AUSTRALIAN POULTRY CRC

FINAL REPORT

Program (2B)

Project No:

05-17

PROJECT LEADER:

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DATE OF COMPLETION: 04/01/2010

Project No: 05-17

Project Title: Transfer of Diagnostic Technologies from Research Laboratories to a Core Diagnostic Facility for the Benefit of the Australian Poultry Industry

ISBN 1 921010 35 5

Transfer of Diagnostic Technologies from Research Laboratories to a Core Diagnostic Facility for the Benefit of the Australian Poultry Industry

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Published in January 2010

Contents

Introduction

Currently, most poultry diagnostic laboratories in Australia rely on serology as a method of disease diagnosis, or other indirect and less sensitive examinations including histology. Few laboratories also utilise conventional PCR for detection of avian pathogens. This project brings 'state of the art' diagnostic technology to the Australian poultry industry for the direct assessment of an agent, together with strain differentiation, which has become a real issue as more and more live vaccines enter the market.

The main aim of this project was to utilise by transfer of technology from the research carried out within APCAH and in other partners of the Poultry CRC, for practical diagnosis and monitoring of the major poultry diseases in Australia.

The major specific outcomes that were targeted during this project for commercialisation were:

1. Rapid diagnosis and concurrent differentiation of the infectious bronchitis virus (IBV) strains,
2. Rapid detection and differentiation of infectious laryngotracheitis virus (ILTV),
3. A multiplex PCR for detection of avian *Escherichia coli* and concurrent distinguishing between opportunistic and virulent *E. coli* strains,
4. Rapid diagnosis of *Mycoplasma gallisepticum* (MG), *M. synoviae* (MS), *M. meleagridis* (MM) and *M. anatis* (MA) by PCR,
5. Rapid differentiation of MG vaccine and field strains by PCR,
6. Rapid differentiation of MS vaccine and field strains by PCR-coupled single-strand conformation polymorphism (SSCP),
7. Rapid diagnosis of chicken infectious anaemia (CIA) by real-time PCR.
8. Rapid detection and differentiation of avian *Eimeria* species,
9. Detection of all avian leukosis viruses (ALV) and differentiation of ALV-A and J from other ALVs by PCR,
10. Rapid and reliable diagnosis of Chlamydiosis by PCR,
11. Rapid detection and typing of the fowl adenoviruses (FAV),
12. Rapid detection of *Avibacterium paragallinarum, Pasteurella multocida*, avian reoviruses, reticuloendotheliosis virus, Avian encephalomyelitis, Marek's disease virus (MDV), Egg Drop Syndrome virus and infectious bursal disease virus (IBDV), and

This project has also aimed to:

- Establish a core national diagnostic platform for investigations of the major poultry diseases.
- Provide rapid, reliable and standardized diagnostic and typing services for the poultry industry.
- Ensure the preparedness for disease outbreaks
- Facilitate determination of the source of future disease outbreaks
- Facilitate interpretation of epidemiological investigations.

- Assist to address important issues regarding eradication of poultry diseases and designing vaccination programs.
- Ultimately reduce the economic losses caused by major poultry diseases as well as the cost of control programs.

General Approach

The extent of the methodology used in this project was based on the development of the diagnostic assays in several phases. Diagnostic assays were classified into six groups according to their development status although a number of assays were developed according to the urgent requirements of the industry at the time:

Group 1 assays were those that were already in use for diagnosis of field cases at APCAH. These included RFLP techniques for differentiation of *M. synoviae* or *M. gallisepticum* strains, for detection of ALV-J and ALV-A isolates, and for differentiation of ILTV isolates. Standard operating procedures were prepared for these techniques and reviewed by an expert in the field.

Group 2 assays were those that had already been designed and tested under experimental as well as field conditions but required optimisation equipment and reagents used at APCAH. These included PCR assays for detection of *A. paragallinarum* in pure cultures or direct clinical samples, for detection of virulent *E. coli* in pure cultures, for detection of *M. gallisepticum* and *M. synoviae* in clinical specimens. These tests were developed in three consecutive phases:

a. *In vitro* using pure cultures.

b. *In vivo* under experimental conditions using several chicken experiments on *A. paragallinarum*, *M. gallisepticum* and *M. synoviae* (mostly funded by poultry CRC) carried out in 2006-2007 in our department. Swabs collected from palatine cleft or upper trachea (for *M. gallisepticum* and *M. synoviae*) or nasal discharge (for *A. paragallinarum*) were collected during these experiments to complete the second phase of the development of these assays.

c. *In vivo* under field conditions. Clinical samples submitted to our laboratory during the course of this study were also used to further optimise the test under field conditions and results were used to complete the final phase of these assays.

In addition to these requirements, sampling directly from clinical specimens (inflammatory exudates for *E. coli* and palatine swabs for mycoplasmas as opposed to bacterial culture)..

Group 3 assays were those that had been developed and tested under experimental conditions but required further testing under field condition as well as preparation of the SOPs. This group includes capillary electrophoretic technique for the specific identification of chicken *Eimeria* species, rtPCR for concurrent detection and differentiation of IBV strains and rtPCR-RLFLP for IBV strain identification. The *Eimeria spp* specific assay had already been tested using pure or mixed *Eimeria spp* cultures and were examined *In vivo* under experimental conditions using specimens collected from CIT experiments (funded by Eimeria Pty Ltd) conducted at APCAH, and under field conditions using clinical samples submitted to our laboratory during the course of this study. Similarly, the IBV rtPCR and rtPCR-RFLP had both been examined extensively under experimental condition, using pure viral cultures, and also been examined under field conditions using limited number of samples submitted to APCAH in early years. Further examinations were performed using clinical specimens submitted to APCAH during the course of this study. In addition sampling technique for these two tests were optimised.

Group 4 assays includes PCR or Real-Time PCR assays (for CIAV and ILTV) that have been developed and tested under *in vitro* experimental conditions but require further examination under *in vivo* experimental and field conditions as well as preparation of standard operating procedures.

a) Tissues or swabs collected from birds used in CRC-funded (CIAV) or AECL-funded (ILTV) experiments conducted in our department were used for completion of the *in vivo* experimental conditions.

b) Examinations of the two tests under field conditions were carried out using a large number of specimens collected at APCAH during the last few years and during the course of this study.

Group 5 assays included Chlamydophila PCR. This test required:

a) Modifications (as to use different targets for amplification) to optimise the test for poultry

b) Modification to Real-Time PCR module.

c) Optimisation using numerous feline and avian samples that had already been processed in our laboratory.

d) Optimisation of sampling techniques (conjunctival, choanal or cloacal swabs or infected tissues or faeces) under field conditions using clinical cases during the course of this study.

Group 6 assays included Real-Time PCR or PCR tests for avian Reovirus, Adenovirus, pathogenic *Salmonella spp*, IBDV, *Pasteurella multocida* (and for differentiation of the *P. multocida* vaccine), MDV, REV, EDSV and AEV. These tests had been adopted from other laboratories but been used in APCAH on a limited basis. Although these assays had been examined extensively under experimental and field conditions in the source laboratories, they required further optimisation and standardisation under condition used at APCAH. Clinical samples from chicken experiments conducted at APCAH or diagnostic cases submitted to APCAH were used to optimise these tests.

Results

Detection and differentiation of *Mycoplasma synoviae* vaccine strain and field isolates using PCR and HRM analysis

Mycoplasma synoviae is an economically important pathogen of poultry worldwide, causing respiratory infection and synovitis in chickens and turkeys (Kleven, 1997). Provisional diagnosis and monitoring of *M. synoviae* infection is usually made using serological assays, while definitive diagnosis is made by isolation and identification of the organism and or PCR. Further identification of the *M. synoviae* isolates (i.e. defining the strain involved) is very useful for epidemiological tracing and is of critical importance in countries in which poultry flocks are vaccinated with the live attenuated *M. synoviae* strain MS-H (Vaxsafe MS, Bioproperties Australia). The conventional technique for identification of *M. synoviae* strains is RFLP of genomic DNA however this technique is time consuming and expensive.

As part of an Australian Research Council (ARC) project, two novel techniques, Single Stranded conformational Polymorphism (SSCP) and High Resolution Melt (HRM) melt curve analysis, were developed in combination with PCR and shown to have potential in differentiation of *M. synoviae* strains (Jeffery *et al.*, 2007). While reliable, the SSCP technique appeared to be time consuming. Thus, HRM curve analysis was chosen as a method of choice for identification of *M. synoviae* strains in this CRC project.

Methodology

During the course of the CRC project, several *M. synoviae* isolates from a range of geographical locations, were tested. All cultures were grown in mycoplasma broth (MB) as modified (Morrow *et al.*, 1998) from the original formulation of Frey et al.(Frey *et al.*, 1968). Total genomic DNA was extracted from mycoplasma cultures as described previously (Sykes *et al.*, 1998). PCR amplification, agarose gel electrophoresis, HRM curve analysis and nucleotide sequencing were all carried out as described previously (Jeffery *et al.*, 2007).

Results

Two additional HRM profiles (K and L) were found in submissions from commercial chickens in Australia (results not shown). However most of the cases M. synoviae strains detected during CRC project were found to indistinguishable or very close in HRM curve profile to class A (that also includes the MS-H vaccine strain, its parent strain and several other field strains). In most cases, nucleotide sequencing of the vlhA gene confirmed HRM curve profiles. In cases where neither HRM curve analysis nor nucleotide sequencing was unable to distinguish the unknown strain from class A strains, temperature sensitivity phenotype (a characteristics of MS-H vaccine) and/or RFLP of genomic DNA was performed as confirmatory tests.

Except for the two cases where M. synoviae strains was attributed to new profiles (K and L), all other M. synoviae strains were found to belong to class A and non-temperature sensitive suggesting that the isolates were most likely to be non-vaccinal (field-) isolates.

Detection and differentiation of infectious bronchitis virus (IBV) strains using RT-Real time PCR and HRM analysis

Conventionally, IBV detection and characterisation involves nucleotide sequencing of the S1 gene. However, the S1 gene is one of the most variable regions of the IBV genome and may be subject to recombination events (Jia *et al.*, 1995; Kottier *et al.*, 1995; Lee & Jackwood, 2000). Also, it is often not rapid enough to allow an adequate response to an IBV outbreak - it can take weeks to complete while an IBV infection only lasts 3-10 days (Cavanagh & Naqi, 2003). The inability to achieve rapid identification severely restricts the implementation of appropriate measures to prevent spread and reoccurrence. As apart of a PhD and an Australian Egg Corporation Limited (AECL) projects recently, a real-time PCR / high resolution melt (HRM) curve analysis protocol was developed in our laboratory to differentiate infectious bronchitis virus (IBV) reference strains (Hewson *et al.*, 2009). In another unpublished study funded by AECL, this method was used to detect and classify IBVs in field submissions.

Methodology

Forty suspected IBV cases were received over an 11 month period. Seventeen submissions were found to be positive for IBV by PCR. High resolution melt (HRM) curve analysis classified each

as either a subgroup 1, 2 or 3 strain (12 submissions), or a strain that was unable to be classified (five submissions). To confirm the classifications made using the HRM curve analysis, and further characterise strains present in the submissions that could not be classified, the 3'UTR and partial S1 gene nucleotide sequences for the 17 IBV strains were determined and their identity with those of the relative reference strains compared.

Results

For 12 IBV strains classified as subgroup 1, 2, or 3 using HRM curve analysis, the 3'UTR and S1 gene nucleotide sequences had identities of ≥ 99 % with the respective subgroup reference strain. Analysis of the 3'UTR and S1 gene nucleotide sequences for the five IBV strains that could not be classified indicated that two were related to subgroup 1, two to subgroup 3 and one was a potential recombinant strain (between strains from subgroups 2 and 3).

Detection and differentiation of *Mycoplasma gallisepticum* strain using PCR and HRM analysis

Recent studies in our laboratory have found that a combination of PCR and high resolution melting (HRM) curve analysis provides a rapid and cost-effective alternative to the direct analysis of nucleotide sequence variation, particularly when large numbers of samples are to be analysed (Hewson *et al.*, 2009; Jeffery *et al.*, 2007; Steer *et al.*, 2009). The *vlhA* intergenic region in MG genome contains conserved and variable regions (Papazisi *et al.*, 2003). Preliminary examinations of the *vlhA* intergenic region through sequences previously available in the GenBank database, suggested the potential of this region for detection and differentiation of MG strains. The aim of the present study was to investigate the potential of HRM curve analysis of PCR amplicons generated from *vlhA* intergenic region to establish a single closed-tube test method for differentiation of MG strains. Both conventional and normalised dissociation plots were generated and genotyping applied to differentiate the MG isolates/strains, and the results compared with DNA sequencing of PCR amplicons.

Methodology

MG strains

Twelve MG strains including three vaccine strains (ts-11, 6/85 and F), the reference strain S6 and eight MG strains (from Australia or the USA) available in our laboratory were used initially in this study (Table 1). All cultures were grown from a single colony in modified mycoplasma broth (Morrow *et al.*, 1998). All mycoplasma cultures were grown in mycoplasma broth containing 10% swine serum, 0.01% (w/v) nicotinamide adenine dinucleotide (NAD) and 0.002% (w/v) herring sperm DNA (Sigma, Castle Hill, Australia). The media base consisted of: 7.5 g trypticase peptone, 2.5 g phytone peptone, 0.5 g thiotone peptone (all from BBL, North Ryde, Australia), 5 g yeast extract (Difco, North Ryde, Australia), 5 g sodium chloride, 0.4 g potassium chloride, 0.35 g magnesium sulphate heptahydrate, 0.05 g disodium hydrogen orthophosphate, 0.1 g potassium dihydrogen phosphate, 1 g glucose, 1.5 ml of a 1.6% phenol red solution, and 10 ml of a yeast autohydrolosate solution prepared from bakers' yeast per liter. Mycoplasma agar was prepared as for mycoplasma broth, with the omission of glucose and phenol red, and solidified

with 1.0% (w/v) Special Noble agar (Difco). Also three groups of MG strains from USA, Europe and Israel were used to evaluate the newly developed PCR-HRM technique for its potential to differentiate MG strains from a wider geographical locations (Table 4). These specimens were compared with each other and with MG strains analysed earlier in this study (Table 1).

DNA extraction

Total genomic DNA was extracted from mycoplasma cultures and from swabs taken from birds using a DNA extraction kit (QIAGEN) according to the manufacturer's instructions. Genomic DNA was extracted from a commercial batch of Vaxsafe MG (Bioproperties Australia Pty Ltd) and from the ts-11 master seed which was previously passaged at least five times *in vitro*. A group of fifteen specific pathogen free (SPF) chickens and two separate commercial layer chicken flocks were vaccinated with ts-11 vaccine strain according to the manufacturer's instructions at 28 days of age within a positive pressure isolator or under field condition respectively. DNA was extracted from fifteen tracheal swabs taken from SPF chickens two weeks postvaccination and from eighteen cultures grown from swabs taken from commercial layer chickens 7-12 weeks post vaccination. Briefly, 0.5 ml of mycoplasma culture was pelleted by centrifugation at 20000 ***g*** for 5 min. The cells were washed twice in phosphate buffered saline (PBS) and resuspended in 500 µl RLT lysis buffer (QIAGEN) and incubated for two hours at room temperature or overnight at 4 ºC. Swabs taken from birds were also placed in 0.5 ml lysis buffer (QIAGEN) and incubated at 4 ºC overnight. Then 15 µl of Qiaex II matrix (QIAGEN) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA), centrifuged for 30 s at 10000 ***g*** and the flow-through discarded. The column was washed with 600 µl RW1 buffer (QIAGEN) and twice with 500 µl RPE buffer (QIAGEN) and subjected to centrifugation at 18000 ***g*** for 90 s. The DNA was eluted from the matrix using 50 µl distilled water and used in PCR immediately or stored at -20 ºC for future use.

PCR

The region preceding the trinucleotide repeat of a member of the *vlhA* gene family was chosen for amplification. This was based on the observation of poor conservation of nucleotides within this region between *vlhA* family members (Papazisi *et al.*, 2003). A pair of oligonucleotide primers, ts-11-F (5'-GTTTGGAGTTGGTGTATAGTTAG -3') and ts-11-R (5'-TCTTCTTCGAAAACAAAAGG -3'), flanking the target region was designed with the PCR amplicon expected to yield a product of 226 bp. Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 5 µM SYTO® 9 green fluorescent nucleic acid stain (Invitrogen), 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 60 s, 35 cycles of 94ºC for 10 s, 50ºC for 10 s and 72 ºC for 10 s, and a final cycle of 72ºC for one min. In each set of reactions, MG ts-11 genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.7% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

DNA from *Mycoplasma synoviae* (strain WVU-1853), a field isolate of *Mycoplasma meleagridis*, *Mycoplasma anatis*, *Mycoplasma gallinaceum* and *Mycoplasma gallinarum* were tested in PCR (as described above) did not produce any detectable band on agarose gel.

High-resolution melting curve acquisition and analysis

HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). In order to determine the optimal melting condition for differentiation of MG strains, the PCR products were subjected to three different rampings of 0.1°C, 0.2°C and 0.3°C/s between 72°C and 82°C. All specimens were tested in triplicate and their melting profiles analysed using Roter Gene 1.7.27 software and the HRM algorithm provided. Normalisation regions of 73.5-74.0 and 79.5-80.0 were used for analysis. Each isolate was set as a 'genotype' and the average HRM genotype confidence percentage (GCP) (the value attributed to each isolate/strain being compared to the genotype with a value of 100% indicating an exact match) for the replicates were predicted by the software. The GCPs for MG ts-11 known isolates were averaged and the standard deviation (SD) calculated and used to establish the GCP range for ts-11 vaccine strain cut off point. The cut off point was applied in HRM analysis to evaluate the differentiation power of the test to discriminate ts-11 strain.

Sequencing and nucleotide sequence analysis of PCR amplicons

PCR amplicons were gel purified using the QIAquick® Gel Purification Kit (QIAGEN) following the manufacturer's instructions. Purified amplicons were subjected to automated sequencing (BigDye® Terminator v3.1, Applied Biosystems) in both directions, using the same primers as used for PCR. The sequences were analysed using ClustalW (Thompson *et al.*, 1994) and DNAdist (Felsenstein, 1989) in BioManager (Australian National Genomic Information Service, Sydney Bioinformatics) and BioEdit Sequence Alignment Editor (version 6.0.9.0). GenBank accession numbers were assigned to the nucleotide sequences of the MG isolates and reference strains (Table 1).

Results

PCR amplicons of different sizes generated from MG strains using oligonucleotide primers ts-11-F and ts-11-R

Amplified PCR products from different MG strains were analysed by gel electrophoresis (Figure 1). MG strains ts-11, S6, F and K1453 generated one major band while 6/85, Ap3AS, K1659, 86134, 87006 and 87081 produced a major band with one or two minor bands visible on the agarose gel. The major amplicon band ranged in size from 145 (87006) to 352 bp (S6).

Conventional and normalised HRM curve analysis differentiates MG strains

The PCR amplicons from 10 different MG strains (Table 1) were subjected to HRM curve analysis (Figure 2). Visual examination of the conventional melt curves at different ramp temperatures revealed that 0.3°C/s resulted in most strains showing distinct conventional melt curves for MG strains. In the conventional melt curve, ten distinct curve profiles were detected (Figure 2a). MG vaccine strains ts-11 and 6/85 each generated a single peak at 76.5 and 74.8ºC respectively (Table 2). The MG strain 86134 and Ap3As generated only two major peaks at 75.3 and 77.3ºC, and 75.7 and 77.1 ºC respectively. The MG strains S6 and K1453 generated a major peak at 78.2 and 77.7ºC respectively and a shoulder peak at a lower temperature than their major peak. MG strains 87006 and K1659 generated a major peak at 74.9 and 75.2ºC respectively and a minor peak at a higher temperature than their major peak. The MG strain 87081 was the only strain that generated one major peak at 74.8ºC and two minor peaks at higher temperatures than the major peak. All MG strains also generated ten distinct normalised HRM curves (Figure 2b). Replicates of each MG strain were genotyped with defined genotypes in normalised HRM graph with equal or greater than 95 GCP (Table 2). HRM curve analysis for PCR amplicons using templates from DNA extractions

and/or PCRs run on different days showed slight shifts in melting temperature; however, the conventional melt curve shapes and normalised HRM graphs were unchanged. The mean and standard deviation of the melting points for the different peaks, and GCP of standard errors resulting from several runs of PCR and HRM curve analysis are shown in Table 2.

Consistent HRM curve profile of MG after passage in vitro and in vivo

In order to evaluate the consistency of the newly-developed PCR HRM curve analysis technique following MG strain passage *in vitro* and *in vivo*, the MG ts-11 vaccine strain was used as a model. Highly similar conventional and normalised HRM curve profiles were generated from Vaxsafe MG (Bioproperties Australia Pty Ltd) and the master seed (results not shown). To assess *in vivo* sensitivity and specificity of the developed PCR test, DNA from swabs taken from SPF and commercial layer chickens were subjected to PCR and HRM curve analysis. Both conventional and normalised curves were highly similar to those of ts-11 (Figure 3) with a mean of 94.5 ± 2.0 and 87.0 ± 14.3 GCP for the fifteen SPF and eighteen commercial layer chicken specimens, respectively.

