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& DEVELOPMENT CORPORATION

Attenuation and characterisation of *Eimeria* spp.

A report for the Rural Industries Research and Development Corporation

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FOREWORD

This publication describes the outcomes of Rural Industries Research and Development Corporation project No DAQ-215AJ "Attenuation and characterisation of *Eimeria* spp. for use in a living vaccine for avian coccidiosis (Stage 2)."

The first of this series of projects with the same title (EIRDC DAQ 25E & CMRDC DAQ 29CM) was initiated in 1993. The main aim of the project was to develop precocious lines of *E. maxima* and *E. acervulina* suitable for use in a live poultry coccidiosis vaccine. The objectives were to isolate and purify field strains of the two species, attenuate the strains by selecting for precocious development and characterise the precocious lines in terms of pathogenicity and protection. This Stage 1 project finished in 1996 with all objectives successfully achieved.

On 4 March 1996 a DPI/RIRDC/poultry industry sponsored poultry coccidiosis vaccine workshop was held at the Animal Research Institute to discuss options for development of a live coccidiosis vaccine in Australia. Further research on coccidiosis vaccines in Australia was supported with activities to concentrate on development of live vaccine lines of *E. tenella* and *E. necatrix*. It was also considered important that a commercial partner be identified and involved with the further development of these vaccine lines.

Hence, the Stage 2 project (RIRDC DAQ-215AJ) was developed. The objectives of the project were to isolate and purify field strains of *E. tenella* and *E. necatrix*, attenuate the strains by selecting for precocious development and characterise the precocious lines in terms of drug sensitivity, reproductive potential, pathogenicity and protection against homologous and heterologous challenge. Other objectives included the development of DNA based techniques to differentiate the seven species of poultry *Eimeria* and creation of a cryopreserved bank of purified field isolates of the seven species. *Eimeria* Pty Ltd was identified as the commercial partner and agreements between *Eimeria*, DPI and RIRDC have been entered into. A productive working relationship has developed between researchers at DPI and Grant Richards of *Eimeria* and the vaccine strains developed and characterised in Stages 1 and 2 are in the process of being registered in Australia.

A proposal for a new project (Stage 3) has been submitted to RIRDC. In that project one strain each of *E. brunetti*, *E. praecox* and *E. mitis* will be selected from the bank of isolates collected during DAQ 215AJ and modified for precocious development. The three precocious lines will then be characterised as for the previous vaccine lines.

The final outcome of the three stages of the project series will be the availability, to the Australian poultry industry, of live precocious vaccines against the seven species of *Eimeria* that cause poultry coccidiosis. Vaccination is now being used routinely to protect flocks in the USA and some European countries including Britain. The benefits of using live coccidiosis vaccines include long term, economical protection against disease; ability to manage existing and developing chemical resistance; and provision of an alternative to chemical control to minimise residue and withholding period problems.

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

This report, a new addition to RIRDC's diverse range of over 450 research publications, forms part of our Chicken Meat and Egg Program Committee's Flock Health and Disease Management R&D sub-programs, the aims of which are to improve and maintain the Australian poultry industry's flocks health status and productivity, and to minimise disease outbreaks and develop containment strategies.

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Managing Director
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Abbreviations

ANOVA	analysis of variance
ARI	Animal Research Institute
bp	base pair
DMSO	Dimethyl sulphoxide
DPI	Department of Primary Industries, Queensland
ITS1	first internal transcribed spacer of rDNA
MEM	minimum essential medium
PCR	polymerase chain reaction
RAPD	randomly amplified polymorphic DNA
rDNA	ribosomal DNA
RIRDC	Rural Industries Research and Development Corporation
SSU	small subunit of rDNA, formerly known as 18S region

Contents

Executive Summary

The RIRDC/DPI funded project entitled "Attenuation and characterisation of *Eimeria* spp. for use in a living vaccine for avian coccidiosis (Stage 2)" was initiated in 1996 with the following five objectives:

1. Isolate and purify field strains of *E. tenella* and *E. necatrix*.
2. Modify prepatent period of one strain of each of these species by selecting for precocious development.
3. Characterise these modified strains in terms of drug sensitivity, reproductive potential, pathogenicity and protection against homologous and heterologous challenge.
4. Develop DNA based techniques to differentiate *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. praecox* and *E. mitis*.
5. Create a cryopreserved bank of purified field isolates of the seven poultry *Eimeria* species.

Project outcomes are summarised below under the five objective headings.

1. Isolate and purify field strains of *Eimeria tenella* and *Eimeria necatrix*.

Parasites used in live coccidiosis vaccines must have the properties of low virulence and high susceptibility to anti-coccidial compounds (coccidiostats). Therefore, parent strains used for vaccine development were collected from small, non-commercial flocks that did not routinely use coccidiostats and in which clinical coccidiosis was not a problem. Three isolates of *E. tenella* and two isolates of *E. necatrix* were collected from flocks of this type. In addition, two virulent isolates each of *E. tenella* and *E. necatrix* were collected as challenge strains to test the immunity induced by the vaccine lines. All isolates were purified by series of single oocyst passages (inoculating a single oocyst into a chicken and allowing it to reproduce). Purified isolates were then stored in liquid nitrogen.

2. Modify prepatent period of one strain of each of these species by selecting for precocious development.

The prepatent period of the parasites was modified by serial passaging of the parasites through susceptible chickens, in each case using the oocysts recovered earliest from the previous passage. The prepatent periods (and virulence) of two isolates each of *E. tenella* and *E. necatrix* were successfully reduced in this way. Passaging of one isolate of *E. necatrix* (Mc Gregor) was discontinued because the prepatent period was not dropping as expected. The Redlands and Darryl strains of *E. tenella* each underwent 15 selection passages with resultant drops in prepatent period of 24 and 23 hours. The Medichick and Gatton strains of *E. necatrix* each underwent eight selection passages with resultant drops in prepatent period of 25 and 24 hours.

3. Characterise these modified strains in terms of drug sensitivity, reproductive potential, pathogenicity and protection to homologous and heterologous challenge.

The Redlands and Darryl strains of *E. tenella* and the Medichick and Gatton strains of *E. necatrix* were characterised in randomised block design cage trials for drug sensitivity, pathogenicity and protection against homologous and heterologous challenge. All the strains are highly susceptible to Toltrazuril with Sulphaquinoxaline having a significant effect on the two *E. tenella* strains and the Medichick strain of *E. necatrix*. An Amprolium based product had little effect on any of the strains except the Darryl strain of *E. tenella*. After selection for precocious development, all the strains demonstrated a reduction in oocyst output compared to the parent strain, but the greatest effect was seen in the *E. tenella* strains. All the strains were highly protective against homologous challenge and against challenge with two different, virulent field isolates (heterologous challenge).

4. Develop DNA based techniques to differentiate *E. tenella, E. necatrix, E. acervulina, E. maxima, E. brunetti, E. praecox* and *E. mitis*.

During the course of the project, six different techniques were evaluated to develop molecular assays to detect and identify the seven species of poultry *Eimeria*. The most useful techniques are PCR tests targeting first internal transcribed spacer (ITS1) rDNA and sequencing of small subunit rDNA. The latter is not suitable for detecting minor contamination of vaccine stocks with other species. It is, however, extremely useful for confirming the identity of purified strains and for investigating the identity of putative new species. The former approach is effective, but the ITS1 region has been found to be more

variable than is ideal for the highly sensitive and species specific PCR test envisaged. Comparison of sequences of this region of DNA from Australian, European and American isolates reveals considerable variation that may affect the ability of these assays to detect all isolates of one species. Overall this approach shows good promise when PCR primers that are appropriate for Australian isolates are used. Once test validation has been completed, the assays will be able to be used both to identify species causing infections and to detect contamination of vaccine stocks with other species.

5. Create a cryopreserved bank of purified field isolates of the seven poultry *Eimeria* species.

Four or more purified isolates of each of the seven species of poultry *Eimeria* have been collected, purified by single oocyst passage and cryopreserved in liquid nitrogen. This library of isolates will prove valuable for future studies of *Eimeria*.

Conclusion

The objectives of this Stage 2 project have been successfully achieved and are reported in detail in the following document. The Redlands strain of *E. tenella* and the Medichick strain of *E. necatrix* have been transferred to *Eimeria* Pty Ltd for incorporation in a new quadrivalent vaccine. A proposal for a new project (Stage 3) has been submitted to RIRDC. That project will include the modification for precocious development of one strain each of *E. brunetti*, *E. praecox* and *E. mitis* selected from the bank of isolates. The efficacy of these three precocious lines will then be evaluated similarly to previous species.

1. Introduction

Coccidiosis is an enteric disease caused by parasitic protozoa. The parasites are transmitted via an infective stage, the oocyst, in the faeces of parasitised animals and, because there is a series of asexual and sexual reproductive cycles in the host, numbers may build up rapidly. Large numbers of the parasites may lead to debilitation and death of the host animal. In domestic fowl, coccidiosis may be caused by seven species in the genus *Eimeria*: *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. All seven species have been recorded in Australia (Callow, 1984; Jorgensen, Stewart, Jeston, Molloy, Blight & Dalgliesh, 1997). All except one, *E. praecox*, have been implicated in commercially important outbreaks of coccidiosis on Australian poultry farms.

The control of coccidiosis in the Australian poultry industry has, to date, largely relied on the routine use of anti-coccidial compounds (coccidiostats). There are, however, several current and emerging problems with the use of coccidiostats. Parasite resistance to currently available coccidiostats has been well documented overseas (Chapman & Shirley, 1989; Stephan, Rommel, Daugschies & Haberkorn, 1997) and is suspected of becoming more common in Australia. As a result, management strategies are becoming more complex and more expensive. This is further complicated by a lack of development of new drugs. The cost of drug development and registration has increased to such an extent that there is little incentive for drug companies to investigate new compounds (Shirley, 1992). An additional and growing problem is the attention and concern from consumers about the routine use of drugs and chemicals in food animals.

Alternative management strategies to avoid or decrease the use of coccidiostats have focussed largely on vaccines. Live vaccines using low-virulence precocious lines of the parasites have been shown overseas to be efficient and cost-effective, but are not yet available in Australia. In a previous RIRDC project, staff from the Department of Primary Industries, Queensland developed precocious vaccine lines of *E. maxima* and *E. acervulina*. These lines were subsequently released to a commercial partner, *Eimeria* Pty Ltd, for commercial development, with the ultimate aim being an effective and comprehensive live coccidiosis vaccine for use in the Australian poultry industry. Hence, the major aim of the

project reported here was to develop precocious lines of the other two species of fowl coccidia that are commonly implicated in disease outbreaks, *E. tenella* and *E. necatrix*.

To be effective, live vaccine lines must exhibit several key characteristics.

1. They must be drug sensitive. Although the low-virulence lines cause little or no disease, management strategies may require control of the parasites.
2. They must have low virulence. The vaccine lines must be able to induce an immune response without causing severe disease.
3. They must maintain a reasonably high reproductive rate. A high oocyst output will enable production costs to be minimised and thus the product will be more cost-effective.
4. They must protect against both parent and other virulent strains of the same species. To be of greatest use vaccine lines must protect against any strains that the poultry are likely to be exposed to.

This project aimed, therefore, not only to produce the precocious lines, but also to characterise the lines to gauge their suitability for use in a vaccine.

The effective use of vaccines also relies on the ability to accurately identify each species. The traditional method of identification requires examination of the morphology of the parasites and biological parameters such as prepatent period and site of infection in the gut of the chicken. This process is time-consuming and, because infections commonly consist of several species, is often extremely difficult. The use of DNA-based technology for diagnostics has been expanding rapidly and is especially useful where, as in this case, there are multi-species complexes. Thus, a further aim of this project was to examine appropriate molecular techniques to assess their usefulness in the identification of *Eimeria* species.

2. Objectives

- Isolate and purify field strains of *E. tenella* and *E. necatrix.*
- Modify prepatent period of one strain of each species by selecting for precocious development.
- Characterise these modified strains in terms of drug sensitivity, reproductive potential, pathogenicity and protection against homologous and heterologous challenge.
- Identify and apply DNA based techniques to differentiate *E. tenella*, *E. necatrix*, *E. acervulina*, *E. maxima*, *E. brunetti*, *E. praecox* and *E. mitis*.
- Collect and purify a bank of field isolates of the poultry *Eimeria*.

3. Methods

3.1. Biosafety and quarantine

3.1.1. Rearing of coccidia-free birds

All birds used in this work were Webster's strain white leghorns, which were supplied from the minimal disease breeding flock on-site at the Animal Research Institute. Day-old chicks were transferred to positive pressure isolators in a designated clean chicken rearing room, which is isolated from all experimental areas. Strict biosafety procedures were in place and appropriate staff training was completed to ensure no contamination of the coccidia-free birds would occur. Faeces from the isolators was monitored weekly to ensure no coccidial infections were present. The birds were reared to at least four weeks of age before they were removed and used for parasite work.

