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Eimeria vaccines and diagnostics

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Executive summary

Coccidiosis of poultry, caused by unicellular parasites called *Eimeria*, represents an intestinal disease of major economic importance worldwide. Seven species of *Eimeria* are recognized to infect chickens, and these taxa differ considerably in their ability to cause disease. Given the major problems linked to genetic resistance in the parasite against anti-coccidial drugs and restrictions of the use of these drugs in many countries, there has been an urgent need to develop new and improved methods for the prevention and control of coccidiosis. The present project made significant advances toward optimizing improved methods for the freezing and storage of *Eimeria* vaccines, and also established highly specific and sensitive diagnostic tests for the diagnosis, prevention and surveillance of coccidiosis. The biotechnological advances made in the production and storage of vaccines and diagnosis have major implications for the prevention and control of chicken coccidiosis. These advances are central to a better understanding of the epidemiology and dynamics of disease in intensive and extensive chicken establishments and underpin effective coccidiosis control programs.

CONTENTS

General introduction

Coccidiosis of poultry, caused by protozoan parasites of the genus *Eimeria* (Apicomplexa: Eimeriidae), represents an enteric disease of major economic importance globally. Currently, seven species of *Eimeria* are recognized to infect chickens, and these taxa differ considerably in their biology and pathogenicity. Given the problems associated with genetic resistance in the parasite against anti-coccidial drugs and restrictions of their use in many countries, there has been an urgent need to develop alternative approaches for the control of avian coccidiosis. Currently, one of the most promising approaches is the use of live, precocious vaccines, such as the one being developed by Bioproperties Pty Ltd (which contains four different species of *Eimeria*). Such an approach has the advantages that it relies on the protective immune responses in chickens against the parasites, it is environmentally safe and there is no drug with-holding period. However, there have been limitations in the use of this vaccine which relate to the quality control of the effectiveness of the live vaccine and to the production of live parasite material contained in the vaccine. Also, there are problems with its storage and transport because of the short 'shelf-life' (~12 weeks).

Also the accurate diagnosis is paramount to the surveillance and control of coccidiosis and for investigating the epidemiology of *Eimeria*. It is also central to the quality control of any *Eimeria* vaccine being produced. Traditionally, member of the genus *Eimeria* have been identified by morphology and/or morphometry of their sporocysts and oocysts as well as their patterns of development, but these criteria are unreliable for species infecting chickens. Polymerase chain reaction (PCR)-based techniques (using appropriate genetic markers) are particularly suited because of their ability to specifically amplify DNA from minute amounts of parasite material. Studies have demonstrated that the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of ribosomal DNA (rDNA) provide genetic markers for the identification of *Eimeria* species or detection of population variation. Approaches have been developed which use species-specific oligonucleotide primers to the ITS for diagnosis based on the presence or absence of an amplicon on an agarose gel. The latter approach has the major disadvantage that individual primer sets are required for the detection of one species per PCR reaction. Also, this approach provides no information on the extent of sequence or length variation (i.e. genetic variation) within a PCR product. We established a diagnostic procedure some years ago (Woods et al., 2000). While useful, this method had disadvantages in that it employed radiolabelled primers in the PCR (which poses a major human health risk to the operator), the amplicons are resolved in a conventional electrophoretic system which is laborious and time-consuming for the analysis of large sample sizes.

The present project addressed these aspects, which all have important commercial implications.

Objective

Focus on improved approaches for the sustainable control of coccidiosis in poultry using non-molecular and genetic approaches.

Methodologies (general)

M1. Improved production and *in vitro* storage commercial vaccines

An improved approach for the cryopreservation of different developmental stages of *Eimeria* was assessed to replace the current vaccine storage protocol, in order to substantially extend the shelf-life of live vaccines. This included establishing optimum conditions, and testing the viability, infectivity and virulence of the different species of *Eimeria* in chickens prior to and after storage or cryopreservation. Also, new methods for the propagation of different species of *Eimeria* in chicken eggs (*in vivo*) and/or *in vitro* were evaluated (using a range of different experimental conditions) and established as an alternative to their production in chickens. Preliminary work (Grant Richards, unpublished) indicated the feasibility of the approaches proposed. In addition to the commercial perspective, this part of the project provided the foundation for the provision of parasite materials which was critical to the success of the project. Grant Richards (Eimeria Pty Ltd) was responsible for conducting this work under the supervision of Greg Underwood and David Tinworth. M1 relates to work described in chapters 1 and 2.

M2. Improved surveillance and control of coccidiosis *via* DNA technology

A high resolution polymerase chain reaction (PCR)-coupled fluorescence capillary electrophoretic (CE) method was developed for the specific identification of all seven species of *Eimeria* infecting chickens, thus overcoming all of the previous limitations. Once evaluated, the method was employed as a tool for the surveillance and control of coccidiosis, the quality control of the vaccine strains being maintained or developed and for epidemiological surveys in Australia and/or overseas. The prerequisite to this goal was the establishment of a bank of DNA samples from parent strains and corresponding attenuated "vaccine strains" of *Eimeria* and from "field strains of *Eimeria*. Using the technology developed, epidemiological surveys were conducted to estimate the prevalence of different species of *Eimeria*. Moreover, investigated genetic variation within each of the four species (contained in the vaccine) for different geographical locations using DNA fingerprinting. The specificity and sensitivity of this assay was established using a panel of well-defined isolates, and its ability to distinguish vaccine from field isolates (for all four species) critically assessed. The development of such an assay has major implications for the monitoring of vaccine efficacy (through coprological examination of chicken flocks prior to, during and after vaccination) and for the detection of outbreaks relating to one or more heterologous species, thus ruling out the inefficacy of any component of the vaccine. The aim was for the approach to be cost- and time-effective as well as 'user-friendly'. Overall, the development of highly specific and sensitive molecular tools contributed significantly toward the objective and will substantially benefit end-producers.

Eimeria Pty Ltd provided the parasite materials (i.e. oocyst isolates representing individual species [produced at different times] contained in the vaccine and a range of monospecific field isolates) from Australia and/or from overseas. One Research Officer, Dr Genevieve Morris, conducted the investigations and was responsible for achieving milestones. This person was responsible for the coordination of the work, collection and preparation of materials, genetic analyses, writing of progress reports, and the presention and dissemination of research findings. Persons responsible for each part of the project were to provide both written and oral reports the project leader of the University of Melbourne every three months, in order to monitor progress. M2 relates to work described in chapters 3-5.

Chapter 1 Cryopreservation of *Eimeria* species

(prepared by Rima Youil and Wayne Woods)

1.1. Summary

Eimeria Pty Ltd (Wayne Woods and Grant Richards) conducted a range of experiments to assess different conditions for the cryopreservation of multiple species of *Eimeria*. Sporozoites were used for these experiments, based on results from a range of preliminary studies. The findings revealed that sporozoites of *E. acervulina*, *E. maxima*, *E. necatrix* and *E. tenella* infect chickens and that oocysts can be recovered from infected chickens. The results also demonstrated that sporozoites of all four species were viable after freezing. However, *E. maxima and E. acervulina* recovery rates were low. It was anticipated that the improved formula developed in this study will allow for better recovery rates. For *Eimeria* species which naturally infect the upper regions of the intestinal tract, oral delivery of the frozen sporozoite preparation is a better option than cloacal inoculation. Consequently, experiments were conducted to determine whether the oral inoculation of sporozoites in the presence of an antacid was a viable approach. Work has continually focused on improving the excystation and recovery of sporozoites for commercial purposes and optimization of the conditions using a comparative assessment of different protocols. This part of the project has been extended from July 1, 2007, with an emphasis on cryopreservation for our commercial vaccine lines of *Eimeria*.

1.2. Introduction

Observations at Eimeria Pty Ltd have indicated that the traditional procedure used for the preservation of sporocysts was unreliable and would not provide for the long-term preservation of Eimeria's master seed stocks. In an effort to establish new methods for cryopreservation, a series of parameters were studied in order to ensure that any changes in the seed characteristics did not impact on the final oocyst output in vaccine manufacture.

Sporocysts are each encompassed in a cyst wall that acts to protect the enclosed sporozoite from harmful substances that may be encountered during the exogenous phase of their life cycles (Long, 1970, Tomley 1997). This wall is effectively impermeable to many of the cryoprotectants that are commonly used. The sporozoites, on the other hand, lack a cell wall. Their cell membrane has been found to be permeable to cryoprotectants. This finding provided an option to cryopreserve sporozoites, as opposed to the two cyst forms. Two methods for freezing of sporozoites were tested. One was a rapid or "flash-freeze" and the other was a gradual freeze or "floating tent" method. Along with freezing down the sporozoites, the rate of viable sporozoites recovered upon thaw is also a critical issue. To ensure that no more than an acceptable loss of sporozoites occurred, a series of freeze/thaw experiments (at various sporozoite concentrations) was warranted.

The method for the delivery of cryopreserved sporozoites was also addressed. The natural life cycle of *Eimeria* relies upon oocysts being ingested by the chicken and passing to the gizzard. Here, the oocysts undergo enzymatic, as well as mechanical, disruption to release sporocysts. The sporocysts subsequently "hatch" to release sporozoites, which are passed down into the gut. The sporozoites undergo asexual and sexual reproduction, whereby the number of oocysts build up in great numbers in the intestines and the cloaca prior to their excretion. Sporozoites are highly susceptible to low pH and osmotic changes. These factors become important when considering oral administration of a sporozoite-based vaccine. For *Eimeria* species, such as *E. tenella* and possibly *E. necatrix*, cloacal administration is considered the best choice for delivery since these species generally colonise the lower part of the intestine. However, for *E. acervulina* and *E. maxima*, oral delivery is likely to be the route of choice, since these species occupy the upper regions of the intestinal tract. It is difficult for sporozoites to travel up the intestinal tract for colonisation.

Consequently, a series of studies was performed in order to test different formulations for oral inoculation. The majority of the method improvements were based on *E. tenella*, with the assumption that they could be extrapolated to the other species.

1.3. Materials and methods.

Hatching and freezing procedure

Viable oocysts (4.4x107) were added to Wash Solution (minimal essential medium (MEM), glycerol (5% v/v), foetal bovine serum (FBS, 6% v/v)], spun and resuspended in glass beads/wash solution at 1:1 w/v. The oocysts are shaken at medium speed, checking every 2 mins (microscopically at 200x magnification) until released sporocysts no longer appeared to visibly increase in number. Sporocysts are hatched in hatching solution (Wash Solution + sodium taurodeoxycholate (2%w/v) and trypsin at 1% w/v) and incubated at 41°C. Sporozoites are then washed, spun down and resuspended in cryopreservation solution (EPL proprietary formula) to give 4.4x106 sporozoites/mL prior to being dispensed into cryovials (1.1 mL) and frozen.

Assessment of caecal samples for oocyst presence

A 0.5 mL tube was tared on a balance and 0.1 - 1.0 g caecal material from a single bird was added and the tube reweighed. Saturated NaCl solution was added to 1.0 g per 0.2 g of caecal material. The sample was homogenized by a vortex shaker for at least 15 seconds and was constantly mixed by hand until dispensing was completed. Using a pipette, the solution was used to fill one chamber of a McMaster slide and the slide left to sit for 5 minutes. The slide was examined at 100x magnification and oocysts counted over 10 microscope fields of view. The final number was divided by 10 to determine the average number of oocysts per field of view. If no oocysts were detected within ten fields, then the rest of the chamber was checked. Each sample was assigned a one-letter code based on the number of oocysts per field: N: No oocysts observed; V: <1 per field; F: 1-10 per field; P: 11-30 per field; H: 31-80 per field); E: 81+ per field.

Comparison of hatching media

Parallel batches of *E. tenella* were hatched in a base solution of either: (i) PBS or (ii) 1xMEM/6% FBS, then frozen using the standard (“floating tent”) method.

Comparison of rapid freezing and "floating tent" methods

The standard "floating tent" method of freezing *E. tenella* sporozoites (group 1) was compared to placing the vials directly into liquid nitrogen and freezing rapidly (group 2). The ‘floating tent’ method entails clipping cryovials into cryocanes and attaching the cryocanes across the top (attached lid) of a small foam box. A large box is taken and 1/3 filled with liquid nitrogen. The small box is carefully floated over liquid nitrogen. The lid of the large box is then closed. After 2 h, cryocanes are removed and rapidly placed in a liquid nitrogen storage tank. In the latter treatment group, vials were placed upright such that the liquid nitrogen could not reach the cap seal. Vials remained in liquid nitrogen for five minutes before being placed in a liquid nitrogen tank for storage. After 2 weeks, vials of each test batch were thawed and inoculated into birds via the cloacal route.

Inoculations

Vials of frozen material were thawed rapidly at 30°C. For *E. tenella*, the material was diluted 1/10 in 1xMEM + 6% FBS before inoculation. Each bird received 0.25 mL material via a tuberculin syringe, either orally, or cloacally.

Comparison of the freezing efficiencies of sporozoites, sporocysts and oocyst

Recovery rates of frozen sporozoites (group 1), sporocysts (group 2) and oocysts (group 3) of *E. tenella* were compared, using identical amounts of starting material for each batch. This was to determine whether freezing of sporozoites was more efficient than the traditional method of freezing sporocysts. The inoculations for this trial were performed via the oral route, with the material being mixed with antacid (0.25 mL of each) prior to treatment. Two more groups were used as a comparison: birds inoculated with thawed sporozoites of the same material that were inoculated either (a) cloacally (group 4), or (b) orally, minus antacid (group 5).

Comparison of the concentration of sporozoites hatched and frozen

We investigated both the hatching and freezing/storage of *E. tenella* sporozoites at a 10x concentration (group 1) and 20x concentration (group 2) and compared this to a standard 1x concentration (group 1). After freezing and thawing, the 10x and 20x batches were diluted to a standard 1x concentration in 1xMEM/6% FBS. All three batches were then diluted by a further 1/10. 0.25 ml aliquots of each treatment were administered cloacally to birds.

