Live vaccines
for three species
of *Eimeria*

**A report for the Rural Industries Research
and Development Corporation**

by Glenn Anderson and Wayne Jorgensen

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Foreword

This publication describes the outcomes of Rural Industries Research and Development Corporation project No DAQ-259J "Attenuation and characterisation of *Eimeria* spp for use in a living vaccine for avian coccidiosis (Stage 3)."

The previous two stages of this series of projects (EIRDC DAQ 25E & CMRDC DAQ 29CM; DAQ-215AJ) have been successfully completed. The main aim of those projects was to develop precocious lines of *E. maxima, E. acervulina, E. tenella* and *E. necatrix* suitable for use in a live poultry coccidiosis vaccine.

The current and final stage 3 project (RIRDC DAQ-259J) had the aim of developing vaccines against the remaining three *Eimeria* species present in Australia, *E. mitis, E. brunetti* and *E. praecox*. The objectives of the project were to attenuate two strains of each species by selecting for precocious development and to characterise the candidate vaccine lines in terms of drug sensitivity, reproductive potential, pathogenicity and protection against homologous and heterologous challenge. The project team successfully achieved the aim of the project and also evaluated a trial procedure to assess resistance to in-feed coccidiostats in field isolates.

A productive and highly cooperative working relationship has developed between researchers at DPI and commercial partners, *Eimeria* Pty Ltd (identified at the beginning of the Stage 2 project). The DPI team have been undertaking additional contract trial work to facilitate the vaccine registration process. The vaccine strains developed and characterised in Stages 1 and 2 are in the final stages of registration in Australia and will be available for unlimited use in 2003.

The final outcome of the three stages of the project series will be the availability, to the Australian poultry industry, of live 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 600 research publications, forms part of our Chicken Meat R&D program which aims to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images, and Egg R&D program which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Abbreviations

ANOVA	analysis of variance
ARI	Animal Research Institute
DMSO	dimethyl sulphoxide
DPI	Department of Primary Industries, Queensland
LSD	Least significant difference
MEM	minimum essential medium
PCR-RFLP	polymerase chain reaction linked restriction fragment length polymorphism
RIRDC	Rural Industries Research and Development Corporation

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Executive Summary

The RIRDC/DPI funded project entitled "Attenuation and characterisation of *Eimeria* spp for use in a living vaccine for avian coccidiosis (Stage 3)" was initiated in 1999 with the following two objectives:
1. Attenuated lines of *E. mitis, E. brunetti* and *E. praecox* to be developed for incorporation in an efficacious live vaccine protective against all seven species of *Eimeria* in Australian chickens.
2. A trial technique to evaluate coccidiostat resistance to be developed.
Project outcomes are summarised below under the two objective headings.

1. **Attenuated lines of *E. mitis, E. brunetti* and *E. praecox* to be developed for incorporation in an efficacious live vaccine protective against all seven species of *Eimeria* in Australian chickens.**

1a. Source of vaccine and challenge isolates of *E. mitis, E. brunetti* and *E. praecox.*
Parasites used in live coccidiosis vaccines must have high susceptibility to anti-coccidial compounds (coccidiostats). Parent strains used for vaccine development were therefore collected from small, non-commercial flocks that did not routinely use coccidiostats and in which clinical coccidiosis had not been a problem. Isolates of *E. mitis, E. brunetti* and *E. praecox* were collected from flocks of this type during the phase 2 project. In addition, isolates of *E. mitis, E. brunetti* and *E. praecox* were collected from commercial operations for use as challenge strains to test the immunity induced by the vaccine lines. All isolates were purified by series of single oocyst passages (i.e. inoculating a single oocyst into a chicken and allowing it to reproduce). Purified isolates were stored in liquid nitrogen.

1b. Modification of the 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 parasites through susceptible chickens, in each case using the oocysts recovered earliest from the previous passage. Two isolates of *E. mitis* were passaged in this way. The prepatent period of the Jorgensen strain was successfully reduced 20 hours over nine passages and a concomitant reduction in virulence was achieved. The strain was later evaluated for use as a vaccine.

Two strains of *E. brunetti* underwent selection for precocious development [Bowden strain (9 passages) and Monarto strain (13 passages)]. Neither was suitable for use as a vaccine strain due to high pathogencity. The Bowden parent strain, however, was found during drug sensitivity trials to have very low pathogenicity. After discussions with the project commercial partners and the RIRDC Research Manager, the Bowden parent strain was subsequentally evaluated for use as a vaccine.

Eimeria praecox is the least pathogenic of the *Eimeria* species present in Australia. Two strains (Jorgensen and Kelly) were evaluated for virulence while being tested for drug sensitivity. Due to its very low pathogenicity, the Jorgensen strain was chosen for further evaluation without passaging for precocious development.

1c. Characterisation of strains in terms of drug sensitivity, reproductive potential, pathogenicity and protection to homologous and heterologous challenge.
The Jorgensen and Kelly strains of *E. mitis*, the Monarto and Bowden strains of *E. brunetti*, and the Jorgensen and Kelly strains of *E. praecox* were characterised for drug sensitivity. The Jorgensen strain of *E. mitis* (precocious), the Bowden strain of *E. brunetti* and the Jorgensen strain of *E. praecox* were characterised for pathogenicity and protection against heterologous challenge. The Jorgensen strain of *E. mitis* (precocious) was also characterised for reproductive potential and protection against homologous challenge. The Bowden strain of *E. brunetti* (precocious) also underwent characterisation for protection against homologous challenge. All characterisation trials were cage trials with randomised block designs.

Drug sensitivity: The Bowden strain of *E. brunetti* and the Jorgensen strain of *E. praecox* were found to be susceptible to manufacturers' recommended treatment doses of Toltrazuril, Amprolium and Sulphaquinoxaline. The Jorgensen strain of *E. mitis* was found to be susceptible to recommended treatment doses of Toltrazuril and Amprolium.

Pathogenicity: Doses of 20 000 oocysts of Jorgensen strain *E. mitis* or 15 000 oocysts of Jorgensen strain *E. praecox* or Bowden strain *E. brunetti* caused no significant increase in feed conversion ratios or decrease in bodyweight gain of experimental birds indicating the proposed vaccine strains had very low pathogenicity.

Homologous challenge: The Jorgensen strain of *E. mitis* provided protection against challenge with the parent strain. Homologous challenge trials were not performed on the Bowden strain of *E. brunetti* or the Jorgensen strain of *E. praecox* as the vaccine strains are unmodified parent strains. It was demonstrated, however, that the precocious line of the Bowden strain of *E. brunetti* provided protection against challenge with the parent strain.

Heterologous challenge: Birds vaccinated with the Jorgensen strain of *E. mitis*, Bowden strain of *E. brunetti* or Jorgensen strain of *E. praecox* were protected against challenge with two field strains of the same species.

Reproductive potential: The attenuated line of the Jorgensen strain of *E. mitis* demonstrated a highly reduced reproductive potential compared with the parent strain, which is an indicator of the success of the attenuation process. Reproductive potential trials were not performed on the Bowden strain of *E. brunetti* or the Jorgensen strain of *E. praecox* as they were unmodified parent strains.

2. A trial technique to evaluate coccidiostat resistance to be developed.
A randomised block design was developed to determine the ability of in-feed coccidiostats (Narasin and Monensin) to protect birds against the harmful effects of infection with various parasite stocks. Parasites used in the new trial format were Eimeriavax 4m (the new vaccine containing *E. necatrix, E. tenella, E. acervulina* and *E. maxima* developed in Stages 1&2), field isolates from a commercial layer farm and a broiler farm, and the Kelly strain of *E. praecox*, which was not significantly affected by Toltrazuril in the standard drug sensitivity trial. There was evidence of some resistance to both Narasin and Monensin.