3.1.2. Housing of experimental birds

All birds were kept in isolated rooms or sheds whilst experimental work was under way. Strains undergoing attenuation were maintained in medium-security isolators. All types of housing and all procedures carried out therein were approved by the ARI Animal Ethics Committee. Strict biosafety and quarantine procedures were in place and appropriate staff training was completed to ensure no cross-contamination of species or strains of parasites would occur. These procedures included restriction of staff movement between rooms, directional movement from clean to infected birds, use of appropriate coats, footwear and disposable gloves, and thorough decontamination of rooms between batches of birds (see below). Uninfected control birds were also sporadically maintained in experimental rooms to ensure these procedures were preventing cross-contamination.

3.1.3. Laboratory procedures

Laboratory work was performed in two laboratories. Any work associated with the strains undergoing attenuation was completed in one laboratory. The remaining work was completed in the other. Strict biosafety and quarantine procedures were in place and appropriate staff training was completed to ensure no cross-contamination of species or strains of parasites would occur. These procedures included restriction of staff movement between laboratories, use of different fume hoods for different species, use of appropriate coats and disposable

gloves, batching of routinely used reagents, sterilisation of glassware, and thorough decontamination of benches and equipment using an ammonia solution.

3.1.4. Disposal and decontamination

On completion of experimental, work birds were euthanased by cervical dislocation and, together with any other contaminated materials such as gloves and leftover feed, secured in plastic bags. Similarly, contaminated waste from the laboratories was also bagged securely. Until January 1999 the bags were then incinerated on-site at the Animal Research Institute. Since that time, a biohazardous waste contractor has disposed of the bags. Decontamination of experimental rooms consisted of hosing the room floors with hot water (where available) and Divosan Q-cide^{TM} (a quarternary ammonia compound). Cages, feeders, faeces trays and tray scrapers were initially washed and then heat-treated at 80°C for at least two hours. Heat treatment has been shown to be the most effective form of treatment for the purposes of oocyst/coccidial sterilisation of cages (Fish, 1932).

3.2. Isolation and purification of field isolates

Strains were obtained from three types of sources: virulent strains used for vaccination, backyard flocks and outbreaks on commercial poultry farms. Most samples from backyard flocks contained a mixture of several species. Samples from commercial outbreaks usually contained one predominant species, but also often included other species. Thus, the standard procedure for isolating a particular species from a field sample started with a passage through a bird that had been vaccinated with all species apart from the desired species. This initial isolation was followed by purification of the strain by a series of two or three single oocyst passages through naïve birds. For initial single oocyst passages, six birds were each inoculated with one oocyst that conformed to the expected morphology for that particular species and was representative of the isolated strain. Each subsequent single oocyst passage used oocysts of the appropriate morphology and prepatent period from one of the infections that were established in the previous passage.

3.3. Selection of strains for vaccine development

Parasite strains used in live coccidiosis vaccines must have low virulence and be susceptible to coccidiostats. Strains collected from commercial outbreaks are mostly highly virulent. In addition, they have usually been exposed to the routine use of coccidiostats, which may have led to some degree of coccidiostat resistance. Thus, potential vaccine strains were selected from relatively small, non-commercial flocks that were not regularly exposed to coccidiostats and did not have clinical coccidiosis.

3.4. Selection for precocious development (attenuation)

Parasite lines were selected for precocious development by serial passaging through naïve chickens. For each passage, 12 or 18 birds were inoculated with between 2 000 and 20 000 (mostly 5 000) oocysts. Faeces collections were made at four hourly intervals starting 8 to 12 hours before the time of patency calculated from the previous passage. Faeces samples were examined for oocysts using a saturated sugar flotation method. Oocysts from the first samples in which oocysts were found were then used to inoculate the birds in the following passage. This process resulted in progressively shorter prepatent periods, with a concomitant decrease in pathogenicity, and was continued until the prepatent period had fallen about 24 hours.

3.5. Characterisation trials

A series of five characterisation trials were completed for each precocious strain: drug sensitivity, reproductive potential (oocyst output), pathogenicity, homologous challenge and heterologous challenge. Each followed a randomised block design consisting of either five or six blocks of six or five treatment groups (total of 30 block/treatment combinations). Each block/treatment combination represented one experimental unit consisting of a single cage of three birds (one male and two females). The birds were about four weeks of age at the start of the trial. Birds were given vaccination and challenge doses of parasites by oral inoculation. Early trials used oocyst output and/or bodyweight gain as the parameters measured to indicate treatment effects. Facilities for measuring feed consumption were installed in 1998, which allowed the calculation of feed conversion ratios, where applicable, for the later trials. All parameters were measured over the 10, 11 or 12 day period following challenge, which encompassed the main reaction period. Oocyst output was measured by collecting and weighing all of the faeces from a cage from that period, taking a subsample and calculating the number of oocysts per gram. Oocysts were counted, with the aid of a microscope, using salt

flotation in a McMaster counting slide. Bodyweight gains and feed conversion ratios (weight of feed consumed/bodyweight gain) were calculated by measuring the weight of each bird and each cage's feed on the day of challenge and at the end of the trial. Two different feed formulations were used over the period in which feed conversion ratios were calculated. The use, in later trials, of a feed lacking growth promotants resulted in feed conversion ratios that were higher than those in the earlier trials.

3.5.1. Drug sensitivity trials

The drug sensitivity trials were used to assess whether the parasites were susceptible to commonly available coccidiostats. Each trial consisted of six blocks of five treatments. The treatment groups included: a negative control group, which received neither parasites nor any drug treatment; a positive control group, which received a parasite challenge but no drug treatment; and three groups that were given three different drug treatments after receiving a parasite challenge. The drug treatments are outlined in Table 1. Challenge doses of parasites consisted of either 10 000 or 15 000 oocysts. Oocyst output, bodyweight gain and, in later trials, feed conversion ratio were measured as indicators of drug treatment effectiveness.

Table 1. Drug treatments used in the drug sensitivity trials.

Trade Name	Constituents	Dose Rate (in drinking water)	Days of Application
Baycox®	Toltrazuril (25 g/l)	3 ml/l	2-3 (8 hr/day) 9-10 (8 hr/day)
Poultro	Sulphaquinoxaline (145 g/kg) Diaveridine (36.3 g/kg) Menadione (3.6 g/kg)	0.56 g/l	1-5 9-12
Coccivet	Amprolium (80 g/l) Ethopabate (5.1 g/l)	1.5 ml/l	2-8

An additional trial was completed to validate the drug sensitivity trial design. The main aim of the validation trial was to determine whether the drug treatments, in the absence of parasites, had any effects on the bodyweight gain or feed conversion of the birds. The trial

consisted of six blocks of four treatment groups. The treatment groups included: a negative control group, which received no drug treatment; and the three different drug treatment groups. Bodyweight gain and feed conversion ratio were calculated for a twelve day treatment period.

3.5.2. Reproductive potential (oocyst output) trials

The reproductive potential trials were used to assess whether the precocious lines had reduced oocyst outputs compared with the parent strains. Each trial consisted of six blocks of five treatments. The treatment groups included: a negative control group, which received no parasites; and four groups that were given four different parasite challenges. Challenge doses of parasites consisted of either 500 or 3 000 oocysts of the parent strain or the precocious line. Oocyst output was measured to indicate any change in reproductive potential.

3.5.3. Pathogenicity trials

The pathogenicity trials were used to assess whether the precocious lines had significantly reduced pathogenicity compared with the parent lines. Each trial consisted of six blocks of five treatments. The treatment groups included: a negative control group, which received no parasites; and four groups that were given four different parasite challenges. Challenge doses of parasites consisted of 5 000, 10 000 or 20 000 oocysts of the precocious line or 10 000 oocysts of the parent strain for *E. tenella* and 2 000, 5 000 or 10 000 oocysts of the precocious line or 10 000 oocysts of the parent strain for *E. necatrix*. Bodyweight gain and, in later trials, feed conversion ratio were measured as indicators of the virulence of the two lines.

3.5.4. Homologous challenge trials

The homologous challenge trials were used to determine if vaccination with the precocious lines would induce protective immunity against the parent strains. Each trial consisted of five blocks of six treatments. The treatment groups included: a negative control group, which received no parasites; a positive control group, which received a parasite challenge but no vaccination; and four groups that were given varying vaccine doses before receiving a parasite challenge. Vaccine doses consisted of 10, 100, 1 000 or 5 000 oocysts of the precocious line. Challenge doses of parent strains were given twenty-one days after vaccination and consisted of 5 000 oocysts for *E. tenella* and 10 000 oocysts for *E. necatrix*. Oocyst output, bodyweight gain and, in later trials, feed conversion ratio were measured as indicators of the effectiveness of the vaccination.

3.5.5. Heterologous challenge trials

The heterologous challenge trials were used to determine if vaccination with the precocious lines would induce protective immunity against other virulent field strains. Each trial consisted of six blocks of five treatments. The treatment groups included: a negative control group, which received no parasites; two positive control groups, which received parasite challenges with two different virulent field isolates but no vaccination; and two groups that were given a vaccination dose before receiving the two different parasite challenges. Vaccination doses consisted of 10 oocysts of the precocious line. Floor trays were placed in cages to simulate a pen trial by allowing recycling of oocysts over a twenty-one day period prior to challenge. Challenge doses of virulent field strains consisted of 5 000 oocysts for *E. tenella* and 10 000 oocysts for *E. necatrix*. Oocyst output, bodyweight gain and, in later trials, feed conversion ratio were measured as indicators of the effectiveness of the vaccination.

3.5.6. Statistical analysis

ANOVA models (1-way and 2-way) appropriate to the trial designs were used to test the treatment effects for statistical significance. The cage of three birds was used as the experimental unit in all analyses. The protected Least Significant Difference procedure was used to compare treatment means at the 5% level of significance. Oocyst numbers were transformed [initially $\log_e(X+1)$ and later $(X+1)^{1/3}$] for further analysis.

3.6. Storage of parasites

Parasite samples are routinely stored at 12°C in 2% Potassium dichromate. This storage method appears to have little effect on infectivity of the oocysts over a six month period. Storage periods of over twelve months, however, lead to significant decreases in infectivity. A series of trials to examine the optimal storage temperature and maximum safe storage time is currently under way.

For long term storage, parasites are maintained frozen in liquid nitrogen. Fresh oocysts are suspended in Eagles medium and shaken with glass beads for two minutes to release the sporocysts. The sporocysts are resuspended in a solution of 7.5% DMSO and 10% foetal calf serum in MEM (pH 7.5) and dispensed into cryovials. The vials are stored at room temperature overnight and then placed in the vapour phase of liquid nitrogen where the

parasites are frozen at a rate of about 10°C per minute. After freezing, the vials are stored in liquid nitrogen. For use, vials are thawed rapidly at 37°C and then stored on ice until use. The parasites are inoculated into birds within 30 minutes of thawing. After freezing, batches of parasites were tested for infectivity by inoculation of a naïve bird with one vial out of the batch. This was replaced in the later part of the project by the quality control procedure outlined below.

3.7. Quality control

A detailed quality control procedure was designed and implemented to ensure the infectivity and purity of the cryopreserved parasite stocks. The procedure involved amplification of the parasites by an initial inoculation of one naïve bird with frozen parasites. Oocysts collected from that bird were then used to inoculate four other naïve birds in doses calculated to cause mild clinical disease (mild diarrhoea). The birds were killed at designated times throughout the reaction period and their guts were examined for the distribution of lesions. Gut scrapings were taken to examine the distribution and morphology of any oocysts that were present. Gut scrapings were also used for DNA extraction so that PCR tests (see below) could be used to confirm the observations. In addition, faeces samples were taken and examined daily to determine the prepatent period. A negative control bird was maintained in the experimental room throughout the entire procedure to ensure that no observations could be attributed to contamination from external sources. The procedure was modified to suit unfrozen samples simply by leaving out the initial amplification step.

3.8. DNA analysis

The aim of molecular differentiation part of the project was to assess available molecular techniques for suitability for differentiating at least five of the species of *Eimeria* that infect chickens and avoid, if possible, the time and costs involved in developing new tests. Thus, a number of techniques were investigated as they became available in the literature. Investigation of techniques that gave inconsistent or non-specific results was discontinued so that resources could be directed towards other possible techniques. The main techniques that were investigated are described below in the chronological order in which they were examined.

3.8.1. DNA extraction

DNA for use in the following procedures was extracted from two sources: oocysts separated from faecal samples and parasite material contained in gut scrapings. Samples of oocysts (about 10^6) were initially shaken with glass beads until approximately 90% were ruptured (about 2 min). Both types of sample were digested using proteinase K and detergent, purified using phenol/chloroform and precipitated in ethanol following standard techniques outlined in Sambrook, Fritsch & Maniatis (1989). DNA samples were subsequently resuspended in distilled water and stored at –20°C.

3.8.2. PCR tests from antigenic sequences

This technique involved the design of PCR (polymerase chain reaction) primers from gene sequences for *E. tenella* and *E. acervulina* that were available on international databases. Nested primer sets were designed from sequences of the sporozoite antigen EASZ240/160 gene of *E. acervulina* and an immunodominant microneme protein gene of *E. tenella*. The lack of gene sequences from other *Eimeria* species precluded the design of other primer sets. Primary amplifications were performed with the outer pair of PCR primers. Secondary amplifications used the inner pair of primers and product from the first reaction as the DNA template. The specificity of the primers was tested by attempting amplification of the other species of chicken *Eimeria*. Sensitivity was tested using serial dilutions of oocysts of the target species mixed with oocysts of another species. Complete details of this technique are given in Molloy, Eaves, Jeston, Minchin, Stewart, Lew & Jorgensen (1998).

