# Title

# The *Eimeria*-host cell interaction in broiler chickens

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## Dedication

**To, Ali, my dear son**

**My kind son, my best friend who always gives me strength and force to tolerate everything....**

**I am so sorry; I may fulfill your wish for going back to your country, Iran, where you told me many people like you and you want to live there. I will never forget that you told me; "Dad please finish your study and backing to Iran". I tried, but failed to grant your wish,**

**It is not your fault but mine. I know a day will come when you can make the decision for yourself and you can choose your country for the rest of your life.**

**I shall never forget you and the way that you put your arm around my neck.**

**I do not know if there will come a day when we can meet each other or not, but Ali, you know that I love you forever. Wherever I will not forget you until my heart stops beating.**

**Your father**

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# Summary

Coccidiosis is an enteric infection of chickens caused by protozoan parasites of the genus *Eimeria*. Coccidiosis is a worldwide disease with an economic impact on broiler chicken production. An outbreak of disease can reduce weight gain and feed digestion in the entire flock, reducing the production of processed meat for market. The major characteristics of *Eimeria* species are the invasion of specific sites in the intestine of chickens and specificity of the immune response.

To date, prophylaxis and vaccination are used to control coccidiosis. However, the continuous use of chemotherapeutics has led to increased drug resistance by *Eimeria*. In the case of vaccination, immunity against *Eimeria* is species-specific, hence, there is a need to vaccinate chickens against all species of *Eimeria* for complete protection. The *Eimeria*-host cell interaction is the first stage in the reproductive cycle in chickens that produces the damage in the chicken intestine. A more complete understanding of the environmental factors within the intestinal tract that influence this interaction will be useful to control the disease. The lack of a suitable method to study the interaction between *Eimeria* and host cells derived from different areas of intestine has hampered our understanding of the disease. The cell type of interest for this study was the chicken enterocyte. A layer of mucus is secreted by goblet cells in the intestinal epithelial to protect the enterocytes. *Eimeria* sporozoites have to cross the mucus layer in order to invade the epithelial cells. It is reasonable to assume that this mucus may have some involvement in *Eimeria*-enterocyte attachment. The objectives of this study were to investigate the roles of the enterocyte and intestinal mucus in the attachment process and the subsequent penetration of host cells by *Eimeria* sporozoites.

Newly hatched, and 3-week-old chickens, were killed and intestinal segments were collected for developing an *in vitro* method *ex vivo* (organ culture system, isolated enterocytes and a frozen section method) to study the *Eimeria* interaction with intestinal epithelial cells. *Eimeria* sporozoites were extracted from oocysts and then labelled with a fluorescent dye (PKH-67). The frozen section model was found to be superior to the use of isolated enterocytes and organ culture systems, and was used for subsequent experiments in this project. This method was used to

investigate the *Eimeria*-enterocyte attachment at preferred and non-preferred sites on the surface of enterocyte membranes. Indeed, the use of this method demonstrated that D-galactose on the surface of sporozoites had an important role in the attachment of *E. tenella* sporozoites to caecal enterocytes, with caecal and duodenal mucus both functioning as a physical barrier to *E. tenella.* In addition, two other major developments resulted from this project, these being; the development of a PCR protocol that can specifically identify different *Eimeria* species in a mixed sample containing at least 0.05 ng/µl of *Eimeria* DNA and a propidium iodide method that is a suitable indicator tool to assess the viability of oocysts and sporocysts. Finally, the inclusion of $MgCl_2$ in the extraction buffer increases the hatchability of sporozoites from sporocysts.

In conclusion, this study led to development of a frozen section method which can be used *ex vivo* to investigate further the role of mucus from vaccinated and non-vaccinated chickens, diets with different compositions, anticoccidial drugs, and the identification of the specific receptors in different areas of the chicken intestine. Finally, the propidium iodide method in combination with the PCR protocols can be used as a quality assurance tool in the production of *Eimeria* vaccines.

# Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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# Abbreviations used in thesis

| | |
|---|---|
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometer |
| °C | Degrees centigrade |
| *C.* | *Cryptosporidium* |
| cells/ml | Cells per millilitre |
| cm | Centimetre |
| $cm^2$ | Square Centimetre |
| DE-52 | Dimethylaminoethyl cellulose |
| D-gal | D-galactose |
| DNA | Deoxyribonucleic acid |
| dNTP | 2'-deoxynucleoside 5'-triphosphate |
| *E.* | *Eimeria* |
| *E.a* | *Eimeria acervulina* |
| *E.t* | *Eimeria tenella* |
| FBS | fetal bovine serum |
| g | Gram |
| g/L | Gram per litre |
| HBSS | Hanks' Balanced Salt solution |
| IU | International unit |
| IU/ml | International units per milliliter |
| MAbs | Monoclonal antibodies |
| MDBK | Madin Darby Bovine Kidney |
| MEM | Minimum Essential Medium Eagle |
| mg/ml | Milligram per millilitre |
| ml | Millilitre |

| | |
|---|---|
| mM | Millimole |
| mm | Millimetre |
| NBF | Neutral buffered formalin |
| ng | Nanogram |
| nm | Nanometre |
| OD | Optical density |
| PCR | Polymerase chain reaction |
| PBS | Phosphate buffered saline |
| pM | Picomole |
| PI | Propidium iodide |
| RAPD | Random amplified polymorphic DNA |
| RNA | Ribonucleic acid |
| rpm | Revolutions-per-minute |
| SD | Standard deviation |
| SCAR | Sequence-characterized amplified regions |
| TAE | Tris acetate-EDTA |
| Taq | Thermostable DNA polymerase |
| Tris | Tris[hydroxymethyl]aminomethane |
| x 40 | A magnification of x 40 |
| xg | Times gravity |
| UV | Ultraviolet |
| v/v | Volume by volume |
| W/v | Weight by volume |

# CHATER 1: REVIEW OF LITERATURE

## 1.1 Introduction

Coccidiosis is an enteric disease of chickens caused by protozoan parasites of the genus *Eimeria* belonging to the phylum *Apicomplexan* (Constantinoiu *et al.*, 2004). Seven species of *Eimeria* (*Eimeria acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella*) produce disease in broiler chickens (*Gallus domesticus* L.) (Lillehoj and Lillehoj, 2000). The most prevalent *Eimeria* species in broilers are *E. tenella* and *E. acervulina* (Karim and Trees, 1990; Lobago *et al.*, 2005). The clinical signs of coccidiosis in broiler chickens are diarrhoea, ranging from mucoid and watery to hemorrhagic; reduction in weight or weight gain; and, in severe cases, mortality (Motha and Egerton, 1984; Yunus *et al.*, 2005). The major characteristics of *Eimeria* species are the invasion of specific sites in the gut of chickens, the species specificity of the immune response and the structure of the oocysts.

Coccidiosis is a worldwide disease with an economic impact on the production of broiler chickens. It occurs essentially in intensively housed poultry (Voeten *et al.*, 1988; Williams, 2006). An outbreak of disease can reduce weight gain, feed conversion and feed digestion in the entire flock (Idris, 1997). The results of an outbreak can be reduced production of processed meat for market with the end result that the broiler industry worldwide loses more than one billion US dollars a year due to coccidiosis (Jenkins *et al.*, 1997). The poultry industry spends millions of US dollars on anticoccidial drugs annually for prophylaxis of the disease. Allen (2002) reported that worldwide prevention of severe outbreaks costs about US$800 million annually.

An *Eimeria* life cycle is characterized by two stages: (1) Endogenous (asexual or schizogony and sexual) and (2) Exogenous (sporogony) (Allen and Fetterer, 2002b). The most important stage for this project is schizogony, which starts when the *Eimeria* sporozoites are released from oocysts and infect the intestinal epithelial cells (Fernando, 1990; Calnek *et al.*, 1997). The infection process of intestinal epithelial cells by sporozoites consists of sequential steps: (1) binding of sporozoites to the surface membrane of intestinal epithelial cells, and (2) invasion of sporozoites into host cells by invagination of the cell membrane. The sporozoites, which have penetrated, develop into large numbers of schizonts following nuclear division. This asexual reproduction repeats between 2 to 4 times depending species and is associated with the onset of the intestinal tissue damage. After asexual reproduction, merozoites form