Conclusion
The candidate vaccine strains selected for field evaluation were drug susceptible, had low pathogenicity and were highly protective against challenge with two virulent field isolates (heterologous challenge). The strains have been transferred to *Eimeria* Pty Ltd for incorporation in new custom vaccines. A proposal for a new project has been submitted to RIRDC to develop the diagnostic tools necessary to support use of the live vaccines in the marketplace and ensure their continued efficacy.

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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) and all have now 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. In two previous RIRDC projects, staff from the Department of Primary Industries, Queensland developed precocious vaccine lines of *E. maxima*, *E. acervulina*, *E. tenella* and *E. necatrix*. These lines were subsequently provided 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 vaccine lines of the other three species of fowl coccidia, *E. mitis*, *E. brunetti* and *E. praecox*.

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 candidate parasite lines, but also to characterise the lines to gauge their suitability for use in a vaccine.

2. Objectives

- Identify at least two candidate parasite strains from each species (*E. mitis*, *E. brunetti* and *E. praecox*) from the isolates held at ARI (collected and purified in previous RIRDC-funded projects).
- Evaluate the drug sensitivity of the candidate strains.
- Modify prepatent period of the strains, as appropriate, by selecting for precocious development.
- Characterise the strains in terms of pathogenicity, reproductive potential and protection against homologous and heterologous challenge.
- Develop and evaluate a drug sensitivity trial protocol for use with in-feed prophylactic coccidiostats.
- Characterise the sensitivity of at least three field isolates to two industry-standard in-feed prophylactic coccidiostats (ionophore and chemical).

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 biosecurity 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. After use, all isolators were disassembled and scrubbed in hot water and Virkon at manufacturers recommended concentration before reassembly and reuse.

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 Review 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, frequent use of insecticides and rodent baits, and thorough decontamination of rooms between batches of birds (see below). Uninfected control birds were also maintained in rooms during experimental procedures to evaluate whether contamination was occurring.

3.1.3. Laboratory procedures

Laboratory work was performed in two laboratories. Work associated with the candidate vaccine strains 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 remaining feed, secured in plastic bags. Similarly, contaminated waste from the laboratories was also bagged securely. Bags were removed from site and destroyed by a biohazardous waste contractor. Decontamination of experimental facilities was aimed at destruction of oocyst contamination. Decontamination of experimental rooms consisted of hosing the room floors with hot water and treatment with Divosan Q-cide™ (a quarternary ammonia compound). Vaccine passaging rooms were also maintained at 40°C for greater than three days prior to use to kill any residual oocysts by desiccation. Decontamination of trial sheds consisted of hosing out the sheds to remove gross contamination followed by treatment with Divosan Q-cide™ and thorough steam cleaning. 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 sterilisation for coccidia (Fish, 1932).

3.2. 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 mainly selected from relatively small, non-commercial flocks that were not regularly exposed to coccidiostats and did not have clinical coccidiosis. The one exception to this was the Monarto strain of *E. brunetti* which was isolated from a commercial flock. It was selected as a candidate strain because it was, at the time, causing significant economic losses. In doing so, it was hoped to minimise antigenic variation between the vaccine line and the field strain to maximise the effectiveness of the vaccine. Use of the strain for vaccine development work was, of course, dependent on it being sufficiently drug sensitive.

3.3. Selection for precocious development

Parasite lines were selected for precocious development by serial passaging through naïve chickens. For each passage, 12 birds were inoculated with between 2 000 and 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 and was continued until the prepatent period had fallen about 24 hours.

3.4. Characterisation trials

Precocious parasites were assessed for vaccine suitability using five characterisation trials: drug sensitivity, reproductive potential (oocyst output), pathogenicity, homologous challenge and heterologous challenge. Unmodified parasites were assessed using drug sensitivity, pathogenicity and heterologous challenge trials. Each trial followed a randomised block design consisting of either five blocks of six treatment groups or six blocks of 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 parasites by oral inoculation. Oocyst output and/or bodyweight gain and feed conversion ratio were the parameters measured to indicate treatment effects. All parameters were measured over a 10, 11 or 12 day period following final inoculation, which encompassed the main reaction period. Oocyst output was measured by collecting and weighing all of the faeces from a cage for the period between expected patency and the end of the trial, 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 final inoculation and at the end of the trial. The genetics of the SPF chickens used in trials and use of a feed lacking growth promotants resulted in feed conversion ratios that were higher than those observed routinely in commercial production.

3.4.1. 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 drug treatment; a positive control group, which received a parasite inoculation but no drug treatment; and three groups that were given three different drug treatments after receiving a parasite inoculation. The drug treatments are outlined in Table 1. Inoculants consisted of either 10 000 or 15 000 oocysts. Oocyst output, bodyweight gain and feed conversion ratio were measured as indicators of drug treatment effectiveness.

Table 1. Drug treatments used in the drug sensitivity trials. Each treatment regime followed the recommendations of the manufacturer.

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)
Coxitrol	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

3.4.2. Pathogenicity trials

The pathogenicity trials were used to assess whether the parasites had sufficiently low pathogenicity. 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 inoculations. Inoculants for trials assessing precocious lines consisted of: 2 000, 5 000 or 20 000 oocysts of the precocious line or 20 000 oocysts of the parent strain for *E. mitis* and 2 000, 5 000 or 10 000 oocysts of the precocious line or 10 000 oocysts of the parent strain for *E. brunetti*. Inoculants for trials assessing unmodified strains (*E. brunetti* and *E. praecox*) consisted of: 5 000, 10 000 or 15 000 oocysts of the candidate strain or 10 000 oocysts of a different (virulent) field strain. Bodyweight gain and feed conversion ratio were measured as indicators of pathogenicity.

3.4.3. Homologous challenge trials

The homologous challenge trials were used to determine if vaccination with 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 21 days after vaccination and consisted of 20 000 oocysts for *E. mitis* and 15 000 oocysts for *E. brunetti*. Oocyst output, bodyweight gain and feed conversion ratio were measured as indicators of the effectiveness of the vaccination.

3.4.4. Heterologous challenge trials

The heterologous challenge trials were used to determine if vaccination with the vaccine candidates would induce protective immunity against other virulent field strains. Challenge strains for each of the three species were isolated from cases of clinical coccidiosis on commercial farms. 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 vaccinated before receiving the two different parasite challenges. Vaccination doses consisted of 2 000 oocysts for *E. mitis* and *E. praecox* and 1 000 oocysts for *E. brunetti*. Challenge doses of virulent field strains were given 21 days after vaccination and consisted of 20 000 oocysts for *E. mitis*, 10 000 oocysts for *E. brunetti* and 30 000 oocysts for *E. praecox*.

Oocyst output, bodyweight gain and feed conversion ratio were measured as indicators of the effectiveness of the vaccination.

3.4.5. Reproductive potential (oocyst output) trials

Reproductive potential trials are used to assess whether precocious lines have reduced oocyst outputs compared with the parent strain. The trial design incorporates six blocks of five treatments. The treatment groups include: a negative control group, which receives no parasites; and four groups given four different parasite challenges. Challenge doses of parasites consisted of either 100 or 10 000 oocysts of the parent strain or the precocious line. Oocyst output was measured to indicate any change in reproductive potential.

3.4.6. 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 using the $(X+1)^{1/3}$ (cube-root) transformation for further analysis. The raw (untransformed) oocyst count data has a typically highly skewed distribution (long tail to the right). For validity of ANOVA, assumptions about the data are required to be approximately met, and these include approximate normality, or at least symmetry, and equality of treatment variances. Experience has shown that for oocyst count data, the cube root transformation performs best among the family of variance-stabilising transformations. It is calculated as follows: X is the original raw data (oocyst count data); a one is added to the oocyst count; and X+1 is then raised to the power 1/3 (taking the cube root of X+1). The transformed means are the usual arithmetic means calculated on this transformed scale.