3.8.3. Small subunit rDNA probes

Merck have lodged a patent on the small subunit (SSU, often in the past called 18S) ribosomal DNA (rDNA) sequences of all species of *Eimeria* that infect chickens as well as species-specific DNA probes designed from those sequences. Due to patent restrictions, this technique was largely evaluated for use in confirmatory testing of other techniques rather than for use as an industry-based test. The technique involved the initial PCR amplification of samples using conserved primers (i.e. primers that will work for all species) that amplify the target DNA region. The amplified product was then blotted onto a membrane and each of the species-specific probes was given the opportunity to hybridise to the target DNA. Adhesion of one particular probe then allowed the identification of the species.

3.8.4. PCR test on 5S intergenic spacer regions

Stucki, Braun & Roditi (1993) described a PCR test for *E. tenella* that was designed from the sequence of a 5S ribosomal RNA gene repeat unit. Two primers were designed for the variable intergenic spacer region of the repeat unit. The PCR primers only worked for *E. tenella* and tests for sensitivity showed that they could detect fewer than 10 oocysts. This test was assessed for specificity on Australian isolates.

3.8.5. RAPD PCR tests

Randomly amplified polymorphic DNA (RAPD) PCR tests were evaluated as a means of identifying species-specific fragments that were suitable for developing normal PCR tests. This was attempted largely because no further published tests for *Eimeria* were available at that stage of the project. RAPD PCR tests rely on small non-specific PCR primers that randomly amplify fragments from anywhere in the genome. Fragments that appeared consistently in all isolates of one species, but not in any other species could be tested for specificity by attempting hybridisation with all of the species. If the fragment hybridised with only one species it could then be sequenced and appropriate species-specific PCR primers could be designed.

3.8.6. PCR tests on first internal transcribed spacer rDNA

These tests have been evaluated in a nested PCR system. The outer pair of primers was designed to anneal in the regions flanking the internal transcribed spacer (ITS1) and thus amplify the entire spacer. They were designed from sequences that appeared to be conserved across all *Eimeria* species so that the initial amplification should work for all species that are present in a sample. The amplified product was then used as template DNA for secondary species-specific tests. Details of inner species-specific primers for four species of *Eimeria*, *E. acervulina*, *E. brunetti*, *E. necatrix* and *E. tenella*, were made available by B. Schnitzler and have now been published (Schnitzler, Thebo, Mattsson, Tomley & Shirley, 1998). The ITS1 of Australian isolates was sequenced so that additional primers for the other three species of chicken *Eimeria* could be designed. Inner primers for *E. praecox* and *E. mitis* were designed from sequence data from three Australian isolates of each species. Primer sets for *E. maxima* were designed from sequence data from one American isolate and from two Australian isolates. Sequences for the ITS1 for these three species from Europe were released and species-specific primers published in 1999 (Schnitzler, Thebo, Tomley, Uggla & Shirley,

1999). The lateness of this availability, however, allowed little time for evaluation work on the primers using Australian isolates.

3.8.7. Small subunit rDNA sequencing

Complete small subunit (SSU) rDNA sequences have been published for all species of *Eimeria* that infect chickens (Barta, Martin, Liberator, Dashkevicz, Anderson, Feighner, Elbrecht, Perkins-Barrow, Jenkins, Danforth, Ruff & Profous-Juchelka, 1997). This allowed the comparison of sequences from Australian isolates with those from overseas to confirm the identity of the species found in Australia. The technique consisted of automated sequencing of DNA fragments produced using nested primer systems. One pair of primers was designed to amplify the entire SSU. The amplified product was then used as the template for secondary amplifications using four pairs of primers that produced overlapping fragments that were short enough to be sequenced. These eight primers are given in Ellis, Morrison & Johnson (1994), but some were modified slightly to perform better when used on *Eimeria*. Initially, one strain each of *E. tenella* and *E. acervulina* were sequenced entirely. Subsequently, only the first of the four fragments was sequenced. This fragment exhibited sufficient interspecific variation to enable positive identification of the species to which each strain belonged.

4. Results

4.1. Isolation and purification of field isolates

The numbers of strains of each of the seven species that have been collected, isolated via passage through an immunised bird, purified via single oocyst passage, cryopreserved in liquid nitrogen and later tested and found to be infective are listed in Table 2. Sources of strains included backyard flocks, virulent strains used for vaccination and outbreaks on commercial poultry farms.

Table 2. Number of isolates of each species held currently in the collection (cryopreserved). Columns two, three and four indicate the number of isolates from the different types of sources: non-commercial (small, non-commercial flocks), commercial (used as virulent vaccine strains or from commercial flocks not showing clinical coccidiosis) and outbreak (outbreaks of clinical coccidiosis on commercial farms).

Species	Non-commercial	Commercial	Outbreak	Total number of isolates
E. necatrix	2	2	1	5
E. tenella	3	1	2	6
E. brunetti	2	0	2	4
E. mitis	3	0	1	4
E. praecox	3	1	0	4
E. maxima	3	1	1	5
E. acervulina	2	1	2	5
Total	18	6	9	**33**

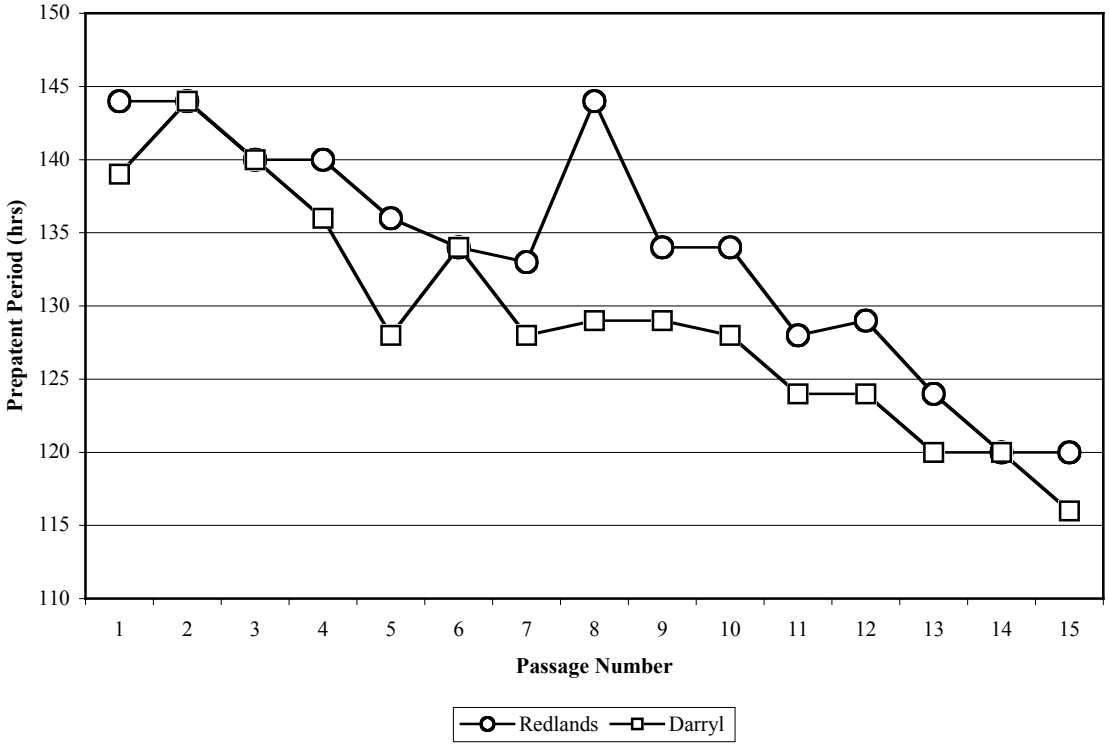
4.2. Selection for precocious development

Selection for precocious development was successful for four strains of the parasites, two strains each of *E. tenella* and *E. necatrix* (Table 3). Each of these strains had a final prepatent period 23 to 25 hours shorter than the starting period (Figures 1 & 2). Passaging of one strain, the McGregor strain of *E. necatrix*, was unsuccessful and was discontinued because the prepatent period was not dropping as expected (Table 3, Figure 2).

Table 3. Summary of passaging data for two strains of *Eimeria tenella* and three strains of *E. necatrix.*

Species	**Strain**	**No. of Passages**	**Prepatent period – start** (hrs)	**Prepatent period – finish** (hrs)	**Prepatent period – drop** (hrs)
E. tenella	Redlands	15	144	120	24
E. tenella	Darryl	15	139	116	23
E. necatrix	Medichick	8	148	123	25
E. necatrix	McGregor	15	156	150	6
E. necatrix	Gatton	8	152	128	24

Figure 1. Prepatent periods throughout passaging of two strains of *Eimeria tenella*



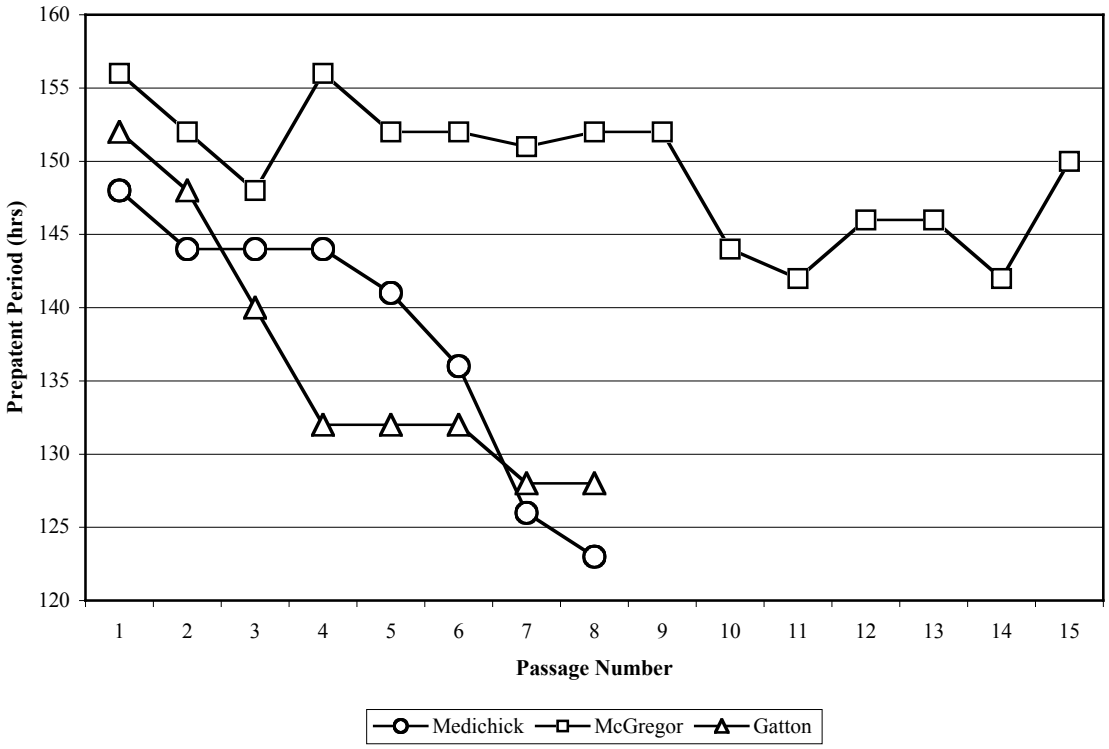


Figure 2. Prepatent periods throughout passaging of three strains of *Eimeria necatrix*

4.3. Characterisation trials – validation of drug sensitivity trial design

Results from the validation trial (Table 4) show no significant differences in bodyweight gain between the groups receiving the drug treatments and the negative control group, which received no drugs. There was some variation in the feed conversion ratios that, although minor, was significant. Two of the drug treatments resulted in feed conversion ratios that were significantly lower than that of the negative control birds, which is the opposite effect to that expected from parasite infections.

Table 4. Results from the drug sensitivity validation trial. There was no challenge dose of parasites used in this trial because the aim was to determine the effects of the coccidiostats alone on the measured parameters. The drugs were given in the drinking water following the manufacturers' recommendations. Parameters were measured for 12 days of treatment. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment (complete details in Table 1.)	**Bodyweight gain** (g/bird)	**Feed conversion ratio**
Poultro (Sulphaquinoxaline)	203	3.11^{a}
Baycox (Toltrazuril)	199	$3.19^{a,b}$
Coccivet (Amprolium)	198	3.14^{a}
Negative control	203	3.26^{b}
LSD (P=0.05)	**9**	**0.12**

4.4. Characterisation trials – Redlands strain of *E. tenella*

4.4.1. Drug sensitivity – Redlands strain of *E. tenella*

Results from the drug sensitivity trial (Table 5) show that the Toltrazuril treatment group had a significantly lower oocyst output than the positive control group, which was challenged but received no medication. There were no significant differences in oocyst output between the Sulphaquinoxaline or Amprolium treatment groups and the positive control group. The Toltrazuril and Sulphaquinoxaline treatment groups had bodyweight gains that were not significantly different from that of the negative control group, but were significantly higher than that of the positive control group. The bodyweight gain of the Amprolium treatment group was not significantly different from that of the positive control group. The feed conversion ratio of the Toltrazuril treatment group was not significantly different from that of the negative control group, but was significantly lower than that of the positive control group. The feed conversion ratio of the Sulphaquinoxaline treatment group, while significantly higher than that of the negative control group, was significantly lower than that of the positive control group. The feed conversion ratio of the Amprolium treatment group was not significantly different from that of the positive control group.

