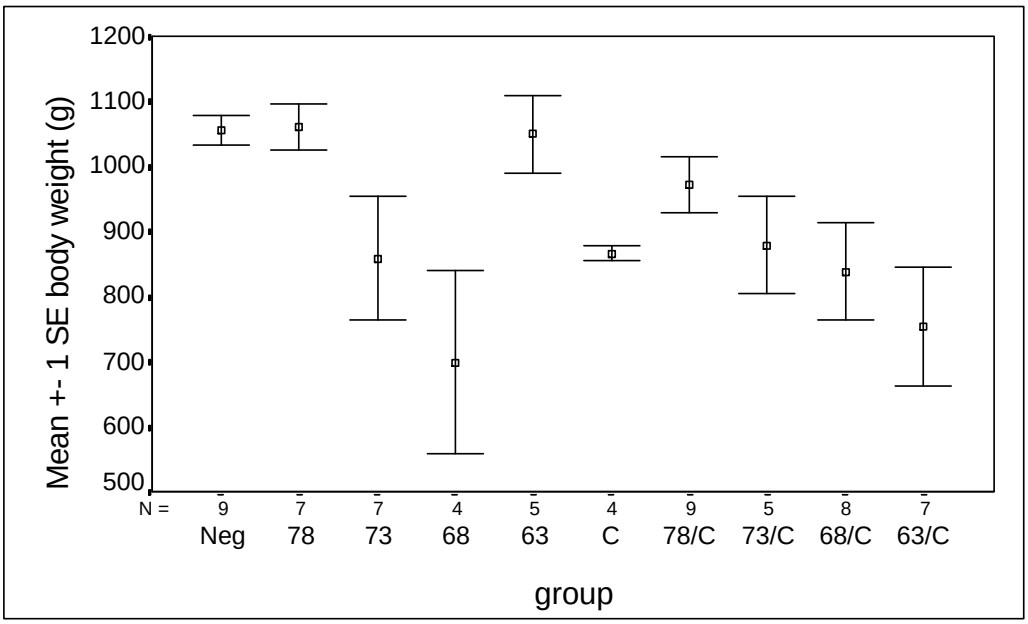


Analysis of variance (ANOVA) results:
Group effects were significant (p0.006)

	68	63/C	73	78	C	73/C	68/C	63	78/C	Neg
68										
63/C										
73	*									
78	*									
C	*									
73/C	*									
68/C	*	*								
63	*	*								
78/C	*	*								
Neg	*	*								

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

Figure 3.8 *Packed cell volume (mean ± SE) for additional passages of the 60/2 clone*



Analysis of variance (ANOVA) results:
Group effects were significant (p0.007)

	68	63/C	68/C	C	73/C	73	78/C	78	63	Neg
68										
63/C										
68/C										
C										
73/C										
73										
78/C	*	*								
78	*	*	*							
63	*	*	*							
Neg	*	*	*							

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

Figure 3.9 *Body weight (mean $\pm$ SE) for additional passages of the 60/2 clone*

3.4.3. Tests for reversion to virulence of the 60/2 clone at passage 78

Safety tests were conducted in accordance with the method described in the British Pharmacopoeia (Veterinary) 1993, Addendum 1995 (Anonymous, 1995). The method describes an *in vivo* test for reversion to virulence and is used for attenuated vaccine strains of MDV.

In the test performed, each of ten day-old SPF chickens (CSIRO) was inoculated with 40,000 PFU/ bird (equivalent to 10 x 4,000 PFU/ bird, the minimum recommended dose for the TMC Rispens vaccine) of the test vaccine 60/2 clone at passage 78, subcutaneously with 0.2 mL in the back of the neck. Chickens were maintained for 7 days in a bubble isolator at RMIT, then killed and bled. Approximately 2 mL of blood per bird was recovered. Lymphocytes were isolated from each blood sample, pooled and then diluted in 5 mL of CFM. Each bird of a new group of day-old SPF chickens was inoculated with 0.2 mL of this suspension (1 mL/ bird is specified in the British Pharmacopoeia method, however, the suspension used was concentrated and 1mL was considered to be too large a volume to be administered to day-old chicks); excess suspension was stored at -80°C. This procedure was repeated four more times so that the vaccine virus had been passaged a total of 6 times. Birds from the final group were observed for 10 weeks and then killed and necropsied for evidence of MD. Lymphocyte samples taken at each backpassage were assayed for viraemia and also for MDV and CAV by PCR. According to the British Pharmacopoeia method, there is no evidence of reversion to virulence if, in addition to the criteria of the safety test, there is no significant difference in microscopic lesions in the brachial and sciatic plexuses and the gonads of birds having received the backpassaged virus to those having received the unpassaged virus.

Results

According to the method described in the British Pharmacopoeia, the final safety test should be performed on the 6^{th} backpassage of virus or the highest passage where virus can be recovered from the birds. Initially, five backpassages of the virus were carried out. Viraemia could not be detected in any of the pooled lymphocytes collected from birds at any backpassage. The difficulty experienced in demonstrating viraemia when using the 60/2 clone has been observed in earlier studies (Sections 3.1.4 & 3.4.2) and will be discussed later.

The test was repeated in a second series, again to five backpassages, however viraemia could still not be demonstrated and therefore the guidelines for the reversion to virulence test were fulfilled and the safety test on the final backpassage no longer required. However, although not required by the test guidelines, a standard 10-week safety test was performed due to the detection of virus by the more sensitive MDV PCR test. By the PCR, virus could be detected in the pooled lymphocytes for each backpassage of both the first and second series of backpassages. Six SPF birds were inoculated intra-peritoneally with 0.2 mL of the pooled lymphocytes from the fifth backpassage of the second series. Birds were bled at 3 weeks after inoculation (approx. 2 mL per bird) and tested for viraemia by cell culture and PCR methods (see Table 3.14).

Table 3.14 *Detection of viraemia by cell culture and PCR following backpassage of the 60/2 clone at passage 78*

Backpassage No.	Viraemia[a]	
	Cell culture	**PCR**
1	-	+
2	-	+
3	-	+
4	-	+
5	-	+
6	-	+

a Pooled lymphocytes. Blood was collected at seven days post-inoculation, except for backpassage 6 where blood was collected at 21 days.
- Not detected
+ Detected

Discussion

Viraemia could not be detected by cell culture methods in any of the backpassages (Table 3.14) which reflects the low levels of viraemia previously observed for the 60/2 clone (Tables 3.3; 3.13). In the initial selection of clones (Section 3.1.4.), only 44% of the 60/2 clone birds were positive for viraemia 6 weeks after inoculation compared with up to 100% for the lower passage clones (Table 3.3). Lower passage clones also obtained higher levels of viraemia (Table 3.3**;** Figure 3.4) compared to the 60/2 clone. In Section 3.4 where various passage levels of the 60/2 clone were tested in chickens, viraemia was only noted in 50% of birds 5 weeks after inoculation for the passage 78 preparation. However, in the backpassage test the blood was pooled which may have resulted in positive samples being diluted to undetectable levels. In addition, the blood was collected only 7 days after inoculation, whereas blood was collected later in other experiments at periods closer to the time of the peak viraemia, usually 2 - 3 weeks after inoculation. It is interesting to note that low levels of viraemia were also demonstrated for the Rispens vaccine strain (Table 3.11). The Rispens vaccine when inoculated at the standard dose of 4,000 PFU, produced viraemia in 33% of birds after 14 days compared to 100% for HVT.

Virus could be detected by PCR at each backpassage (Table 3.14). PCR is more sensitive than cell culture for virus detection and allows the detection of very few copies of the MDV genome. In addition, PCR can detect virus that may be latent. The greater sensitivity of this method allowed the detection of MDV in all of the backpassage samples which were negative by cell culture methods (Table 3.14).

Although not a requirement of the test, a safety test at the sixth backpassage was performed to more fully characterise the safety of the vaccine. Although the number of birds used for this test were less than that specified in the method, none of the 6 birds at the sixth backpassage developed any evidence of MD over the 10-week observation period or at autopsy, which indicated that the 60/2 clone at passage 78 did not show any tendency to revert to virulence. In addition to this, earlier experiments (Sections 3.1.4 & 3.4.2) showed no evidence of contact transmission using the 60/2 clone so that bird-to-bird passage in the field is an unlikely event, which is a desirable property for a live attenuated vaccine such as this.

4. Australian Serotype 1 vaccine: Experimental challenge studies

4.1 Large scale chicken tests of the 60/2 clone at passage 78

4.1.1 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 SPF birds were vaccinated with 2000 PFU or 40 000 PFU of clone 60/2 at passage 78 and were maintained for 10 weeks. At the end of the 10 week period, gross lesions were observed and measurements were made of individual bursa and body weights and thymus score. Birds were also examined histologically.

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 another small-scale trial (Section 3.4.2) to assess attenuation of the 60/2 clone after additional passage in cell culture (Table 3.13).

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; although not statistically significant, vaccinated groups showed slightly lower ratios. Thymus scores 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 (Sections 3.3.4. & 4.1.3.), 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). 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.

4.1.2 Determination of 50% Protective Dose

Day-old SPF chicks were inoculated with 1000, 200, 40, 8 or 1.6 PFU/0.2 mL dose of the RMIT vaccine (clone 60/2 passage 78) and maintained for 10 weeks after challenge with the cell-culture prepared MPF 57 challenge virus. At the end of the period, birds were examined according to the same criteria as in Section 3.1.4.

The 50% Protective Dose (PD_{50}) of the RMIT vaccine was calculated to be 97.7 PFU/dose and 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 expect, 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.

4.1.3 Comparison of the RMIT vaccine with commercial vaccines

This experiment was conducted to compare the efficacy of the RMIT and Rispens vaccines and other commercial vaccines in large numbers of birds. Day-old SPF birds were vaccinated subcutaneously (s.c) in the back of the neck with 0.2 mL of the appropriate vaccine and dose (Table 4.1). Nine days after vaccination all groups, except for the contact control (negative) group, were challenged with the cell culture-grown MPF 57 challenge virus and then maintained for 10 weeks. 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.

Table 4.1 *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.

Results and Discussion

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%; Table 4.2). 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 4.1). The validity of the challenge using cell culture-grown MPF 57 challenge virus (Chapter 3.2) is apparent from the 92% incidence of MD in the positive controls, confirming the results obtained in earlier experiments (Sections 3.3.4 & 4.1.2)

Table 4.2 *Protection results for large scale comparison of RMIT and commercial vaccines in SPF chickens challenged with MPF 57.*

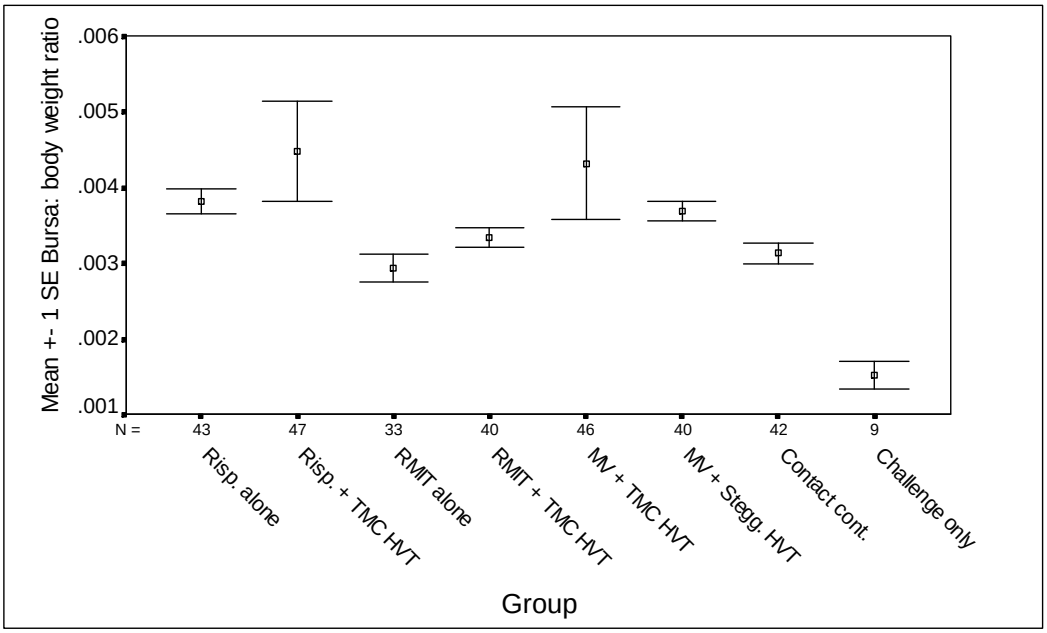
Group	MD			Group size	MD Total	Protective Index[a]
	Deaths	Tumours	Total		%	(PI)%
Rispens alone	1	0	1	45	2.2[a]	97.6
Rispens + TMC HVT	2	0	2	49	4.1[a,b]	95.5
RMIT alone	6	1	7	40	17.5[b]	81.0
RMIT + TMC HVT	3	0	3	43	7.0[a,b]	92.4
MV + TMC HVT	3	4	7	49	14.3[a, b]	84.5
MV + Stegg HVT	8	11	19	48	39.6[c]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

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

a,b,c, Different superscripts when in the same column indicate significant differences ($p<0.05$)

As expected from the experimental design, the contact control group experienced a decrease in bursa: body weight ratio (Figure 4.1) 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 4.1.1.).

The thymus scores (Figure 4.2) indicated 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.

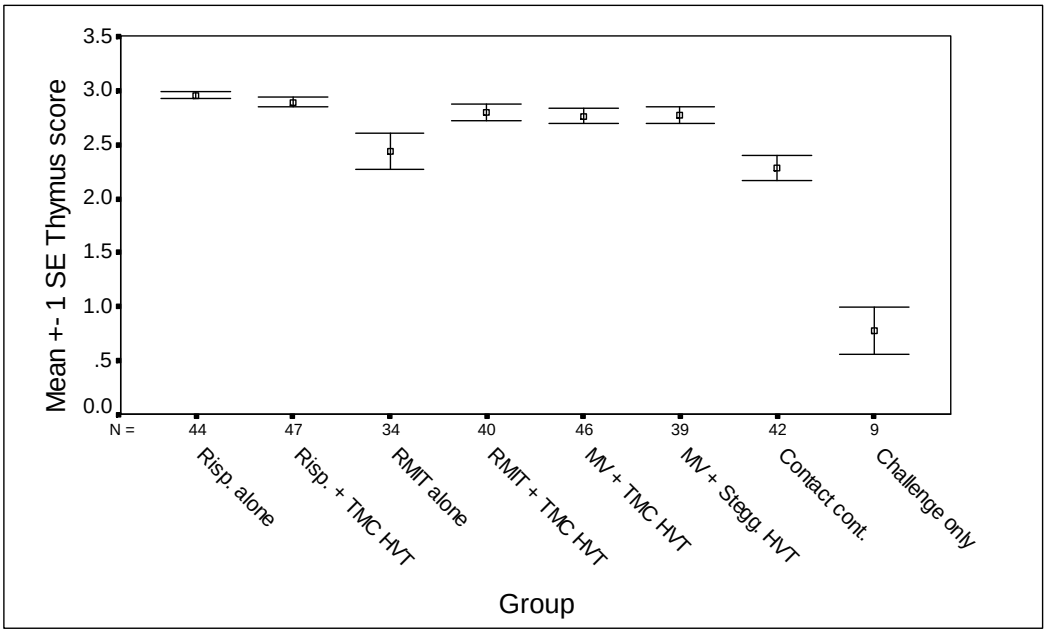


Analysis of variance (ANOVA) results:
Group effects were significant (p0.024)

	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 4.1 *Bursa: body weight ratio (mean ± SE) for large scale comparison of RMIT and commercial vaccines in SPF chickens.*



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 4.2 *Thymus scores (mean $\pm$ SE) for large scale comparison of RMIT and commercial vaccines in SPF chickens.*

4.1.4 Discussion

In Section 3.4, it was shown that various passage levels of the 60/2 clone (RMIT vaccine) 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 4.1.1.), 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.

Bursa:body weight ratios were only moderately lower than the negative control birds and 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. In protection trials using commercial birds (see Section 3.3), no dermatitis was reported, probably because commercial birds are usually less susceptible to MDV than SPF birds and often possess maternal antibodies (see Section 4.1.1.). However, no tumours were detected in the large-scale safety test of the RMIT vaccine.

Although the Rispens vaccine appeared to perform marginally better than the RMIT vaccine (Section 4.1.3.), 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 environmental factors, 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.

4.2 Evaluation of the RMIT serotype 1 vaccine in commercial birds and its comparison with commercially available vaccines

4.2.1 Introduction

In Section 4.1, the RMIT serotype 1 MD vaccine was shown to be relatively safe and efficacious, giving a comparable rate of protection in SPF birds as the commercially available Rispens vaccine. However, the efficacy of the vaccine under field conditions may depend upon many other factors such as genetic characteristics of a chicken and its maternal antibody status.

Because of this, a trial involving commercial birds was undertaken to assess the RMIT vaccine and to compare it with commercially available vaccines.