Comparison of differing amounts of antacid in an oral inoculum and cloacal inoculation

Recent results suggested that oral inoculation with antacid was not as efficient as cloacal inoculation. To examine further, *E. tenella* sporozoites were tested for recovery after freezing and thawing. Each batch was diluted to a 1/10 concentration in 1xMEM/6%FBS, and 0.25 mL was inoculated into each bird. For the oral inoculation groups, however, the bird was administered antacid at ratios ranging from 1:1 to 12:1 (antacid:inoculum) immediately prior to inoculation. Testing was also performed on birds that had been fasted for up to 24 h prior to inoculation.

1.4. Results

Comparison of two hatching media

In both cloacally administered groups, inoculated birds yielded extreme levels of oocysts. However, birds inoculated with the second treatment (1xMEM/6%FBS) produced faeces that contained more densely packed oocysts. In birds that were orally inoculated, few to no oocysts were detected in the first treatment group, while moderate levels were detected in the second. *These results indicated that the MEM/FBS-based hatching mix yields sporozoites that are more likely to survive freezing.*

Comparison of rapid freezing vs the "floating tent" method

Birds in the “floating tent” group produced extreme levels of oocysts in their caeca. No oocysts were detected in the caecal material of birds of the second group which received the rapidly frozen sporozoite preparation. *Rapid freezing of sporozoites is not a viable approach.*.

Comparison of the freezing efficiencies of sporozoites, sporocysts and oocysts

Birds inoculated cloacally yielded high to extremely high levels of oocysts using frozen sporozoites only. Oral delivery resulted in few to high levels of oocysts using frozen/thawed sporozoites or sporocysts. No oocysts were found in the frozen/thawed oocyst and the negative control groups. The recovery of sporocysts was observed to be slightly lower than (but comparable to) that of sporozoites when each were mixed with antacid and inoculated orally. However, the recovery of sporozoites via the cloacal route was confirmed to be more efficient than the traditional sporocyst freezing method by at least an order of 10. *These results confirm that the cryopreservation of sporozoites, and subsequent recovery via cloacal inoculation, is the most conservative use of material for storage.*

Comparison of the concentration of sporozoites hatched and frozen

The results from hatching indicated the starting oocyst concentration yielded the corresponding 10x and 20x sporozoite levels with no loss of potency. This indicates that the hatching method does not suffer any loss of efficiency if the concentration of starting oocyst is increased by as much as 20-fold. All three products (1x, 10x and 20x) were found to produce caecal oocysts of a comparable (extreme) level. *This indicates that no loss of freezing efficiency occurs as the sporozoite concentration is increased by as much as 20-fold. The density of the material, however, suggests that the procedure may not be very workable for concentrations above 20x. Furthermore, the number of 'doses' per vial would increase to the point where much infective material would be wasted after thawing.*

Comparison of differing amounts of antacid in an oral inoculum and cloacal inoculation

For a cloacal inoculation control, extreme levels of oocysts were detected in the caecal material. For oral inoculation with no antacid, no oocysts were detected in the majority of birds. In studies where varying ratios of commercial antacid was used, comparable levels of oocysts were detected in each group, suggesting that no particular amount of antacid was important, above the minimum dose tested (0.25 mL). However the individual oocyst output results within each group varied from extreme levels to very few. Oral inoculation of birds which had been fasted and/or received antacid made no observable difference to the results. Birds which received a concentrated solution of sodium bicarbonate instead of commercial antacid also gave variable results. However birds which were both fasted and received sodium bicarbonate gave results which were consistent and approaching that of cloacal inoculation. *These results indicate that there are other influences in oral inoculation that are acting without a detectable pattern and may explain why some inoculation trials have yielded very good outputs, while others have suggested that oral inoculation is poor. The combination of fasting and sodium bicarbonate solution is a promising approach which may eliminate the need for difficult cloacal inoculations.*

Investigation of the freezing of *E. necatrix*

Using this procedure, *E. necatrix* sporozoites have been successfully hatched, and frozen at a 1x concentration. Recovery has been achieved, but results indicate that the number of oocysts produced is approximately 10-fold lower than that of *E. tenella*. Since *E. necatrix* initially infects higher up in the intestine (and subsequently migrates to the caeca), it is hoped to greatly increase recovery through oral inoculation improvements.

Investigation of the freezing of *E. acervulina*

Using this procedure, *E. acervulina* sporozoites have been successfully hatched, and frozen at a 1x concentration. Recovery has been achieved, with results suggesting that oral inoculation is much more effective than cloacal. But the results indicate that the number of oocysts produced is approximately 10-fold lower than that of *E. necatrix*. It is hoped to greatly increase this through improvements in oral inoculation and hatching / freezing solutions for this species.

Investigation of the freezing of *E. maxima*

Using this procedure, *E. maxima* sporozoites have been successfully hatched, and frozen at a 1x concentration. Recovery has been achieved at low levels, with results suggesting that oral inoculation is much more effective than cloacal. Hatching at room temperature (in case of temperature sensitivity in this species) failed to work. It is hoped to greatly increase this through improvements in oral inoculation and hatching / freezing solutions for this species.

1.5. Discussion

The recovery of sporozoites from sporulated oocysts is clearly critical in the overall success of cryopreservation. The mechanical disruption of the oocysts may be a significant rate limiting step in the release of maximal viable sporozoites. At present, we have utilized glass beads to assist in the mechanical disruption. Other methods, such as sonication, exist that are worthwhile testing. Despite this limitation, our studies have clearly provided evidence that the hatching process (in the presence of 1xMEM/6%FBS) and the gradual freezing down of sporozoites in the presence of the cryoprotectant formulation 5% glycerol + 4.5% DMSO as well as a rapid thawing process provided the best opportunity to obtain viable sporozoites. Our studies also showed that the recovery of sporozoites was more effective (when administered via the cloacal route) compared with sporocyst inoculation. In fact, sporozoite freezing was confirmed to be more efficient than the traditional sporocyst freezing method by an order of 10.

For practical considerations, the current hatching and freezing procedure requires a large investment of time to convert a batch of fresh oocysts into frozen stocks. For example, a batch of $1x10^9$ *E. tenella* oocysts would require approximately 200 freezing runs to completely store. Furthermore, it would yield approximately 6000 vials of frozen product per batch, at a standard concentration, which would accommodate a large storage space. Our studies showed that batches of sporozoites could be prepared and stored at concentrations of 20x. In fact, preparations of *E. tenella* sporozoites (three different batches each of 10x [90 vials] and 20x [80 vials] have been stored frozen. Alone, these could collectively inoculate an estimated 50-100,000 birds to produce high levels of daughter oocysts. This demonstrates the power of this technology for large scale manufacture, long term storage potential and ease of usage, commercially. Our studies aimed to achieve replication indices of frozen sporozoites that were comparable to that of fresh oocysts. While for *E. tenella*, a species that lends itself quite well to cryopreservation, the replication index has shown to be 2000. This means that each oocyst that was used in the initial preparation of the sporozoite replicates to produce 2000 oocysts following inoculation. *E. necatrix*, on the other hand, was recovered after cloacal inoculation at a rate that was approximately 10 fold lower than that of *E. tenella*. Before accepting that this may be the limit for this species, further experimentation with alternative cryoprotectant formulations and further improvements to oral delivery are warranted. *E. acervulina and E. maxima* sporozoites were also successfully hatched. The sporozoites were recovered in birds via oral inoculation, but recovery was low. It is anticipated that the use of the optimal cryopreservative formulation (which is not yet been tested on these species) is likely to improve upon recovery. Thus far, *E. maxima* has proven to be the most difficult to cryopreserve. However, *E. maxima* has yet to be tested using the optimal cryopreservation formula and may very

well prove to provide better recovery rates. Delivery is also an important aspect of vaccine development which was addressed. While cloacal inoculation works exceedingly well, oral delivery is an attractive option for large scale, rapid inoculations. This is important not only for commercial usage but also in large scale vaccine manufacture. Studies performed indicate that, due to inherent lack of robustness of sporozoites, fasting the birds and administering the sporozoite preparation along with a concentrated sodium bicarbonate solution (as an acid neutralizer) may be a viable option.

Chapter 2 *In ovo* propagation of *Eimeria tenella*

2.1. Summary

The reproductive characteristics of a precocious line (designated *E. tenella* Rt3+15) from Australia were investigated in chicken embryos and the implications of the findings appraised. While it was possible to reproduce *E. tenella* in chicken embryos, other species could not be propagated efficiently.

2.2. Introduction

Eimeria tenella is the most pathogenic species, causing haemorrhagic typhlitis in chickens (McDougald and Reid 1997). *Eimeria* species reproduce asexually (schizogony or merogony) and sexually (gametogony) in the intestinal cells to produce large numbers of progeny (oocysts), which are excreted in the faeces and subsequently undergo sporulation (sporogony) in the environment to become infective to susceptible chickens (McDougald and Reid 1997).

The ability to complete the life cycle of various species of *Eimeria* in embryonated chicken eggs has significant implications for investigating fundamental aspects of their biology and development, for establishing methods for the testing of anti-coccidial drugs and for the production of live vaccines against avian coccidiosis. Long (1965, 1966) first demonstrated that some species of *Eimeria* could complete their endogenous development in the chorio-allantoic membrane of the chicken embryo. Subsequently, Long (1972, 1973) also showed that the continuous passage of *E. tenella* in embryos resulted in an adaptation, associated with a reduction of pathogenicity, size of the schizonts (asexual stages) ~~stage~~ and a change in the site of development, compared with the original, 'parent' line of the parasite. Serial passage of *E. tenella* in embryonating chicken eggs usually results in the development of a line of parasite which is significantly less pathogenic to chickens compared with wild-type strains while remaining immunogenic (Long et al. 1982). For these reasons, an egg-adapted line of *E. tenella* (derived after >100 passages) has been included in a commercial vaccine (Livacox®) (Shirley and Bedrnik 1997; Williams 2002a,b). Recently, a live vaccine, Eimeriavax®, containing multiple species of *Eimeria*, containing a precocious line (for definition see Shirley and Bedrnik 1997) of *E. tenella*, was developed and has been released in Australia (http://www.apvma.gov.au/gazette/gazette0302p17.shtml). In the present study, the reproductive characteristics of this 'precocious' line (designated *E. tenella* Rt3+15) were investigated in chicken embryos and the implications of the findings discussed.

2.3. Materials and methods

A precocious line (Rt3+15) of *E. tenella* isolated from naturally infected chickens in Australia (cf. Jorgensen et al. 2006) was maintained in specific pathogen-free (SPF) chickens, held in custom-built isolators under stringent conditions to prevent cross-contamination. The parent line of *E. tenella* was originally identified to species based on the morphometry of sporulated oocysts, prepatent period and location of gross lesions in the caeca (McDougald and Reid 1997). The biological parameters of the precocious line Rt3+15 conformed to those (i.e. shortened prepatent period, smaller and fewer generations in schizogony, lower virulence and reproductive potentials) defined by Bedrnik et al. (1995), and the monospecificity of this line was verified by molecular analysis (Woods et al. 2000).

Oocysts produced in SPF chickens were allowed to sporulate in the caecal content under constant aeration at 30°C for a minimum of 48 h, isolated by flotation using saturated NaCl, washed extensively in water, pelleted by centrifugation (1000 *xg* for 10 min) and resuspended and stored in

phosphate-buffered saline (PBS) supplemented with benzyl-penicillin (200 IU) and dihydro-streptomycin sulphate (250 mg/ml) at a concentration of ~10^4 oocysts per ml (estimated using a Neubauer haemocytometer; Gasser et al. 1987). Approximately ~10^5 oocysts were washed three times in PBS (by centrifugation) in a tube and then resuspended in 3.3 ml of PBS; a sub-aliquot (~300 µl) of oocyst suspension was plated onto horse blood agar (Oxoid®) and incubated aerobically at 37°C for 24 h to examine whether any bacteria were capable of growing (after exposure to antibiotics). The same volume (3 ml) of glass beads (2 mm in diameter) was added to the tube which was vortexed for ~2 min until >90% of the oocysts had ruptured to release sporocysts (verified by light microscopic examination at 100-times magnification). To release sporozoites from the sporocysts, the suspension (without glass beads) was transferred to a fresh conical 50 ml tube and incubated (under gentle inversion every 20 min) at 41°C for 60-90 min in 25 ml of PBS containing 1% w/v porcine trypsin and 2% w/v sodium taurocholate (Sigma). The tube was centrifuged, the supernatant aspirated and 50 ml of foetal calf serum (FCS) containing antibiotics (same concentration as used previously) added. The sporozoites were sedimented by centrigation and re-suspended in 50% v/v FCS in PBS to achieve a final concentration of ~$5x10^4$ per ml. This suspension, pre-incubated at 41°C, was used for the inoculation of embryonating chicken eggs.

Fertile eggs from SPF chickens incubated to produce 10-day old embryos were used for the inoculation with different doses of sporozoites (Table 2.1). The eggs were candled, the air sac marked, and a site free from blood vessels selected for the inoculation. The injection site on the shell was disinfected with iodine, allowed to dry, and a small hole (~2 mm; above the air sac) drilled into which a circular dentist drill prick-punch was inserted. The sporozoite suspension was then injected into the allantoic cavity using a sterile 25-guage needle. The hole was sealed with a non-toxic glue, and the eggs incubated at 37.8°C for 4 h (rotating them every 30 min) and subsequently at 41°C for 7 days (rotating them two times daily). Within 24 h of inoculation, the eggs were 'candled' to verify the viability of the embryos, and daily thereafter.

After ~168 h, eggs were cooled to 4 °C and the shell over the air sac was fractured and removed to the level of the air sac/allantoic cavity region using sterile forceps. The membrane separating the air sac from the embryo was peeled off. The embryo was removed with the yolk sac and albumen bolus, and the allantoic fluid discarded. The remaining chorio-allantoic membranes (with meconium and urate deposits) were excised and placed into a sterile container. The membranes were pooled, diced finely using scissors, placed into a 50 ml tube and centrifuged at 2000 *g* for 15 min. The membranes were washed in PBS, followed by two washes in PBS containing 2% Tergitol® (BDH Chemicals). They were then transferred to a fresh tube, digested for 60 min in 1% trypsin (in PBS) and centrifuged at 2000 *g* for 10 min. The supernatant was discarded and the sediment resuspended in 1% v/v potassium dichromate (in 30-40 ml). The remaining tissue residues were removed by sieving (1 mm mesh size). Oocysts were allowed to sporulate (at 30°C under constant aeration) for ≥ 48 h, after which sporulation was verified microscopically. The sporulated oocysts were sedimented, washed two times in 40 ml of water, incubated for 20 min with 3% v/v sodium hypochlorite, washed extensively in PBS containing antibiotics (at the same concentration as used previously) and stored at 4°C. The oocysts in the suspension were enumerated, and the sporulation established (see Table 2.1).