Table 5. Results from the drug sensitivity trial for the Redlands strain of *E. tenella*. The challenge dose of parasites consisted of 15 000 oocysts. The drugs were given in the drinking water following the manufacturers' recommendations. Parameters were measured for 12 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment (complete details in Table 1.)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)	**Feed conversion ratio**
	Geometric mean	**$(X+1)^{1/3}$ transformed**		
Parasites only	154.0×10^6	536^a	159^b	2.436^a
Parasites +Poultro (Sulphaquinoxaline)	139.9×10^6	519^a	182^a	2.335^b
Parasites + Baycox (Toltrazuril)	0.5×10^6	81^b	191^a	2.196^c
Parasites +Coccivet (Amprolium)	128.7×10^6	505^a	159^b	2.490^a
Negative control	-	-	197^a	2.146^c
LSD (P=0.05)		**74**	**16**	**0.096**

4.4.2. Reproductive potential (oocyst output) – Redlands strain of *E. tenella*

Results from the reproductive potential trial (Table 6) show that the group challenged with 500 oocysts of the precocious line had a significantly lower oocyst output than the other three groups. There were no significant differences in oocyst output between groups challenged with 500 oocysts of the parent strain, 3 000 oocysts of the parent strain or 3 000 oocysts of the precocious line. There appears, however, to be a trend for the group receiving 3 000 oocysts of the parent strain to have a higher oocyst output than the other two groups.

Table 6. Results from the reproductive potential (oocyst output) trial for the Redlands strain of *E. tenella*. Oocyst output was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Oocyst output per bird**	
	Geometric mean	**$(X+1)^{1/3}$ transformed**
500 oocysts, parent strain	155.6×10^6	538^a
500 oocysts, precocious line	38.2×10^6	337^b
3 000 oocysts, parent strain	189.2×10^6	574^a
3 000 oocysts, precocious line	143.8×10^6	524^a
LSD (P=0.05)		**75**

4.4.3. Pathogenicity – Redlands strain of *E. tenella*

Results from the pathogenicity trial (Table 7) reveal no significant differences in bodyweight gain between any of the groups challenged with the precocious line and the negative control group, which received no parasites. The group that was challenged with the parent strain, however, had a significantly lower bodyweight gain than the other four groups.

Table 7. Results from the pathogenicity trial for the Redlands strain of *E. tenella*. Bodyweight gain was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Bodyweight gain** (g/bird)
5 000 oocysts, precocious line	113[a]
10 000 oocysts, precocious line	111[a]
20 000 oocysts, precocious line	103[a]
10 000 oocysts, parent strain	82[b]
Negative control	108[a]
LSD (P=0.05)	**15**

4.4.4. Homologous challenge – Redlands strain of *E. tenella*

Results from the homologous challenge trial (Table 8) show that the groups vaccinated with 10 or 100 oocysts had oocyst outputs that were not significantly different from that of the positive control group, which was challenged but not vaccinated. There appears, however, to be a trend for those two groups to have lower oocyst outputs than the positive control group. The other two vaccinated groups had significantly lower oocyst outputs. Oocyst output for the group vaccinated with 5 000 oocysts was also significantly lower than that for the group vaccinated with 1 000 oocysts. Groups vaccinated with 100 or 1 000 oocysts had significantly lower bodyweight gains than the negative control group. Bodyweight gains for the other two vaccinated groups and the positive control group were not significantly different from that of any other group.

Table 8. Results from the homologous challenge trial for the Redlands strain of *E. tenella*. Parameters were measured for 10 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$Log_e(X+1)$ transformed**	
10	5 000	173×10^6	18.97^a	169^{ab}
100	5 000	144×10^6	18.78^a	143^b
1 000	5 000	71×10^6	18.08^b	147^b
5 000	5 000	29×10^6	17.19^c	159^{ab}
Nil	5 000	209×10^6	19.16^a	159^{ab}
Nil	Nil	-	-	175^a
	LSD (P=0.05)		**0.47**	**27**

4.4.5. Heterologous challenge – Redlands strain of *E. tenella*

Results from the heterologous challenge trial (Table 9) show that the groups that were vaccinated before challenge had significantly lower oocyst outputs than the groups that were not vaccinated. The vaccinated groups had bodyweight gains that were not significantly different from that of the negative control group, which received no parasites. The unvaccinated groups, however, had bodyweight gains that were significantly lower than that of the negative control group.

Table 9. Results from the heterologous challenge trial for the Redlands strain of *E. tenella*. Parameters were measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts, strain)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$(X+1)^{1/3}$ transformed**	
10	5 000, Medichick	0.1 X 10^6	39^b	181^{ab}
10	5 000, Inghams	0.0 X 10^6	10^b	196^a
Nil	5 000, Medichick	149.1 X 10^6	530^a	151^{bc}
Nil	5 000, Inghams	163.2 X 10^6	546^a	129^c
Nil	Nil	-	-	195^a
	LSD(P=0.05)		**51**	**32**

4.5. Characterisation trials – Darryl strain of *E. tenella*

4.5.1. Drug sensitivity – Darryl strain of *E. tenella*

Results from the drug sensitivity trial (Table 10) show that the Toltrazuril treatment group had a significantly lower oocyst output than the positive control group, which was challenged but received no medication. There were no significant differences in oocyst output between the Sulphaquinoxaline and Amprolium treatment groups and the positive control group. The Sulphaquinoxaline and Toltrazuril treatment groups had bodyweight gains that were not significantly different from that of the negative control group. The Amprolium treatment group had a significantly lower bodyweight gain than those three groups. The positive control group had a bodyweight gain that was significantly lower than those of the other four groups.

Table 10. Results from the drug sensitivity trial for the Darryl strain of *E. tenella.* The challenge dose of parasites consisted of 10 000 oocysts. The drugs were given in the drinking water following the manufacturers' recommendations. Parameters were measured for 11 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment (complete details in Table 1.)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
	Geometric mean	**$Log_e(X+1)$ transformed**	
Parasites only	248 X 10^6	19.3^a	170.0^c
Parasites +Poultro (Sulphaquinoxaline)	64 X 10^6	18.0^a	219.4^a
Parasites + Baycox (Toltrazuril)	0.7 X 10^6	13.4^b	217.8^a
Parasites +Coccivet (Amprolium)	319 X 10^6	19.6^a	196.1^b
Negative control	-	-	221.1^a
LSD (P=0.05)		**1.9**	**15.6**

4.5.2. Reproductive potential (oocyst output) – Darryl strain of *E. tenella*

The results from the reproductive potential trial (Table 11) show oocyst outputs that are significantly different for each of the groups. The lowest is that of the group challenged with 500 oocysts of the precocious line, followed by the group challenged with 3 000 oocysts of the precocious line. The next highest is that of the group challenged with 500 oocysts of the parent strain and the highest is that of the group challenged with 3 000 oocysts of the parent strain.

Table 11. Results from the reproductive potential (oocyst output) trial for the Darryl strain of *E. tenella*. Oocyst output was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Oocyst output per bird**	
	Geometric mean	**$(X+1)^{1/3}$ transformed**
500 oocysts, parent strain	62.5×10^6	397^b
500 oocysts, precocious line	0.9×10^6	98^d
3 000 oocysts, parent strain	113.9×10^6	485^a
3 000 oocysts, precocious line	7.8×10^6	199^c
LSD (P=0.05)		**50**

4.5.3. Pathogenicity – Darryl strain of *E. tenella*

Results from the pathogenicity trial (Table 12) show that there were no significant differences between the bodyweight gains of any of the groups challenged with the precocious line and that of the negative control group, which received no parasites. The group challenged with the parent strain had a significantly lower bodyweight gain than the other four groups. The feed conversion ratios of the groups challenged with the precocious line were not significantly different from that of the negative control group whereas that of the group challenged with the parent strain was significantly higher than those of the other four groups.

Table 12. Results from the pathogenicity trial for the Darryl strain of *E. tenella*. Parameters were measured for 11 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Bodyweight change** (g/bird)	**Feed conversion ratio**
5 000 oocysts, precocious line	193[a]	3.07[b]
10 000 oocysts, precocious line	188[a]	3.05[b]
20 000 oocysts, precocious line	189[a]	3.04[b]
10 000 oocysts, parent strain	143[b]	3.78[a]
Negative control	187[a]	3.10[b]
LSD (P=0.05)	**15**	**0.20**

4.5.5. Homologous challenge – Darryl strain of *E. tenella*

Results from the homologous challenge trial (Table 13) show that the groups that were vaccinated with 10 or 100 oocysts had oocyst outputs that were not significantly different from that of the positive control group, which was challenged but not vaccinated. There appears, however, to be a trend for the group vaccinated with 100 oocysts to have a lower oocyst output than the positive control group. The other two vaccinated groups had significantly lower oocyst outputs than the positive control group. All of the vaccinated groups had bodyweight gains that were not significantly different from that of the negative control group, which received no parasites. The bodyweight gain of the positive control group was significantly lower than those of the other five groups.

Table 13. Results from the homologous challenge trial for the Darryl strain of *E. tenella*. Parameters were measured for 10 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$(X+1)^{1/3}$ transformed**	
10	5 000	30.5×10^7	673^a	201^a
100	5 000	16.4×10^7	547^a	183^a
1 000	5 000	3.2×10^7	318^b	189^a
5 000	5 000	1.4×10^7	242^b	179^a
Nil	5 000	24.8×10^7	628^a	53^b
Nil	Nil	-	-	184^a
	LSD (P=0.05)		**127**	**29**

4.5.5. Heterologous challenge – Darryl strain of *E. tenella*

Results from the heterologous challenge trial (Table 14) show that the two vaccinated groups had significantly lower oocyst outputs than the two unvaccinated groups. Of the two vaccinated groups, the group challenged with the Medichick strain had a significantly lower oocyst output than that of the group challenged with the Inghams strain. No significant differences were observed in bodyweight gains.

Table 14. Results from the heterologous challenge trial for the Darryl strain of *E. tenella*. Parameters were measured for 10 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts, strain)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$(X+1)^{1/3}$ transformed**	
10	5 000, Medichick	42.1×10^6	17.55^c	165
10	5 000, Inghams	65.5×10^6	18.00^b	193
Nil	5 000, Medichick	123.1×10^6	18.63^a	172
Nil	5 000, Inghams	138.0×10^6	18.74^a	151
Nil	Nil	-	-	203
	LSD (P=0.05)		**0.40**	**64**

4.6. Characterisation trials – Medichick strain of *E. necatrix*

4.6.1. Drug sensitivity – Medichick strain of *E. necatrix*

The results from the drug sensitivity trial (Table 15) show that birds were withdrawn from the trial (= mortalities) from both the Amprolium treatment group and the positive control group, which was challenged but received no medication. In addition, both these groups had significantly lower bodyweight gains than the other three groups, the positive control group having a significantly lower bodyweight gain than the Amprolium treatment group. There were no significant differences between the bodyweight gains of the Toltrazuril or Sulphaquinoxaline treatment groups and the negative control group, which received no parasites.

Table 15. Results from the drug sensitivity trial for the Medichick strain of *E. necatrix.* The challenge dose of parasites consisted of 10 000 oocysts. The drugs were given in the drinking water following the manufacturers' recommendations. Bodyweight gain was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment (complete details in Table 1.)	**Mortalities**	**Bodyweight gain live birds only** (g/bird)	**Bodyweight gain live and dead birds** (g/bird)
Parasites only	5	77[c]	76 [c]
Parasites +Poultro (Sulphaquinoxaline)	0	219[a]	219 [a]
Parasites + Baycox (Toltrazuril)	0	222 [a]	222 [a]
Parasites +Coccivet (Amprolium)	7	142[b]	132 [b]
Negative control	0	231 [a]	231 [a]
LSD (P=0.05)		**45**	**28**

4.6.2. Reproductive potential (oocyst output) – Medichick strain of *E. necatrix*

Results from the reproductive potential trial (Table 16) reveal no significant differences in oocyst output between any of the challenge groups.