Detection of minor variations in vlhA nucleotide sequence by the newly-developed PCR HRM curve analysis technique

To confirm if classification of MG strains by HRM curve analysis is correlated with variation in nucleotide sequence of the amplicons, nucleotide sequences of amplicons from each distinct curve profile as well as from one MG isolate, from a SPF chicken and one from a commercial layer chicken specimen were determined and compared. Some of MG strains generated more than one DNA band on agarose gel (Figure 1). In order to compare the nucleotide sequences of the amplicons from different MG strains, the major DNA band of each strain was gel purified, sequenced and used for calculation of sequence identities. Only major DNA bands were examined since contribution of the other (minor) bands towards the overall sequence identities was difficult to determine especially because the intensity of staining varied between the bands most likely due to different number of amplicons per band. The PCR amplicon for the S6 reference strain was composed of 352 bp, in comparison the ts-11 amplicon was 226 bp in length with a 126 bp deletion at the 5' end (Figure 4). Other strains with a distinct HRM curve profile contained deletions from 33 to 207 nucleotides in their sequences with a number of nucleotide substitutions through out each of these sequences (Figure 4). Comparison of the nucleotide sequences further revealed that PCR amplicons generated from isolates from the SPF and commercial layer chickens vaccinated with ts-11 strain were identical to the MG ts-11 vaccine strain sequence (data not shown). The extent of sequence variability in PCR amplicons for the representative of each distinct group was determined and compared with the mean GCP of HRM analysis (Table 3). The highest sequence identity (100%) was observed between amplicon sequences from 6/85, Ap3AS and 86134 strains while the lowest (36.1%) belonged to those of K1659 and 87081 strains. The GCPs for the HRM curves related to 6/85/Ap3AS, 6/85/86134 and 86134/Ap3AS were 0.0, 0.0 and 13.2 respectively. The GCP for the HRM curves of K1659 and 87081 was 0.4. MG strains F and 87081 that also had high sequence identity (99.1%) however, generated two different conventional melt curves with three peaks and one peak respectively and this difference was reflected in their normalised melt curves and GCP (0.1). Since MG F strain produced one amplicon band while MG 87081 generated two, the difference in the shape of the normalised and conventional melt curves or the number of peaks in conventional melt curves was likely to be due to the contribution of additional DNA amplicons. The highest GCP (66.1) was found between S6 and F strain while the lowest (0.0) was between ts-11 and a number of specimens including F, S6 and 6/85 strains. The sequence identity for the S6 and F strains was 98.7% while those for the ts-11 and other strains such as F, S6 and 6/85 were 79.2, 81.0 and 56.4% respectively. A poor correlation (0.2) was observed when GCP and sequence identity of all MG strains were compared. The correlation between sequence identity and GCP of MG strains (ts-11, S6, F and K1453) (that generated a single amplicon) was found to be 0.78. Thus the poor correlation between

sequence identities and GCPs when all strains were used in comparison was due to the contribution of extra amplicons generated by MG strains 6/85, Ap3AS, K1659, 86134, 87006 and 87081.

A mathematical calculation for non-subjective grouping of MG strains using GCPs

Using GCPs for ts-11 vaccine strain, a cut-off value was generated as a mathematical model to assess the relationship of the field isolates without visual interpretation by the operator (non-objective). The average of the 125 genotype confidence values for ts-11 strain was 93.7 with a SD of 10.7. A value of 3 SD (32.1) was then calculated and subtracted from the average GCP to determine a cut off point. Thus the GCP range for ts-11 vaccine strain and related field samples was determined to be 61.6 - 100. The cut off point of 61.6 was then applied for genotyping MG strains and isolates. All amplicons generated from ts-11 vaccinated SPF and commercial layer chickens had GPCs between 63.1-100 and were therefore genotyped automatically as ts-11. All the other MG strains had GCPs between 0.0-19.5 and were therefore automatically identified as "variation" (Figure 5). Thus the gap between the highest non-ts-11 and lowest ts-11 specimens was approximately 43 GCP.

Assessment of the potential of the PCR-HRM technique for differentiation of MG isolates from diverse geographical locations

The PCR amplicons from each three groups of MG isolates from USA, Europe and Israel (Table 4) were subjected to HRM curve analysis and isolates with conventional/normalised curves similar to each other or to those characterised earlier in this study (Table 1) were selected for further examinations by nucleotide sequencing. The shape of the curves and number, relative height and temperature of the peaks and subsequently the GCPs were considered in this initial screening.

Visual examination of the conventional and normalised melt curves of the MG isolates from USA (Figure 6a) revealed that MG isolate K6216D had a single peak at 76.8 °C highly similar to the ts-11 and was genotyped as ts-11 with 95 GCP. The nucleotide sequence of the amplicon (226 bp) was also identical to that of ts-11. MG isolates K5917 and K6096 had two similar conventional melt curve peaks at 76.5 and 79.7 °C and similar normalisation curves at GCP of 99. The sequence of the PCR amplicons (323 bp) was also identical. The MG isolates K5037A and K5792D also generated similar normalised and conventional melt curves with one peak at 78.3°C and a shoulder peak at a lower temperature. These two isolates produced a major DNA band (319 bp) with identical nucleotide sequences. The MG isolate K4181C and K4094 generated slightly different pattern to those of K5917 and K6096 with two peaks at 76.6 and 79.5°C, and 76.6 and 79.8 °C respectively. The nucleotide sequence analysis of these two isolates revealed 99.7% identity to each other and 93.7-100% identity to K5917/K6096. The rest of the isolates from USA (K4158C, K4355, K4705, K503 and K5104) had curves that were distinct to the other USA and Australian MG strains/isolates used in this study. Visual examination of conventional and normalised melt curves of MG isolates from Europe revealed that MG isolate B40/95 and F strain generated amplicons with similar melt curve with one peak at 78.4°C and a shoulder peak at a lower temperature. Nucleotide sequences of amplicons revealed 99.7% sequence identity (one nucleotide substitution in 319 bp). This similarity was also reflected in similar normalised melt curves with GCP of 88. All other MG isolates from Europe did not have similarity to each other (Figure 6b) or to any other reference strains/isolates characterised earlier in this study. Examination of the Israeli MG isolates revealed that the isolate MSA-9 and MKT-6 generated highly similar conventional melt curves with two peaks at 74.6 and 77.8 °C (Figure 6e). The normalized melt curves for the amplicons of these isolates also were highly similar and had GCP of 92 (Figure 6f). The nucleotide sequence of the respective amplicons (355 bp) were found to be identical. Similarly the MG isolates BCV-6 and VR-5 generated amplicons with similar conventional melt curves with one peak at 78.0°C and a shoulder peak at a lower temperature. Nucleotide sequences (319 bp) of amplicons were identical to each other and to the MG strain K1453 (examined earlier in this study). This similarity was also reflected in similar normalised melt curves with GCP of 90.

Table 1. *Mycoplasma gallisepticum* strains/isolates used in this study and their origin

Isolate/strain	Origin	Reference	GenBank Accession No.
ts-11	Australia	(Whithear *et al.*, 1990)	FJ654144
F	USA	(Carpenter *et al.*, 1979)	FJ654142
S6	USA	(Adler *et al.*, 1957)	FJ654143
6/85	USA	(Evans & Hafez, 1992)	FJ654146
Ap3AS	Australia	(Soeripto *et al.*, 1989)	FJ844437
K1659	USA	(Kleven *et al.*, 1988)	FJ654145
K1453	USA	(Kleven *et al.*, 1988)	FJ654137
86134	Australia	This study	FJ654139
87006	Australia	This study	FJ654140
87081	Australia	This study	FJ654141
94001	Australia †	This study	N/A*
93148	Australia †	This study	N/A

* Not applicable: nucleotide sequence was identical to ts-11

† Flock vaccinated with ts-11

Table 2. Mean ± SD of the melting points and genotype confidence percentage (GCP) for each strain following PCR and HRM curve analysis

Genotype/Number of times tested	No. of isolates/batches tested	Peak 1 (ºC)	Peak 2 (ºC)	Peak 3 (ºC)	GCP ± SD
ts-11 (125)	3	76.5 ± 0.3			96.1 ± 3.6
K1659 (30)	1	75.2 ± 0.1	76.9 ± 0.1		97.9 ± 2.5
K1453 (28)	1	77.7 ± 0.1			98.1 ± 1.5
87006 (21)	1	74.9 ± 0.1	79.1 ± 0.1		98.9 ± 0.3
87081 (18)	1	74.8 ± 0.9	76.4 ± 0.7	77.6 ± 0.4	95.0 ± 2.1
F (58)	1	78.2 ± 0.1			98.4 ± 1.6
S6 (28)	1	78.2 ± 0.1			99.2 ± 0.7
6/85 (38)	1	74.8 ± 0.1			96.9 ± 2.2
86134 (42)	1	75.3 ± 0.8	77.3 ± 0.3		98.4 ± 1.4
Ap3AS (21)	1	75.7 ± 0.1	77.1 ± 0.4		98.2 ± 1.2

Table 3. Percentage of sequence identity of MG isolates/strains compared with confidence percentage of HRM analysis

Sequence Identity (%)

Strain	S6	F	K1453	87081	ts-11	6/85	K1659	86134	87006	Ap3AS
S6	-	98.7	95.0	98.4	81.0	56.4	44.9	56.4	47.6	56.4
F	66.1	-	93.7	99.1	79.2	55.7	47.6	55.7	46.9	55.7
K1453	36.2	22.1	-	92.8	88.9	56.4	44.9	56.4	47.6	56.4
87081	1.5	0.1	0.8	-	79.2	56.4	36.1	56.4	42.8	56.4
ts-11	0.0	0.0	0.0	0.4	-	56.4	44.9	56.4	47.6	56.4
6/85	0.0	0.0	0.0	0.0	0.00	-	66.7	100.0	68.3	100.0
K1659	0.0	0.0	0.0	0.4	0.08	1.9	-	68.7	77.9	68.7
86134	4.8	0.4	5.6	64.2	0.57	0.0	0.0	-	68.3	100.0
87006	0.0	0.0	0.0	0.3	0.00	3.9	28.3	0.0	-	66.9
Ap3AS	0.1	0.0	0.0	39.2	0.69	0.0	2.3	13.2	3.2	-

Genotype confidence percentage

Table 4. *Mycoplasma gallisepticum* isolates obtained from USA, Europe and Israeil

Isolate	Host	Origin	GenBank Accession No./Reference
K415830	Turkey	USA	NRA*
K4181C	Chicken	USA	(Ferguson *et al.*, 2005)
K4355	Chicken	USA	(Ferguson *et al.*, 2005)
K4705	Chicken	USA	(Ferguson *et al.*, 2005)
K5037A	Turkey	USA	(Raviv *et al.*, 2007)/GU133050
K503	Chicken	USA	(Ferguson *et al.*, 2005)
K6216D	Chicken	USA	NRA/GU133047
K5792D	Chicken	USA	(Raviv *et al.*, 2007)/GU133051
K5917	Chicken	USA	NRA/GU133048
K6096	Chicken	USA	NRA/GU133049
K5104	Turkey	USA	(Ferguson *et al.*, 2005)
K4049	Housefinch	USA	NRA
B18/86	Turkey	England	NRA
B5/94	Pheasant	England	NRA
B40/95	Turkey	England	NRA/GU166688
B41/02	Chicken	England	NRA
B134/03	Pheasant	England	NRA
B7/04/50	Not known	Germany	NRA
B19/04/5	Turkey	Germany	NRA
B90/05	Turkey	England	NRA
B114/06	Partridge	Scotland	NRA
B40/07/6	Chicken	England	NRA
MYZ-8	Chicken	Israel	(Lysnyansky *et al.*, 2008)
MSA-9	Chicken	Israel	(Lysnyansky *et al.*, 2008)/ GU133053
RV-6	Chicken	Israel	NRA
VR-5	Chicken	Israel	NRA/GU133052
BCV-6	Chicken	Israel	NRA
DSD-14	Turkey	Israel	NRA
BNC-10	Turkey	Israel	NRA
MKT-6	Turkey	Israel	NRA/GU133054

* No reference available

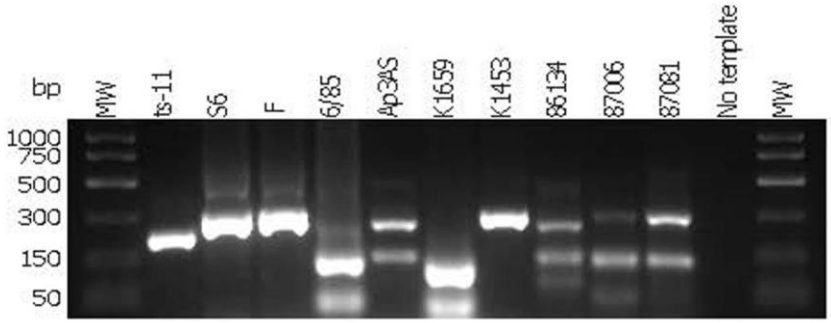

Figure 1. Agarose gel electrophoresis of PCR products of the *vlhA* gene promoter region from different MG isolates/strains. MW, molecular weight marker (PCR Marker, Sigma).

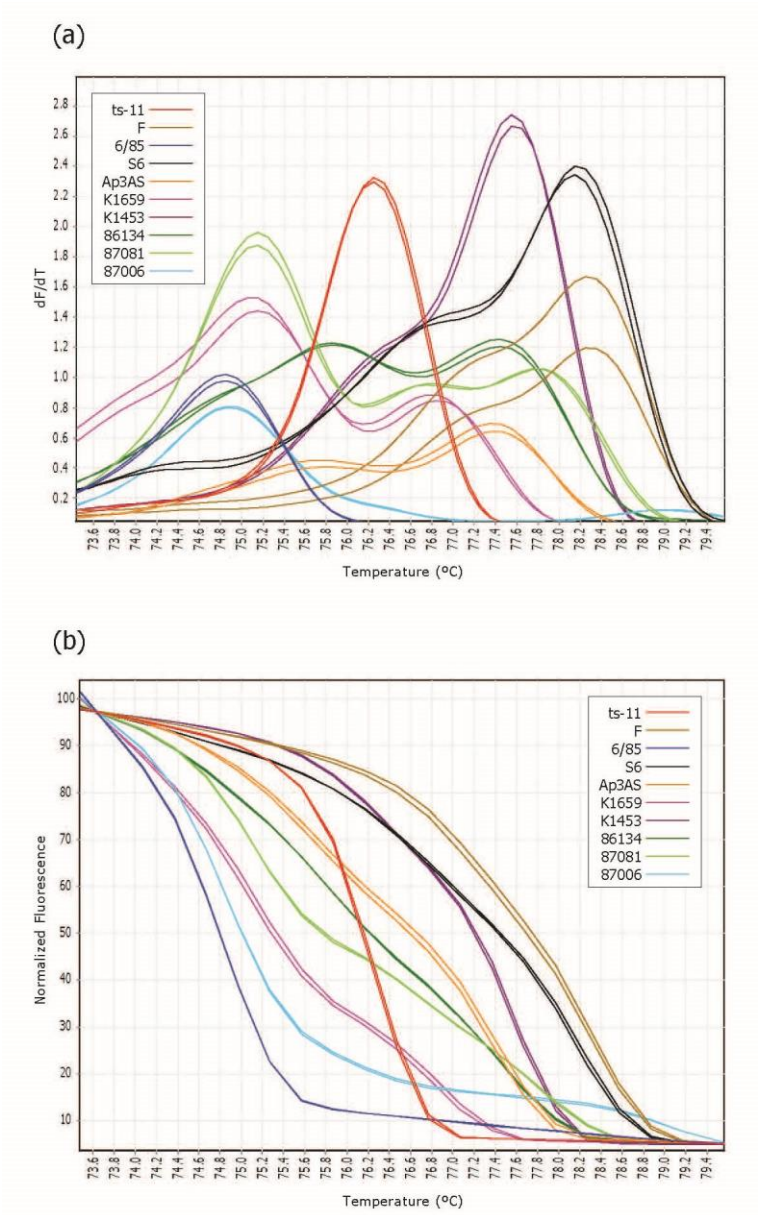

Figure 2. (a) Conventional melting-curve analysis of PCR products induplicate of the *vlhA* gene promoter region from different MG isolates/strains.

(b) Normalised HRM curve analysis of PCR amplicons of the *vlhA* gene promoter region from different MG isolates/strains

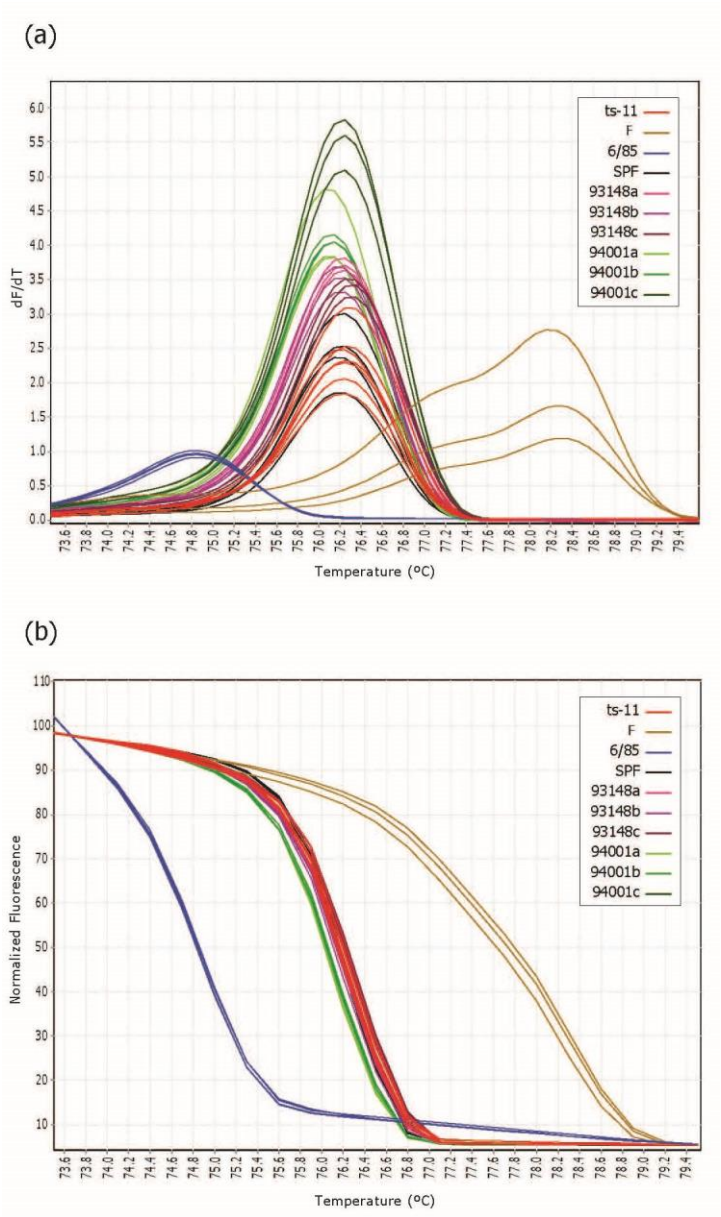

Figure 3. (a) Conventional melting-curve analysis of PCR products of the *vlhA* gene promoter region from ts-11 and MG isolates from ts-11 vaccinated birds.

(b) Normalised HRM curve analysis of PCR amplicons of the *vlhA* gene promoter region from ts-11 and MG isolates from ts-11 vaccinated birds. The isolates 93148a, b, c and 94001a, b, c were field specimens collected 7-12 weeks postvaccination with ts-11.

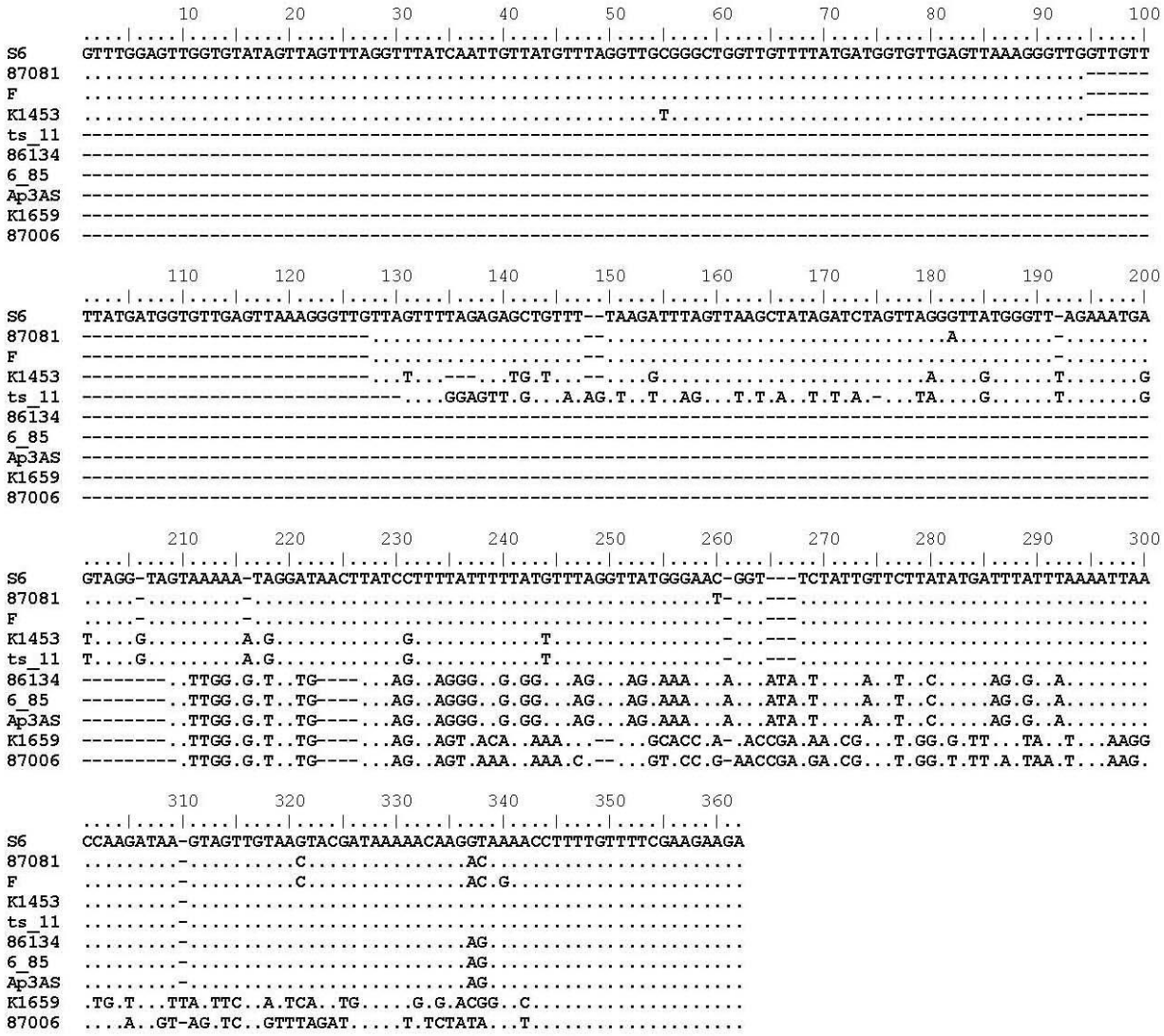

Figure 4. Comparison of the nucleotide sequences of MG isolates/strains *vlhA* gene promoter region amplicons using CLUSTALW. Identical nucleotides and deletions are shown by '.' and '-', respectively.