4.2.2 Materials and Methods

Sixty-three female day-old Cobb chickens (supplied by BAIADA Hatchery, Kootingal, NSW) were assigned to each of eight vaccine groups (Table 4.3.) and were identified by an aluminium tag inserted through the wing web. The parent flock was more than 40 weeks of age and had been vaccinated with the Rispens vaccine. Each bird was vaccinated at day-old s.c in the back of the neck with 0.2 mL of the appropriate vaccine and dose (Table 4.4). The vaccine was diluted in Cell Culture Medium and mixed vaccines were combined in the one 0.2 mL dose. The two control groups were inoculated with diluent alone.

All birds, with the exception of the negative control group; were housed together on the floor of a single controlled environment room to inlet and outlet airflow at the Victorian Institute of Animal Science (VIAS), Attwood. In accordance with standard commercial practice, birds were controlled fed to limit their growth to the industry standard.

One week after vaccination, 10 birds per group selected at random were killed and tested for chicken anaemia virus (CAV) and their packed cell volume (PCV) determined.

Nine days after vaccination all groups, except for the negative control, were challenged intra-peritoneally with the standard dose (50 PFU/0.2 mL) of a cell-culture preparation of MPF 57 (De Laney *et al.* 1998, Morrow *et al*. 1997).

Birds were maintained for 10 weeks after challenge and any that died or required euthanasia were examined for gross and histological lesions. After ten weeks, all remaining birds were euthanased, examined for gross lesions and assigned a thymus score; measurement of bursa and body weights were then taken. Thymus scores were graded 0-3 where a score of 3 was normal and one of 0 indicated total atrophy.

Histology was performed on 10 randomly chosen birds per group and on any suspect tissues; tissues examined included brachial, sciatic and caeliac nerves, left gonad, kidney, liver, heart, lung and brain. Assessment of lesions was by the criteria previously used in Section 3.1.4 (Table 3.2), and scores of each bird were summed and averaged per group. The final histology score was derived by subtracting the mean score of the control group from the mean score of the vaccine group. Gross and histological examination was used to confirm the presence of MD for birds that died during the experiment.

***Table 4.3**. Vaccine groups for protection comparison using commercial Cobb birds challenged with MPF 57.*

Vaccine	No. of birds
RMIT alone	52
RMIT + The Marek's Company (TMC) HVT	52
Rispens alone	52
Rispens + TMC HVT	52
TMC HVT alone	52
Maravac + TMC HVT	52
Negative control (nonvacc./non challenged)	52
Positive control challenge (nonvacc.)	52
Total	416

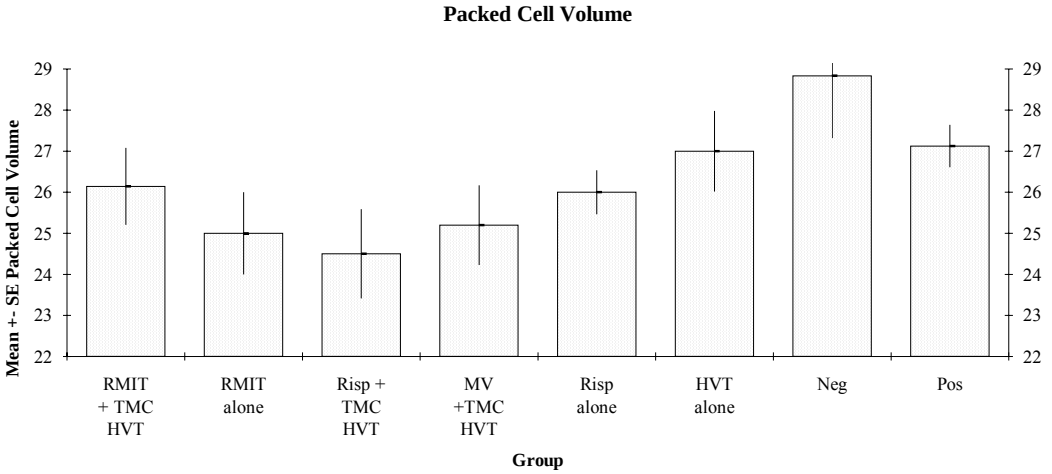
***Table 4.4.** Dose of vaccine given to day-old birds.*

Vaccine	Batch	Dose
RMIT (Woodlands 60/2 passage 78)	02/06/97	4,000 PFU*
The Mareks' Company (TMC) Rispens	M7101	4,000 PFU*
TMC HVT	H7301	8,000 PFU*
Fort Dodge Maravac (MV)	70470	2,000 PFU

*Titre determined by the RMIT plaque-assay method and the vaccines administered at the minimum recommended dose.

4.2.3 Results

All birds tested negative for CAV by PCR at eight days of age. The remaining results are presented in table and graph form.



Analysis of variance (ANOVA) results:

Group effects were not significant (p0.087)

PCV

	RMIT+ HVT	RMIT	Rispens+ HVT	MV+ HVT	Rispens	HVT	Pos	Neg
RMIT+HVT								
RMIT								
Rispens+HVT								
MV+HVT								
Rispens								
HVT								
Pos								
Neg		*	*	*	*			

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

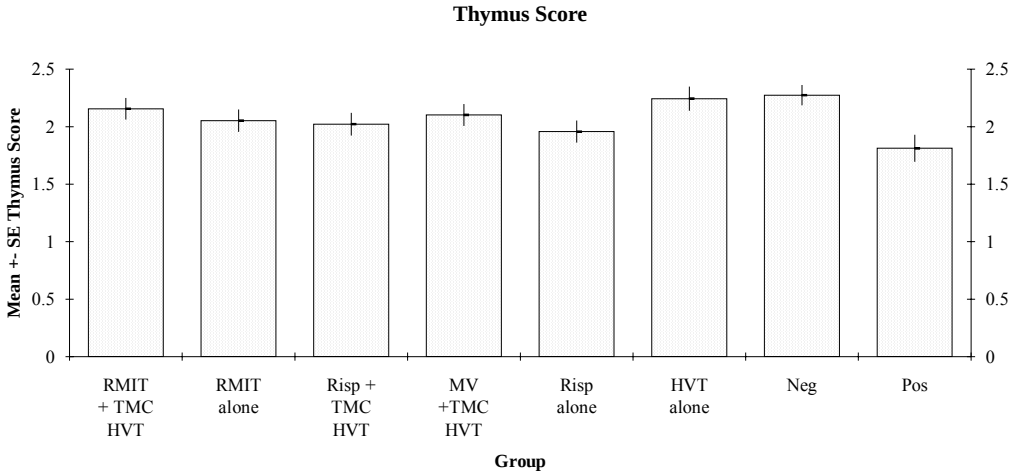
Figure 4.3*. Packed Cell volume. One week after vaccination, blood samples were taken from 10 random commercial birds per group, heparinised and loaded into capillary tubes. Tubes were then placed in a microhaematocrit centrifuge and then the PCV read by placing the capillary tube against the appropriate sized segment of a haematocrit grid and the percentage of packed cells was determined. The results are presented as the mean percentage haematocrit reading +/- the standard error of the mean (SE).*

Table 4.5 *Protection results of large-scale comparison of RMIT and commercial vaccines in commercial chickens challenged with MPF 57. Birds that died prior to challenge were not included in the protection calculations.*

Group	MD			Group size	MD Total %	Protective Index[b] (PI)%
	Deaths	Tumours[a]	Total			
RMIT alone	11	1	12	50	24.0	66.5
RMIT + TMC HVT	1	1	2	49	4.1	94.3
Rispens alone	1	2	3	49	6.1	91.5
Rispens + TMC HVT	1	0	1	45	2.2	96.9
TMC HVT alone	0	4	4	45	8.9	87.6
MV + TMC HVT	0	6	6	49	12.2	83.0
Negative control	0	0	0	51	0.0	100
Positive control (Challenge only)	18	15	33	46	71.7	

[a] Tumours do not include perivascular cuffs and do not include tumours of birds that died during experiment (these are represented under deaths).

[b] Protective Index (PI%)= $\frac{\%\text{MD Positive control} - \%\text{ MD observed group}}{\%\text{MD Positive control}} \times 100$



Analysis of variance (ANOVA) results:

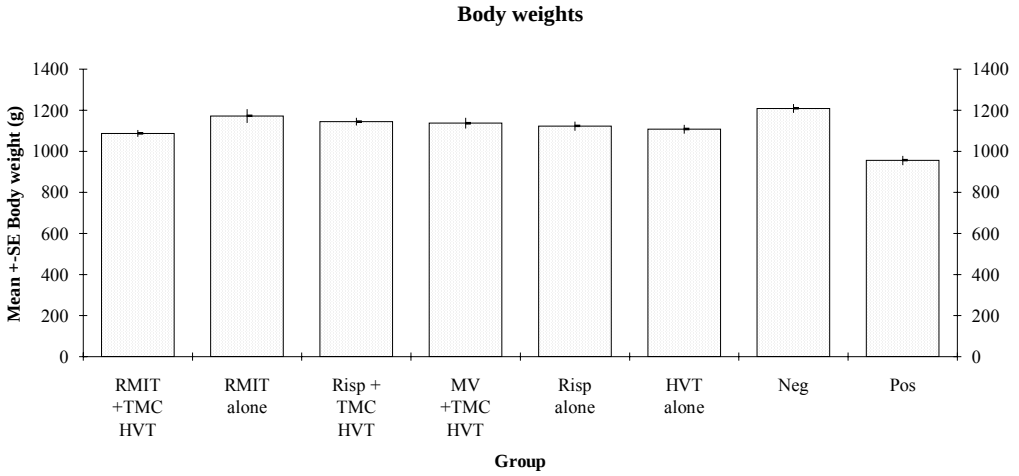
Group effects were not significant (p0.064)

Thymus score

	RMIT+HVT	RMIT	Rispens+HVT	MV+HVT	Rispens	HVT	Pos	Neg
RMIT+HVT								
RMIT								
Rispens+HVT								
MV+HVT								
Rispens								
HVT					*			
Pos	*					*		
Neg					*		*	

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

Figure 4.4 *Thymus Score of commercial chickens. At ten weeks after challenge all remaining birds in the experiment were euthanased and assigned a thymus score. Thymus scores were graded 0-3 where a score of three was normal and one of zero indicated total atrophy. The results are presented as the mean thymus score per group +/- the standard error of the mean (SE).*



Analysis of variance (ANOVA) results:

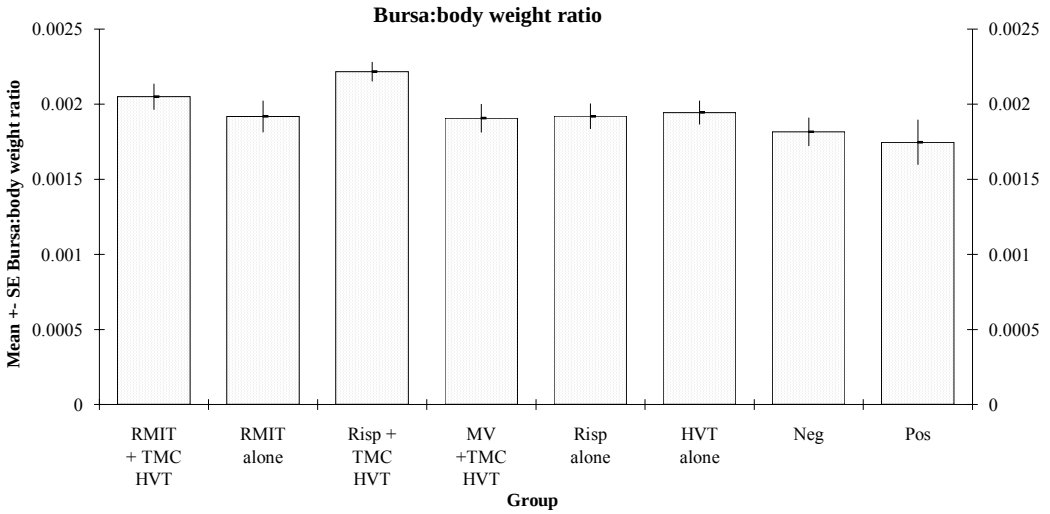
Group effects were significant (p0.00)

Body weights

	RMIT+HVT	RMIT	Rispens+HVT	MV+HVT	Rispens	HVT	Pos	Neg
RMIT+HVT								
RMIT	*							
Rispens+HVT								
MV+HVT								
Rispens								
HVT								
Pos	*	*	*	*	*	*		
Neg	*		*	*	*	*	*	

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

Figure 4.5 *Body weights of commercial chickens. At the end of the experiment the body weights of each bird in each group were taken and averaged. The results are presented as the mean bird body weight +/- the standard error of the mean (SE).*



Analysis of variance (ANOVA) results:

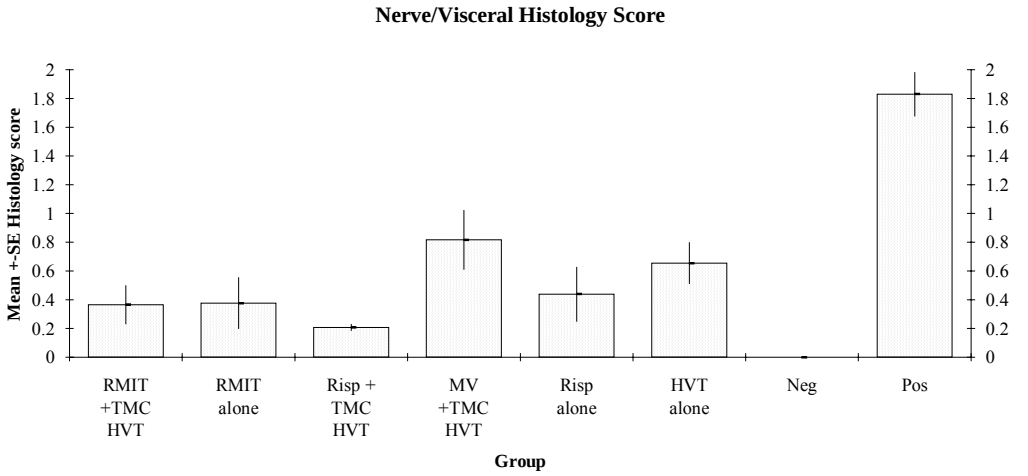
Group effects were significant (p0.035)

B:B weight ratios

	RMIT+HVT	RMIT	Rispens+HVT	MV+HVT	Rispens	HVT	Pos	Neg
RMIT+HVT								
RMIT								
Rispens+HVT		*						
MV+HVT			*					
Rispens			*					
HVT			*					
Pos	*		*					
Neg			*					

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

Figure 4.5 *Bursa:body weight ratios of commercial chickens of various vaccine groups. After euthanasia at ten weeks after challenge, the body and bursa weights of the birds were taken and graphed as a mean ratio +/- the standard error of the mean (SE).*



Analysis of variance (ANOVA) results:

Group effects were significant (p0.002)

Histology Score

	RMIT+HVT	RMIT	Rispens+HVT	MV+HVT	Rispens	HVT	Pos	Neg
RMIT+HVT								
RMIT								
Rispens+HVT								
MV+HVT								
Rispens								
HVT								
Pos	*	*	*	*	*	*		
Neg				*			*	

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

Figure 4.6 *Nerve/Visceral Histology Score of commercial chickens. Histology was performed on 10 randomly chosen birds per group and on any suspect tissues. The assessment of the lesions was by the criteria in Table 3, and scores of each bird was summed and averaged for the group. The final histology score (as plotted) was derived by subtracting the mean score of the negative control group from the mean score of the vaccine group +/- the standard error of the mean (SE).*

4.2.4 Discussion

The packed cell volume of a blood sample is a useful objective correlate of the incidence of MD in challenged birds. However, the PCV results of this trial are only indicative of the residual virulence in the vaccines. From Figure **1**, the PCV of birds following vaccination was reduced, although the difference with unvaccinated birds was not significant.

Table 4.6 indicates the highest rate of protection was obtained by the Rispens vaccine when used in combination with TMC HVT (PI 97). The RMIT vaccine also used in combination with HVT produced a comparable rate of protection, although when used alone, provided poor protection. The incidence of MD in the positive control, which was derived from a vaccinated flock, was lower than obtained for SPF birds in the previous study (Section 4.1) indicating that maternal antibodies alone can provide protection, albeit small in this instance.

With respect to thymus score, TMC HVT vaccine performed better than the others (Figure 4.4) The RMIT vaccine when used in combination with HVT also performed well. The positive control group showed an unusually high thymus score with only two vaccinated groups (HVT and RMIT + HVT) significantly higher.

Body weights (Figure 4.5) for the challenge group were less than the negative controls, which were housed separately. They were the highest for the group vaccinated with the RMIT vaccine and were the lowest for the RMIT vaccine when used in combination with HVT. These results do not correlate with the thymus score or protection data obtained when the RMIT vaccine was used in combination with HVT. Birds vaccinated with Rispens + HVT also performed well by this criterion. These results do correlate with results from other sections of the experiment. Statistically, body weights for the RMIT vaccine and Rispens + HVT were not significantly different than those from other vaccine groups.

The bursa:body weight ratios (Figure 4.5) show a similar trend to that reflected by the protective index figures. Once again, the RMIT vaccine in combination with HVT performed well. Rispens in combination with HVT performed best. Groups receiving these two vaccines also performed well and are the only two groups that differ significantly from the positive control.

