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Molecular Diagnostics for Marek's Disease

(Molecular diagnostic techniques to detect both vaccine and wild type Marek's disease viruses)

A report for the Australian Egg Corporation Limited

by Dr Graham W. Burgess

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Molecular Diagnostic Tools for Wild Type and Vaccine Strains of Marek's Disease Virus
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Foreword

Marek's disease is a disease of chickens produced by a herpesvirus that results in reduction in the immune response in acutely infected birds followed by the production of tumours in many of the infected birds. This disease significantly limits the productivity of both egg producing and meat producing birds. Vaccination of all breeding and egg laying birds is necessary to reduce the effects of this disease. There has been a progressive increase in the effects of this herpesvirus on all classes of chickens and effective vaccination has become a necessity in most sectors of the industry.

There are several vaccines available for this disease. However, they are relatively expensive and as they rely on the inoculation of day-old chickens with live cells infected with one of the vaccine viruses, the probability of failure is relatively high. Monitoring of vaccination is an essential part of effective farm management. It is necessary to monitor the presence of the vaccine virus as it grows in cells in the bloodstream of the vaccinated chicks. This is both difficult and expensive.

This project aimed to develop simple cost-effective sample collection techniques combined with a series of assays that are able to detect the genes of the vaccine viruses in the blood of vaccinated birds. This has the potential to significantly reduce the cost of monitoring vaccination and allows monitoring of commercial flocks on a routine basis to be carried out. This will significantly improve the productivity of all sectors of the industry. The project met its objectives and developed simple sample collection techniques combined with a series of cost-effective assays that can be semi automated for all three classes of Marek's disease vaccine viruses.

The project also developed simple and effective techniques for demonstrating the presence of the wild type virus responsible for the production of the disease. Imported vaccine strains of Marek's disease virus closely resemble the wild type viruses. This project was able to identify subtle differences between the imported vaccine strain and the Australian wild type viruses and exploit these differences in the development of diagnostic tests that can readily differentiate the two classes of virus.

The assays developed by this project are being further developed into commercial kits that are being transferred to the relevant parts of the industry and they have the potential to generate revenue on the export market.

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

This report is an addition to AECL's range of research publications and forms part of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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One of the major financial contributions to this project has been made by TropBio (Townsville) and the assistance offered by Dr Jan Smith has been invaluable to the success of this project.

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Abbreviations

Abbreviation	Full name
General Reagents	
ABTS	2,2-azino-di-(3-ethyl-benzthizoline sulphonic acid)
Anti-Dig-POD	Antidigoxigenin-horseradish peroxidase conjugated antibody
ATV	Antibiotic Trypsin Versine
BCIP (x-phos)	5-bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
CEF	Chicken embryo fibroblast cells
CPE	Cytopathic effect
Dig-UTP	Digoxigenin-11-deoxyuricil triphosphate
DMEM	Dalbecio's Modified Eagle media
DNA	Deoxyribonucleic acid
ddH_2O	deionised water
dNTP	Deoxynucleotide
dTTP	Deoxythimidine triphosphate
EDTA	Ethylenediaminetetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ELOSA	Enzyme linked oligosorbent assay
FBS	Foetal bovine serum
gA	Glycoprotein A
gB	Glycoprotein B
gC	Glycoprotein C
HVT	Herpesvirus of Turkeys
MD	Marek's disease
MDV	Marek's disease virus
$MgCl_2$	Magnesium chloride
NBT	Nitro Blue Tetrazolium
PBS	Phosphate buffered saline complete
PBS-A	Phosphate buffered saline without calcium
PCR	Polymerase chain reaction
TAE	Tris-Acetate EDTA
T_m	Melting temperature of DNA. The temperature at which 50% of the DNA sample denatures to become single stranded
Units of Measure	
Abs	Absorbance value
bp	Base pairs, refers to the length of a DNA fragment
g	Times gravity
g	Grams
mg	milligrams
µg	micrograms
ng	nanograms
pg	picograms
fg	femptograms
ag	attograms
l	Litres

ml	millilitres
μl	microlitres
mol	moles
mmol	millimoles
μmol	micromoles
M	molar (moles l^{-1})
mM	millimolar
μM	micromolar
nM	nanomolar
°C	degrees celcius
sec	seconds
min	minutes
hr	hours
v	volts

Executive Summary

Introduction

Marek's disease is a disease of chickens produced by a herpesvirus that produces a reduction in the immune response in acutely infected birds followed by the production of tumours in many of the infected birds. This disease significantly limits the productivity of both egg producing and meat producing birds. Vaccination of all breeding and egg laying birds and in some cases broilers is necessary to reduce the effects of this disease.

A progressive increase in virulence of Marek's disease type 1 has been noted in Australia and coupled with the introduction of genetic strains of birds with an increased susceptibility to Marek's disease, problems are very likely to increase.

Vaccination

Existing vaccines have been based on the use of Type 2 Marek's disease virus and Turkey Herpesvirus (HVT, Type 3). Recent introduction of imported and locally produced Type 1 Marek's disease vaccines has complicated monitoring of vaccine efficacy. *In ovo* vaccination has provided new options that must be evaluated.

Vaccination is relatively expensive and relies on the inoculation of chicken embryos or day-old chickens with one of the vaccine viruses as a cell associated or cell free vaccine. The probability of failure is higher in cell associated vaccines where fibroblast cells infected with attenuated virus need to be delivered to the chick in a viable state. Monitoring of vaccination is an essential part of effective farm management. It is necessary to monitor the presence of the vaccine virus as it grows in cells in the bloodstream of the vaccinated chicks. This is both difficult and expensive.

Diagnostics and Monitoring of Viraemia

Current techniques for the monitoring of serotype 1vaccine efficacy during suspected outbreaks have severe limitations. This project aimed to develop simple cost-effective sample collection techniques combined with a series of assays that are able to detect the genes of the vaccine viruses in the blood of vaccinated birds. This has the potential to significantly reduce the cost of monitoring vaccination and allows monitoring of commercial flocks on a routine basis to be carried out.

The use of effective diagnostics will significantly improve the productivity of all sectors of the industry. The project met its objectives and developed simple sample collection techniques combined with a series of cost-effective assays for all three serotypes of Marek's disease vaccine viruses, that have the potential be semi automated.

The project also developed simple and effective techniques for demonstrating the presence of the wild type virus responsible for the production of the disease. Imported vaccine strains of Marek's disease virus (MDV) closely resemble the wild type viruses. This project was able to identify subtle differences between the imported vaccine strain and the Australian wild type viruses and exploit these differences in the development of diagnostic tests that can readily differentiate the two viral strains.

The assays developed by this project are being developed further into commercial kits that are being transferred to the relevant parts of the industry and they have the potential to generate revenue on the export market.

The monitoring of vaccine viraemias can be achieved by collecting a three to five millilitre sample of whole blood with a suitable anticoagulant and propagating the virus in cell culture following the purification of lymphocytes from the blood. This requires the sample to be transported in a way that the lymphocytes will remain viable until they can be inoculated into the cell cultures. Molecular diagnostics are based on the detection of viral genome and it is not necessary to maintain viability of lymphocytes in transit.

Three different serotypes of vaccine viruses are being used on the Australian market. At the time that this project commenced, research groups overseas published sequence information on all three groups of viruses. Using this information we were able to develop sets of primers for use in the polymerase chain reaction (PCR) for each of the three vaccine viruses. These primers targeted areas of the genome that were specific for each of the three MDV serotypes. This ensured that the diagnostics developed were specific for each of the three serotypes. This was confirmed by rigorous testing with representatives of each of the three serotypes of MDV.

Sample Collection and Processing

This project extended the simplification of the collection techniques by developing a system for placing small quantities of whole blood onto an absorbent paper that could be dried and simply sent in the mail to the testing laboratory. This required the development of a suitable absorbent paper that was treated with a preservative to ensure the viability of the DNA in transit. We evaluated commercial treated papers and were able to develop a simple treated paper that performed at least as well as commercial products and proved to be a lot more cost-effective.

The samples collected onto dried filter paper contain relatively small numbers of infected lymphocytes. It was therefore necessary to develop genome detection assays that were sufficiently sensitive to detect the low copy number of vaccine virus present in the sample. This was achieved by using nested polymerase chain reaction for each of the assays. The sensitivity of the assays was confirmed using serial dilutions of virus as well as samples collected from vaccinated birds.

Polymerase Chain Reaction

In order to make the assay simple and cost-effective, a system was developed to allow the nested PCR to be carried out in 96 well trays. The nested PCR is a two-step process that requires the addition of a second set of reagents after the first amplification step. This provides an opportunity for contamination of the samples resulting in false positives being produced. We developed a technique for overcoming this problem resulting in a PCR that was capable of being automated and retaining the additional sensitivity and specificity afforded by the nested PCR configurations PCR.

The traditional way of detecting the product from a PCR is to carry out electrophoresis in an agarose gel. Small products migrate much more quickly through the gel than do larger products. The position of the product can be compared with a set of controls to determine whether the product produced is of the expected size. We evaluated a commercial 96 well agarose gel electrophoresis system that produced results for all 96 products in a much shorter time than in the conventional agarose gel systems.

Colorimetric Detection

The detection of PCR products in agarose gel is not a technique normally used in routine diagnostic laboratories. We have designed an additional set of probes that can be attached to the PCR products and this attachment adds specificity to the reaction. The combined probe and PCR product is anchored to the bottom of a 96 well plate and the PCR product is detected using a label that is incorporated during the final step of the nested PCR reaction. The final indicator system is an enzyme labelled antibody that results in the production of coloured product in the wells containing the specific PCR product. This procedure of coloured end product detection adds both additional sensitivity and specificity to the reaction. The procedure is very similar to a range of assays that are normally carried out in a diagnostic laboratory. This makes the use of coloured end product detection a very attractive option in most diagnostic laboratories.

Detection of Field Strains of Serotype 1

The project also developed a series of assays aimed at the detection of birds infected with wild type virus. Emphasis was placed on the selection of the most appropriate samples that would allow the detection of wild type virus in infected birds without the need to kill birds in order to collect tissue samples. In the early stages of the infection we demonstrated that wild type virus was present in relatively high concentrations in the blood of the infected birds. As the disease progressed the levels of virus in the blood decreased significantly. However, there was then a substantial increase in the levels of virus in the feathers of the infected birds. We developed a simple and reliable system of collecting samples from a limited number of feathers from each of the infected birds.

Using the wild type virus detection techniques we were able to demonstrate that many of the birds that were vaccinated with the conventional vaccines especially the turkey herpesvirus vaccine were also infected with wild type virus that could be detected as the early as 10 days of age.

Strain Differentiation within Serotype 1

The most recently introduced serotype 1 vaccine, CVI 988 or Rispens, is based on a mildly virulent virus that has been modified to make it less virulent by growing it for some time in chicken embryo fibroblast cell cultures. This modified or attenuated virus is very similar to the original wild type virus. Several publications have described a change that occurs in the virus where there is an increase in a part of the genome that is repeated known as the 132 bp repeat. We have confirmed that the vaccine viruses do have an increase in the number of 132 bp repeats. However, once the vaccine virus is inoculated into birds the increase in the number of 132 bp repeats reverts to the two copies that are normally found in the wild type virus. We have therefore verified the findings of some other workers that there is a change in the virus during attenuation and some of the changes that occurred during attenuation are rapidly removed once the virus is exposed to the immune system of the chicken.

We have exploited a number of the other genes that are candidates for controlling the process of tumour formation and related functions in the bird. We have identified three genes where there are changes that can be exploited to differentiate wild type and the vaccine viruses. We have developed assays that will exploit these changes and allow us to differentiate between birds that have been vaccinated with attenuated type 1 Marek's disease virus and those birds that are infected with wild type virus.

These strain differentiation assays are being rigorously evaluated and we are comparing vaccine viruses with extended panels of wild type viruses from both Australia and a range of other countries.

In situ PCR

The project also aimed to develop assays that would allow the detection of the genes of Marek's disease viruses, both vaccine and wild type, in tissue sections so that the distribution of infected cells could be demonstrated. This technique is known as *in situ* PCR. Considerable progress has been made in developing reagents that will allow us to carry out *in situ* PCR on a variety of tissues from vaccinated birds and from embryos. The full procedure to date has been elusive. However, much of the initial development has been carried out and it is anticipated that the technique will be applied in the relatively new future.

The assays that have been developed have been applied to samples collected from birds vaccinated with a range of different vaccine protocols. Some of the sets of samples were collected from the same birds from which samples for viral isolation were also collected. A comparison was made between their results for cell culture isolation and genome detection combined with the simple sample collection and semi-automated testing techniques. The cell culture isolation techniques failed to detect any additional samples and in some cases the cell cultures were contaminated and they failed to give an answer while the genome detection produced a definitive result.

1 Introduction

1.1 Marek's Disease

1.1.1 Brief History

Marek's disease (MD) in a lymphoproliferative disease that affects commercial and domestic chickens. It was first reported as a paralysis of roosters in 1907 and the first reported outbreaks occurring in the USA as early as 1914 (Calnek & Witter, 1991). Since then there have been many reported MD outbreaks in the USA, Europe and Australia. Work by Churchill and Biggs, (1967) determined that the etiological agent of MD was a herpesvirus, Marek's disease virus (MDV). It was subsequently found that MDV was a group of three closely related viruses, distinguishable using immunoprecipitation methods as Serotypes 1, 2 and 3. Serotype 3 is also known as Turkey herpesvirus, and like serotype 2 MDV is in non-pathogenic in chickens. The development of an effective *in vitro* culture system for MD by Churchill *et al.*, (1969) paved the way for the attenuation of serotype 1 viruses by serial passage in chick embryo fibroblast cell culture. The first vaccines produced by this method were introduced in the early 1970's and significantly reduced MD losses incurred by poultry producers. Since the introduction of attenuated vaccines there has been a steady increase in the virulence of MDV evidenced by losses due to the disease in vaccinated birds in Australia (Zerbes *et al.*, 1994) and internationally (Witter, 1997). Field strains of serotype 1 MDV are now separated into distinct pathotypes on their ability to cause lesions in vaccinated birds. The pathotypes range from mildly pathogenic (mMDV) to the virulent strains (vMDV) and the newer very virulent plus (+vvMDV) isolates. The latest terminology to be used in pathotyping is hypervirulent although this name has not yet been given an acronym in scientific publications.

Today there are a number of MDV vaccines in use throughout the world that have been derived from all three serotypes. These vaccines are administered singly, as a bivalent vaccine containing serotype 2 and HVT (USA and Europe) or HVT and attenuated serotype 1 (Australia) or as a trivalent vaccine (Witter, 1985) using all three serotypes. In spite of the extensive use of vaccines, MD continues to be problematic to producers and veterinarians within the poultry industry.

1.1.2 Infection and Pathogenesis

MDV is spread through the inhalation of infective dander in the immediate environment of commercial sheds where birds are housed at high densities. Enveloped viral particles in the dander (feather follicle epithelium) are able to gain entry to the birds lymphatics either through alveolar macrophages or direct entry into respiratory epithelial cells. Following inhalation, MDV can be recovered from spleen tissue within two days and is released from feather follicle epithelium at around two weeks.

Early infection with MDV produces a short cytolytic phase, predominantly in B-cells. Following the early cytolytic phase, MDV becomes latent in activated T-cells. This latent state is generally well established within five to seven days post infection. Although there has been a great deal of research into the mechanisms involved in the shift from lytic to latent infection, latency in MDV infected cells is not well understood. However, it is well established that latency is required for the MDV infected lymphocytes to undergo transformation and subsequently form tumours of peripheral nerves and visceral organs.

There has been little work done on elucidating the mechanisms required for the switch from latent infection in lymphocytes to a productive lytic infection in feather follicle epithelium. It is likely that there are numerous virus host interactions required, given that MDV must overcome the latent state before it can migrate to epithelial cells.

1.1.3 Vaccination

Vaccination has traditionally been done at one day post hatch. However, more recently *in ovo* vaccination at 18 to 19 days of incubation has become popular with broiler producers because of its lower cost. In Australia, *in ovo* vaccination is done using cell associated HVT at various manufacturers doses or part thereof. There is some dispute at present as to the efficacy of *in ovo* vaccination with respect to the vaccine deposition site. The inference being that only vaccine deposition in the embryo produces an effective early immune response in the bird. Although live animal trials have been carried out to investigate this assertion, the mechanisms of viral uptake likely to play a role in this are not understood.

1.1.3.1 HVT Vaccination

HVT was first used as a vaccine following the first breakthrough by field strains of serotype 1 with increased virulence, in birds vaccinated with an attenuated serotype 1. In Australia, two strains of HVT are used regularly and effectively as vaccines. FC 126 (Bioproperties and Fort Dodge) is an imported strain sold within Australia. NSW 1/70 is a local isolate produced in Australia by Intervet. Vaccination with HVT is given in the full manufacturer's recommended dose (viral titre not given by manufacturers) together with attenuated serotype 1 CVI 988 (Rispens) as a bivalent vaccine in layer birds. Broilers are generally vaccinated with HVT at much lower dosages via the *in ovo* route.

1.1.3.2 Serotype 2 Vaccination

There is only one serotype two vaccine used within Australia at present. This is an Australian isolate marketed under the traded name "Maravac" by Fort Dodge Ltd. Although very popular in the 1980's, outbreaks following vaccination in the 1990's caused it to lose popularity to the extent that few producers routinely vaccinate with type 2.

1.1.3.3 Serotype 1 Vaccination

Vaccination with attenuated serotype 1 vaccine in Australia is done exclusively with CVI 988, also commonly called Rispens after the researcher who isolated the virus for the first time (Rispens *et al.*, 1972). As stated previously, CVI 988 is generally used in conjunction with HVT as a bivalent vaccine in layer birds. An Australian MDV serotype 1 was isolated from commercial birds in NSW and attenuated by serial passage in cell culture. This isolate BH16 has been tested in birds challenged with local virulent strains (Delaney *et al.*, 1998) and the results indicate that it offers good protection against MD. However, BH16 is not being offered as a vaccine at this stage.

1.1.3.4 Polyvalent Vaccination

Polyvalent vaccines were composed of blends of all three types of vaccines, the serotype 1 component was found not to be required for vaccine efficacy after a series of trials (Witter, 1985). Today most polyvalent vaccines are bivalent (Witter, 1985). Trials of polyvalent vaccines (FC-126, Md11/75c and SB-1) delivered by *in ovo* injection resulted in higher protection rates than vaccination one day post-hatch (Sharma & Witter, 1982). Other trials using bivalent vaccines (HVT/SB-1) produced similar increases in the protection against virulent challenge virus (Sarma *et al.*, 1995). Interestingly, the manufacturer of INOVOJECT® use a monovalent HVT at slightly higher titres when trialing their machines (Marsh *et al.*, 1997).