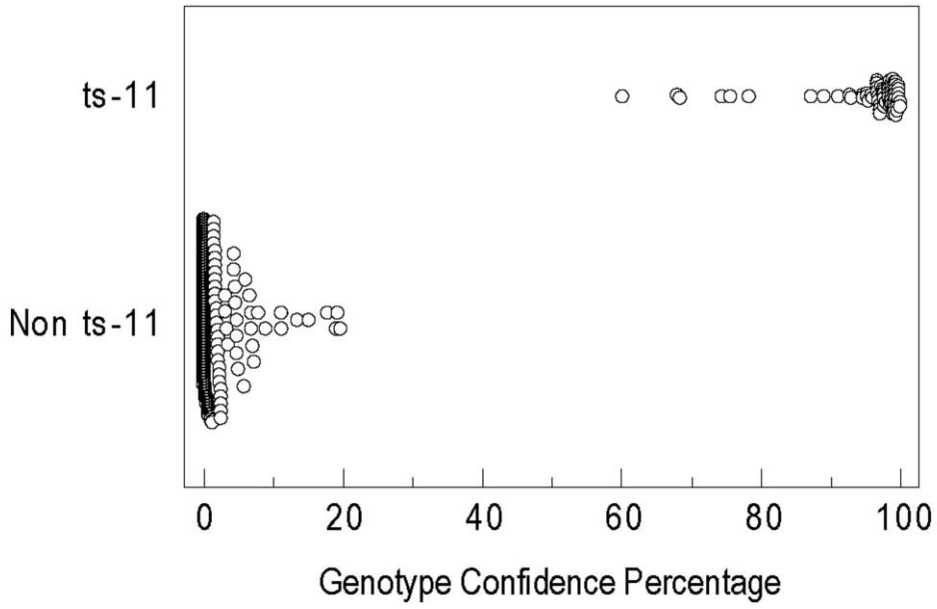

Figure 5. Comparison of the distribution of genotype confidence percentages (GCP) from ts-11 and non-ts-11 strains/isolates using dot plot.

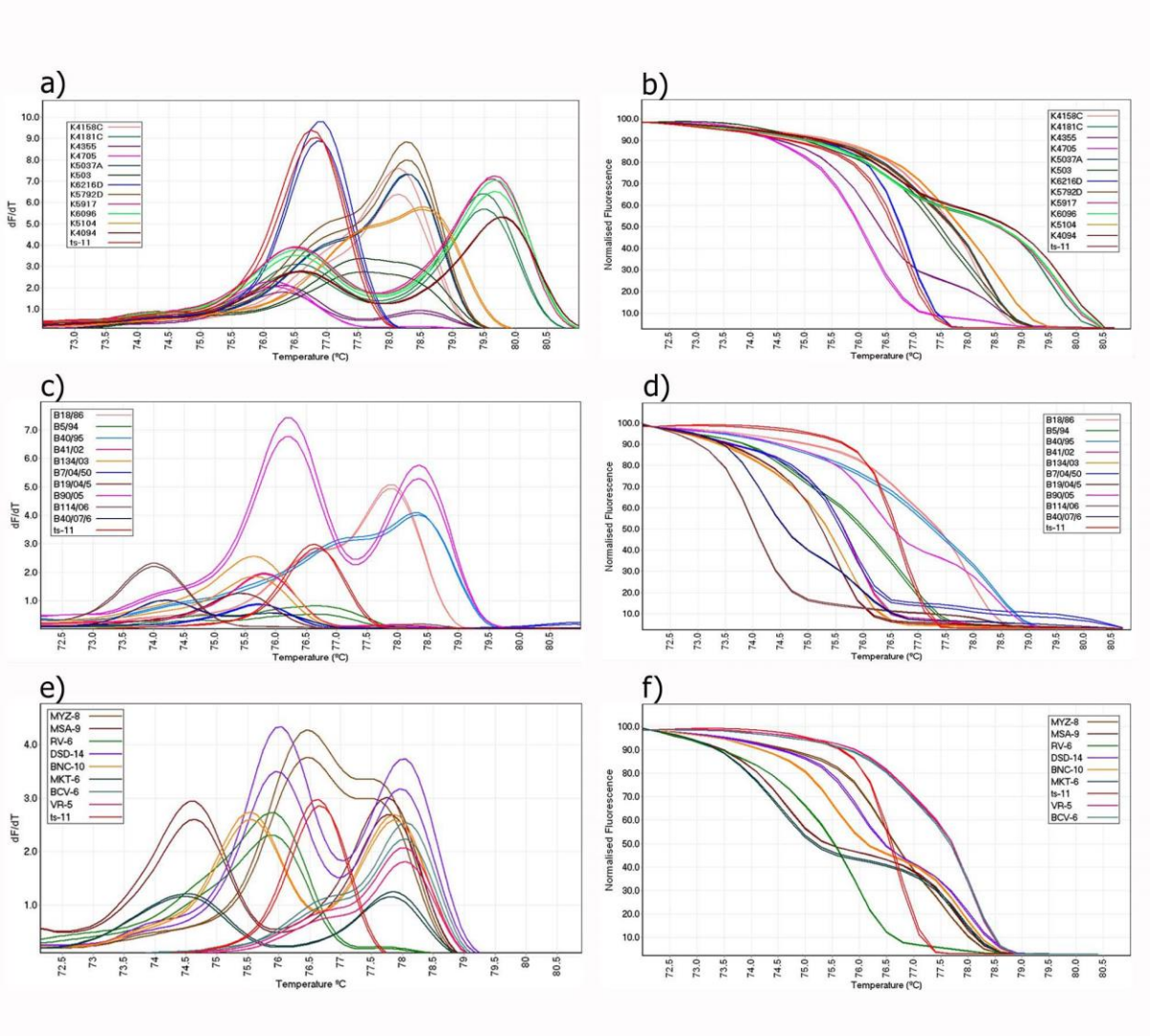

Figure 6. (a, c and e) Conventional melting-curve and (b, d and f) normalised HRM curve analysis of PCR products of the *vlhA* gene promoter region from ts-11 and MG isolates from USA (a and b), Europe (c and d) and Israel (e and f).

Detection and Differentiation of Infectious Laryngotracheitis Viruses (ILTV)

Recently under a research project funded by AECL, a combination of RFLP patterns for a number of ILTV genes were used for classification of ILTVs in Australia (Kirkpatrick *et al.*, 2006). Application of this methodology on historical ILTVs isolated from different locations in Australia resulted in identification of 5 different ILYV classes, with the SA2 and A20 vaccine strains classified as class 1 (Kirkpatrick *et al.*, 2006). The main purpose of the current study was to use this methodology to identify the ILTV classes responsible for the recent outbreaks of ILTV in Australia, to establish if there is any links between the oubreaks occouring in different states and to investigate if the vaccine strains may be linked to these outbreaks.

Methodology

Viruses

The commercial ILTV vaccine strains SA2, A20 and Serva, along with ILTV field isolates collected over a-three-year period were used in this study (Table 6). All field isolates were isolated from the upper respiratory tract and/or conjunctiva of infected birds during outbreaks of ILT in Australia. All but two submissions for this study were made from commercial layer and broiler farms experiencing mortalities associated with ILT. Two submissions were from flocks of game birds (Table 6). Typing which revealed solely a vaccine strain associated with morbidity from vaccine reactions were not included in the study. A total number of 92 ILTV isolates were examined in this study, of which 52 were from NSW, 36 were from VIC and 4 were from Queensland (QLD).

Extraction of viral DNA

DNA was extracted from infected CEK cell supernatant, commercial vaccines, or directly from swabs taken from infected trachea using a method described previously (Kirkpatrick *et al.*, 2006; Sykes *et al.*, 1997). Where insufficient amplification was achieved in PCR, virus isolates were propagated on chicken embryo kidney (CEK) cells using standard techniques (Tripathy & Purchase, 1980). Scrapings of affected trachea and/or conjunctiva were diluted in cell culture medium and inoculated onto CEK cells. Once infected, CEK cells were frozen at -80ºC, thawed, centrifuged at 1500 RPM for 5 min and then the supernatant was removed and used for DNA extraction. The extracted DNA samples were used immediately or stored at -20ºC before use in the PCR.

Polymerase chain reactions

Primers for the genes thymidine kinase (TK), infected cell protein 4 (ICP4) and infected cell protein 18.5 (ICP18.5) were used in PCR as described previously by Kirkpatrick (Kirkpatrick *et al.*, 2006) with some modifications. The ICP4 and ICP18.5 PCRs were carried out by subjecting the reactions to 94ºC for one minute followed by 35 cycles of 94ºC for 15 sec, 64ºC for 45 sec and 68ºC for 5.5 minutes, and a final extension of 68ºC for 10 min. All PCRs were performed using Platinum *Taq* DNA polymerase high fidelity (Invitrogen, Carlsbad, CA). A control tube containing distilled H_2O, instead of extracted DNA, was included as negative control in all series of PCR. The PCR products were separated by electrophoresis in 1% agarose gels, stained with GelRed (Biotium, Hayward, CA), and exposed to ultraviolet light for

visualization using the Kodak Gel Logic 1500 Imaging System with Kodak Molecular Imaging Software (Version 4.0.5, 2005) system.

RFLP and staining

Ten µl volumes of PCR products were digested separately with the restriction endonucleases at 37ºC for 2 hr as described previously (Kirkpatrick *et al.*, 2006). The restriction endonuclease enzyme *Msp*I was used for the TK gene products and *Hae*III for the ICP4 and ICP18.5 PCR products. After digestion, the resultant DNA fragments were separated on a 15% polyacrylamide gel. Restriction DNA fragments on polyacrylamide gels were visualized by staining with GelRed (Biotium, Hayward, CA), and subjected to digital imaging using the Kodak Gel Logic 1500 Imaging System with Kodak Molecular Imaging Software (Version 4.0.5, 2005) system.

Cluster Analysis

ILTV RFLP pattern combinations were subjected to a cluster analysis using the Free-Tree software available at http://www..natur.cuni.cz/~flegr/programs/freetree (Pavlicek *et al.*, 1999). The data input was established by the presence or absence of a particular restriction site and designated either a 1 or a 0 at each position. Similarity coefficients were calculated using the method described by Nei and Li (Nei & Li, 1979) and DICE. An unrooted dendogram was constructed using the unweighted pair group method and testing for robustness was obtained by bootstrapping using a count of 500 repetitions.

Results

This study identified the appearance of a new digestion pattern (Figure 7) in the ICP-4 gene RFLP analysis not found in our previous work (Kirkpatrick *et al.*, 2006). Pattern D, as depicted in figure 7, displays a differing fragment just over 150 bp in length and was first recognised when the Nobilis ILT vaccine was typed using our technique. This has imposed the designation of further viral genotypes which include this unique fragment pattern. Classification of the ILTV isolates using RFLP patterns generated from genes TK, ICP4 and ICP18.5 revealed that ILTV isolates fell into nine different classes, each designated chronologically (Tables 5 and 6). Four new classes of ILTV; 6, 7, 8 and 9, (Table 5) were found in addition to those reported in our previous study (Kirkpatrick *et al.*, 2006). The classification of the commercial ILT vaccine strains using this process has designated both the SA2 and A20 strains as class 1 ILT viruses (Kirkpatrick *et al.*, 2006) and the Serva strain as a class 7 virus (refer to Table 5) based on RFLP pattern combinations. During the sampling period of the current study, New South Wales isolates (Figure 9) exhibited class 2, 3, 7, 8 and 9 RFLP patterns, while Victorian isolates (Figure 10) displayed class 2, 6, 7 and 8 RFLP patterns and Queensland isolates (Figure 11) included only class 2 or 3 patterns. The most common Victorian isolate typed by our laboratory was class 2 (N= 31) whereas the most common single NSW isolate was class 8 (N=37). Isolates displaying the class 2 ILTV pattern combination were found in NSW, Victoria and Queensland. Historical data from our laboratory indicates that this strain has been in circulation in Victoria since 1999, Queensland since 2001 and NSW since 2004. The Free-Tree based cluster analysis (Figure 8) revealed a close relationship of classes 4 and 6 with the class 2 virus in a separate cluster not containing any vaccine strain classes. Class 3 isolates were found on two separate occasions, in game bird flocks from both NSW and Queensland. Records from our laboratory indicate that this genotype was in circulation in Victoria and South Australia in 2004. A number of isolates from a discrete region in central Victoria were found to belong to class 6, which was distinct from all previously-described classes. This outbreak case was confined to a single broiler company and this particular class has not been identified in our laboratory since. A case in 2007 (Table 6) revealed a virus which was

indistinguishable from the Nobilis ILT vaccine strain (class 7) and was associated with mortalities at a layer farm in the Sydney basin area. Investigation into the history of this isolate indicated an increased mortality and upper respiratory symptoms in unvaccinated birds that had been placed in the same shed as birds vaccinated two weeks earlier with the Nobilis ILT vaccine. The appearance of isolates showing a class 8 RFLP pattern combination began in NSW in mid 2008 and has persisted to date. Many submissions of class 8 isolates were supplied with a case history including vaccination (with Intervet or Fort Dodge vaccines) some time earlier, suggesting that this particular strain of ILTV is less susceptible to the host's immune response when challenged with the virus. Class 8 ILTV has only been found in Victoria on one occasion, in conjunction with a class 7 isolate. This case involved a layer farm that had been vaccinated with the Nobilis vaccine three weeks prior to the outbreak involving significant mortalities. Isolates displaying class 9 RFLP patterns have been identified on 10 occasions, all of which were submitted from cases in the Sydney basin area. A number of these cases were in concurrence with a class 7 or 8 virus in the same sample (Table 6). The Free-Tree based dendogram (Figure 8) revealed three distinct clusters. Classes 1 and 5 (which are indistinguishable by this technique alone (Table 5) share a close relationship with class 3 ILTV. Classes 8 and 9 convene with class 7, as do classes 4 and 6 with class 2.

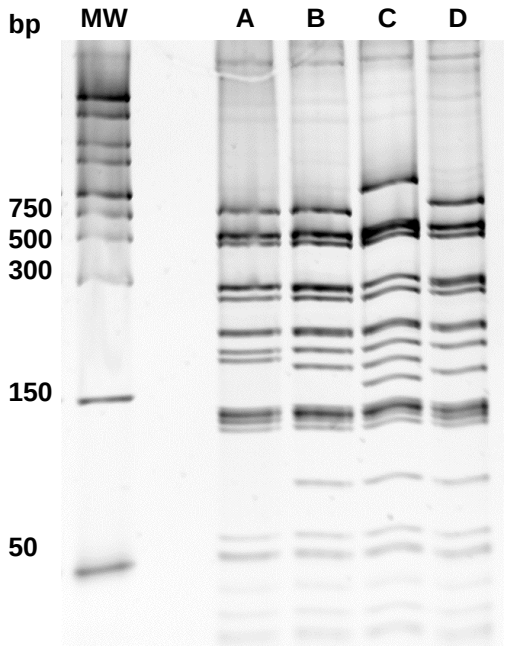

Figure 7. Polyacrylamide gel electrophoresis of DNA fragments formed by restriction digestion of ICP4 PCR products using enzyme *Hae*III. Genomic DNA from virus isolates representing patterns A, B, C and D were used as template in the PCR. RFLP fragments were separated on a 15% polyacrylamide gel and visualized by staining with GelRed (Biotium, Hayward, CA), and subjected to digital imaging using the Kodak Gel Logic 1500 Imaging System.

Table 5. Classification of ILTV strains based on RFLP digestion pattern combinations

PCR products				
TK	ICP4	ICP18.5		
Restriction Enzymes				
*Msp*I	*Hae*III	*Hae*III		
RFLP PATTERNS			**CLASS**	**Vaccine strain equivalent**
A	A	A	1	A20 / SA2 (Fort Dodge)
B	B	B	2	-
B	A	C	3	-
B	C	C	4	-
A	A	A	5*	-
B	B	C	6	-
B	D	C	7	Serva (Intervet Nobilis)
A	D	C	8	-
A	D	A	9	-

* PCR-RFLP of PCR product ORFB-TK using *Fok*I, produced pattern B, which represents class 5. This is different for that produced by SA2 being pattern A, representing class 1

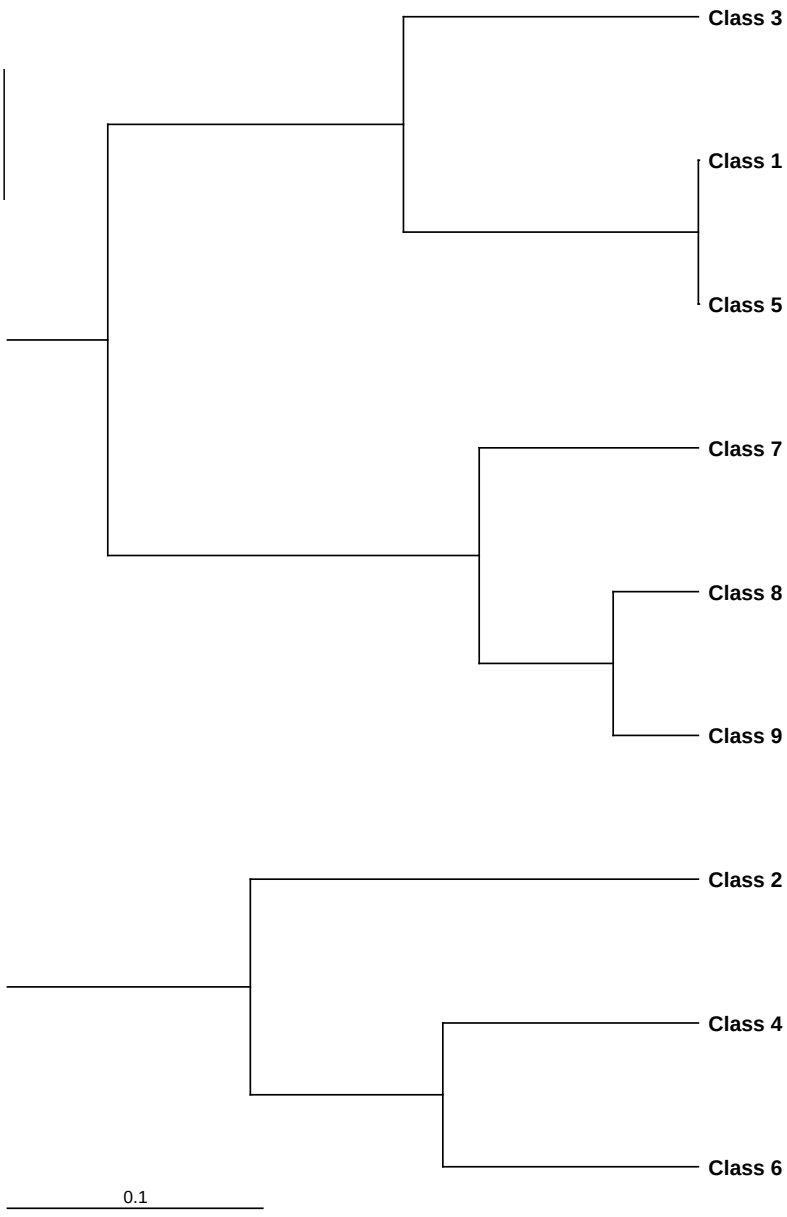

Figure 8. Dendogram constructed using cluster analysis of restriction endonuclease digestion pattern data for the nine classes of ILTV. The branch lengths represent the genetic distances between the classes. Similarity coefficients were calculated based on Nei-Li and DICE and a bootstrap resampling method was used for 500 repetitions.

Table 6. Field isolates typed in the current study over a three-year period.