Table 16. Results from the reproductive potential (oocyst output) trial for the Medichick strain of *E. necatrix*. Oocyst output was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	Oocyst output per bird	
	Geometric mean	$(X+1)^{1/3}$ transformed
500 oocysts, parent strain	9.6×10^5	99
500 oocysts, precocious line	3.5×10^5	70
3 000 oocysts, parent strain	7.7×10^5	92
3 000 oocysts, precocious line	3.7×10^5	72
LSD (P=0.05)		**34**

4.6.3. Pathogenicity – Medichick strain of *E. necatrix*

Results from the pathogenicity trial (Table 17) show that there were no significant differences between the bodyweight gains of any of the groups challenged with the precocious line and that of the negative control group, which received no parasites. The group challenged with the parent strain had a significantly lower bodyweight gain than the other four groups. The feed conversion ratios of the groups challenged with the precocious line were not significantly different from that of the negative control group whereas that of the group challenged with the parent strain was significantly higher than those of the other four groups.

Table 17. Results from the pathogenicity trial for the Medichick strain of *E. necatrix*. Parameters were measured for 12 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Bodyweight gain** (g/bird)	**Feed conversion ratio**
2 000 oocysts, precocious line	217[a]	3.04[b]
5 000 oocysts, precocious line	218[a]	3.03[b]
10 000 oocysts, precocious line	222[a]	3.05[b]
10 000 oocysts, parent strain	90[b]	5.91[a]
Negative control	222[a]	3.10[b]
LSD (P=0.05)	**18**	**0.79**

4.6.4. Homologous challenge – Medichick strain of *E. necatrix*

Results from the homologous challenge trial (Table 18) show that the group vaccinated with 10 oocysts had an oocyst output that was significantly higher than those of the other three vaccinated groups. Although the oocyst output for that group was not significantly different from that of the positive control group, which was challenged but not vaccinated, there appears to be a trend for it to be lower. There were no significant differences in oocyst output between birds in the groups vaccinated with 100, 1 000 or 5 000 oocysts. There were no significant differences in bodyweight gains between the groups vaccinated with 100,

1 000 or 5 000 oocysts, and the negative control group, which received no parasites. The group vaccinated with 10 oocysts had a bodyweight gain that was significantly lower than those of those four groups. The positive control group had a bodyweight gain that was significantly lower than those of the other five groups.

Table 18. Results from the homologous challenge trial for the Medichick strain of *E. necatrix*. Parameters were measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$(X+1)^{1/3}$ transformed**	
10	10 000	5.1×10^6	172^a	139^b
100	10 000	0.2×10^6	54^b	185^a
1 000	10 000	0.1×10^6	52^b	185^a
5 000	10 000	0.1×10^6	46^b	191^a
Nil	10 000	7.1×10^6	192^a	99^c
Nil	Nil	-	-	179^a
	LSD (P=0.05)		**25**	**35**

4.6.5. Heterologous challenge – Medichick strain of *E. necatrix*

Results from the heterologous challenge trial (Table 19) show that the two vaccinated groups had significantly lower oocyst outputs than those of the two unvaccinated groups. For both vaccinated and unvaccinated groups, the groups challenged with the Groves strain had oocyst outputs that were significantly lower than those of the groups challenged with the McGregor strain. The bodyweight gains of the vaccinated groups were not significantly different from that of the negative control group, but were significantly higher than those of the unvaccinated groups. In the unvaccinated groups, the bodyweight gain of the group challenged with the Groves strain was significantly lower than that of the group challenged with the McGregor strain.

Table 19. Results from the heterologous challenge trial for the Medichick strain of *E. necatrix*. Parameters were measured for 10 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts, strain)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)
		Geometric mean	**$(X+1)^{1/3}$ transformed**	
10	10 000, McGregor	0.78×10^6	92^c	83^a
10	10 000, Groves	0.19×10^6	57^d	115^a
Nil	10 000, McGregor	23.57×10^6	287^a	38^b
Nil	10 000, Groves	7.56×10^6	196^b	-47^c
Nil	Nil	-	-	97^a
	LSD (P=0.05)		**32**	**33**

4.7. Characterisation trials – Gatton strain of *E. necatrix*

4.7.1. Drug sensitivity – Gatton strain of *E. necatrix*

Results from the drug sensitivity trial (Table 20) show that the Toltrazuril treatment group had a significantly lower oocyst output than the positive control group, which was challenged but received no medication. There were no significant differences in oocyst output between the Sulphaquinoxaline or Amprolium treatment groups and the positive control group. The Toltrazuril treatment group had a bodyweight gain that was not significantly different from that of the negative control group. The Sulphaquinoxaline and Amprolium treatment groups had bodyweight gains that were not significantly different from that of the positive control group, but were significantly lower than that of the negative control group. The Toltrazuril treatment group had a feed conversion ratio that was not significantly different from those of the negative and positive control groups. The Sulphaquinoxaline and Amprolium treatment groups had feed conversion ratios that were not significantly different from that of the positive control group, but were significantly higher than that of the negative control group.

Table 20. Results from the drug sensitivity trial for the Gatton strain of *E. necatrix*. The challenge dose of parasites consisted of 10 000 oocysts. The drugs were given in the drinking water following the manufacturers' recommendations. Parameters were measured for 11 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment (complete details in Table 1.)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)	**Feed conversion ratio**
	Geometric mean	**$(X+1)^{1/3}$ transformed**		
Parasites only	14.9×10^6	246^a	154^b	4.31^a
Parasites +Poultro (Sulphaquinoxaline)	14.6×10^6	244^a	141^b	4.43^a
Parasites + Baycox (Toltrazuril)	0	0^b	182^a	3.89^{ab}
Parasites +Coccivet (Amprolium)	14.5×10^6	244^a	156^b	4.39^a
Negative control	-	-	196^a	3.65^b
LSD (P=0.05)		**53**	**24**	**0.57**

4.7.2. Reproductive potential (oocyst output) – Gatton strain of *E. necatrix*

The results from the reproductive potential trial (Table 21) show that the group challenged with 500 oocysts of the precocious line had an oocyst output that was significantly lower than those of the other three groups. The oocyst output of the group challenged with 500 oocysts of the parent strain was significantly lower than those of the groups challenged with 3 000 oocysts. There was no significant difference between the oocyst outputs of the two groups receiving 3 000 oocysts, even though there appears to be a trend for the group receiving the precocious line to have a lower output than the group receiving the parent strain.

Table 21. Results from the reproductive potential (oocyst output) trial for the Gatton strain of *E. necatrix*. Oocyst output was measured for 12 days following challenge. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Oocyst output per bird**	
	Geometric mean	**$(X+1)^{1/3}$ transformed**
500 oocysts, parent strain	2.4×10^6	133^b
500 oocysts, precocious line	1.1×10^6	102^c
3 000 oocysts, parent strain	6.6×10^6	187^a
3 000 oocysts, precocious line	4.8×10^6	168^a
LSD (P=0.05)		**25**

4.7.3. Pathogenicity – Gatton strain of *E. necatrix*

Results from the pathogenicity trial (Table 22) show that there were no significant differences between the bodyweight gains of any of the groups challenged with the precocious line and that of the negative control group, which received no parasites. The group challenged with the parent strain had a significantly lower bodyweight gain than the other four groups. The feed conversion ratios of the groups challenged with the precocious line were not significantly different from that of the negative control group whereas that of the group challenged with the parent strain was significantly higher than those of the other four groups.

Table 22. Results from the pathogenicity trial for the Gatton strain of *E. necatrix*. Parameters were measured for 12 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Treatment	**Bodyweight gain** (g/bird)	**Feed conversion ratio**
2 000 oocysts, precocious line	167[a]	3.70[b]
5 000 oocysts, precocious line	166[a]	3.59[b]
10 000 oocysts, precocious line	169[a]	3.64[b]
10 000 oocysts, parent strain	117[b]	4.73[a]
Negative control	159[a]	3.85[b]
LSD (P=0.05)	**21**	**0.48**

4.7.4. Homologous challenge – Gatton strain of *E. necatrix*

Results from the homologous challenge trial (Table 23) show that the group that was vaccinated with 10 oocysts had a significantly higher oocyst output than the other three vaccinated groups. The oocyst output for that group was significantly lower than that of the positive control group, which was challenged but not vaccinated. There were no significant differences in oocyst output between birds in the groups vaccinated with 100 or 1 000 oocysts, but the group vaccinated with 5 000 oocysts had an oocyst output that was significantly lower than those of the other four challenged groups. There were no significant differences in bodyweight gains between any of the vaccinated groups and the negative control group, which received no parasites. The positive control group had a bodyweight gain that was significantly lower than those of the other five groups. There were no significant differences in feed conversion ratio between any of the vaccinated groups and the negative control group. The positive control group had a feed conversion ratio that was significantly higher than those of the other five groups.

Table 23. Results from the homologous challenge trial for the Gatton strain of *E. necatrix*. Parameters were measured for 12 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)	**Feed conversion ratio**
		Geometric mean	**$(X+1)^{1/3}$ transformed**		
10	10 000	2.9×10^6	143^b	169^b	3.66^b
100	10 000	0.3×10^6	67^c	177^{ab}	3.68^b
1 000	10 000	0.1×10^6	50^c	189^a	3.40^b
5 000	10 000	0.0×10^6	7^d	188^a	3.47^b
Nil	10 000	6.6×10^6	187^a	136^c	4.32^a
Nil	Nil	-	-	179^{ab}	3.70^b
	LSD (P=0.05)		**35**	**19**	**0.36**

4.7.5. Heterologous challenge – Gatton strain of *E. necatrix*

Results from the heterologous challenge trial (Table 24) show that the two vaccinated groups had significantly lower oocyst outputs than the two unvaccinated groups. The bodyweight gains of the vaccinated groups and the unvaccinated group challenged with the McGregor strain were not significantly different from that of the negative control group, but were significantly higher than that of the unvaccinated group challenged with the Medichick strain. The feed conversion ratios of the vaccinated groups and the unvaccinated group challenged with the McGregor strain were not significantly different from that of the negative control group, but were significantly lower than that of the unvaccinated group challenged with the Medichick strain.

Table 24. Results from the heterologous challenge trial for the Gatton strain of *E. necatrix*. Parameters were measured for 12 days following challenge. Feed conversion ratio = weight of feed consumed/bodyweight gain. LSD = least significant difference. Within columns, means with different superscript letters are significantly different at the 5% level (F-test in the ANOVA).

Vaccination (oocysts)	**Challenge** (oocysts, strain)	**Oocyst output per bird**		**Bodyweight gain** (g/bird)	**Feed conversion ratio**
		Geometric mean	**$(X+1)^{1/3}$ transformed**		
10	10 000, Medichick	0.4×10^6	75^c	260^a	2.96^b
10	10 000, McGregor	0	0^d	262^a	2.99^b
Nil	10 000, Medichick	6.6×10^6	188^b	184^b	3.60^a
Nil	10 000, McGregor	15.3×10^6	248^a	244^a	3.08^b
Nil	Nil	-	-	248^a	3.14^b
	LSD (P=0.05)		**39**	**24**	**0.21**

4.8. Molecular differentiation

4.8.1. PCR tests from antigenic sequences

The outer primers designed for *E. acervulina* successfully amplified a fragment that was 741 base pairs long. The inner primers produced a fragment that was 544 base pairs long. Specificity testing of the primers revealed no non-specific amplification of fragments from the other species of chicken *Eimeria*. As few as 10 oocysts in a mixed sample of 10^6 oocysts were successfully detected by the nested PCR system. These results are detailed in Molloy *et al.* (1998). The primers designed for *E. tenella* did produce some non-specific amplification, amplifying fragments from *E. necatrix* as well as *E. tenella*. Further validation work on that set of primers was discontinued.

4.8.2. Small subunit rDNA probes

The results obtained from using the seven SSU rDNA probes were variable. Probes for three species, *E. acervulina*, *E. praecox* and *E. tenella* maintained specificity when used on Australian isolates and hybridised to all appropriate samples. The probe for *E. brunetti* hybridised to the only *E. brunetti* sample available at the time and maintained specificity. The probe for *E. maxima* maintained specificity, but hybridised to only two of the three *E. maxima* samples. The probe for *E. mitis* did not hybridise to any isolates. The probe for *E. necatrix* was found to hybridise non-specifically.

4.8.3. PCR test on 5S intergenic spacer regions

Assessment of the test described by Stucki *et al.* (1993) showed that the species-specificity was maintained for the Australian isolates of *E. tenella*. All Australian samples of *E. tenella* produced positive results when tested.

4.8.4. RAPD PCR tests

Results from the RAPD PCR tests were variable and lacked the consistency between samples that is required to develop specific PCR tests. Thus, this approach was discontinued in favour of assessing the ITS1 PCR tests that had become available later in the project.