microgametocytes and macrogametocytes that continue to further the intestinal damage (Williams, 1998). The intestinal damage from *Eimeria* infection occurs after the sequence of event, leading to the release of the sporozoite from oocysts. Therefore, the initial binding of *Eimeria* sporozoites to intestinal epithelial cells and their subsequent enormous reproduction are prerequisites for the onset of pathophysiological events in the disease. Thus, the determination of the factors involved in the attachment of *Eimeria* sporozoites to target cells may assist in understanding how to control the disease.

Each *Eimeria* species completes its life cycle within one host and in a specific region of that host's intestinal tract (Doran and Augustine, 1973). For example, *Eimeria acervulina* infects the duodenum, whereas *E. tenella* infects the caecum (Augustine, 2001a). In contrast, *in vitro* studies of the site and host specificity of *Eimeria* species are not clearly defined compared with the situation *in vivo*. Some *in vitro* studies show the ability of sporozoites to invade various cell cultures derived from different organs and animals. For example, *E. tenella*, like *Toxoplasma*, has been shown to invade many different cell types *in vitro* (Augustine, 2001b); however, other species demonstrate a degree of site specificity (Augustine, 2001b). Such ambiguity that arises from *in vitro* studies causes difficulties when trying to determine the mechanism involved in the attachment of *Eimeria*, particularly for determining the site specificity of the infection. This body of evidence shows that a system that exploits the *ex vivo* use of original host cells maintained in conditions similar to that observed *in vivo* is necessary to achieve a meaningful assessment of the *Eimeria*-host cell binding. Thus, the cell type of interest for this study is the epithelial cell of the chicken intestine.

The use of anticoccidial drugs has played a major role in the control and cure of coccidiosis in poultry production, but overuse of these drugs has led to the appearance of drug resistance by some species of *Eimeria*. This has become an increasing concern to production and profitability in the poultry industry (Allen and Fetterer, 2002b). Although, new anticoccidial compounds are currently effective in addressing the drug resistance of *Eimeria* species (Williams, 2006), their continued usefulness is questionable. The potential of parasites to develop tolerance and drug residues in chicken meat are the most serious limitations for anticoccidial chemoprophylaxis and treatment (Chapman, 1989; Wang *et al.*, 2006).

Anticoccidial vaccines have been developed in order to provide drug-free alternatives for coccidiosis control, especially for drug resistant *Eimeria*. Vaccines work by stimulating chicken immunity against *Eimeria* oocysts (Brake, 2002; Dalloul and Lillehoj, 2006). This immunity is species specific, and chickens vaccinated with one species of *Eimeria* remain susceptible to infection by other species and strains. In addition to species-specific immunity, the methods of administration of vaccines can affect the immunization of chickens. For example, the oral vaccination can be resulted different imunity in chickens due to obtain unlike doses of oocysts. Thus, some vaccinated chickens may exhibit signs of coccidiosis, whereas others show no immunity against disease, suggesting that an adequate immune stimulation for a given flock is questionable (Williams *et al.*, 2001). Despite these problems, vaccination is the preferred method of preventing coccidiosis and negates the problem of drug resistance ( Williams *et al.*, 2000; Rojs *et al.*, 2002). The attachment of *Eimeria* sporozoites to target cells is a key requirement in order to improve vaccination and prevention of the disease. A better understanding of the interaction can assist to increase the attachment of sporozoites to host cells for producing adequate immunity in the vaccination, or to reduce the binding for the control of the disease. Thus, the elucidation of the *Eimeria*-host cell attachment mechanism is essential for improved means of coccidiosis control.