Year	ILTV Class	Region	Flock type	Number of Isolates
2007	2	Mornington Peninsula / Gippsland, Vic	Broilers	8
2007	2	North East Melbourne, Vic	Layers	1
2007	6	North Central Vic	Broilers	4
2007	7	Sydney basin, NSW	Layers	1
2008	2	Mornington Peninsula / Gippsland, Vic	Broilers / Layers	14
2008	2	North East Melbourne, Vic	Broilers	2
2008	2	Geelong outskirts, Vic	Broilers	2
2008	2	Central NSW	Unknown	2
2008	8	Sydney basin, NSW	Broilers / Layers	29
2008	9	Sydney basin, NSW	Broilers	2
2008	7+8	Geelong outskirts, Vic	Layers	1
2008	7+8	Sydney basin, NSW	Layers	1
2008	8+9	Sydney basin, NSW	Layers	1
2008	7+8+9	Sydney basin, NSW	Unknown	1
2009	2	Mornington Peninsula / Gippsland, Vic	Broilers	4
2009	2	Gold Coast Hinterland, Qld	Broilers	3
2009	3	Wide Bay-Burnett region, Qld	Game birds	1
2009	3	Central NSW	Game birds	1
2009	8	Sydney basin, NSW	Broilers	8
2009	9	Sydney basin, NSW	Broilers	4
2009	8+9	Sydney basin, NSW	Broilers	1
2009	7+8+9	Sydney basin, NSW	Unknown	1
		Total		92

*Vic, Victoria; NSW, New South Wales; Qld, Queensland.

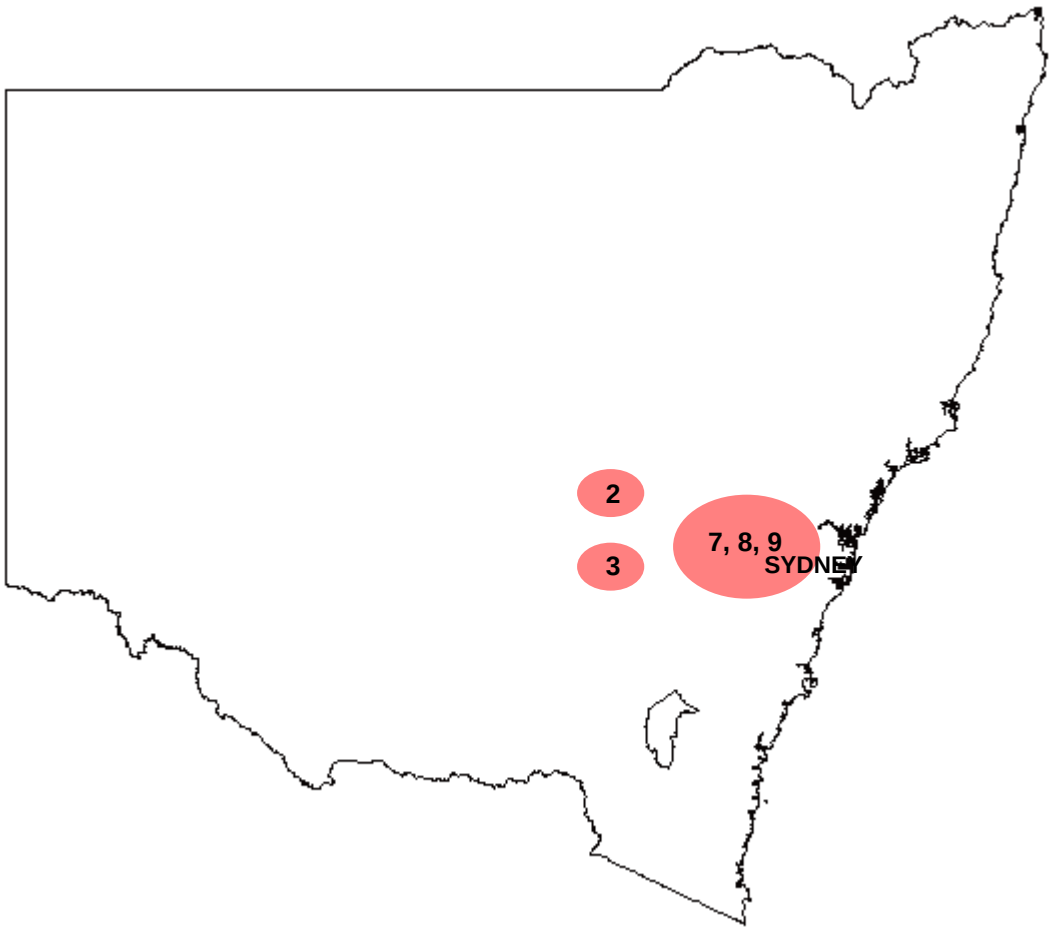

Figure 9. Map of New South Wales depicting outbreak areas in the Sydney basin area and central NSW.

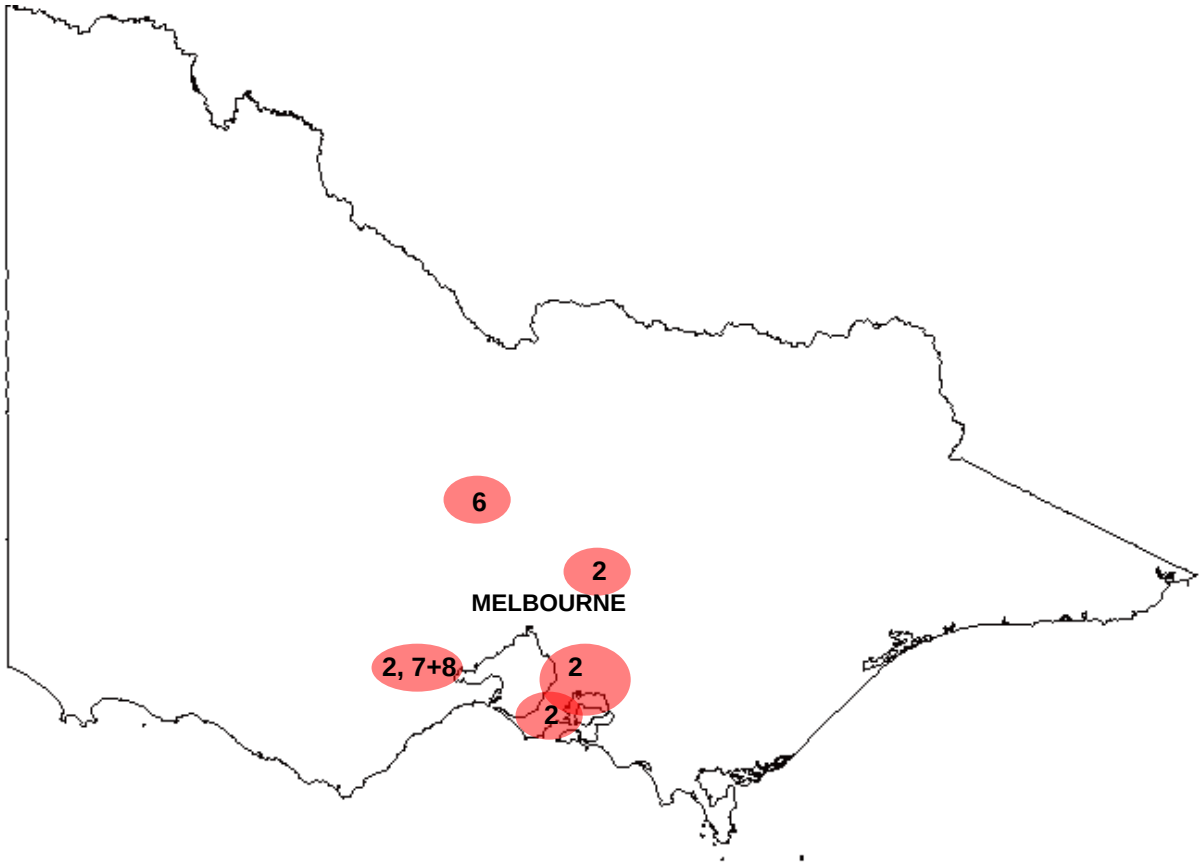

Figure 10. Map of Victoria showing location of outbreaks on the Mornington Peninsula, in the Gippsland region, Geelong, Central Victoria and North-Eastern Melbourne.

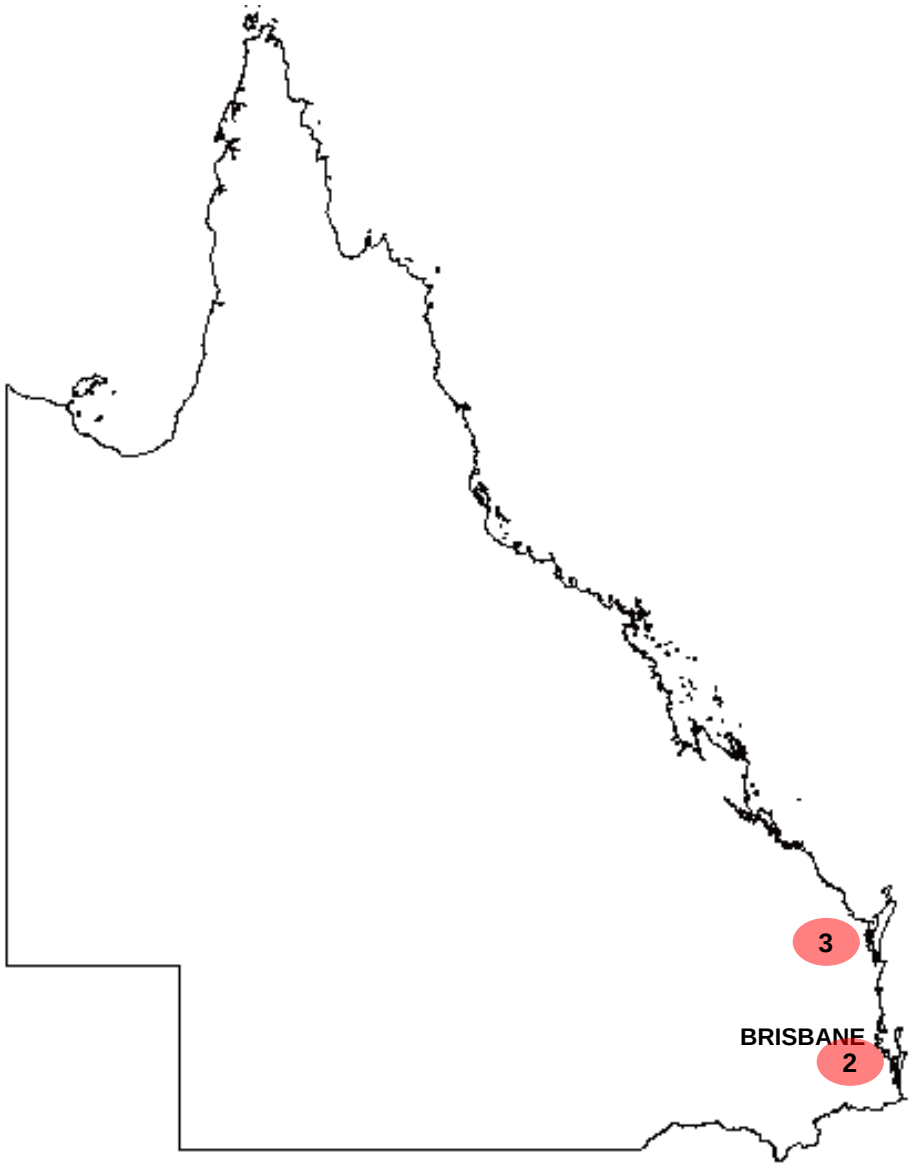

Figure 11. Map of Queensland showing location of outbreaks in the Wide Bay-Burnett region and the Gold-Coast hinterland.

Characterisation of Chlamydiaceae species using PCR and high resolution melt curve analysis of the 16S rRNA gene

The most important zoonotic chlamydiosis is psittacosis, a systemic disease in psittacine birds with acute, protracted, chronic or subclinical manifestations. It is caused by *Chlamydophila. psittaci* and is a notifiable disease in Australia and several other countries. Historically, there have been several suspected outbreaks of chlamydiosis in Australian broiler, broiler breeder, backyard, layer flocks (personal communications with Drs George Arzey, Peter Scott, Liam Morrisroe), as well as in squab pigeon (Forsyth, 2005) and duck flocks (Gilchrist, 2005). The economic damage in connection with these outbreaks is considerable, but of particular concern is the possibility that some of these outbreaks were associated with clinical symptoms in personnel who were in contact with the affected birds.

Recently, under a research project funded by RIRDC Chicken Meat, a PCR protocol (16SG), targeting 16S rRNA gene coupled with high resolution melt (HRM) curve analysis was developed in our laboratory and shown to reliably detect and identify the seven different *Chlamydiaceae spp*. In another study also funded by RIRDC Chicken Meat, the capacity of this protocol was assessed for detection and identification of *Chlamydiaceae spp*. in routine diagnostic specimens from different animals particularly chickens. Also its sensitivity with that of CTU/CTL PCR (targeting the *ompA* gene) was evaluated.

Methodology and Results

A total number of 733 specimens from a range of animal species, 199 (27%) were found positive by 16SG PCR while only 72 (<10%) were positive by CTU/CTL. In some animal species (including koalas and sheep) the infecting *Chlamydiaceae spp*. was only detectable by 16SG PCR while in some others (chickens) 16SG PCR was significantly more sensitive than CTU/CTL PCR for detection of infecting *Chlamydiaceae spp*.

Where a sufficient amount of DNA was available (64 submissions), amplicons generated by the 16SG PCR were subjected to HRM curve analysis and results were compared to that of nucleotide sequencing. In all instances, the infecting *Chlamydiaceae spp*. was genotyped according to the identity of its nucleotide sequence to a reference species. Analysis of the HRM curves and nucleotide sequences from 16SG PCR amplicons also revealed the occurrence of a *Chlamydophila*-like, a *Parachlamydia*-like and a variant of *Chlamydophila psittaci* in chickens.

A PCR-coupled high resolution melting (HRM) curve analytical approach for the monitoring of monospecificity of avian *Eimeria* species

Coccidiosis of chickens is an intestinal parasitic disease caused by one or more species of the protozoan parasite genus *Eimeria* (Apicomplexa: Eucoccidia: Eimeriidae) and is one of the commonest and economically most important diseases of poultry worldwide (Shirley *et al.*, 2005). This disease causes production losses, and sometimes leads to high mortality. While the control of coccidiosis has relied mainly on the preventative use of anti-coccidial drugs (coccidostats), together with the induction of species-specific natural immunity in chicken flocks (Shirley *et al.*, 2004; Shirley *et al.*, 2005), this widely used approach is costly and has led to serious problems with drug resistance in *Eimeria* populations (Williams, 1998). Thus, live attenuated or precocious vaccines are now finding widespread application, particularly in

intensive establishments (Shirley *et al.*, 2004; Shirley *et al.*, 2005). Since the immunity in chickens is specific to a particular species of *Eimeria*, vaccines need to contain each relevant species (usually multiple) in order to be effective. Live, attenuated (sporulated oocyst) vaccines containing 3-7 species of *Eimeria* have been released. These include Livacox^{R} Q, Livacox^{R} T, Paracox^{R}, Paracox^{R} 8 and Eimeriavax^{R} 4m (Shirley & Bedrnik, 1997; Williams, 1998; Williams, 2002a; Williams, 2002b). A number of Eimeria vaccine candidates have been attenuated by selection for the ability to replicate in chicken embryos (Gore *et al.*, 1983; Long, 1974; Long *et al.*, 1982; Norton & Joyner, 1981; Shirley, 1979) but most attenuated lines have been selected for rapid passage (i.e., having a shortened pre-patent period) through the chicken host (Shirley, 1979). Consequently, they have a low reproductive (Shirley & Bedrnik, 1997) potential and have a reduced virulence, but still have a strong immunogenicity in chickens (Shirley & Bedrnik, 1997; Williams, 1998). Therefore, such vaccines (with or without preventative, chemotherapeutic treatment) provide the best prospects for the control of coccidiosis in the immediate future (Chapman *et al.*, 2002; Shirley *et al.*, 2005). Central to the production of such live vaccines is the maintenance (by serial passage) of monospecific lines of *Eimeria* in chickens under stringent conditions. The production of live vaccines *via* propagation and passage in chickens is expensive and they can be readily contaminated during production and/or processing. Therefore, the production of such vaccines on a commercial scale requires the rigorous monitoring of quality, including species composition at each step of production. Traditionally, conventional coproscopic methods were used for the detection and enumeration of *Eimeria* oocysts purified from the faeces from infected chickens. However, given that there is significant overlap in the size and shape of oocysts between/among some species of *Eimeria (Long & Joyner, 1984)*, a range of molecular-diagnostic methods have been developed (Morris & Gasser, 2006). A number of previous studies (Fernandez *et al.*, 2003; Schnitzler *et al.*, 1998; Schnitzler *et al.*, 1999) have developed polymerase chain reaction (PCR)-based techniques for detection and identification of *Eimeria* species. However these techniques are relatively time consuming, as they require agarose gel electrophoresis for visualisation of the PCR products (Fernandez *et al.*, 2003; Schnitzler *et al.*, 1998; Schnitzler *et al.*, 1999), or lack the capacity to detect non-specific (false positive) amplifications (Schnitzler *et al.*, 1999). Rapid real-time PCR assays specific to four species of *Eimeria* have recently been described (Blake *et al.*, 2008) but require separate reaction tubes for detection of each of the species tested. Also other PCR-coupled techniques such as high-throughput capillary electrophoresis, high-resolution electrophoretic procedures and single-strand restriction fragment length polymorphism analysis have found applicability (Gasser *et al.*, 2005; Morris *et al.*, 2007a; Morris *et al.*, 2007b; Woods *et al.*, 2000a; Woods *et al.*, 2000b). While these approaches have been useful and effective, they have relied on the high resolution analysis of denatured PCR products on electrophoretic gels, taking a day to carry out. The advent of high resolution melting (HRM) curve analysis circumvents the use of such gels, substantially decreasing analysis time, and has been reported to achieve high mutation detection rates for amplicon sizes of 100-300 bp (Herrmann *et al.*, 2006; Varga & James, 2006; Wittwer *et al.*, 2003). In the present study, we report a simple and effective PCR-coupled HRM, using the ITS-2 region, for the verification of monospecificity of *Eimeria* lines maintained in chickens specifically for commercial anti-coccidia vaccine production and discuss the implications of such a tool.

Methodology

Eimeria species and isolates

Monospecific lines of *Eimeria* (originally isolated from naturally infected chickens in Australia; Table 7) were passaged in specific pathogen-free chickens held in custom-built isolators under stringent conditions to prevent cross-contamination. *Eimeria* were originally identified to species based on the morphometry of sporulated oocysts, prepatent period, location of gross lesions in the intestine(s) and using PCR-coupled capillary electrophoretic analysis of second internal transcribed spacer of nuclear ribosomal DNA (ITS-2), as described previously (Gasser *et al.*, 2005). Between four to eight isolates from each of the seven known

species of *Eimeria* were used in this study. The isolates were labelled as follows: *E. acervulina* (A4, A14, A16, A23, A35, A38, A65); *E. brunetti* (B2, B12, B15, B17, B21, B29); *E. maxima* (M5, M20, M25, M48, M50, M51, M91); *E. mitis* (Mt2, Mt4, Mt8, Mt9); *E. necatrix* (N4, N7, N13, N16, N32, N42, N52, N90); *E. tenella* (T2, T5, T8, T11, T14, T15, T16); and *E. praecox* (P2, P3, P7, P11, P12, P33, P67).

Extraction of genomic DNA

Oocysts were isolated from faeces using saturated NaCl, washed extensively in 50 ml volumes of H_20 and made up to a final aqueous suspension (10 ml containing 5 x 10^6 oocysts). The oocysts were then purified using a sucrose-gradient centrifugation method (Gasser *et al.*, 1987), which removed faecal components inhibitory to the PCR, washed (as previously) and then resuspended in 1 ml of H_20. Glass beads, proteinase K and Sodium dodecyl sulphate were added, the sample was vortexed and incubated overnight at 37ºC. DNA was isolated from oocysts using a Wizard® Genomic DNA Purification Kit (Promega) and eluted in 50 µl of dH_2O (thus containing DNA from approximately 10^5 oocyts per µl). The concentration of eluted DNA ranged from 30-150 ng/µl as determined by spectrophotometry at A_{260}.

PCR amplification

The PCR was carried out as reported previously (Kirkpatrick *et al.*, 2009), using oligonucleotide primers WW2 (5'-ACGTCTGTTTCAGTGTCT-3') and WW4r (5'-AAATTCAGCGGGTAACCTCG-3'). The PCR was performed in 25 µl volumes, containing 200 µM each dATP, dCTP, dGTP and dTTP, 2mM $MgCl_2$, 250 µM each primer, 0.5 U GoTaq (Promega), 5x Buffer, 5 uM SYTO®9 green fluorescent nucleic acid stain (Invitrogen) and, with exception of no template control, 2 µl of genomic DNA (from approximately 2 x 10^5 oocysts). The detection limit of GoTaq was 1-2 pmol of DNA, as previously shown (Gasser *et al.*, 2001) The amplification mixture was incubated at 94º C for 3 min, then 35 cycles of 94º C for 30 sec, 55º C for 30 sec, 72 ºC for 60 sec, with a final incubation at 72 ºC for 3 min. In each set of reactions dH_2O instead of template was included as a negative control. Amplification of target sequences was carried out in triplicate on a RotorGene thermal cycler (RG6000, Corbett Research Pty Ltd). Optical measurements in green channel (excitation at 470 nm and detection at 510 nm) were recorded during the extension step. Following the PCR, samples were subjected to agarose gel electrophoresis, CE analysis and.or HRM curve analysis.

Agarose gel electrophoresis

After PCR, 10 µl of individual amplicons were mixed with 2 µl of loading buffer (10 mM NaOH, 95% formamide, 0.05% of both bromophenol blue and xylene cyanole) and the intensity of selected samples verified on SYBR safe™ (Invitrogen) stained 2% agarose gels using 0.5x TBE (65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9; Bio-Rad) as the buffer and molecular weight marker (PCR marker, Sigma) as a size marker. After electrophoresis, gels were photographed (Kodak) upon ultraviolet transillumination.