"Back-transformation" means that the steps involved in calculating $(X+1)^{1/3}$ are applied in reverse to the transformed means. Firstly, the transformed means are cubed (raised to the power 3), then a one is subtracted. Back-transformed data are provided in the results tables of this report to provide an indication of raw data values, but were not used in the analyses.

3.5. Storage of parasites

Parasite samples were stored routinely 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. Only parasites that had been stored for a period less than four weeks since production were used for trials.

For long term storage, parasites are maintained frozen in liquid nitrogen. Samples for freezing are prepare as follows. 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 15 minutes of thawing. After freezing, batches of parasites were tested for infectivity and purity using the quality control procedure outlined below.

3.6. Quality control

A detailed quality control procedure was used 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 and thawed 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. 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.

DNA analyses were performed on oocyst samples suspected of being contaminated or misidentified. Analyses were done by Eimeria Pty Ltd using the PCR-RFLP technique described by Woods, Whithear, Richards, Anderson, Jorgensen and Gasser (2000).

3.7. Evaluation of resistance to prophylactic coccidiostats

3.7.1. Trial design
Each trial consisted of five blocks of six treatments. The treatment groups included: a negative control group, which received no parasites and no prophylactic; a positive control group, which received a parasite challenge but no prophylactic; two groups that each received one of the prophylactics in addition to a parasite challenge; and two groups that each received one of the prophylactics, but no parasites. Challenge doses consisted of 20 000 oocysts. Oocyst output, bodyweight gain and feed conversion ratio were measured as indicators of the effectiveness of the prophylactic.

Statistical analyses were the same as those outlined above for the vaccine candidate characterisation trials.

3.7.2. Selection of parasites for evaluation
A range of different parasites were used to validate the trial design. The Eimeriavax 4m live coccidiosis vaccine was used initially because it would provide data for parasites that have been shown previously to be susceptible to coccidiostats used for treatment of coccidiosis. Field isolates with suspected coccidiostat resistance were obtained from two sources. One (the Kelly strain of *E. praecox*, which was isolated from a backyard flock with no history of coccidiostat use) was identified during testing of vaccine candidates for sensitivity to coccidiostat treatments. Two others were collected from commercial flocks following outbreaks of clinical coccidiosis attributed to parasite drug resistance.

4. Results

4.1. Drug sensitivity trials

4.1.1. Drug sensitivity - Kelly strain of *E. mitis*

Results from the trial (Table 2) show that treatment groups receiving Toltrazuril or Amprolium after infection produced significantly fewer oocysts than the positive control group, which received parasites only, whereas there was no significant difference in oocyst output between the group receiving Sulphaquinoxaline treatment and the positive control group. There were no significant differences in bodyweight gain or feed conversion between the positive control group and the negative control group, which received no parasites. Similarly, there were no significant differences in those two parameters between the control groups and the groups receiving Sulphaquinoxaline and Toltrazuril treatments. The Amprolium treatment group, however, had significantly lower bodyweight gain and significantly higher feed conversion ratio than the other groups.

Table 2. Drug sensitivity trial for the Kelly strain of *E. mitis*
The drugs were given in the drinking water following the manufacturers' recommendations. Parameters were measured for 10 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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	130.4×10^6	507[a]	163[a]	4.542[b]
20 000	Poultro: Sulphaquinoxaline	67.4×10^6	407[ab]	171[a]	4.353[b]
20 000	Baycox: Toltrazuril	24.9×10^6	292[c]	168[a]	4.304[b]
20 000	Coccivet: Amprolium	59.3×10^6	390[bc]	146[b]	4.992[a]
Nil	Nil	-	-	179[a]	4.229[b]
LSD	**(*P*=0.05)**		**102**	**16**	**0.362**

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4.1.2. Drug sensitivity - Jorgensen strain of *E. mitis*

Results from the trial (Table 3) show that groups receiving Toltrazuril or Amprolium treatment following infection produced significantly fewer oocysts than the positive control group, which received parasites only, and the Sulphaquinoxaline treatment group. No significant differences between treatment groups were found in the parameters of bodyweight gain or feed conversion ratio.

Table 3. Drug sensitivity trial for the Jorgensen strain of *E. mitis*

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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	28.2×10^6	304^a	136	3.00
20 000	Poultro: Sulphaquinoxaline	21.4×10^6	278^a	136	2.96
20 000	Baycox: Toltrazuril	4.5×10^6	165^b	146	2.96
20 000	Coccivet: Amprolium	5.4×10^6	176^b	145	2.96
Nil	Nil	-	-	148	2.97
LSD	**(*P*=0.05)**		**34**	**13**	**0.17**

4.1.3. Drug sensitivity - Monarto strain of *E. brunetti*

Results from the trial (Table 4) show that oocyst output was significantly reduced in those groups receiving parasites plus Toltrazuril or Sulphaquinoxaline treatment compared with the positive control group, which received parasites only, but there was no significant difference in oocyst output between the group receiving Amprolium treatment and the positive control group. The bodyweight gain of the Toltrazuril treatment group was not significantly different from that of the negative control group, which received no parasites, but was significantly higher than that of the positive control group. The bodyweight gains of the Sulphaquinoxaline and Amprolium treatment groups were significantly lower than that of the negative control group, but significantly higher than that of the positive control group. The Toltrazuril treatment group had a feed conversion ratio that was not significantly different from that of the negative control group, but was significantly lower than that of the positive control group. The Sulphaquinoxaline and Amprolium treatment groups had feed conversion ratios that were significantly higher than that of the negative control group, but significantly lower than that of the positive control group.

Table 4. Drug sensitivity trial for the Monarto strain of *E. brunetti*

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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
10 000	Nil	18.9 X 10^6	266[a]	110[c]	3.703[a]
10 000	Poultro: Sulphaquinoxaline	4.3 X 10^6	162[b]	137[b]	3.206[c]
10 000	Baycox: Toltrazuril	2.3 X 10^6	132[b]	163[a]	2.955[d]
10 000	Coccivet: Amprolium	13.1 X 10^6	236[a]	128[b]	3.418[b]
Nil	Nil	-	-	171[a]	2.861[d]
LSD	**(*P*=0.05)**		**36**	**11**	**0.210**

4.1.4. Drug sensitivity - Bowden strain of *E. brunetti*

Results from the trial (Table 5) show that oocyst output was significantly reduced in all three coccidiostat treatment groups compared with the positive control group, which received parasites only. The Toltrazuril treatment had a significantly higher impact on oocyst output than the other two coccidiostats. No significant differences were detected between bodyweight gains or feed conversion ratios of the treatment groups.

Table 5. Drug sensitivity trial for the Bowden strain of *E. brunetti*
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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
10 000	Nil	9.6×10^6	212[a]	127	3.143
10 000	Poultro: Sulphaquinoxaline	5.5×10^6	176[b]	132	2.870
10 000	Baycox: Toltrazuril	0.1×10^6	41[c]	135	2.977
10 000	Coccivet: Amprolium	4.2×10^6	162[b]	137	2.909
Nil	Nil	-	-	137	2.989
LSD	(*P*=0.05)		**25**	**14**	**0.235**

4.1.5. Drug sensitivity - Kelly strain of *E. praecox*

Results from the trial (Table 6) show that there was no significant difference between the oocyst outputs of the positive control group, which received parasites and the groups receiving parasites plus Amprolium. The oocyst outputs of the groups receiving Sulphaquinoxaline or Toltrazuril treatment were significantly higher than that of the positive control group. There was no significant difference in feed conversion ratio between the positive control group, which received parasites only, and the three groups that received drug treatment following infection. All of those four groups, however, had a feed conversion ratio that was significantly higher than that of the negative control group, which received no parasites. The positive control group and the groups receiving Toltrazuril and Sulphaquinoxaline treatment had bodyweight gains that were significantly lower than that of the negative control group. There was no significant difference between the bodyweight gains of the Amprolium treatment group and the negative control group.