The intestinal mucus protects the intestinal epithelium cells that are located at the interface between the intestinal contents and the absorptive system of the intestine (Fernando *et al.*, 1983; Yagi *et al.*, 1990). The mucus is variable in thickness, composition, and elasticity in different areas of chicken intestine (Smirnov *et al.*, 2005). *Eimeria* sporozoites have to cross this mucus layer successfully in order to invade the epithelial cells ( Smirnov *et al.*, 2004; Smirnov *et al.*, 2005) and may affect the *Eimeria*-enterocyte attachment. Buret *et al.* (1990) suggest that "the environment occupied by the intestinal coccidian is a complex and changing one and that it can affect not only the cells of the host that make up the environment, but the parasites that come to utilize it". Specific carbohydrate epitopes found in mucus have been previously implicated as potential receptors for *Cryptosporidium parvum* adhesion to epithelial cells ( Joe *et al.*, 1998; Joe *et al.*, 1994; Cevallos *et al.*, 2000). Based on the role of mucus in *Cryptosporidium* attachment and the *Eimeria* binding to various cell lines derived from different tissues and animals, the signals that the coccidians use to find an appropriate site to infect may be located in the complex layer of mucus.

*In vivo* studies cannot be performed to determine the function of each part of the complex intestinal environment and the reasons for the *Eimeria* sporozoite binding in a specific region of the chicken intestine. To study this attachment, an *in vitro* model is needed which is analogous to the situation of the host-parasite interaction *ex vivo*. As the *Eimeria*-host cell attachment is the first stage in chicken infection, an understanding of the environmental factors within the intestinal tract that influence this interaction will be useful to control the disease. The objectives of this study were to identify the role of enterocytes and intestinal mucus that may be relevant to the attachment and the penetration of *Eimeria* sporozoites into host cells by using an *in vitro* intestinal model. Understanding the binding factors related to the site-specific invasion by *Eimeria tenella* may lead to ways of inhibiting the disease or increasing the attachment of sporozoites for greater immunity in chickens after vaccination.

## 1.2 Life cycle of *Eimeria*

*Coccidia* of the genus *Eimeria* are members of the family *Eimeriidae*, belonging to the subphylum *Apicomplexa* (Calnek *et al.*, 1997). The various species of *Eimeria* are common protozoan parasites in chicken intestine. Seven species of *Eimeria* are recognized in the chicken: *Eimeria acervulina*, *E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. The life cycles of *Eimeria* species are homoxenous and completed in exogenous and endogenous phases (Fernando, 1990).

The endogenous phase of *Eimeria* begins, when chickens ingest sporulated oocysts. After the release of sporocysts from oocysts, sporocyst walls are digested by gastro-intestinal enzymes releasing individual sporozoites. The sporozoites invade the intestinal epithelial cells (Fernando, 1990; Allen and Fetterer, 2002a) and, following nuclear divisions, develop into schizonts. This cycle of asexual reproduction is repeated 2-4 times in different species of *Eimeria*. These are primary events in the initial *Eimeria*-host cell attachment that are prerequisites for the reproductive phases of schizonts that result in cellular damage to intestinal epithelial cells (Williams, 1998).

After several generations of asexual reproduction, merozoites form microgametocytes and macrogametocytes. Macrogametocytes are fertilized by microgametocytes to produce zygotes. The zygotes mature into unsporulated oocysts encased by thick walls. These oocysts are released from intestinal tissue and pass out with the faeces (Jeurissen *et al.*, 1996; Shirley *et al.*, 2005). The potential of *Eimeria* species for enormous reproductive capability during the endogenous phase and the related intestinal damage causes a serious problem for poultry industries, and the chickens are rendered more susceptible to other diseases.

The exogenous phase starts when the correct environmental conditions (temperature, oxygen and moisture) allow the nuclear division of unsporulated oocysts to form four sporocysts, with each sporocyst containing two sporozoites (Jeurissen *et al.*, 1996; Allen and Fetterer, 2002a). After oocyst sporulation, they become infective to chickens (Jeurissen *et al.*, 1996).

*Eimeria tenella* is a pathogenic species that has economical significance making it a subject for continuing research to understand its interaction with host cells (Karim and Trees, 1990; Lobago *et al.*, 2005). After release from the oocysts, *E. tenella* sporozoites attach and peneterate host cells as the first step of schizogony. Subsequent repetitive attachment by merozoites results in more damage to intestinal tissues. Thus, understanding attachment and penetration can lead to

better prevention of coccidiosis in chickens through the design of improved vaccines or their usage, anticoccidial drugs, and specialised diets. The life cycle of *E. tenella*, including the asexual, sexual, and sporogony phases is shown in Figure 1.1.