2.4. Results and discussion

After preliminary experiments, in which it was shown that the precocious vaccine line (Rt3+15) of *E. tenella* could be propagated in chicken embryos, seven independent experiments were conducted employing different inoculation doses. The results of these experiments are shown in Table 2.1. Using inoculation doses of 2600-8700 sporozoites per egg, 16000-360000 oocysts were produced per egg. The oocyst yields equated to reproductive indices varying from 17-577. The mortality of

infected chicken embryos ranged from 3.7-25.4%. There was no evidence of bacterial growth before inoculation of eggs with sporozoites or during experimentation. Between 0-72 after inoculation, the mean chicken mortality was ~7%; *post mortem* examination of dead embryos indicated trauma due to needle damage during inoculation (results not shown). Between 96-168 h after inoculation, the mean embryo mortality was ~4%. Examination of dead embryos at ~120 h after inoculation showed chorio-allantoic haemorrhage (the allantoic fluid being dark red), which was interpreted to represent damage due to the asexual replication (shizogony) of the parasite.

For all seven experiments, the mean oocyst yield was ~169,000 oocysts per egg (with a mean mortality of 11.1% for a period of 168 h). Sporozoites from oocysts produced in egg embryos (stored for ~28 days) were used to inoculate 34 eggs (at a dose of 8800 sporozoites per egg). None of the embryos died during the entire experimental period of 168 h, and the yield and reproductive index were ~22000 oocysts per egg and ~20, respectively. Sporozoites from this passage were also successfully passaged again in eggs (results not shown).

Oocysts representing *E. tenella* Rt3+15 were propagated in embryonating chicken eggs after inoculation with 2600 to 8700 sporozoites (per egg), achieving yields of 16000-360000 oocysts per egg after 7 days. Eggs were rotated after inoculation, with the rationale of achieving an even distribution of the sporozoite inoculum and minimizing the high levels of embryo mortality recorded in preliminary studies when eggs were not rotated. Mortality in embryos in eggs not rotated showed extensive haemorrhage in the chorio-allantoic membranes. Hence, current evidence suggests that egg rotation achieves improved reproduction in the chorio-allantoic membranes. Further experiments should compare the effects of different types/speeds of egg rotation on embryo mortality and on the reproductive index for *E. tenella* Rt3+15. A subsequent observation has been that the inoculation of sporozoites into the opposite end of the air sac of 18-day-old embryos (aimed to penetrate the yolk sac just prior to its withdrawal into the abdominal cavity) did not result in any significant mortality (Richards, unpublished observation). Hence, the age of the embryo and its ability to withstand trauma and/or the site of injection of sporozoites are considered central to achieving the best reproductive rates.

Eimeria tenella Rt3+15 is presently being used as an effective vaccine at a dose rate of 150 oocysts per chicken. With the mean inoculation dose of ~7500 sporozoites, one chicken egg can produce ~1250 doses of vaccine. This equates to ~800 eggs per one million vaccine doses. However, compared with the reproductive index (~5600) of this line of *E. tenella* in chickens (unpublished data), the propagation in eggs is presently less efficient and yields are variable. In the present study, there was considerable variation in the oocyst yields among experiments, although the yields within an experiment among individual embryos did not vary greatly (data not shown). Nonetheless, it should be possible to achieve higher oocyst yields and enhanced embryo-adaptation (leading to decreased embryonic mortality) through increased serial passage in eggs, thereby maintaining immunogenicity in the vaccinated host. For instance (using an inoculation dose of ~10000 sporozoites per egg), Long (1972) reported embryonic mortalities of ~29%, 7% and 0% during passages one, 37 and 38, respectively. The corresponding reproductive indices were ~500, 1800 and 1300 from respective passages. It is likely that the lower reproductive indices achieved in the present study compared with that of Long (1972) relate to an insufficient adaptation to the growth in chicken embryo and precocious nature of Rt3+15 *E. tenella* line. Further experimentation is required to verify whether the pathogenicity or/and immunogenicity of *E. tenella* Rt3+15 in chickens change as it adapts to chicken embryos over a large number of passages and to establish its reproductive characteristics in embryos and, subsequently, in chickens. Since the serial passage of *E. tenella* in embryonating chicken eggs commonly results in the emergence of a line significantly less pathogenic in chickens compared with 'field strains' but immunogenic (Long et al. 1982) an embryo-adapted line (after more than 100 passages) has been incorporated into the Livacox® vaccine (Shirley and Bedrnik 2002). *Eimeria tenella* Rt3+15 has the potential to cause moderate caecal lesions in young chickens (Richards, unpublished). Thus, a reduction in its pathogenicity and

increase in its reproductive index during chicken embryo-adaptation are likely to enhance its suitability as a vaccine constituent, but these aspects require testing.

The propagation of *E. tenella* Rt3+15 in embryonated chicken eggs may also have implications for fundamental investigations of the developmental biology of this parasite, the parasite-host relationship and, importantly, for investigating the molecular biology of reproduction and development. This "closed" experimental system (i.e. the chicken embryo) could provide opportunities for functional genomic studies of *E. tenella*, having advantages over the use of chickens. This is particularly pertinent, given the *Eimeria* genome projects presently underway (Shirley et al. 2004). For example, it may be possible to establish and subsequently utilize double-stranded RNA interference (RNAi or gene silencing) (cf. Campbell and Choy 2005) to investigate the functions of specific genes in *E. tenella in ovo*. There is a number of reports of the applicability of RNAi to protozoan parasites (Ullu et al. 2004), including apicomplexans, although there is some debate as to whether *Plasmodium* species have the genetic make-up or machinery for gene-silencing). Given that the *in vitro* propagation of *Eimeria* has major limitations compared with the *in vivo* propagation in chicken embryos, there appears to be scope for developing a gene-silencing tool for *E. tenella*, provided that this species has the appropriate molecular machinery (e.g., argonaute proteins; reviewed by Peters and Meister, 2007). Hence, while some improvements need to be made to the propagation approach for the production of *E. tenella* Rt3+15 in chicken embryos, it has the potential to lead to the development of a functional genomic tool. Also, future work should to focus on the *in ovo* propagation of other species of *Eimeria*.

Table 2.1. Propagation of oocysts in embryonating chicken eggs inoculated with different doses of sporozoites from the Australian precocious line (Rt3+15) of *Eimeria tenella*. Chicken embryo mortalities, oocyst yields and reproductive indices are tabled

Sporozoites inoculated per egg	Number of eggs used	Embryo mortality 0-168 h	No. of sporulated oocysts per egg	Reproductive index[a]
2600	27	1 (3.7%)	19400	60
4300	29	3 (10.3%)	310000	577
5100	124	8 (6.5%)	90000	141
6600	58	4 (6.9%)	200000	242
7700	59	15 (25.4%)	16000	17
8400	30	7 (23.3%	190000	181
8700	33	2 (6.0%)	360000	331
Totals:	360	40 (11.1%)	Means: 169343	221

[a] Reproductive index = (number of oocysts produced multiplied by eight sporozoites each) divided by the total number of sporozoites inoculated.

Chapter 3 Development of a high throughput capillary electrophoresis for the identification and differentiation of seven species of *Eimeria* from chickens

3.1. Summary

A capillary electrophoretic approach was developed for the identification of seven currently-recognised species of *Eimeria* infecting chickens. In this technique, the second internal transcribed spacer (ITS-2) of ribosomal DNA is PCR-amplified from any of the seven species using a single set of oligonucleotide primers (one of which is fluorescently labelled). The amplicons are heat denatured and subjected to capillary electrophoresis in a MegaBACE^{TM} 1000 (Amersham). The chromatograms captured are stored electronically and then analysed using MegaBACE^{TM} Fragment Profiler software. Using control DNA samples representing monospecific lines of *Eimeria*, specific peaks in the chromatograms were defined for the unequivocal identification of each of the seven species and their differentiation. Electrophoretic reading and analysis are carried out automatically, thus making it a time- and cost-effective method. This procedure is finding applicability as a tool for the quality control of *Eimeria* vaccines, the monitoring of coccidiosis outbreaks and the high throughput analysis of oocyst samples for epidemiological surveys.

3.2. Introduction

Coccidiosis of chickens is an enteric disease caused by protozoan parasites of the genus *Eimeria* (Apicomplexa: Eimeriidae) with major economic impact globally (McDougald et al., 1997). Seven species of *Eimeria* are recognised to infect chickens, and these differ in their biology and pathogenicity (McDougald et al., 1997). The accurate identification of *Eimeria* species and 'strains' has important implications for diagnosis and control, as well as for epidemiology and population biology studies. Traditionally, *Eimeria* species have been identified by morphology and/or morphometry of their sporocysts and oocysts as well as their patterns of development, but these criteria can be unreliable for species infecting chickens (Eckert et al., 1995). Molecular techniques can overcome such limitations (Gasser, 1999). Polymerase chain reaction (PCR)-based techniques (using appropriate genetic markers) are particularly suited because of their ability to specifically amplify DNA from minute amounts of parasite material. Studies have demonstrated that the first (ITS-1) and second (ITS-2) internal transcribed spacers of ribosomal DNA (rDNA) provide genetic markers for the identification of *Eimeria* species or detection of population variation (e.g., Barta et al., 1998; Schnitzler et al., 1998, 1999; Woods et al., 2000a,b; Gasser et al., 2001). Some workers have developed PCR approaches, using species-specific oligonucleotide primers to these spacers, for diagnosis based on the presence or absence of an amplicon on an agarose gel (Schnitzler et al., 1998, 1999). This latter approach has a disadvantage that single primer sets are employed for the detection of only one species per PCR reaction. Also, this approach provides no information on the extent of sequence and/or length variation within a specific amplicon. To overcome these limitations, we developed isotopic and non-isotopic slab gel electrophoretic procedures for the identification of *Eimeria* species from chickens and detection of population variation using sets of genus/order-specific primers flanking the ITS (Woods et al., 2000a,b; Gasser et al., 2001). In the last few years, there have been substantial advances in electrophoretic procedures due to the increased demand for high-speed, high throughput diagnosis and genetic analysis as well as computerized data handling and *in silico* analysis. This applies particularly to the different types of capillary electrophoresis (Righetti et al., 2002; Mitchelson, 2003). In this report, we describe the application of the MegaBACE^{TM} capillary electrophoresis system to the specific identification and differentiation of all seven species of *Eimeria* infecting chickens, thus overcoming previous

limitations and leading to a cost effective alternative for high throughput analysis.

3.3. Materials and methods

Monospecific lines of *Eimeria* representing (originally isolated from naturally infected chickens in Australia) were passaged in specific pathogen-free chickens held in custom-built isolators under stringent conditions to prevent cross-contamination. *Eimeria* were originally identified to species based on the morphometry of sporulated oocysts, prepatent period and location of gross lesions in the intestine(s); to rule out putative contamination of these monospecific lines with one or more heterologous species, the 18S rRNA gene, which also provides species-level identification, was sequenced from amplicons from selected oocyst isolates. The 18S sequences determined for individual species had 99-100% identity with those published previously. In addition to the monospecific isolates, oocysts were also isolated from faecal samples from chickens with naturally-acquired *Eimeria* infections from a range of different poultry establishments in eastern Australia (hence designated "field samples").

Prior to isolation, *Eimeria* oocysts were allowed to sporulate in the faecal samples under constant aeration at 30°C for a minimum of 48 h. They were then isolated using saturated NaCl (Shirley, 1995), washed extensively in 50 ml volumes of water and made up to a final aqueous suspension (10 ml containing $5x10^6$ oocysts). The oocysts were then purified over a sucrose-gradient (Gasser et al., 1987) to remove faecal components inhibitory to the PCR, washed and then resuspended in 0.5 ml of water. The same volume of glass beads (2 mm in diameter; Sigma) was added and the tube vortexed vigorously for 3-5 min until >90% of the oocysts had ruptured (monitored by microscopic examination at 400x magnification). The suspension containing sporocysts was then transferred to a fresh Eppendorf tube, proteinase K (150 mg/ml) and sodium dodecyl-sulphate (5%) added, and then incubated at 37°C overnight. After centrifugation at 13000*g* for 5 min, genomic DNA was column-purified from the supernatant (Wizard™ DNA Clean-Up, Promega, WI, USA).

The ITS-2 region (plus flanking sequence; ~400-650 bp, depending on species) was PCR-amplified from individual genomic DNA samples using primers WW2 (forward: 5'-ACGTCTGTTTCAGTGTCT-3') and WW4r (reverse: 5'-AAATTCAGCGGGTAACCTCG-3') designed specifically to the 5.8S and 28S rRNA gene sequences of members of the genus *Eimeria* (Gasser et al., 2001). Primer WW2 is specific for the genus *Eimeria*, whereas primer WW4r is specific for the family Eimeriidae. Primer WW2 was 5'-end-labelled with the fluorescent dye 5-carboxyfluorescein (FAM). PCR reactions were performed in 25 ml volumes usually containing 1-20 ng of genomic DNA template, 50 pmol primer, 200 mM of each dNTP, 3.5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Promega, WI, USA) under the following thermocycling conditions: 94°C, 15 s (denaturation); 60°C, 15 s (annealing); 72°C, 30 s (extension) for 30 cycles in a Thermal Cycler 480 (Perkin Elmer). Samples without DNA (no-DNA) or with chicken DNA (purified as for *Eimeria* DNA) were also included as negative control samples. The smallest amount of genomic template from which the ITS-2 could be amplified and which was detectable on an agarose gel was estimated at ~5-10 pg (equivalent to ~5-50 oocysts).