1.1.4 Diagnosis

1.1.4.1 Cell Culture

The primary method for the diagnosis of MDV is viral isolation in cultures of chicken kidney cells (CK), duck embryo fibroblasts (DEF) or chicken embryo fibroblasts (CEF). Veterinarians, when diagnosing MD, also use gross pathology and histopathology following necropsy of suspect birds. Field strains of serotype 1 MDV are generally isolated using CK and DEF cultures while CEF culture is very useful for serotype 2 and HVT isolation. Typically MDV plaques develop in cell culture after five to fourteen days post inoculation. There are sufficient morphological differences between the plaques produced by MDV 1, MDV 2 and HVT to allow cell culture to be used as a serotype diagnostic. Fluorescently tagged antibodies specific for each serotype are also sometimes used as a confirmatory procedure.

1.1.4.2 Sampling, Collection and Transport

Samples for cell culture diagnosis generally consist of lymphocytes isolated from buffy coat or spleen of suspect birds. The purified lymphocytes are used to inoculate monolayers and the resulting plaques enumerated. Samples of this type require collection by highly trained staff in aseptic conditions if diagnosis is to be effective. In addition, samples need to be transported to the diagnostic laboratory rapidly and under refrigerated conditions. This makes the diagnostic procedure expensive and susceptible to contamination. Furthermore, the amount of blood required for buffy coat preparation or the removal of the spleen as samples results in the sacrifice of one chick per sample.

1.2 PCR

1.2.1 Introduction

The polymerase chain reaction (PCR) has been used with great success in the diagnostics of bacterial and viral diseases in humans for many years and in its various forms has cemented a place in the modern pathology laboratory. PCR is the technique where very specific fragments of target genome are amplified *in vitro* to the exclusion of all other sequences present in a sample. In effect, this means that viral DNA in infected birds can be selectively amplified and identified amongst the milieu of chicken DNA.

1.2.2 PCR Technology in Diagnostics

Like most diagnostic techniques, PCR can be used in any of several formats. Standard single amplification series PCR uses a single set of two matched primers to amplify a specific fragment that lies between the primer binding sites on the genome of the target organism. Variations on single series are nested and semi-nested PCR. Nested PCR uses two sets of primers, one set binding to the amplification product of the other set. Semi-nested works in a similar manner but has one primer common to both amplification series. In both cases, the product from the first amplification series is used as template in the second. Both nested techniques offer vastly superior sensitivity to single series reactions. However, nesting of PCR reactions is very prone to carryover contamination from previous reactions and under normal conditions is not able to be used in conjunction with the Uricil-N-glycosylase (UNG) system (Roche, USA).

1.2.2.1 PCR in MD Diagnostics

PCR's designed specifically for the diagnosis of MDV have been described on several occasions by other researchers with primer sets published for all three serotypes (Davidson *et al.*, 1995; Aminev *et al.*, 1998; Handberg *et al.*, 2001). In spite of the specificity reported for these PCR's, none have been adopted as standard diagnostic techniques within the poultry industries of their respective countries. One of the possible reasons for the reluctance to adopt PCR as a routine tool is that it does not offer significant improvements in sensitivity over the existing cell culture techniques. However, all the previously reported PCR's were single series reactions with inherent limitations in sensitivity.

1.2.2.2 Monitoring for Postvaccinal Viraemia

Probably the most widespread use of MDV diagnostics is in the area of monitoring the efficacy of vaccination. Producers need to know whether the vaccine virus is actively replicating in the bird and hence eliciting the required immune response.

1.2.3 Serotype and Strain Differentiation

1.2.3.1Differentiation of Serotype

The traditional cell culture based methods of diagnosis rely heavily on the ability of the technician to recognise morphological differences in cytopathic effect (CPE). This requires highly trained staff and can be very time consuming and expensive. As a confirmation step, immunofluorescence is sometimes used on the monolayer to detect viral antigens. Although this method can readily differentiate between serotypes, it is not able to differentiate between strains of serotype 1.

The recent publication of the complete genome sequence of two serotype 1 isolates, the GA strain (Lee *et al.*, 2000a) and the Md5 strain (Tulman *et al.*, 2000) and single sequences for serotype 3 (Afonso *et al.*, 2001) and serotype 2 (Izumiya *et al.*, 2001) have given researchers the information required to develop highly specific serotypic PCR's. In addition it is now possible to compare the genomes of two serotype 1 isolates to look for exploitable sequence differences.

1.2.3.2 Marker of Attenuation

The ability to differentiate between isolates of serotype 1 is critical given the trend towards higher virulence in wild strains. The lack of effective differentiation techniques means that breaks in vaccination with attenuated type 1 could be due to more highly virulent field strains or ineffective vaccination technique. The accepted molecular marker used to identify attenuated serotype 1 virus has been the expansion of a 132 base pair direct repeat within the terminal and inverted repeats flanking the unique long region (Silva, 1992; Becker *et al.*, 1993). PCR results indicate that there can be anywhere up to 40 copies of the 132 bp sequence following cell culture attenuation. This differs from the field strains that generally contain only two copies of this fragment. However, this marker has been called into question as a useful tool for the specific detection of vaccine strains following the evaluation of attenuated type 1 MDV following *in vivo* passage (Young and Gravel, 1996). These researchers found that the repeat returned to the original two copies following single *in vivo* passage.

1.3 Shortcomings of the Existing Diagnostics

Although the current cell culture based assay is successful in the monitoring of vaccine efficacy, they do have some limitations. The sample system required involves the aseptic collection of blood or spleen tissue followed by refrigerated overnight transport. The sample collection needs to be done by a veterinarian on site and processing of the samples requires an experienced technician. Simple cost effective sample systems would be of great advantage in this area. In particular, systems that alleviate the need for refrigeration would reduce costs significantly.

Currently there is no established method for differentiation between strains of serotype 1. Field strains can be isolated in cell culture but the CPE are not always evident in the first few passages. Rapid and specific diagnostics for serotype 1field strains will be a useful tool that at present does not exist.

Further reductions in cost of vaccine monitoring and wild type detection systems would be achieved with adaptation of new assays to high throughput screening. Many immunological assays and PCR based systems have been successfully conducted in 96 well micro plates. With significant reductions in cost and reduced reporting time, these new assays may encourage producers to monitor the MD status of flocks more regularly.

2 Objectives of this Project

This project is aimed at developing technologies that will form the basis of a suite of assays and systems for the monitoring and diagnostics of MDV in Australia. Ultimately, the assays will be capable of being transferred to the poultry industry or allied sciences.

More specifically, the objectives of the project are to:

- Develop improved procedures, which can detect the genome of serotype 1 MDV in the blood of infected or vaccinated birds.
- Develop sensitive and specific PCR procedures for detecting serotypes 2 and 3 MDV.
- Identify sequences that can be used to differentiate wild type and vaccine strains of serotype 1 MDV
- Develop and evaluate simplified sample collection and indicator systems that match the requirements and capabilities of the Australian poultry industry and transfer these to the relevant Australian laboratories.

3 General Materials and Methods

3.1 Viruses Used for Experimental Work

The viral strains and serotypes used for the optimisation of PCR's were obtained from vaccine suppliers and collaborating institutions and can be seen in Table 3.1.

Table 3.1: Viruses used in the optimisation of experimental PCR's

Strain	Serotype	Supplier
CVI 988 (Rispens)	1	Bioproperties Ltd
BH 16 Pass 15	1 (challenge)	Intervet Ltd
BH 16 High Passage	1 (vaccine)	Intervet Ltd
Woodlands #1 Pass 14	1 (challenge)	RMIT
Woodlands #1 high passage	1	RMIT
BC 1 (MSB 1)	1 (lymphoblastoid cell line)	TropBio Ltd
(Maravac)	2	Fort Dodge Pty Ltd
FC 126	HVT	Fort Dodge Pty Ltd
NSW 1/70	HVT	Intervet Ltd

3.2 Cell Culture

3.2.1 MDCC MSB 1 Cell culture

MDCC-MSB 1 cells (TropBio, Townsville) at passage level 19 were removed from liquid nitrogen storage and thawed rapidly at 37°C. Thawed cells were pelleted by centrifugation at 1500*g* for ten minutes. The supernatant was removed and resuspended in 10 ml of RPMI containing 10% FBS (high growth rate media) before being placed in a 25 cm^2 culture flask (Starstead. Australia) and grown in an incubator at 37°C and 5% CO_2 .

Cells were passaged by dividing the contents into 5 ml aliquots and placing into 75 cm^2 culture flasks containing RPMI with 2% FBS (maintenance media) at 37°C.

3.2.2 Chick Embryo Fibroblast Cells

Primary cell culture was prepared using variations to the method described by Freshney (1983). Chicken embryos were removed from the egg after nine days incubation at 38°C and rinsed once in sterile PBS-A. The head and limbs were removed prior to dissection into three pieces. The embryonic tissue was washed again in PBS-A to remove blood and other cell debris. The washed tissue was emolliated by squeezing once through a sterile 2 ml syringe before being disaggregated in 20 ml of pre-warmed (37°C) ATV for 15min with constant gentle stirring. Cells suspended in ATV were poured off and centrifuged at 1500 *g* for 10 min. ATV treatment of the tissue was repeated to give two harvests of cells suitable for primary culture. After centrifugation the cell pellets were washed (× 2) by resuspension in PBS-A and centrifugation at 1500 *g* for 10 min. Cell pellets were placed in 20 ml of DMEM with 10% FBS and transferred to 75 cm^2 culture flasks and incubated at 37°C.

3.3 DNA Extraction

3.3.1 Standard Phenol/Chloroform Method

All DNA extractions for the isolation of template to be used in optimisation of PCR were carried out using modifications to the method described by Asusbel *et al*., (1995). Two methods were employed, one being a standard high yield method while the other being low yield with higher quality to prevent shear of long base pair (over 40000 bp) templates.

Each cell pellet was suspended in 2 ml of lysis buffer (100 mM NaCl; 10 mM Tris Cl pH 8; 25 mM EDTA pH8; 0.5%W/V SDS; 0.1 mg ml^{-1} proteinase K) per 100 mg of tissue or 10^6 cells and kept at 37°C for 2 hr with gentle agitation (high yield), or overnight without agitation (low yield). Lysed cell solutions were divided into 500 µl aliquots and proteins and lipids were removed using an equal volume of phenol:chloroform:isoamyl-alcohol pH 8.0 at 25:24:1 v/v (Sigma-Aldrich) by gentle mixing and centrifugation at 2000 *g* for 10 min in room temperature conditions. The aqueous phase was removed by pipetting (wide bore tip for high quality method) and placed in a clean microcentrifuge tube. Final clean up was carried out using chloroform: isoamyl-alcohol (24:1) with centrifugation as above. The DNA was precipitated in two volumes of ice cold 100% ethanol. The precipitation mixture was centrifuged at 22500 *g* for 30 min at room temperature. The DNA pellet was washed twice in 70% ethanol before being vacuum dried at 37°C. Template DNA was stored as a dry pellet at -20°C or resuspended in 10 mM Tris Cl and stored at 4°C prior to use.

3.3.2 Commercial DNA Extraction Methods

DNA was extracted using commercial kits in some specific cases where large numbers of samples or rapid reporting was required. Extractions were carried out using Dnasol (Invitrogen), Dneasy (Invitrogen), Genomic Tip Columns (Qiagen) and Qiaprep blood and tissue spin kits (Qiagen). In each case the protocols were carried out following the manufacturers instructions.

3.3.3 Recovery of PCR Product DNA from Agarose Gels

DNA bands in agarose gels were recovered using a simple spin column Ultrafree-DA (Millipore, USA) as per manufacturers instructions. Where longer term storage was required, DNA bands were extracted from gels using Qiaprep Gel Extraction kit (Qiagen, Germany) using the manufacturers instructions.

3.3.4 Plasmid DNA Recovery

Plasmid DNA for sequencing was recovered using Qiaprep plasmid (Qiagen) using the manufacturers instructions.

3.4 Agarose Gel Electrophoresis

PCR products were visualised using agarose gel containing 0.5 µg ml^{-1} ethidium bromide. Gels were between 0.6 and 2% agarose (Progen molecular biology grade agarose) (W/V) in TAE buffer depending on the size of the DNA product. Smaller products (> 1000 bp) were run on 2% gels of 50 ml volume (Hoeffer HE 33, Phamacia Biotec) at 85 v or in a 125 ml gel volume (SciPlas HU13, Crown Scientific Australia) at 100 v. Larger products were visualised using 0.6 % agarose gels of 200 ml volume (GNA 200, Pharmacia) at 115 v. 10 µl of PCR product was added to 2 µl of 6 × loading buffer and placed in preformed wells in the agarose gels.

Fluorescent signals from ethidium bromide intercalated in DNA bands under UV light were visualised and captured using Genesnap (Synoptics Ltd., UK). The size of DNA products was estimated

empirically by comparison of migration distances using 0.1 µg mm $^{-1}$ of lane width using 1Kb Plus DNA Ladder™ (Invitrogen, Australia).

A rapid electrophoresis system (E-Gel96, Invitrogen USA) was trailed during the latter stages this project but it could not be fully evaluated for high throughput screening of PCR products and so it is not mentioned in the results section.

3.5 PCR

3.5.1 Primer and Probe Design

The DNA sequences used in the design of primers and probes was obtained from Genbank (USA). Suitable oligonucleotides were generated using the OLIGO 6.60 software (Molecular Biology Insights, USA) before being evaluated manually. Oligonucleotides used as primers and probes were synthasised by Sigma Genosys (Australia) and resuspended in nuclease free water to a concentration of 100 µM. A full list of all primer and probe sequences can be seen in Appendix 1.

3.5.2 PCR Conditions

All PCR's were carried out in a Mastercycler Gradient (Eppendorf, Germany). Optimisation of each PCR was done using a 10°C gradient to determine the primer annealing temperature. Generally, PCR consisted of an initial denaturing step of 10 min at 94°C followed by thirty five cycles of denaturing at 94°C for 1 min, annealing at between 47 and 63°C (see specific reactions for exact temperatures) for 50 sec and an elongation step at 72°C for 1 min per 1000 bp in the expected product. A final elongation step at 72°C for 7 min preceded the reaction being held at 4°C until electrophoresis.

Each reaction consisted of 1 × reaction buffer (supplied by manufacturer), 2.5 mM $MgCl_2$, 200 µM each dNTP, 1µM each primer, 0.02 units µl^{-1} Red Hot Polymerase (ABgene, UK) or 0.03 units µl^{-1} recombinant *taq* polymerase (Sigma, USA) and 10 ng DNA template (unless otherwise stated). Reaction volumes were made up to either 25 or 50 µl using nuclease free water (Sigma, USA)

3.5.3 Sequencing PCR

Sequencing of PCR or cloned PCR products was done using the CEQ-DTCS-Quick Start Kit (Beckman Coulter, USA) with minor modifications to the manufacturers instructions. Precipitation of the sequencing reaction product was carried out in 100% molecular biology grade ethanol by centrifugation at 21,000 *g* and 4°C for 20 min. This modification to the manufacturers protocol resulted in slightly longer read lengths during sequencing. Sequences were read on a CEQ 2000XL (Beckman Coulter, USA). The resulting sequence and chromatographs were analysed using Sequencher 4.0.5 (Gene Codes Corp., USA) software.

3.5.4 Single tube nested PCR

Single tube nested or Semi-nested PCR was performed to reduce the possibility of contamination from carry over PCR product. The reaction conditions and cycling remained the same as used in standard PCR. Initial amplification was done in a reaction volume of 15 µl and the reaction was covered with molecular biology grade mineral oil (Sigma Aldrich. USA). The nested round of PCR was done in a 10 µl volume and delivered to the reaction by deposition on top of the mineral oil layer. The second round amplification mix was moved through the mineral oil using pulse centrifugation at 400 *g*. The second round amplification was then performed under normal conditions.

3.5.5 Southern Blots

When gels were run, bands of interest were marked by nicking the side of the gel. The gels were washed briefly in ddH_2O then the DNA was denatured by washing twice in 10x volumes denaturing solution (1.5 M NaCl; 0.5 M NaOH) then washing twice in 10x volumes of neutralising buffer (1.5 M NaCl; 1.0 M Tris pH 8.0). The DNA bands were bound to a positively charged nylon membrane (Boehringer Mannheim, Germany) overnight using capillary transfer. The membrane was removed from the tank and the location of wells marked with a ball point pen, the gel was than checked under UV light for residual DNA. The membrane was washed briefly in 2x SSC and the DNA fixed at 85°C for 2hr. Five washes in post fix buffer (3x SSC: 0.5% SDS) for 10 min each and once in post fix buffer at 65°C for 1 hr was used to remove any excess agarose. The membrane was prehybridised in prehybridisation buffer (6x SSC; 5x Dernhardts solution; 0.05% (w:v) Na pyrophosphate; 100 $\mu g\ ml^{-1}$ Boiled herring sperm DNA; 0.5% SDS) for 2 hr at 35°C. Hybridisation of the oligonucleotide probe was done in hybridisation buffer (6x SSC; 1x Dernhardts solution; 100$\mu g\ ml^{-1}$ boiled herring sperm DNA; 0.05% N_a pyrophosphate; 5.0 ng (0.1 –1ρM) oligo probe) for16 hr at 65°C (depends on T_m of probe). Three stringency washes of 30 min each (2x SSC at 55°C, 1x SSC at 51°C and 0.5x SSC at 46°C) were used to strip excess probe from the membrane before equilibrating in detection buffer #1 (0.1M malic acid; 0.15M NaCl pH 7.5) for 5 min at room temperature. The membrane was washed in detection buffer #2 (#1 + 1% membrane blocker) for 30 min at room temp then 30 min at room temp in detection buffer #2 with 1:5000 dilution of strep/AP conjugate. Following binding of the conjugate the membrane was washed twice for 15 min each in buffer #1 then equilibrated in buffer #3 (100mM Tris; 100mM NaCl pH 9.5) for 3 min. The membrane was placed in detection buffer #3 containing 3.5 $\mu l\ ml^{-1}$ each of NBT and BCIP and colour allowed to develop (five hours to overnight). The blot was washed briefly in ddH_2O and photographed using a digital scanner (Optic Pro, Plustek USA).

4 Results

4.1 Serotype specific PCR

4.1.1 Serotype 1 PCR

4.1.1.1 Materials and Methods

Primers for the serotype 1 diagnostic PCR were modifications of those described by Young and Gravel (1996). Three primers designated 41, 25 and 24 are run in a semi-nested format targeting a segment of the glycoprotein C gene within the unique long region. The outside amplification series gives an amplicon of 650 bp and the nested series a 201 bp amplicon. The initial primer sequences published by Schmidt (1997) had a G-C mismatch at the 3' end of the nested forward primer (24). Two primer sequences were trailed. One set had the G-C mismatch corrected and was designated as primer 24, the second primer (pers comm, Walkden-Brown, 2000) had the 3' base deleted from the oligo and was designated 24UNE. Detailed information on the primers can be seen in Appendix 1. The PCR was run under the standard cycling conditions with an optimum annealing temperature of 60°C and $MgCl_2$ concentration of 2.5 mM.