Table 6. Drug sensitivity trial for the Kelly strain of *E. praecox*
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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	19.5×10^6	269[b]	143[b]	3.247[a]
20 000	Poultro: Sulphaquinoxaline	37.2×10^6	334[a]	141[b]	3.277[a]
20 000	Baycox: Toltrazuril	38.3×10^6	337[a]	143[b]	3.332[a]
20 000	Coccivet: Amprolium	19.1×10^6	267[b]	146[ab]	3.229[a]
Nil	Nil	-	-	153[a]	3.060[b]
LSD	**(P=0.05)**		**47**	**9**	**0.165**

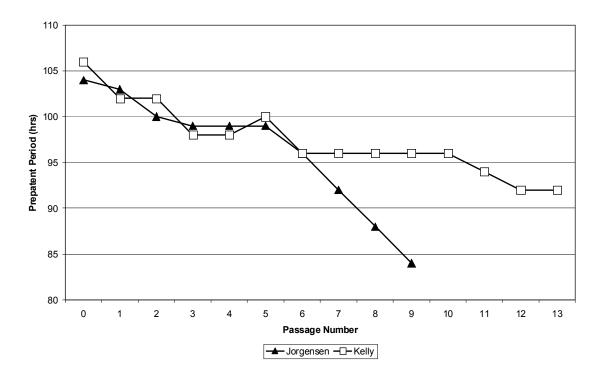### 4.1.6. Drug sensitivity - Jorgensen strain of *E. praecox*

Results from the trial (Table 7) show that there were no significant differences in the feed conversion ratio data. There was no significant difference in bodyweight gain between the positive control group, which received no drug treatment following infection, the negative control group, which received no parasites, and the group receiving Toltrazuril treatment following infection. The bodyweight gains of the other two drug treatment groups were significantly lower than the bodyweight gain of the negative control group. The oocyst outputs of the three groups receiving drug treatment following infection were significantly lower than that of the positive control group. That of the Toltrazuril treatment group was reduced to zero.

Table 7. Drug sensitivity trial for the Jorgensen strain of *E. praecox*
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). No oocysts were detected in negative control groups (result not included in analysis).

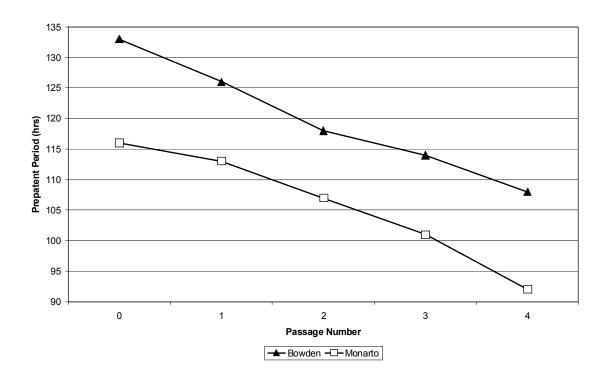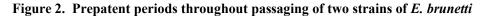| Challenge (oocysts)	Treatment	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	39.0 X 10^6	339[a]	168[ab]	3.304
20 000	Poultro: Sulphaquinoxaline	8.8 X 10^6	206[b]	163[b]	3.353
20 000	Baycox: Toltrazuril	0	0[c]	170[ab]	3.153
20 000	Coccivet: Amprolium	2.8 X 10^6	142[b]	163[b]	3.312
Nil	Nil	-	-	179[a]	3.209
LSD	**(*P*=0.05)**		**88**	**14**	**0.216**

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4.2. Selection for precocious development

Two strains of *Eimeria mitis* underwent selection for precocious development. The Jorgensen strain underwent nine passages with a 20 hour decrease in prepatent period being achieved (104 hr → 84 hr) (Figure 1). The Kelly strain underwent 13 passages with a resultant 14 hour decrease in prepatent period (106 hr → 92 hr). The Jorgensen strain was chosen for further characterisation studies because of the greater reduction in prepatent paeriod compared with the Kelly strain.

Figure 1. Prepatent periods throughout passaging of two strains of *E. mitis*

Two strains of *Eimeria brunetti* underwent selection for precocious development. Both strains underwent four passages. A 24 hour decrease in prepatent period was achieved for the Monarto strain and a 25 hour decrease for the Bowden strain (Figure 2). The Monarto strain retained high pathogenicity and the passaged line was clearly unsuitable for further evaluation as a vaccine line. The Bowden parent strain was a mild isolate, but the selected line became more virulent with passaging and therefore was also not suitable for use as a vaccine line. The parent Bowden strain clearly demonstrated low virulence and, following consultation with the project commercial partners and RIRDC Research Manager, was chosen for further characterisation studies.

Figure 2. Prepatent periods throughout passaging of two strains of *E. brunetti*

The Kelly strain of *E. praecox* was not suitable for passaging because of its demonstrated drug resistance. Selection for precocious development was not attempted for the Jorgensen strain of *E. praecox* because of the already low pathogenicity demonstrated in the drug sensitivity trial.

4.3. Characterisation trials – Jorgensen strain of *E. mitis*

4.3.1. Pathogenicity - Jorgensen strain of *E. mitis*

Results from the trial (Table 8) show that there were no significant differences in bodyweight gain between treatment groups. There were no significant differences in feed conversion ratio between the three groups that received different doses of the precocious line and the negative control group, which received no parasites. In contrast, the feed conversion ratio of the group that received parent strain parasites was significantly higher than that of the negative control group.

Table 8. Pathogenicity trial for the Jorgensen strain of *E. mitis*

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).

Challenge (oocysts)	Bodyweight gain (g/bird)	Feed conversion ratio
2 000 precocious line	180	3.30[b]
5 000 precocious line	172	3.44[ab]
20 000 precocious line	174	3.34[b]
20 000 parent strain	164	3.51[a]
Nil	178	3.32[b]
LSD (*P*=0.05)	**17**	**0.17**

4.3.2. Homologous challenge - Jorgensen strain of *E. mitis*

Results from the trial (Table 9) show that the oocyst outputs of the groups that received the parasite challenge were all significantly different. Although all of the vaccinated groups produced significantly fewer oocysts than the positive control group, which was unvaccinated prior to challenge, there was a clear linear relationship between the vaccination dose and the reduction in oocyst output. No significant differences in bodyweight gains or feed conversion ratios were detected between the positive control group and the negative control group, which received no parasites. None of the vaccinated groups demonstrated any significant differences in those two parameters from the control groups apart from the group vaccinated with 5 000 oocysts, which had a significantly higher bodyweight gain and significantly lower feed conversion ratio.

Table 9. Homologous challenge trial for the Jorgensen strain of *E. mitis*

Parameters were measured for 10 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). No oocysts were detected in negative control groups (result not included in analysis).

Vaccination (oocysts)	Challenge (oocysts)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed Conversion Ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
Nil	20 000	104.0×10^6	470^a	187^b	3.918^a
10	20 000	31.6×10^6	316^b	189^b	3.933^a
100	20 000	10.4×10^6	218^c	190^b	3.853^a
1 000	20 000	1.6×10^6	117^d	204^{ab}	3.834^{ab}
5 000	20 000	0.2×10^6	56^e	213^a	3.624^b
Nil	Nil	-	-	195^b	3.877^a
LSD	**(*P*=0.05)**		**32**	**17**	**0.219**

4.3.3. Heterologous challenge - Jorgensen strain of *E. mitis*

Results from the heterologous challenge trial (Table 10) show that the oocyst output was significantly lower in the vaccinated group than in the unvaccinated group for both challenge strains. No significant differences were found in the bodyweight gains or feed conversion ratios.