The quality and intensity of amplicons were examined on ethidium bromide-stained 2.5% agarose gels, using 65 mM Tris-HCl, 27 mM boric acid, 1 mM EDTA, pH 9 (TBE) as the buffer and a 100 bp ladder (Promega, WI, USA) as a size marker. Subsequently, each sample was diluted 1/30 with water and 1 µl thereof mixed with 9 µl loading solution containing 97% v/v water, 0.1% v/v Tween 20 (Sigma) and 2.9 % v/v 900 bp ladder size standard (Amersham). Samples were denatured at 95°C for 2 min and 10 µl volumes electrokinetically injected into LPA matrix

(Amersham) capillaries at 4 kV for 50 sec and run in a MegaBACE^{TM} 1000 DNA Sequencer (Amersham) at 10 kV for 120 min. Electrophoretic profiles representing individual samples were captured, peak values imported into an Excel spreadsheet, and chromatograms analysed and compared using Fragment Profiler^{TM} software. Each peak represented the position of the sense strand for each denatured amplicon. The intensity (vertical axis) scale of the chromatograms was adjusted, such that background fluorescence was displayed, and hence no peaks remained undetected.

3.4. Results and discussion

A bank of genomic DNA reference samples representing monospecific oocyst isolates (and/or distinct passages) of *Eimeria acervulina* (n = 25), *E. brunetti* (n = 23), *E. maxima* (n = 25), *E. mitis* (n = 9), *E. necatrix* (n = 18), *E. praecox* (n = 12) and *E. tenella* (n = 15) from the states of Victoria, Queensland and South Australia was available. Agarose gel electrophoretic analysis of ITS-2 PCR products amplified (individually) from all monospecific samples revealed that one band was detected for *E. tenella*, whereas two bands were displayed for the other species (not shown). The resolution on agarose gels of multiple bands for some species indicates the existence of different sequence types within an amplicon, which is consistent with previous findings (Gasser et al., 2001). This has been confirmed previously by sequencing of cloned ITS-2 amplicons (multiple clones sequenced) for selected samples (codes: A7, B1, M1, N1 and T6) representing each species. Also, the ITS-2 sequences determined for isolates M1 *(E. maxima)* and T6 *(E. tenella)* showed concordance with sequences deposited in GenBank™ (accession numbers AF027722, AF027723, AF027724, AF027725, AF027726 and AF026388). Some novel ITS-2 sequence types have been identified for *E. mitis* and *E. praecox*, which were not unexpected, as intra-specific or intra-isolate sequence variability for the ITS-2 region has not yet been examined rigorously.

Amplicons produced from all of the monospecific *Eimeria* DNA samples (n = 127) were subjected to capillary electrophoretic analysis. For each *E. acervulina*, *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella*, there were no appreciable differences in the number and location of peaks in chromatogram profiles between samples (selected samples shown), although some differences in relative peak intensity were detected between some samples representing a species (not shown). Within *E. brunetti*, *E. mitis* and *E. praecox*, minor variation in the position(s) of peaks, indicative of population variation, was detectable. Irrespective of such variation, the position of particular 'dominant' peaks remained consistent for all samples of each species in Australia. Since all samples subjected to analysis originated from Australia, it is possible that population variation may be detected upon analysis of samples from a broader range of countries. Nonetheless, species-specific chromatographic peaks could be defined for each of the seven species of *Eimeria* (asterisks), allowing their identification and delineation (see Table 3.2).

As naturally infected chickens can be infected simultaneously with multiple species of *Eimeria*, the ability of the capillary electrophoretic approach to specifically detect DNA in oocyst samples containing more than one species needed to be assessed. Thus, amplicons produced from 109 "field" samples (unknown species compositions) originating from various groups of multiple chickens from different Australian poultry farms were tested. By comparison with selected monospecific reference samples, the species compositions within individual samples were determined, even for samples containing *E. brunetti*, *E. mitis* and *E. praecox*, whose profiles overlap significantly. All seven species of *Eimeria* were represented in the 109 field samples (Table 3.1). A single species was detected in the majority (46.8%) of samples, two species in 35.8% and three species in 14.7%, whereas four or five species were detected in the minority (0.9-1.8%). Capillary electrophoretic results were consistent with those achieved (for a subset of samples tested previously) using an isotopic electrophoretic approach. In 'blind' tests, the reproducibility of the method to correctly identify all species present in mixed oocyst isolates and in deliberately-mixed

genomic DNA samples was evaluated by different human operators. No significant variation in chromatogram profiles was detected among different runs on different days, and the species compositions of mixed samples was as expected. These findings demonstrate that the technique is applicable to detecting single- and mixed-species infections in chickens.

The capillary electrophoretic approach was evaluated using amplicons produced on different days and subjected to electrophoresis on different days, and this was done over an extended time period (months). Importantly, internal size standards were incorporated into each sample, ensuring the accuracy of sizing and the reproducibility of electrophoretic separation. Also, amplicons representing well-defined reference samples (for each of the seven species of *Eimeria*) were included in each sets of samples subjected to analysis. The present capillary electrophoretic approach has significant advantages compared with previous techniques (Woods et al., 2000a,b; Gasser et al., 2001, particularly in relation to human health and safety (being non-isotopic), throughput, and data storage and analysis capacities. The scoring of profiles is performed automatically, which eliminates the need for the pouring, handling and/or exposure of electrophoretic gels, thus significantly reducing time and cost overall. Also, the chromatograms representing different samples (run in different gels and days) can be stored electronically (also in a spreadsheet format) as well as retrieved for comparative analysis at any time. Hence, this high throughput electrophoresis approach is suited for the screening of large numbers of samples, and should provide a useful epidemiological tool for monitoring coccidiosis outbreaks and for investigating the prevalence of different species of chicken *Eimeria* in particular geographical regions. The method will now be employed for the monitoring of the monospecificity of vaccine lines of *Eimeria* (between and after passages in chickens), and for epidemiological investigations and disease surveillance. Similar approaches will be applicable to a range of other prokaryotic and eukaryotic pathogens, provided appropriate species-specific DNA markers are available.

Table 3.1. Results of the capillary electrophoresis-based analysis of oocyst DNA samples from chickens originating from various poultry establishments in eastern Australia

Species detected	Number of samples tested	(Subtotal)
E. acervulina	12	
E. brunetti	4	
E. maxima	12	
E. mitis	3	
E. necatrix	3	
E. praecox	6	
E. tenella	11	(51)
E. acervulina and *E. maxima*	12	
E. acervulina and *E. mitis*	5	
E. acervulina and *E. praecox*	1	
E. brunetti and *E. necatrix*	1	
E. maxima and *E. mitis*	2	
E. maxima and *E. praecox*	3	
E. maxima and *E. tenella*	11	
E. mitis and *E. tenella*	1	
E. praecox and *E. tenella*	1	(39)
E. acervulina, *E. maxima* and *E. mitis*	4	
E. acervulina, *E. maxima* and *E. praecox*	2	
E. acervulina, *E. maxima* and *E. tenella*	3	
E. acervulina, *E. necatrix* and *E. tenella*	1	
E. brunetti, *E. necatrix* and *E. tenella*	1	
E. maxima, *E. mitis* and *E. tenella*	2	
E. maxima, *E. necatrix* and *E. tenella*	2	
E. maxima, *E. praecox* and *E. tenella*	1	(16)
E. acervulina, *E. maxima*, *E. mitis* and *E. praecox*	1	
E. acervulina, *E. maxima*, *E. mitis* and *E. tenella*	1	(2)
E. acervulina, *E. maxima*, *E. mitis*, *E. praecox* and *E. tenella*	1	(1)
Total number tested:	109	

Table 3.2. The positions of the diagnostic peaks determined for each species of *Eimeria* from chickens by capillary electrophoresis of ITS-2 amplicons in a MegaBACE^{TM} 1000 (Amersham)

Species	**Position of diagnostic peak (range)**
Eimeria acervulina	411-412
E. brunetti	471-472
E. maxima	355-356
E. mitis	435-436
E. necatrix	568-569
E. praecox	466-467
E. tenella	550-551

Chapter 4 Application of PCR-coupled capillary electrophoresis for investigating persistent coccidiosis problems

4.1. Summary

The PCR-coupled capillary electrophoresis (CE) approach was employed to investigate the epidemiology of *Eimeria* species on a broiler-breeder farm in Victoria, Australia. The *Eimeria* populations of two flocks vaccinated against coccidiosis were followed over an eleven week period. All seven recognized *Eimeria* species of chickens were detected in both flocks. One flock suffered increased morbidity and mortality in its eighth week, and had consistently higher *Eimeria* oocyst counts, species prevalences and rates of co-infections. Four *Eimeria* species included in the vaccine administered occurred at higher prevalences prior to the disease outbreak in the flock. Using the CE approach, two new, previously undescribed *Eimeria* genotypes were discovered in both chicken flocks, one of which dominated toward the end of the study period. The molecular approach proved versatile and capable of providing detailed epidemiological data applicable to investigating and interpreting coccidiosis outbreaks.

4.2. Introduction

Chickens ingest sporulated oocysts from contaminated litter or the environment, the parasite passes to the intestine where it invades the wall and undergoes several phases of replication, and ultimately new oocysts are produced and excreted in the faeces. Individual birds may suffer morbidity, diarrhoea (which may be bloody) or death, or show no discernible clinical signs at all. Birds that survive the initial infection rapidly develop immunity, which is species-specific (Williams 1998; McDougald 2003). The seven species of *Eimeria* vary in their pathogenicity; *E. necatrix* and *E. tenella*, in particular, are known to be associated with spectacular bloody lesions/enteritis and mass morbidity and mortality in naïve chickens. *E. acervulina*, *E. brunetti* and *E. maxima* are also widely recognized to cause clinical disease. The other two species, *E. praecox* and *E. mitis*, while considered to be relatively non-pathogenic, do cause a reduced feed conversion efficiency and growth rate (Gore and Long 1982; Fitz-Coy and Edgar 1992); these latter factors can cause massive financial losses in the low profit-margin business of intensive poultry farming. Therefore, all species are important economically.

Under commercial production systems, the disease is managed by controlled exposure to infective *Eimeria* oocysts to stimulate immunity in the flock, without the risk of clinical disease. This is achieved by hygiene, the preventative application of anticoccidial drugs and/or live vaccines. All seven species of *Eimeria* are found in Australia (Newton and Green 1965; Jorgensen et al. 1997), and four of them are contained within the commercial vaccine 'Eimeriavax 4m' (Bioproperties Australia Ltd). To maximize the efficiency of poultry management, including the correct timing of application and composition of vaccines, it is essential to understand the epidemiology of individual species of *Eimeria*.

Epidemiological studies indicate that each of the seven species has a worldwide distribution, with chicken flocks on many individual farms harbouring at least six (Kucera 1990; Williams et al. 1996; Thebo et al. 1998; Mattiello et al. 2000). Such surveys have usually been restricted by the limitations of the diagnostic methods employed. Morphological characters, such as the size and shape of *Eimeria* oocysts from faeces, region of the intestinal tract affected and pre-patent period of the parasite(s) after passage through experimentally infected chickens give an indication as to which species is/are causing coccidiosis. However, such approaches may not achieve a species-specific

diagnosis, because there is an overlap in these characters or parameters among some species (Long and Joyner 1984).

Recently, we established polymerase chain reaction (PCR)-coupled electrophoretic techniques which allow the specific identification and delineation of all seven poultry *Eimeria* species (Woods et al. 2000; Gasser et al. 2001; Gasser et al. 2005). These techniques employ genetic markers in the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA, and have been shown to be effective and achieve reproducible results (Morris and Gasser 2006). The oligonucleotide primers used in the PCR are family- or genus-specific, designed such that they may also hybridize to and amplify DNA from genetic variants of presently recognized species of *Eimeria*, new operational taxonomic units (OTUs) or cryptic species. In the present study, we applied PCR-coupled CE to conduct a detailed survey of the temporal changes in *Eimeria* populations infecting broiler breeder-chickens and causing persistent coccidiosis problems in an intensive poultry establishment. Using this technique, we also discovered two new OTUs of *Eimeria* which are genetically distinct from all of the seven currently recognized species.

4.3. Case history, materials and methods

Two sheds of broiler-breeder chickens on one farm in central Victoria, Australia, were studied. In one shed (-A), there was a history of increased and repeated morbidities and mortalities in chickens of five to eight weeks of age. In another shed (-B), there was no evidence of disease. Shed-B was age-matched as closely as possible to shed-A, except that its flock was 18 days older. All sheds were run as "all-in, all-out", deep litter systems. At one week of age, chicks were vaccinated with 'Eimeriavax 4m' (Bioproperties) by eyedrop (containing live, attenuated strains of *E. acervulina, E. maxima, E. necatrix* and *E. tenella*). Before placement, the chicks were also vaccinated against Marek's Disease and Infectious Bronhcitis at one day of age in the hatchery. No anticoccidial drugs were administered preventatively.

From each of the two sheds, 19 or 20 fresh faecal samples were collected immediately from the litter surface into clean 10 ml tubes at random sites throughout each shed at weekly intervals for 11 weeks (from 13th December 2005 to 22nd February 2006). At one time point (7th February 2006), no samples were collected, resulting in a two-week gap between samplings. All faecal samples were stored at 4^{o}C until processed (within 30 days of collection). Each sample was subjected to a faecal examination procedure to enumerate *Eimeria* oocysts, followed by molecular analysis of the oocysts (see below). The farm manager reported on the health of the chicken flock at each sampling period. Increased mortalities were only recorded on one occasion in shed-A, when the birds were 54 days old (i.e., week 8). A course of the anticoccidial drug Baycox (Bayer) was applied to the flock in shed-A for 3 days from the time at which increased fatalities were first recorded (from 30th January to 1st February 2006, inclusive). The farm manager reported a significant improvement in the health of the flock by the second day of treatment, and the flock was considered clinically almost normal by the third day of treatment. At no time were anticoccidials used in shed-B.