4.1.1.2 Sensitivity of Serotype 1 PCR

The sensitivity of the serotype 1 PCR was tested in Single series and semi-nested format. The semi-nested PCR was found to be 1000 times more sensitive than single round PCR (see Figure 4.1).

4.1.1.3 Specificity of Serotype 1 PCR

The specificity of the serotype 1 primers was tested against serotype 2 and HVT using all the strains available within Australia at the time. No significant cross-reactions were observed with any of the strains tested (see Figure 4.2)

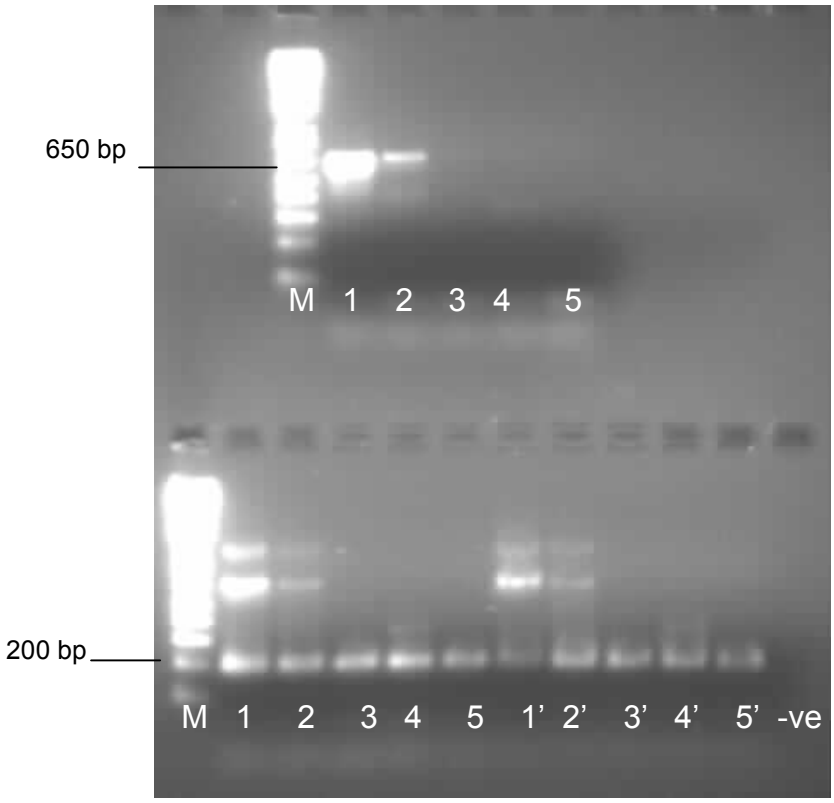

Figure 4.1: Sensitivity of serotype 1 specific semi-nested PCR compared to single series amplification.
Template was a serial dilution of DNA extracted from MSB 1 cells (strain BC 1). Top row, outside primer set amplification. Bottom row, nested primers set using two modifications of primer 24. Lanes M; marker, 1: 47 ng, 2: 4.7 ng, 3: 470 fg, 4: 47 fg, 5: 4.7 fg. Lanes 1' to 5' are amplification products using the 3' deletion primer (24UNE)

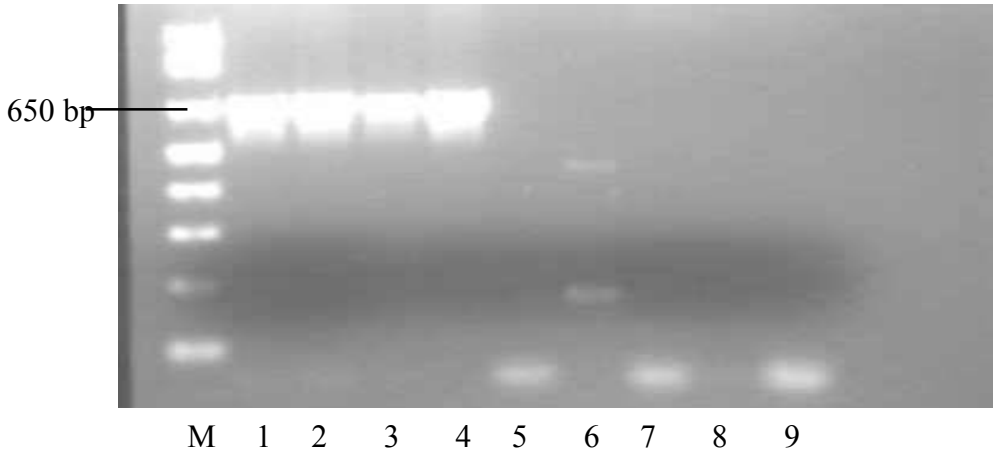

Figure 4.2: Specificity of the serotype 1 PCR. Lanes M: marker, 1: CVI 988, 2: BH16 (high pass), 3: Woodlands #1challenge, 4: BC1, 5:MDV 2 (Maravac), 6: HVT (Intervet), 7: HVT (FC126), 8: H_2O, 9: CEF DNA.

4.1.2 HVT Specific PCR

4.1.2.1 Materials and Methods

Three primer sets were trialed for use as the standard HVT diagnostic PCR. Two sets, one semi-nested (HVT) and a second in fully nested format (HVTFN) were designed to amplify fragments of the glycoprotein B gene within the unique long region. This area was chosen due to the low homology between HVT and serotype 1 in this area (Afonso *et al.*, 2001) and has been targeted by others

(Aminev *et al.*, 1998) to produced serotype specific products. The third set of primers (HVT2) tested amplified a fragment of the repeat region between the two unique regions. Full details of the primer sequences and binding areas can be seen in Appendix 1. Standard cycling conditions used for the HVT PCR's were with the annealing temperature being 60°C and 2.5 mM $MgCl_2$.

4.1.2.2 Sensitivity Testing

All three primer sets HVT, HVT2 and HVTFN were run and the PCR products compared directly using Agarose gel electrophoresis. The FNR primer sets targeting the repeat region failed to produce a product under these conditions and also during subsequent optimisation reactions (data not shown). Both semi-nested primers sets, HVT and HVT2 performed well during the initial amplification round with the HVT2 PCR giving slightly better results. The nested primer sets also both produced the predetermined amplicon during second round amplification. However the HVT glycoprotein B specific set produced significantly higher yields than the set targeting the repeat region. Further optimisation of the HVT2 PCR was unable to give any further improvements in the amplification products. A single round amplification reaction using the HVT2 inside primer set produced a very low-grade response (data not shown) and it is the nested primers that are believed to be causing the reduction in efficiency. The amplification products resulting from the sensitivity testing can be seen in Figure 4.3.

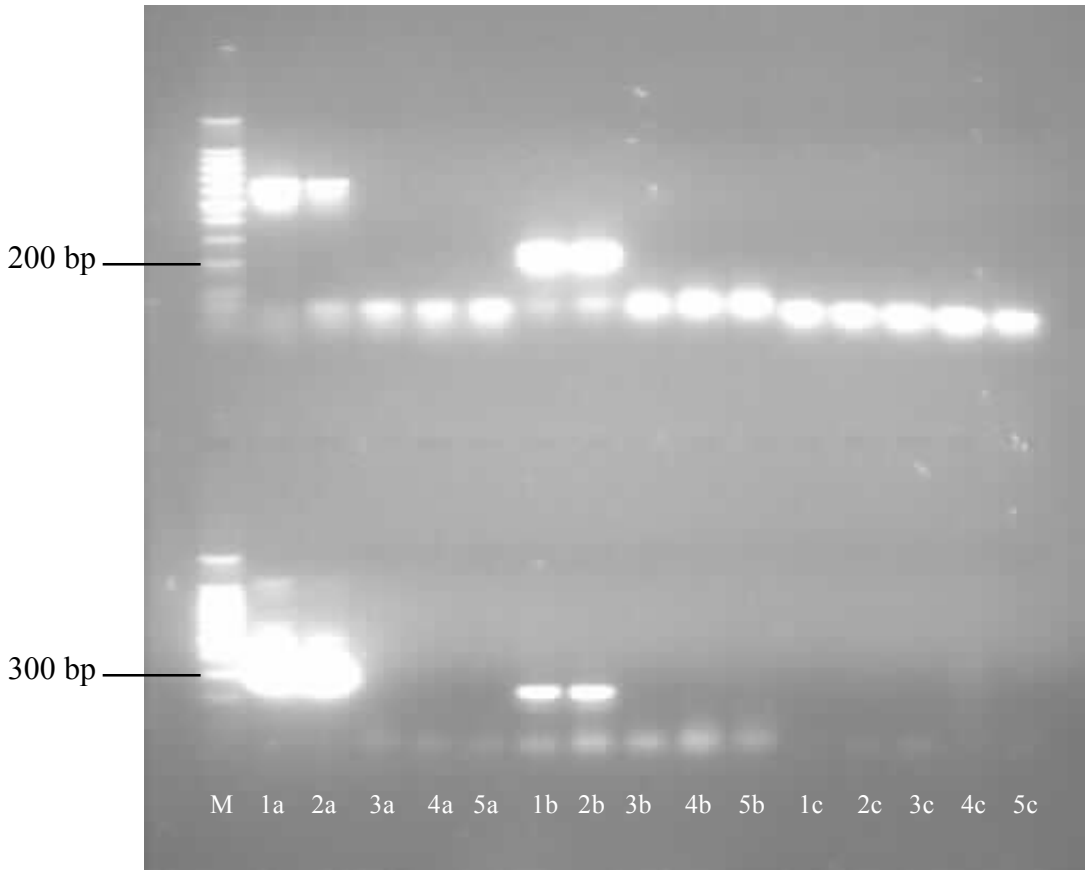

Figure 4.3: Sensitivity of three primer sets designed for the HVT diagnostic PCR.
Reactions in lanes b and c are semi-nested and fully nested reactions respectively. Lanes a are semi-nested reactions targeting the glycoprotein B gene. Top row are outer primer sets, bottom row are nested primer set products. Lanes M: marker, 1: HVT (Intervet), 2: HVT (strain FC 126), 3: BH16 challenge, 4: CEF DNA, 5: H_2O

4.1.2.3 Specificity testing of the HVT primer Set

The HVT primer set used was selected as the best of the three sets trialed and used in a specificity reaction where they were run against other serotypes. Some very weak bands of different sizes were produced during the nested round of reactions with one from serotype 2 being within 50 bp of the expected HVT product size. However, there was no consistent size band produced across the serotype 1 isolates used in testing. The amplification products can be seen in Figure 4.4.

Figure 4.4: Specificity of the HVT PCR targeting the glycoprotein B gene. Lanes M: marker, 1: CVI 988, 2: BH16 (high pass), 3:Woodlands#1 challenge, 4: BC 1, 5: MDV 2 (Maravac), 6: HVT (Intervet) 7: HVT (strain FC126), 8: CEF DNA, 9: H_2O

4.1.3 Serotype 2 PCR

4.1.3.1 Materials and Methods

Primer sets targeting the glycoprotein B gene of serotype 2 MDV have been previously described and one set has been designed previously in this lab (Schmidt, 1997). However, all primers have cross-reacted strongly with BC 1 DNA extracted from MSB 1 cells. Following the publication of the complete genome sequence of the serotype 2 strain HPRS 24 (Izumiya *et al.*, 2001), primers targeting different regions were designed and evaluated.

The primers were designed to bind within the repeats flanking the unique long region in areas that indicated the least homology with serotype 1 and HVT. Three fully nested sets designated S1, S2 and S3 were trialed. The full description of the primer sets can be seen in Appendix 1.

4.1.3.2 Selection of Serotype 2 Primers

All three nested primer sets were tested using DNA template extracted from Serotype 2 Vaccine virus (Maravac, Fort Dodge) and the resulting bands compared using ethidium bromide stained agarose gel electrophoresis. The results can be seen in Figure 4.5. The S1 primer set was selected as the preferred set, offering slightly higher levels of sensitivity over the S2 set. The S3 primer set failed to produce an amplicon in first round PCR.

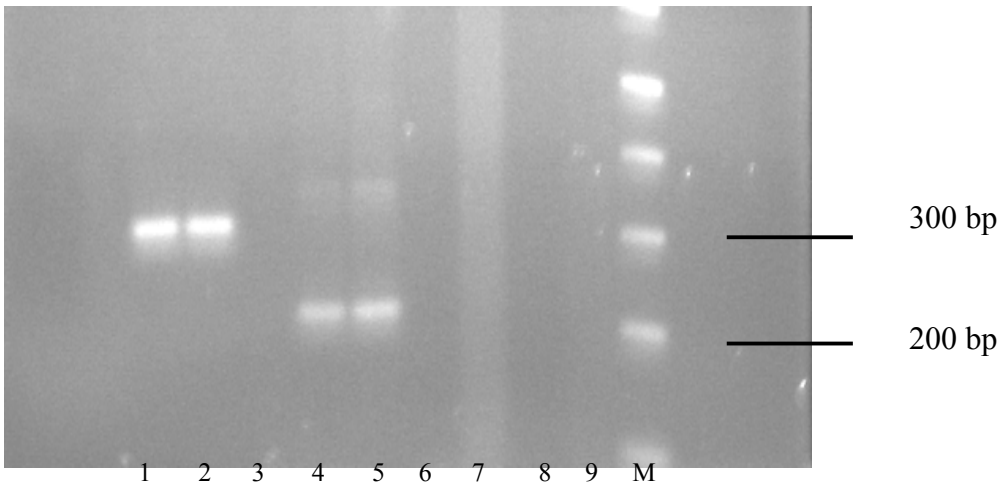

Figure 4.5: Comparison of the three primer sets designed for Serotype 2 MDV PCR. Lanes 1 & 2: S1 set, 3: H_2O, 4 & 5: S2 set, 6: H_2O, 7 & 8: S3 set, 9: H_2O, M: marker.

4.1.3.3 Specificity of Selected Serotype 2 Primers

The S1 primer set was used in a PCR using the panel of serotype 1, 2 and HVT and the results can be seen in Figure 4.6. The primers failed to react with the type 1, HVT and CEF derived DNA template. The Serotype 2 primer set did have a very mild crossreaction with BC1 DNA from MSB-1 cells in other PCR's (data not indicated).

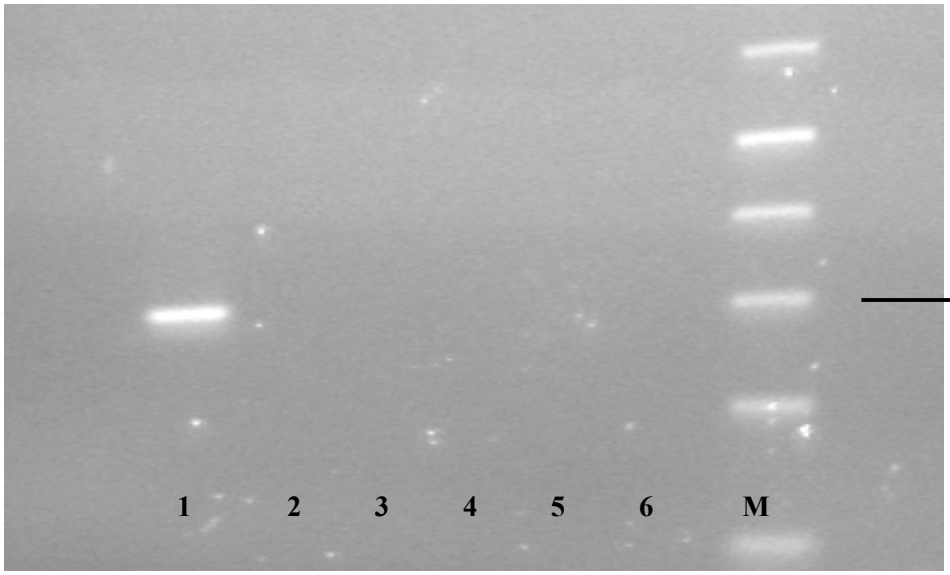

Figure 4.6: Specificity of the S1 primer set for serotype 2 MDV. Lanes 1; Serotype 2 (Maravac), 2: BH 16 challenge, 3 HVT (Intervet), 4: CVI 988 (Bioproperties), 5: CEF DNA, 6: H_2O, M: marker

4.2 Sample Collection and Processing

4.2.1 Filter Paper Collection

4.2.1.1 Commercial Products

Commercial collection systems are available that use a variety of formats for blood collection. All these systems use a similar collection volume of approximately 10 µl collected directly onto the paper media. Most of the available products use a chemically impregnated paper that preserves DNA

and lyses the blood cells upon contact with the paper. Two commercial products were tested in conjunction with a system developed as part of this project. Isocode® PCR Preparation Dipsticks (Schleicher & Schuell, Germany) are a treated filter paper product, used in accordance with the manufacturers instructions. The templates extracted from Isocode papers were used in the HVT PCR. Untreated 903 collection paper (Schleicher & Schuell, Germany) was used in accordance with the manufacturers instructions and the template subjected to identical PCR amplification. Agarose gel analysis of the reactions indicated that the PCR produced detectable bands using the Isocode method but no products were visible using the untreated paper. The results can be seen in Figure 4.7. Collection of blood in capillary tubes (Heparinised and with EDTA) was undertaken by collecting blood from a wing stab into the tube. The tubes were placed in an upright orientation overnight to allow settlement of the cellular component. The buffy coat was removed by breaking the tube just below the plasma layer and soaking a collection lobe with buffy coat. No detectable PCR product could be visualised on agarose gels (data not indicated).

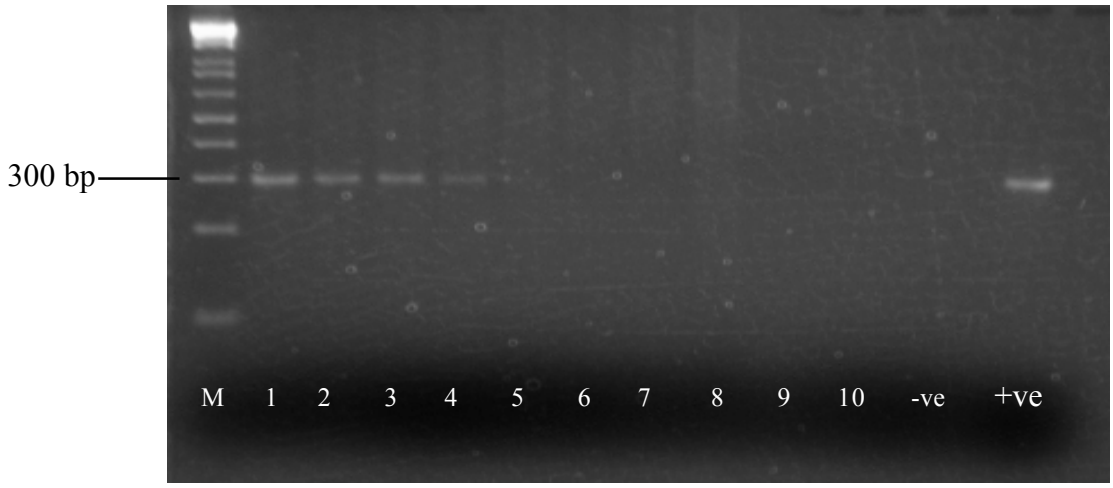

Figure 4.7: PCR amplification of DNA template extracted from Isocode Dipsticks and untreated 903 using single round primers designed for the HVT PCR. Lanes M; marker, 1: 2000 cells, 2: 1500 cells, 3: 1000 cells, 4: 800 cells, 5: 500 cells. Positive control was Phenlo/chloroform extracted DNA. Lanes 1-5 are from Isocode paper while lanes 6-10 are from untreated paper.