Table 10. Heterologous challenge trial for the Jorgensen strain of *E. mitis*

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). No oocysts were detected in negative control groups (result not included in analysis).

Vaccination (oocysts)	Challenge (oocysts, strain)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	**$(X+1)^{1/3}$ transformed**		
2 000	20 000, Redlands	0.5×10^6	79[c]	168	3.968
2 000	20 000, Beerwah	1.2×10^6	105[c]	167	3.989
Nil	20 000, Redlands	6.5×10^6	187[b]	157	4.078
Nil	20 000, Beerwah	29.8×10^6	310[a]	160	4.122
Nil	Nil	-	-	166	3.959
LSD	(***P*=0.05**)		**39**	**16**	**0.334**

4.3.4. Reproductive potential (oocyst output) - Jorgensen strain of *E. mitis*

Results from the trial (Table 11) show that significant differences in oocyst output were found between all treatment groups. Groups receiving the parent strain had oocyst outputs that were significantly higher than those of the precocious line at both dose levels. The group inoculated with 100 oocysts of the parent line had an oocyst output that was significantly higher than that of the group inoculated with 10 000 oocysts of the precocious line.

Table 11. Reproductive potential (oocyst output) trial for the Jorgensen strain of *E. mitis*
Oocyst output was 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). No oocysts were detected in negative control groups (result not included in analysis).

Challenge (oocysts)	Oocyst output per bird	
	Back-transformed	**$(X+1)^{1/3}$ transformed**
100 precocious line	0.1×10^6	52^d
10 000 precocious line	3.7×10^6	154^c
100 parent strain	6.4×10^6	185^b
10 000 parent strain	46.0×10^6	358^a
LSD (P=0.05)		**23**

4.4. Characterisation trials – Bowden strain of *E. brunetti*

4.4.1. Pathogenicity - Bowden strain of *E. brunetti*

Results from the pathogenicity trial for the precocious line (Table 12) show that there were no significant differences in bodyweight gain or feed conversion ratio between treatment groups receiving 10 000 oocysts of the parent strain or 2 000 oocysts of the precocious strain, and the negative control group, which received no parasites. Treatment groups receiving 5 000 or 10 000 oocysts of the precocious strain had significantly lower bodyweight gains and significantly higher feed conversion ratios than the other three treatment groups.

Table 12. Pathogenicity trial for the precocious line of the Bowden strain of *E. brunetti*
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).

Challenge (oocysts, strain)	Bodyweight gain (g/bird)	Feed conversion ratio
2 000, precocious	176^{ab}	2.845^{b}
5 000, precocious	164^{bc}	3.022^{a}
10 000, precocious	159^{c}	3.083^{a}
10 000, parent	190^{a}	2.763^{b}
Nil	189^{a}	2.777^{b}
LSD (*P*=0.05)	**11**	**0.163**

Results from the pathogenicity trial for the parent strain (Table 13) show that the bodyweight gains of groups receiving the Bowden parent strain were not significantly different from or were significantly higher than that of the negative control group, which received no parasites. Feed conversion ratios of the groups receiving the Bowden parent strain were not significantly different from or were significantly lower than that of the negative control group. The group receiving the Monarto parent strain had a significantly lower bodyweight gain and a significantly higher feed conversion ratio than the other treatment groups.

Table 13. Pathogenicity trial for the Bowden (parent) strain of *E. brunetti*
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).

Challenge (oocysts, strain)	Bodyweight gain (g/bird)	Feed conversion ratio
5 000, Bowden	199[a]	2.805[c]
10 000, Bowden	183[c]	2.858[bc]
15 000, Bowden	194[ab]	2.829[c]
10 000, Monarto	149[d]	3.209[a]
Nil	184[bc]	3.003[b]
LSD (*P*=0.05)	**11**	**0.146**

4.4.2. Homologous Challenge - Bowden strain of *E. brunetti*

Results from the trial (Table 14) show that oocyst outputs of the vaccinated groups were significantly lower than that of the positive control group, which received no vaccination prior to challenge. No significant differences were detected in bodyweight gain results and no meaningful differences were detected in feed conversion ratio data.

Table 14. Homologous challenge trial for the precocious line of the Bowden strain of *E. brunetti*
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). No oocysts were detected in negative control groups or groups vaccinated with 1 000 or 5 000 oocysts (results not included in analysis).

Vaccination (oocysts)	Challenge (oocysts)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed Conversion Ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
Nil	15 000	10.0×10^6	216^a	168	3.800^{ab}
10	15 000	0.2×10^6	56^b	174	3.723^{ab}
100	15 000	0.0×10^6	6^b	166	3.831^a
1 000	15 000	0.0×10^6	0	166	3.814^a
5 000	15 000	0.0×10^6	0	176	3.604^b
Nil	Nil	-	-	174	3.749^{ab}
LSD	**(*P*=0.05)**		**74**	**13**	**0.202**

4.4.3. Heterologous Challenge - Bowden strain of *E. brunetti*

Results from the trial (Table 15) show that there was no significant difference between the oocyst outputs of the vaccinated and unvaccinated groups challenged with the Monarto strain, but that there was a significantly lower oocyst output in the vaccinated group than in the unvaccinated group in the groups challenged with the Anderson strain. There was no significant difference in bodyweight gain in the groups challenged with the Anderson strain and the negative control group, which received no parasites. The bodyweight gains of the groups challenged with the Monarto strain were significantly lower than that of the negative control group, but that of the vaccinated group was significantly higher than that of the unvaccinated group. There was no significant difference in feed conversion ratio in the groups challenged with the Anderson strain, the vaccinated group challenged with the Monarto strain and the negative control group. The feed conversion ratio of the unvaccinated group challenged with the Monarto strain was significantly higher than those of the other treatment groups.

Table 15. Heterologous challenge trial for the Bowden strain of *E. brunetti*
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). No oocysts were detected in negative control groups (result not included in analysis).

Vaccination (oocysts)	Challenge (oocysts, strain)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
1 000	10 000, Monarto	26.8×10^6	299^b	118^b	4.73^b
1 000	10 000, Anderson	23.5×10^6	286^b	186^a	3.40^b
Nil	10 000, Monarto	32.7×10^6	320^{ab}	78^c	7.64^a
Nil	10 000, Anderson	39.4×10^6	340^a	176^a	3.50^b
Nil	Nil	-	-	170^a	3.47^b
LSD	**(P=0.05)**		**36**	**25**	**2.31**

4.5. Characterisation trials – Jorgensen strain of *E. praecox*

4.5.1. Pathogenicity - Jorgensen strain of *E. praecox*

Results from the trial (Table 16) show that no significant differences in bodyweight gains or feed conversion ratios were found.

Table 16. Pathogenicity trial for the Jorgensen strain of *E. praecox*

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).

Challenge (oocysts, strain)	Bodyweight gain (g/bird)	Feed conversion ratio
5 000, Jorgensen	146	2.535
10 000, Jorgensen	151	2.472
15 000, Jorgensen	155	2.403
10 000, Inghams	153	2.415
Nil	144	2.513
LSD (*P*=0.05)	**16**	**0.183**

4.5.2. Heterologous Challenge - Jorgensen strain of *E. praecox*

Results from the first heterologous challenge trial (Table 17) show that there was no significant difference between the oocyst outputs of the vaccinated and unvaccinated groups challenged with the Pfizer strain, but that there was a significantly lower oocyst output in the vaccinated group than in the unvaccinated group in the groups challenged with the Inghams strain. There was no significant difference in bodyweight gain in the groups challenged with the Inghams strain and the negative control group, which received no parasites. There was no significant difference in bodyweight gain between the vaccinated and unvaccinated groups challenged with the Pfizer strain, but bodyweight gains for those groups were significantly lower than that of the negative control group. None of the feed conversion ratios of the treatment groups receiving parasites was significantly different from that of the negative control group. The feed conversion ratio of the unvaccinated group was significantly lower than that of the vaccinated group for the groups challenged with the Inghams strain.