Each faecal sample was weighed. Five ml of saturated NaCl solution (specific gravity of 1.2) were added per 1 g of faeces and homogenized by vortexing. Then, 0.3 ml of this suspension was pipetted into a McMaster chamber (Hodgson 1970), and examined using a microscope at 100x magnification. The *Eimeria* oocysts in ten fields of view were counted, and the mean number of oocysts per gram (opg) of faeces calculated.

After oocyst quantification, each sample was made up to 10 ml with saturated NaCl solution. *Eimeria* oocysts were separated from faecal matter by two flotations on this solution using a centrifugation approach, washed twice (in 10 ml) and then suspended in 2 ml of water. The

oocysts were then purified over a sucrose-gradient (Gasser et al. 1987); this method has been demonstrated to remove faecal components inhibitory to the PCR (unpublished findings). The oocysts were washed again (as above), and suspended in 0.5 ml of water in a 1.5 ml Eppendorf tube. The oocysts were ruptured using glass beads (0.71-1.18 mm in diameter, Sigma; 0.5 ml) by vortexing in the tube for 5 min (verified by microscopy). Proteinase K (150 mg/ml) and sodium dodecyl-sulphate (5 % w/v) were added, the tube was incubated at 37°C for 12-16 h and vortexed again. After centrifugation at 13,000 *g* for 5 min, genomic DNA was column-purified (Wizard™ DNA Clean-Up, Promega) from the supernatant.

The CE method was carried out essentially as described in chapter 3. The quality and amount of individual amplicons were verified on ethidium bromide-stained agarose gels (Gasser et al. 2005). They were then diluted 1/6 with water, and 1 µl of each mixed with 10 µl of loading solution containing Hi Di Formamide and the LIZ 500 size standard (1:0.006 ratio) (Applied Biosystems). Samples were denatured at 95°C for 5 min, and 1 µl volumes electrokinetically injected into POP-7™ polymer matrix capillaries at 2 kV for 10 sec and run in a 3730 DNA Analyzer (Applied Biosystems) at 230 V for 1 h. Electrophoretic profiles for individual samples were captured, and the program Genemapper (v.3.7, Applied Biosystems) used to automatically analyse the chromatograms. Flagged data were then examined manually, and the final selections exported into a file in the program Excel (Microsoft Office Professional Edition 2003). Each of the seven recognized species were identified based on the diagnostic peaks defined previously (Gasser et al. 2005) but adjusted slightly due to the use of a different analyser. All data were stored in Excel ® spreadsheets (Microsoft Office Professional Edition 2003, © 1985 – 2003 Microsoft Corporation). All descriptive statistics were carried out using Minitab 15 ® (LEADTOOLS ©1991-2004). The prevalence of each species of *Eimeria* was defined as the proportion of relevant samples found to be positive for that species.

4.4. Results

The faecal oocyst counts for shed-A peaked in the third week (median opg of 4020) (Table 4.1), following the vaccination with 'Eimeriavax 4m' at seven days of age. Shed-B was not sampled until chickens were four weeks of age, at which time oocyst counts were very high (median opg of 9700). From this time, oocyst counts in shed-B decreased rapidly and remained low, although they rose slightly in the last two weeks of the sampling period (median opg of 500 and 570, respectively; Table 4.1). Shed-A showed a different pattern, with oocyst counts remaining considerably higher than in shed-B at the same age, until the eighth week of age (the time point of anticoccidal application; Table 4.1). Counts then decreased in shed-A between weeks 8 and 10, before increasing again in the last sampling period (median opg of 800).

The CE analysis of samples repeatedly detected two unique electrophoretic profiles, designated pX and pY, which did not match those of any of the seven recognized species of *Eimeria*. While samples with pX had a minor presence in both sheds, those with pY became the most prevalent *Eimeria* genotype in shed-A at the last two ages sampled (Table 4.1). Until the application of Baycox at the eighth week, shed-A had a higher prevalence of each *Eimeria* species than did shed-B, when compared with the same age group (Table 4.1; excepting single sampling periods for the genotypes producing pX and pY, and two periods for *E. praecox*). For the first measurement taken after the anticoccidial drug was withdrawn from shed-A (10 weeks), prevalences in shed-A decreased, and were comparable with those in shed-B. This continued through to the end of the sampling period. The exception was the *Eimeria* genotype with the electrophoretic profile pY, which was present in 100% of chickens examined from shed-A at eleven weeks, but only peaked at 10% (in the tenth week) in shed-B (Table 4.1).

E. acervulina was the most prevalent species in both sheds, and was detected in 100% of birds examined in their fourth week of life. This species remained highly prevalent, particularly in shed-A, but decreased to 5% prevalence in both sheds by the last sampling period (Table 4.1). *E. mitis* was highly prevalent in shed-A, peaking in 100% of birds sampled in the fourth week and remaining high for the entire study period. This species represented a much less prominent part of the *Eimeria* population in shed-B, remaining low until the nineth week (75%) and then staying high until the end of the sampling period (Table 4.1). *E. maxima* formed a prominent part of the coccidial population in both sheds, but was particularly abundant in shed-A, remaining at between 35% and 75% from the third to the tenth week of age. It was present at consistently moderate levels in shed-B, peaking at 40% at ten weeks. *E. brunetti* was much more prominent earlier in shed-A than shed-B, peaking at 80% in the sixth week for shed-A, but reaching 5% in the first eight weeks for shed-B. There was a late prominence of *E. brunetti* in shed-B, with a peak of 50% occurring at nine weeks. *E. necatrix* represented a prominent part of the *Eimeria* population in shed-A, peaking at 60% in the seventh week, and remaining at moderate levels even after the application of the anticoccidial compound (Table 4.1). By contrast, it formed a minor part of the coccidial population in shed-B, although it did increase a little by the last sampling period (21% at 11 weeks; Table 4.1). *E. praecox* represented a minor part of the population in both sheds until the peaks commencing at eight weeks in shed-A (60%) and 11 weeks in shed-B (79%). *E. tenella* was consistently more prominent in shed-A than in shed-B, peaking at 60% in the sixth week in the first shed, but not being present at more than 25% in shed-B (at nine weeks; Table 4.1).

The occurrence of infections with multiple species of *Eimeria* in individual chickens remained higher for each age class in shed-A compared with shed-B, throughout the entire sampling period (Fig. 4.1). In shed-A, the median number of species peaked at five per bird in the sixth week, and while this decreased to 2.5 by the tenth week, it decreased to no lower a level than shed-B at the same age. The number of multiple species peaked in shed-B at nine weeks, with a much lower median of 2.5 species per sample.

4.5. Discussion

The primary motivation behind this investigation was a recurring disease problem in one of the sheds (-A) on the farm, in which flocks were repeatedly suffering from increased morbidity and mortality at between five to eight weeks of age, with signs consistent with coccidiosis. However, all chickens on the farm had been vaccinated at one week of age with 'Eimeriavax 4m', a live vaccine containing attenuated strains of *E. acervulina, E. maxima, E. necatrix* and *E. tenella*. Therefore, the question arose as to whether the disease outbreaks were indeed due to *Eimeria*. If so, which species was/were involved, and were they contained in the vaccine? Were there any complicating factors? To study this problem, post mortem examinations of affected birds might detect disease due to *E. necatrix* and/or *E. tenella*, which cause/s prominent, characteristic lesions. However, this would neither provide information about changing *Eimeria* populations in the lead up to the disease outbreak, before pathological changes became apparent, nor about other contributing factors or complications. Other methods have been used previously to investigate patterns of *Eimeria* infection; faecal samples are collected, oocysts isolated, and then specific pathogen free (SPF) laboratory chickens infected experimentally. Diagnosis is carried out by the analysis of a combination of distinctive characters, including prepatent period of the infection/s, oocyst size and shape, and lesion location and type in the intestine/s (e.g., Kucera, 1990; Williams et al. 1996; McDougald et al. 1997; Thebo et al. 1998; Mattiello, 2000). However, these characters are often unreliable under the circumstance of mixed infections, due to an overlap in the distribution of the *Eimeria* species in the gut, and size and shape of oocysts among species (Long and Joyner, 1984). The expense and time-consuming nature of these traditional diagnostic approaches also means that, for practical purposes, multiple samples from the same flock are combined (e.g., McDougald et al. 1997), resulting in a loss of detailed information about the species presence in individual birds and

distribution within the flock. In contrast, the CE method employed provides a rapid and relatively inexpensive means of distinguishing among all species of *Eimeria* (see Morris and Gasser, 2006), making practical analysis of many samples from individual birds and repeated samples of flocks through time possible, thus being perfectly suited for application on commercial properties.

All seven recognized species of *Eimeria* occurred in both sheds examined in the present study (cf. Table 4.1); frequently, individual chickens harboured more than one species simultaneously (cf. Fig. 4.1). Shed-A had consistently higher species prevalences, higher oocyst numbers per gram of faeces, and higher numbers of species per sample than shed-B at the same age (Fig. 4.1 and Table 4.1). This information suggests that the chickens in shed-A were not developing effective immunity against the *Eimeria* species (vaccine and 'wild') present in the shed. For example, for *E. acervulina* and *E. maxima* (both included in the vaccine used), prevalences in shed-A remained very high (≥ 75% and 35% respectively) from three through to eight weeks of age, while, over the same age period, prevalences in shed-B decreased to 10% and 5%, respectively. There was no evidence that 'Eimeriavax 4m' was applied incorrectly, as three of the *Eimeria* species contained in the vaccine were at high prevalence by week three (Table 4.1), and *E. necatrix* appeared to "cycle" later (first appeared in shed-A at five weeks, and shed-B at six weeks). The decreasing oocyst excretion rates between the fourth and fifth week of age (median opg of 9700 and zero, respectively; Table 4.1) suggest that the birds in shed-B were developing protective immunity against *Eimeria* species. This finding may be particularly associated with the development of protective immunity against *E. acervulina*, which was uniformly present at four weeks of age, but decreased in prevalence to 30% at five weeks. A sudden decline in faecal oocyst counts would be expected if the vaccine were stimulating immunity through the natural 'cycling of oocysts' via the environment (Williams 1998). Vaccinated chickens should develop infections as they would from natural exposure, but without clinical disease; the parasite replicates in the intestine and the bird releases oocysts in the faeces, which then reinfect the flock. Such cycling is essential to achieve protection; as the birds develop immunity, the oocyst shedding should decrease, with "background oocyst levels" assisting in maintaining immunity by providing a constant low-grade challenge, further boosting immunity over time. By contrast, oocyst levels in shed-A remained much higher than those in shed-B at the same age until the time of the administration of the anticoccidial drug (Baycox) in the eighth week (Table 4.1), suggesting that, in this case, *Eimeria* infections were not becoming self-limiting.

The prevalence of *E. brunetti* was relatively high, peaking at 80% in shed-A (sixth week) and 50% in shed-B (nineth week). While considered to be a significant pathogen, *E. brunetti* has not yet been included in the *Eimeria* vaccine ('Eimeriavax 4m') employed in Australia. Therefore, the spread of this natural infection in the flocks would take place in a relatively uncontrolled manner from bird-to-bird, as compared with a universal exposure to the species in the vaccine at one week of age. While the prevalence of *E. brunetti* decreased somewhat before the outbreak of disease in the eighth week, it is unclear whether it represented a primary, contributing or a predisposing factor, due to the concurrent "cycling" of species from the vaccine at the time of the outbreak. Nonetheless, *E. brunetti* may need to be included in the vaccine in the future.

The positive response of the health of the flock in shed-A to the course of Baycox suggests that *Eimeria* was the main immediate factor in the outbreak of disease. The eighth week-sample from shed-A was taken in the middle of the anticoccidial drug application, and, already, faecal oocyst counts had decreased compared with the last period (medians of 870 and 80 opg in the seventh and eighth weeks, respectively). The faecal oocyst count remained low in shed-A in the tenth week, but, by the eleventh, it had increased to a level comparable with that in shed-B at the same chicken age (Table 4.1). Leading up to the outbreak of disease in shed-A, there was a high prevalence of *E. necatrix* compared with that in shed-B in age-matched chickens (Table 4.1). In addition, independent molecular testing carried out on intestinal material taken from three autopsied chickens at the time of the outbreak identified *E. necatrix* as the dominant species (W. Woods,

unpublished). *E. necatrix* is well known to have a lower fecundity and to be a 'poor competitor' relative to other species (Williams, 1998). As a result, in naturally infected, unvaccinated flocks, disease caused by *E. necatrix* is rare in chickens of less than 12-14 weeks of age. In this case, this species was applied to birds of one week of age via the 'Eimeriavax 4m' vaccine, but it does not appear to have 'cycled' effectively in young birds in either shed (Table 4.1; although there was no information from shed-B for the first three weeks). However, the present stratified data across shed-A revealed that other species were also at high prevalences at this time (i.e., the last time period before drugs were applied [i.e., the seventh week], *E. acervulina* and *E. maxima*, as well as the less pathogenic *E. mitis* and *E. praecox* were all at a prevalence of at least 50%). Therefore, the situation was more complex than would be suggested based on the initial finding of *E. necatrix* to be the dominant species. The findings suggested a difference in the development and degree of immunity between sheds–A and –B. While the reasons for this difference are unclear, the possibilities include host (e.g., underlying subclinical non-parasitic infections) and environmental (e.g., crowding, air quality and stress) factors having a significant negative effect on the health of chickens in shed-A but not in shed-B. If chickens in shed-A were more susceptible due to one or more of these factors, then the administration of the live Marek's Disease (MD) vaccine, which can increase the susceptibility of chickens to coccidiosis (Biggs et al. 1968, Rice and Reid, 1973), could have precipitated or exacerbated the disease problem. While a course of an anticoccidial drug can achieve a reduction of fatalities, it does not address the underlying problem. Hence, the application of the CE technique has major merit, by providing detailed temporal 'snap shots' of the specific composition of *Eimeria* populations prior to, during and after outbreaks.