4.2.1.2 In House Filter Paper Collection System

Pre-punched filter paper (Whatman 3MM) (TropBio, Townsville, Australia) sample discs, each having 6 × 6 mm removable lobes (see Figure 4.3) were spiked with MDV genome by absorption of 5 dilutions of MDCC-MSB 1 cells in sterile PBS-A (Table 4.1). Spiked discs were dried for 1 hr at room temperature before being loaded with 10 µl of whole chicken blood from free-range White Leghorn Chickens with no previous history of MD. Papers were dried at room temperature for 1 hr and placed in a sealed ziplock plastic sample storage bag (TropBio, Townsville, Australia) for DNA extraction the following day or stored at room temperature for use four days later. Template DNA was extracted from loaded lobes using one of two methods prior to PCR.

DNA extraction by detergents and proteolytic digestion was carried out using the method described by DeVange Pantaleeff *et al*. (1999) with modifications to the centrifugation step. Each disc was detached

from the sample plate and placed in a 1.5 ml microcentrifuge tube containing 100 µl of extraction buffer (10 mM Tris-Cl [pH 8.3], 50 mM KCl, 0.1 % w/v gelatin, 0.45 % w/v Tween-20, 0.45 % w/v Nonidet P-40, 60 µg ml $^{-1}$ proteinase K). Lysis was accomplished by heating to 56°C for 90 min followed by 94°C for 20 min to denature the proteinase K. Each sample was spun at 10,000 *g* for 10 min to pellet cellular debris. Samples were cooled to room temperature and 5 µl of each supernatant was used as PCR template.

Filter paper discs were removed as described above and prepared for PCR using a chelating resin, Chelex-100 (Biorad, Australia) as described by Polski *et al*. (1998). Samples were vortexed for 10 min in 500 µl of ddH_2O. The supernatant was removed and added to 200 µl of 5% w/v Chelex-100 (BioRAD, USA). Samples were incubated at 56°C for 90 min followed by 99°C for 10 min. Samples were briefly vortexed and centrifuged with 5 µl of supernatant being used as PCR template.

Table 4.1: Dilutions of MSB 1 cells used in simulation of clinical samples.

Lobe Number	MSB 1 Cells
1	50,000
2	25.000
3	10,000
4	5000
5	1000
6	Negative control

The PCR produced visible bands down to 5000 MSB 1 cells from the untreated paper using the extraction buffer method at 24 hours after spiking the lobes (Figure 4.8). The PCR also reacted very strongly when the lobe spiked with 25000 cells was used directly in the PCR reaction. However, the lobe spiked with 50000 cells failed to produce a product. The DNA isolation using Celex-100 failed to produce any visible products following semi-nested PCR.

Following four days storage at room temperature, spiked collection lobes were extracted using both methods. The period of four days was selected because it represented a reasonable time in which samples could be expected to be in transit with couriers or the Postal services. None of the samples using either extraction method tested produced bands on agarose gels following PCR with the semi-nested serotype 1 primers (data not indicated).

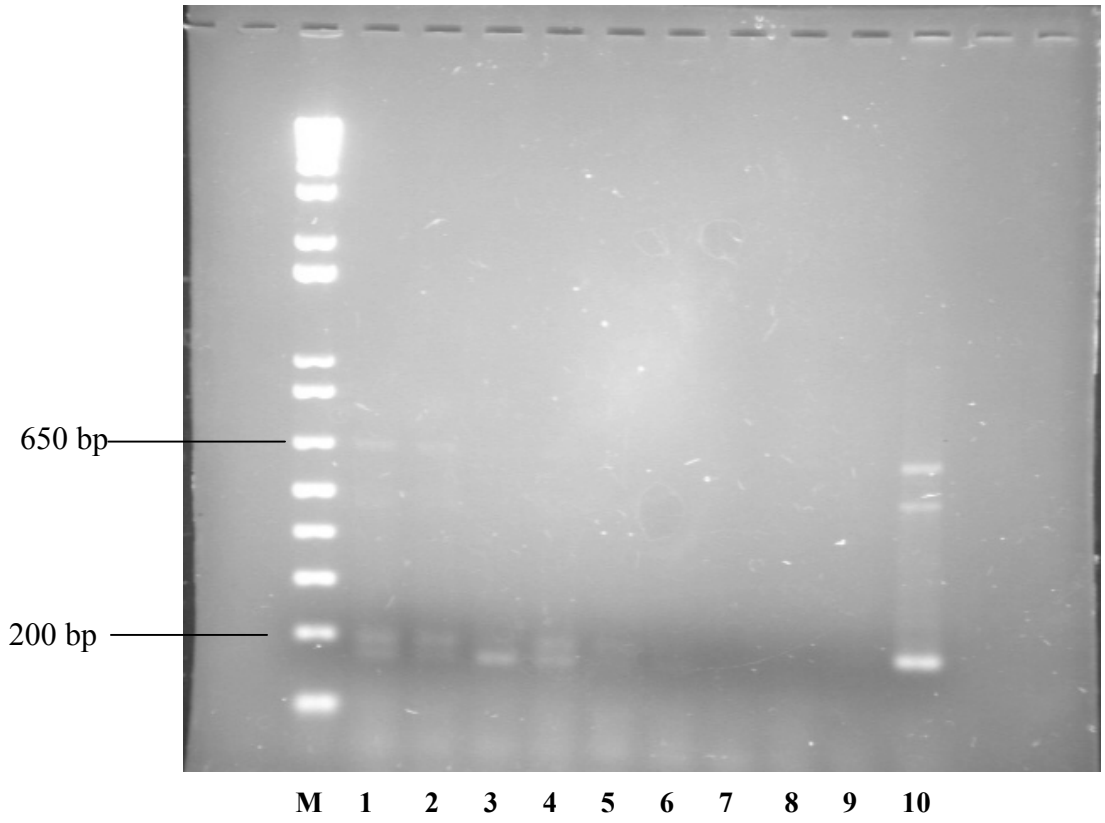

Figure 4.8: PCR using serotype 1 semi-nested primers from template prepared from MSB 1 cells on in house untreated filter paper collection discs. Lanes M: marker, 1: 50000 cells, 2: 25000 cells, 3: 10000 cells, 4: 5000 cells, 5: 1000 cells, 6: 0 cells, 7: CEF DNA, 8: H_2O, 9: 50000 cells using disc in PCR, 10: 25000 cells using disc in PCR

4.3 Internal Control for Diagnostic PCR

4.3.1 Fragment Synthesis

Internal controls need to have some inherent features that make them suitable for co-amplification. There need to be exploitable differences between the sequences of the target and control DNA's. The reasons for this are twofold, firstly, non-specific binding of primers is not likely to occur, and secondly, amplification products from control and target DNA's can be detected separately using oligonucleotide probes. This difference can also be extended to the size of the control in cases where the products will be detected using agarose gel electrophoresis. The T_m of control DNA should be comparable to that of the target sequence to allow for effective co-amplification using a single set of cycling parameters (Pham *et al*., 1998). However, T_m of the control should not be such that it out-competes the target sequence during the PCR process.

Although few publications are available on the construction of internal controls, those that are tend to favour the hybrid primer method of synthesis and use lambda phage DNA as template (Pham *et al*., 1998 and Helweg-Larsen *et al*., 1998). Briefly, primers specific for a fragment of control (phage) DNA of appropriate size and T_m are designed using normal criteria (see section 3.4). The hybrid primers are generated by adding the target specific primer, forward or reverse, to the 5' end of its respective control primer in a co-linear manner. Extension from the 3' end of the primer results in an oligonucleotide with target specific primer sequences at the 5' end. Subsequent rounds of amplification produce double stranded fragments capable of being amplified using target specific primers alone.

The internal control was synthesised using modifications to the hybrid primer method described by Helweg-Larsen *et al.* (1998). Plasmid DNA derived from the cloning vector pBR322 (Promega WI. USA) was utilised as template, heterologous to MDV genomic DNA. An area of sequence between bases 2480 and 3370 (numbered sequentially from Promega data sheet, Promega web site) was selected by subjecting the plasmid sequence information to alignment searches (http://vega.igh.cnrs.fr/bin/nph-align) with each of the MDV primers and oligonucleotide probe individually. The selected area indicated that no binding of MDV specific primers would take place during subsequent PCR's.

Hybrid primers were designed by combining plasmid specific primers with the outside primer set used in the semi-nested PCR, specific for MDV. In each case the MDV specific sequence comprised the 5' region with the 3' region derived from the plasmid primers. Lists of possible forward and reverse plasmid specific primers were generated by entering the suitable sequence data (Promega) into the primer design software. The sequence and binding locations of the plasmid specific component and the hybrid primers for all serotypes can be seen in Appendix 1. Hybrid primer sequences were an addition of each forward and reverse set respectively. The indicated binding locations for the primers gives an expected amplicon of 769 bp.

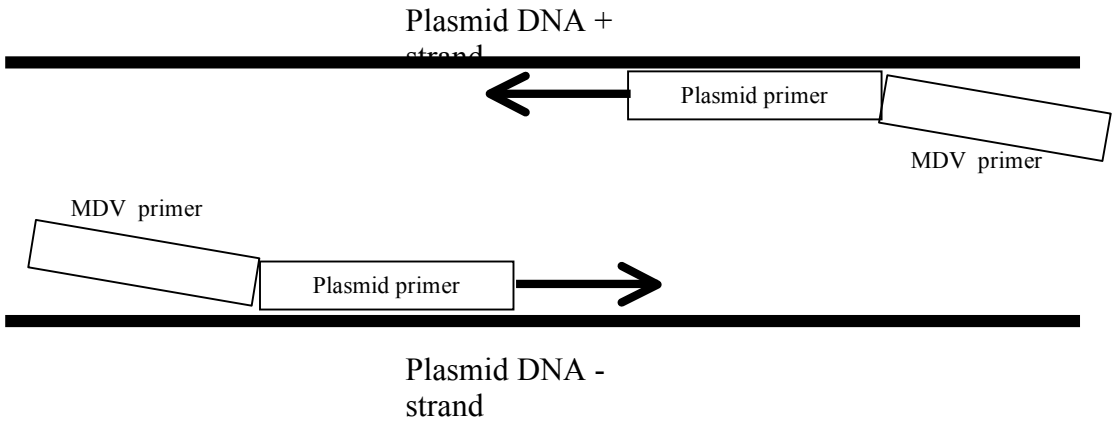

Figure 4.9: Design of internal control using hybrid primers and DNA from pBR 322. Amplification produces a product with the MDV specific primers in the flanking regions of the internal control fragment. This allows subsequent amplification of control DNA with MDV primers.

4.3.2 Amplification and Testing of the Internal Control

4.3.2.1 Amplification and Quantitation

Internal control DNA was synthesised by PCR using 4 ng of pBR322 template (Promega, USA). The cycling parameters were those used for the Diagnostic PCR. Following PCR, the product was visualised on agarose gel and the required band excised and recovered using centrifugation (Ultrafree-DA, Millipore USA). Control DNA was ethanol precipitated before being reconstituted and stored at -20°C in 10 mM Tris-Cl. The yield was estimated qualitatively using UV spectrophotometry and a series of dilutions made for testing with MDV specific primers.

Dilutions of the control DNA were used in an amplification reaction with the outside primers 41 and 25. The amplified DNA was run on an agarose gel and bands were detectible from 6 x 10^{-17} g of control DNA. This level of detection is equal to 68 copies of the control fragment (see Figure 4.10).

4.3.2.2 Co-amplification of Control DNA and MDV Template

Control DNA was co-amplified with 10 ng of DNA extracted from MSB 1 cells using the outer serotype 1 primers 41 and 25. Under these conditions, 680 copies of internal control DNA was required for effective amplification of both templates (see Figure 4.11)

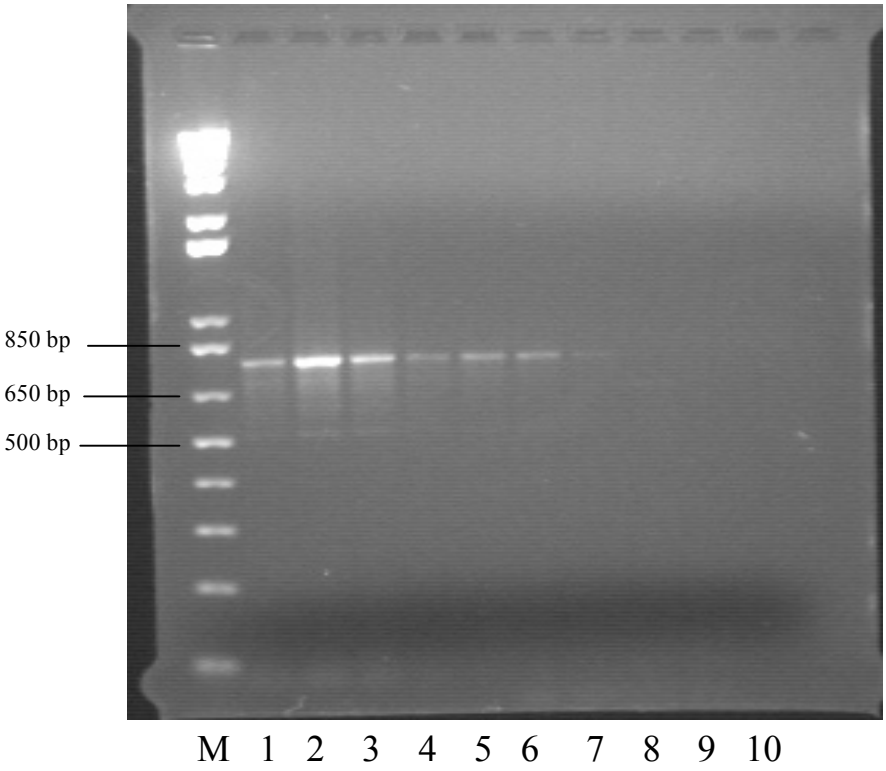

Figure 4. 10: Amplification of the internal control construct using the outside primers 41 and 25 from the serotype 1 PCR. Lanes M; marker, 1 to 8: serial dilution of construct from 6 x 10^{-11} g to 6 x 10^{-18}g, 9: CEF DNA, 10: H_2O

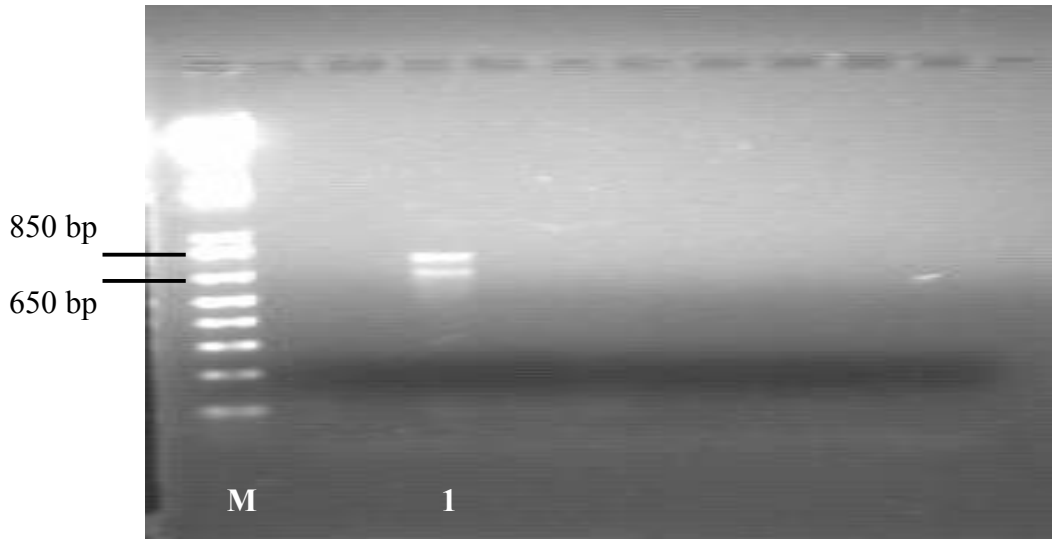

Figure 4. 11: Co-amplification of constructed internal control DNA and serotype 1 template using primers 41 and 25. Lanes M: marker, 1: 6 x 10^{-11} g of internal control and 10 ng of DNA extracted from BC 1 strain MDV.

4.4 Colorimetric PCR Product Detection

Colorimetric detection using 96 well microtitre plates and soluble chromogen systems (Kemp *et al*., 1989) are proving to be a popular alternative to conventional electrophoresis. Two formats, enzyme-linked immunosorbent assay (ELISA) (Kemp *et al*., 1989) and enzyme-linked oligosorbent assay (ELOSA) (Keller *et al*., 1991) are used. Both systems use PCR product labelled by the incorporation of digoxigenin -11- dUTP (DIG-UTP) during amplification. Labelled amplicon is detected using anti-DIG antibodies conjugated to horseradish peroxidase (Antidig-POD) and chromogens such as ABTS. Capture of product on the surface of wells is usually accomplished via a protein/protein interaction, the most popular being the binding of streptavidin and biotin. However, the basic principal of both assay systems is similar. While ELOSA offers high specificity by using two probes specific for the amplicon, ELISA can give equal specificity and higher sensitivity.

A number of variations on the PCR-ELISA have been described with roughly similar results. Some systems use wells pre-coated with specific capture probe (Keller *et al*., 1991 & Mallet *et al*.,1995). More recently, methods describing hybridisation of probes and amplicon prior to placement in streptavidin coated wells have been employed with some success (Muramatsu *et al*., 1997).

4.4.1 Standardising for Streptavidin Coating

4.4.1.1 PCR and Probes used in the ELISA

DNA product used in the PCR/ELISA was generated using the inside primer sets and all PCR conditions were as described previously with modifications to the dNTP concentrations. Labelling was carried out using dTTP and DIG-11-dUTP (Boehringer Mannheim) at 19:1 in a 25 µl reaction volume.