Capillary Electrophoresis

Capillary electrophoresis was performed according to the method described before (Gasser *et al.*, 2005). Briefly the primer WW2 was labelled with 6-fluorescein (FAM) was used in the PCR. The PCR products were subjected to electrophoresis on Long- Ranger gels in an ABI Prism 377 DNA Sequencer. The resultant gel image was captured and chromatograms analysed and compared using GelScan software.

HRM curve analysis

After completion of the PCR cycles, the melt curve was generated by increasing the temperature from 71ºC to 90ºC at different ramp speeds from 0.1 to 0.8 ºC/sec and recording fluorescence. HRM curve analysis was performed using the software Rotor-Gene 1.7.27 with normalisation regions of 72-73 and 88-89 and an average confidence threshold of approximately 90%.

Results

Adjustment of the yield of PCR products

In order to adjust the yield of the PCR products prior to HRM analysis, PCR was performed and the amount of products generated from each *Eimeria* species visualised by gel electrophoresis. Adjustments to the dilution of the DNA template were made to obtain approximately the same visual staining density for the PCR products. Figure 12 shows examples of PCR products generated from a representative isolate from each of the *Eimeria* species. As expected, amplicons of approximately 490 base pairs (bp) were generated for *E. tenella*, 340 bp for *E. maxima*, 390 bp for *E. acervulina*, 450 bp for *E. mitis*, 530 bp for *E. preacox*, 560 bp for *E. necatrix* and 430 bp for *E. brunetti*.

Correlation of the HRM melt-curve profiles of the ITS-2 PCR products with those of CE profiles

A subset of monospecific *Eimeria* species vaccine lines, as determined by their capillary electrophoresis (CE) profile and morphological analysis were selected and subjected to PCR of the ITS-2 region and high resolution melt (HRM) curve analysis. Preliminary optimisation revealed that melting between temperatures 75ºC and 90ºC with an optimal ramp of 0.2ºC/sec resulted in detectable and consistent differences between species (Figure 13). Lower ramps resulted in variation in isolates from *E. brunetti* (Figure 14).

Using an optimal ramp of 0.2ºC/sec, one, two or three peaks were generated between 75°C and 90°C depending on the species tested (Table 7 and Figure 13. A). *E. maxima*, *E. mitis* and *E. tenella* all generated one major peak. *E. acervulina* generated two major peaks, with the right peak being slightly smaller. *E. praecox* generated two peaks, as well, with one being a minor shoulder peak at higher temperature than the major peak. *E. brunetti* generated three peaks, with two smaller shoulder peaks at higher temperature than the major peak. *E. necatrix* generated three peaks, one smaller shoulder peak on either side of the major peak. Analysis of the conventional (Figure 13. A) or normalised curves (B) revealed seven distinct HRM genotypes in agreement with the CE profiles (Table 7). *Eimeria* species with identical CE profiles generated identical HRM genotypes. For example, *Eimeria* isolates A4, A14 and A16 produced the same HRM curve profile (A). Similarly isolates B2 and B5, and B12 also produced the same profile (B). Several HRM runs (performed on different days) using at least one representative from each profile resulted in only minor variations in melting temperatures of the peaks (Table 8). Following normalisation of data, genotypes were consistent with those described in Table 8.

Sensitivity of the HRM curve analysis for the detection of impurities in Eimeria species

In order to assess the capacity of HRM curve analysis for the detection of specimens containing mixture of species, all combinations of two species of *Eimeria*, at similar concentrations, were assessed. Visual assessment of the conventional and normalised curves could distinguish both species within each mixture with the genotype of the mixture represented as ‘variation’ after analysis of the melt curve by the software

(Table 9). Figure 15 shows an example of conventional (A) and normalised (B) melt curves resulted from an *E. acervulina* and *E. necatrix* mixture.

To further assess the capacity of the HRM curve analysis for detection of small amounts of contaminating *Eimeria* species, DNA from four representative isolates of the known *Eimeria* species (*E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*) were spiked (simulating contamination) with other *Eimeria* species and subjected to CE and HRM curve analysis. Different combinations of DNA from two species of *Eimeria* were mixed into one PCR reaction at various concentrations and subjected to ITS-2 PCR followed by either CE or HRM curve analysis (Table 10). Examples of the melt curve and HRM analysis for *E. tenella* and *E. acervulina* mixtures (reactions 6-9, Table 10) and the pure species are shown in Figures 16 A and B. Both CE and HRM curve analysis could detect the dominant species in all samples. The presence of the exact minor species was detected in 4 and 3 specimens by CE and HRM curve analysis respectively. Variation in HRM curve analysis was detected where the exact minor species could not be defined.

Table 7. Melting points for the peaks and HRM curve profiles of ITS-2 PCR products from selected *Eimeria* isolates.

Species	Isolate	CE profile	Peak 1 (ºC)	Peak 2 (ºC)	Peak 3 (ºC)	Melt genotype	HRM genotype	Confidence %
E. acervulina	A4	A	79.90	81.17	-	A	A	97.33
E. acervulina	A14	A	79.80	81.24	-	A	A	87.49
E. brunetti	B2	B	79.06	81.56	83.80	B	B	99.50
E. brunetti	B15	B	79.30	81.26	83.30	B	B	99.75
E. maxima	M5	M	83.13	-	-	M	M	91.73
E. maxima	M51	M	83.17	-	-	M	M	91.82
E. mitis	Mt2	Mt	81.42	-	-	Mt	Mt	99.79
E. mitis	Mt4	Mt	81.40	-	-	Mt	Mt	61.84
E. mitis	Mt8	Mt	81.34	-	-	Mt	Mt	90.46
E. necatrix	N4	N	82.34	83.46	85.00	N	N	82.15
E. necatrix	N13	N	82.40	83.46	85.17	N	N	93.78
E. praecox	P2	P	78.50	-	-	P	P	91.21
E. praecox	P5	P	78.16	-	-	P	P	89.94
E. praecox	P15	P	78.40	83.60	-	P	P	91.41
E. tenella	T2	T	84.54	-	-	T	T	92.28
E. tenella	T7	T	84.46	-	-	T	T	91.37

Table 8. Average and standard deviation (SD) of the melting points for the peaks of different profiles resulted from several runs of ITS-2 PCR followed by HRM curve analysis.

			Peak 1		Peak 2		Peak 3	
Species	Pattern	No. samples	Average	SD	Average	SD	Average	SD
E. acervulina	A	7	79.84	0.13	81.31	0.22	-	-
E. brunetti	B	6	79.26	0.19	81.59	0.29	83.58	0.24
E. maxima	M	7	82.42	0.14	-	-	-	-
E. mitis	Mt	4	81.35	0.39	-	-	-	-
E. necatrix	N	8	82.42	0.09	83.54	0.08	85.11	0.11
E. praecox	P	7	78.55	0.43	83.61	0.08	-	-
E. tenella	T	7	84.54	0.13	-	-	-	-

Table 9. Capacity of HRM curve analysis for the detection of two species of *Eimeria* mixed at similar concentrations. Seven pure preparations from each of the species of *Eimeria* were used.

Reaction	Sample combinations	HRM genotype
1	A (*E. acervulina*)	A
2	A + B	Variation
3	A + M	Variation
4	A + Mt	Variation
5	A + N	Variation
6	A + P	Variation
7	A + T	Variation
8	B (*E. brunetti*)	B
9	B + M	Variation
10	B + Mt	Variation
11	B + N	Variation
12	B + P	Variation
13	B + T	Variation
14	M (*E. maxima*)	M
15	M + Mt	Variation
16	M + N	Variation
17	M + P	Variation
18	M + T	Variation
19	Mt (*E. mitis*)	mt
20	Mt + N	Variation
21	Mt + P	Variation
22	Mt + T	Variation
23	N (*E. necatrix*)	N
24	N + P	Variation
25	N + T	Variation
26	P (*E. praecox*)	P
27	P + T	Variation
28	T (*E. tenella*)	T

Table 10. Detection limits of mixed sample examples (dominant and minor amounts) using the CE and the HRM methods.

Species		Ratio	CE - species detected		HRM - species detected	
			Dominant	Minor	Dominant	Minor
E. necatrix	*E. maxima*	10^{-2} : 1	Yes	No	Yes	Variation
E. necatrix	*E. maxima*	10^{-3} : 1	Yes	No	Yes	Variation
E. necatrix	*E. maxima*	1 : 10^{-2}	Yes	Yes	Yes	Yes
E. necatrix	*E. maxima*	1 : 10^{-3}	Yes	Yes	Yes	Yes
E. necatrix	*E. maxima*	1 : 10^{-4}	Yes	Yes	Yes	Yes
E. tenella	*E. acervulina*	10^{-2} : 1	Yes	Yes	Yes	Variation
E. tenella	*E. acervulina*	10^{-3} : 1	Yes	No	Yes	Variation
E. tenella	*E. acervulina*	1 : 10^{-3}	Yes	No	Yes	Variation
E. tenella	*E. acervulina*	1 : 10^{-4}	Yes	No	Yes	Variation

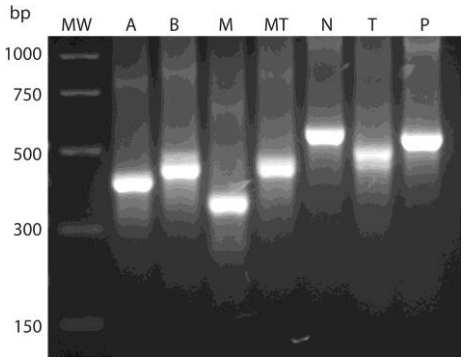

Figure 12. Agarose gel electrophoresis of PCR products of the ITS-2 gene from different *Eimeria* species. *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. tenella and E. praecox* (A, B, M, MT, N, T and P, respectively). MW is molecular weight marker (PCR marker, Sigma).

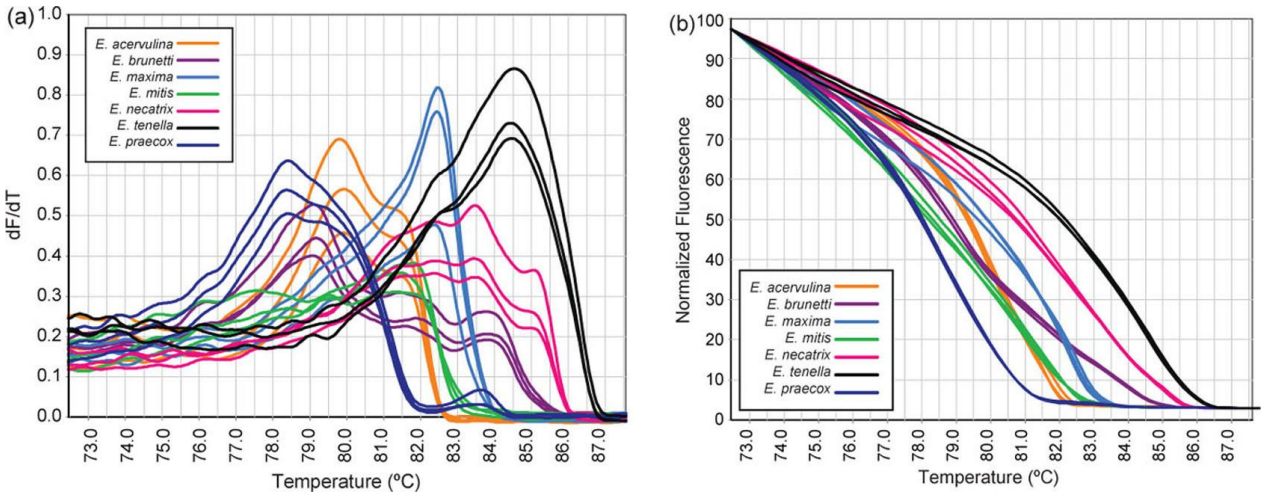

Figure 13. Representative of conventional (2a) and normalised (2b) HRM melt curves of PCR products of the ITS-2 gene from different *Eimeria* species.

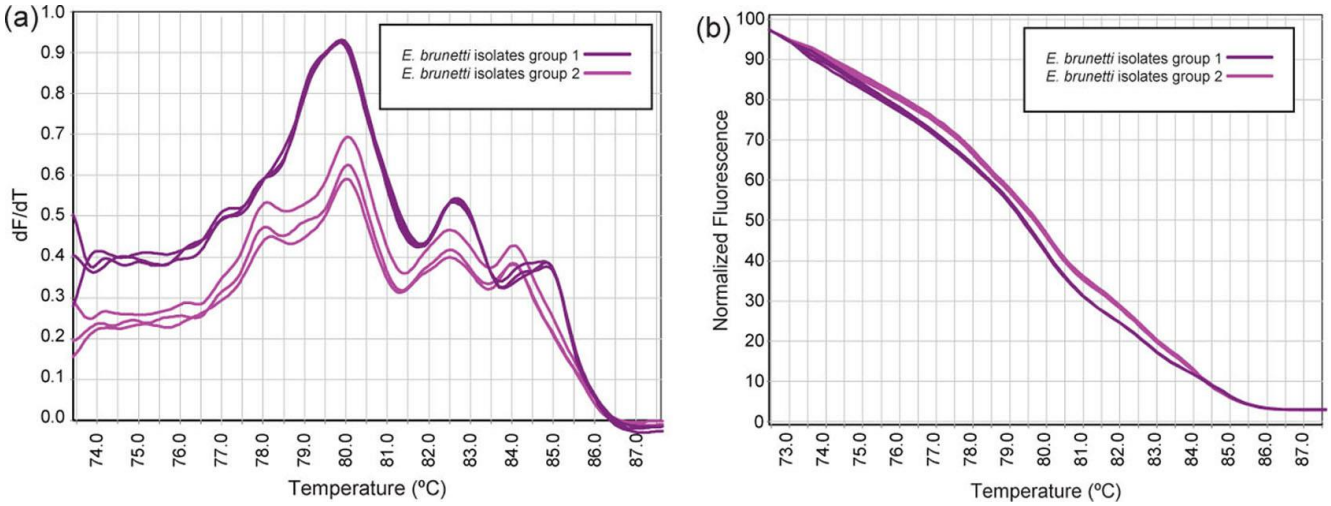

Figure 14. Replicates of conventional (3a) and normalised (3b) HRM melt curves of PCR products of the ITS-2 gene from *E. brunetti* isolates.

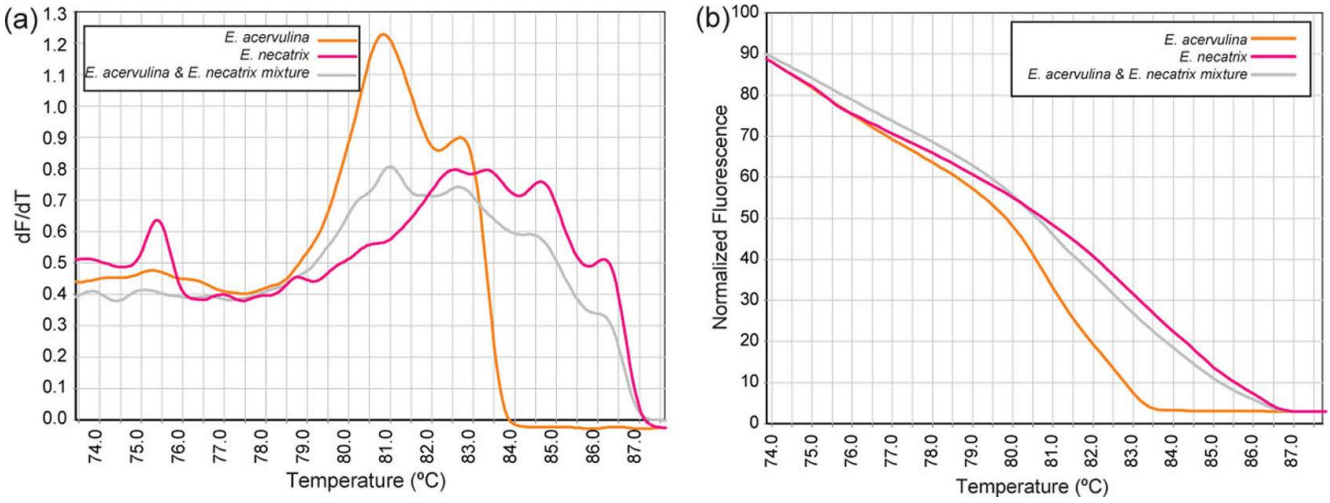

Figure 15. Conventional (4a) and normalised (4b) HRM melt curves of *E. acervulina*, *E. necatrix* and a mixed sample (1:1 ratio).

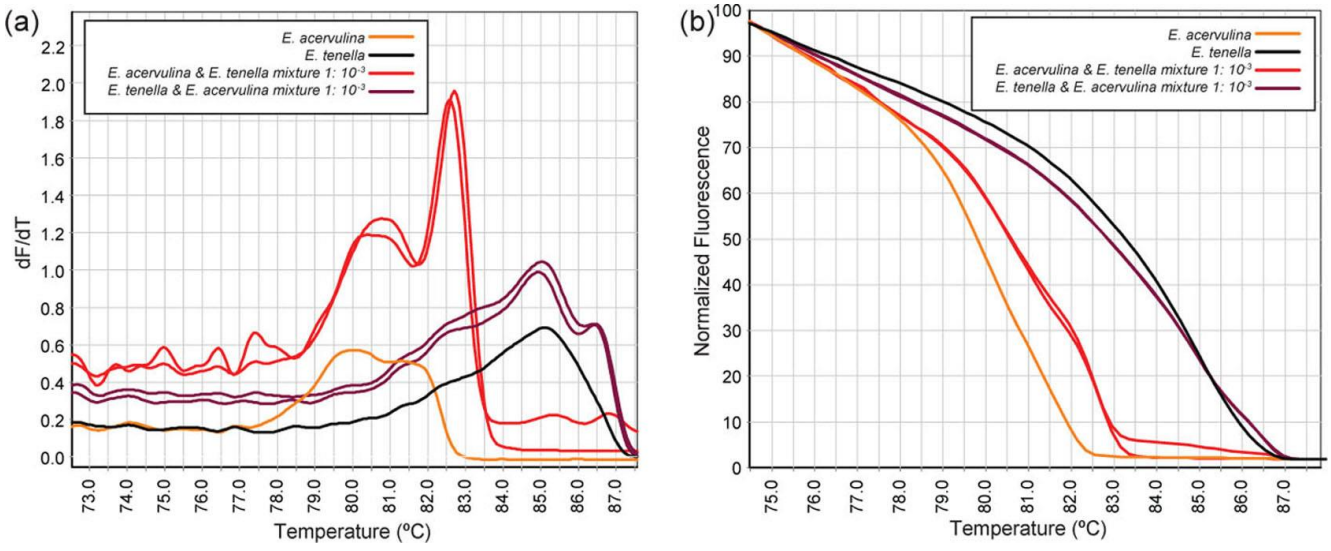

Figure 16. Conventional (5a) and normalised (5b) melt curves of *E. acervulina* and *E. tenella* mixed samples at 1:1, $1:10^{-3}$ and 10^{-3}:1 ratios.

Classification of fowl adenovirus serotypes using high resolution melting curve analysis of the hexon gene region

Identification of the serotype(s) involved in IBH outbreaks is very useful for epidemiological tracing and is of critical importance where vaccination is to be used for control of disease (Balamurugan & Kataria, 2004; Jadhao *et al.*, 2003). Typing of strains conventionally by virus micronuetralisation is cumbersome, requiring reference materials, and results often require extensive interpretation (El-Attrache & Villegas, 2001).

In Australia, IBH is controlled by the use of a live FAdV-8b vaccine that was developed in 1989. However, in recent years there have been sporadic occurrences of IBH in Australian broilers. The number and serotype of the FAdVs responsible was unknown. In a recent study funded by RIRDC Chicken Meat, HRM curve analysis on PCR amplicons of the Loop 1 region of the hexon gene was used to accurately genotype reference strains from each of the twelve FAdV serotypes (Steer *et al.*, 2009). In a separate study (unpublished), also supported by RIRDC Chicken Meat, the capacity of the assay was assessed for routine typing of field specimens in Australia.

Methodology and results

A total of 26 cases were screened by this method, with FAdV-8b and FAdV-11 identified in 13 cases each. In one case FAdV-1 was also identified, in association with FAdV-11. No evidence of Chicken Anaemia Virus (CIAV) or Infectious Bursal Disease Virus (IBDV) was detected in association with any of these outbreaks.

For comparison of diagnostic techniques, representative field isolates from each serotype were also assessed by virus microneutralisation and nucleotide sequence analysis of the hexen gene. Cross-neutralisation was observed between field FAdV-11 strain virus and reference FAdV-2 and 11 antisera, a result also seen with type 2 and 11 reference viruses. Field strains 1 and 8b were neutralised only by their respective type antisera. The FAdV-8b field strain was identical to the Australian FAdV vaccine strain (type 8b) in the hexon loop 1 (L1) gene region. The FAdV-11 field strain hexon L1 gene sequence had the highest identity to FAdV-11 (93.2%) and FAdV-2 (92.7%) reference strains.