Table 17. First heterologous challenge trial for the Jorgensen strain of *E. praecox*
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). No oocysts were detected in negative control groups (result not included in analysis).

Vaccination (oocysts)	Challenge (oocysts, strain)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	**$(X+1)^{1/3}$ transformed**		
2 000	20 000, Pfizer	378×10^6	723[a]	169[b]	3.64[a]
2 000	20 000, Inghams	3×10^6	143[c]	180[ab]	3.70[a]
Nil	20 000, Pfizer	528×10^6	808[a]	168[b]	3.63[a]
Nil	20 000, Inghams	65×10^6	403[b]	198[a]	3.27[b]
Nil	Nil	-	-	191[a]	3.51[ab]
LSD	**(P=0.05)**		**142**	**19**	**0.30**

Results from the second heterologous challenge trial (Table 18) show that the oocyst output of the vaccinated group was significantly lower than that of the unvaccinated group for both of the challenge strains. There was no significant difference in bodyweight gain between the groups challenged with the Inghams strain, the vaccinated group challenged with the Medichick strain and the negative control group, which received no parasites. The bodyweight gain of the unvaccinated group challenged with the Medichick strain was significantly lower than those of the other treatment groups. No significant differences were detected between feed conversion ratios.

Table 18. Second heterologous challenge trial for the Jorgensen strain of *E. praecox*
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). No oocysts were detected in negative control groups (result not included in analysis).

Vaccination (oocysts)	Challenge (oocysts, strain)	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	**$(X+1)^{1/3}$ transformed**		
2 000	30 000, Medichick	0.4×10^6	76[b]	154[a]	3.816
2 000	30 000, Inghams	0.0×10^6	34[c]	161[a]	3.690
Nil	30 000, Medichick	3.1×10^6	146[a]	147[b]	3.824
Nil	30 000, Inghams	3.1×10^6	146[a]	161[a]	3.740
Nil	Nil	-	-	159[a]	3.664
LSD	(*P*=0.05)		37	28	0.196

4.6. Assessment of resistance to prophylactic coccidiostats

4.6.1. Sensitivity to prophylactic coccidiostats – Eimeriavax 4m

Results from the trial (Table 19) show that the oocyst outputs of the two groups receiving a prophylactic in addition to parasites were significantly lower than that of the positive control group, which received parasites only. The bodyweight gain of the group receiving Monensin only was significantly lower than that of the negative control group, which received no parasites and no prophylactic, but was significantly higher than that of the positive control group. The bodyweight gain of the group receiving Narasin only was not significantly different from that of the negative control group. The bodyweight gain of the group receiving both parasites and Monensin was significantly lower than that of the group receiving Monensin only and not significantly different from that of the positive control group. The bodyweight gain of the group receiving both parasites and Narasin was not significantly different from that of the group receiving Narasin only. There was no significant difference in feed conversion ratio between the groups receiving Narasin (parasites or not), the group receiving only Monensin and the negative control group. The feed conversion ratio of the group receiving parasites and Monensin was significantly higher than that of the negative control and not significantly different from the group receiving Monensin only and the positive control group.

Table 19. In-feed drug sensitivity trial for Eimeriavax 4m
The drugs were given in the feed following the manufacturer's 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). No oocysts were detected in negative or treatment control groups (results not included in analysis).

Note: This trial was funded by Eimeria Pty Ltd and the results have been reported here with permission from that company.

Challenge (oocysts)	Prophylactic	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	64.4 X 10^6	401^a	119^d	4.88^a
20 000	Monensin	15.1 X 10^6	247^b	137^{cd}	4.59^{ab}
20 000	Narasin	19.9 X 10^6	271^b	150^{bc}	4.06^{bc}
Nil	Monensin	-	-	162^b	4.07^{bc}
Nil	Narasin	-	-	171^{ab}	3.71^c
Nil	Nil	-	-	189^a	3.52^c
LSD	**(*P*=0.05)**		**112**	**21**	**0.55**

4.6.2. Sensitivity to prophylactic coccidiostats – McLean Farms isolate

Results from the trial (Table 20) show that the oocyst outputs of the two groups receiving a prophylactic in addition to parasites were significantly lower than that of the positive control group, which received parasites only. The bodyweight gains of the groups receiving Monensin or Narasin only were not significantly different from that of the negative control group, which received no parasites and no prophylactic. The bodyweight gains of the groups receiving parasites in addition to Monensin or Narasin were significantly lower than those of the groups receiving the coccidiostats only. There was no significant difference in feed conversion ratio between the groups receiving Narasin (parasites or not), the group receiving only Monensin and the negative control group. The feed conversion ratio of the group receiving parasites and Monensin was significantly higher than that of the group receiving Monensin only, but was not significantly different from that of the negative control group.

Table 20. In-feed drug sensitivity trial for the McLean Farms isolate
The drugs were given in the feed following the manufacturer's 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). No oocysts were detected in negative or treatment control groups (results not included in analysis).

Challenge (oocysts)	Prophylactic	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	118 X 10^6	491[a]	127[c]	4.53[a]
20 000	Monensin	40 X 10^6	342[b]	126[c]	4.44[ab]
20 000	Narasin	35 X 10^6	327[b]	135[bc]	4.18[abc]
Nil	Monensin	-	-	153[ab]	3.83[c]
Nil	Narasin	-	-	159[a]	3.82[c]
Nil	Nil	-	-	147[abc]	3.89[bc]
LSD	(*P*=0.05)		100	24	0.56

4.6.3. Sensitivity to prophylactic coccidiostats – Kelly strain of *E. praecox*

Results from the trial (Table 21) show that no significant differences were detected for any parameter.

Table 21. In-feed drug sensitivity trial for the Kelly strain of *E. praecox*
The drugs were given in the feed following the manufacturer's 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). No oocysts were detected in negative or treatment control groups (results not included in analysis).

Challenge (oocysts)	Prophylactic	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	35.4×10^6	328	187	3.55
20 000	Monensin	24.1×10^6	289	186	3.55
20 000	Narasin	41.3×10^6	346	198	3.42
Nil	Monensin		-	204	3.44
Nil	Narasin		-	200	3.48
Nil	Nil		-	201	3.35
LSD	**(*P*=0.05)**		**66**	**21**	**0.21**

4.6.4. Sensitivity to prophylactic coccidiostats – Pfizer isolate

Results from the trial (Table 22) show that no significant differences were detected for the parameters of oocyst output and feed conversion ratio. There were no significant differences in bodyweight gain apart from between the group receiving parasites and Monensin and the negative control group which received no parasites and no prophylactic.

Table 22. In-feed drug sensitivity trial for the Pfizer isolate

The drugs were given in the feed following the manufacturer's 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). No oocysts were detected in negative or treatment control groups (results not included in analysis).

Challenge (oocysts)	Prophylactic	Oocyst output per bird		Bodyweight gain (g/bird)	Feed conversion ratio
		Back-transformed	$(X+1)^{1/3}$ transformed		
20 000	Nil	116×10^6	488	183[ab]	3.32
20 000	Monensin	64×10^6	399	167[b]	3.45
20 000	Narasin	100×10^6	464	175[ab]	3.40
Nil	Monensin	-	-	177[ab]	3.41
Nil	Narasin	-	-	177[ab]	3.39
Nil	Nil	-	-	187[a]	3.31
LSD	**(P=0.05)**		**133**	**17**	**0.18**

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5. Discussion

5.1. Drug sensitivity trials

Some difficulty was experienced in generating sufficient disease to cause measurable effects on bodyweight gain and feed conversion ratio in the trials due to the low pathogenicity of the strains used. In those instances, however, oocyst output figures could be used to determine if the coccidiostats were having an impact on the ability of the parasite to multiply.