A 5' biotinylated oligonucleotide capture probe (Invitrogen, USA) with specificity for a region within the nested amplicon produced by the inside set of primers was used to bind PCR product to the microtitre wells. Details describing all the serotype specific capture probe are given in Appendix 1.

4.4.1.2 Standising and Coating of Streptavidin Microtitre Plates

A 5' biotinylated 25 base oligonucleotide linker (Life Technologies) was labelled using a 3' DIG end labelling kit (Boehringer Mannheim). Titration of the labelled linker was done using 80 µl of a serial dilution (1 mM to 0.16 fM) of 3' DIG labelled linker placed in 2 × eight well microtitre strips (Boehringer Mannheim) pre-coated with streptavidin and incubated at room temperature for 2 hr. Anti-Dig-POD fragments (Boehringer Mannheim) were diluted in ELISA diluent (Tropbio, Townsville Australia) to the manufacturer's recommended concentrations and 100 µl aliquots were placed in each well then incubated at 38°C for 30 min. 100 µl of one step ABTS colour indicator (Kirkegaard & Perry Laboratories, USA) was added to each well, incubated for 30 min at room temperature and the optical densities were read using a Multiscan EX plate reader and Labsystems Genesis V3.00 software at dual wavelengths of 414 and 492 nm. The results can be seen in Figure 4.12. . A concentration of 400 pM was chosen as a standard probe concentration because higher concentrations offered no advantage in detecting bound streptavidin.

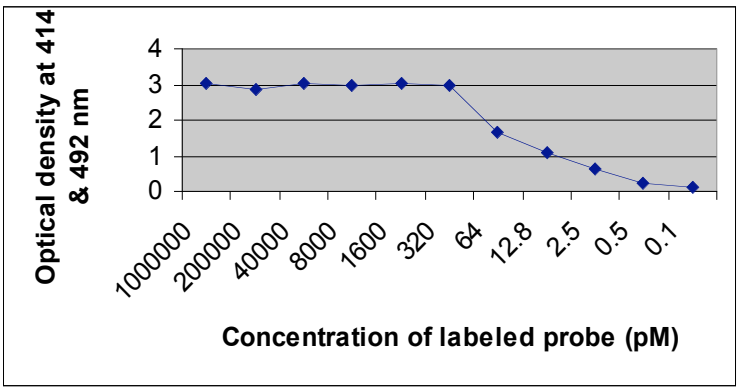

Figure 4.12: Binding affinity of the labelled oligonucleotide linker for commercial streptavidin coated microtitre plates.

100 µl aliquots of each of a serial dilution (50 µg ml $^{-1}$ to 1.5 ng ml $^{-1}$) of streptavidin (ICN Biochemicals Inc) in 50 mM carbonate coating buffer (pH 9.0) were placed in 8 well microtitre strips (Costar, Cambridge, MA. USA) and incubated overnight at 4°C (Harlow and Lane, 1988). The optimum probe concentration (determined from the results of the previous step) was used to determine the optimum amount of streptavidin required for noncovalent coating. The optimum streptavidin concentration of 200 ng ml^{-1} was chosen as this produced the highest absorption reading with higher concentration indicating lower viable binding (see Figure 4.13) The chosen streptavidin concentration was then used to coat 8 well micro strips for use in the PCR/ELISA

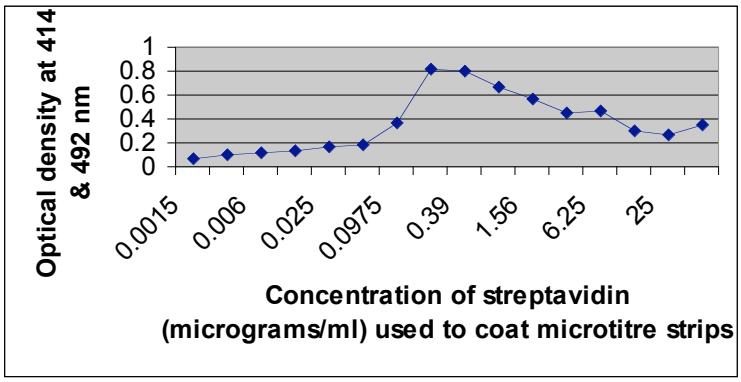

Figure 4.13: Optimisation of streptavidin coating of microtitre strips using a labelled oligonucleotide linker.

4.4.1.3 PCR/ELISA

The PCR ELISA was carried out following the methods described by Muramatsu *et al*, (1997). Ten microlitres of labelled PCR product was mixed with 90 µl of hybridisation buffer (4 × SSC, 20 mM EDTA, 20 mM HEPS and 0.15% w/v Tween 20) containing 0.01 mM biotinylated capture probe. The mixture was heated to 95°C for 5 min to denature the PCR product and cooled to 55°C for 5 min to anneal the probe. Annealed probe was placed in noncovalently bound streptavidin coated microtitre strips and incubated at room temperature for 2 hr prior to detection using anti-Dig-POD (Boehringer Mannheim, Germany) fragments and ABTS single pack colour indicator system (Kirkegaard & Perry Laboratories, USA). Following 30 min incubation time with the indicator system, no colour change was detectable (see Figure 4.14). Overnight incubation produced very low absorption readings and may represent background from residual anti-Dig-POD fragments. Further testing indicated that the streptavidin was stripped off the plated during the wash steps involved in the ELISA component of the assay.

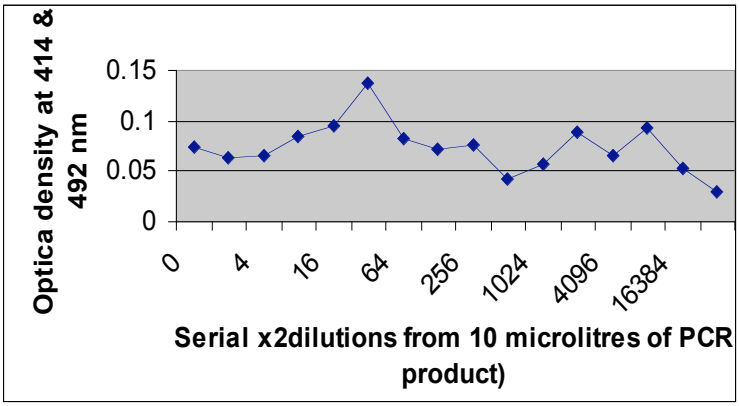

Figure 4.14: Colorimetric detection of PCR product using the ELISA on noncovalently bound streptavidin coated plates.

4.4.2 Modified PCR/ELISA

The PCR/ELISA was used with commercially available streptavidin coated plates. The commercial products use a covalent binding process that results in a coating that is resistant to ELISA wash steps. The procedure was carried out using the serotype 1 primers using the protocol described by Muramatsu *et al*. (1997) with minor modifications. During the probe hybridisation step, the hybridised probe/PCR amplicon was held at 28°C for ten minutes before being placed in the microtitre plates. Template for the PCR was a serial 5 x dilution of BH 16 DNA from 50 ng to 26 fg. The results can be seen in Table 4.2 and Figure 4.15

Table 4.2: Results of PCR/ELISA using modified method and commercial streptavidin coated plates.

Template	OD	Template	OD
50 ng	0.78	3.2 pg	2.266
10 ng	1.73	0.64 pg	0.806
2 ng	2.376	0.13 pg	2.656
0.4 ng	2.173	26 fg	2.551
80 pg	2.328	CEF DNA	0.059
16 pg	2.667	H_2O	0.119

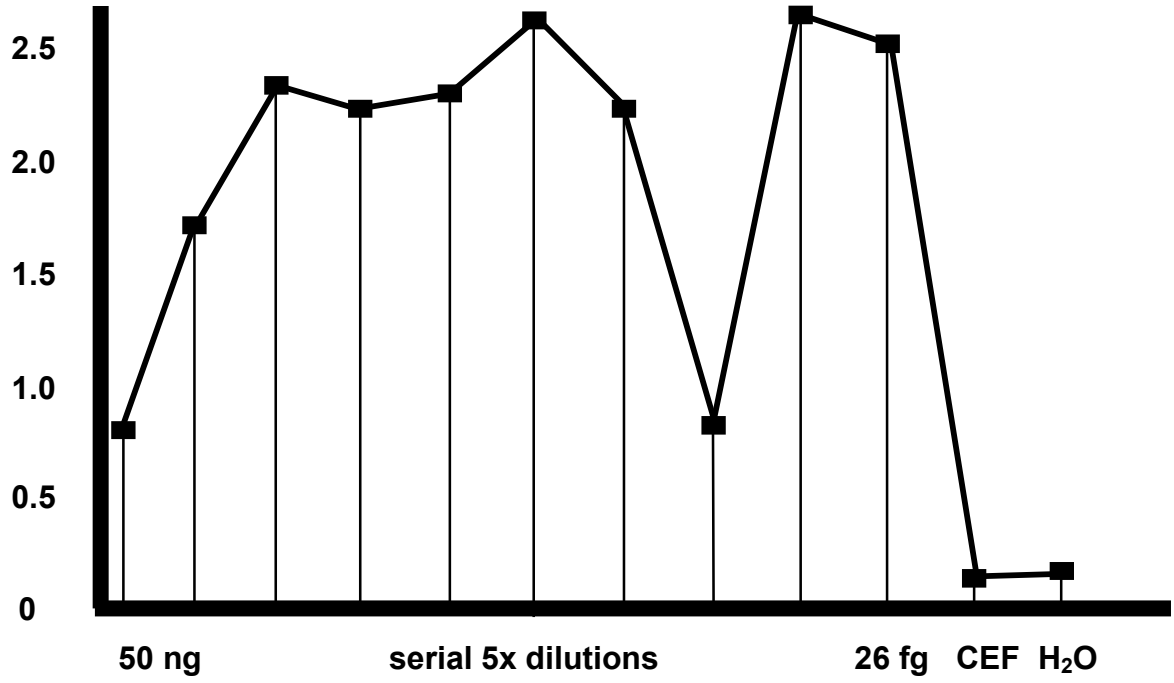

Figure 4.15: Results of the PCR ELISA using dilutions of BH 16 DNA

4.5 Application of Diagnostic PCR to Clinical Samples

4.5.1 Testing of Layer Birds

4.5.1.1 Samples and Collection

Commercial layer birds vaccinated with HVT and CVI 988 (Bioproperties, Australia) as a bivalent vaccine were obtained at two days post hatch. The birds were housed in negative pressure isolators and blood was collected onto filter paper discs at seven, fourteen and twenty one days post vaccination. Bleeding of the birds was initiated by wing stab with a 20_G needle. The collection lobe was then saturated with blood and allowed to dry before being stored overnight. Samples taken at seven days were stored until the second set of samples were taken and both sets were processed together.

4.5.1.2 Collection Media

Paper collection discs as described previously (4.2.1.2) were chemically treated with a cell lysis and protein denaturant by addition to the collection lobes. The discs were air dried in a laminar flow cabinet and stored in a ziplock bag. Papers were stored for one week prior to use.

4.5.1.3 Processing and PCR

The samples were processed following the protocol used for the Isocode Sticks 5 μl of the extracted template used in both the HVT semi-nested and serotype 1 semi-nested PCR's. The HVT PCR produced strong bands on an agarose gel at both seven and fourteen days (see Figure 4.16). No response could be detected at either time period for the serotype 1 PCR (data not indicated). Samples taken at 21 days post vaccination were stored overnight and subjected to PCR for serotype 1 and HVT. At 21 days the response to the HVT PCR was less intense than at previous sampling points. However, the serotype 1 PCR produced visible bands of the correct size in all samples (see Figure 4.17).

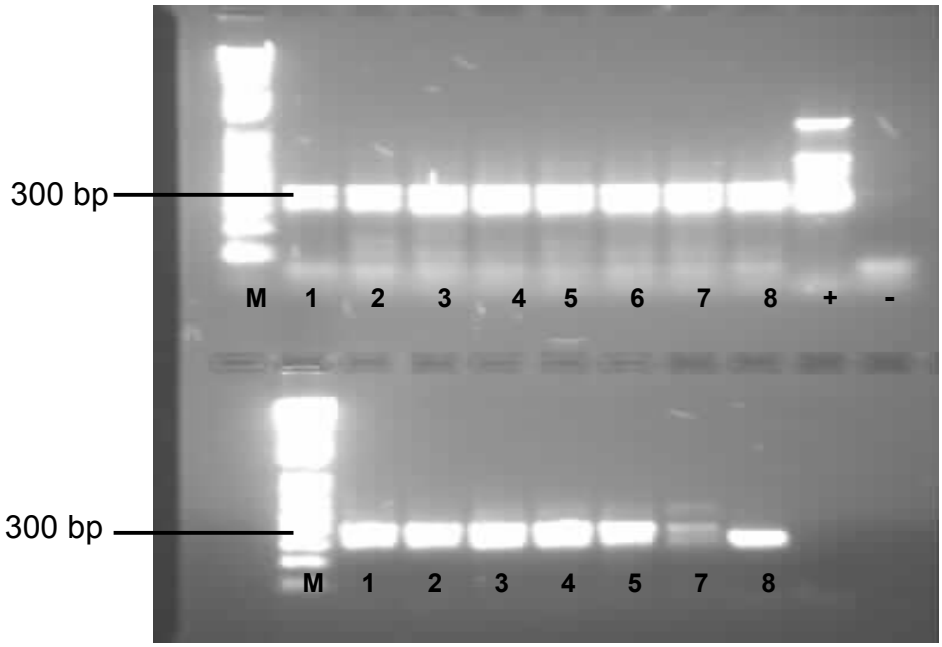

Figure 4.16: HVT PCR results from vaccinated layer birds using filter paper. Blood samples taken at seven days (top row) and fourteen days (bottom)

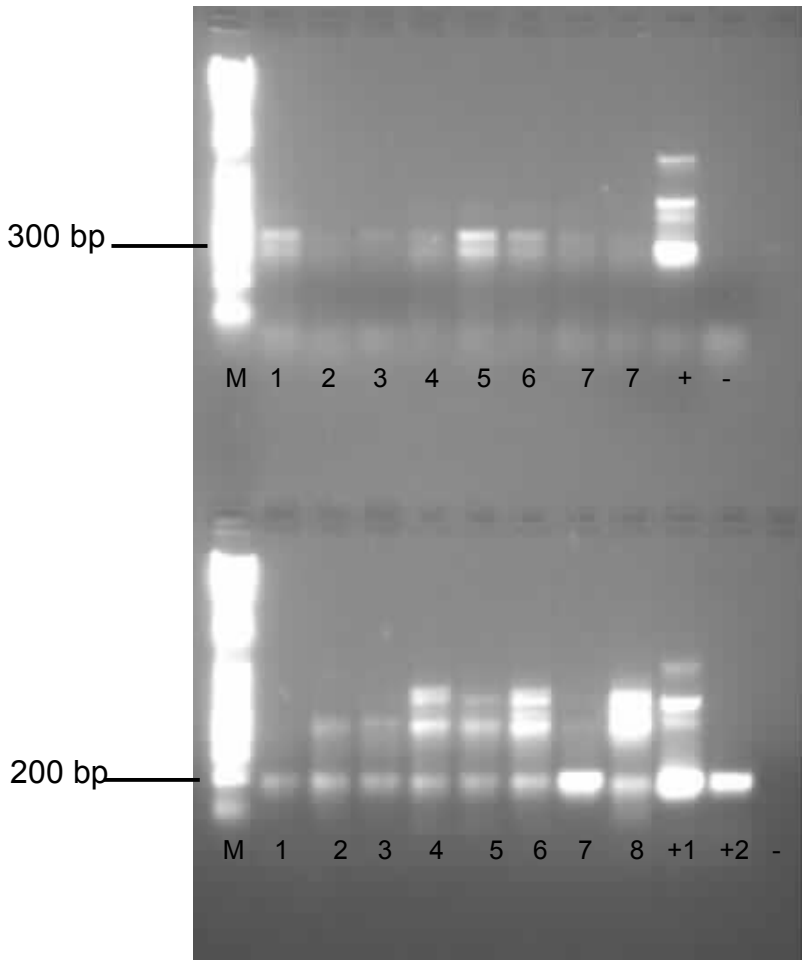

Figure 4.17: PCR detection of MDV vaccines from commercial layer birds. HVT semi-nested primers (top row), serotype 1 semi-nested primers (bottom row). Two positive controls were included in the serotype 1 PCR, +1 is an Australian strain BH 16 and +2 is CVI 988

4.5.2 Testing of Broiler Birds

Commercial broiler flocks from Queensland and New South Wales were used to assess the viability and acceptability of the sample collection technique. Where possible, duplicate samples were sent for cell culture evaluation at other laboratories.

4.5.2.1 Sample Collection and Processing

Treated filter paper discs were sent to consulting veterinarians as prototype collection kits and samples were collected in the hatcheries at 21 days post hatch from broilers vaccinated with half the recommended dose of HVT. The collection time of 21 days was used as the samples were also sent to other labs for cell culture evaluation. Dried blood samples were sealed in ziplock bags and sent to our lab by overnight post at room temperature. Each sample was processed as stated previously in section 4.5.1.3 and subjected to PCR using the semi-nested HVT primer set. Following PCR, the samples were run on agarose gels and visualised using ethidium bromide staining.

4.5.2.2 Samples from Queensland

A set of 29 samples was taken from a broiler flock in Southeast Queensland and tested for HVT viraemia. Of the samples tested, 22 were judged to have reacted to the primers. Most of the resulting bands were very light indicating a reasonably low response (see Figure 4.18). A similar result was obtained from the cell culture assay.

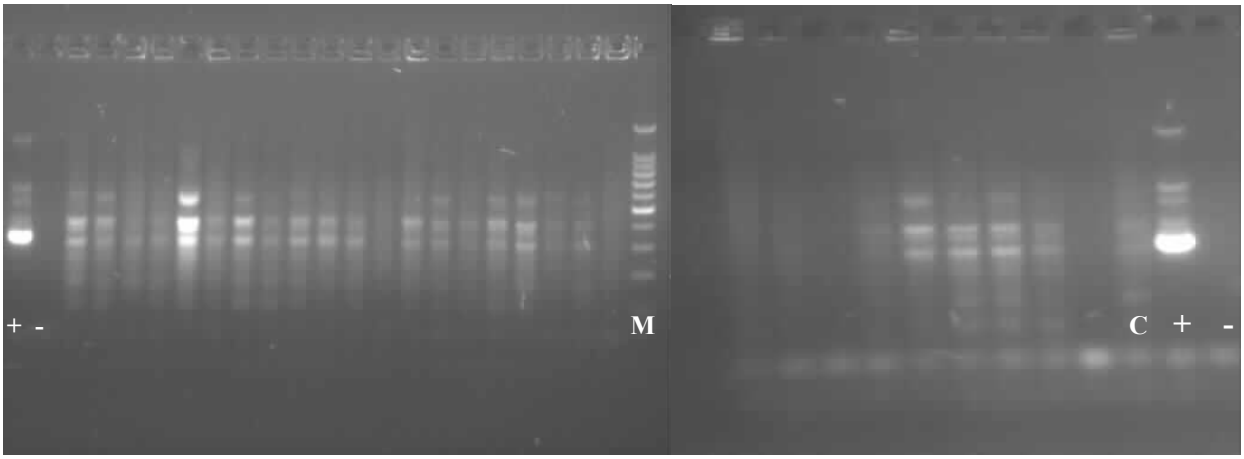

Figure 4.18: PCR amplified bands form commercial broilers vaccinated with HVT. Lanes +: positive control, M: marker, C: CEF DNA, -: H_2O.