Diagnostic PCR for the detection of *Avibacterium paragallinarum*, the causative agent of Infectious Coryza

Infectious coryza is an upper respiratory tract disease of chickens caused by the bacterium *Avibacterium paragallinarum*. The economic impact of the disease is mainly associated with a significant reduction (10%-40%) in egg production, especially on multi-age farms. The traditional method of confirming infectious coryza in a chicken flock is the isolation and biochemical characterisation of *A. paragallinarum*. This is a technically demanding task as most *A. paragallinarum* isolates have stringent nutritional requirements, so that the normal media used in bacteriology laboratories does not support the growth of these organisms. The PCR test appears to be a useful diagnostic tool for the detection of infectious coryza. The test can also be used as confirmatory test following the isolation of a haemophilic organism. As well, the HPG-2 PCR test is an alternative to culture (Chen *et al.*, 1996). This PCR was adopted from Chen *et al.* (1996).

Methodology

DNA extraction. Total genomic DNA was extracted from *A. paragallinarum* cultures and from swabs taken from birds using a DNA extraction kit (QIAGEN) according to the manufacturer's instructions. Briefly, 0.2 ml of *A. paragallinarum* culture was added to 500 µl RLT lysis buffer (QIAGEN) and incubated for two hours at room temperature or overnight at 4ºC. Swabs taken from experimentally infected birds were also placed in 0.5 ml lysis buffer (QIAGEN) and incubated at 4ºC overnight. The rest of the DNA extraction process was carried out as described for *M. gallisepticum* cultures (page 6).

PCR. A pair of oligonucleotide primers, N1 (5'- TGA GGG TAG TCT TGC ACG CGA AT -3') and R1 (5'- CAA GGT ATC GAT CGT CTC TCT ACT -3'), reported previously (Chen *et al.*, 1996) were used in the PCR. Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl extracted genomic DNA, 25 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 98ºC for 150 s, 30 cycles of 94ºC for 30 s, 66ºC for 20 s and 72ºC for 20 s, and a final cycle of 72ºC for 7 min. In each set of reactions, *A. paragallinarum* genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

Results

PCR amplicons of expected size generated from *A. paragallinarum* using oligonucleotide primers N1 and R1

Amplified PCR products from *A. paragallinarum* were analysed by gel electrophoresis. Specimens contained *A. paragallinarum* generated a 511-bp DNA band visible on agarose gel (Figure 17).

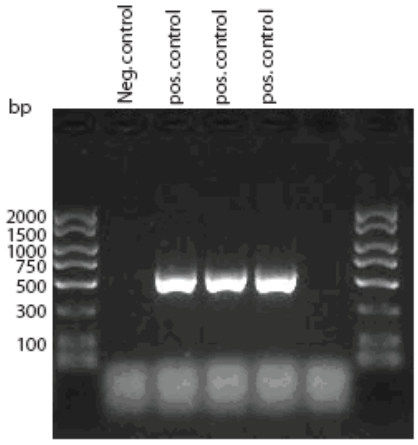

Figure 17. Agarose gel (1%) electrophoresis of PCR products from swabs taken from infraorbital sinuses of three infected birds (positive controls) and of an un-infected bird (negatibve control).

Diagnostic PCR for the detection of chicken anaemia virus (CIAV)

Chicken anemia virus (CIAV) is a member of the genus Gyrovirus within the family Circoviridae and causes immunosuppression in chickens. CIAV infection is ubiquitous in poultry flocks worldwide and significant production losses can occur as a consequence of both clinical and subclinical infections of commercial poultry. Infections are of increasing concern because of their role in vaccination failure, reduced production performance and potentiation of other infectious diseases, including Marek's disease.

DNA extraction

Total genomic DNA was extracted from CIAV MDCC-MSB1 cell culture using a DNA extraction kit (QIAGEN) as described above for *Avibacterium paragallinarum*.

PCR

The CIAV ORF-2 region was chosen for amplification. A pair of oligonucleotide primers, QF1 (5'-GAATGTGCCGGACTTGAGGA -3') and QR1 (5'- GGGTCGCAGGATCGCTT -3'), flanking the target region was designed with the PCR amplicon expected to yield a product of 65 bp (Kaffashi *et al.*, 2006). Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 2 µl extracted genomic DNA, 10 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 95ºC for 120 s, 30 cycles of 95ºC for 30 s, 62ºC for 20 s and 72ºC for 5 s, and a final cycle of 72ºC for 5 min. In each set of reactions, CIAV genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

PCR amplicon generated from CIAV

Amplified PCR products from CIAV specimens were analysed by gel electrophoresis. Specimens that contained CIAV generated a 65-bp DNA band on the agarose gel (Figure 18).

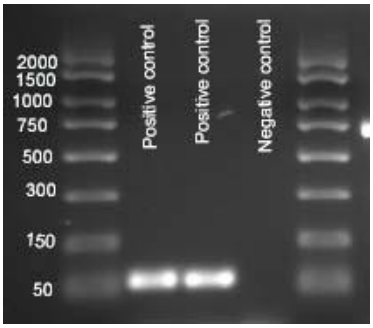

Figure 18. Agarose gel (2%) electrophoresis of PCR products from positive controls (CIAV-infected MSB1 cells) and negative control (sterile distilled H_2O).

Diagnostic PCR for the detection of *Pasteurella multocida,* the causative agent of fowl cholera

Respiratory diseases are a major cause of economic losses to the Australian chicken meat and egg industries. One of the bacterial diseases that is important contributor to the respiratory disease complex is fowl cholera. *Pasteurella multocida*, the causative agent of fowl cholera, is a member of a very extensive family of closely related organisms, many of which have only recently been recognised. The conventional method for the identification of a suspect isolate as *P. multocida* requires that the isolate be subjected to an extensive range of biochemical tests. The PCR test has significant advantages over this conventional approach. Therefore, this PCR was adopted from a previously published report (Townsend *et al.*, 1998) and optimised for detection of the agent.

DNA extraction

Total genomic DNA was extracted from *Pasteurella multocida* culture or swabs taken from experimentally infected birds using a DNA extraction kit (QIAGEN) as described for *Avibacterium paragallinarum*.

PCR

A pair of oligonucleotide primers, KMT1SP6 (5'- GCT GTA AAC GAA CTC GCC AC -3') and KMT1T7 (5'- ATC CGC TAT TTA CCC AGT GG -3') were used in PCR as described before (Townsend *et al.*, 1998). Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 95ºC for 4 min, 30 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of reactions, *Pasteurella multocida* genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.8% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

PCR amplicon generated from *Pasteurella multocida*

Amplified PCR products were analysed by gel electrophoresis. Specimens that contained *Pasteurella multocida* generated a 460-bp DNA band on the agarose gel (Figure 19).

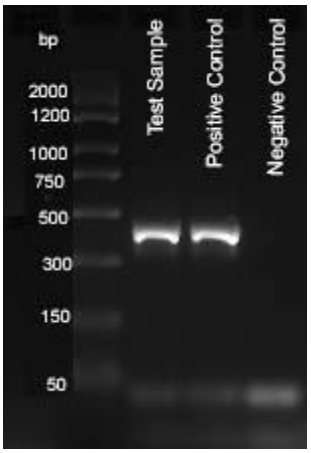

Figure 19. Agarose gel (1.8%) electrophoresis of PCR products from positive control (a known *Pasteurella multocida* culture), from test sample (a swab taken from liver of a bird infected with *Pasteurella multocida*) and negative control (sterile distilled H_2O).

Diagnostic PCR for the detection of Marek's disease virus (MDV)

Marke´s disease virus (MDV) is a lymphoproliferative disease of chickens characterized by malignant transformation of $CD4^+$ helper T lymphocytes in various organs. The disease is an economically important disease of chickens. The MDV belongs to strongly cell-associated avian herpesviruses that have been subdivided in to three serotypes. Serotype 1 viruses are the pathogenic viruses and their cell-culture-attenuated variants. Serotype 2 viruses are the naturally occurring non–pathogenic chicken viruses, and serotype 3 viruses are designated as the non – pathogenic turkey herpesviruses. This PCR was adopted from a previously published report (Zhu *et al.*, 1992) and optimised for detection of MDV1 strains.

DNA extraction

Genomic DNA was extracted from a commercial batch of Vaxsafe® RIS Vaccine (Bioproperties Australia Pty Ltd).Total genomic DNA was also extracted from tissue specimens or swabs taken from tissues of infected birds using a DNA extraction kit (QIAGEN) as described for *Avibacterium paragallinarum*.

PCR

A pair of oligonucleotide primers, MDV-gA1-F (5'- CATGCAAGTCATTATGCGTGAC -3') and MDV-gA1-R (5'- TGTTTCCATTCTGTCTCCAAGA -3') were used in PCR as described before (Zhu *et al.*, 1992). Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 95ºC for 3 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of reactions, MDV genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.8% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

PCR amplicon generated from MDV

Amplified PCR products were analysed by gel electrophoresis. Specimens that contained MDV generated a 200-bp DNA band on the agarose gel (Figure 20).

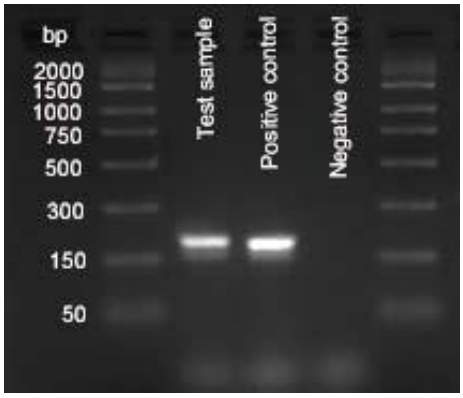

Figure 20. Agarose gel (1.8%) electrophoresis of PCR products from test sample (skin homogenate of an infected chicken), positive control (Rispens vaccine- Vaxsafe® RIS Vaccine) and negative control (distilled H_2O)

Diagnostic PCR for the detection of Egg drop syndrome (EDS) virus

Egg drop syndrome (EDS), caused by an adenovirus, is an economically important disease of laying hens characterized by depressed egg production, laying of soft-shelled or shell-less eggs, and failure to reach peak production. The causative EDS virus is the sole member of Group III avian adenoviruses. Only one serotype of the EDS virus has been recognized. The virion contains DNA which varies in size from 25 to 43.8 kbp. Due to the widespread occurrence of antibodies to avian adenoviruses, serological methods are only of minor relevance to diagnose most avian adenoviruses. Therefore, this PCR was adopted from a previous report (Raj *et al.*, 2001) and optimised for direct detection and identification of the agent.

DNA extraction

Total genomic DNA was extracted from inoculated chicken embryo liver cell culture using a DNA extraction kit (QIAGEN) as described for *Avibacterium paragallinarum*.

PCR

A pair of oligonucleotide primers, EDS-238-F (5'- TTG GCG TCT TCA AGG CAC TG -3') and EDS-238-R (5'- CAC ACA ACT GCA TCT GAC TG -3'), from the J fragment of EDS was selected with the PCR amplicon expected to yield a product of 238 bp (Raj *et al.*, 2001). Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 3 µl extracted genomic DNA, 25 µM of each primer, 1.5 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 3 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of reactions, EDS genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight

marker, were analysed by electrophoresis in 1.8% agarose gels stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

PCR amplicons generated from EDS virus

Amplified PCR products from specimens containing EDS virus were analysed by gel electrophoresis. Specimens that contained EDS generated a 238-bp DNA band on the agarose gel (Figure 21).

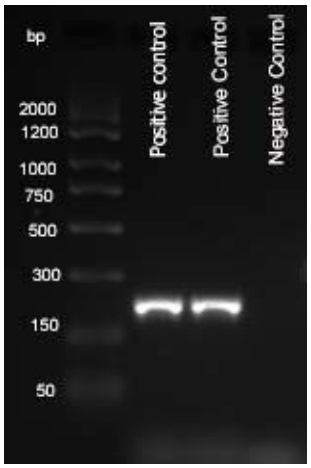

Figure 21. 1.8% Agarose gel electrophoresis of PCR products from positive controls (inoculated chicken embryo liver cell culture) and negative control (distilled H_2O)

RT-PCR for the detection of avian leukosis virus

Avian leukosis virus (ALV) infections in commercial chickens affect adversely poultry and egg production since infected chickens may exhibit reduced growth rates, decreased egg production, and produce eggs of reduced size and quality. In addition, ALV causes tumors and sporadic deaths in sexually mature chickens. Congenital transmission accounts for the spread of ALV virus from one generation to the next, and the virus can also spread horizontally from chick to chick. To prevent transmission, the poultry industry must identify infected eggs and chickens, and remove them from breeding populations. The exogenous and oncogenic avian leukosis: sarcoma subgroups A, B, C, and D are horizontally and vertically transmitted in chickens. Subgroup E ALV viruses are non-oncogenic endogenous viruses that are transmitted in a noninfectious form from one generation of chickens to the next in a Mendelian fashion along with the host genes. This RT-PCR was adopted from a previous report (Hauptli *et al.,* 1997) and optimised for the detection of ALV (subgroups A to E) in clinical samples.

RNA extraction

Total RNA was extracted from ALV-A, ALV-B and ALV-J grown in chicken embryo fibroblast (CEF) cells. Two hundred µl of inoculated CEF cell culture was lysed and lysed in 450 µl RLT buffer (Qiagen, Victoria, Australia) with 1% β-mercaptoethanol for two hours at room temperature or overnight at 4ºC. Then 15 µl of Qiaex II matrix (QIAGEN) and 300 µl 70% ethanol were added and mixed, and the lysate was loaded into a multispin MSK-100 column (Axygen Inc., Hayward, CA, USA), centrifuged for 30 s at 10000 ***g*** and the flow-through discarded. The column was washed with 600 µl RW1 buffer (QIAGEN) and twice with 500 µl RPE buffer (QIAGEN) and subjected to centrifugation at 18000 ***g*** for 90 s. The RNA was

eluted from the matrix using 50 µl DEPC-dH_2O and used in RT-PCR immediately or stored at -70 ºC for future use.

RT-PCR

The oligonucleotide primers used in RT-PCR detection of ALV included the forward primer ALVgp85U3 (5'- ACG GAT TTY TGC CTY TCT -3') and the reverse primer ALVgp85L3 (5'- ATT GTG YCT RTC CGC TGT C -3'). To detect ALV subgroups A to E, primers were selected from regions of gp85 env gene, showing the highest degree of conservation between different ALV strains as published before (Hauptli *et al.*, 1997). To produce single stranded cDNA, one µl (25 µM) of oligo dt primer was added to 5 µl of extracted RNA and the mixture heated at 100°C for 1 min, then chilled on ice for 5 mins. Nineteen µl of cDNA master mix containing 1 U RNasin® ribonuclease inhibitor (Promega), 1 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), 5 µl MMLV 5× buffer, 9.5 µl DEPC-H_2O and 50 µM each dATP, dTTP, dCTP, dGTP, was added to each tube, resulting in a final volume of 25 µl. The mixture was incubated at 42°C for 1 hour, then 100°C for 5 mins, and then subjected to PCR. DEPC- dH_2O was used, instead of extracted RNA, as a negative control.

PCR was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer ALVgp85U3 and ALVgp85L3, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 95 ºC for 4 min, 35 cycles of 95ºC for 30 s, 50ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, ALV specimens and DEPC- H_2O were included as positive and negative controls, respectively.

The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.8% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from ALV

Amplified PCR products from ALV specimens were analysed by gel electrophoresis. Specimen that contained ALV-A and ALV-B generated a 466-bp DNA band on the agarose gel (Figure 22).

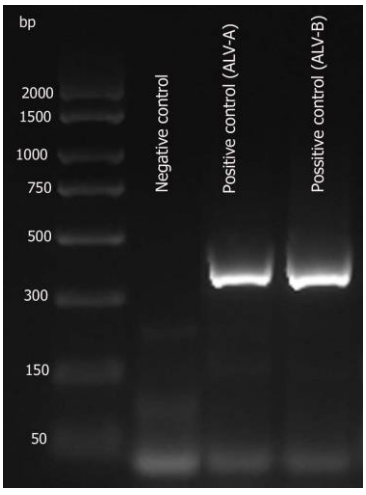

Figure 22. Agarose gel (1.8%) electrophoresis of PCR products from positive controls (inoculated CEF with ALV-A and ALV-B respectively) and negative control (DEPC- H_2O).

RT-PCR for the detection of avian leukosis virus subgroup J

Avian leukosis virus (ALV) is the most common naturally occurring avian retrovirus and causes neoplastic diseases and other production problems in chickens. Historically, Avian leukosis caused by ALV-J has been a significant problem in the Australian boiler breeders. The ALVs which belong to genus *Alpharetrovirus* within the *Retroviridae* family, are presently classified into 10 sub-groups (A-J) of which A, B, C, D and J are exogenous, that is, possess complete infectious particles while E, F, G, H and I are endogenous. Subgroups A, B, C, D, E and J have been detected in chickens, F and G in pheasant, H in partridge and I in quail. Sub-group classification of the ALVs is based on differences that occur in the viral glycoprotein antigens of the viral envelope.

This RT-PCR was adopted from Bagust *et al.* (2004) and optimised for the detection of ALV-J in clinical samples.

RNA extraction

Total RNA was extracted from ALV-J grown in chicken embryo fibroblast (CEF) cells. Two hundred µl of inoculated CEF cell culture was lysed and lysed in 450 µl RLT buffer (Qiagen, Victoria, Australia) with 1% β-mercaptoethanol for two hours at room temperature or overnight at 4ºC. RNA extraction was then performed as described above for ALV.

RT-PCR

The oligonucleotide primers used in RT-PCR detection of ALV-J included the forward primer ALV-J-H5 (5'-GGATGAGGTGACTAAGA -3') that anneals just upstream from the 3' region of the

pol gene and is conserved across several ALV subgroups. The reverse primer ALV-J-H7b (5'-GAACCAAAGGTAACACACGT-3') anneals specifically in the gp85 region of ALV-J (Bagust *et al.*, 2004). This pair of primers is known to be specific for ALV-J and results in a 544 bp PCR product. To produce single stranded cDNA, one µl (25 µM) of oligo dt primer was added to 5 µl of extracted RNA and the mixture heated at 100°C for 1 min, then chilled on ice for 5 mins. Nineteen µl of cDNA master mix containing 1 U RNasin® ribonuclease inhibitor (Promega), 1 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), 5 µl MMLV 5× buffer, 9.5 µl DEPC-H_2O and 50 µM each dATP, dTTP, dCTP, dGTP, was added to each tube, resulting in a final volume of 25 µl. The mixture was incubated at 42°C for 1 hour, then 100°C for 5 mins, and then subjected to PCR. DEPC- dH_2O was used, instead of extracted RNA, as a negative control.

PCR was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer ALV-J-H5 and ALV-J-H7b, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 3 min, 35 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, ALV-J and DEPC-H_2O were included as positive and negative controls, respectively.

The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.8% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from ALV-J

Amplified PCR products from ALV-J specimens were analysed by gel electrophoresis. Specimen that contained ALV-J generated a 544-bp DNA band on the agarose gel (Figure 23).

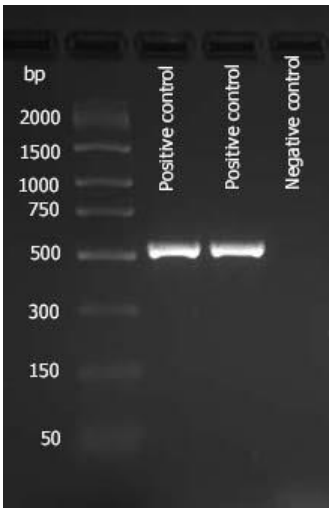

Figure 23. Agarose gel (1.8%) electrophoresis of PCR products from positive controls (inoculated CEF with ALV-J) and negative control (DEPC- H_2O).

Diagnostic RT-PCR for the detection of avian reovirus (ARV)

Avian reovirus (ARV) is an important cause of diseases in poultry. In particular, reovirus-induced arthritis, chronic respiratory diseases, and malabsorption syndrome provoke considerable economic losses. ARV belongs to the genus *Orthoreovirus*. ARV has segmented genomes consisting of 10 genome segments of double-stranded (ds) RNA. The RNA is packaged into a non-enveloped icosahedral double capsid. Diagnosis is dependent on the detection of the virus in clinical samples, although the presence of the virus does not necessarily confirm that this is the cause of the disease, except where reoviruses are detected in affected joints. This RT-PCR was adopted from a previous report (Bruhn *et al.*, 2005) and optimised for the detection of ARV in clinical specimens.

RNA extraction

Total RNA was extracted from avian reovirus grown in chicken embryo kidney cells. Two hundred µl of inoculated cell culture was lysed in 450 µl RLT buffer (Qiagen, Victoria, Australia) with 1% β-mercaptoethanol for two hours at room temperature or overnight at 4 ºC. RNA extraction was then performed as described above for ALV-J.

RT-PCR

A pair of oligonucleotide primers, ARV-S2-P4 (5'- CCCATGGCAACGATTTC -3') and ARV-S2-P5 (5'- TTCGGCCACGTCTCAAC -3'), selected from the conserved region of S2 genome segment as described

before (Bruhn *et al.*, 2005) with the PCR amplicon expected to yield a product of 399 bp. The S2 cDNA was prepared as described above for ALV-J.

PCR was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 60 s, 30 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, ARV and DEPC- H_2O were included as positive and negative controls, respectively.

The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.5% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from ARV

Amplified PCR products from ARV cell culture was analysed by gel electrophoresis. Specimens that contained ARV generated a 399-bp DNA band on the agarose gel (Figure 24).