4.8.5. PCR tests on first internal transcribed spacer rDNA

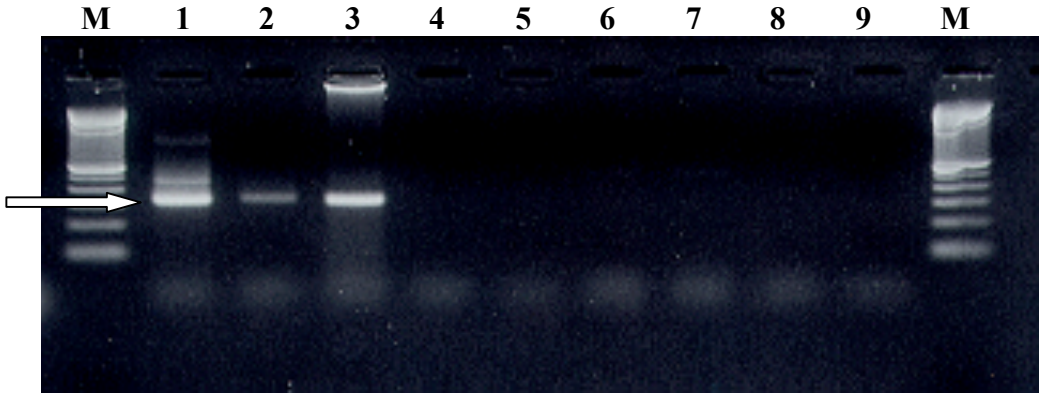
The outer primers have, so far, successfully amplified all of the samples tested. Results from testing using the inner primer sets are detailed below by species.

E. acervulina

The primers designed by Schnitzler *et al.* (1998) successfully amplified all three Australian isolates of *E. acervulina* that were tested, producing a fragment 321 base pairs in length (Figure 3). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*.

Figure 3. Results of PCR tests using *E. acervulina* specific ITS1 PCR primers.

M: Molecular weight 100 bp ladder; Lanes 1-3: *E. acervulina* isolates (321 bp fragment amplified – see arrow); Lane 4: *E. brunetti*; Lane 5: *E. maxima*; Lane 6: *E. mitis*; Lane 7: *E. necatrix*; Lane 8: *E. praecox*; Lane 9: *E. tenella.*

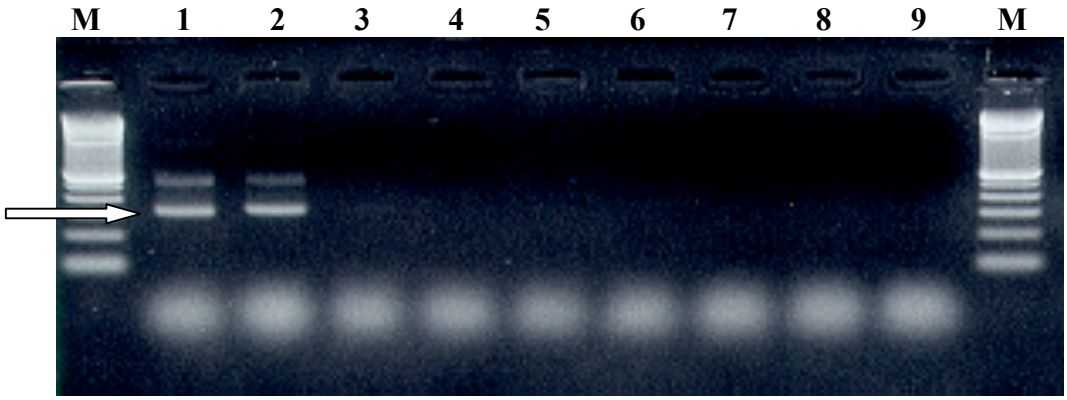


E. brunetti

The primers designed by Schnitzler *et al.* (1998) successfully amplified the two Australian isolates of *E. brunetti* that were tested, producing a fragment 311 base pairs in length (Figure 4). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*.

Figure 4. Results of PCR tests using *E. brunetti* specific ITS1 PCR primers.

M: Molecular weight 100 bp ladder; Lanes 1-3: *E. brunetti* samples (2 isolates, 311 bp fragment amplified – see arrow, Lane 3 very feint); Lane 4: *E. acervulina*; Lane 5: *E. maxima*; Lane 6: *E. mitis*; Lane 7: *E. necatrix*; Lane 8: *E. praecox*; Lane 9: *E. tenella.*

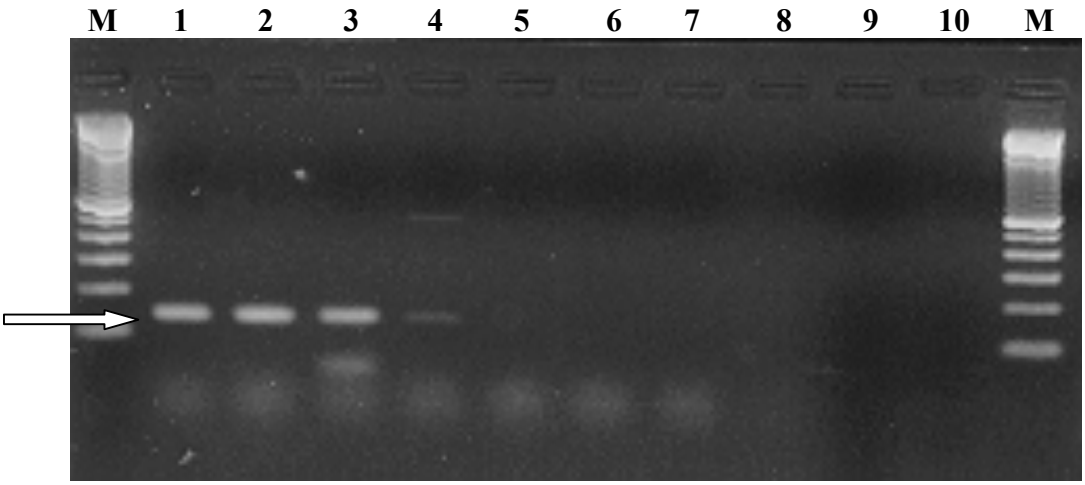


E. maxima

Sequencing of the ITS1 of two Australian isolates revealed significant differences between the Australian and American isolates. Primers designed from the sequences obtained for the Australian isolates successfully amplified all four Australian isolates that were tested, producing a fragment 145 base pairs in length (Figure 5). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*. Differences between the primers designed by Schnitzler *et al.* (1999) and the sequences obtained from Australian isolates suggest that those primers may not detect at least one Australian isolate.

Figure 5. Results of PCR tests using *E. maxima* specific ITS1 PCR primers.

M: Molecular weight 100 bp ladder; Lanes 1-4: *E. maxima* isolates (145 bp fragment amplified – see arrow); Lane 5: *E. acervulina*; Lane 6: *E. brunetti*; Lane 7: *E. mitis*; Lane 8: *E. necatrix*; Lane 9: *E. praecox*; Lane 10: *E. tenella.*

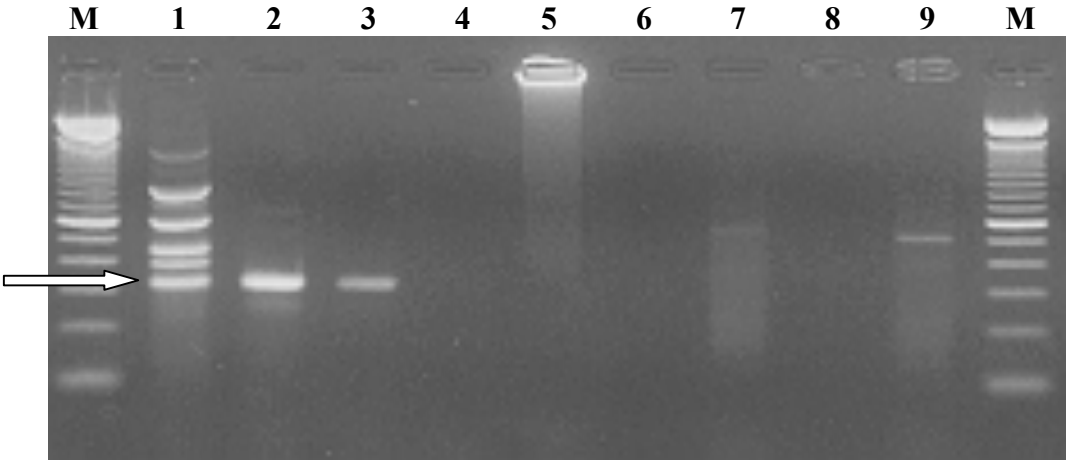


E. mitis

Two different sequences were obtained from Australian isolates of *E. mitis*. Thus, two sets of primers were designed. One set successfully amplified all three Australian isolates of *E. mitis* that were tested, producing a fragment 328 base pairs in length (Figure 6). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*. The second set of primers amplified some isolates of *E. acervulina* in addition to the *E. mitis* isolates and was therefore discarded. The primers recently designed by Schnitzler *et al.* (1999) are yet to be evaluated. Visual comparison of the primers and the sequences obtained from Australian isolates suggests that there are no differences that would prevent the primers from detecting Australian isolates.

Figure 6. Results of PCR tests using *E. mitis* specific ITS1 PCR primers.

M: Molecular weight 100 bp ladder; Lanes 1-3: *E. mitis* isolates (328 bp gragment amplified – see arrow); Lane 4: *E. acervulina*; Lane 5: *E. maxima*; Lane 6: *E. brunetti*; Lane 7: *E. necatrix*; Lane 8: *E. praecox*; Lane 9: *E. tenella*.

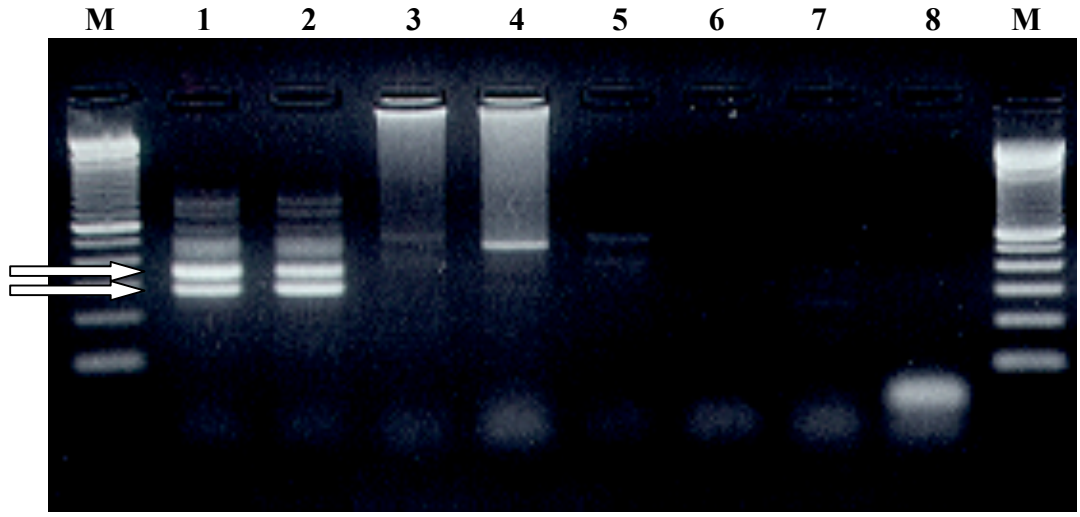


E. necatrix

The primers designed by Schnitzler *et al.* (1998) successfully amplified all three Australian isolates of *E. necatrix* that were tested, producing fragments 307 and 383 base pairs in length (Figure 7). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*.

Figure 7. Results of PCR tests using *E. necatrix* specific ITS1 PCR primers.

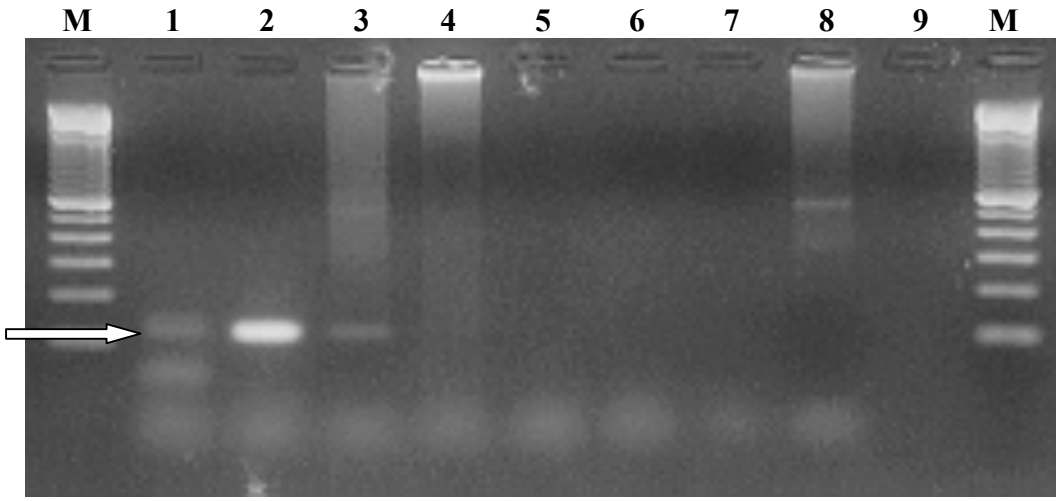
M: Molecular weight 100 bp ladder; Lanes 1-2: *E. necatrix* isolates (307 and 383 bp fragments amplified – see arrows); Lane 3: *E. tenella*; Lane 4: *E. acervulina*; Lane 5: *E. mitis*; Lane 6: *E. brunetti*; Lane 7: *E. praecox*; Lane 8: *E. maxima.*



E. praecox

The primers designed from sequences from Australian isolates successfully amplified all three Australian isolates of *E. praecox* that were tested, producing a fragment 116 base pairs in length (Figure 8). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*. Differences between the primers designed by Schnitzler *et al.* (1999) and the sequences obtained from Australian isolates suggest that those primers may not detect at least one Australian isolate.