4.5.2.3 Samples from NSW.

A similar set of 20 samples was obtained from a commercial hatchery in NSW and subjected to PCR with HVT primers as described previously. All 20 samples reacted in the PCR and gave a strong band on an agarose gel (see Figure 4.19). A duplicate set of samples was sent for cell culture evaluation. No results were obtained from the cell culture assay due to bacterial contamination.

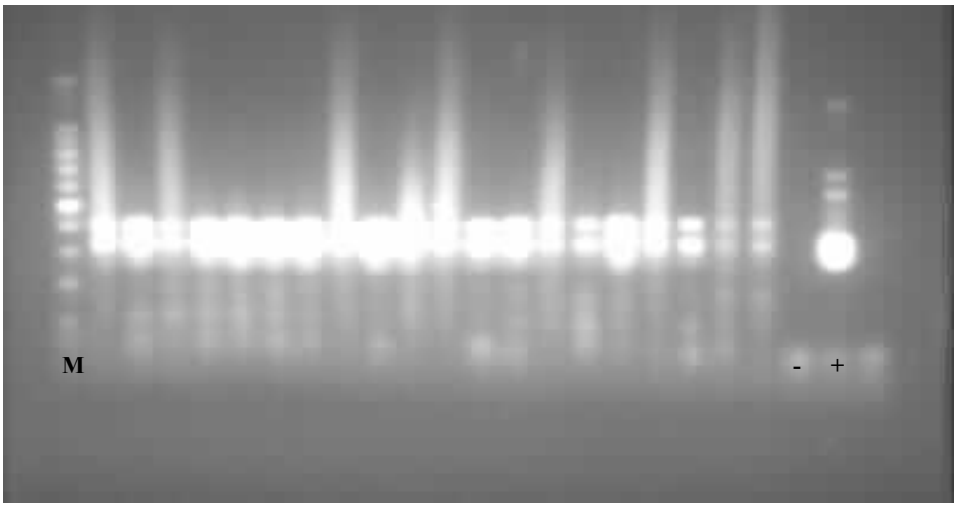

Figure 4.19: PCR products following reaction with the HVT primers in commercial broilers vaccinated with HVT. Lanes M: marker, -: negative control, +: positive control

4.5.3 Testing for Wild Type MDV in Commercial Birds

4.5.3.1 Sample Types and Processing

The conventional method for the detection of viraemia uses three to five millilitres of peripheral blood or spleen tissue as a clinical sample. These samples have been found to be unsuitable for the detection of oncogenic field strains. MDV is known to replicate in the feather follicle epithelium of infected chickens (Calnek *et al.*, 1970) and both antigens and DNA have been recovered from this sample type (Davidson *et al.*, 1986). Whole feathers have been used as samples for MDV diagnosis with some success (Handberg *et al.*, 2001) and represent an alternative to viral isolation in CEK and CEF cells in the diagnosis of outbreaks.

Samples of blood and feather (three large feathers per bird tested) were taken from a flock of unvaccinated, 60 day old broilers suffering from poor feed conversion, lethargy and diarrhoea. At necropsy, the birds were found to have proventricular lesions. The peripheral blood lymphocytes were separated using Ficoll Paque Plus (Amersham Biosciences). Feather tip pulp was pressed from the tips of feathers and homogenised in a microcentrifuge tube. Three DNA extraction kits were tested on each of the samples. DNAzol (Invitrogen), Easy DNA (Invitrogen) and DNeasy Tissue Kit (Qiagen, Germany). The DNeasy Tissue Kit was more convenient and provided a very rapid extraction process with DNA ready for PCR in less than two hours. Ten nanograms of Template DNA from seven blood samples and fifteen feather pulp samples were subjected to PCR using the serotype 1 primer set. Eight of the blood samples were not used due to clotting during transport. The matched samples can be seen in Table 4.3.

Table 4.3: Samples used to evaluate the use of feather tip pulp in the detection of field strains of MDV serotype 1.

Sample Number	DNA From Feather Tip Pulp	DNA From Whole Blood
1	+	-
2	+	-
3	+	-
4	+	-
5	+	-
6	+	-
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
12	+	+
13	+	+
14	+	-
15	+	-

Examination of Figure 4.20 indicated a stronger response to PCR from feather pulp samples than whole blood. All the feather samples corresponding to blood samples reacted to the primers but two of the blood samples failed to produce the expected band. Some non-specific bands were produced in these PCR's and also in subsequent PCR on clinical samples but did not react to the serotype 1 probe in Southern Blotting (data not indicated). This protocol was used on samples from a flock suffering from late mortalities with all samples reacting to the serotype 1 primers. Subsequent samples from flocks on the same broiler farm reacted to the type 1 primers in 30% of cases (data not indicated).

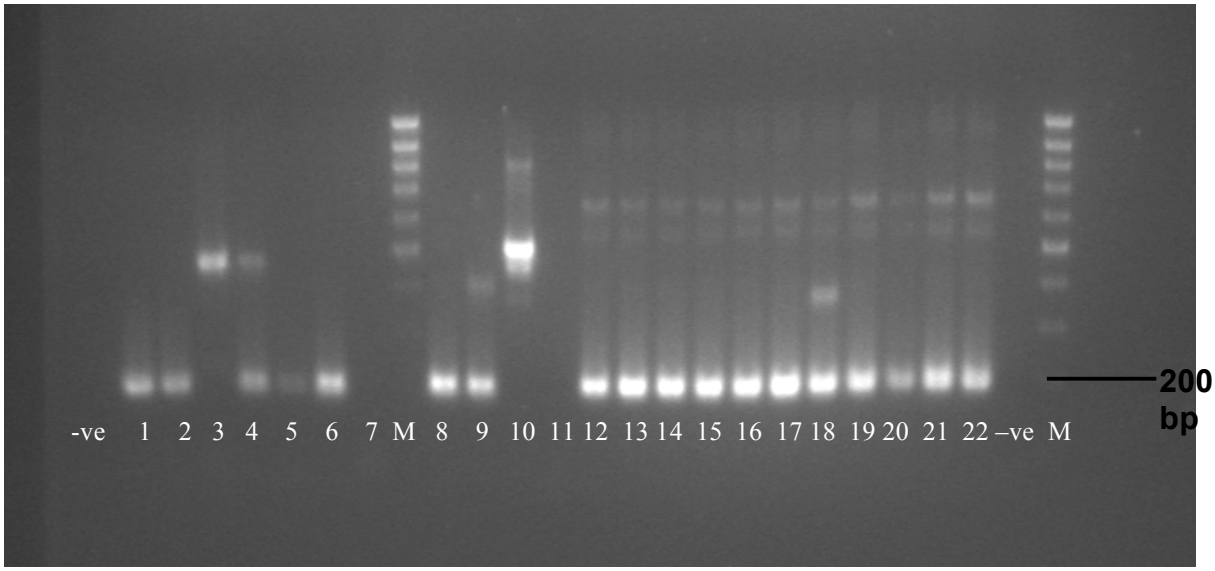

Figure 4.20: PCR amplification products from the serotype 1 primer set using DNA extracted from blood and feather tip pulp. Lanes M; marker, -ve: negative control. Lanes 1 to 7 (blood samples) are from the same birds as lanes 14 to 20 (feather samples),

4.6 Strain Differentiation in Serotype 1 MDV

4.6.1 Current Molecular Marker

4.6.1.1 132 Base Pair Repeat

The current molecular marker for the differentiation of attenuated and field isolates of serotype 1. PCR primers (Witter *et al.*, 1997) were used to amplify the repeats in Australian attenuated and challenge strains, BC1 strain and CVI 988 with modifications to the original protocol. The published annealing temperature of 65°C failed to produce any products. The reaction was optimised for annealing temperature and found to give the best results at 49°C. DNA from birds vaccinated with CVI 988 was recovered at 21 days post vaccination and subjected to the 132 bp repeat PCR. Amplification of the repeat sequence was detected in the attenuated strains from Australia and CVI 988 extracted from vaccine virus. Two copies predominated in the DNA extracted from the Australian Challenge strain, BC 1 from lymphoblastoid cells and CVI 988 following 21 days *in vivo* passage (see Figure 4.21).

The expansion of the repeats appears as a smear in the lane and is representative of a mixed population with variable iterations of the 132 base motif. A probe specific for the central area of the repeat was designed to establish the identity of the smear and used in a Southern Blot (Figure 4.22)

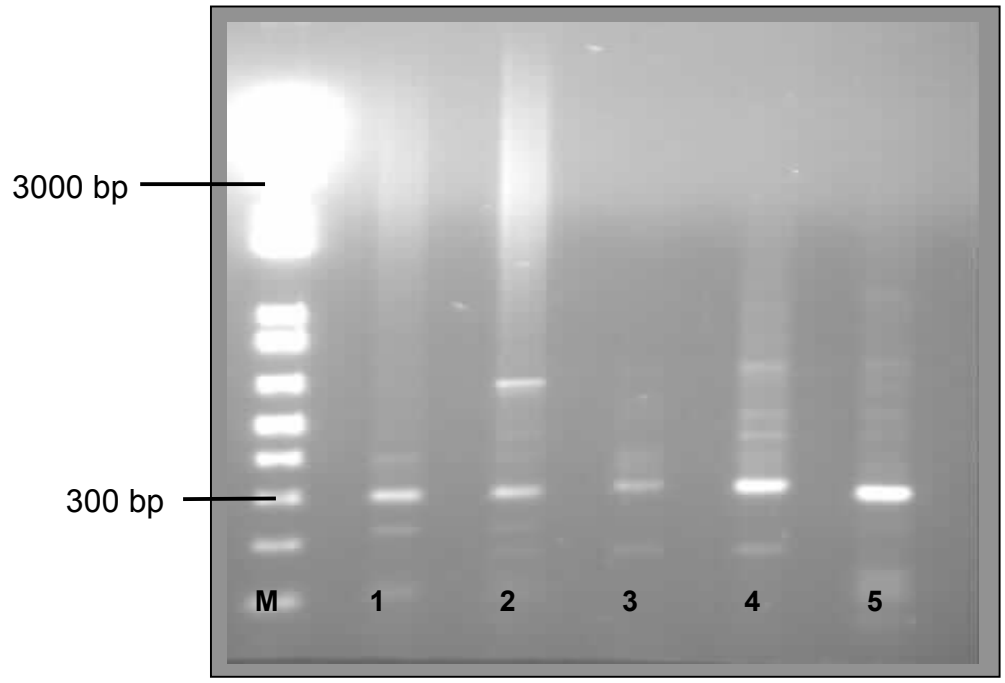

Figure 4.21: 132 bp repeat PCR results. Lanes M: marker, 1: CVI988 attenuated, 2: BH 16 attenuated, 3: Woodlands # 1 challenge strain, 4: BC 1 strain, 5: CVI 988 recovered 21 days post vaccination

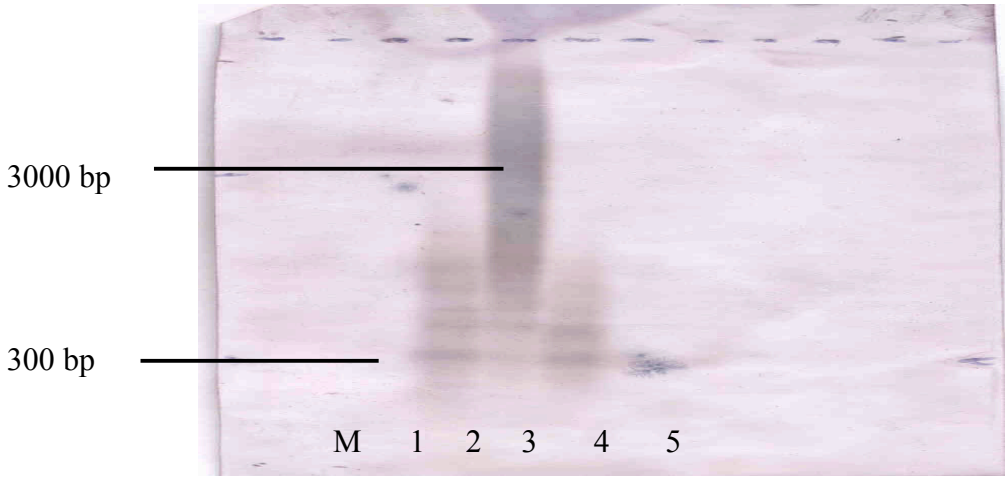

Figure 4.22: Southern blot using probe specific for the 132 bp repeat. Lanes M: marker, 1: CEF DNA, 2: BC 1 DNA, 3: BH 16 attenuated, 4: Woodlands #1 challenge strain, 5: H_2O

4.6.2 New Strain Specific Molecular Markers

4.6.2.1 The meq Gene

The *meq* gene of serotype 1 MDV encodes an oncogene that contains transactivating domains and a basic leucine Zipper domain (Qian *et al.*, 1995; Liu *et al.*, 1998). A 178 bp insertion in the *meq* transactivating domain has been reported to be specific for the CVI 988 vaccine strain (Lee *et al.*, 2000b) and could possibly be useful in differentiating between the vaccine and field strains in vaccinated birds. Lee *et al*. (2000) reported that CVI 988 vaccine virus had two distinct copies of the *meq* gene, one with the insert and one in the original form.

Primers were designed to amplify a region spanning the complete open reading frame of the *meq* gene. PCR was carried out on template extracted from CVI 988 vaccine and cell culture adapted serotype 1 isolates. The results indicate that all the Australian isolates tested carry a similar insert (Figure 4.23). Sequencing of the PCR product indicated that with the exception of a reiteration of five G's in the CVI 988, the insertion was identical (data not included).

Samples were obtained from commercial broilers that had reacted to the serotype 1 diagnostic PCR as indicated previously (section 4.5.3.1) were used in a reaction with the *meq* primers. The results clearly indicate that Australian field strains produce the same band pattern on agarose gels as CVI 988 (see Figure 4.24).

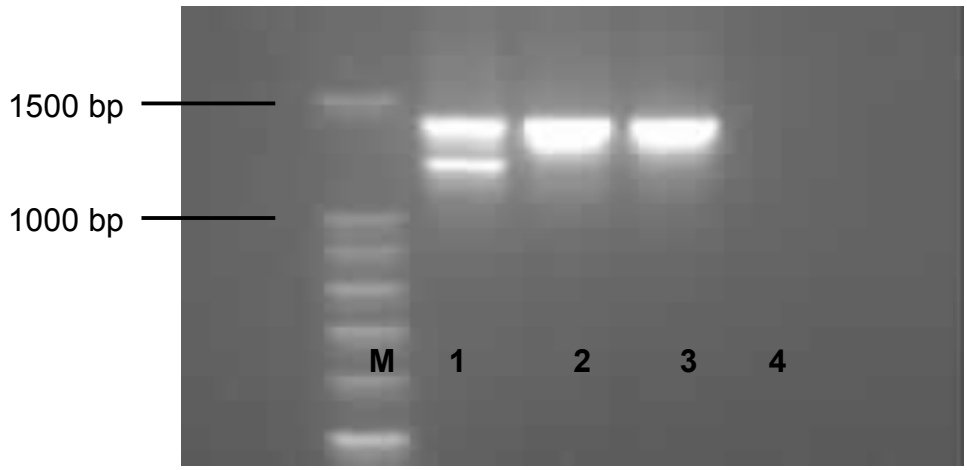

Figure 4.23: PCR products produced by the *meq* primers. Lanes M: marker, 1: CVI988 attenuated, 2: BH 16 attenuated, 3: Woodlands #1 challenge strain, 4: H_2O

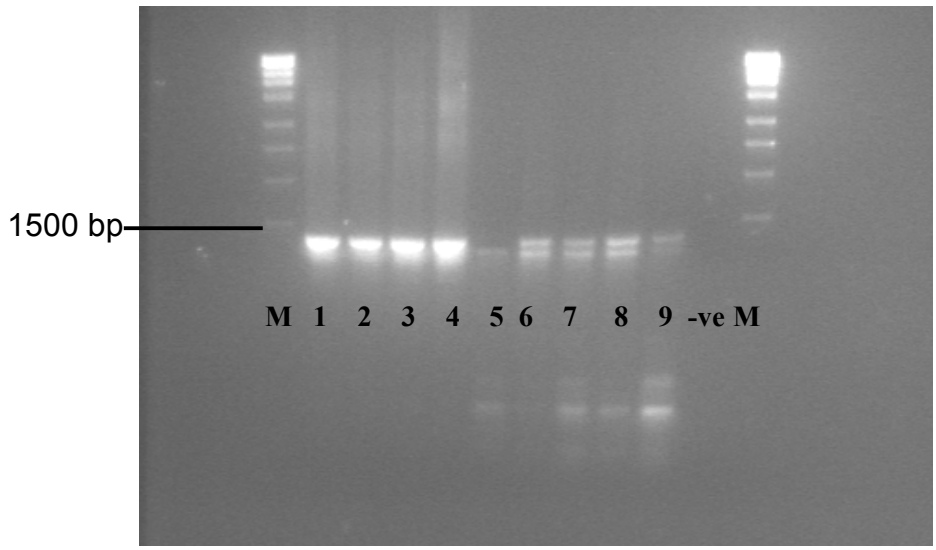

Figure 4.24: *meq* gene PCR using Australian cell culture adapted strains and field strains recovered from feather tip pulp. Cell culture strains 1: BH 16 attenuated, 2: BH 16 challenge strain, 3: Woodlands#1 attenuated, 4: Woodlands#1 challenge strain, lanes 5 –9 field strains

4.6.2.2 Other Genes Investigated for Strain Differentiation

Several genes that may have been subject to selective pressure due to their antigenic nature or their role in the regulation of transcription were investigated in an attempt to locate exploitable sequence differences. Primers were designed to amplify the complete coding regions of the glycoprotein B, pp38 and vIL8 genes (see Appendix 1 for full details of primers).

None of the PCR's designed for these genes produced a detectable difference when the amplicons were run on agarose gels.