The results of the drug sensitivity trials were somewhat variable, but, in general, followed the trends observed during the previous two projects. Most of the strains (Kelly and Jorgensen strains of *E. mitis*, Monarto and Bowden strains of *E. brunetti*, Jorgensen strain of *E. praecox*) were susceptible to treatment with Toltrazuril, but showed varying degrees of susceptibility to Sulphaquinoxaline and Amprolium. Unusually, the Jorgensen strain of *E. praecox* showed complete susceptibility to Toltrazuril (i.e. no oocysts were produced following treatment with Toltrazuril). In contrast, the Kelly strain of *E. praecox* demonstrated significant resistance to all three of the coccidiostats and was clearly not suitable for further vaccine work.

5.2. Selection for precocious development

Two strains of *E. mitis* were attenuated by selecting for precocious development. The reductions in prepatent period (20 hr for the Jorgensen strain and 14 hr for the Kelly strain) (see Fig 1) were achieved over a number of passages that was comparable with other species such as *E. necatrix*. Both strains appeared suitable for further vaccine development work. The Jorgensen strain was chosen for further work because of the greater reduction in prepatent period.

Two strains of *E. brunetti* underwent selection for precocious development. In both instances, the decrease in prepatent period (25 hr for the Bowden strain and 24 hr for the Monarto strain) occurred unexpectedly rapidly (four passages for each) (see Fig 2). At the same time, there appeared to be no reduction in pathogenicity. Further investigation of the precocious line of the Bowden strain showed that the pathogenicity of the precocious line was actually higher than the pathogenicity of the parent strain. This also appeared to be the case for the Monarto strain. These results were contrary to all previous work involving selection for precocious development. The outcome may be attributable to the unusual biology of *E. brunetti*. As an infection with the species progresses, it migrates down the gut of the host bird. The collection of faster developing parasites may therefore have selected for parasites that inhabit the gut at the upper end of the distribution rather than parasites that were actually developing faster than normal. As each passage occurred the population would have been skewed further so that by the end of four passages most of the parasites would invade the gut in one small area rather than be spread across a large area. This would cause more serious damage to the gut in that particular spot and have a greater impact on the bird. If this is the case, there are implications for the isolation and purification of *E. brunetti* strains as well as for the level of selection pressure that should be applied while selecting for precocious development. Neither of the precocious lines was suitable for further vaccine development work because of their high pathogenicity.

Selection for precocious development was not attempted for *E. praecox*. In light of the low pathogenicity of the species in general and the candidate strain in particular, it was decided that the benefits of maintaining a high reproductive potential (greater immunogenicity and cheaper vaccine production) and wider antigenic mix outweighed any benefit from reducing the prepatent period.

5.3. Characterisation of vaccine strains

5.3.1. Jorgensen strain of *E. mitis*

The Jorgensen strain has low pathogenicity and it was therefore difficult to induce sufficient disease to produce significant differences in bodyweight gain and feed conversion ratio, even when using the parent strain. Nevertheless, the results from the characterisation trials demonstrate that the precocious line is suitable for use in a live vaccine.

The Jorgensen strain is susceptible to both Toltrazuril and Amprolium based on oocyst output figures (Table 3). In contrast, there is no indication that Sulphaquinoxaline had a significant impact on reducing oocyst output. That is consistent with previous results that showed the drug limited the impact of the infection through maintaining bodyweight gain and feed conversion ratio while not reducing the oocyst output. Sulphaquinoxaline could not, however, be recommended for control of this strain.

The pathogenicity of the precocious line is clearly reduced compared with the parent strain as demonstrated by results from the pathogenicity trial (Table 8). While there were no significant differences in bodyweight gains, all of the treatment groups receiving the precocious line, even at a dose of 20 000 oocysts, maintained a feed conversion ratio at a level not significantly different from the negative control group. In contrast, a 20 000 oocyst dose of the parent strain resulted in a feed conversion ratio that was significantly higher than that of the negative control group.

The precocious line effectively protected birds against challenge with the parent strain. Results from the homologous challenge trial (Table 9) show that vaccination with as few as 10 oocysts of the precocious line could significantly reduce the oocyst output from a subsequent challenge of the same strain. There was a clear linear relationship between dose size and subsequent oocyst output with increased vaccination doses producing a greater reduction in oocyst output. Although there were no significant differences in bodyweight gain or feed conversion ratio between the positive and negative control groups, the treatment group that was vaccinated with 5 000 oocysts of the precocious line had a significantly higher bodyweight gain and a significantly lower feed conversion ratio, suggesting a beneficial effect of vaccination beyond protection against subsequent challenge.

There is good evidence that the precocious line also protects birds against challenge with other field strains. The heterologous challenge trial demonstrated a clear reduction in oocyst output for vaccinated birds compared with unvaccinated birds for both challenge strains (Table 10). There were no significant differences in bodyweight gain or feed conversion ratio, which was unexpected given that the Beerwah challenge strain was isolated from a commercial flock demonstrating clinical symptoms and increased flock heterogeneity. This is a good example of how strains purified using the single oocyst passage technique can vary in pathogenicity from the parent isolate. Unfortunately, the length of time required to produce purified parasite strains from field isolates using single oocyst passaging precluded the production of more purified strains from the Beerwah isolate, especially when the pathogenicity of any new strains could not be predicted .

The reduction in reproductive potential of the precocious line was marked. The reproductive potential trial showed that even a dose of 10 000 oocysts of the precocious line produced fewer oocysts from the resulting infection than 100 oocysts of the parent strain (Table 11). This is an excellent result in terms of selection for precocious development and reduction in pathogenicity, but further evaluation of production of the parasites in eggs rather than live chickens may be required to ensure that the parasites can be produced economically for use in commercial vaccines.

5.3.2. Bowden strain of *E. brunetti*

The Bowden strain was isolated from a backyard flock that was free of clinical disease and appears to have very low pathogenicity. A pathogenicity trial for the precocious line showed clearly that the precocious line was more pathogenic than the parent strain with significant effects seen in both bodyweight gain and feed conversion ratio (Table 12). A pathogenicity trial with the parent strain, however, showed that doses of up to 15 000 oocysts of the parent strain had no significant negative effect on bodyweight gain or feed conversion ratio and a dose of 5 000 oocysts had a positive effect on both parameters (Table 13). After discussion with the project's commercial partners, Eimeria Pty Ltd, and the RIRDC research manager, it was decided that the parent strain should be evaluated for use as a vaccine. Characterisation trials demonstrated that the strain is suitable for use in a live vaccine.

The Bowden strain is susceptible to all three coccidiostats that were tested, though Toltrazuril had a greater impact than the other two (Table 5). No significant differences in bodyweight or feed conversion ratio were found in the drug sensitivity trial, supporting the contention that the parent strain has low pathogenicity.

The homologous challenge trial for the precocious line demonstrated that vaccination was highly protective against challenge with the same strain as demonstrated by significant reductions in oocyst output in vaccinated birds compared with unvaccinated birds (Table 14). In fact, a vaccine dose of 1 000 oocysts or more completely stopped oocyst production. It is expected that the parent strain, because of its greater antigenic diversity, would perform as well or better than the precocious line in that situation.

The heterologous challenge trial using the parent strain demonstrated that vaccination also gives good protection against challenge with other field isolates. Vaccination with the Bowden strain reduced oocyst output following challenge with the Anderson strain (Table 15). Although vaccination did not reduce oocyst output for the Monarto strain challenge, it significantly retarded the large impacts on bodyweight gain and feed conversion ratio seen in the unvaccinated birds. It should be noted that the Monarto challenge strain is a particularly virulent strain that caused major economic losses in commercial flocks. The lack of reduction in oocyst output may be attributable to the saturation effect noted previously for other species of *Eimeria*.