Figure 8. Results of PCR tests using *E. praecox* specific ITS1 PCR primers.
M: Molecular weight 100 bp ladder; Lanes 1-3: *E. praecox* isolates (116 bp fragment amplified – see arrow); Lane 4: *E. brunetti*; Lane 5: *E. acervulina*; Lane 6: *E. maxima*; Lane 7: *E. mitis*; Lane 8: *E. necatrix*; Lane 9: *E. tenella.*

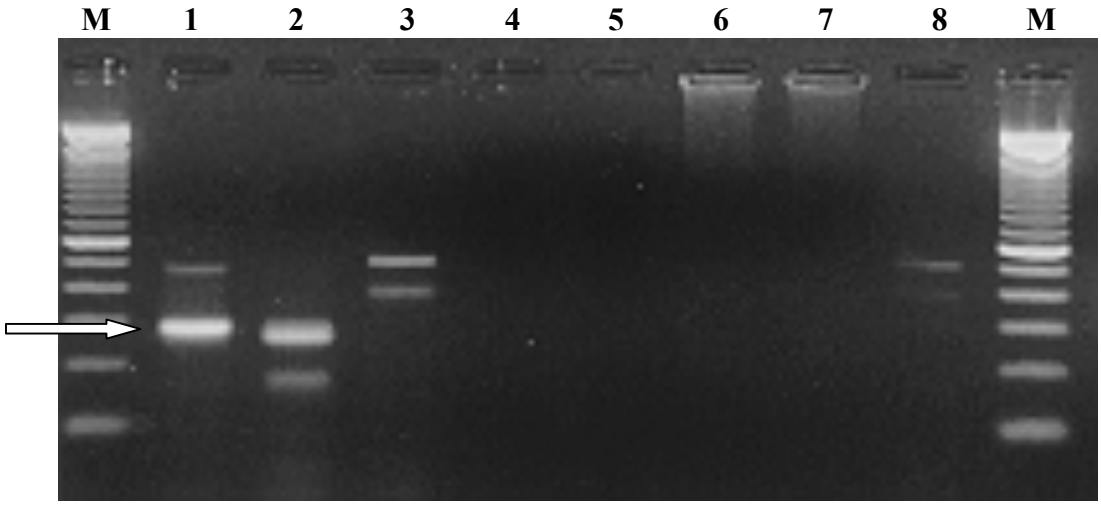


E. tenella

The primers designed by Schnitzler *et al.* (1998) successfully amplified all four Australian isolates of *E. tenella* that were tested, producing a fragment 278 base pairs in length (Figure 9). No non-specific amplification was detected when the primers were tested on isolates of the other six species of chicken *Eimeria*.

Figure 9. Results of PCR tests using *E. tenella* specific ITS1 PCR primers.

M: Molecular weight 100 bp ladder; Lanes 1-2: *E. tenella* isolates (278 bp fragment amplified – see arrow); Lane 3: *E. necatrix*; Lane 4: *E. acervulina*; Lane 5: *E. maxima*; Lane 6: *E. mitis*; Lane 7: *E. brunetti*; Lane 8: *E. praecox*.



4.8.6. Small subunit rDNA sequencing

Almost complete sequences of the SSU were produced for one isolate each of *E. tenella* and *E. acervulina*. In addition, the first 500 nucleotides have been determined for *E. mitis*, *E. praecox* and *E. maxima* (Figure 10). Comparison of the sequences of Barta *et al.* (1997) with those of the Australian isolates reveals no differences in the first five hundred nucleotides between American and Australian sequences of *E. tenella* or *E. maxima*. There is a single nucleotide difference in the fragment between isolates of *E. acervulina* or *E. mitis*, and two nucleotide differences between the isolates of *E. praecox* from the two continents.

Figure 10. Aligned DNA sequences of the first 500 nucleotides of the small subunit (SSU) rDNA of five species of *Eimeria*. Dots indicate nucleotides identical to those in the top sequence. Dashes indicate alignment gaps (inferred insertions/deletions). Nucleotides that have been underlined differ from the sequences of Barta *et al.* (1997).

```
E. tenella          TAGTCATATG CTTGTCTCAA AGATTAAGCC ATGCATGTCT
E. acervulina       .......... .......... .......... ..........
E. mitis            .......... .......... .......... ..........
E. praecox          .......... .......... .......... ..........
E. maxima           .......... .......... .......... ..........

E. te.   AAGTATAAGC TTTTATACGG TGAAACTGCG AATGGCTCAT TAAAACAGTT
E. ac.   .......... .......... .......... .......... ..........
E. mi.   .......... .......... .......... .......... ..........
E. pr.   .......... .......... .......... .......... ..........
E. ma.   ........A. .......... .......... .......... ..........

E. te.   ATAGTTTATT TGATGGTCTC ATTTTACATG GATAACCATG GTAATTCTAT
E. ac.   .......... .......... -......... .......... ..........
E. mi.   .......... ..........- T......... .......... ..........
E. pr.   .......... ..........- T......... .......... ..........
E. ma.   .......... ..........- T......... .......... ..........

E. te.   GGCTAATACA TGCGCAAAGG TCACCTCCTT TGGAGGGGCT GTGTTTATTA
E. ac.   .......... .......G.. C.T......C .......... ..........
E. mi.   .......... ......T... C.T......C .......... ..........
E. pr.   .......... .-.......A. CT....T..C .......... ..........
E. ma.   .......... ........A. CT....T... ......A... ..........
```

```
E. te.  GATACAAAAC CAACCCAC-T TTGTAGTGGA GTCTTGGTGA TTCATAGTAA
E. ac.  .......... .........C ....-..... .......... ..........
E. mi.  .._....... .......... ..-.-..... .C........ ..........
E. pr.  .......... .........T ..-.-..... ...A...... ..........
E. ma.  .......... ..G.....AA ..C.T..... .......... ..........

E. te.  CCGAACGGAT CGCAGTTGGT TCTTTTGGGC CCGCGATGGA TCATTCAAGT
E. ac.  .......... .........- -......... .......... ..........
E. mi.  .......... .........- -......... .......... ..........
E. pr.  .......... .........- -......... .......... ..........
E. ma.  .......... .........- -......... .......... ..........

E. te.  TTCTGACCTA TCAGCTTTCG ACGGTAGGGT ATTGGCCTAC CGTGGCAGTG
E. ac.  .......... .......... .......... .......... ..........
E. mi.  .......... .......... .......... .......... ..........
E. pr.  .......... .......... .......... .......... ..........
E. ma.  .......... .......... .......... .......... ..........

E. te.  ACGGGTAACG GGGAATTAGG GTTCGATTCC GGAGAGGGAG CCTGAGAAAC
E. ac.  .......... .......... .......... .......... ..........
E. mi.  .......... .......... .......... .......... ..........
E. pr.  .......... .......... .......... .......... ..........
E. ma.  .......... .......... .......... .......... ..........

E. te.  GGCTACCACA TCTAAGGAAG GCAGCAGGCG CGCAAATTAC CCAATGAAAA
E. ac.  .......... .......... .......... .......... ..........
E. mi.  .......... .......... .......... .......... ..........
E. pr.  .......... .......... .......... .......... ..........
E. ma.  .......... .......... .......... .......... ..........

E. te.  CAGCTTCGAG GTAGTGACGA GAAATAACAA TACAGGGCAT TTTATGCTTT
E. ac.  ...T...... .......... .......... .......... ._........
E. mi.  ...T...... .......... .......... .......... ..........
E. pr.  ...T...... .......... .......... .......... ..........
E. ma.  ...T...... .......... .......... .......... ..........

E. te.  GTAATTGGAA
E. ac.  ..........
E. mi.  ..........
E. pr.  ..........
E. ma.  ..........
```

5. Discussion

5.1. Isolation of field isolates

The identification of strains of *E. brunetti* and *E. mitis* as the causes of commercial outbreaks of coccidiosis demonstrates that these species have economic impacts on the Australian poultry industry. Although these species are unlikely to have impacts as large overall as the four species commonly implicated in outbreaks, *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella*, on a shed basis the impact may be just as great. It is not yet confirmed what impact *E. praecox* may have. Although it has been found to be widespread, *E. praecox* has not been identified as the cause of any clinical outbreaks in commercial flocks. Although apparently less pathogenic than the other six species of chicken *Eimeria,* large numbers of *E. praecox* can cause subclinical coccidiosis. Subclinical coccidiosis may cause economic losses through decreased bodyweight gains, increased feed conversion ratios and, because of inherent variation in the number of oocysts ingested by individual birds, uneven grow-out in broilers and onset of lay in layers.

5.2. Selection for precocious development

The method initially employed in project DAQ 25E/29CM to produce precocious lines of *E. acervulina* and *E. maxima* has again proved effective for producing two precocious lines each of *E. tenella* and *E. necatrix*. The number of passages required to produce a similar fall in prepatent periods was remarkably consistent between the two strains of each species (Figures 1 and 2). The difference between the species is a clear reflection of the difference in biology of the two species. It is unclear why the process failed for the McGregor strain of *E. necatrix*. The number of oocysts used in each passage should have ensured that sufficient genetic diversity was present to allow selection for faster developing parasites. There are no other obvious factors that should have influenced the process. We therefore conclude that the attenuation process is not always successful.

5.3. Design of characterisation trials

The characterisation trials have proven to be effective in gauging the suitability of strains for use in a live coccidiosis vaccine. The introduction of feed conversion ratio as an

additional measured parameter, as requested by the RIRDC, has given greater sensitivity in detecting treatment effects. Validation of the drug sensitivity trial design, requested by R. Jenner, showed that the drugs themselves had no significant effect on bodyweight gain. Thus, any significant differences in bodyweight gain in drug trial results can be interpreted as being caused by the parasites rather than the drugs. The drugs did have a small effect on feed conversion ratios. There were significant decreases in feed conversion ratio for birds receiving the Sulphaquinoxaline or Amprolium treatments compared with the negative control birds. The differences were minor, however, and any significant increases in feed conversion ratio would clearly demonstrate decreased drug susceptibility.

5.4. Characterisation of precocious vaccine lines

5.4.1. Redlands strain of *E. tenella*

The Redlands strain is a relatively mild strain and differences in pathogenicity between the parent and precocious lines are not as clear cut as those in the other strains. Nevertheless, the precocious line exhibits all of the appropriate characteristics for use in a live vaccine. The strain appears to be highly susceptible to Toltrazuril, which reduced oocyst output and prevented a decrease in bodyweight gain or an increase in feed conversion ratio when compared to the negative control group. Whilst the Sulphaquinoxaline treatment had no significant effect on the oocyst output of the parasites, it appeared to reduce the impact of the infection on the birds by maintaining bodyweight gain and reducing the increase in feed conversion ratio compared with the positive control birds. The Amprolium treatment produced no significant differences from the positive control group and could not be recommended for control of vaccine reactions.

There was a clear drop in oocyst output in the precocious line. Birds challenged with 500 oocysts of the precocious line produced significantly fewer oocysts than birds challenged with 500 oocysts of the parent strain. In contrast, although there appeared to be a trend for birds receiving 3 000 oocysts of the precocious line to produce fewer oocysts than those receiving 3 000 oocysts of the parent strain, there was no significant difference in oocyst outputs between the two groups. This is probably due to the oocyst dose/oocyst output saturation effect that has been examined recently in some detail (Jeston, Anderson & Jorgensen, 1998). The drop in oocyst output in the precocious line should not be sufficient to prevent the strain's use in commercial production.

The pathogenicity of the precocious line was also decreased significantly. Challenges of as many as 20 000 oocysts of the precocious line had no significant effect on bodyweight gains whereas challenges of 10 000 oocysts of the parent strain resulted in significantly decreased bodyweight gains.

The precocious line successfully induced immunity to the parent strain. A single vaccine dose of greater than 100 oocysts was required to cause significant reduction in oocyst output upon challenge, but there was a trend for vaccine doses as low as 10 oocysts to cause a reduction in oocyst output. No meaningful differences in bodyweight gains were observed in the homologous challenge trial because of the mild nature of the parent strain.

The precocious line also induced immunity to two other virulent field strains. There were significant differences in oocyst output between vaccinated and non-vaccinated groups for both challenge strains. Similarly, for both challenge strains, vaccinated birds had similar bodyweight gains to the negative control birds whereas non-vaccinated birds had significantly lower bodyweight gains than the negative control birds.

5.4.2. Darryl strain of *E. tenella*

The precocious line of the Darryl strain exhibits all of the appropriate characteristics for use in a live vaccine. The strain appears to be susceptible to Toltrazuril, which reduced oocyst output and prevented a decrease in bodyweight gain when compared to the negative control group. Whilst the Sulphaquinoxaline treatment had no significant effect on the oocyst output of the parasites, it maintained bodyweight gain at a level comparable to the negative control group. The Amprolium treatment had no significant effect on the oocyst output of the parasites and, although it kept the reduction in bodyweight gain below that seen for the positive control group, it did not prevent a reduction occurring.