Following the publication of the second complete MDV 1 genome (Tulman *et al.*, 2000), the open reading frames of all the genes of the two serotype 1 sequences was aligned using Clustal X and the alignments analysed using GENEDOC version 2.6.002. Significant amounts of heterologous sequence were located in the UL36 open reading frame. This gene encodes the major tegument protein and is approximately 10000 base pairs in length. There is an eighteen base pair motif that is repeated three times in the GA strain and found in the Md5 strain as six copies. Primers were designed to bind in flanking regions of the repeats and a section of some 300 base pairs that differs between the two strains.

PCR was carried out on all the available Australian templates and CVI988 using the UL36 primer set. There was a significant difference in the size of amplicon produced by the isolates tested. In particular, the Woodlands strains (vaccine and challenge) gave considerably different products. The BH 16 strain was consistent between vaccine and challenge strains and gave a similar size product to CVI 988. However, there is a detectable difference between CVI 988 and the Australian strains (see Figure 4.25).

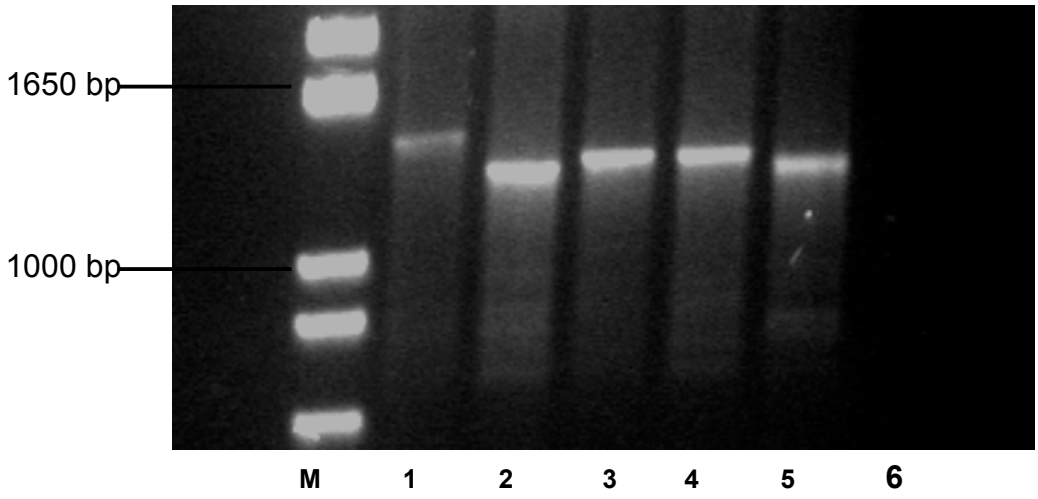

Figure 4.25: Results of the UL36 PCR. Lanes M: marker, 1: Woodlands #1 attenuated, 2: Woodlands#1 Challenge, 3: BH 16 attenuated, 4 BH16 challenge, 5 CVI 988 attenuated, 6: negative control.

The ICP 4 gene of serotype 1 MDV has been studied extensively as a possible regulator of latency and possibly oncogenesis. Recently a 116 base pair insertion in the ICP 4 gene of CVI 988 was reported (Majerciak *et al.*, 2001). Primers (see Appendix 1) were designed to amplify a region of the ICP4 gene that contained the 116 base pair insert. PCR was carried out on this region using all available templates and the amplification products visualised using agarose gels and ethidium bromide staining. The CVI 988 template from vaccine virus and recovered from vaccinated birds gave the expected band size while the Australian isolates produced a band size consistent with an ICP 4 gene without an insert (see Figure 4.26).

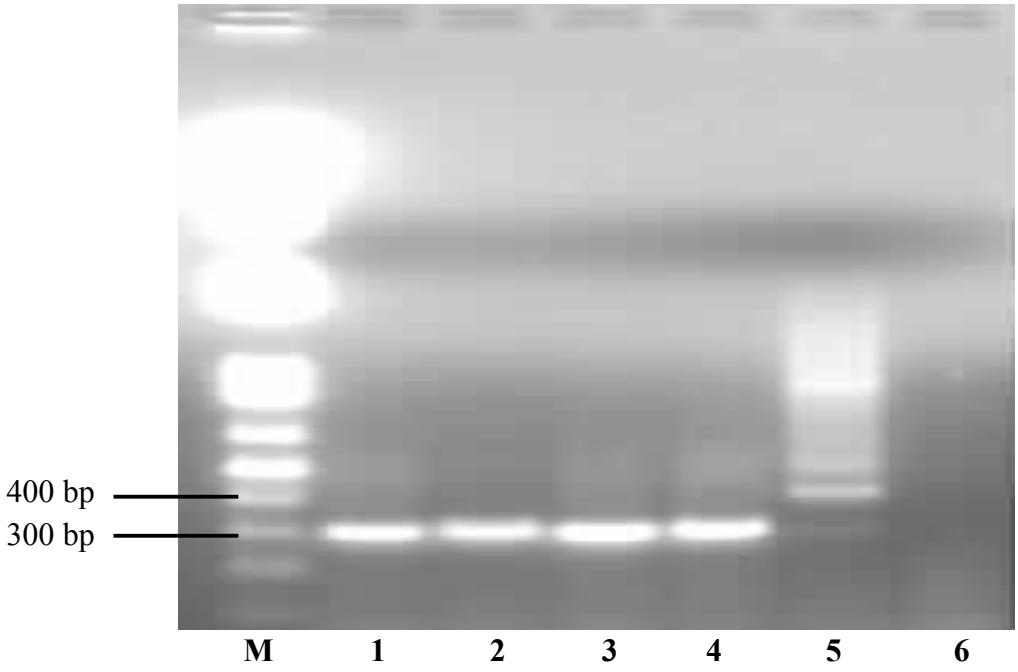

Figure 4.26: Results of the ICP 4 primer set specific for the flanking regions of the CVI 988 insert. Lanes M; marker, 1 Woodlands #1 challenge strain, 2: Woodlands #1 attenuated, 3: BH 16 challenge, 4: BH 16 attenuated, 5: CVI 988 attenuated, 6: H_2O

4.6.2.3 in situ PCR

The *in situ PCR* was carried out using a compilation of a number of published protocols (Walter *et al.*, 1994; Martinez *et al.*, 1995; Levin *et al.*, 1996) that used materials and equipment available in our laboratories.

Paraffin fixed and sectioned specimens were placed onto silanated (AES saline) glass slides and incubated at 60°C for 1 hr to melt the paraffin. Slides were dipped in xylene (benzene free) for 5 min and dehydrated by immersion in 100% EtOH for 5 min. This was repeated a further 2 -3 times to remove all the Xylene and the slides dried at 80°C for 30 min. Rehydration in 0.1M Tris pH 7.4 for 10 min at room temp was followed by washing with 0.3% Tween 20, 0.3% Nonidet P40 in 0.1M Tris pH 7.4 for 10 min at room temp. Permeablisation of the cells was done in 10µg ml^{-1} proteinase K /10 mM EDTA and 0.1M Tris pH 8.0 at 37°C for 10, 15 or 20 min (separate slides) then sections were washed twice in 0.1M Tris pH 7.4 at room temp for 5 min. The tissues equilibrated in PCR buffer for 5 min at room temp. A 2 x PCR reaction mix was made up and diluted 1:1 with Easyseal reagent. A reaction volume of 50µl was placed over each tissue section and covered with a glass cover slip. The PCR cycling was the same as that used for a solution phase reaction (serotype 1 PCR). Once the reaction was complete the cover slips were removed by soaking the slides in dd H_2O or 1 x PBS.

The slides were washed twice more in PBS at room temp for 5 min each then dipped in 2% gelatin and soaked in 10% glutaraldehyde for 20 min at room temp in the dark to cross-link the amplified DNA.

The slides were dehydrated in 0.3M ammonium acetate/95% EtOH for 5 min and air dried at room temp for 5 min before being stored overnight if required.

A gene frame (Abgene, USA) was placed around the tissues and 50 µl of hybridisation buffer (50 %v/v formamide, 1 × SSC, 5% w/v dextran sulphate. 0.05% v/v Tween 20 and 0.1 % w/v skimmed milk powder) containing 200 ng ml^{-1} of DNA probe was pipetted onto the sections. Slides were placed in a thermocycler fitted with an *in situ* block (Eppendorf, Germany) and heated at 94°C for 10 min to denature the PCR product and hybridised overnight at 42°C. The sections were then washed in 2× SSC for 5 min at room temp and 10 min at 60°C, 0.2 × SSC for 5 min at room temp, 10 min at 38°C followed by 0.1× SSC at room temp for 2 min. the slides were incubated at 37°C for 30 min in buffer #2 (0.1M Tris pH 7.5, 0.1M NaCl, 2 mM $MgCl_2$, 0.05% v/v Triton X-100 and 1%w/v skimmed milk). The sections were covered with 150µl of buffer #1 (0.1 M malic acid, 0.15 M NaCl pH7.5) containing 1.5:1000 extravidin-AP (Sigma Aldrich, USA) conjugate or dilution recommended by the manufacturer and incubated at room temp for 15-30 min. Following the binding of the conjugate the sections were washed briefly (5 min) in buffer #1 and then allowed to equilibrate in buffer #3 (0.1M Tris pH 9.5, 0.1M NaCl and 50 mM $MgCl_2$) for a further 5 min. The sections were then incubated under dye solution (132µl NBT and 100µl BCIP per 30 ml of buffer #3) for between 30 min and 2 hr in the dark or over night at room temp, alternatively, use a single pack NBT kit (Sigma Aldrich, USA) at the manufacturers recommended concentrations. The sections were then counter stained with nuclear fast red, covered with DPX (BDH Laboratories, UK) and a cover slip applied.

None of the tissue samples treated in this way produced a visible product within cells. The PCR itself is working satisfactorily in the solid phase amplification and extractions from sections having undergone PCR gave an appropriate size though weak band on agarose gels (data not indicated). Several modifications to the permeablisation of cells, with only ten seconds in proteinase K based buffer, were made in an attempt to localise PCR product within cells with little or no improvement in results. Staining of the sections following hybridisation of the serotype 1 probe resulted in large areas of diffuse colouration. Tissue sections infected with MDV but having the PCR mix replaced with PBS as negative controls, gave occasional areas of diffuse staining following the PCR cycling . This further indicates that the tissues are not able to retain a localised PCR product within the individual cells.

5 Discussion

5.1 General Diagnostic PCR

5.1.1 Serotype 1 Specific PCR

The primer set used for the serotype 1 PCR gives good specificity for the intended target as indicated in Figure 4.2. There was no cross reaction with serotype 2 MDV. The local strain of HVT (Intervet) gave two very faint bands of different size to those expected by with the type 1 primers. Although this weak cross-reaction is evident it would not hinder the PCR process or produce confusing results in the diagnostic setting. The PCR's performed during the testing and optimisations were done under ideal conditions using 10 ng of purified template. The 10 ng used is much more than the amount of target DNA expected in clinical samples and has a higher number of PCR targets relative to the total mass of DNA. Furthermore, the Phenol/Chloroform extracted DNA is clean and free of impurities and PCR inhibitors. It is very unlikely given the level of reaction that the non-specific amplicons seen in Figure 4.2 would be capable of amplification in the clinical setting.

The sensitivity of the Serotype 1 primer set when used in the semi-nested format gives better results than a single amplification series PCR. Figure 4.1 clearly indicates that nesting of PCR primers can give a one thousand fold increase in sensitivity. The ability of PCR to detect target DNA at this level is essential in diagnosing MDV in peripheral blood. The target gene for the type 1 PCR is the glycoprotein C gene, located in the unique long region of the MDV genome. Unlike the repeat regions where two copies of each gene are represented, there is only one copy of this gene. Further gains in sensitivity may be achieved by targeting genes from the repeat regions. However, investigation of many genes from the repeats has indicated that the PCR product from primers targeting these regions often gives a laddered set of amplicons as opposed to a single band. Products of this nature make it difficult to interpret results on agarose gels and may even be detrimental to the overall sensitivity of the assay.

The clinical material tested using the serotype 1 primer set has consisted of filter paper collected peripheral blood from CVI 988 vaccinated layer chicks and feather tips collected from broiler birds suspected of carrying field strains of MDV. The primers performed well whether using relatively unprocessed or highly purified template. In particular the ability of the primers to work on unprocessed template is an indication of the robust nature of the PCR.

5.1.2 Serotype 2 PCR

Several sets of serotype 2 primers were designed both previously (Schmidt, 1997) and during the current project. One recurring problem found in all serotype 2 primers was a strong cross-reaction with DNA extracted from BC 1 strain serotype 1 MDV (data not indicated). This is the strain found in MSB 1 cells and is commonly used as a source of serotype 1 template during PCR design and optimisation. The reason for this is as yet unknown and does make the choice of primers difficult. However, BC 1 strain MDV does not occur naturally in Australian flocks and so the cross-reactivity with serotype 2 primers was not considered as relevant to the developmental process.

Unlike the serotype 1 and HVT primer sets, the type 2 primers were unable to be trialed in the clinical setting. This was due to the unavailability of birds (layers or broilers) vaccinated with serotype 2 vaccine. Although spiking filter paper lobes with vaccine could have tested the primers, it was not considered to be truly representative of a clinical sample. Similarly, hand vaccination of SPF chicks was not undertaken because it does not truly represent the vaccination process as conducted routinely in hatcheries.

In spite of the limitation faced when designing and optimising the serotype 2 primers, the level of specificity and sensitivity would be adequate in the clinical setting. They also represent the first set of type 2 nested primers to be published.

5.1.3 HVT PCR

The semi-nested primer set chosen for the HVT PCR, like the serotype 1 set gave very good specificity and sensitivity for their target. The template used in optimising the reactions is very pure and high in target copy number relative to clinical material. Any observable differences in the response to the primers under these conditions is likely to be exacerbated when running the PCR on clinical samples. Although other sets were trialed (Figure 4.3) the primers targeting the glycoprotein B gene gave consistently higher levels of reaction product than other sets tested.

This set of primers was used to amplify DNA from clinical samples derived from full dose vaccination in layer birds and lower dose vaccination in broilers. In both cases it was able to detect MDV genome in relatively unprocessed template material (Figures 4.16 to 4.19). Once again this attests to the robust nature of the PCR in the semi-nested form. Figures 4.18 and 4.19 indicate a considerable difference in the response to the HVT primers given that the samples were taken at the same time relative to vaccination (21 days) and on the same collection media. The reaction observed in Figure 4.19 is much higher than that indicated by Figure 4.18. The reason for this is unknown and could be an indication of better vaccination as opposed to inconsistencies in the collection system. The possible improvements that could be made with respect to the collection system will be dealt with later in this section.

There were a number of instances where clinical samples from HVT vaccinated broilers failed to produce a reaction to the HVT primer set. Matching samples were sent for cell culture evaluation and the results indicated an adequate viraemia to HVT. The reason for the failure of PCR to detect the viraemia was perplexing in light of previous successes with this PCR. The only difference between this trial and previous trials with the HVT primers had been a change from a recombinant *taq* polymerase (Sigma Aldrich, USA) to a native polymerase (Promega, USA). The reason for the change in amplification enzyme was due to a significant increase (150%) in the cost of the recombinant *taq* polymerase. Comparisons of other recombinant enzymes and the native *taq* indicated that the latter was unsuitable for amplification of genome from unprocessed clinical samples (data not indicated). Another recombinant polymerase "Red Hot Polymerase" (Abgene, USA) derived from *Thermus icelandicus* has been used successfully in subsequent experiments and is capable of generating PCR products at very low concentrations. The reason for the difference in amplification efficiencies between native and recombinant enzymes is not clear. However, it is strongly recommended that high quality recombinant polymerase enzymes be used when the template is prepared from filter paper collection systems.

5.1.4 Objective One and Two

The first objective outlined in Chapter 2 was the development of a serotype 1 specific PCR reaction capable of detecting vaccine and field strains of MDV in birds. The results presented indicate that the semi-nested PCR used in type 1 diagnostics is capable of detecting field strains and vaccine virus in commercial birds. The PCR's designed for serotype 2 and HVT have proved to be both specific and sensitive. In addition, the HVT PCR is capable of being used in the clinical setting. This result meets the second objective outlined in Chapter 2.

5.2 Differentiation of Serotype 1 MDV Strains

5.2.1 Genes Selected for Investigation

A number of genes were identified as possible candidates for the differentiation between strains of serotype 1 MDV. The ability to differentiate between vaccine and field strains in particular is seen as desirable due to the possibility of co-infection of vaccinated birds with wild type viruses. The expansion of the 132 bp repeat region in attenuated strains has been the standard molecular marker for many years. Our data supports the assertion by Young and Gravel (1996) that this expansion is not a permanent marker in vaccinated birds. Figure 4.21 clearly indicates that the expansion in CVI 988 is reduced to the initial two copies found in wild type viruses. BH 16 also underwent the same reversion following three passages *in vivo* (pers comms, Dr Roberta Karpathy, Intervet Vaccine Laboratory. 2000). Although the reason for this is yet to be proven conclusively, current thinking is that isolates of MDV exist as a population of mixed genotypes. Cell culture adapted and attenuated MDV represents a genotype that replicated well in cell culture. When the attenuated virus is passaged *in vivo* there is a shift back towards the wild type genotype that replicated better in the host. Irrespective of the mechanism of this dynamic shifting between populations, it presents the research community with problems with respect to differentiation between strains.

Our work has focused on looking for differences in other areas of the genome and in particular, differences between isolates of MDV that may be exploitable. The genes selected for this work were done so on the basis of either possible changes due to selective pressure from their antigenic nature, or their ability to regulate replication, latency or oncogenesis.

None of the genes encoding proteins found in the envelope of infective MDV that were investigated using PCR or by alignment search were found to have any significant areas of heterologous sequence.

The *meq* gene of MDV has been the subject of much research in recent years. The work done by Lee *et al.* (2000) indicated a possible difference in the *meq* gene of attenuated CVI 988 compared to the original isolate. There was a reported insertion of 178 bp in addition to the gene of original length. Subsequent PCR of this gene in CVI 988 using primers designed to amplify the complete open reading frame, confirmed these observations. The cell culture adapted Australian strains also produced a product of the same size as the attenuated CVI 988 (Figure 4.23). This had not been reported in other strains of type 1 elsewhere at that time. Furthermore, when a field strain recovered from feather tip pulp was subjected to the *meq* PCR, the products more closely resembled the attenuated CVI 988 (Figure 4.24). The variability of this gene is interesting in the context of regulation of oncogenesis and certainly warrants further investigation. However, with respect to this project it is an unreliable marker for differentiation between strains. In particular, it will not distinguish between the most popular type 1 vaccine in use in Australia and the local field strains.

With the publication of the complete genome of Md5 and a complete genome for the GA strain available by collation of numerous sequences, it has been possible to directly align the open reading frames of many of the type 1 MDV genes. There is almost perfect homology between the genes of the unique long region and in many of the genes within the long repeats. In addition, a number of the genes from the repeat regions from other strains have been published.