Using the CE approach, two previously undescribed genetic variants of *Eimeria* were discovered. The detection of profiles pX and pY was possible because the oligonucleotide primer set WW4r-WW2 is specific in the PCR for the family Eimeriidae or the genus *Eimeria* (see Woods et al. 2000). The discovery of these two OTUs (i.e., OTU-X and OTU-Y) is not unique to the farm studied herein, as they have both been detected recently and independently in other commercial chicken production establishments in Victoria, New South Wales and Western Australia (W. Woods, unpublished findings). Also, in addition to OTU-X and OTU-Y, another genetic variant of *Eimeria*, designated OTU-Z, has been detected multiple times in all of these three states of Australia (unpublished findings). In the present study, OTU-Y was one of the dominant species (by prevalence) in shed-A towards the end of the sampling period (Table 4.1). Its prevalence increased to prominence after the application of the anticoccidial drug (Baycox) in this shed, which suggests that this species may have propagated opportunistically after the drug-induced decrease or removal of *Eimeria* species. Alternatively, it is possible that the flock had not been completely exposed to the oocysts, and, hence, some birds remained relatively naïve immunologically. The detection of such new OTUs raises concerns regarding the effectiveness of some commonly-used, traditional diagnostic approaches, and leads to an increased awareness of their potential disease and economic significance. While there is no published information on the biology of these new OTUs, preliminary infection experiments of each of them indicate their biological distinctiveness from the seven currently recognized species of *Eimeria* (unpublished findings). Work is planned to genetically and biologically characterize OTU-X, OTU-Y and OTU-Z in detail and to establish their specific status and importance as pathogens in chickens. The hypothesis that each of these OTUs represents a unique ('cryptic') species of *Eimeria* will be tested.

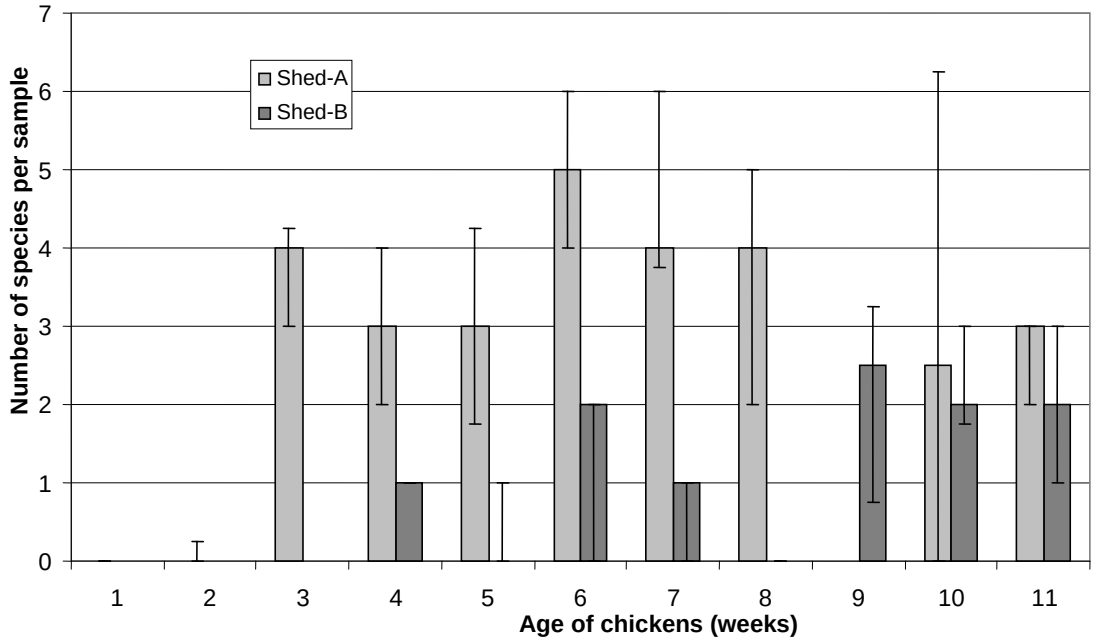
The discovery of these new OTUs demonstrates the advantages and benefit of the analytical/diagnostic CE approach used. In addition, this is the first time that the epidemiology of new genetic variants (OTUs) of *Eimeria* has been studied through time. The CE method is applicable to any biological sample that may be expected to contain *Eimeria*, including gut samples from necropsied chickens. Since this technique does not rely on clinical signs for diagnosis, even non-pathogenic species can be detected; this is important under intensive farming conditions, because all species may have a negative effect on growth rate and feed conversion efficiency (Gore

and Long, 1982; Fitz-Coy and Edgar, 1992; Williams, 1998), particularly in mixed-species infections. While the development and application of anticoccidial drugs to control coccidiosis has facilitated modern intensive farming of poultry (Chapman 1997; Williams et al. 1999), there are significant problems with drug resistance, and a growing reluctance from the public to use drugs in food-producing animals (e.g., banning of the use of preventative anticoccidials in the EU; Bedford, 2000). In the future, an understanding of the epidemiology of each *Eimeria* species will be central to formulating the most effective vaccination regimes, particularly in a situation in which anticoccidial drugs are not used preventively. Further surveys of different flocks in different poultry establishments using the CE approach should give improved insights into the dynamics of *Eimeria* populations and coccidiosis, which should assist in the design and implementation of future prevention and control programmes.

Table 4.1. Oocysts per gram of faeces and prevalence of different species and operational taxonomic units (OTUs) of *Eimeria* in broiler-breeder chickens in sheds-A and -B. All calculations derive from 19 or 20 faecal samples. Oocysts counts per gram of faeces are presented as 'median (1st quartile/3rd quartile)' for each shed for each sampling period. Prevalences are presented as percentage of relevant samples that were positive. Age is weeks old (1 to 11). '-' indicates no data collected at this time period for that shed. During week 8 the flock in shed-A suffered from increased morbidity and mortality, at which point a course of the anticoccidial drug Baycox (Bayer) was applied to this flock for 3 days. Species present in the 'Eimeriavax4m' vaccine are highlighted in bold. OTU-X and OTU-Y represent the two new genotypes of *Eimeria* discovered.

	Age / shed	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Oocyst counts	A	0 (0/0)	0 (0/4)	4020 (801/6870)	2990 (1987/6180)	450 (68/2580)	1320 (761/3180)	860 (49/2140)	80 (50/190)	-	50 (12/190)	800 (357/3350)
	B	-	-	-	9660 (4250/33400)	0 (0/150)	20 (0/140)	130 (20/240)	0 (0/8)	110 (10/490)	510 (270/2200)	570 (70/1230)
Prevalence (%) of species												
E. acervulina	A	10	10	90	100	80	100	80	75	-	65	5
	B	-	-	-	*100*	*30*	*53*	*63*	*10*	*40*	*20*	*5*
E. brunetti	A	0	0	55	35	35	80	35	0	-	25	0
	B	-	-	-	*0*	*5*	*5*	*5*	*5*	*50*	*45*	*21*
E. maxima	A	0	5	60	35	55	75	60	50	-	40	10
	B	-	-	-	*5*	*10*	*26*	*11*	*5*	*20*	*40*	*16*
E. mitis	A	0	0	95	100	60	95	75	65	-	50	60
	B	-	-	-	*0*	*0*	*5*	*11*	*5*	*75*	*75*	*58*
E. necatrix	A	0	0	0	0	30	50	60	35	-	35	10
	B	-	-	-	*0*	*0*	*5*	*0*	*5*	*5*	*10*	*21*
E. praecox	A	0	0	0	0	0	0	5	60	-	25	80
	B	-	-	-	*0*	*0*	*11*	*11*	*5*	*15*	*20*	*79*
E. tenella	A	0	10	45	25	20	60	45	20	-	25	0
	B	-	-	-	*0*	*5*	*16*	*11*	*0*	*25*	*0*	*0*
OTU-X	A	0	10	5	5	0	15	20	25	-	0	0
	B	-	-	-	*0*	*0*	*21*	*0*	*5*	*10*	*0*	*5*
OTU-Y	A	0	0	5	0	10	0	5	10	-	60	100
	B	-	-	-	*0*	*0*	*5*	*0*	*0*	*10*	*10*	*0*

Fig. 4.1. Bar graph depicting the median number of species (-/+ 1st/3rd quartiles) detected in faecal samples using the CE approach, according to the shed (-A or -B) and the age (weeks) of the chickens. (NB samples neither were taken from shed-A at week 9 nor from shed-B for weeks 1-3 inclusive)



Chapter 5 Application of PCR-coupled capillary electrophoresis to provide detailed insights into *Eimeria* populations in intensive poultry establishments

5.1. Summary

The PCR-based capillary electrophoresis (CE) approach was used to conduct an epidemiological survey of *Eimeria* species in seven Australian broiler flocks, varying in age from 18 to 42 days. We confirmed that all seven recognized *Eimeria* species of poultry were present. *Eimeria acervulina* and *E. maxima* were the most common, followed by *E. mitis* (i.e., 89%, 87% and 64% of chickens, respectively). *E. praecox* was present in 44% of birds, whereas *E. brunetti* and *E. tenella* were uncommon, being found in 36% and 26%, respectively. *E. necatrix* was rarely detected (10%). Even the least common species were present in more than 70% of sheds. The prevalence of individual species was higher in older than younger chickens. Most of the chickens sampled were simultaneously infected with multiple *Eimeria* species (mean = 3.6). The number of *Eimeria* oocysts excreted per gram of faeces reached a peak at 36 days of age, before declining to a considerably lower level by 42 days. As anticoccidial drugs were permanently withdrawn at 36 days, the decreasing *Eimeria* oocyst excretion rates indicated the development of protective immunity in the chickens. The present study showed that even healthy chickens usually harbour numerous species of *Eimeria*. The CE technique proved to be a time and cost effective means of investigating the epidemiology of *Eimeria* in commercial establishments.

5.2. Introduction

Avian coccidiosis is one of the most important diseases affecting the intensive poultry industry worldwide. An intestinal disease caused by protozoan parasites of the genus *Eimeria* (Apicomplexa), it is usually the replicative (schizogony) phase of these agents in the intestinal wall which causes most of the damage to the avian host (McDougald, 2003). Currently, there are seven recognized species that infect chickens; they vary in their ability to induce diarrhoea (which may be bloody), morbidity and mortality (Williams, 1998; Allen and Fetterer, 2002). However, even relatively non-pathogenic species are important economically, because their infections can result in significantly reduced feed conversion, weight gain and productivity even in clinically normal birds (Gore and Long, 1982; Fitz-Coy and Edgard, 1992; Williams, 1998). Infections are self-limiting in surviving chickens, as species-specific immunity develops rapidly. *Eimeria* species are extremely widespread in the poultry industry, and coccidiosis is usually controlled by the application of anti-coccidial drugs and/or administration of live vaccines (Shirley et al., 1995). The specific diagnosis of infections plays a crucial role in the prevention, surveillance and control of coccidiosis (Morris and Gasser, 2006). In addition, the application of live vaccines has it own complications. As the immunity induced is species-specific (Williams, 1998; McDougald, 2003), the composition of a vaccine must be appropriate for each geographical region, production system and age of the chickens to maximize its efficacy.

All seven species of *Eimeria* infecting chickens are frequently detected in surveys of commercial poultry farms in many countries, for example, Czechoslovakia, Sweden and Argentina (Kucera et al., 1990; Thebo et al., 1998; Matiello et al., 2000), and all of them have been recorded in Australia in earlier studies (Newton ad Green, 1965; Jorgensen et al., 1997). Naïve birds are exposed to oocysts present in their environment, acquire infection orally and start to shed oocysts in faeces within four to five days, depending on *Eimeria* species (Allen and Fetterer, 2002). Peak oocyst excretion rates typically occur at around four to eight weeks of age (Williams, 1998; Lunden

et al., 2000); thereafter, species-specific immunity develops in healthy birds, and oocyst excretion rates decline. *E. necatrix* has relatively low fecundity, is a poor 'competitor', and tends to appear in older birds of up to 12 weeks of age (Williams, 1998). Therefore, it rarely causes a problem in broiler flocks which are slaughtered before this age. Individual birds are frequently infected simultaneously with more than one *Eimeria* species (McDougald et al., 1986), and up to six have been recorded on single farms (Williams et al., 1994).

Previously, the collection of large-scale epidemiological data for chicken coccidiosis has involved the initial gathering and examination of faecal or litter samples, microscopic examination and isolation of oocysts, and the subsequent inoculation of experimental birds. Observations are then made of traditional diagnostic characters, such as prepatent period, oocyst morphology, and the location and appearance of intestinal lesions (e.g., Williams et al., 1996; McDougald et al., 1997). However, this methodology is not only costly and time-consuming, but also unreliable under the circumstances of mixed field infections, particularly because the overlap in biological and morphological characters makes the unequivocal identification and differentiation of *Eimeria* species impossible (Long and Joyner, 1984).

To overcome the limitations of traditional methods, various polymerase chain reaction (PCR)-based detection techniques have been developed (Morris and Gasser, 2006). These methods usually involve the use of a different oligonucleotide primer pair for each species in the PCR (e.g., Schnitzler, 1998, 1999; Fernandez et al., 2003; Su et al., 2003). Recently, we developed a PCR-based capillary electrophoretic (CE) approach (Gasser et al., 2005), utilizing genetic markers in the second internal transcribed spacer (ITS-2) region of nuclear ribosomal DNA, for the specific identification of all seven recognized *Eimeria* species from chickens. In contrast to previously developed PCR-based approaches, this analytical/diagnostic approach allows the rapid, accurate and simultaneous differentiation of all species in a single sample, as well as the possibility of detecting new operational taxonomic units (OTUs) (i.e., genetic variants or 'cryptic species'). This CE approach has considerable advantages over previous slab gel electrophoretic methods (Woods et al., 2000; Gasser et al., 2001). It is non-isotopic, an internal size standard is included in each sample (allowing the direct comparison of samples run on different days) and it is more rapid and less laborious to carry out. In the present study, we applied this method to conduct a detailed epidemiological survey of a series of broiler farms, sampling chickens of a range of ages, and demonstrate the usefulness of this technique as a powerful tool to the poultry industry and producers.