As expected, the positive control group had the highest histology score (Figure 4.6), indicating that those birds had a greater number of lesions than any vaccinated group. Birds vaccinated with Rispens in combination with HVT had the least lesions followed by RMIT in combination with HVT. From histological scores alone, the RMIT vaccine when used alone gave the highest rates of protection, however, scores from all vaccine groups were not statistically different from one another.

5. Experimental challenge studies of commercially available vaccines in Australia

The Australian poultry industry has traditionally relied upon Australian-developed vaccines to protect the industry. However, at a time of increasing Marek's problems and apparent failure of existing vaccines, there was no commercially available, Australian-derived type 1 vaccine. The importation of CR6 was inevitable and called for a series of trials to evaluate, firstly, the performance of existing local vaccines and the newly imported serotype 1 vaccine.

5.1 Evaluation of Australian serotype 2 and 3 vaccines in commercial birds

5.1.1 Introduction

Most of the vaccine studies reported in the international literature use SPF birds. The main advantage of these birds is that they do not have maternal antibody, since the parental stock are not vaccinated. However, the conduct of challenge trials in such birds does not represent what is happening in a commercial situation. Firstly, they are often of a genetic type no longer found in common commercial use and, secondly, commercial birds do have maternal antibody which is known to interfere with vaccination at day old. This study investigates the responses of layer birds (Isa) and broiler breeder hens (Cobb) to currently available Australian type 3 (HVT) and type 2 (Maravac) vaccines. Commercial birds were used to establish how these existing Australian vaccines perform in the presence of maternal antibody, resembling a commercial situation (as opposed to SPF birds).

This experiment was carried out at the Animal Research Institute (ARI), Qld. by Dr. Peter Young and included the selection of three, commercially available, vaccines: Webster HVT (NSW 1/70) cell free type 3 vaccine; Webster Maravac (MD19) cell associated type 2 vaccine; and Steggles Vaccine Laboratories HVT cell-associated type 3 vaccine.

5.1.2 Experimental design

Twenty-five commercial Isa or Cobb birds per treatment group were used, with two additional control groups unvaccinated. The vaccine treatment given to each group is listed in Table 5.1. The vaccines were given at the manufacturers recommended dose using diluents supplied by same. Both Isa and Cobb birds were provided by Baiada Poultry and were vaccinated at their hatchery s.c. into the back of the neck at day-old. Twenty-four day-old chicks from the same batch as the experimental group were euthanased and blood samples collected; the serum was frozen and stored. Additional vials of vaccine (sister vials) were supplied by the manufacturers and after vaccination, were returned for titration.

Birds in groups A-F were challenged at day 9 with an intra-peritoneal (ip) injection of a lymphocyte preparation of the Woodlands No. 1 strain. The birds were observed for ten weeks after challenge, and were culled if they appeared severely depressed or moribund. All surviving chicks were bled and their sera submitted to assess their immunological status with respect to CAV, IBDV and REV. The birds were then euthanased and necropsied for MD lesions.

5.1.3 Results and Discussion

The Isa birds showed signs of MD earlier than the Cobb birds and all vaccinated groups appeared to be depressed and doing poorly. Autopsy results showed that the unvaccinated, non-challenged birds were exposed to MDV. Unfortunately this was due to management problems.

The Protective Indices (PI) for all vaccination treatments were disappointingly low (Table 5.1). It is generally accepted that the PI should be greater than 70 if the vaccine is to have any protective effect in the field. However, it should be remembered that nearly all the vaccine studies reported in the literature were undertaken with SPF birds which are free of maternal antibody. In this trial, commercial layer and broiler breeds were used and the presence of maternal antibody undoubtedly had a marked effect.

The non-vaccinated Isa birds had 90% lesions which is quite satisfactory. However, the Cobb birds had only 69% lesions. This indicates that the Cobb birds may have some innate resistance since the same challenge virus was used for both groups.

For the Isa birds, each of the vaccinated groups was sufficiently different from the non-vaccinated control group; that is, there was a significant effect of vaccination. However, there was no significant difference among the different vaccine groups even though the PIs ranged from 20 to 52. Of the Cobb birds, three of the vaccinated groups were not statistically different from the non-vaccinated controls (HVT cell-free, Maravac alone and Maravac + HVT cell-free). Groups receiving HVT cell-associated and Maravac + HVT cell-associated were not significantly different from each other.

Overall, the results indicate that there may be some benefit from HVT cell-associated vaccine compared with HVT cell-free, at least in Cobb birds. Results between these two groups could have been anticipated, as cell-associated HVT is recognised in literature world-wide as more protective (Witter, 2001), perhaps due to the greater susceptibility of cell-free HVT to neutralisation by maternal antibody (Prasad, 1978; Witter & Burmester, 1979). These observations can now hold true for Australian HVT vaccine strains, however, further studies need to be carried out to determine if this preliminary result is valid.

Table 5.1 *Protection evaluation of Australian MD vaccines in commercial birds by virulent challenge with Woodlands No. 1 MDV.*

Vaccine/Treatment Group	**Isa**		**Cobb**	
	Percent lesions	**Protective Index* (PI%)**	**Percent lesions**	**Protective Index (PI%)**
A - Webster HVT (cell-free)	60	33 (a**)	49	30 (a)
B - Steggles HVT (cell-assoc.)	44	52 (b)	41	41 (b)
C - Webster Maravac	63	31 (a)	72	0 (a)
D - Maravac + Webster HVT (cell free)	53	43 (b)	60	13 (a)
E - Maravac + Steggles HVT (cell-assoc.)	73	20 (a)	24	66 (c)
F - non-vaccinated	90	NA (a)	69	NA (a)
G - non-vaccinated and non-challenged				

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

**No significant difference among groups with common letters

5.2 Evaluation of CR6 and Australian serotypes 2 and 3 vaccines in commercial birds

5.2.1 Introduction

Government approval to allow the importation of CR6 into Australia facilitated its inclusion into the vaccine efficacy trials. The following experiment was performed at the ARI by Dr. Peter Young and investigated the efficacy of local and CR6 vaccines in commercial birds of known maternal antibody status. The test vaccines were the recently imported Webster Clone CR6 type 1, Webster Maravac (MD19) cell-associated type 2, Webster HVT (NSW 1/70) cell free type 3 and Steggles Vaccine Laboratories cell associated HVT type 3.

5.2.2 Experimental design

The experiment consisted of two components that were run simultaneously. Part A consisted of four treatments and two control groups by two different (maternal antibody status) bird types conducted in isolators. Part B consisted of two treatments and two control groups conducted in group pens in a Hepa-filtered shed. The non-vaccinated, non-challenged control group was housed in a separate shed. All treatment and control groups were replicated. The treatment groups are listed in Table 5.2.

A total of 520 day-old female Isa chicks from HVT-vaccinated dams (hereafter referred to as "HVT maternal antibody") and 320 day-old female Isa chicks from CR6 vaccinated dams (hereafter referred to as "CR6 maternal antibody") were supplied by Baiada Poultry. The chicks were sexed at the hatchery prior to their dispatch to ARI. Twenty day-old chicks from each of the two antibody backgrounds and from the same hatch as the experimental chicks, were euthanased and blood samples were taken at ARI prior to vaccination. The serum from these samples was frozen and stored. Blood was also collected from a random sample of 20 breeder hens from each of the two donor flocks. Sera were submitted for testing for CAV, IBDV, REV, HVT, MDV and BLS.

The chicks were vaccinated sc into the back of the neck at the manufacturers' recommended dose using Maravac diluent. Additional vials of vaccine (sister vials) were supplied by the manufacturers and after vaccination, were returned for titration.

Birds were challenged with an i.p. injection of a lymphocyte preparation of the Woodlands No. 1 strain nine days after vaccination (Part A birds) or 13 days post-vaccination (Part B birds). All birds were observed for ten weeks post-Part A challenge, and were culled if they appeared severely depressed or moribund.

Six days after the initial vaccination, chicks in Group M of Part B only were re-vaccinated with a specified vaccine (Table 5.2).

At five weeks of age, the numbers of birds in each isolator or replicate group was reduced to 20. Blood samples were collected and peripheral blood leucocytes prepared for inoculation onto primary chicken kidney cells (CK) and secondary chicken embryo fibroblast cells (CEF) for virus isolation.

At the end of the observation period, all surviving chicks were bled and their sera submitted to assess their immunological status with respect to CAV, IBDV and REV. The birds were then euthanased and necropsied for MD lesions.

5.2.3 Results and Discussion

In the first few weeks of the trial, difficulties arose with the water supply to isolators 4, 5, 6, 15 and 18. This resulted in a loss of replicates for treatment groups A, E and L. The remaining birds in isolators 4 and 6 (Group K, non-vaccinated; challenged) were combined. These birds had a lower lesion score (72) than the comparable group E (85). The elimination of Group K birds from the trial slightly improved the PI for the vaccine treatment groups, as shown in the column entitled 'Revised PI' (Table 5.2).

Non-vaccinated and non-challenged birds of both maternal antibody groups were successfully protected from challenge even though they were in the same shed as birds in the challenged groups. Unfortunately, the non-vaccinated, non-challenged group of Part B (which were housed in a quadrangle pen), was exposed to MD at some point, despite the fact that this location had been used for similar control groups in the past without any problems. There were several potential sources of exposure, including the post-mortem room and adjacent commercial birds used in other studies.

In Part A of the trial, the effect of the source of maternal antibody (either HVT or CR6 vaccination of the parent flock) was examined. Only three vaccines gave adequate levels of protection against challenge. These were: HVT + Maravac in HVT maternal antibody birds, HVT + Maravac in CR6 maternal antibody birds and the trivalent HVT + Maravac + CR6. The latter trivalent vaccine combination provided the best PI observed in these trials and is clearly worth trialing in field situations, provided that it is not too expensive. It is interesting to note that the bivalent HVT + Maravac outperformed the bivalent HVT + CR6 combination in birds with either HVT or CR6 maternal antibody. CR6 alone provided a very poor result, as seen in previous trials. The CR6 result was no better in chicks with HVT maternal antibody than in chicks with CR6 maternal antibody. The results from this trial indicate that the influence of maternal antibody is not due to the vaccine used in the parent flock.

In Part B of the trial the effect of a second dose of vaccine was examined. There was no benefit for a second vaccination with HVT following an initial bivalent vaccination with HVT + Maravac. The PIs were similar to those for HVT + Maravac treatments in Part A.

Table 5.2 *Protection evaluation of CR6 and Australian MD vaccines in commercial birds of known antibody status by virulent challenge with Woodlands No. 1 MDV.*

		Group	Isolator	Treatment	Total No. of birds	Lesions	% Lesions	Revised PI*	Sig **
PART A	**HVT maternal antibody**	A	5 24	CR6	2 20	2 14	73	23	a
		B	21 19	c.a. HVT + Maravac	20 20	6 3	23	76	b
		C	1 14	c.a. HVT + CR6	20 20	9 8	43	55	b
		D	12 22	c.a. HVT + Maravac + CR6	20 20	8 7	38	60	b
		E	15 2	non-vacc. + challenge	7 20	4 19	85	NA	a
		F	9 10	non-vacc. + non-chall.	20 20	0 0	0	NA	NA
	CR6 Maternal antibody	G	23 16	CR6	20 20	16 15	78	18	a
		H	13 8	c.a. HVT + Maravac	20 20	2 7	23	76	c
		I	11 7	c.a. HVT + CR6	20 20	9 7	40	58	b
		J	3 20	c.a. HVT + Maravac + CR6	20 20	0 4	10	90	c
		K	6+4	non-vacc. + challenge	18	13	72	NA	a
		L	17 18	non-vacc. + non-chall.	20 2	0 0	0	NA	NA
PART B	**HVT Maternal antibody**	M	MSI	HVT + Maravac + **2nd dose** c.f. HVT	45	10	22	73	c
		N	MSI[†]	c.a. HVT + Maravac	45	8	18	78	c
		O	MSI	non-vacc. + challenge	45	36	80	NA	a
		P	Quadrangle[‡]	non-vacc. + non-chall	44	12	27	NA	NA

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

**No significant difference among groups with common letters
[†]Medium Security Isolation Building: birds were housed in open pens within this building.
[‡]Birds were housed in an insect-proof pen in the quadrangle at ARI.

5.2.4 Conclusions

CR6 does not seem to be useful in Australian conditions as a sole vaccine, however, it may have some potential in combination with current Australian vaccines. The trivalent combination of HVT + Maravac + CR6 appeared to be particularly useful.

Current Australian vaccines in a bivalent combination (HVT + Maravac) provided adequate protection. The results from this combination were particularly consistent (PI = 76, 76, 73 and 78) in this trial, although perhaps not as high as required in commercial situations with high burdens of MDV.

It would have been useful to have been able to evaluate HVT alone to determine what contribution the Maravac component made to the protection afforded by the HVT + Maravac combination. Unfortunately, there were insufficient isolator/shed combinations to include this treatment in this trial. In the previous trial, HVT provided a PI of only 52 (Table 5.1) but given the variability of results between trials, it is difficult to make valid comparisons.

6. Laboratory studies

Various laboratory studies were carried out on aspects relating to MD in Australia. All of these studies were peformed at RMIT University and also formed part of academic studies. MD laboratory studies are essential to assist the chicken industry in the maintainence of MD control, whether they form part of newer and better vaccines, or better MDV detection methods.

Also supported by RIRDC, but not detailed here, is work performed by Dr. Peter Young at ARI, Qld. Attempts were made to develop a serotype specific enzyme-linked immunosorbent assay (ELISA) to measure post-vaccination antibody status which would provide an assessment of vaccine efficacy and coverage. However, this proved to be quite difficult. Considerable success was achieved with the development of a serotype specific PCR for serotype 1 and 3 viruses. Techniques were developed to rapidly detect MDV in blood, tumours, organs and feather follicles. In particular, techniques were developed to detect virus in blood samples collected onto filter paper. This means that only a pin prick of blood is required to detect virus and non-destructive blood samples can be obtained, even from very young birds.

6.1 Cell culture growth studies of RMIT and Rispens vaccine strains

6.1.1 Introduction

Factors such as growth rate, yield, viability and storage are important when considering the economic viability and final cost of a vaccine. As such, a vaccine strain that grows to higher titre in a shorter period of time may be considered to have an advantage over one that does not. In this chapter, a short series of experiments was conducted to determine the growth characteristics of the RMIT vaccine (60/2 clone, passage 78) and the Rispens vaccine strains.

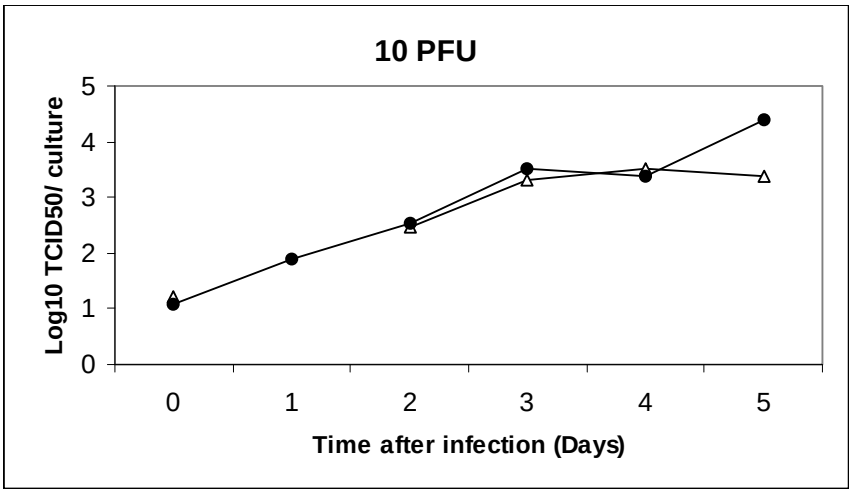
6.1.2 Methods

Day-old CEF cultures in 6-well plates (9.6 cm^2/well) were prepared for inoculation. For each vaccine (Table 6.1), dilutions were made in MEM Growth Medium so that an inoculum volume of 0.4 mL/ well contained either 10, 100, 1,000, or 10,000 PFU. Sufficient replicates of each dilution were set up so that duplicate wells inoculated with each virus concentration could be harvested after 1, 2, 3, 4 and 5 days of incubation at 37^oC in a 5% CO_2 incubator. Cultures were harvested at different times by decanting the medium, washing with PBS, then adding 0.2 mL of trypsin-versene solution and incubating at 37^oC for 5 minutes. Once cells had detached from the plate, cells were resuspended in 1.0 mL of CFM and then stored at -80^oC. After all cultures had been harvested and stored, samples were thawed and tested by the quantal assay. The quantal assay method was preferred as it required fewer cultures for the large number of tests to be performed. The results shown in Figures 6.1 – 6.4 were determined from a single assay per sample.