The ICP 4 (infected cell protein number four) has been extensively studied with respect to its role in latency. Recent work by Majerciak *et al.* (2001) found an insert in the ICP 4 gene of CVI 988. Like the differences in the *meq* gene, this had not been reported in other type 1 strains. Alignments of the ICP 4 sequence of CVI 988, GA and Md5 indicated that the insert did not exist in other isolates. The PCR designed during this project was able to confirm that the insert is not present in Australian strains. The results in figure 4.26 clearly demonstrate a detectable difference in the amplicon from CVI 988 and the

Australian strains. The relatively small size of the PCR products will allow the reaction to be run in a nested format thus making it suitable to use in the diagnostic setting.

This PCR now gives the Australian poultry industry the ability to differentiate between the current choice in type 1 vaccine and Australian strains. However, it must be emphasised that there has not yet been the opportunity to fully evaluate this PCR on field strains.

A gene that had not been previously considered as a candidate for strain differentiation is the UL 36 (unique long open reading frame number 36). This gene encodes the large tegument gene and is just under 10000 base pairs in length. The alignment of this gene in the GA and Md5 strains indicated a significant difference in an area approximately 1200 bases down stream from the start codon. There was a repeated motif of 18 bases in both sequences, present as three copies in the GA strain and six copies in the Md5 strain. The PCR designed to amplify the repeats and a section of heterologous sequence just upstream from the repeats, produced significantly different bands on an agarose gel. The Woodlands strain gave different results between the attenuated strain and the challenge strain. The BH 16 strains remained consistent in their products and CVI 988 gave slightly different results from the Australian isolates. This is the first report of differences in the genome of Australian isolates.

The results obtained give the industry the ability to differentiate between two Australian strains of serotype 1. However, at this time it can only be done on the basis of product size and PCR's would need to be run with a set of standards. Further sequencing of these products may produce a probe set that can differentiate between isolates by sequence homology.

5.2.2 Objective Three

In terms of the third objective from Chapter 2, the results of the project meet the objective in that we have been able to identify areas of the type 1 genome that allow for the differentiation of serotype 1 isolates. In particular, the industry now has the ability to monitor the efficacy of CVI 988 against challenge from virulent field isolates.

5.3 Collection Media, Techniques for PCR Product Detection

5.3.1 Collection Media

The primary choice for the collection of clinical material has been filter paper in either untreated or treated form. Both commercial and in house developed media was tested. In terms of sensitivity there was little difference between the commercial products and the in house system. Given the relatively low number of MDV genomes in lymphoblastoid cells compared to vaccine CEF cells, the in house method may even be slightly better than the commercial products. In terms of cost the in house method is much more cost effective than commercial products.

With the exception of the first group of layer birds, the clinical trials undertaken to date have been conducted with the assistance of consulting Veterinarians working in the field. In these cases, a prototype collection kit was supplied to the vets and all the collections were done in the hatcheries. All of the cooperating veterinarians considered the collection system to be easy to use and convenient. In particular, those veterinarians who travel long distance to the hatcheries in remote areas found the system preferable to refrigerated transport.

The inconsistencies observed in the clinical samples tested (Figures 4.18 and 4.19) using the HVT primers may be a result of the collection technique. Either the inclusion of proteins inhibitors or the DNA binding capacity of the filter paper may be contributing factors. However, the presence of inhibitors is unlikely due to the treatment process. The concentration of chemical used is effective in lysing cells and denaturing proteins on contact with whole blood. The high binding affinity of paper

for DNA may be the main contributing factor. Although further work needs to be done to increase the consistency of the assay, there is no system available to quantitatively assess the amount of DNA being released from the sample. Improvements in this area will only come from high volume testing.

The amount of template available for PCR amplification may be capable of being improved with the use of capillary tube as the principal collection device. The filter paper would then be used as the transport media. Use of capillary tubes would have two main advantages. Firstly there is a volume advantage in that capillary tubes hold six times the volume capable of being absorbed directly onto paper lobes. Only the buffy coat component of whole blood is spotted onto the paper. Secondly, the elimination of the erythrocytes would vastly reduce the amount of inhibitors in the PCR. Although commercial capillary tubes have been tested, the anticoagulants used may have inhibited the subsequent reactions. Other anticoagulants such as sodium citrate would offer an alternative to heparin or EDTA but not interfere with PCR. However, no commercial products of this nature are available.

In spite of the few limitations experienced to date, the treated filter paper collection system developed here offers great potential as a routine sampling method. It offers a more attractive option in that birds being tested for viraemia following vaccination do not need to be sacrificed while sampling, making it more attractive to producers where profit margins are very low. The need for refrigerated transport is eliminated making it more convenient for veterinarians using it in the field. The cost of transport is reduced because there is no need to ship bulky packages via overnight freight. Samples can simply be placed in ziplock bags and sent by regular post or overnight satchel. Furthermore, on arrival at the diagnostic lab, the samples need not be processed immediately. Our results indicate that there is sufficient stability in the system to safely allow seven to ten days between collection and processing.

5.3.2 PCR Product Detection

The conventional method of PCR product detection using agarose gel electrophoresis, although reliable, is very time consuming and not cost effective when considering the time needed for trained technicians to run gels. PCR ELISA offers an alternative to electrophoresis, especially in cases where high throughput is required. The protocol used here takes approximately 2.5 hours to complete from the time the reactions are taken out of the thermocycler. The time taken to run an agarose gel is 1.5 to 1.75 hours plus the time taken to cast the required gel and load the samples. In the case of gels the required time increases with the number of samples to be run. ELISA takes a consistent amount of time irrespective of the sample number. Furthermore, PCR/ELISA is capable of being automated in some labs where gel electrophoresis is not.

The initial work on the ELISA was done using polycarbonate microtitre plates. Although streptavidin was capable of being passively bound to the plate, little or no signal could be detected following the ELISA. Subsequent investigation indicated that the streptavidin was washed from the plates during the ELISA process. Commercial streptavidin coated plates use covalently bound streptavidin, which withstands the numerous washing steps involved in the ELISA protocol. Being a proprietary product, no information is available on the concentration of streptavidin used in the coating process. The project investigated the possibility of an in house covalent coating system but found that it would not be a cost effective alternative to the commercial products. The main benefit to come from the attempt at our own coating system is a protocol that allows us to evaluate the binding capacity of commercial products before they are used in diagnostic assays.

A number of the ELISA formats tested during the project produced disappointing results and have not been included in the results. One commercial system (DIAPOPS, Nunc USA) assessed gave results comparable to blocking ELISA when used on the serotype 1 PCR but gave adequate results for the HVT PCR. This system uses a primer that is covalently linked to the side of the PCR tube. All PCR and ELISA are carried out in a single tube. Investigation of the type 1 PCR product indicated the presence of sequences allowing the possible formation of hairpin loops in the probe binding region, not present in the HVT product. Modification to the protocol resulted in only slight improvements in results. Redesign of the PCR would have needed to be done in order to use this system. The use of this

system would have required the redesign of all existing primers and probes to be effective. Further complications are that nested PCR cannot be performed in this system making the sensitivity a limiting factor. Furthermore, using the UNG system to prevent carryover contamination would prevent any second round amplification.

One of the objectives of the project was to produce tools that could be used in a wide variety of situations. The PCR/ELISA format chosen and reported here is capable of such flexibility and has been used by other researchers on bacterial assays (Muramatsu *et al*.,1997) and in diagnostics for aquatic pathogens (Cullen & Owens, unpublished data).

One possible disadvantage with the PCR/ELISA is cost. The incorporation of Dig-11-dUTP into a PCR product is expensive due to the cost of Dig. At present, the labelled nucleotide is only available from one supplier. There may be further gains to be made in the improvement in cost by reducing the ratio of labelled UTP to dTTP. Other possibilities remain such as multiple peroxidase conjugates, multiple probes or more freely available labels such as fluorescein. Although all of these are possibilities they need to be tested against the standard we have produced here.

5.3.3 Objective Four

The fourth stated objective of this project was to adapt the PCR's to simple, cost effective sample collection and product detection techniques. The collection technique developed during this project certainly meets these criteria for both simplification and reduction of costs over the present techniques.

5.4 *in situ* PCR

Although the results for the *in situ* PCR were disappointing, there has been some progress made during the period of this project. Some of the reasons for this are that there were no protocols available for this technique on avian tissues. Moreover, there were no protocols for spleen or liver tissues from mammals or other similar organs from higher animals. All the protocols used were compilations of other procedures adapted to suit the available materials and equipment.

The protocol has made some considerable progress in spite of the limitations in available guide lines. The PCR using the outer primer set of the type 1 PCR have worked in the solid phase and are capable of producing a band when extracted from post reaction sections. The indicator system used, although not ideal does demonstrate higher levels of staining of type 1 infected tissue sections. However, localisation of the PCR product to a single cell has not been accomplished.

Further optimisation of the treatment process may result in better results. Another indicator system is under investigation but has not yet been used on infected tissues. Although other tissue types may offer a more robust platform for the PCR, the objective of the *in situ* protocol was to look at viral replication with respect to vaccine deposition in *in ovo* vaccination.

Although the objectives of this component of the project have not been fully met, we are very close to being able to report a positive outcome.

6 Implementations

MDV continues to be one of the most economically significant pathogens of commercial poultry both here in Australia and internationally. The rapid evolution of the type 1 field strains toward greater virulence presents the industry with highly elusive goals and objectives.

There are two areas central to the ongoing control and understanding of MDV in our local industry. Firstly we need the ability to effectively monitor the response to vaccination with the current panel of vaccines in commercial poultry. Secondly, we need to be able to monitor the extent of challenge by field strains in young birds. Monitoring for vaccine efficacy has been successfully carried out in this country for some years using cell culture assays.

While these present systems are effective in detecting viraemia, there are very limited numbers of laboratories and technical staff with the experience and training capable of undertaking this work. These techniques are cumbersome and expensive to conduct. They require a coordinated effort from veterinarians, producers, transport and laboratory staff so that all phases of the system work to their optimum level. Molecular diagnostics with simplified sampling methods does not suffer the limitation imposed by these requirements.

This project has demonstrated the viability of a new set of tools to effectively monitor the efficacy of vaccination against MDV. With more developmental work these new tools would offer a cost effective and convenient alternative to the current methods used in monitoring post-vaccinal viraemia. One significant advantage is the ability of producers to take samples in the hatcheries. The standard paper collection system with agarose gel electrophoresis can be run for $10.00 per sample in our laboratory in a low (20 sample batch) throughput situation. This is likely to offer a cost advantage over current techniques because of the lower cost of transport (less than $6.00 in an overnight post satchel). The cost of the assay is higher when the ELISA detection system is used but it has benefits in interpretation of results and speed, although not yet fully evaluated in the area of high throughput screening. The E-gel 96 system from Invitrogen does show potential in reducing the cost of 96 well assay formats. If it proved viable, the gel system could reduce the cost of rapid product detection in 96 well format from $4.00 to 50 cents per sample. Significant reductions in time also result with the gel system taking only 20 minutes to load and run.

With a significant reduction in costs and added convenience, more producers may be encouraged to monitor for effective viraemia on a regular basis.

The monitoring for challenge by field strains has not been routinely undertaken by most producers in Australia. The reason for this may lie in the difficulty in isolating field strains in cell culture and the inability of standard PCR to detect wild type virus in the conventional blood or spleen samples. The method outlined here is able to detect serotype 1 MDV in the feather follicle of infected birds. This assay has proven to be both rapid and effective and can be carried out for $15.00 per sample using commercial DNA extraction kits. The results can be available in as little as 24 hours following delivery of the samples. This represents a significant improvement in our ability to monitor flocks for field strains and will contribute to improvements in shed hygiene and allow producers to make more informed management decisions.

In addition to the diagnostic assays, this project has provided the industry with the ability to monitor the protection offered by the current serotype 1 vaccine. The cost is slightly higher than the field strain diagnostic because two separate PCR's are run on a single sample and standards are also run for comparative analysis. Further research in this area may lead to a significant reduction in costs if new primer and probe sets are developed. This is an area where cell culture has not been effective. CPE from all serotype 1 viruses have similar morphology. We are now in a position to effectively monitor for any increase in virulence in Australian field strains.

The future of viral diagnostics for the poultry industry within Australia and internationally will see many changes over the next decade. New technologies using microarrays or genechips have already been used to detect multiple respiratory viruses in humans. This type of technology may offer the diagnostic lab the opportunity to screen for all of the avian viral pathogens in a single assay. Furthermore, this type of technology is suited to the development of therapeutic agents by monitoring the immune response to vaccination and challenge. The probes designed during the course of this project are not suitable for this type of system. Oligonucleotides for microarrays are generally 70 bp to 100 bp in length while probes for blots and PCR/ELISA work well at 20 bp to 25 bp. However, the PCR's and genome information gathered may be of use in the future when designing genechips.

7 Recommendations

Implementation of the technologies that resulted from this project will largely be accommodated in a subsequent project UJC-10J jointly funded by both chicken meat and egg industry programs.

The objectives of the new program include:

- to develop and transfer to the industry molecular techniques for monitoring blood samples for the presence of Marek's disease virus
- to use a panel of simple, cost-effective molecular diagnostic techniques to describe the effects of various management strategies on Marek's infection
- to develop and validate quantitative assays for Marek's disease viral DNA based on real-time PCR
- to assemble a comprehensive collection of DNA for Australian wild type Marek's disease viruses

It is anticipated that with the collaboration of a commercial partner (TropBio) kits will be developed incorporating the reagents required to carry out these assays. The assays to date have been developed on an Eppendorf gradient cycler. The cycling parameters can be transferred to a magnetic card that will allow the assays to be carried out on a conventional 96 well PCR cycler.

Initially the assays will be carried out at James Cook University in the configuration that it is anticipated will be transferred to other laboratories in the poultry industry.

Selected laboratories throughout Australia will be set up with an appropriate 96 well PCR cycler and be supplied with kits that will allow them to carry out routine testing of layer and broiler birds.

In order to implement these techniques on international scale it will be necessary to further develop and validate the kits.

The major intellectual property involved in this project involves the sequence of the primers developed throughout the project. Discussions will be held with RIRDC regarding the publication, patenting or technology transfer of this intellectual property.

Appendix

All oligonucleotides designed and used as primers or probes.

Name	Sequence	Use	Gene	Product Size
	Serotype 1 specific oligos			
41	CGGGCAAGAACGCATACATCC	primer	gC	650
25	TGTTTCCATTCTGTCTCCAAGA	primer	gC	650/201
24	CATGCAAGTCATTATGCGTGA	primer	gC	201
Gap	Biotin-CTGCATCAGTGGATGTACTGG	probe	gC	N/A
F132	GCGATGAAAGTGCTATGGAGGA	primer	132 bp repeat	311 two copies
R132	ATCCCTATGAGAAAGCGCTCGA	primer	132 bp repeat	311 two copies
P132	Biotin-GAGGCTCGTGTGAAGAACCCT	probe	132 bp repeat	N/A
meq-f1	TCTTCCCGAAACTATGAAAA	primer	meq	1198/1376
meq-r1	AATAAATTCCGCACACTGAT	primer	meq	1198/1376
UL36f	TTA CAC TAT CCG GTT TAG TTA	primer	UL36	unknown
UL36r	ATT GCC TTA TGT CGA TG	primer	UL36	unknown
GB-CLN for	ATGAATTCTGAACGCCCTATGCC	primer	gB	3108
GB-CLN rev	ATGTCGACAGATCAGGTGCGAGT AGAAC	primer	gB	3108
pp38-CLN for	ATGGATCCGATAGTCTCGGGTAT AGAAA	primer	pp38	1296
pp38-CLN rev	ATAAGCTTAACTTCTTCGCCTGAT	primer	pp38	1296
vIL8-CLN for	ATGAATTCTGTGCGAGTCCTGCGGGT AG	primer	vIL8	1053
vIL8-CLN rev	ATAAGCTTTGGTTCGGGTAAGGCGTT CA	primer	vIL8	1053
ICP For	ATTGGTTCGCGACAAGATAACACA	primer	ICP 4	300/416
ICP Rev	GCCCAGGCAAGTGCAATGT	primer	ICP 4	300/416
	Serotype two specific oligos			
S1FO-MDV2	TGCGTGCGATTAGTGT	primer	repeat regions	619
S1RO-MDV2	TCGCAGGAAAGTAAGTTC	primer	repeat region	619
S1FI-MDV2	GCACGCGCATCATAAG	primer	repeat region	304
S1RI-MDV2	TCCTGATTCCGTAGCACT	primer	repeat region	304
SI Prb MDV2	Biotin-AACGAAGCGAGGCTAAGT AGCAGG	probe	repeat	N/A
S2FO-MDV2	AACTCGGGACTGTTCGTA	primer	repeat region	637
S2RO-MDV2	GAGTCGGCCGTAGGAG	primer	repeat region	637
S2FI-MDV2	GGTCGCTGCGGTCTTT	primer	repeat region	232
S2RI-	GCGGAGGTGTAACGATT	primer	repeat	232

MDV2			region	
S3FO-MDV2	CCTTCGCCTGGACAATAG	primer	repeat region	530
S3RO-MDV2	TCCTGCGCGAATGTG	primer	repeat region	530
S3FI-MDV2	GCCGTCGATCGCCTAAC	primer	repeat region	251
S3RI-MDV2	CCAAACGTCAGCCGAGTC	primer	repeat region	251
	HVT specific oligos			
HVT-lf	CTCTATGGCAAAGTGGAAAG	primer	gB	639
HVT-sf	GAGAGTTGTTGCGGGACA	primer	gB	314
HVT-cr	TGTTGCACTCGCAGTTACAC	primer	gB	639/314
HVT-prb	Biotin-CCGGCAAGGCCATCTTGA ATGACT	probe	gB	N/A
HVT2-F	GGAAATGGCAAAGGAATTAGT	primer	repeat region	405/218
HVT2-smallR	CGCCTGAGAATTATATCTACT	primer	repeat region	218
HVT2-bigR	TGTATCGCCACGTTAGCAC	primer	repeat region	405
HVTFN-of	GCTGCTCTCCAGGAATCA	primer	gB	657
HVTFN-or	GTTCTGAGAACAACCGAATG	primer	gB	657
HVTFN-if	GGCATCGCTTTGTTTCCA	primer	gB	189
HVTFN-ir	TGGGGATGTACGGGGGTA	primer	gB	189
	Plasmid component of the hybrid primers used in the synthesis of internal control DNA fragments			
Forward	ACGCTCAAGTCAGAGGTG	primer	pBR 322	726*
Reverse	CATTGGTAACTGTCAGACC	primer	pBR 322	726*

* the length of the internal control DNA fragment in the sum of the plasmid DNA and the two MDV primer sequences

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