5.3. Materials and methods

Fresh faecal samples were collected on November 18th, 2005 from chickens from seven broiler sheds from six separate commercial broiler "grow-out" operations located between Sydney and Newcastle, New South Wales, Australia. All farms represented "all-in, all-out" systems, using deep litter. The seven individual sheds were selected to represent as wide a chicken age range as possible. The age classes were 18, 21, 23, 29, 36, 38 and 42 days. The chickens of 18 and 21 days old were kept in separate sheds on the same farm. No farm had overt signs of coccidiosis or abnormal rates of morbidity or mortality. No other health problems were apparent at the time of faecal collection. Ten fresh individual faecal samples were collected from chickens (immediately after defaecation) from the litter surface into clean 10 ml tubes at random sites in each shed. All faecal samples were stored at 4^{o}C until processed (within 30 days of collection). Each sample was subjected to a quantitative faecal examination procedure to enumerate *Eimeria* oocysts, followed by molecular analysis. None of the farms had used anticoccidial vaccines. Each farm followed the same preventative anticoccidial drug treatment program. From placement at 'day-old' to 26 days, birds received 60 ppm of salinomycin and 125 ppm of dinitolamide in the feed. From 27 to 36 days of

age, this program was changed to between 75 and 125 ppm of lasalocid. All drugs were withdrawn after 36 days, and from that time until final culling at 56 days, the chickens were not treated.

Each faecal sample was weighed and 5 ml of saturated NaCl solution (specific gravity 1.2) added per 1 g of faeces, then homogenized using a vortex mixer, and 0.3 ml of the suspension transferred to a McMaster chamber (Hodgson, 1970) for microscopic examination at 100 x magnification. *Eimeria* oocysts were counted in ten fields of view, and the mean number of oocysts per gram of faeces calculated.

Following oocyst quantification, saturated NaCl was added to the remainder of the sample and made up to 10 ml. *Eimeria* oocysts were isolated by two flotations on saturated salt. Oocysts were then washed twice by centrifugation (1500 *g*) in 10 ml and resuspended in 2 ml of water. The oocysts were purified over a sucrose-gradient (Gasser et al., 1987) to remove faecal components known to be inhibitory to the PCR (unpublished findings), washed (as before) and then resuspended in 0.5 ml of water in a sterile Eppendorf tube. The same volume of glass beads (~1 mm in diameter, Sigma) was added and the tube vortexed vigorously for 5 min to rupture the oocysts. Proteinase K (150 mg/ml) and sodium dodecyl-sulphate (5 % w/v) were added, and the tube was incubated at 37°C for 12-16 h and then vortexed again. After centrifugation at 13000 *g* for 5 min, genomic DNA was column-purified (using Wizard^{TM} DNA Clean-Up, Promega) from the supernatant.

PCR-based capillary electrophoretic (CE) analysis was carried out essentially as described in chapter 3, with only minor modification. After verifying the quality and intensity of individual amplicons on ethidium bromide-stained agarose gels (Gasser et al., 2001), they were diluted 1/6 with water, and 1 µl of each mixed with 10 µl of loading solution containing Hi Di Formamide and the LIZ 500 size standard (1:0.006 ratio) (Applied Biosystems). Samples were denatured at 95°C for 5 min, and about 1 µl volumes electrokinetically injected into POP-7^{TM} polymer matrix capillaries at 2 kV for 10 sec and run in a 3730 DNA Analyzer (Applied Biosystems) at 230 V for 1 h. Electrophoretic profiles representing individual samples were captured, and chromatograms analysed automatically using the program Genemapper (v.3.7, Applied Biosystems). Flagged data were then examined manually, the final selections exported into a file in the program Excel (Microsoft Office Professional Edition 2003) and processed. Each of the seven recognized species were identified based on the diagnostic peaks defined previously (Gasser et al., 2001) and adjusted slightly due to the use of an alternative analyser.

All data were stored in Excel spreadsheets. All descriptive statistics were carried out using the program Minitab 15 (LEADTOOLS 1991-2004). The prevalence of each *Eimeria* species was defined as the proportion of relevant samples found to test positive for a particular species.

5.4. Results

The numbers of oocysts per gram of faeces varied across age groups (see Fig. 5.1). Oocyst excretion rates increased from 18 to 23 days, reaching a plateau lasting from 23 to 38 days (peaking at 36 days) and then starting to decrease between 39 and 42 days.

Utilizing the PCR-based CE approach, all seven recognized *Eimeria* species were detected (Tables 5.1 and 5.2), and no intraspecific profile variation was detected. The prevalences of individual *Eimeria* species were recorded across age groups (from seven sheds, Table 5.1), in all samples (from 70 birds; Table 5.1) and within each age group (from ten samples, Table 5.2). *E. acervulina* and *E. maxima* were the most prevalent species, being detected in 87% and 89% of all samples, respectively, and 100% of sheds (Table 5.1). *E. mitis* was also highly prevalent, being found in 64% of all birds sampled and 86% of sheds. While *E. praecox* was present in all sheds, it was found in less than 50% of the chickens (Table 5.1). *E. brunetti* and *E. tenella* were relatively

uncommon (being present in 36% and 26% of samples, respectively), and *E. necatrix* was rare (10 % of samples) (Table 5.1). However, even the least common species were found in more than 70% of sheds (Table 5.1).

The prevalences of individual species varied with the age of the chickens, (Table 5.2). Mostly, higher prevalences of each species were found in older compared with younger birds. For example, prevalences of *E. acervulina, E. maxima* and *E mitis* were 40% initially, and increased to 100% by 23, 29 and 36 days, respectively. For *E. acervulina* and *E. mitis*, prevalences then decreased in the oldest age group (Table 5.2). *E. praecox, E. brunetti* and *E. tenella* reached their highest prevalence in the oldest age group of chickens tested, but did not reach 100%. For *E. necatrix*, higher prevalences were usually recorded in the younger age groups, but were still low overall.

The occurrence of mixed infections increased as the birds aged (Fig. 5.2). All age classes harboured a mean of more than two *Eimeria* species per host, making infections with multiple species the rule. Chickens from the oldest three age classes averaged more than four species per host. One chicken (29 days old) carried all seven *Eimeria* species. Overall, the mean number of species per sample (n=70) was 3.6 (*S.D.* = 1.73). Individual flocks harboured between four (at 21 days) and seven (at 18, 29 and 42 days) species of *Eimeria* (Table 5.2).

5.5. Discussion and conclusions

Previous epidemiological studies have almost invariably relied on the expensive and time-consuming methodology of isolating oocysts and infecting experimental birds to subsequently establish the presence of the different species of *Eimeria*. The substantial cost of this approach precludes the analysis of individual samples from single birds; rather, samples from one shed are pooled together, providing information about population-level prevalence, but not stratified data on the variation across the flock. In the event of an outbreak of coccidiosis, post mortem examinations may indicate a primary disease-causing agent (e.g., *E. necatrix* or *E. tenella*) but give no information regarding the importance of less prominent species or indeed the epidemiology of highly pathogenic species leading up to the problem. Jenkins et al. (2006) utilised a PCR-based diagnostic tool to distinguish among *Eimeria* species found in naturally infected chickens in the field. Their method used sequence differences in ITS-1 to distinguish among species, but required a different primer pair for each, necessitating seven separate PCR reactions to test each sample. The present approach has the advantage of utilizing a single primer pair (i.e., WW2-WW4r) which is able to amplify genomic DNA from all seven *Eimeria* species in a single PCR reaction. Since the primers used are able to hybridize in the PCR to the template from all *Eimeria* species, this CE assay has the capability of detecting new OTUs (e.g., different genetic variants or new species not previously described), which are unlikely to be identified by other means. Importantly, this assay is applicable worldwide, as even population variation in species (reflected in mutations within the ITS-2 but not in the conserved flanking rDNA regions) in different parts of the world should not affect the function of this primer pair. The present CE method also has significant technical advantages over manual electrophoretic techniques, not requiring the use of isotopes and eliminating the need to pour slab gels. The use of an internal size marker in each test sample eliminates the need to use reference control samples (for each of the seven *Eimeria* species) every time a gel is run. In addition, the chromatograms produced by CE analysis are readily transformed into spreadsheet data, and can be stored electronically and retrieved at any time to compare profiles representing samples run on different days. In a separate study, using a selected number of samples, it has been demonstrated that the results achieved using the CE approach (Gasser et al., 2001) are essentially the same as those achieved using a previous isotopic electrophoretic method (Morris et al., unpublished findings), confirming the appropriateness of this method for the rapid and accurate screening of large sample sizes.

Therefore, the assay was perfectly suited for the analysis of individual faecal samples, allowing an insight into the detailed temporal and spatial changes in the 'population structure' of individual *Eimeria* species. The present study was designed to sample flocks of different ages on the same day, and the data do not represent the same flock through time. Therefore, we have gained a valuable 'snap-shot' of the distribution of *Eimeria* species in Australian broilers of varying ages. This molecular epidemiological survey in a limited geographical area and single production system support the observation that each of the seven species of *Eimeria* are present in chickens in Australia (as reported earlier by Newton and Green, 1965; Jorgensen et al., 1997), although there had been no previous studies from the state of New South Wales, where the present investigation was carried out. In the study area, each species was ubiquitous, as even the least common (*E. necatrix*) was present in more than 70% of the sheds examined (Table 5.1). This study revealed the complexity of *Eimeria* infections in clinically healthy commercial broiler flocks, not previously reported from Australia. On average, each sample (representing a single chicken), contained more than three species of *Eimeria*, and each shed contained a minimum of four (cf. Table 5.2). These data are in accordance with the diversity found in other flocks worldwide (Kucera et al., 1990; McDougald et al., 1997; Mattielo et al., 2000), but, as far as we are aware, all seven species have not been recorded previously in an individual chicken with a natural infection.

The fact that *Eimeria* species were widespread, with even the most rare being found in more than 70% of sheds examined (Table 5.1), does not necessarily suggest that coccidiosis is a welfare concern in the chickens or is limiting the efficiency of the poultry operation studied. These chickens were asymptomatic. The correct, judicious use of anticoccidial drugs, in combination with good animal husbandry practices, appears to have enabled the producer to maintain the low level of infection necessary to ensure that a protective level of specific immunity develops in the flocks without the occurrence of clinical coccidiosis. The initial, very low oocyst excretion rate (median of zero and of 13.5 oocysts per gram of faeces, at 18 and 21 days, respectively) rapidly increased to a higher plateau which lasted from days 23 to 38 (cf. Fig. 5.1; peaks at 36 days with median of 1269 oocysts per gram). This reveals a relatively late increase in the oocyst excretion rate compared with the early, lower peak expected in flocks uniformly vaccinated at one day of age, but corresponds well to that described from other preventatively medicated flocks (e.g., Williams et al., 1999). By 42 days of age, the faecal oocyst count had fallen again to lower levels, with a median of only 106 oocysts per gram of faeces, indicating the development of specific immunity to an increasing number of species.

The transmission of *Eimeria* species through the flock is reflected in the diversity of species detected, with older birds harbouring more species than younger chickens (Fig. 5.2). For each species except *E. necatrix*, a rise in prevalence was recorded in young compared with older chickens, as infections spread through the flock (Table 5.2). *E. acervulina* and *E. mitis* decreased in prevalence in the oldest chickens sampled, which may reflect the development of specific immunity to these species. It is interesting that, while oocyst counts decrease in the oldest chickens (42 days), there was no corresponding drop in species diversity (see Table 5.2; Figs. 5.1 and 5.2). As specific immunity develops, the shedding of oocysts in the faeces is reduced, but the host may still continue to harbour the parasites. Considering that the CE method can detect the presence of *Eimeria* species, even as their oocyst production reduces significantly, older animals may still have high species diversity, representing remnants of earlier, larger *Eimeria* populations, while a decreasing faecal oocyst count indicates the development of protective immunity (Figs. 1 and 2). Anticoccidial drugs were permanently withdrawn after the chickens had reached 36 days of age. After this time, there was no sudden rise in the oocyst counts; rather, the overall oocyst count started to reduce, and decreased to considerably lower levels by 42 days of age (Fig. 5.1), providing further evidence of a healthy flock having developed protective immunity and thus not requiring preventative anticoccidial treatment.

In the current study, the most prevalent species were *E. acervulina* and *E. maxima* (see Tables 5.1 and 5.2), both of which are frequently recorded as being dominant species in commercial flocks. They are important pathogens, and their dominance combined with the health of the flocks provides further evidence that the current management strategy employed in the poultry establishments studied was effective. *E. brunetti* had a relatively minor presence on these farms (in 36% of all samples), and has been recorded in broiler flocks elsewhere (e.g., Argentina; see McDougald et al., 1997). Although present at low levels compared with the dominant *E. acervulina* and *E. maxima*, *E. brunetti* is considered to be pathogenic; while obviously controlled under the present anticoccidial management regime, if a vaccine were to replace drug use in the future, this species should be included. Some surveys have shown the most pathogenic species, *E. tenella*, to be highly prevalent (Kucera et al., 1990; Williams et al., 1994; Mattielo et al., 2000), but it was rare in the present study. This is possibly because firm faecal samples were taken; *E. tenella* oocysts are derived from the caecum and would be expected to occur in higher numbers in caecal rather than faecal deposits. Also, *E. necatrix* was uncommon, probably because of the age of the birds sampled. Having a low reproductive index and being a relatively 'poor competitor', the oocyst excretion rate for *E. necatrix* does not usually reach a peak until birds have reached 9–14 weeks of age, and, therefore, this species does not typically cause a problem in broiler flocks which are slaughtered before reaching this age (Williams, 1998; McDougald, 2003). However, in this study, *E. necatrix* was (rarely) detected predominantly in younger birds (see Table 5.2); this apparent aberration may simply be due to the fact that it was rare. *E. praecox* and *E. mitis* are generally considered to be non-pathogenic, but have been shown to have a negative effect on growth rate and feed conversion efficiency (e.g., Gore and Long, 1930; Fitz-Coy and Edgar, 1992; Williams, 1998). Given the high prevalence of these two species in the present study (see Tables 5.1 and 5.2), the importance of their presence in commercial flocks may have to be reconsidered.