Table 6.1 *Vaccine batches used to estimate growth curves in cell culture*

Vaccine	Batch No.
Rispens (TMC)	M7101
RMIT (Woodlands No.1, 60/2 clone, passage 78)	08/04/97

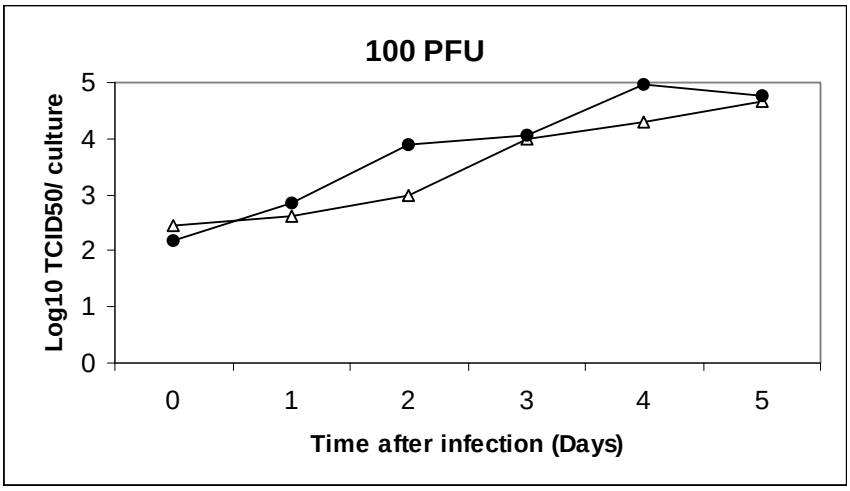
6.1.3 Results



—Δ— RMIT vaccine

—●— Rispens vaccine

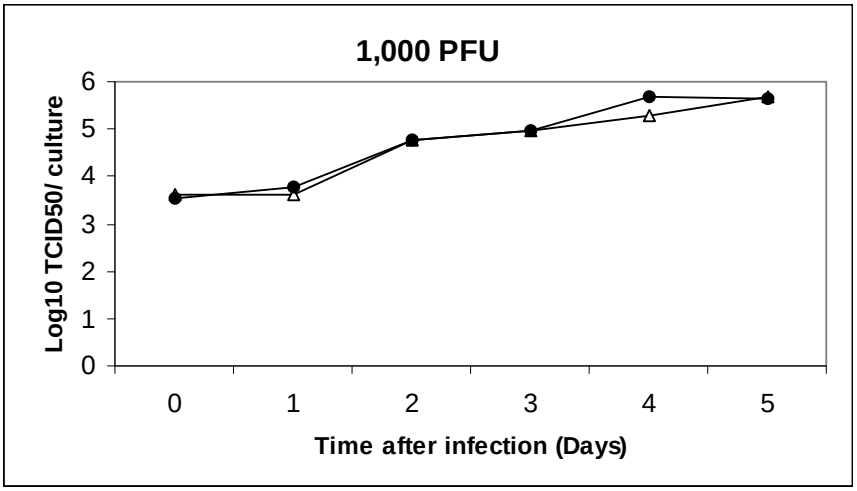
Figure 6.1 *Growth curves for the RMIT and Rispens vaccines using an inoculum of 10 PFU per culture*



—Δ— RMIT vaccine

—●— Rispens vaccine

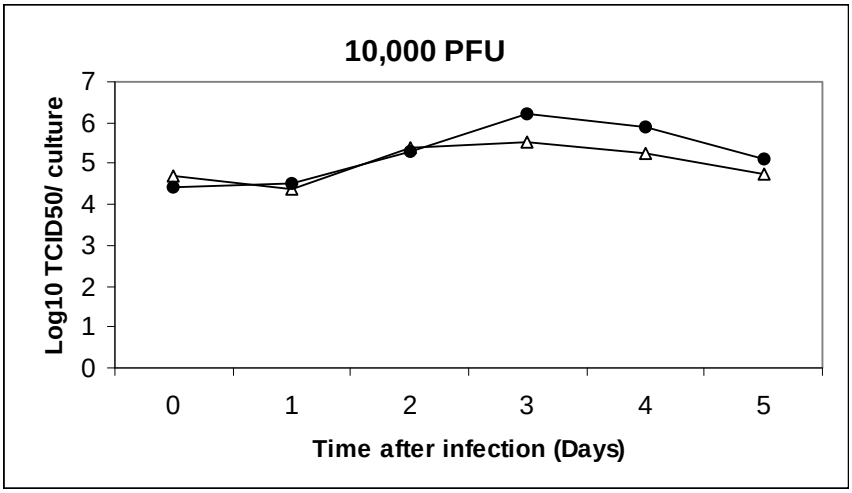
Figure 6.2 *Growth curves for the RMIT and Rispens vaccines using an inoculum of 100 PFU per culture*



—Δ— RMIT vaccine

—●— Rispens vaccine

Figure 6.3 *Growth curves for the RMIT and Rispens vaccines using an inoculum of 1,000 PFU per culture*



—Δ— RMIT vaccine

—●— Rispens vaccine

Figure 6.4 *Growth curves for the RMIT and Rispens vaccines using an inoculum of 10,000 PFU per culture*

6.1.4 Discussion

From the Figures 6.1 - 6.4, the growth characteristics of both vaccine strains appear to be comparable. There was a steady exponential rate of growth for both viruses until day 3, after which a plateau was encountered, especially for the highest inoculum dose (10,000 PFU). The exponential phase represents that part of the growth curve where most cells are actively producing virus and the relative loss due to thermal inactivation is small. After 3 days, fewer cells release virus and losses due to thermal inactivation are greater, giving rise a decrease in virus titre (Figure 6.4, day 5). Later, decreases in titre are a prominent feature when higher input titres of virus are used (Figure 6.4) as the conditions are closer to that of one-step-growth. One-step-growth occurs where there is sufficient virus titre in the inoculum to simultaneously infect all of the cells in the culture, resulting in synchronous viral growth (Howes, 1959). As such, all cells become infected and produce progeny virus within a short time, giving rise to rapid cell death.

For a cell-free inoculum, the amount of virus input relative to the number of cells in the culture is referred to as the Multiplicity of Infection (Howes, 1959). In this study where cell-associated virus containing infected cells with an unknown amount of infectious virus has been used, only virus input and output titres per culture can be examined.

It appears from this study, that the optimum time for harvest is 3 days when peak titres have been reached and before a decline in titre occurs due to thermal inactivation. An input of around 100 PFU/ culture seems to provide the best compromise between one-step-growth and optimum yield. Serotype 1 MD vaccine viruses are highly cell associated and infectious titres are dependent upon the presence of intact cells. If high numbers of intact infected cells are maintained, then minimal loss of the virus will be experienced during freezing, long-term storage, thawing and handling of the vaccine.

6.2 The development of a MDV serotype 1 specific DIG-labelled probe for the detection of MDV by dot-blot hybridization

6.2.1 Introduction

Dot-blot hybridization assays are sensitive techniques, which involve the extraction and denaturation of nucleic acid, which is spotted onto a nylon membrane and treated with a probe consisting of a labelled stretch of DNA complementary in sequence to the specific region of interest (Collier & Oxford, 2000). A number of methods are available for labelling probes using both radioactive and non-radioactive reporter molecules. A non-isotypic labelling method is the digoxigenin (DIG) system that involves the incorporation of DIG-11-dUTPs to a nucleic acid fragment corresponding to the region of interest. Labelling of the probe using DIG can be performed by a number of methods including the incorporation of DIG during a PCR reaction or using the random-primer method.

Dot-blot hybridization is a useful technique because it can screen a large number of samples on the one blot, thereby greatly reducing costs and offering greater specificity than PCR because a larger region of DNA is needed to hybridise target DNA.

The use of the DIG labelling system for the detection of target DNA using a dot-blot hybridization technique has been successfully used as a rapid detection technique for a number of viruses, including hepatitis B virus in serum (Kejian & Bowden, 1991), human parvovirus B19 infection in plasma (Hicks *et al.,* 1995) and cytomegalovirus DNA in urine samples (Musiani *et al.,* 1990).

Because of limitations associated with current detection techniques for MDV serotype 1 virus, MDV 1-specific probes used in a rapid identification assay such as a dot-blot technique, which is less expensive and more specific than those currently available, would be very useful for field diagnosis and important in vaccine evaluation.

6.2.2 Preparation and labelling of the 132 bp repeat PCR probe

A passage 13 preparation of the Woodlands No. 1 strain (De Laney *et al*., 1995) was grown in CEF cell culture, harvested and the DNA extracted by phenol/chloroform (Aly *et al*., 1993) for use as a template. The template used for the probe was the 132 bp repeat region which was amplified using the 132 bp primers described by Silva (1992). A PCR reaction was performed in which a proportion of the dTTP's were substituted with DIG-11-dUTP's using the Roche PCR DIG Probe synthesis kit according to manufacturers instructions.

Results

The gel electrophoresis photograph (Figure 6.5) shows the increase in molecular weight of the labelled 132 bp repeat PCR product (Lanes 3-6) compared with the unlabelled PCR product (two copies of 132 bp repeat and 53 bp primer sequence, giving a 317 bp product; Figure 6.5, Lane 2). This increase is due to the higher molecular weight of DIG uridine nucleoside indicating the incorporation of DIG-11-dUTP into the reaction product.

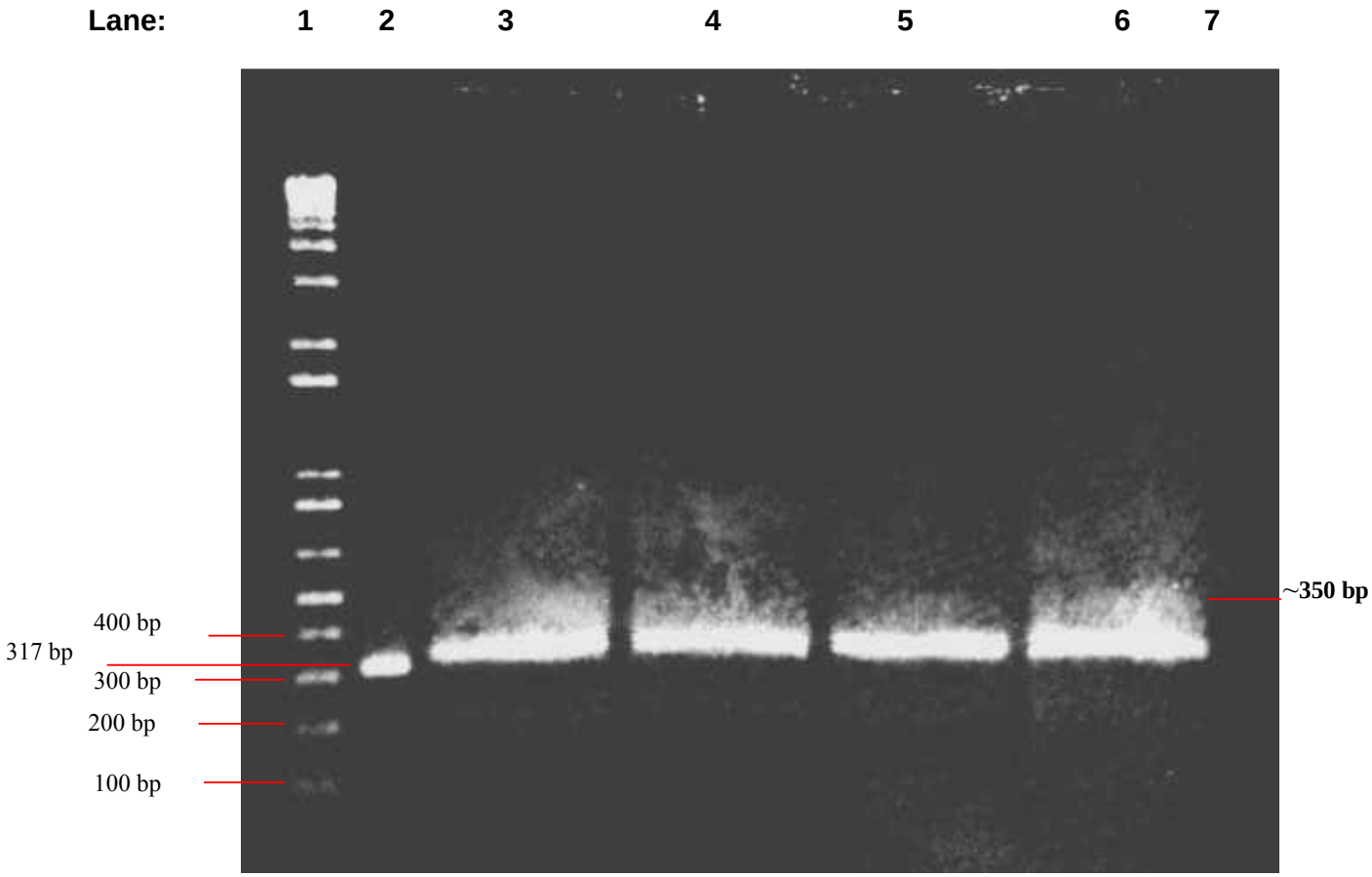


Figure 6.5 *Gel electrophoresis of 132 bp PCR product labelled with DIG*

Lane 1: 1kb plus MW markers, Lane 2: unlabelled 132 bp PCR product control, Lanes 3-6: Labelled 132 bp PCR products, Lane 7: negative control.

Determination of concentration of product yield

The concentration of labelled probe was performed after every labelling reaction, so that a standard amount could be used in each dot-blot hybridization assay. Newly labelled probe was diluted and blotted against control DNA (DIG random primed labeled pBR328 DNA) of known concentration. An estimate of the labelled DNA concentration was made according to its colour intensity in comparison with control DNA of known concentration (Figure 6.6).

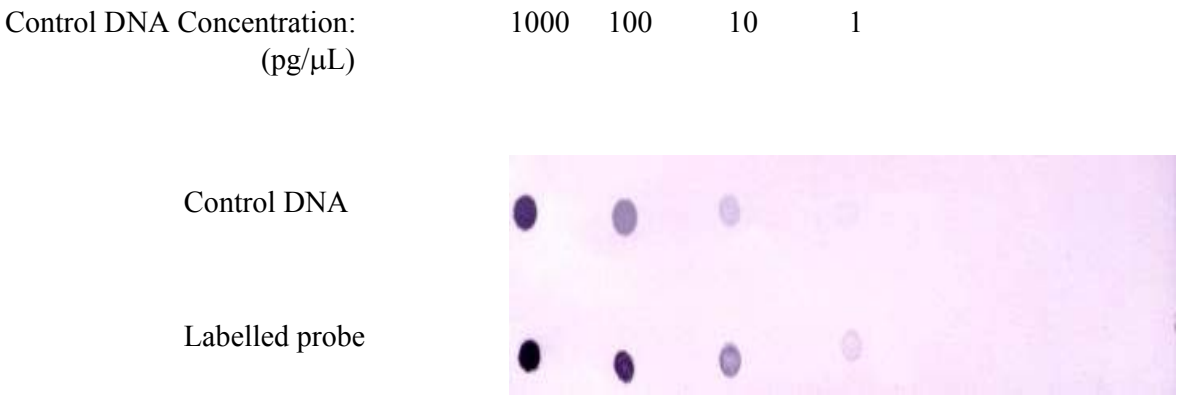


Figure 6.6 *Blot of estimation of the yield of labelled probe*

Discussion

The labelling technique was rapid and easy to perform. However, a disadvantage existed with respect to the yield of the DNA probe. The generation of high quantities of labelled probe proved difficult as the 132 bp region is relatively small and, despite the use of 35 cycles of amplification, the yield of labelled DNA was low. In order to increase the concentration, several 50 μL labelling reactions were performed simultaneously (Figure 6.5) and the DNAs excised from the gels were pooled. An estimation of the yield was then performed by comparing the DNA concentration of the probe against known DNA standards, thereby ensuring that a standard concentration of probe was used in each dot-blot hybridization (Figure 6.6).

A major problem with the PCR labelling technique was in the recovery of the labelled product from the agarose gel. High quantities of labelled products were often observed under the UV transilluminator but, after excision and extraction, recovery was small (Figure 6.5). In an attempt to overcome this problem, extraction was performed using the Bio 101® Geneclean® Kit. Based upon the intensity of the band under the UV transilluminator, increased recoveries were noted but yields were still lower than expected. In a further attempt to avoid losses associated with the extraction of DNA from agarose, a Qiagen PCR purification kit was used which eliminated the need for electrophoresis. The kit allowed purification of the PCR product by removing unused dNTP's and primers. Unlike the electrophoresis technique, however, the purification kit did not remove unlabelled template DNA and cellular DNA in the reaction mixture.

6.2.3 Sensitivity and specificity of dot-blot hybridization compared with PCR and isolation and identification in cell culture

Techniques available for the detection of MDV include virus isolation in cell culture, serology and PCR. All possess disadvantages, which limit their use for screening large numbers of field samples (De Laney *et al*., 1998; Davidson *et al.,* 1986; Pereira, 1994). In this section the sensitivity and specificity of the 132 bp probe was compared with existing techniques, by comparing the detection rates of each in field samples of flocks showing possible signs of MD infection.