5.3.3. Jorgensen strain of *E. praecox*

The Jorgensen strain had sufficiently low pathogenicity that it was decided to evaluate the parent strain without undertaking selection for precocious development. The virulence of the strain was clearly demonstrated in the pathogenicity trial where doses of up to 15 000 oocysts had no significant impact on bodyweight gain or feed conversion ratio (Table 16). The other characterisation trials demonstrated that the strain also has the other key characteristics required of a vaccine strain.

The strain is sensitive to the three coccidiostats tested, as demonstrated by reduced oocyst outputs in treated birds compared with untreated birds (Table 7). The level of susceptibility was, however, somewhat variable. In contrast to the complete control afforded by Toltrazuril (oocyst output reduced to zero), Amprolium and Sulphaquinoxaline provided only limited control, as shown by their failure to prevent decreased bodyweight gains.

The strain protected birds against challenge with other field strains. The first heterologous challenge trial provided little useful information, particularly because the Pfizer challenge strain was not *E. praecox*, but a mixture of *E. acervulina* and *E. mitis* that had been misidentified during isolation. Consideration of the Inghams challenge, however, shows a reduced oocyst output in vaccinated birds (Table 17). Although the unvaccinated birds had a significantly lower feed conversion ratio than the vaccinated birds, neither result is significantly different from the negative control birds and the cause is not clear. The second heterologous challenge trial used the Medichick strain to replace the Pfizer isolate. It should be noted that the Medichick strain represents the first strain of *E. praecox* that has been implicated in an outbreak of clinical disease in a commercial flock in Australia. The bodyweight gain data from this trial support the contention that *E. praecox* can have production effects. The trial

demonstrated significant reductions in oocyst output in vaccinated groups compared with unvaccinated groups for both challenge strains (Table 18). It also demonstrated that vaccination could prevent the decreased bodyweight gain seen in unvaccinated birds challenged with the Medichick strain.

5.4. Probiotic effect of vaccination

It was noted in the previous project that vaccination with *Eimeria* oocysts can provide a benefit to birds beyond what is expected. In this project, there were two instances where this apparently probiotic effect has occurred (the homologous challenge trial for the Jorgensen strain of *E. mitis* and the pathogenicity trial for the Bowden parent strain of *E. brunetti*). The mechanism for improved bird performance following vaccination is not clear, but further investigation of the phenomenon is required to determine if the effect can be reproduced consistently.

5.5. Assessment of resistance to prophylactic coccidiostats

The design of the trial to assess resistance to prophylactic coccidiostats was validated using the Eimeriavax 4m vaccine and three field isolates. That Eimeriavax 4m trial showed that the drugs may have a significant effect on bodyweight gains in the absence of parasites, though the effect was not significant in the other trials. The treatment groups where birds receive only the prophylactics were therefore included as controls in all trials of this type.

The Eimeriavax 4m trial demonstrated that the vaccine is susceptible to both prophylactics based on the decreased oocyst outputs of birds receiving the prophylactics in addition to parasites compared with the birds receiving parasites only (Table 19). There is evidence of some resistance to Monensin, however, as shown by the significantly lower bodyweight gains of birds receiving both Monensin and parasites compared with birds receiving Monensin only. Which species contained in the vaccine contributed to the reduced susceptibility could not be determined.

The McLean Farms isolate trial again showed that the parasites are, in general, sensitive to the two compounds based on oocyst output data (Table 20). In this case, however, there is evidence of some resistance to both compounds. Bodyweight gain is significantly higher and feed conversion significantly lower in birds receiving Monensin alone compared with birds receiving parasites as well as Monensin. The bodyweight of the treatment group receiving Narasin only is significantly higher than that of the group receiving parasites as well as Narasin.

The trial assessing resistance in the Kelly strain of *E. praecox* demonstrates that the strain is not susceptible to either of the two compounds. There is no significant difference in oocyst output between the positive control group and the groups receiving the prophylactics in addition to the parasites (Table 21). Unfortunately, the strain is not sufficiently virulent at the oocyst dosage used to reveal significant differences in the parameters of bodyweight gain and feed conversion ratio. It is not surprising that this strain demonstrated resistance to the prophylactics as it also demonstrated resistance to the three treatments tested in the standard drug sensitivity trial. It is, however, surprising that such a high level of resistance was detected in this particular strain since it was isolated from a backyard flock with no history of coccidiostat usage.

The Pfizer isolate also showed no susceptibility to the two compounds based on oocyst output data (Table 22). Again, the strain was insufficiently pathogenic to provide meaningful results in the bodyweight gain and feed conversion ratio data. It is less surprising that this isolate demonstrated resistance since it was collected from a commercial farm during a clinical outbreak of coccidiosis.

In conclusion, it appears that the trial design outlined above is suitable for detecting resistance to prophylactic coccidiostats and, because it incorporates coccidiostat only controls, it should be suitable for use with any coccidiostats without further validation. It also appears that resistance to prophylactic compounds may be more widespread in Australia than thought previously.

6. Implications

- The vaccine lines that were produced in this project, in addition to those produced in the previous two projects, will allow the production of low virulence live coccidiosis vaccines to protect chickens against all seven species of *Eimeria*. Use of the vaccine should allow significant reductions in the use of coccidiostats and effectively reduce production costs and losses, especially if further coccidiostats are withdrawn from use.

- A trial has been designed that will allow the assessment of parasite drug resistance to any coccidiostats. This will provide a valuable tool in the investigation of suspected resistance problems, allowing poultry producers to make informed decisions about their coccidiosis control programs.

7. Recommendations

- The application of live coccidiosis vaccines will require ongoing research to develop supporting diagnostic technologies to optimise the performance of the vaccines and identify potential problems. Serological methods that allow investigation of immune responses and quantitative diagnostic methods should both be developed.

- Results from the trials investigating resistance to prophylactic coccidiostats raise serious concerns over how widespread drug resistance may be in Australia. 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.

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Project Title:	**Attenuation and characterisation of chicken *Eimeria* for live vaccines**

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Objectives	• Attenuated lines of *E. mitis, E. brunetti* and *E. praecox* to be developed for incorporation in an efficacious live vaccine protective against all seven species of *Eimeria* in Australian chickens.
	• A trial technique to evaluate coccidiostat resistance to be developed.
Background	Coccidiosis is one of the more economically important disease problems in Australia's intensive poultry industries. Control of the disease is based on the use of chemicals and drugs that cost the poultry industries more than $10 million P.A. Vaccination, used alone, or in combination with coccidiostats is the most sustainable avenue for control of coccidiosis. Seven species of *Eimeria* can cause coccidiosis. The previous two stages of this series of projects with the same title have been successfully completed with the development and evaluation of vaccine strains of *E. maxima, E. acervulina, E. tenella* and *E. necatrix* suitable for use in a live poultry coccidiosis vaccine.
Research	Vaccine strains of *E. mitis, E. brunetti* and *E. praecox* have been isolated purified and demonstrated, in trials, to be drug susceptible, have low pathogenicity and be highly protective against challenge with two different, virulent field isolates. The proposed vaccine strains have been transferred to commercial partners *Eimeria* Pty Ltd for incorporation into new custom vaccines. A trial format has been developed and evaluated to monitor developing resistance to in-feed coccidiostats.
Outcomes	This project completes the development of a suite of live vaccines against all the currently known species of *Eimeria* causing coccidiosis in Australia. Vaccines from the two previous projects are available from our commercial partners as Eimeriavax 4m. The strains developed in this project will be available to the Australian industry through Eimeria Pty Ltd once field trials and registration are complete.
Implications	The final outcome of the three stages of the project series is 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.

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