There was a clear drop in oocyst output in the precocious line. Birds challenged with 500 or
3 000 oocysts of the precocious line produced significantly fewer oocysts than birds challenged with the same number of oocysts of the parent strain. The drop in oocyst output in the precocious line should not, however, be sufficient to prevent the strain's use in commercial production.

The pathogenicity of the precocious line was also decreased significantly. Challenges of as many as 20 000 oocysts of the precocious line had no significant effect on bodyweight gains or feed conversion ratios whereas challenges of 10 000 oocysts of the parent strain resulted in decreased bodyweight gains and increased feed conversion ratios.

The precocious line successfully induced immunity to the parent strain. A single vaccine dose of greater than 100 oocysts was required to cause significant reduction in oocyst output upon challenge, but there was a trend for vaccine doses of 100 oocysts to cause a reduction in oocyst output. Vaccination with as few as 10 oocysts prevented decreases in bodyweight gain after challenge.

The precocious line also induced immunity to two other virulent field strains. There were significant differences in oocyst output between vaccinated and non-vaccinated groups for both challenge strains. The reduction in oocyst output is, however, less marked than that in the Redlands strain, which suggests that this strain does not protect as well against the two challenge strains as does the Redlands strain. No significant differences in bodyweight gains were found in the heterologous challenge trial because the challenge doses, in this case, proved insufficient to have an effect on the bodyweight gains of the birds.

5.4.3. Medichick strain of *E. necatrix*

The parent Medichick strain is obviously more virulent than the two strains of *E. tenella* selected for vaccine development. Nevertheless, the precocious line of the Medichick strain exhibits all of the appropriate characteristics for use in a live vaccine. The strain is susceptible to Toltrazuril and Sulphaquinoxaline, which prevented any deaths and prevented a decrease in bodyweight gain when compared to the negative control group. The Amprolium treatment did not prevent bird deaths and, although it kept the reduction in bodyweight gain below that seen for the positive control group, it did not prevent a reduction occurring.

There appears to be a trend for a drop in oocyst output in the precocious line, but the differences in oocyst output between the precocious and parent lines are not significant for birds challenged with 500 or 3 000 oocysts. Interestingly, birds inoculated with 3 000

oocysts did not produce more oocysts than those receiving 500 oocysts. Again, this is probably due to a saturation effect (Jeston, Anderson & Jorgensen, 1998).

In contrast to the oocyst output, the pathogenicity of the precocious line was clearly decreased. Challenges of up to 10 000 oocysts of the precocious line had no significant effect on bodyweight gains or feed conversion ratios whereas challenges of 10 000 oocysts of the parent strain resulted in decreased bodyweight gains and increased feed conversion ratios.

The precocious line successfully induced immunity to the parent strain. A single vaccine dose of greater than 10 oocysts was required to cause significant reduction in oocyst output upon challenge, but there was a trend for vaccine doses of 10 oocysts to cause some reduction in oocyst output. Vaccination with as few as 10 oocysts resulted in a lower reduction in bodyweight gain than that seen in the positive control group and vaccine doses of 100 oocysts prevented any decrease in bodyweight gain after challenge.

The precocious line also induced immunity to two other virulent field strains. There were significant differences in oocyst output and bodyweight gain between vaccinated and non-vaccinated groups for both challenge strains. Bodyweight gains in the vaccinated birds were not significantly different from those of the negative control group.

5.4.4. Gatton strain of *E. necatrix*

The Gatton strain is quite a mild strain and the precocious line exhibits all of the appropriate characteristics for use in a live vaccine. The strain is highly susceptible to Toltrazuril, which reduced oocyst output to zero and prevented a decrease in bodyweight gain or increase in feed conversion ratio when compared to the negative control group. In contrast, the Sulphaquinoxaline and Amprolium treatment groups had oocyst outputs, bodyweight gains and feed conversion ratios that were not significantly different from those of the positive control group.

There was a clear drop in oocyst output in the precocious line. Birds challenged with 500 oocysts of the precocious line produced significantly fewer oocysts than birds challenged with 500 oocysts of the parent strain. In contrast, although there appeared to be a trend for birds receiving 3 000 oocysts of the precocious line to produce fewer oocysts than those

receiving 3 000 oocysts of the parent strain, there was no significant difference in oocyst outputs between the two groups. This, again, is probably due to a saturation effect.

The pathogenicity of the precocious line was also clearly decreased. Challenges of up to 10 000 oocysts of the precocious line had no significant effect on bodyweight gains or feed conversion ratios whereas challenges of 10 000 oocysts of the parent strain resulted in decreased bodyweight gains and increased feed conversion ratios.

The precocious line successfully induced immunity to the parent strain. A single vaccine dose of 10 oocysts produced a significant reduction in oocyst output upon challenge. Vaccination with 10 oocysts also prevented any decrease in bodyweight gain or increase in feed conversion ratio after challenge.

The precocious line also induced immunity to two other virulent field strains. There were significant differences in oocyst output and bodyweight gain between vaccinated and non-vaccinated groups for both challenge strains. In the case of the vaccinated group challenged with the McGregor strain, oocyst output was reduced to zero. Bodyweight gains and feed conversion ratios in the vaccinated birds were not significantly different from those of the negative control group whereas the group that was unvaccinated and challenged with the Medichick strain had decreased bodyweight gain and increased feed conversion ratio. Although there were trends for the McGregor strain to produce the same effects in the unvaccinated birds, the challenge dose, in this case, proved insufficient to produce significant differences from the negative control birds.

5.5. Molecular differentiation

5.5.1. PCR tests from antigenic sequences

The test developed for *E. acervulina* was highly successful, having appropriate specificity and high sensitivity. In contrast, the test for *E. tenella* also detected *E. necatrix* and was therefore not specific enough to be useable. It is not entirely clear why this was the case. It is thought that *E. tenella* and *E. necatrix* are closely related. The test may therefore have been detecting sequences for antigens that are the same or very similar in the two species. Overall, the approach shows good promise. It is, however, limited at present by the lack of availability of antigen gene sequences. The approach could be further investigated by

starting with base level research looking for appropriate species-specific antigens. The process would, however, be expensive and time consuming.

5.5.2. Small subunit rDNA probes

The results for this approach were variable. It is unclear why some probes did not work on Australian isolates whereas others did. Further investigation of why this was occurring was beyond the scope of this study. Overall, the technique shows reasonable promise, but its usefulness in Australia is limited by several factors. More work is required to investigate differences between American and Australian isolates so that probes could be modified if necessary. The need for the combination of PCR and hybridisation into one assay to obtain adequate sensitivity would also make the test cumbersome and time consuming. Finally, the limitations and costs associated with the patented probes would limit the application of the method as a routine test in commercial laboratories.

5.5.3. PCR test on 5S intergenic spacer regions

The one test that was available worked well on Australian isolates, giving appropriate specificity and high sensitivity. The approach appears suitable for the other species of chicken *Eimeria*, but would require further work sequencing the appropriate DNA fragments and designing appropriate PCR primers.

5.5.4. RAPD PCR tests

RAPD PCR tests have, in themselves, been largely abandoned as useful tools in diagnostics because of a lack of reproducability. The aim in this study was to avoid this problem by using the RAPD data to design specific PCR tests. The lack of reproducability encountered, however, meant that species-specific fragments could not be identified reliably and the approach was abandoned. There is no obvious way in which this technique could be applied further.

5.5.5. PCR tests on first internal transcribed spacer rDNA

DNA sequences of the ITS1 of the seven species of chicken *Eimeria* indicate that the spacer is sufficiently variable for this approach to work. It is obvious, however, that the ITS1 is more variable than is ideal for species-specific tests. Comparison of sequences from Australian, European and American isolates reveals large intraspecific differences in some species that may result in the inability of primers to successfully amplify all of the strains of

that species. It is therefore imperative that the tests be validated with more than one isolate of each species. Validation of the primer sets developed in Europe by Schnitzler *et al.* (1998, 1999) and developed in this project using at least two Australian strains of each species from geographically distant collection sources should ensure that these tests are appropriate for identifying most Australian isolates. Further validation, however, is required to ensure that the tests will detect and identify all Australian isolates to species.

Some of the variation in the ITS1 could be explained by the presence of cryptic species (i.e. species that have the same morphology as other species, but are genetically distinct). This possibility is currently being investigated using SSU rDNA sequencing in a parallel study being funded by the DPI. Overall, this approach shows good promise when PCR primers that are appropriate for Australian isolates are used. The tests should be able to be applied as routine diagnostic tests once validation has been completed and can be used both to identify species causing infections and to detect contamination of vaccine stocks with other species. The variability of the rDNA spacers may also be appropriate for differentiation of strains within a species. Research looking at this aspect is currently under way in a collaborative program between *Eimeria* Pty Ltd and the University of Melbourne Veterinary School.

5.5.6. Small subunit rDNA sequencing

The SSU is a conserved part of the rDNA that, in general, shows consistent variation between species, but little variation within species. The differences between the *Eimeria* isolates from Australia and their counterparts from America can be explained by the occurrence of several point mutations (single nucleotide changes). In three of the four differences (one each from *E. acervulina*, *E. mitis* and *E. praecox*), the Australian isolate shares the same nucleotide as the other six species (both Australian and American isolates). This suggests that the nucleotide found in the Australian isolate represents the ancestral state and the nucleotide in the American isolate is a mutation. These three differences could also be explained by nucleotide reading errors in the sequencing of the American isolates. In the fourth instance, the nucleotide seen in the Australian isolate of *E. praecox* is apparently a mutation that is shared with *E. maxima*. Regardless of whether the differences are real or represent artifacts, however, the results demonstrate that little divergence has occurred between the populations on the two continents.

Although the presence of interspecific variation and lack of major intraspecific variation in the SSU appears ideal for designing species-specific PCR tests, the level of interspecific variation is not sufficient to allow the design of PCR primers that will differentiate between all of the seven species of chicken *Eimeria*. Without species-specific primers (which could, in themselves, be used as PCR tests), this test will only identify the dominant species in a mixed sample and only then if that species is highly dominant in number. Thus, it is not suitable for detecting minor contamination of vaccine stocks with other species. This test is, however, extremely useful for confirming the species identity of purified strains and for investigating the identity of putative new species.

6. Implications

- The vaccine lines that were produced in this project, in addition to those produced in the previous project DAQ 25E/29CM, will allow the production of a low virulence quadrivalent live coccidiosis vaccine to protect chickens from the four species of *Eimeria* that commonly cause outbreaks of clinical coccidiosis. Use of the vaccine should allow significant reductions in the use of coccidiostats and effectively reduce production costs and losses.

- Species-specific DNA tests will be available to diagnose coccidial infections in Australia after validation of the tests that were evaluated and developed in this project has been completed. These tests will allow more accurate identification of species that are causing problems and improved surveillance and refinement of management strategies.

7. Recommendations

- Although there are now vaccine lines of the four species of *Eimeria* that are known to cause major problems in the Australian poultry industry, there are none available for the remaining three species. Clinical coccidiosis may not be a common result of infection with these species, but all are likely to cause production losses. These problems are likely to become more evident as the vaccine incorporating the other four species is used more commonly throughout the industry.
  → Detailed investigation using species-specific molecular tests would allow assessment of the impact of the three species on the Australian industry.
  → Development of vaccine lines for the three species would allow the use of a comprehensive coccidiosis vaccine and a significant reduction in use of coccidiostats.

- Results from the drug sensitivity trials raised some concerns over how widespread drug resistance in the seven species of chicken *Eimeria* may be, even amongst non-commercial flocks. Drug resistance in Australian isolates of *Eimeria* should be investigated in detail to determine how widespread resistance to various coccidiostats, particularly those now in routine use in the poultry industry, is. Further investigation of the problem may enable modification of management strategies to reduce costs, improve results and extend the useful life of particular coccidiostats.

- The heterogeneity of *E. maxima*, previously noted because of problems with variability in cross protection between strains, has become even more evident with genetic analysis. The taxonomic status of the nominal species needs to be investigated so that appropriate vaccination strategies can be used. Further genetic analysis using SSU rDNA should enable detailed study of this problem.

8. Intellectual Property

Intellectual property from the project is in the form of a product that is commercially desirable. A commercialisation strategy was developed prior to the start of the project and commercial agreements between DPI, RIRDC and *Eimeria* Pty Ltd have been signed and are in force.

9. Communications Strategy

The vaccine lines of the Redlands strain of *E. tenella* and the Medichick strain of *E. necatrix* have been provided to the project commercial partner, *Eimeria* Pty Ltd. These lines are currently undergoing registration trials so that they can be incorporated into a non-virulent quadrivalent live coccidiosis vaccine that will be available to the poultry industry throughout Australia. The vaccine lines developed from the Darryl strain of *E. tenella* and the Gatton strain of *E. necatrix* are available from the Department of Primary Industries, Queensland for future release, if needed.

Publications and presentations arising from this project

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