Sensitivity is determined from the percentage of specimens that are positive when tested against a reference procedure (the *gold standard*). Specificity is determined by the percentage of specimens correctly identified as negative. A test with low specificity would be expected to produce a high percentage of false-positive samples (Mendenhall, 1993). Generally, the *gold standard* for the evaluation of new viral detection techniques, are based on virus isolation procedures. Unfortunately, many virulent strains of MDV grow poorly in cell culture and, in consequence, field samples are often not detected (De Laney *et al.,* 1998). It was, therefore, not possible to determine sensitivity and specificity using virus isolation as the reference technique. PCR was therefore chosen as the *gold standard* because it is the most sensitive of the available techniques and can detect non-viable MDV, an important consideration since MDV is a highly labile virus.

Detection of MDV 1 from problem flocks by PCR, virus isolation and identification by IP, IFA and dot-blot hybridization using the 132 PCR probe

Whole blood from 109 samples was collected from 6 sites throughout Victoria and New South Wales from vaccinated and unvaccinated problem flocks exhibiting possible signs of MD infection. The samples were then transported to the laboratory in heparinised tubes at 4^{o}C within 24 hr of collection. Lymphocytes were isolated by Ficoll-Paque® centrifugation and the samples screened for MDV by PCR (Aly *et al.,* 1993); IFA, Immunoperoxidase (IP) and dot-blot hybridization using the 132 bp PCR probe.

Determination of sensitivity and specificity of the dot-blot hybridization procedure and virus isolation against PCR (the gold standard).

In order to determine the sensitivity and specificity of the dot-blot hybridization technique against the PCR (*gold standard*) a 2 x 2 contingency table was used to compare the isolation rates of the field samples for the two techniques (Mendenhall, 1993).

		Gold Standard (PCR) +	Gold Standard (PCR) -
Test Assay	+	**a**	**b**
	-	**c**	**d**

Where:
a = number of field samples positive for both test and *gold standard* assays
b= number of field samples positive for the test assay and negative for the *gold standard* assay
c= number of field samples negative for the test assay and positive for the *gold standard* assay
d= number of field samples negative for both test and *gold standard* assays

a + b + c + d = total number of field samples

Thus:

$$\text{Sensitivity} = \frac{a}{a + c}$$

$$\text{Specificity} = \frac{d}{b + d}$$

Results

Detection rates of 109 field samples using PCR, dot-blot hybridization and virus isolation.

Table 6.2 shows the number of positive samples detected by each technique. Of the 109 field samples screened, dot-blot hybridization showed the highest rates of detection, with the 132 bp PCR probe detecting 92.6%. Only 50% of the samples were positive by PCR while isolation and identification using IP and IFA, detected 34.8% and at 13.8% of the samples, respectively.
The detection results of PCR were then used to calculate the sensitivity and specificity of virus isolation and dot-blot hybridization as shown in Table 6.3.

Table 6.2 *Positive samples detected from 109 samples using PCR, virus isolation, and dot-blot hybridization.*

Technique	**PCR**	**Isolation and Identification by:**		**Dot-Blot Hybridization**
Flock # (# samples in flock)		**IFA**	**IP**	**132 bp product Probe**
1(30)	24	0	9	28
2(14)	5	0	6	13
3(8)	7	0	3	8
4(19)	8	12	17	16
5(28)	1	0	0	27
6(10)	10	3	3	9
Total Positive/109	**50.5%**	**13.8%**	**34.9%**	**92.6%**

S*ensitivity and specificity calculations of virus isolation and dot-blot hybridization against PCR*

The sensitivity and specificity of IP, IFA and the dot-blot hybridization using the 132 bp product probe was determined against the isolation results by PCR (Section 6.2.3). Results in Table 6.3 indicate highest sensitivity rates were achieved by dot-blot hybridization, compared with virus isolation and detection by IP or IFA. Despite the higher sensitivity of the dot-blot, higher specificity was obtained by both culture detection methods.

Table 6.3 *Sensitivity and specificity of virus isolation and dot-blot hybridization against PCR; which was used as the gold standard.*

Technique	**Isolation by IP**		**Isolation by IFA**		**132 bp product probe**	
Flock #	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
1	89	70	0	100	95	12.5
2	50	25	0	100	100	11
3	100	80	0	100	88	NA
4	52	N/A	78	50	88	20
5	0	NA	0	NA	100	NA
6	100	NA	30	NA	90	NA
TOTAL	**65%**	**58%**	**19%**	**87%**	**94%**	**14.5%**

Note: NA refers to flocks, from which there were no negative samples for both test and reference tests and, therefore, it was not possible to determine specificity.

Discussion

The results presented in Table 6.2 show a much higher detection rate by dot-blot hybridization tests than by any of the other techniques, with PCR detecting little over half the samples detected by blots. These results indicate that the dot-blot is the most sensitive of the available techniques, but in order to determine the sensitivity and specificity against a gold standard, the isolation rates of one of the available techniques had to be used as a reference. PCR was selected as the *gold standard* as it is the most sensitive of the available techniques and because of the problems associated with virus isolation in cell culture.

The results indicated that the dot-blot hybridization technique was much more sensitive than virus isolation in cell culture and detection by either IP or IFA. A much lower sensitivity by virus isolation was expected due to the difficulties in growing many wild-type MDV-1 isolates in cell culture (De Laney *et al.,* 1998; McKimm-Breschkin *et al.,* 1990). Detection by IP was more sensitive then by IFA, which was unexpected since the same monoclonal antibody was used in both techniques. Possible reasons include the presence of background staining of cells that sometimes made it difficult to determine a positive sample, leading to possible false-positive results based on operator error.

Because of the difficulties in determining positive samples by IP, the lymphocyte samples were then tested by IFA. Fluorescent staining of antigen made positive samples much easier to detect. However, between the time of testing by IP and IFA, the lymphocyte samples had been frozen down, which could have ultimately reduced the chances of isolation in cell culture. A more accurate comparison of the two techniques would have involved co-inoculation of the cell culture plates and identification by the two techniques on the same day.

Despite the high sensitivity, the results showed low specificity for the dot-blot procedure when tested reactions were noted with serotype 2 or 3 viral DNA prepared from infected CEF cells. The low specificity of dot-blot hybridization could be the result of non-specific binding of the probe to host cell DNA or it could be due low sensitivity of the PCR which gave rise to false negative results.

Generally, DNA probes are considered more specific under stringent hybridization conditions than PCR primers, since the target fragment in a DNA probe is much larger than a primer sequence. Although it seems highly unlikely that a large region of non-specific DNA would be homologous to a target region, this probability could be increased when only a small region of DNA is needed to hybridise, as is the case with primer sequences.

Despite the unlikely possibility, the presence of exogenous DNA in the samples may increase the risks of non-specific binding. When DNA was extracted and purified from infected lymphocytes, it contained both host cellular DNA and viral DNA. The probe may, therefore, have bound non-specifically to a region of the chicken DNA. A sequence analysis was performed on the probe to determine the extent of homology with regions other than the MDV 1 target site. The results showed closest homology with MDV 2 and 3 followed by low homology with human DNA (Unpublished observation). Nevertheless, the possibility that probe sequences were similar to a region of the chicken chromosomal DNA cannot be discounted, as there is little data available on the sequence of chicken nucleic acid regions. This probability would seem unlikely, however, since negative controls consisting of MDV 2 and 3 virus grown up in CEF cells were used. The fact that the controls were harvested in CEF cells means that both viral and chicken DNA would be present in the samples and yet these controls were never positive. To eliminate the possibility of the probes binding to a component of the serum or the lymphocyte, a negative control consisting of lymphocytes extracted from specific pathogen free (SPF) birds should have been used.

Although vaccination status of the flocks was variable, most were vaccinated with serotype 3. The probe may, therefore, have detected the vaccine, although this would be very unlikely since each blot contained a serotype 2 and 3 negative control, which were never positive. Negative control strains were harvested from cell culture and passaging of the vaccine in the chicken may have altered the genome sequence from that of the cell culture control, thereby increasing its homology for the target region. This would also seem highly unlikely and no data has been published indicating such changes. A negative control consisting of lymphocytes extracted from an SPF chicken that had been vaccinated with HVT should have been used to confirm the probe was not binding with the serotype 3 vaccine after being passaged in a chicken.

If non-specific hybridization was occurring, it would be likely that the PCR would also be non-specific since the same primers were used to develop the 132 bp repeat PCR probe and yet low specificity for the PCR was not obtained.

Previous work using dot-blot hybridization as a detection technique for viruses indicated that most difficulties arose from concerns about low sensitivity and not low specificity. Specificity was generally ensured by the selection of probes with high homology for the target region and by the use of stringent hybridization conditions (Musiani *et al.,* 1990; Landry, 1990). Determination of sensitivity and specificity of a new detection test is heavily reliant upon the correct identification of samples by the *gold standard*. Despite its great theoretical sensitivity when carried out under optimum conditions, a number of problems have been associated with the use of PCR in the detection of MDV. Many of the samples may have contained inhibitory factors that affect PCR reaction conditions, as is suggested by the detection of MDV in 9 samples by culture, that were negative by PCR (Kramvis *et al.,* 1996; Klein *et al,* 1997). Field samples were collected in heparin-lined tubes to inhibit the blood from clotting. Unfortunately, the heparin may also have been acting as a PCR inhibitor, a problem also experienced by others (Izraeli *et al.,* 1991). Other possible PCR inhibitors arise from the use of blood samples that include haemin, which reversibly binds to *Taq* polymerase and acts as an inhibitor. Traces of denatured albumin have also been shown to greatly affect the outcome of a PCR reaction (Klein *et al,* 1997, Vandenvelde *et al.,* 1993). The presence of these PCR inhibitors may have been

overcome by the pretreatment of the nucleic acid with NaOH denaturation or octanoate thermoprotection (Vandenvelde *el al.,* 1993). Despite the introduction of such treatment methods to avoid the action of PCR inhibitors, the development of a nested PCR which would use a set of internal controls that would bind to a region of the chicken cellular DNA would ensure negative results were not caused by such inhibitory factors. Samples that did not amplify the internal control would indicate the presence of inhibitory factors. However, because of time restraints a nested PCR was not attempted.

Another problem that may have been associated with the PCR was that the samples were, in fact, positive but the 132 bp bands were not of sufficient intensity to be detected under the UV transilluminator. This seems likely because at least 30% of the samples were weakly positive by dot-blot hybridization, suggesting the presence of low concentrations of target DNA. Another study found this to be a problem, as detection of some samples were only possible after the performance of a Southern blot hybridization (Pereira, 1994). The presence of virus-specific DNA could, therefore, have been confirmed by Southern blot hybridization but, if used routinely, would be a very time consuming and expensive addition to the test.

Previously, studies have shown that the PCR, when carried out at an annealing temperature of 50^{o}C, produces non-specific products ranging in size from 300 to 1000 bp in samples not containing MDV (Pereira, 1994). Accordingly, an annealing temperature of 55^{o}C was selected which ensured that primers would only anneal to the binding sites within the IR_L region. However, DNA from an MDV population with variation in the 132 bp repeat primer binding region will not be detected because of the highly stringent conditions selected for primer annealing (Pereira, 1994).

All of these problems indicate that samples negative by PCR should be looked at critically. Samples containing MDV DNA, which were not detected by PCR, would ultimately affect the results for sensitivity and specificity in Table 6.3. In Flock No. 5, for example, only one sample was positive by PCR, whereas all were positive by the 132 bp PCR dot-blot procedure. More work needs to be done to ensure that the samples being detected by the dot-blot hybridization are, in fact, positive and not the result of non-specific binding which could be determined by the use of appropriate negative controls. Improvement is also needed for the PCR technique in order to increase its sensitivity by ensuring that there are no false-negative samples so that there can be more confidence in its use as a reference technique.

6.2.4 Conclusions

Dot-blot hybridization allows the screening of large numbers of samples within a flock at the same time at a greatly reduced unit cost. With PCR, the unit cost is directly related to the number of samples tested because of the need for a standard amount of *Taq* polymerase in each test. Equipment required for PCR is moderately expensive and reactions must be performed in a containment facility that is often not available in smaller diagnostic laboratories. In this project a dot-blot hybridization procedure for the detection of MDV 1 was evaluated using a probe labelled with DIG that was derived from a region within the MDV 1 genome. The major problems associated with the hybridization test involved low specificity when determined against detection rates of PCR, the *gold standard.* Low specificity may have been the result of the probes binding non-specifically to host cell DNA, resulting in false positive results. This could have been determined by the inclusion of a negative control consisting of lymphocytes extracted from the whole blood of an SPF chicken. If the negative control was detected by the blot then the test should be rendered invalid. Another explanation for the low specificity of the probes was the possibility of the PCR producing false negative results.

Although PCR is potentially the most sensitive technique available for the detection of MDV 1, several factors can affect its ability to detect viral DNA in clinical samples. Variation in the 132 bp fragment within the IR_L region between different strains could limit its capacity to hybridise with the

primers due to the need for highly stringent annealing conditions (Pereira, 1994). In addition, the variable presence of inhibitors of PCR within samples can affect the performance of the test (Kramvis *et al.,* 1996; Klein *et al,* 1997). Visualization of weak bands under the UV transilluminator is often difficult, which could have affected the sensitivity of the test. In a previous study, a probe specific for the 132 bp region, detected bands in samples by Southern blot hybridization, which initially were negative under the UV transilluminator (Pereira, 1994). Another potential problem with the large-scale use of PCR is cross-contamination of the reagents with MDV which often require individual tests to be repeated. All of these factors would have affected sensitivity and specificity results of the dot-blot hybridization tests. It is therefore imperative that further optimisation of the dot-blot hybridization test is performed to increase specificity, including appropriate negative controls and repetition of samples, before it can be adapted as a detection technique for MDV 1. Improvement of the sensitivity of the PCR is also required to ensure its validity as a *gold standard*. This could be done by implementing a number of strategies to avoid the presence of PCR inhibitors and by including a control set of primers which would amplify a region of the host chromosomal DNA, ensuring PCR inhibitors are not present.

In order to make the dot-blot hybridization technique as rapid and economical as possible, further optimisation of probe labelling is required. The 132 bp product probe presented difficulties with respect to obtaining high yields of labelled product. These difficulties were probably due to the small size of the product being amplified. To overcome the problem, a number of PCR labelling reactions were performed and extracted together, but the labelling procedure was relatively expensive due to the increase in the concentration of Taq polymerase and labelled dNTP's required.

A problem associated with both the dot-blot hybridization and PCR techniques is their inability to differentiate between pathogenic and attenuated MDV 1 strains because it is currently not possible to identify specific regions of the genome that are associated with pathogenicity. Such a region, if identified, would be a useful target sequence for the probe as a test for detection of pathogenic strains. By successfully optimising the labelling procedure and implementing a number of the above considerations, the dot-blot hybridization technique using the DIG-labelled probe could potentially be used as a rapid and inexpensive detection method for MDV 1 with particular application for the large-scale screening of flocks.

6.3 Collection and characterisation of MDV field isolates

6.3.1 Introduction

MDVs have evolved with increasing virulence since the introduction of the HVT vaccine against oncogenic serotype 1 viruses. If it can be assumed that recent very virulent MDVs arose due to selection following the widespread use of HVT, it seems likely that further selection will occur following the use of serotype 1 vaccines that may give rise to even more virulent field strains. Therefore it is highly desirable that a repository of strains that have been collected at different times from various parts of Australia and characterised by both molecular and other tests be available for comparative purposes. Unfortunately, representative serotype 1 MDVs, isolated at the time vaccination was first introduced, which could serve as a reference for later changes, are no longer available.

Polymorphism between MDV strains can be studied after isolation of their DNA by pulse-field gel electrophoresis (PFGE) and treatment with restriction endonucleases. The presence of specific fragments can be determined by Southern blot analysis using a) end-labelled whole virus DNA prepared from Australian strains or b) DNA from a gene library prepared from the prototype GA strain. Deletions and expansions of oncogenic and attenuated strains can also be compared using cell-free DNA probes by the use of Random Amplified Polymorphic DNA (RAPD)-PCR.

The molecular and biological characterisation of Australian strains of MDV in the above manner can assist in determining trends in the evolution of very virulent MDV strains and may allow differentiation possible within the same serotype.

6.3.2 Methods and Results

Close to 300 blood samples were obtained from flocks in different parts of Eastern Australia (Table 6.4) in order to achieve a collection of MDV strains representative of this part of the country for characterisation. Upon arrival, the blood samples were processed and their lymphocytes isolated according to the method of De Laney *et al*., (1995). Lymphocyte samples were diluted in 1.5 mL of cell freezing medium (CFM) prior to storage in liquid nitrogen.

The samples were screened for MDV1 by the polymerase chain reaction (PCR). Samples that were positive for MDV1 were passaged twice in SPF CEKs in attempt to increase the virus titre and negative samples were discarded. Those samples producing a cytopathic effect (CPE) were serotyped with serotype-specific monoclonal antibodies (Mabs) by an immunoperoxidase method and/or an immunofluorescence antibody assay. Duplicate cultures were harvested and stored until required for characterisation by PFGE and RAPD PCR. A flow chart summarises the path for isolation and characterisation of a MDV field isolate (Figure 6.7).

Very few samples obtained proved to be positive for MDV 1 by the criteria outlined (Table 6.4). The procedure adapted for this project was based on the selection of PCR as the *gold standard* which is widely considered to be the most sensitive of the available isolation and identification techniques because of the problems associated with virus isolation in cell culture. However, several difficulties were noted with PCR with respect to sensitivity and specificity (Cipriani, 2000). Previously, all samples were screened for MDV 1 by PCR before attempted culture, and those that tested negative were discarded. These problems with PCR suggest that some PCR negative samples could have been culture positive.