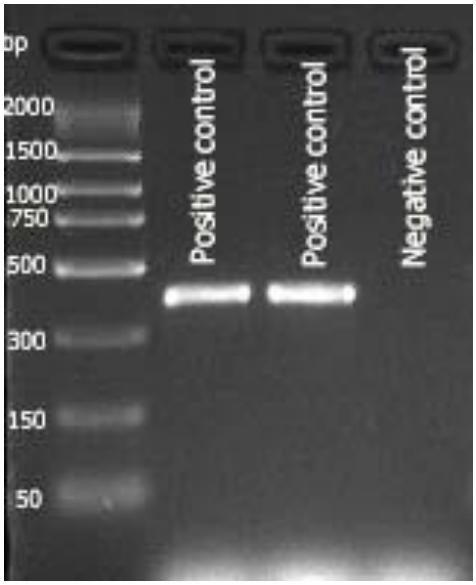

Figure 24. Agarose gel (1.5%) electrophoresis of PCR products from positive controls (inoculated chicken embryo kidney cells with ARV) and negative control (DEPC- H_2O).

RT-PCR for the detection of infectious bursal disease virus (IBDV)

Infectious bursal disease virus (IBDV) is a member of Birnaviridea and the causative agent of IBD or Gumboro disease. Viral genome is dsRNA and segmented. IBD is characterized by destruction of lymphocytes in the bursa of Fabricius (BF) and to a lesser extent in other lymphoid organs. The disease is a major problem in concentrated poultry production areas throughout the world. This RT-PCR was adopted from a published report (Sapats & Ignjatovic, 2002) and optimised in our laboratory for the detection of IBVD in bursal tissues.

IBDV strains

Three vaccine strains and a number of Australian field isolates available in our laboratory were used in this study. The IBDV vaccine strains were V877 (Websters Pty Ltd), D-78 (Intervet Australia Pty Ltd) and Vaxsafe IBD® V877 (, Bioproperties Australia Pty Ltd.). A number of bursal tissue specimens from infected flocks were also used in this experiment.

RNA extraction

All IBDV vaccines were diluted with dH_2O to 4 doses / µl before viral RNA was extracted. Bursal tissue samples were homogenised in DEPC-dH_2O using pestle and mortar and homogenised tissue was clarified by centrifugation (5000 g, 5 min). Two hundred µl of diluted vaccine or 250 µl homogenised bursal tissue was lysed in 450 µl RLT buffer (Qiagen, Victoria, Australia) with 1% β-mercaptoethanol and used for RNA extraction as described for ALV-J..

RT-PCR

A pair of oligonucleotide primers, J1 (5'- GGCCCA GAG TCT ACA CCA TAA C -3') and J2 (5'- CCC GGA TTA TGT CTT TGA AGC -3'), flanking the hypervariable (HV) region of VP2 gene was used as described before (Sapats & Ignjatovic, 2002) with the PCR amplicon expected to yield a product of 743 bp. To produce single stranded cDNA of the hypervariable region of the VP2 gene, one µl (25 µM) of reverse primer (J2) was added to 5 µl of extracted RNA and the mixture was treated for RT-step as described for ALV-J..

Amplification of HV region of VP2 gene was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 60 s, 30 cycles of 94ºC for 30 s, 60ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, IBDV (V877 vaccine strain) and DEPC- H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.5% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from IBDV

Amplified PCR products from IBDV specimens were analysed by gel electrophoresis. Specimens that contained IBDV generated a 743-bp DNA band on the agarose gel (Figure 25).

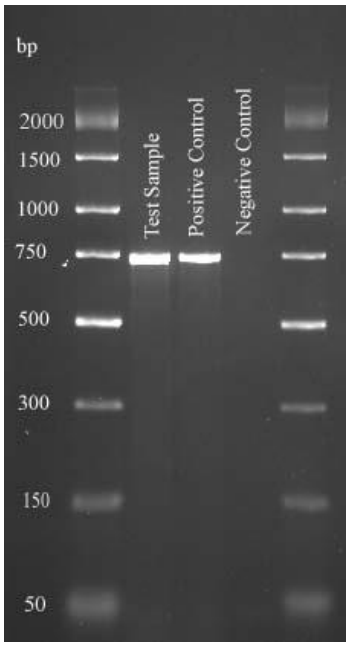

Figure 25. Agarose gel (1.5%) electrophoresis of PCR products from test sample (bursal tissue specimens from an infected flock), positive control (IBDV vaccine strain V877- Vaxsafe IBD® V877) and negative control (DEPC-dH_2O)

Diagnostic RT-PCR for the detection of avian encephalomyelitis virus (AEV)

Avian encephalomyelitis (AE) is a viral infection that occurs in young chickens, pheasants, quail, and turkeys. Young chickens infected with this virus can have clinical signs of ataxia, incoordination, paralysis, or rapid tremors of the head and neck, with high morbidity and variable mortality. In adult laying birds, AE infection causes no neurologic signs, but it can cause a slight reduction in egg production. AEV is mainly an egg-transmitted disease, but infection of poultry with AEV by the fecal–oral route is not uncommon. AEV, a member of the family Picornaviridae, contains a single-stranded RNA genome of positive polarity. This RT-PCR was adopted from Xie *et al.* (2005) and optimised in our laboratory for the detection of AEV.

RNA extraction

Total RNA was extracted from avian encephalomyelitis grown in chicken embryo. Two hundred µl of brain homogenate of inoculated chicken embryo was processed for RNA extraction as described earlier for ALV-J.

RT-PCR

A pair of oligonucleotide primers, MK AE 1 (5'- CTT ATG CTG GCC CTG ATC GT -3') and MK AE 2 (5'- TCC CAA ATC CAC AAA CCT AGC C -3'), selected from the VP2 gene as described before (Xie *et al.*, 2005) with the PCR amplicon expected to yield a product of 619 bp. VP2 cDNA was prepared as described earlier for ALV-J earlier.

PCR was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 60 s, 25 cycles of 94ºC for 30 s, 62ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, AEV and DEPC- H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 1.5% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from AEV

Amplified PCR products from brain homogenate containing AEV was analysed by gel electrophoresis. Specimens that contained AEV generated a 619-bp DNA band on the agarose gel (Figure 26).

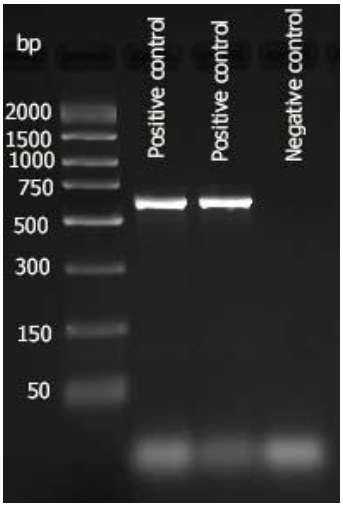

Figure 26. Agarose gel (1.5%) electrophoresis of PCR products from positive controls (brain homogenate of inoculated chicken embryo) and negative control (DEPC- H_2O).

Diagnostic RT-PCR for the detection of avian reticuloendotheliosis virus (REV)

Reticuloendotheliosis viruses (REV) are a group of avian type-C retroviruses that induce reticular and lymphoid tumours in chickens, turkeys and quail. Members of this group include: defective, transforming virus (REV-T), replication competent helper, REV-A, spleen necrosis virus (SNV); chick syncytial virus (CSV), and duck infectious anaemia virus (DIAV). Unlike other RNA viruses, retroviruses replicate through RNA-dependent reverse transcription which leads to chromosomally integrated proviruses. The presence of proviral copies of the retroviral genome can be directly detected from host cell DNA and obviates the preparation of complementary DNA. However, this study is carried out for detection of REV in tissue specimens using RT-PCR. The test was adopted from Aly *et al.* (1993) and can also be performed on DNA extracted from tissue specimens for detection of provirus using PCR protocol.

RNA extraction

Total RNA was extracted from REV grown in chicken embryo fibroblast (CEF) cells. Two hundred µl of of inoculated CEF cell culture was used for RNA extraction as described earlier for ALV-J.

RT-PCR

A pair of oligonucleotide primers, REV LTR 1F (5'- CAT ACT GGA GCC AAT GGTT -3') and REV LTR 2R (5'- AAT GTT GTA CCG AAG TACT -3'), selected from a portion of the unique 3' repeat and unique 5' region of the long terminal repeat (LTR) as described before (Aly *et al.,* 1993) with the PCR amplicon expected to yield a product of 291 bp. LTR region cDNA of REV was prepared by RT as described earlier for ALV-J.

PCR was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 5 µl cDNA, 50 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTp, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). PCR conditions were one cycle of 94ºC for 3 min, 30 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final cycle of 72ºC for 10 min. In each set of RT-PCR reactions, REV and DEPC- H_2O were included as positive and negative controls, respectively.

The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

RT-PCR amplicon generated from REV

Amplified PCR products from CEF cell culture containing REV was analysed by gel electrophoresis. Specimens that contained REV generated a 291-bp DNA band on the agarose gel (Figure 27).

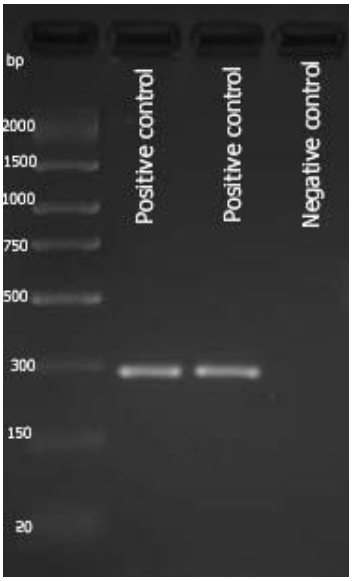

Figure 27. Agarose gel (2%) electrophoresis of PCR products from positive control (CEF inoculated with REV) and negative control (DEPC- H_2O).

Diagnostic PCR for the identification of virulent Avian Pathogenic *Escherichia coli* (APEC)

Avian pathogenic *Escherichia coli* is an economically important respiratory pathogen of chickens worldwide. The development of a PCR test to detect specific strains of the pathogen common in Australian flocks significantly expedites the detection and thus treatment of outbreaks. This test was originally developed in our laboratory (Tivendale *et al.*, 2004) but was further optimised for the detection of pure APEC cultures.

DNA extraction

Total genomic DNA was extracted from 0.2 ml of culture using a DNA extraction kit (QIAGEN) as described for *M. gallisepticum.*

PCR

A part of *iucA, iss and* 16S rRNA genes were chosen for PCR amplification. Four pairs of oligonucleotide primers, iucA Forward (5'- ATGAGAATCATTATTGACATAATT -3'), iucA Reverse (5'- CTCACGGGTGAAAATATTTT -3'), iss Forward (5'- GTGGCGAAAACTAGTAAAACAGC -3'), iss Reverse (5'- CGCCTCGGGGTGGATAA -3') tsh Forward (5'- GGTGGTGCACTGGAGTGG -3'), tsh Reverse (5'- AGTCCAGCGTGATAGTGG -3') 16S Forward (5'- GCTGACGAGTGGCGGACGGG -3'), 16S Reverse (5'- CTCACGGGTGAAAATATTTT -3') flanking the target regions were selected as described before (Tivendale *et al.*, 2004) with expected PCR products of 1482, 762, 642 and 253 bp respectively. Amplification of DNA was performed in 25 µl reaction volume on I-Cycler thermal cycler (Bio-Rad). The reaction mixture contained 2 µl extracted genomic DNA, 2.5 µM of each primer, 2 mM $MgCL_2$, 1250 µM of each dNTP, 1× GoTaq® Green Flexi Reaction Buffer (Promega) and 1 U of Go Taq DNA polymerase (Promega). The 16S rRNA primers were used as a positive control for the presence of *E. coli.* DNA in PCR amplifications. PCR conditions were one cycle of 94ºC for 3 min, 26 cycles of 94ºC for 60 s, 55ºC for 60 s and 72ºC for 60 s, and a final cycle of 72ºC for 10 min. In each set of reactions, E Coli genomic DNA and distilled H_2O were included as positive and negative controls, respectively. The PCR products together with standard molecular weight marker, were analysed by electrophoresis in 2% agarose gel stained with GelRed™ (Biotium, Hayward, CA, USA), and visualised by UV transillumination.

Variable levels of PCR amplicons generated from *E. Coli* in multiplex PCR

Multiplex PCR examined using pure *E. coli* cultures was found to be variable from one run to run specially in relation to the intensity of the DNA bands produced (results not shown). However, when each primer pair was used in separate PCR, the intensity of bands remained consistent from one run to another. Adjustment of the concentrations of the primer pairs appeared to improve the reproducibility of the results to an extent.

In addition, when specificity of this test was assessed against a panel of intestinal bacteria, a number of bacteria were found to produce a visible band with the 16S primer pairs used as internal control (results not shown).

Discussion

Diagnostic assays currently available to the poultry industry through this CRC project

Diagnostic assays already available to the Australian Poultry Industry and the stage of their assessment are outlined in Table 11.

Table 11. Diagnostic assays available to the Australian Poultry Industry and the stage of their assessment

Diagnostic assay	Purpose	Developed	Tested using laboratory specimens	SOP prepared	Used for field diagnostic cases
AE PCR	detection	Y	Y	Y	N
ALV-J-PCR	detection	Y	Y	Y	N
ALV-PCR	detection	Y	Y	Y	N
***Avibacterium paragallinarum* PCR**	detection	Y	Y	Y	Y
CIAV PCR	detection	Y	Y	Y	Y
Chlamydophila PCR	detection	Y	Y	Y	Y
Chlamydia PCR-HRM-16S rRNA	detection and species ID	Y	Y	Y	Y
Avian Pathogenic *E. coli* PCR	detection and virulent strain	Y	Y	Y	Y
EDS PCR	detection	Y	Y	Y	N
***Eimeria* PCR Cap. Elect.**	detection and species ID	Y	Y	Y	Y
***Eimeria* PCR HRM curve analysis**	detection and species ID	Y	Y	Y	Y
FAdV PCR HRM curve analysis	detection and strain ID	Y	Y	Y	Y
Fowl/pigeon POX PCR	detection	Y	Y	Y	Y
IBDV-PCR	detection	Y	Y	Y	Y
IBV PCR	detection	Y	Y	Y	Y
IBV-PCR-HRM curve analysis	detection and strain ID	Y	Y	Y	Y
ILT PCR	detection	Y	Y	Y	Y
ILT-PCR-RFLP	detection and strain ID	Y	Y	Y	Y
MA PCR	detection	Y	Y	Y	Y
MDV-PCR	detection	Y	Y	Y	Y
MG PCR	detection	Y	Y	Y	Y
MG PCR-HRM	detection and strain ID	Y	Y	Y	Y
MM PCR	detection	Y	Y	Y	Y
MS PCR	detection	Y	Y	Y	Y
MS PCR-HRM	detection and strain ID	Y	Y	Y	Y
***Pasteurella multocida* PCR**	detection	Y	Y	Y	Y
Reovirus PCR	detection	Y	Y	Y	N
Reticuloendotheliosis virus PCR	detection	Y	Y	Y	N
***Salmonella spp* multiplex PCR**	detection	N	N	N	N

Number of clinical submissions processed using assays developed under this project (2005-2009)

During the length of CRC project a range of clinical specimens were submitted to our laboratory for diagnostic tests made available to the poultry industry under this project (Table 12). The highest number of specimens related to ILT submitted from Victoria and NSW. These cases were submitted primarily for typing (by PCR-RFLP) of the ILTV involved. Also a significant number of submissions from NSW, Victoria and QLD were related to typing of FAdV and IBV by HRM curve analysis. The number of submissions for other pathogens were relatively lower during the course of this project.

Table 12. Clinical specimen submissions for diagnostic purposes during this CRC project (2005-2009)*

Diagnostic assay	Number of submissions	Number of specimens tested
FAV PCR-HRM	37	111
CIAV PCR	<10	30
Chlamydophila PCR-HRM	<10	<10
Eimeria PCR-HRM	<10	<10
IBV PCR-HRM	25	75
IBDV PCR	<10	<10
E. coli multiplex PCR	<10	20
ILTV PCR-RFLP	150	450
M. gallisepticum PCR	<10	<10
M. synoviae PCR	<10	50
M. meleagridis PCR	<10	<10
MDV PCR	<10	<10
POX PCR	<10	<10

* Excludes submissions for Pathology and/or histopathology and/or bacteriology only

These clinical submissions obviously assisted in assessment of the assays and also resulted in important revelations in relation to a number of poultry diseases that proved problematic during the project. This was particularly evident with epidemiological clues provided to the industry by the use of ILTV PCR-RFLP, FAdV PCR-HRM curve analysis and IBV PCR-HRM curve analysis. The following discussion provides a brief overview on the performance and benefits of the assays from our perspective.

Mycoplasma synoviae *PCR-HRM curve analysis*

The PCR-HRM curve analysis appears to be a reliable tool for detection and differentiation of Australian *M. synoviae* strains from overseas ones. A number of laboratories around the world have adopted this technique for detection and strain identification of *M. synoviae* strains. As part of this project, a collaborative research was carried out with researchers in Netherlands to assess the efficacy of MS-H vaccine for prevention of egg shell abnormalities induced by European *M. synoviae* field strains. This study concluded that that vaccination with MS-H significantly reduces the occurrence of *M. synoviae*-induced egg shell abnormality. The results of this study was published in Avian pathology (Feberwee *et al.*, 2009).

However, due to the dominance of class A *M. synoviae* strains in Australia, differentiation of class A field isolates from MS-H vaccine was found to be challenging, requiring determination of temperature sensitivity phenotype and/or RFLP of genomic DNA. It may be necessary to explore the possibility of other targets as PCR-HRM curve analysis for further differentiation of *M. synoviae* strains in Australia.

Mycoplasma gallisepticum *PCR HRM curve analysis*

Increasing use of the MG live vaccines in poultry has led to a need for a reliable technique that can differentiate MG vaccine strains from field isolates. This is primarily for epidemiological investigation but may also be a requirement of registration authorities when a new MG vaccine is introduced to a country. The use of PCR alone (Evans & Leigh, 2008; Feberwee *et al.*, 2005b) or combined with sequencing (Ferguson *et al.*, 2005; Raviv *et al.*, 2007), RFLP (Khan & Yamamoto, 1989; Klein *et al.*, 1988; Lysnyansky *et al.*, 2005) or AFLP (Feberwee *et al.*, 2005a; Hong *et al.*, 2005) and of RAPD (Feberwee *et al.*, 2005a; Ferguson *et al.*, 2005; Geary *et al.*, 1994), and PFGE (Marois *et al.*, 2001; Mettifogo *et al.*, 2006) have been described for differentiation of MG isolates/strains. Although these techniques may currently be used in some laboratories, they have limitations such as low reproducibility, lengthy procedure and need for extensive interpretation, particularly when a large number of specimens are to be tested.

PCR developed in this project demonstrated a discriminatory power to differentiate ts-11 vaccine strain and MG field isolates based solely on the amplicon size. The ts-11 vaccine strain produced a single DNA band (226 bp) while some isolates produced two (Ap3AS, K1659) or three bands (86134, 87006 and 87081). It has been shown that MG has a large number of *vlhA* gene copies distributed in five distinct loci throughout the genome (Baseggio *et al.*, 1996; Papazisi *et al.*, 2003). Thus, generation of more than one amplicon is expected since a relatively high sequence identity exists between promoter regions of the *vlhA* gene family members. Irrespective of the basis for amplification of more than one amplicon, variation in the number of the amplicons may indeed have contributed to the power of the HRM technique in differentiating different MG strains. Variation in size and number of amplicons could have sufficed for differentiation of a number of MG strains by examination of the PCR products on an agarose gel. However, some strains were found to generate the same number of bands with similar sizes. For example strain K1453 and F both generated a single band of similar size (Figure.1). Thus discrimination of these strains using agarose gel electrophoresis may not be possible. In contrast, these strains were readily distinguishable from each other in conventional and normalised HRM curve analysis. The melting profile of a PCR product is dependent upon length, sequence, GC content, and heterozygosity (Reed *et al.*, 2007). There was no correlation between the number of *vlhA* bands and the number of peaks. Although the number of peaks could be influenced by the number of bands and distribution of the nucleotides in the amplicon. The first shoulder peak of S6, F and K1453 is likely to be due to A-T rich region which melts at lower temperature and the second peak is likely to be due to melting G-C rich region at a higher temperature. For specimens such as 87006 with 3 PCR

bands, the major band produces one peak and the 2 extra bands may perhaps contribute to the shape of the curve. In this study, ts-11 was used as a model to demonstrate the consistency of HRM curve profiles after passage *in vitro* and *in vivo*. All ts-11 vaccine reisolates generated one peak at 76.5 ± 0.3 in conventional melt curve and genotyped as ts-11 with 63.1 or higher GCP. In contrast the highest GCP between ts-11 and other strains used in this study was 19.5%. This illustrates the discriminatory power of HRM curve analysis for differentiation of ts-11 from other MG strains. The PCR HRM curve analysis could also differentiate MG strains that generated amplicons with high sequence identities. For example, the MG strains 6/85, 86134 and Ap3AS had a 100% sequence identity but had distinct conventional and normalised melt curves. Likewise, strains 87081 and K1453 generated distinct melt curve profiles although had only a relatively small difference (7.2%) in their similar size (319 bp) amplicons.

The capacity of PCR-HRM technique in differentiating MG strains/isolates was further evaluated by testing additional thirty MG isolates from different geographical locations including Europe, USA and Israel. Interestingly a field isolate from USA (K6216D) was found to generate similar conventional and normalised melt curves to those of ts-11 with 95 GCP. This isolate had been isolated from progenies of ts-11 vaccinated broiler breeders and found to be indistinguishable from ts-11 by RAPD (personal communication with Dr Naola Ferguson, University of Georgia, USA). The USA MG isolates K5917 and K6096 were found identical by HRM curve analysis. These two isolates had similar mgc2 sequences and were found undistinguishable in the host laboratory (personal communication with Dr Naola Ferguson, University of Georgia, USA). The MG isolates K5037A and K5792D were also found to have similar HRM curves however were reportedly different in their IGSR sequences (Raviv *et al.,* 2007). The cause of this discrepancy is unknown and may require further examination by nucleotide sequencing of other genes. The two Israeli MG strains VR5 and BCV-6 that were found indistinguishable by HRM melt curve analysis, had been isolated from broiler breeders located at different farms but, epidemiologically appeared to be linked (personal communication with Dr Inna Lysnyansky, Kimron Veterinary Institute Israel). No apparent epidemiological links existed between the other two Israeli strains (MSA-9 and MKT-6) that were also indistinguishable by HRM curve analysis. These isolates are currently under further examinations in the host laboratory.