Being present at a high prevalence is only one aspect of a parasite's possible impact on its host. While the CE methodology is sensitive enough to detect small number of oocysts (Gasser et al., 2003), the chromatograms obtained provide information regarding only the presence or absence of *Eimeria* species. The ability to quantify the number of oocysts representing each species contributing to a sample could provide a better understanding of the practical significance of a field sample testing 'positive' for certain species. The development of a real-time PCR assay using genetic markers within the ITS-2 would provide the quantitative data necessary for a more detailed understanding of the level of infection. Real-time PCR has an additional advantage in the analysis, as fluorescence in the sample is measured within the thermocycler, removing the need for the pouring and running of gels. However, such an assay can be relatively expensive to run, and any system developed may necessitate the running of multiple PCRs to test each sample for all seven species. Therefore, a real-time PCR may not replace the current analytical/diagnostic methodology for routine monitoring, but would be used in conjunction with it, under particular circumstances in which the quantitation of faecal oocyst numbers is required.

In conclusion, the application of the diagnostic PCR-based CE technique allowed the rapid and inexpensive acquisition of detailed epidemiological information regarding the prevalence and composition of *Eimeria* populations infecting chickens on commercial farms. We have shown that even a healthy broiler flock can have a high diversity and prevalence of *Eimeria* species. For this study, we used samples taken on the same day from flocks at different stages of development to gain, for the first time, an understanding of changes in *Eimeria* populations through time. In the future, we plan to follow individual flocks throughout their development. Management strategies, including accurate diagnostic tools, will become much more important in the future as the use of preventative anticocccidial drugs is reduced in certain regions of the world (e.g., European Union; Bedford, 2000). Under these circumstances, an understanding of the intricacies of changes in *Eimeria* populations will become increasingly important to enable poultry producers to make informed decisions regarding management practices, such as the use of anticoccidial vaccines, the

species composition thereof and the timing of vaccination. The present CE technique is not limited to known *Eimeria* species, or just the Australian situation, but is applicable world-wide to detect currently known species, genetic variants and/or even new species.

Table 5.1. Prevalence of each *Eimeria* species, analysed by: (a) total number of chickens sampled across all sheds, and (b) total number of sheds

Species	(a) % all samples positive (n=70)	(b) % sheds positive (n=7)
E. acervulina	89	100
E. maxima	87	100
E. mitis	64	86
E. praecox	44	100
E. brunetti	36	71
E. tenella	26	86
E. necatrix	10	71

Table 5.2. Prevalence of each *Eimeria* species, according to the age class of chickens (= shed sampled), and total number of species detected. Ten samples were examined for each age class

Species	% positive in each shed (d = days old) (n=10)						
	18 d	21 d	23 d	29 d	36 d	38 d	42 d
E. acervulina	40	90	100	100	100	100	90
E. maxima	40	80	90	100	100	100	100
E. mitis	40	0	70	80	100	100	60
E. praecox	40	20	60	30	60	20	80
E. brunetti	20	0	0	10	50	80	90
E. tenella	20	0	20	10	30	20	80
E. necatrix	20	20	10	10	0	0	10
Total number of species detected	7	4	6	7	6	6	7

Fig. 5.1. Box plot depicting the relationship between oocysts per gram of faeces (log10 transformed) and the age of chickens examined.

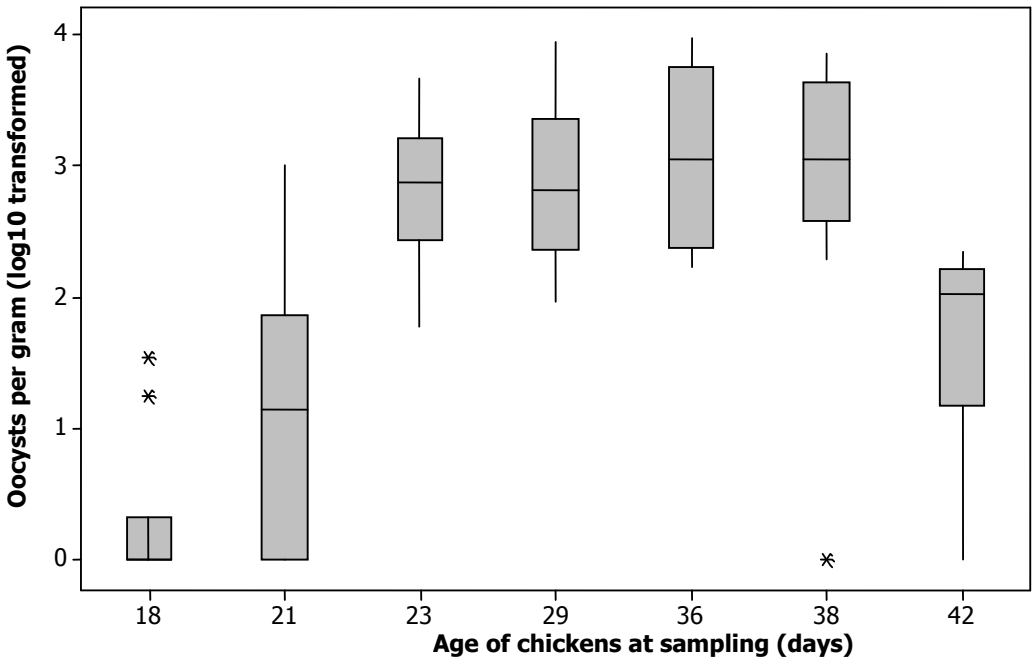
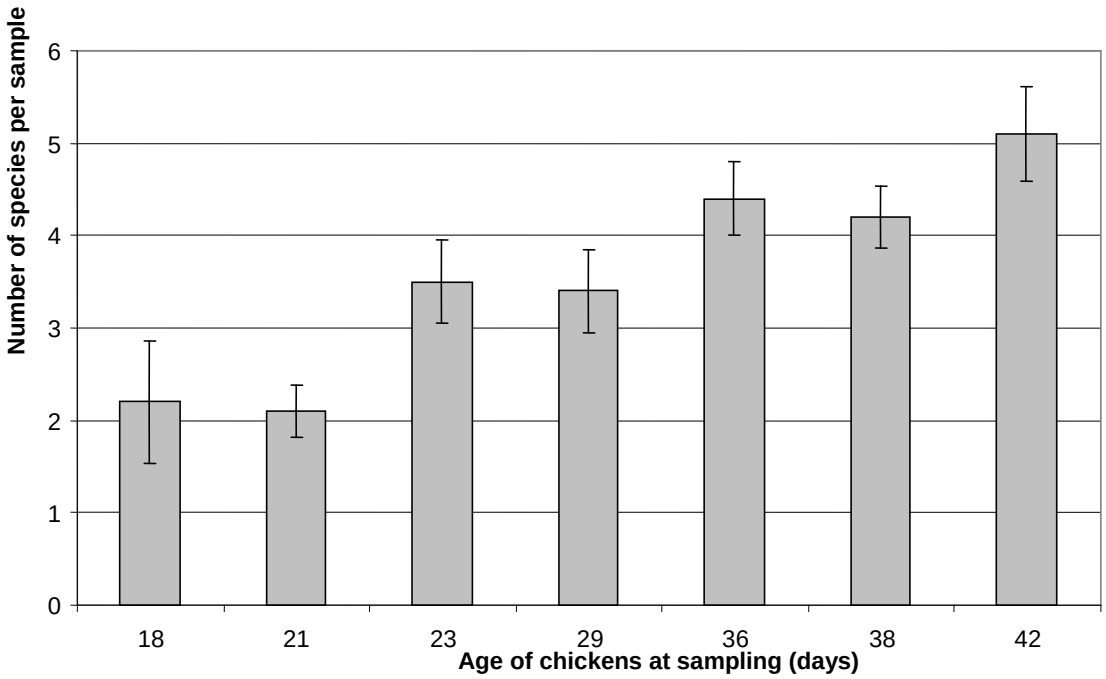


Fig. 5.2. A bar graph depicting the mean number of species (+/- standard error) detected in faecal samples using the CE approach, according to the ages of the chickens.



Implications

Biotechnological advances have been achieved in the control of coccidiosis, through the development of live vaccines and molecular-diagnostic methods. Progress in this area is of major relevance because of the serious problems with resistance in *Eimeria* populations against anti-coccidial compounds (due to their excessive, preventative use and the rapid generation times of *Eimeria*) and residue problems in chicken meat, eggs and the environment. Given these key issues, the specific diagnosis of *Eimeria* infections in chickens is clearly central to a better understanding of the epidemiology and dynamics of disease in intensive and extensive chicken establishments, which underpins the effective prevention and control of coccidiosis. The research in this project has shown clearly that PCR-based approaches utilizing genetic markers in nuclear ribosomal DNA provide rapid and powerful complementary diagnostic and/or analytical tools. In particular, the PCR-coupled CE provides a platform for high throughput and high resolution diagnosis and genetic analysis. While, in the future, the real-time PCR (using specific probes to individual species of *Eimeria*) or high resolution melt assay is likely to become less expensive than is currently the case, such that an inexpensive quantitative assay could be developed, such an assay would not have the analytical capacity of the PCR-coupled CE for the direct display of genetic variation. Currently, the CE approach is affordable to the poultry producer and industry, as it employs only a single set of primers in a standard PCR for all recognized species of *Eimeria*. Given its advantages, the CE approach is now being employed routinely to conduct epidemiological surveys and to investigate, for the first time, the abundance/intensity and dynamics of *Eimeria* infections in selected poultry establishments in Australia, thus logically complementing various prevention, vaccination and control programs. The advances made in the production and storage of vaccines also have substantial implications, but further work is required to optimise the preservation of live *Eimeria* vaccines.

Recommendations

1. The present project has made significant advances toward improved methods for the freezing of some species of *Eimeria*. The recovery of sporozoites from sporulated oocysts is clearly critical to the success of a commercially viable cryopreservation procedure. The mechanical disruption of the oocysts is considered a rate limiting step. *Other methods, such as sonication, require testing.*

2. Studies aimed at achieving replication indices of frozen sporozoites comparable to those achieved for fresh oocysts. For *E. tenella*, a species which lends itself well to cryopreservation, the replication index was 2000. This means that each oocyst used in the initial preparation of the sporozoite replicates to produce 2000 oocysts following inoculation. In contrast, *E. necatrix* was recovered after cloacal inoculation at a 10-fold lower rate. Also, *E. acervulina and E. maxima* sporozoites were successfully recovered in birds, but it was low. Thus far, *E. maxima* has proven to be the most difficult to cryopreserve. *Therefore, substantial further work is required to assess cryoprotectant formulations, freezing profiles and delivery.*

3. PCR-coupled CE provides a powerful platform for high throughput and high resolution diagnosis and genetic analysis. While the CE approach is affordable and useful, it does not allow the vaccine to be differentiated from field strains of *Eimeria*. Being able to track the vaccine (and different species within the vaccine) in chicken a chicken flock following vaccination would be a major advantage. Also, the affordability of any molecular assay could be reduced by using a PCR-coupled high resolution melt analytical method. *Future work should focus on ensuring the affordability of any molecular test and working toward a test which can "follow the vaccine" in the field.*

Therefore, an emphasis should be placed on (1) further developing a practical cryopreservation approach for all species of *Eimeria* included in the commercial vaccine and (2) developing an affordable and highly specific/sensitive molecular method for the delineation of the vaccine from field strain of *Eimeria*. It appears that these areas will be tackled in the extension to project 03-15 from July 1 2007.

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Appendices Dissemination of scientific findings (approved by CRC)

Articles published or in press in international scientific journals:

Gasser RB, Skinner R, Fadavi R, Richards DG, Morris G (2005) High throughput capillary electrophoresis for the identification and differentiation of seven species of *Eimeria* from chickens. *Electrophoresis* 26, 3479-3485.

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Gasser RB (2005) Application of molecular biology to the diagnosis of parasitic diseases: molecular tools - progress, opportunities and prospects. Guangxi University, Nanning, Guangxi Province, China (Professor Huang Weiyi) (20-21.09.05).

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25/07/07 (R1)

Plain English Compendium Summary

Project Title:	*Eimeria* vaccines and diagnostics
Poultry CRC Project No.:	03-15
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Project Overview	Focus on improved approaches for the sustainable control of coccidiosis in poultry using non-molecular and genetic approaches.
Background	Coccidiosis of poultry, caused by unicellular parasites called *Eimeria*, represents an intestinal disease of major economic importance worldwide. Currently, seven species of *Eimeria* are recognized to infect chickens, and these taxa differ considerably in their ability to cause disease. Given the problems linked to genetic resistance in the parasite against anti-coccidial drugs and restrictions of the use of these drugs in many countries, there has been an urgent need to develop new and improved methods for the prevention and control of coccidiosis.
Research	The development of substantially improved methods for the production and storage of live *Eimeria* vaccines and new and accurate diagnostic tests for coccidiosis.
Project Progress	Significant advances were made toward optimising improved methods for the freezing and storage of *Eimeria* vaccines. In addition, a highly sensitive and specific technique was developed for the diagnosis of coccidiosis (due to all species of *Eimeria*), and this approach was used to tackle outbreaks as well as gain, for the first time, accurate insights into disease processes.
Implications	The biotechnological advances made in the production and storage of vaccines and diagnosis have major implications for the prevention and control of chicken coccidiosis. These advances are central to a better understanding of the epidemiology and dynamics of disease in intensive and extensive chicken establishments and underpin effective coccidiosis control programs.
Main publications	Gasser RB, Skinner R, Fadavi R, Richards DG, Morris G (2005) *Electrophoresis* 26, 3479-3485. Gasser RB (2006) *Veterinary Parasitology*. 36, 69-89. Richards DG, Whithear KG, Scott PC, Gasser RB (2006) *Acta Parasitologica*. 51, 156-159. Morris GM, Gasser RB (2006) *Biotechnology Advances* 24, 590-603. Morris GM, Woods WG, Richards DG, Gasser RB (2007) *Parasitology Research* Submitted 090207. In Press 060307. Morris GM, Woods WG, Richards DG, Gasser RB (2007) *Molecular and Cellular Probes* In Press 090307.