Only a small number of blood samples (279 in total) were submitted to the laboratory throughout the time of the project. Eighty-six samples tested positive for MDV 1 but these did not progress past storage in liquid nitrogen. Their low number and their slow accumulation could not justify the

commencement of characterisation studies within the project's time frame. It is intended that these samples will be characterised by the molecular procedures mentioned when facilities at the research laboratory allows. Despite their pre-characterisation status, the samples remain as a viable repository of MDV isolates and will be available for comparative purposes in the future if very virulent MDVs arose due to selection following the widespread use of serotype 1 vaccines.

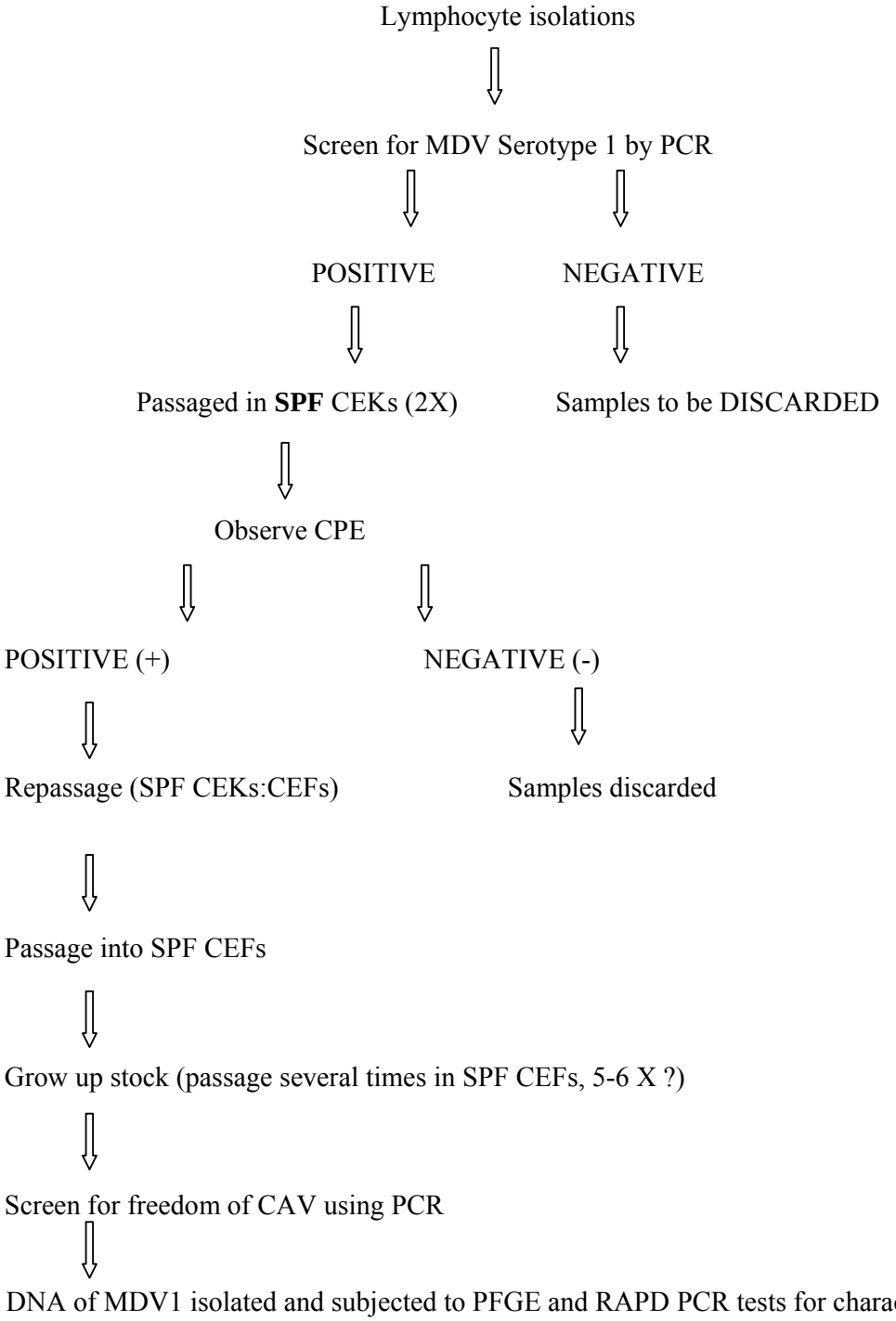


Figure 6.7 *Flow chart of isolation and characterisation of MDV field isolates.*

Table 6.4 *. Summary of Marek's disease virus isolations from vaccinated flocks in Eastern Australia.*

MPF No.	Area	Date Samples Received	No. of Samples Processed	Isolation Procedures[a]	MDV 1 PCR Positive Results	MDV Serotype isolated by cell culture			Vaccine Serotype[b]	Age at Sampling	Clinical Notes
						1	2	3			
98	Not stated	08/04/98		A, B	5	0	0	0	HVT/ca & cf	14 1/2 w.o.	Clinical MD
99	Not stated	08/04/98		A, B	0	0	0	0	Not provided	Not provided	No clinical signs
100	Wallan, Victoria	08/04/98	10	A,B	8	0	0	0	1, 2, 3	Not provided	Clinical MD, 9% mortality
101	Hastings, Victoria	24/04/98	6	A	4				1,3	10-11 w.o	Clinical MD
102	Murchison, Victoria	07/05/98	8	A	8				1, 3	15 d.o	Clinical MD
103	Mitchell, Birchip, Victoria	21/05/98	9	A	5				1, 3	15 d.o	Normal
104	Hubbard, Victoria	26/05/98	10	A	0				1, 3	12 d.o	Normal
105	Drew, Victoria	29/05/98	5	A	4				1, 3	14 w.o	Clinical MD
106	Milroy, Victoria	29/05/98	2	A	2				1, 3	14 w.o	Clinical MD
107	Kinross, Victoria	04/06/98	4	A	1				1, 3	14 d.o	Normal
108	Kinross, Victoria	04/06/98	3	A, B	1	0			Not provided	14 d.o	Normal
109	Colgoa, Victoria	12/06/98	12	A	9				1	3 w.o	Normal
110	Huntly, Victoria	24/06/98	6	A	2				1, 3	14 d.o	Normal
111	Moyeness, Victoria	02/07/98	12	A, C		2	0	1	1,3	15 w.o	Clinical MD; 6% mortality
112	Landman, Victoria	03/07/98	10	A	9				Not provided	8 d.o	Normal
113	Queensland	21/10/98	10	A, C		1		2	1,3	8 w.o	Clinical MD
114	Broiler farms, NSW	30/10/98	7 pools	A	0				Not provided	Not provided	Not provided
115	Tamworth, NSW	28/07/99	11	A	0				Not vaccinated		

MPF No.	Area	Date Samples Received	No. of Samples Processed	Isolation Procedures[a]	MDV 1 PCR Positive Results	MDV Serotype isolated by cell culture			Vaccine Serotype[b]	Age at Sampling	Clinical Notes
						1	2	3			
116	Tamworth, NSW	09/09/99	15	A	0				Not vaccinated	35 d.o	Normal
RMI-8A/3	Sydney, NSW	14/03/00	30	A, D	22	0			3	49-53 do	Normal
RMI-8A/4	Menangle, NSW	22/03/00	14	A, D	5	0			Not provided	4 w.o	Proventriculitis trial
RMI-8A/5	Sydney, NSW	24/03/00	8	A, D	7	0			3	53 d.o	MD tumours & haemangioma in 1 bird
117	Menangle, NSW	30/05/00	28	A, D	0	0			Not provided	28 d.o	Proventriculitis trial
118	Kinross, NSW	07/07/00	10	A	10				1	30-35 w.o	Clinical MD
120	Salvotorie (grower)	07/08/00	10	A	0				1, 3	Not provided	Healthy flock
123	Morwell, Lyndale Poultry	29/09/00	15	A, C	2	4			1, 3	Not provided	Not provided
124	Morwell, Lyndale Poultry	20/10/00	9	A, C	2	2			1,3	32 weeks	Loss 90-100 birds weekly
125	Loddon Valley Bridgewater	26/10/00	8	A, C	4	4			1, 3	20 weeks	Nil
126	Loddon Valley, Bridgewater	26/10/00	7	A, C	2	3			1, 3	20 weeks	Nil

[a]Lymphocyte Preparation Method A - lymphocyte samples diluted with 1.5 mL of CFM
Cell Culture Method B - Two passages in SPF CEKs in 24 well plates, identification by IP?
Cell Culture Method C - One passage in SPF CEKs, identification by IP &/or IFA
Cell Culture Method D - First CEK passage centrifuged in 96 well plates, two subsequent passages in SPF CEKs, and identification by IP
[b]Serotype 1, 2 and /or 3 (HVT) vaccines were administered to day old chickens

6.4 Characterisation of MDV after adaptation to the Vero continuous cell line

6.4.1 Introduction

Two major difficulties in working with MDV have been the highly cell-associated nature of the virus and the lack of continuous cell lines for virus growth. MDV and HVT can be propagated in avian cell cultures prepared from chicken embryo fibroblasts (CEF), chicken embryo kidneys (CEK), chicken kidneys (CK) and duck embryo fibroblasts (DEF) (Biggs and Milne, 1972; Schat and Calnek, 1978; De Laney *et al*., 1995). These cultures have a life span of 2-3 weeks and must be prepared from primary tissues. By contrast, cell lines can be subcultured indefinitely or maintained as a seed-lot for storage in liquid nitrogen. For MD vaccine production a continuous supply of fertile eggs from specific-pathogen-free (SPF) flocks is required, adding significantly to the cost of vaccines.

A continuous cell line that would support MDV replication which could be stored in liquid nitrogen and rapidly expanded for production would have many advantages. The selection and use of such a line was the basis of an investigation carried out by Jaikumar *et al*. (2001). The successful adaptation of MDV to the Vero continuous cell line has allowed new possibilities for the characterisation of MDV. The adaptation of MDVs to growth in a continuous cell line could be useful for vaccine production, compared with CEF cultures that are currently used.

6.4.2 Adaptation and growth of MDV in the Vero continuous cell line

Adaptation of MDV was attempted in the baby hamster kidney (BHK_{21}) and the Vero (African green monkey kidney) epithelial cell lines. BHK_{21} cells have shown to be sensitive to several DNA and RNA viruses (Lennette *et al.,* 1979; Matthews, 1982) but initial attempts to adapt serotype 1 and -3 MDVs to growth in BHK_{21} cells were not successful. The Vero line has been used for the propagation of human viruses like poliomyelitis and rabies (Montagon *et al*., 1985; Montagon, 1989) and animal viruses like rabies (Montagon, 1989), rinderpest and peste des petits ruminants viruses (Tozzini *et al*., 1987; Diallo *et al*., 1987), bluetongue virus (Wechsler and McHolland, 1988) and ovinepox virus (Tozzini *et al*., 1987) and was selected for the growth and adaptation of MDV (Jaikumar *et al.,* 2001).

6.4.3 Characterisation of MDV

Yields

After adaptation of MDV to the Vero continuous cell line, yields of virus were determined by the microtitre technique in primary CEF cultures (Witter, 1983; Schat, 1985; Sharma, 1989). Infectious titres of serotype 1 MDV after 5, 10 and 20 passages were 2.09, 2.20 and 2.15 $\log_{10}$ $TCID_{50}$ per mL, respectively. Titres of HVT after 5, 10 and 20 passages were 3.26, 3.58 and 3.55 $\log_{10}$ $TCID_{50}$ per mL. Neither the Vero-grown serotype 1 virus nor HVT at passages 5,10 and 20 produced plaques in Vero monolayers when tested by the procedure of Thornton (1985).

Indirect immunofluorescent antibody test

The presence of virus in infected Vero cells was confirmed by indirect immunofluorescent antibody test (IIFAT) (Spencer and Calnek, 1970) using serotype 1 and -3 specific monoclonal antibodies (Lee *et al*., 1983). Serotype-specific fluorescent foci were produced after passage by either virus and could be detected in both the cytoplasm and nuclei of the infected cells. The proportion of cells containing

antigen increased to a maximum of 50 % for the serotype 1 virus after 10 days and 90 % for HVT after 10 days. No fluorescence could be detected in uninfected controls that were cultured in parallel.

PCR to detect 132 bp direct repeat of serotype 1 MDV after adaptation to the Vero line

A PCR developed by Silva (1992) was used to detect the presence of 132 bp direct repeat (DR) sequence specific for serotype 1 MDVs. Three hundred ng of total DNA from Vero cells infected with 20^{th} passage preparations of the serotype 1 MDV and an uninfected control culture were used as templates for PCR amplification using primers flanking the 132 bp DR region of serotype 1 MDVs. DNA of the expected sizes of approximately one (185 bp), two (317 bp) and three (449 bp) copies of 132 bp direct repeat (plus a 53 bp flanking repeat) were amplified in the reaction product of DNA isolated from serotype 1-infected Vero cells.

Reaction mixtures containing DNA isolated from HVT-infected and uninfected Vero cells did not produce serotype 1-specific bands. DNA of serotype 1-infected CEF cells and uninfected CEF cells were used as positive and negative controls. Although these results suggest that MDV DNA is present within infected Vero cells, PCR analysis may have detected DNA from residual unadapted virus inoculum, although the original inoculum would seem unlikely to have been present after 20 passages.

PFE to detect intact viral DNA prepared from serotype 1 and HVT after adaptation to cells of the Vero line

Since PCR analysis does not allow discrimination between intact viral DNA and subgenomic fragments in infected cells, attempts were made to detect the presence of intact intracellular viral DNA in infected cells by isolating and obtaining a size estimate of DNAs from serotype 1- and HVT-infected Vero cells. Isolation was achieved using pulsed field gel electrophoresis (PFE) and applying the contour-clamped homogeneous electric field (CHEF) technique described by Chu *et al.* (1986) and Wilson and Coussens (1991).

Vero cells were infected with a 10^{th} passage preparation of serotype 1 virus and 5th passage preparation of HVT and incubated until cell monolayers appeared fully infected and cells began to detach from the culture vessel (5-7 days). Infected cells were treated as outlined by Jaikumar *et al*., (2001), and subjected to PFE and CHEF techniques. Four bands of 1900, 1400, 700 and 180 kb were observed from the extracts prepared from serotype 1- and HVT-infected cells. Lanes containing extracts prepared from uninfected Vero cells and uninfected CEFs contained the same bands in lanes for infected cells except for the 180 kb band which is the approximate size of intact serotype 1 and HVT DNA.

6.4.4 Conclusions

HVT grew more rapidly and produced more extensive CPE and higher virus yields than the serotype 1 virus. These patterns of growth were consistent with the amount and distribution of serotype-specific antigen in infected cells as detected by IIFAT. With serotype 1-infected Vero cells a CPE was not apparent until the 5th passage and a diffuse pattern of immunofluorescent foci was then noted. These observations indicate that the Vero cell line appears to be more susceptible to HVT than to serotype 1 viruses.

An expansion of the 132 bp DR sequence specific for MDV serotype 1 confirms that the infected cell line contained MDV DNA. The presence of intact DNA with a size of approximately 180 kb in both serotype 1- and HVT-infected Vero cells after isolation and subsequent analysis in a pulsed-field gel indicates that whole copies of both types of DNA were present and provides further support for

adaptation to growth of the serotype 1 virus. Yields of the serotype 1 viruses in Vero cells are probably too low for Vero cells to be considered as a substrate for the growth of serotype 1 vaccine viruses. Yields of HVT are higher and, if further increased, should be evaluated in chickens with CEF-grown vaccines.

6.5 Efficacy of γ-inulin as an adjuvant for live caHVT vaccination

6.5.1 Introduction

HVT vaccines, unlike other MD vaccines, are very safe, cheap and can be readily transported without liquid nitrogen. However, in recent years HVT vaccines have shown to be much less effective against emerging field strains of MDV of increasing virulence. Adjuvants are used to improve the immunogenicity of a vaccine without increasing the amount of infectious virus in the vaccine. An adjuvant enhancing the performance of the HVT vaccine would be a value-added benefit to a cheap and readily available existing vaccine.

Algammulin and γ-inulin comprise a novel class of vaccine adjuvant. Their use in vaccines is to exploit the humoral defence known as the alternate complement pathway (ACP). γ- inulin is a potent enhancer of the Th1 immune response pathway, boosting seroconversion rates and immunological memory in protective antibody classes and enhancing cell-mediated immunity. Cooper and Steele (1988) successfully used γ-inulin to activate the ACP of mice (50-100 μg/mouse), resulting in an increase in secondary IgG responses 5- to 28- fold ($p<0.001$) based on experiments using keyhole limpet hemocyanin (KLH) as the immunogen.

γ-inulin is a very soluble polymorphic form of inulin, a neutral polysaccharide. Inulin is the storage carbohydrate of *Compositae* and is readily extracted in large yield from dahlia tubers (Cooper, 1995). γ-inulin-based adjuvants therefore comprise new, safe, potent, and attractive candidates for enhancing responses to veterinary vaccines.