The newly developed PCR HRM technique provides a rapid and reliable means for detection and differentiation of MG strains from both culture and clinical swabs.

IBV PCR-HRM curve analysis

Assessment of this test under routine clinical conditions demonstrated the capacity of the IBV HRM curve analysis to reliably detect and differentiate IBV strains from field submissions. A novel recombinant strain was also detected.

ILTV PCR-RFLP

Application of this test for examination of the clinical specimens from NSW and Victoria raised questions as to the origin of the class 8 virus. It may simply be a true wild-type isolate which by chance, shows molecular similarity to the class 7 virus. As was the case with Guy, Barnes and Smith's work (Guy *et al.,* 1991) where a vaccine virus was found to undergo an increase in virulence, class 8 may be a reverted form of the Nobilis Serva strain which is only rendered by sequential *in vivo* passages in chickens. Or, it may be a viral subpopulation within the Nobilis ILT vaccine preparation. Garcia and Riblet (Garcia & Riblet, 2001) discovered the presence of quasispecies within chicken embryo origin vaccines in the US, using viral plaque-purification techniques. Selection of these viral sub-populations may occur in the field and create a

more complex viral population in an outbreak situation, especially when the virus can establish a state of latency in infected birds. Studies in Northern Ireland (Graham *et al.*, 2000), Taiwan (Chang *et al.*, 1997) and the US (Garcia & Riblet, 2001) have each speculated that since the introduction of live attenuated ILTV vaccines, vaccine strain viruses have displaced wild-type viruses and have been responsible for many of the ILT outbreaks occurring in these regions.

Application of PCR-RFLP for typing ILTVs during this project also revealed that the outbreak involving a single broiler company in central Victoria was short-lived and related to class 6 ILTV genotype. The geographical isolation of this particular company probably evaded a larger outbreak situation. Biosecurity measures may not be as effectively implemented in smaller-scale farm environments, such as game bird flocks. The presence of a class 3 ILTV isolate in game bird flocks in both NSW and Queensland may suggest a movement of this class 3 virus with game-bird transport. This emphasises the fact that ILTV can also be a problem for "back-yard" and other small-scale poultry operations, which are not usually vaccinated and may act as a reservoir for the virus in the Australian poultry population.

Fulton, Schrader and Will (2000) found that vaccine administration by eye-drop provides more consistent flock immunity than aerosol or drinking water administration. A lack of complete flock immunity results in the increased risk of horizontal transmission to naïve birds and can result in vaccine-related outbreaks such as the case described in this study involving a class 7 isolate in the Sydney basin area (Table 6). Paradoxically, it is the density of farms in certain regions such as Victoria's Mornington Peninsula and New South Wales' Sydney basin area that is one of the reasons that this disease is so hard to eradicate. Further investigation into the involvement of air/wind and insects may be necessary to fully understand the route of transmission of ILTV in these provincial outbreaks (Goodwin & Waltman, 1996; Johnson, 2005). Dufour-Zavala (Dufour-Zavala, 2008) describes an industry-wide cooperative management system implemented by the United States Animal Health Association, which involves a decision to vaccinate a zone of farms based on a vote amongst committee members of companies involved upon the diagnosis of a first case of ILT in the area. A coordinated uniform vaccination approach such as this one over a 14-16 week period will ensure complete flock immunity and may be an effective measure against ILT in Australia's endemic regions.

Genotyping of ILTV isolates can aid in the understanding of the behavior and spread of this endemic virus. The appearance of a new fragment in a RFLP digestion pattern means that there has been a detectable nucleotide change to a restriction recognition site, demonstrating the need to gain further information from other areas of the genome to achieve a greater understanding of the conserved and variable regions of the ILTV genome and how this is linked to phenotypic viral diversity. Examination of other genes such as gM/UL9 and gG/UL47 (Oldoni & Garcia, 2007) may be useful to confirm the relatedness of the classes designated in this study. Complete sequence analyses of individual genes may reveal further information on where additional single nucleotide polymorphisms (SNP) lie and which may be used to design faster classification techniques such as real time PCR with high resolution melt curve analysis (HRM) or single strand conformation polymorphism (SSC) analyses. Creelan (Creelan *et al.*, 2006) used a real-time PCR assay to amplify a region of the ICP-4 gene and RFLP to identify the presence of a SNP, but the HRM technique alone would be able to achieve this is a single reaction vessel in less time.

Chlamydiaceae spp *PCR-HRM curve analysis*

Application of this technique during the course of this project revealed its potential for rapid and simultaneous detection and identification of *Chlamydiaceae spp*. in animals particularly chickens and demonstrate the capacity of this system for rapid identification of new *Chlamydiaceae spp*. in animals during routine diagnostic testings.

Eimeria spp *PCR-HRM curve analysis*

This project applied, for the first time, real-time PCR followed by melt curve analysis for detection and classification for *Eimeria* species and also provided a comparison between this newly developed method and CE for detection of impurities in the *Eimeria* vaccine batches. Current molecular methods for differentiation of pathogenic chickens *Eimeria* species include nucleotide sequence analysis and CE. Nucleotide sequencing is now available to many diagnostic facilities at a low cost and CE is also relatively inexpensive to perform. However both these techniques require sophisticated equipment, are time consuming procedures and require skill for interpretation of the results. Performing HRM melt curve analysis also requires relatively expensive equipment (real-time thermocycler) although in recent years with the introduction of real-time PCR assays for varieties of pathogens, a real-time thermocycler has become an inevitable and essential piece of equipment in every diagnostic laboratory. In contrast to nucleotide sequencing and CE, the HRM curve analysis is rapid and convenient, with all relevant procedures including PCR and melt curve analysis able to be performed in a single tube and one machine. An additional advantage of the HRM curve analysis is that it can be performed in an automated module obviating the need for extensive interpretation of results. Furthermore, with each unknown specimen, a library of prototype profiles can be used to facilitate determination of the identity of the profile and possible inter-species variation or facilitate the discovery of a new species. Research within our laboratory has shown that such a library of prototype PCR products (which include SYTO®9) can be reused several times without a detectable effect on the melting curve temperature (results not shown). This is in agreement with previous studies (Herrmann *et al.*, 2006; Morris *et al.*, 2007b) in which SYBR Green I was compared with SYTO®9, with SYTO®9 shown to produce highly reproducible DNA melting profiles and to be less selective in its incorporation into particular amplicons. Experience with HRM curve analysis in our laboratory has shown that a higher level of consistency and reproducibility of HRM curve profiles are achieved when similar quantities of PCR products are used for comparison (unpublished data). In this study, adjustment of the quantity of PCR products prior to HRM curve analysis was carried out by a preliminary PCR, visualization and quantification of the PCR bands on an agarose gel and subsequent adjustments of the template DNA. Alternatively, adjustment of the quantity of PCR products prior to HRM curve analysis can be carried out rapidly by spectrophotometry and adjustment of the template DNA. Results from this study show that the HRM method can detect species impurities (variations / contaminants) more reliably than CE, although visual examination of the HRM curves could only determine which species was contaminating the dominant species in some cases. Further research and analysis may be required to investigate the criteria for interpretation (visual and software based genotyping) of curves of mixed species samples. Once these criteria are established, determining the contaminations in an otherwise 'pure' species will be feasible. Only two combinations of *Eimeria* species. (*E. maxima* and *E. necatrix*; *E. acervulina* and *E. tenella*) were used in this study to investigate the capacity of the HRM curve analysis for detection of minor populations. The combination of *E. maxima* and *E. necatrix* were chosen since both these species inhibit mainly the same part of the intestinal tract (mid-intestine). The combination of *E. acervulina* and *E. tenella* was also chosen to examine the combination of two other *Eimeria* species in the Eimeriavax^{R} 4m that was available for this study. Results from this study also showed that the HRM curve analysis can detect small differences between specimens identified by other techniques as the same species (e.g. samples of *E. brunetti*). Whether the difference detected was a reflection of interstrain ITS-2 sequence variation or due to impurities in some of the specimens was not investigated in this study. However, this highlights an additional advantage of HRM curve analysis that may also be useful for detecting new or mutated species in the field. Although quantification of *Eimeria spp* was not a primary aim of this study, the HRM curve analysis described here demonstrated some capacity to do so. A number of previous studies (Blake *et al.*, 2006; Blake *et al.*, 2008; Swinkels *et al.*, 2006; Swinkels *et al.*, 2007) have developed assays for quantification of *Eimeria spp* but they lack the cross-species breadth of the real-time PCR HRM curve analysis described here. Further studies are necessary to fully investigate the capacity of the real-time PCR HRM curve analysis for quantification of *Eimeria spp*. The neat (extracted

Eimeria-) DNA used in PCR contained DNA from approximately 2 x 10^5 oocyts per 2 µl. Considering a 10^{-4} dilution of the template for *E. maxima* and *E. acervulina* used as examples to examine the capacity of the HRM curve analysis for detection of minor oocyst population (see Table 10), it can be postulated that the HRM curve analysis can potentially detect as few as 20 contaminant oocysts in 2 x 10^5 alternative oocyst population. In summary, the combination of PCR and high resolution melting curve analysis is a rapid and specific technique for the characterisation of *Eimeria* species. The entire process including PCR and HRM curve analysis can be completed within 2-3 h. This time-frame obviously excludes the time required for recovery of oocysts and extraction of DNA prior to PCR. Studies are currently under way in our laboratory to adopt and adapt this assay as the first stage tool for rapid direct identification of *Eimeria* in clinical specimens collected from diseased birds.

FAdV PCR-HRM curve analysis

Application of FAdV PCR-HRM curve analysis for typing of Australian FAdVs revealed that this test is a superior diagnostic method for FAdV identification to virus neutralisation and direct sequence analysis, with regards to accuracy and speed. Furthermore, it provided evidence that IBH in Australian broiler flocks is a primary disease resulting from two alternative FAdV strains from difference species groups, in the absence of the immunosuppressive viruses IBDV and CIAV.

Other tests

A number of diagnostic assays for other important poultry pathogens were optimised and tested using laboratory and/or field specimens. These included PCR assays for the detection of CIAV, *Pasteurella multocida*, *Avibacterium paragallinarum*, MDV, IBDV and ALV-J. Optimisation process included evaluation of different pairs of primers from previously published papers to achieve maximum specificity and sensitivity.

The PCR assay for detection of *Avibacterium paragalinarum* could detect the pathogen in pure cultures as well as swabs taken from experimentally-infected birds.

The CIAV PCR assay was optimised with three different pairs of primers and their sensitivities were compared. Two pairs of primers appeared most sensitive and selected and further optimised for the detection of CIAV in tissue specimens. These two tests performed satisfactorily (in terms of sensitivity) for CIAV in cell cultures but found to have less sensitivity for the virus in thymic tissues. In order to increase the sensitivity of the test for tissue samples, a third set of primers were designed and used in a semi-nested PCR. The latter test was found to be ten-fold more sensitive than the two former ones but may require further optimisation to determine its reproducibility and specificity.

The PCR assay for detection of *Pasteurella multicida* was optimised with two different pairs of primers but both assays were capable to detect the cultured bacteria.

The MDV PCR assay was carried out using three different pairs of primers and one pair producing consistent results was selected and optimised further. The test was capable of detecting MDV in tissue specimens as well as swabs taken from affected tissues.

The IBDV RT-PCR assay was evaluated and optimised with two different pairs of primers from VP2 gene and both were capable of detecting IBDV in bursal homogenates and swabs taken from bursal tissue. Since the primers amplify a hypervariable region of VP2 gene, genotyping of IBDV using amplicons generated in this PCR may be possible.

The PCR / RT-PCR assays for EDS, ALV, ALV-J, ARV, AEV and REV were also optimised but could not be further evaluated due to lack of clinical specimen submissions.

Real-time PCR assays for the detection of *Salmonella spp* was not developed in this project as a collection of reference species/strains was not available in our laboratory.

Recommendations for future work

Assays developed during this project are already in use by the Australian poultry industry. However, accreditation of the tests by a National Association of Testing Authorities (NATA) is required to bring the tests to the standard acceptable by all users and purposes specially vaccine companies and quality control of the pharmaceutical products related to poultry. Prerequisites for NATA accreditation may be establishment of internal controls for all the tests as well as determination of sensitivity of the tests. A further step towards full accreditation of these assays should involve in obtaining an international accreditation authorities possibly to OIE standards.

The sensitivity and specificity of the tests (specially as it relates to specimens containing mixed species/strains of the same organism) is particularly important for assays involving HRM curve analysis. There is no standard procedure for this task since HRM curve analysis is a relatively new technology. This means that future work towards determination of sensitivity and specificity of HRM curve analysis may be extensive.

A number of assays designed or adopted from previously published works were found to require further optimizations. These include *Pasteurella multocida* PCR that appeared to perform satisfactorily when pure cultures used but found somehow less reproducible when swabs from infected tissues were directly examined. Also, a new semi-nested CIAV PCR was developed in this project and on preliminary assessments using cultured viruses found to be highly sensitive. However, the sensitivity of this assay will need to be evaluated for infected tissues including blood samples. Also further work may be required to optimise APEC PCR for a multiplex set up.

It is also suggested that any future work should involve in development/adoption of detection assays for less significant disease of poultry that may prove significant in future. This is beneficial for two reasons: 1) the Australian poultry industry will have an immediate access to diagnostic assays that emerge at a particular point of time and 2) the tests will be used on a regular basis as differentials for other more common diseases. As an example, a test for detection of Avian Nephritis Virus (ANV) is highly desirable at this point of time. Our laboratory received a number of suspected ANV cases from NSW and Victoria in 2009. These cases proved challenging for conventional poultry diagnostic laboratories as they were very similar in lesions to the renal form of IB. Use of IBV PCR-HRM curve analysis proved in our laboratory that field IB strains were not involved in these cases. Similarly, diagnostic assays for Big Liver Spleen (BLS) disease will be useful for the poultry industry.

Detection and strain identification assays for exotic diseases such as ND (Newcastle Disease), highly pathogenic AI (Avian Influenza), turkey Rhinotracheitis (TRT) and *Ornithobacterium rhinotracheale* (ORT) were not included in the current project. However depending upon approval of the Department of Primary Industry, these tests can be developed /adopted in future.

Acknowledgments

Funding to support *Mycoplasma synoviae* research was provided by the Australian Research Council. Also Dr Youssef Abs EL-Osta is thanked for assistance with SSCP techniques and Elchrom Scientific for providing equipment and reagents for SSCP.

Funding to support infectious bronchitis virus research was provided by the Australian Egg Corporation Limited (AECL). We also thank Naomi Kirkpatrick and Denise O'Rourke for technical assistance throughout this study and Mr Garry Anderson for help with statistical analyses.

Funding for *Mycoplasma* research project was directly provided by the Australian Poultry CRC. We also like to thank Professor Janet Bradbury (University of Liverpool, UK), Dr Naola Ferguson-Noel (University of Georgia, USA) and Dr Lysnyansky Inna (Kimron Veterinary Institute, Israel) for providing *mycoplasma gallisepticum* isolates and also Mr Garry Anderson for providing advice on statistical analysis of the data.

Funding for infectious laryngotraceitis virus research project was also directly provided by the Australian Poultry CRC. We would like to thank Dr. Peter Scott for scientific input, Peter Cowling, Rebecca Agnew and Kylie Hewson for technical support, the Victorian and New South Wales Department of Primary Industries (DPI) for the supply of some ILTV isolates, and field veterinarians for submission of clinical cases that were suspected to involve ILT viruses.

The Chlamydia research project was jointly supported by the Australian Rural Industries Research and Development Corporation (RIRDC) Chicken Meat Branch and the Australian Poultry CRC. We also thank Professor Daisy Vanrompay, University of Ghent, Belgium, for providing the Cp. psittaci genomic DNA used in this study.

Funding for Eimeria research project was directly provided by the Australian Poultry CRC. We would like to thank Tony Belfiore and Penelope Steer for technical support.

Fowl adenovirus research project was supported by Chicken Meat Program grant UM-73A, from the Rural Industries Research and Development Corporation (RIRDC) of the Australian Government. We would like to thank Intervet International (Boxmeer, Netherlands) for providing the FAdV reference serotypes, and Tom Grimes for his assistance and input.

We would also like to than Dr Pat Blackal (Department of Primary Industries and Fisheries, QLD) for providing methodology for *Pasteurella multocida* and *Avibacterium paragallinarum* PCRs, Dr Fakhrul Islam (university of New England) for providing MDV strains for testing, Denice O'Rourke (University of Melbourne) for providing various viral and bacterial organisms and Dr Jagoda Igniatovic for advising on IBDV detection and strain differentiation.

Appendix 1: published or submitted articles presenting results directly related to this project

1. Feberwee A, Morrow CJ, Ghorashi SA, Noormohammadi AH, Landman WJ. (2009) Effect of a live Mycoplasma synoviae vaccine on the production of eggshell apex abnormalities induced by a M. synoviae infection preceded by an infection with infectious bronchitis virus D1466. Avian Pathol. 38(5):333-340

2. Ghorashi SA, Noormohammadi AH, Markham PF (2009) Differentiation of Mycoplasma gallisepticum strains using PCR and High Resolution Melting Curve Analysis. Microbiology. Dec 24. [Epub ahead of print]

3. Kirkpatrick NC, Blacker HP, Woods WG, Gasser RB, Noormohammadi AH. (2009) A polymerase chain reaction-coupled high-resolution melting curve analytical approach for the monitoring of monospecificity of avian Eimeria species. Avian Pathol. 38(1):13-19

4. Blacker HP, Kirkpatrick NC, Rubite A, O'Rourke D, Noormohammadi AH. Emergence of new Infectious Laryngotracheitis Viruses in Australia (Submitted to AVJ)

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Plain English Compendium Summary

Project Title:	Transfer of Diagnostic Technologies from Research Laboratories to a Core Diagnostic Facility for the Benefit of the Australian Poultry Industry
Project No.:	05-17
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Objectives	The main objectives of this study were to standardise and make available the previously developed molecular techniques (through CRC- or non-CRC funded projects) in a NATA accredited reference diagnostic laboratory for express and cost effective diagnostic service and epidemiological analysis of major poultry diseases in Australia.
Background	Conventional poultry diagnostic laboratories in Australia rely on serology as a method of disease diagnosis, or other indirect and less sensitive examinations including histology. This proposal sought to bring previously developed 'state of the art' diagnostic technology to the Australian poultry industry for the DIRECT assessment of an agent, together with strain differentiation, which has become a real issue as more and more live vaccines enter the market.
Research	The extent of the methodology used in this project was based on the development of the diagnostic assays in several phases. Diagnostic assays were classified into six groups according to their development status although a number of assays were developed according to the urgent requirements of the industry at the time.
Outcomes	The outcomes of this project facilitated the establishment of a core diagnostic centre for poultry diseases in Australia. This laboratory can provide rapid and reliable world-class diagnostic services for the Australian poultry industry. Examples of the diagnostic assays are overnight diagnosis and strain identification for infectious bronchitis virus, *Chlamydophila psittaci*, *M. synoviae*, *M. gallisepticum*, avian adenoviruses, etc. The cost of the tests would be minimal compared to the currently available services. This will enable the core centre to rapidly solve at a low cost epidemiological question related to major poultry diseases in Australia. The socio-economic benefits are: (i) enhanced focus on avian health through the developed biotechnological diagnostic assays; (ii) improved and sustainable control of important pathogens; (iii) further applications to pathogens of national and global significance using developed technologies; (iv) strengthening the links between fundamental and applied research; and (v) enhancing the quality and quantity of scientifically skilled people.
Implications	Nineteen diagnostic assays are available to the Australian Poultry Industry and the stages of their assessment are outlined. During the length of CRC project a range of clinical specimens were submitted to our laboratory for diagnostic tests made available to the poultry industry

	under this project. The highest number of specimens related to ILT submitted from Victoria and NSW. These cases were submitted primarily for typing (by PCR-RFLP) of the ILTV involved. Also a significant number of submissions from NSW, Victoria and QLD were related to typing of FAdV and IBV by HRM curve analysis. The number of submissions for other pathogens were relatively lower during the course of this project.
Publications	1. Feberwee A, Morrow CJ, Ghorashi SA, Noormohammadi AH, Landman WJ. (2009) Effect of a live Mycoplasma synoviae vaccine on the production of eggshell apex abnormalities induced by a M. synoviae infection preceded by an infection with infectious bronchitis virus D1466. Avian Pathol. 38(5):333-340 2. Ghorashi SA, Noormohammadi AH, Markham PF (2009) Differentiation of Mycoplasma gallisepticum strains using PCR and High Resolution Melting Curve Analysis. Microbiology. Dec 24. [Epub ahead of print] 3. Kirkpatrick NC, Blacker HP, Woods WG, Gasser RB, Noormohammadi AH. (2009) A polymerase chain reaction-coupled high-resolution melting curve analytical approach for the monitoring of monospecificity of avian Eimeria species. Avian Pathol. 38(1):13-19 4. Blacker HP, Kirkpatrick NC, Rubite A, O'Rourke D, Noormohammadi AH. Emergence of new Infectious Laryngotracheitis Viruses in Australia. (Submitted to AVJ)