Embrex Inc. pioneered the development and use of *in ovo* injection technology. The delivery system is the Inovoject® automated egg injection system which punches a tiny hole into the egg shell and then lowers a needle through the hole which delivers the therapeutic product to the embryo. This delivery system was commercialised in 1993 and is now the standard delivery method for MD vaccine in the United States (www.embrex.com, 2001).

This study was conducted to assess the performance of HVT vaccination using γ-inulin as an adjuvant in broiler chickens and involved the use of the automated delivery of adjuvant and vaccine to chicken embryos.

6.5.2 Method

Commercial broiler chickens were assigned to 12 treatments which consisted of three types of vaccination (by hand into or missing the embryo, Inovoject® at day 17.8 and 18.8 of incubation and sc at day old), with or without cell-associated-HVT (caHVT; 4000 pfu) vaccine and with or without doses of γ-inulin, that, based on other studies, were calculated to enhance protective responses (Table 6.5). A total of 150 eggs for Treatments 1-8 were set to ensure that at least 120 hatchlings and treatments were treated by the Inovoject® machine or were vaccinated by hand. Treatments 9-12 were applied to day old chickens by a commercial vaccinator. Initial chicken numbers are shown in Table 6.5.

On day three of the trial, all chickens were challenged with 50 pfu i.p of MPF 57 (a highly virulent strain of MDV; De Laney *et al.,* 1998). Birds were maintained and observed for 66 days for signs of MD. Blood samples were taken from 8 – 10 birds per treatments 1-4, 7 and 8 on day 17 and tested for the presence of a HVT viraemia. All chickens were individually weighed and sexed on day 49. Routine post-mortem examination of all chickens dying during the experiment was performed and

gross MD lesions recorded. On the final day of the trial all remaining birds were euthanased and post-mortemed for gross MD lesions.

6.5.3 Post-vaccinal HVT viraemia

Post-vaccinal viraemia isolated from blood lymphocytes in CEF cell culture was affected by site of vaccine deposition ($p<0.005$) and dose of vaccine ($p<0.001$) (Table 6.5). All birds were derived from a commercial strain and thus maternal antibody to HVT, most probably present, was especially evident in unvaccinated groups (e.g. Treatment Groups 1 and 3).

When nil vaccine treatments were excluded from analyses, the route of vaccination had significant influence on vireamia ($p<0.001$). Analyses of contrast between Inovoject® and hand, hit/miss treatments showed that, there is no significant difference between missing the embryo by hand and the Inovoject® treatment ($p=0.22$) and hitting the embryo by hand and Inovoject® treatment ($p=0.63$) but there was a significant difference between hitting and missing the embryo by hand vaccination ($p<0.001$).

Table 6.5 *. Treatments applied to groups of chickens to study the adjuvant effect of γ-inulin and Inovoject® inoculation to vaccination; the percentage of birds exhibiting a post-vaccinal HVT viraemia on day 17 of the trial (n=8 – 10). Inovoject vaccination was carried out on day 17.8 and 18.8 of incubation, hand vaccination (in ovo) was carried out on the 17.8th day of incubation and a subcutaneous injection was given to birds of 1 day of age.*

Treatment group number	Route of vaccination		Inulin (mg)	HVT (pfu)	Initial chick number	% HVT viraemia Day 17
1	Inovoject®	At 18.8 days	nil	0	101	100
2				4000	80	57[ab]
3		At 17.8 days	nil	nil	109	75
4				4000	96	70[ab]
5			0.5	nil	120	
6				4000	120	
7	Hand	Missed embryo	nil	4000	96	70[a]
8	At 17.8 days	Hit embryo		4000	83	77[b]
9	Day old (subcutaneous)		Nil	nil	125	
10				4000	123	
11			1	nil	116	
12				4000	113	

[ab]Treatments sharing the same superscript were statistically non-significant, [a](p=0.22) [b](p=0.63)

6.5.4 Live weight of chickens

The average live weight of chickens subjected to each treatment is presented in Table 6.6. Analyses showed that there was a significant effect of sex ($P<0.001$) and vaccine dose ($P<0.005$) but not site of vaccination ($P=0.34$) on live weight.

The mean live weights of males and female chickens were 3580±26 and 2903±24g, respectively. Male birds are less susceptible to MD than females and it could be expected that, in comparison with female birds, they would be heavier as less birds would be affected by MD. It could also be assumed that males are generally the heavier gender of the species. Vaccinated chickens (3278±29g) weighed more than unvaccinated chickens (3112±38g). The occurrence of MD was less in vaccinated chickens, and these birds were thus healthier and heavier than in chickens showing signs of MD. γ-Inulin had no effect on live weight.

***Table 6.6** . Live weight of broiler chickens on day 49. All treatment groups were challenged with 50 pfu of MDV1 on day 3.*

Treatment group number	Route of vaccination		Inulin (mg)	HVT (pfu)	n	Average live weights (Day 49)		
						male	female	average
1	Inovoject®	At 18.8 days	0	nil	65*	3587	2802	3195
2				4000	43	3688	2920	3304
3		17.8 days	0	nil	65*	3631	2867	3249
4				4000	49	3669	3046	3358
5			0.5	nil	67	3628	2837	3233
6				4000	74	3563	3026	3295
7	Hand	Missed embryo	0	4000	49	3492	2909	3201
8	At 17.8 days	Hit embryo		4000	57	3598	2958	3278
9	Day old (subcutaneous)		0	nil	65*	3453	2807	3130
10				4000	93	3596	2964	3280
11			1	nil	71	3527	2907	3217
12				4000	72	3639	2919	3279

* pooled average of chickens remaining in the 3 unvaccinated groups.

6.5.5 MD mortality

The first death from MD occurred on day 33 (i.e. 30 days post-challenge). MD-positive chickens included those that died with gross MD lesions and those that exhibited gross MD lesions after euthanasia at day 63 post-challenge. The numbers of MD-positive chickens in different groups are presented in Table 6.7. Analyses revealed that, the presence of MD was significantly influenced by the dose of vaccine ($P<0.001$), the sex of the chickens ($P<0.001$) and the route of vaccination ($P<0.005$). As expected, some protection against MD was obtained by the use of caHVT vaccine alone. The incidence of MD correlated well with the live weights of challenged birds (Table 6.6).

In the absence of γ-inulin, HVT administered via the inovoject® route provided protection not significantly different at 18.8 or 17.8 days or from that provided by hand. However, the inovoject® route of vaccination did perform slightly better than vaccination sc at day old.

Groups treated with γ-inulin were not significantly different ($p<0.05$) from the corresponding untreated groups (e.g. Treatments 4 and 6; 10 and 12). Differences in the percentages of MD in groups administered γ-inulin by the Inovoject® method or sc at day 1 (Treatment Groups 6 and 12) were also non-significant ($p<0.05$). γ-inulin alone (treatments 9 and 11), appeared to provide a non-specific but beneficial effect in reducing the incidence of MD in chicks treated sc at day old. No adverse effects due to γ-inulin were noted.

Table 6.7. *Percentage of MD in broiler chickens treated with different vaccine delivery systems.*

Treatment group number	Route of vaccination		Inulin (mg)	HVT (pfu)	n	% MD
1	Inovoject®	At 18.8 days	0	nil	73*	50.5^{f}
2				4000	46	11.2^{ab}
3		17.8 days	0	nil	73*	39.4^{def}
4				4000	56	7.8^{a}
5			0.5	nil	83	48.1^{f}
6				4000	82	16.7^{ab}
7	Hand	Missed embryo	0	4000	52	42.3^{ef}
8	At 17.8 days	Hit embryo		4000	59	18.5^{abc}
9	Day old (subcutaneous)		0	nil	73*	50.3^{f}
10				4000	92	21.5^{bc}
11			1	nil	83	29.7^{cde}
12				4000	92	24.0^{bcd}

^{abcdef} Treatments sharing the same superscript were statistically non-significant ($p<0.05$).
* pooled average of chickens remaining in the 3 unvaccinated groups.

6.5.6 Conclusions

The inovoject® route of vaccination, at either 18.8 or 17.8th day of incubation, provided adequate protection from MD. However, protection was not significantly different than that offered by vaccination *inovo* by hand at the 17.8th day of incubation.

Based on the live weights of chickens, the route of vaccination and the incidence of MD, no significant differences were observed between groups of birds administered γ-inulin and those that were not. Therefore, γ-inulin did not appear to function as an adjuvant when administered with the caHVT vaccine. This could indicate that γ-inulin levels used were too low to enhance immune responses to MDV. However, a significant difference was observed between non-vaccinated chicks treated sc at day old with γ-inulin and those without, which indicated a beneficial effect, although non-specific, in reducing MD.

7. Marek's disease vaccine assay facility

7.1 The need for a vaccine assay facility

Since their first introduction in the early 1970's, there has been an increase in the number of Australian MDV vaccines available to the poultry industry. Between 1970 and 1990, standardisation of HVT vaccines were conducted by the Therapeutic Goods Administration Laboratories (TGAL) but this facility is no longer available and, for the most part, assays have been carried out by individual manufacturers in-house and without access to standard reference preparations. With the likely availability of even more vaccines (both locally produced and imported), it was considered highly desirable by large sectors of the industry that an independent and reliable assay facility be available to evaluate their effectiveness in relation to one another.

Different vaccine viruses of a particular serotype are likely to provide diverse levels of protection when used in challenge experiments using birds vaccinated with the same dose. These differences have already been noted in Australia for serotype 1 vaccines and for cell-associated and cell-free HVT. Serotype 1 vaccine viruses in particular, are likely to grow to different titres in CEFs, which means that the particular immunising dose set by manufacturers for different viruses is likely to vary.

At the commencement of this project discussions were held with representatives of vaccine manufacturers and the industry to determine the type of assay to be used for the routine testing of vaccines and other important issues regarding such a facility. The following recommendations were agreed to:

1. Reference viruses were to be used with the assays performed. A HVT (serotype 3) reference was to be run in parallel with each HVT batch vaccine and, similarly, a Rispens (serotype 1) control was to be set up with all Rispens and Maravac vaccines.

2. The need for long-term stability testing of reference preparations was recognised.

3. RMIT University was to develop standard methodology for vaccine assays.

4. The reference laboratory was to be accredited with the National Association of Testing Authorities (NATA).

At present there are three procedures in use for the routine testing of vaccines (Table 7.1): i) The Semi-Solid (agarose) Method was first devised by TGAL for assays of cell free HVT and was later adapted for Maravac vaccines.

ii) The Microtitre Assay utilises primary or secondary chicken embryo fibroblasts (CEFs), and is suited to vaccine viruses that do not plaque readily. This procedure is less mathematically precise. The determination of endpoints by this method is very labour intensive and requires the use of many replicates.

iii) The Liquid Overlay method uses secondary CEFs and is the preferred internationally recognised method and is employed in the laboratory. Secondary plaque formation can lead to an overestimation of virus titre, however, RMIT University has not had this problem whilst performing these assays.

Table 7.1 *. MDV Vaccine assays currently used in Australia*

Assay	Advantages	Disadvantages
Agarose	Quantitative; no secondary foci	Suited to cell-free virus; enumeration difficulties
Microtitre	No secondary foci; Useful for low titre viruses	Less sensitive; many replicates required; labour intensive
Liquid Overlay	Easy to read; internationally recognised assay	Secondary plaques; Overestimation of titres

7.1.2 Vaccine assay

Four assays were developed in consultation with MDV vaccine manufacturers. Three involved the use of liquid overlays type assays and one was a semi-solid (agarose) procedure. Vaccine manufacturers usually provided their own diluent for stability assays.

Over 1000 assays were conducted since the establishment of the facility in January 1998 (Table 7.2). The majority of these were core assays in which vaccine and diluent batches produced yearly are tested immediately after preparation and were retested on a three-monthly basis. Research and developmental (R&D) assays formed part of developmental work, including work performed as part of this project.

Core assays represent four individual suppliers of vaccine, with the majority (about 90%) performed for one of these.

Table 7.2 *. Marek's disease vaccine assays conducted from January 1998 to March 2001*

Company	1998						1999						2000						2001					
	Core[d]			R&D[e]			Core			R&D			Core			R&D			Core			R&D		
	V[a]	D[b]	A[c]	V	D	A	V	D	A	V	D	A	V	D	A	V	D	A	V	D	A	V	D	A
The Marek's Company (TMC)	142	31		66			197	47		89			220	25		26	20		58					
Hy-Line	17			18			16						8						4					
Fort Dodge	5												6			16	4	12	4					
Inghams		2																						
TOTAL	**164**	**33**		**84**			**213**	**47**		**89**			**234**	**25**		**42**	**24**	**12**	**66**					

[a]V = Vaccine assays (Liquid pfu)
[b]D = Diluent assays (Liquid pfu)
[c]A = Agarose assays (ffu)

[d] assays which tested vaccine and diluent batches upon initial production and retested on a three-monthly basis
[e] assays which form part of research and developmental work.

7.1.3 Preparation and maintenance of reference vaccine viruses

Establishing a nominal titre and cell count for each reference virus

For the vaccine assays, a HVT reference was titrated in parallel with each HVT batch and a Rispens control assay was carried out with all Rispens and Maravac vaccines. The Rispens (CVI 988) and HVT (FC 126) reference preparations were prepared in 1997 by The Marek's Company (TMC). Rispens batch number (BN) M7101 and HVT BN H7301 were nominated as reference preparations. Initially, approximately 20 assays of ampoules from each batch were performed by the liquid-overlay method. Cell counts and virus titres of the reference preparations were analysed in order to monitor test sensitivity and reproducibility. Data obtained from these assays were used to derive a nominal (mean) titre and cell count with standard deviations for each reference preparation during the establishment phase. The nominal titre and cell count were used to monitor the reference preparations during the monitoring phase.

The maximum and minimum limits set were ± 2 standard deviations for the cell count and ± 1 standard deviation for the virus titre. These limits assume that the cell count is less precise than the virus count. A moving average (i.e. the average of the current result and the two proceeding results) was also determined as it is a better indicator of trends rather than the use of individual data.

Subsequent testing of reference preparations

Nominal cell counts and titres were revised for the two reference viruses after their substantial use over a period of three and a half years.

The cell counts of the first 20 assays of both reference preparations were quite variable (Figures 7.1 & 7.3) but seem to have stabilised over time. The revised cell counts support this observation, as they were slightly lower than those observed in the first 20 assays and the standard deviation decreased for both preparations. This trend was probably due to operator difference or to a loss in titre of the reference vaccine.

Virus titres for H7301, (Figure 7.2) appear to be less variable than cell counts (Figure 7.1). The serotype 1 reference virus, M7101 was less stable than H7301 but most results still fall within one standard deviation (Figure 7.4), however, the revised virus titres did indicate stability of both reference preparations and thus validated the nominal virus titres.

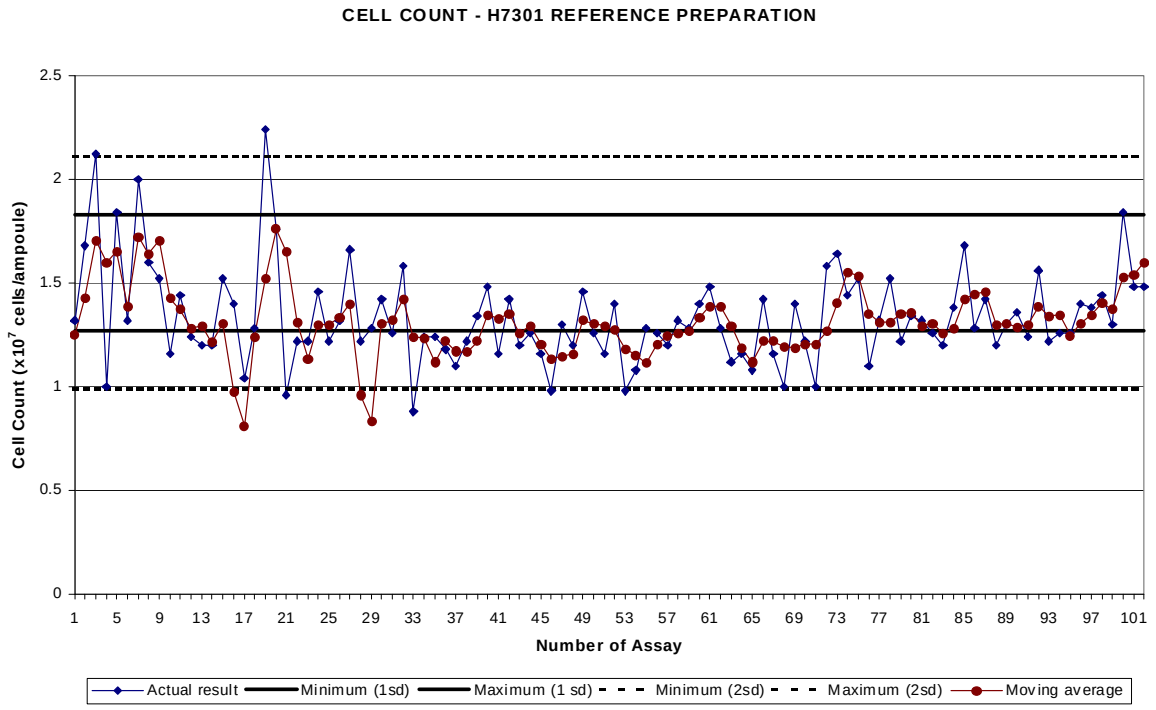


Figure 7.1 *Cell counts of H7301 reference virus preparations used in vaccine assays conducted at RMIT University*

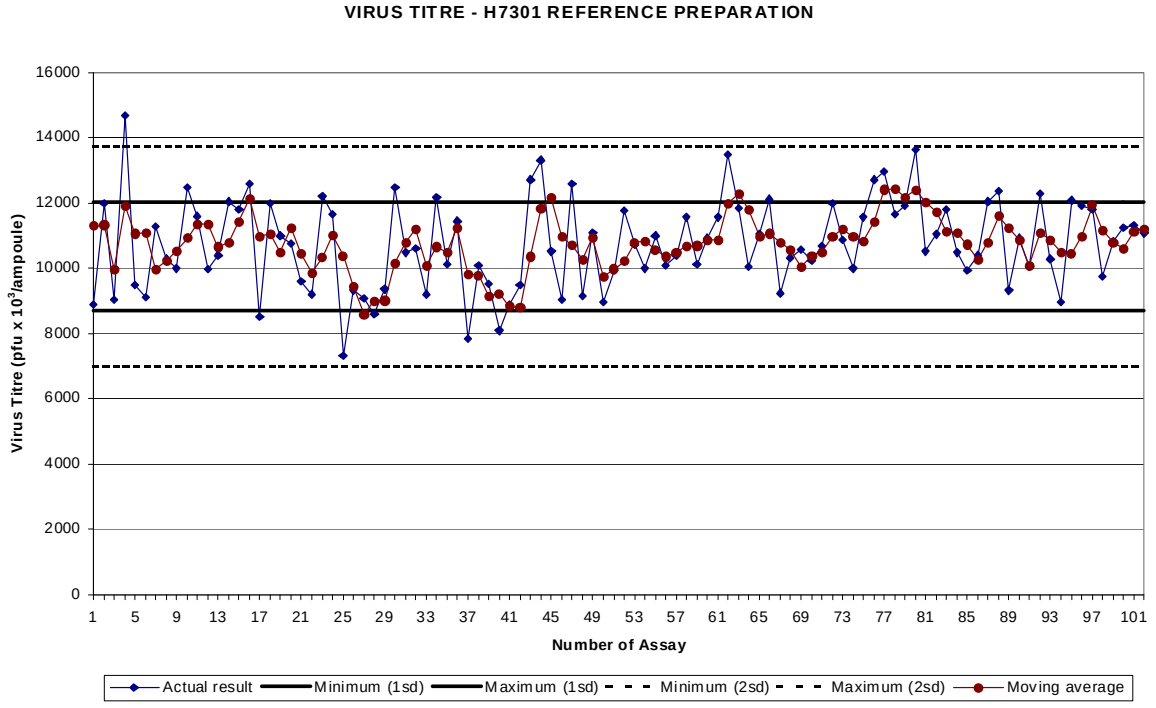


Figure 7.2 *Virus titre of H7301 reference virus preparations used in vaccine assays conducted at RMIT University*

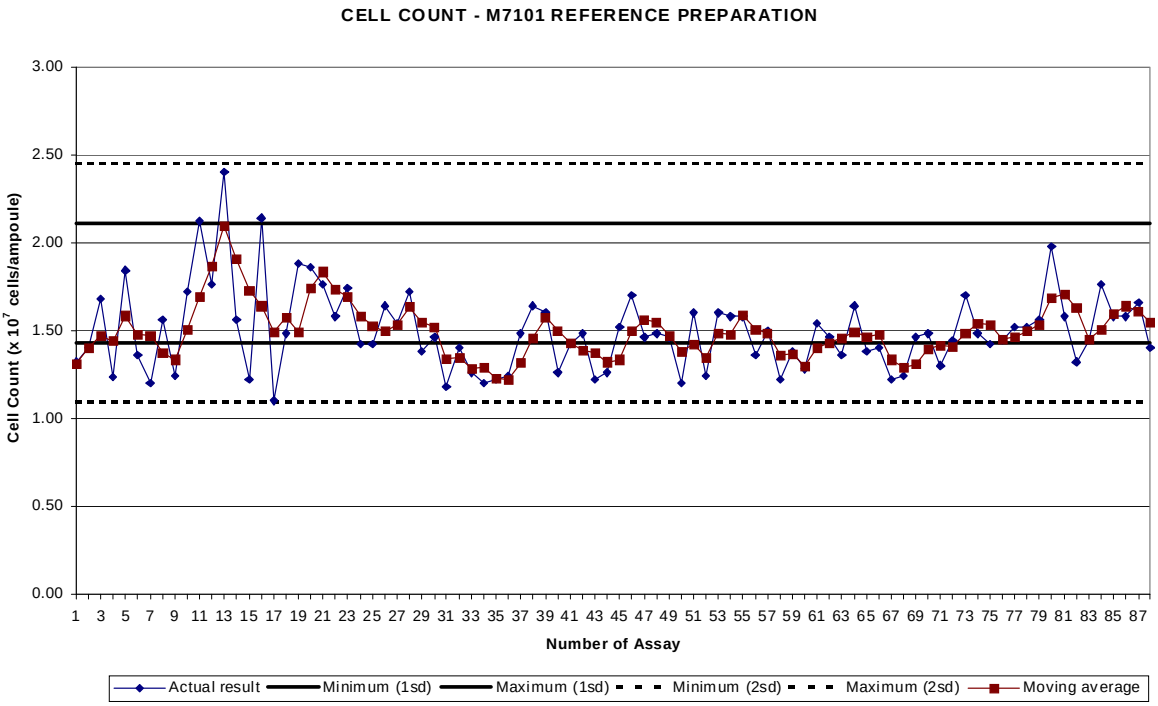


Figure 7.3 *Cell counts of M7101 reference virus preparations used in vaccine assays conducted at RMIT University.*

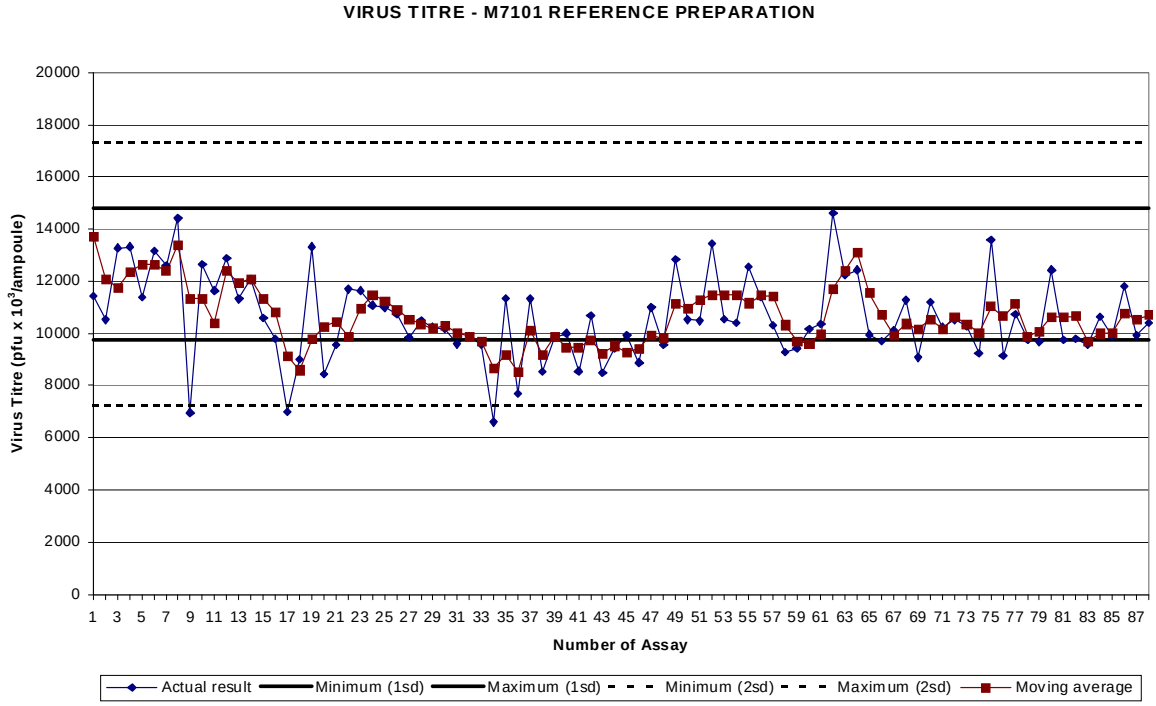


Figure 7.4 *Virus titre of M7101 reference virus preparations used in vaccine assays conducted at RMIT University*

7.1.4 NATA accreditation

The Virology Laboratory already has accreditation with NATA and the Royal College of Pathologists of Australasia as a facility for human influenza vaccines.

The quality manual of the Virology Laboratory (influenza) was revised in late February 2001 to incorporate the new NATA requirements AS ISO/IEC- 17025-1999 and the Marek's disease section incorporating the vaccine assay facility. Standard operating procedures (SOPs) and methods were completed for the purpose of registration.

The Marek's disease section of the Virology Laboratory, following an advisory visit from the regional coordinator for Biological testing from NATA, and external auditors from the Therapeutics Goods Administration, was accredited in June 2003. It has accreditation under 8.02 Tests on veterinary pharmaceutical and biological products and 0.20 Potency of immunological products.

7.2 Optimisation of methods for standardising MDV vaccines

7.2.1 Effect of temperature of diluent on Marek's disease vaccine titre

Studies were carried out in response to industry concerns about possible losses to vaccine potency from the widely used practice of injecting it into chilled diluent. Such practices had been shown to result in losses of MD vaccine titre of about 25% (Lumbardi *et al*, 1982).

In these investigations the standard method for measuring potency losses from diluent, which involves reconstituting thawed vaccine in room temperature (RT) diluent and incubating the diluent-virus mixture for 2 hours in an ice bath, was compared with a slight variation of the method which involved reconstituting thawed vaccine into cold, refrigerated diluent and holding for 2 hours in an ice-bath before retitrating.

Table 7.3 clearly outlines a significant loss of 37% in vaccine titre when vaccine is reconstituted in diluent held at 4°C (Student's *t*-test; P< 0.05) opposed to using diluent held at RT. The table also demonstrates that there is a loss in titre (an average loss of 23.4%) over two hours, whether RT or cold diluent is used to initially reconstitute vaccine. Hence, it is suffice to state that in order to retain the optimal immunising power of a vaccine, it is critical to use RT diluent and to preferably vaccinate without delay.

Table 7.3 . *Loss of frozen Marek's disease vaccine titre in relation to temperature of diluent.*

Time point	**0 hours**		**2 hours**	
Diluent	**A**	**B**	**A**	**B**
Temp. of diluent	**+22-+25°C**	**+4°C**	**0°C (ice)**	**0°C (ice)**
PFU x 10^3/	9 200[a]	4 240[a]	8 040[a]	3 200[a]
ampoule	7 480[a]	5 440[a]	5 760[a]	4 280[a]
	9 680[b]	6 400[b]	6 800[b]	4 480[b]
	8 160[b]	5 760[b]	7 300[b]	3 840[b]
Average	8 630	5 460	6 975	3 950
Loss (%)	**-**	**36.7**[c]	**19.1**	**27.7**

[a] vaccine strain HVT FC 126, batch # H7301, exp. Nov 98 used; diluent batch # 084800901-200 mL, exp. 15/01/00 used.
[b] vaccine strain HVT FC 126, batch # H7301, exp. Nov 98 used; diluent batch # 084800903-400 mL, exp. 15/01/00 used.
[c] Student's *t*- test P=0.011 (P<0.05), highly significant.

7.2.2 Effects of differences in operator

Further investigations explored the significance of operator difference on MD vaccine titre following a change of the operator performing the vaccine assays during the period of the project. This changeover resulted in a 10-20% decrease in vaccine potency for both reference preparations.

Several parallel assays were set up between two operators and substantial losses were still observed (Table 7.4). Operator 2 used a slight variation to the standard method from Operator 1, in which dilution of thawed vaccine was carried out with cold diluent. It was shown in Section 7.2.1 that cold diluent significantly reduces the estimated titre of the vaccine. The discrepancy between the two operators was rectified and parallel assays were performed using the same method. Differences in vaccine titre between the operators were not significant (Table 7.5).

Table 7.4 *. Effect of operator difference on Marek's disease vaccine titre; use of cold diluent by Operator two.*

Assay Number	**Titre** pfu x 10^3 cells/ampoule			
	HVT		**Rispens**	
	Operator 1	**Operator 2**	**Operator 1**	**Operator 2**
1	12080	9720	11240	8600
2	9440	8680	9720	7360
3	10320	10080	10040	9280
Average	10613	9493	10333	8413
Loss (%)	-	**10.6**[a]	-	**18.6**[a]

[a] Student's *t*- test P=0.017 pooled (P<0.05), highly significant.

Table 7.5 . *Effect of operator difference on Marek's disease vaccine titre by the same method using diluent held at room temperature.*

Assay Number	**Titre** pfu x 10^3/ampoule			
	HVT		**Rispens**	
	Operator 1	**Operator 2**	**Operator 1**	**Operator 2**
1	9000	11320	9320	8760
2	11520	11160	8320	9880
3	11200	10120	9360	9600
4			10880	10920
Average	10573	10867	9470	9584
Gain (%)	-	**2.8**[a]	-	**1.2**[b]

[a] Student's *t*-test P=0.78 (P<0.05), not significant.
[b] Student's *t*-test P=0.53 (P<0.05), not significant.

7.3 MDV tests and assays available at RMIT University

RMIT University provides a national independent assay facility for MD vaccines for the general use by all Australian manufacturers. Several other diagnostic tests for MDVs are also available (Table 7.6).

In addition to these diagnostic services, RMIT provides and maintains a repository of MDV serotype 1 challenge viruses. Strain MPF 57, has been supplied to the industry for use in several vaccine efficacy trials. This virus was originally isolated in 1994 from a vaccinated flock of chickens in the Sydney region (De Laney *et al*., 1998) and has been passaged to a limited extent in cell culture. It is supplied to the industry at passage 12.

Table 7.6 *. Summary of diagnostic tests and vaccine assays currently available at RMIT University Virology Laboratory.*

Test	Time taken	Sensitivity	Advantages	Limitations
Isolation of MDV in cell culture	2 weeks (1 passage) 3-4 weeks (2 or 3 passages)	Low	Can discriminate between Serotype 1, 2, 3 vaccine and virulent MDV	Time consuming, Tissue Culture (TC) intensive
PCR for detection of Serotype 1	3 days	High	Rapid	Can **not** discriminate between Serotype 1, 2, 3 vaccine and virulent MDV
PCR for detection of HVT	3 days	High	Rapid	Can **not** discriminate between Serotype 1, 2, 3 vaccine and virulent MDV
AGP test[##]	1 week	Low		Not serotype specific
Marek's Potency Assays				
Liquid overlay	2 weeks	High	Precise	TC intensive
Vaccine	2 weeks	High	Precise	TC intensive
Diluent	2 weeks	High	No 2° plaques	TC intensive
Agarose overlay	2 weeks	Low	Suited to low titre viruses	TC intensive
Quantal assay				

[#] Marek's disease reagents: feather tip antigen and chicken antisera are also provided.

12. Implications

During the course of the project, concerning the development of the RMIT serotype 1 vaccine, the Rispens derivative CVI988/C/R6 was introduced into Australia. The importation of the master seed of the C/R6 clone was the first live poultry vaccine virus introduced into Australia in over 50 years (Jackson & Groves, 1996). Cyanamid Websters secured Australian Quarantine Inspection Service (AQIS) permission in 1994 to import the seed under an Emergency Permit because of the serious outbreaks of MD that were not being effectively controlled by existing Australian manufactured vaccines. The performance of the C/R6 strain was poor in the field and has since been withdrawn from the market. In 1997, the original CVI988 strain was obtained for use in Australia by The Marek's Company, Fort Dodge and Intervet. Control of MD with this strain seems to be effective to date (C. A. W. Jackson, personal communication).

The RMIT vaccine developed as part of this project has proven effective and, despite the introduction of the two Rispens strains, may still have application in the field. The RMIT vaccine may prove more effective than the Rispens vaccine since it was derived from a recently isolated, highly virulent Australian field strain and may therefore provide greater protection under current Australian conditions than the Rispens strain which was derived from a mildly virulent strain in The Netherlands over 30 years ago. The RMIT vaccine has been stored for future reference and/or use.

Interestingly, despite the development and importation of new serotype 1 vaccines in Australia, a serotype 3 vaccine (strain FC126) still attracts the largest share of the vaccine market. Other MD vaccines registered in Australia, but not yet launched include a bivalent CVI988/HVT (strain FC126) and a monovalent CVI988 vaccine by Fort Dodge. Production of the serotype 2 vaccine, Maravac, is likely to cease as demand for it lessens.

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