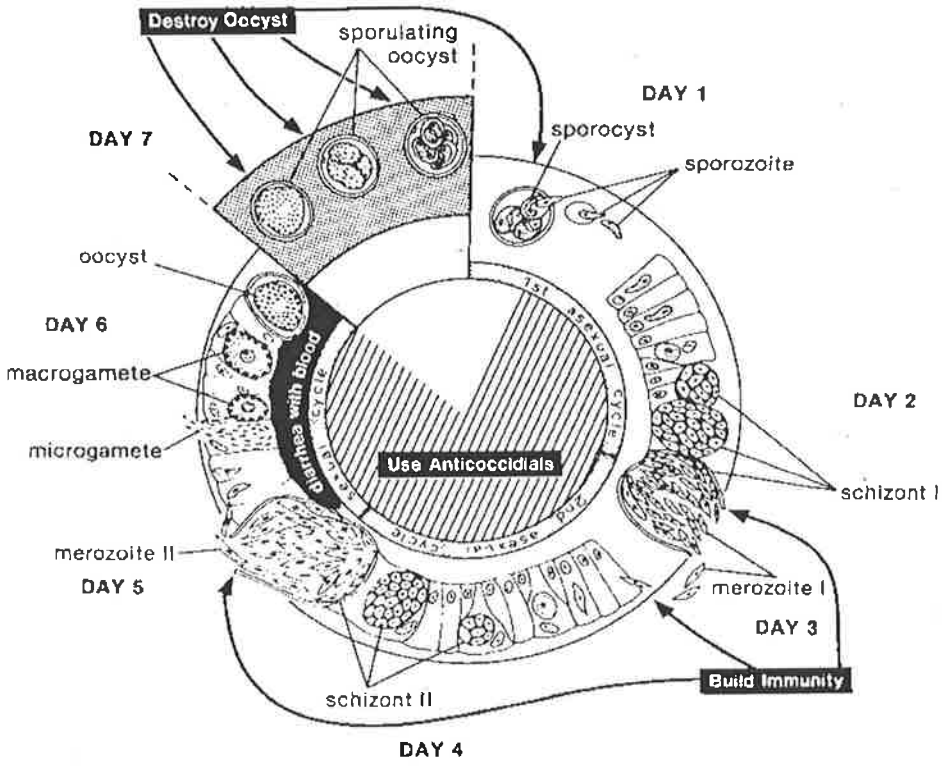


**Figure 1.1** The life cycle of *Eimeria tenella*, including the asexual, sexual and sporogony steps (Calnek *et al.*, 1997).

## 1.3 *Eimeria* pathogenicity and damage

The pathogenicity of coccidiosis is a combination of parasite or host factors (Hungerford, 1969). These factors include the innate pathogenicity of any given species of *Eimeria*, reproductive potential of the different species of *Eimeria*, numbers and age of ingested oocysts, and the susceptibility of the chickens (Fernando, 1990; Calnek *et al.*, 1997). The different species of *Eimeria* invade the intestinal epithelium and produce a certain degree of epithelial cell damage, inflammation and villous atrophy (Pout, 1967). While young chickens are more susceptible to coccidiosis and show more clinical signs of disease, older chickens are more resistant to infection

(Lillehoj, 1998). This difference indicates that changes in the intestinal environment, including composition of cell membrane and intestinal mucus may influence the binding of *Eimeria* to host cells.

The pathogenicity of the seven species of *Eimeria* are varied, with *E. tenella, E. necatrix* and *E. maxima* causing wide-spread damage, *E. acervulina* and *E. brunetti* causing moderate damage (Calnek *et al.*, 1997), and *E. mitis* and *E. praecox* causing minor damage in broiler chickens (Gore and Long, 1982; Fitz-Coy and Edgar, 1992).

Each species of *Eimeria* is strongly site-specific in the intestinal tract of chickens (Augustine, 2001a). This site-specificity causes each species damage in a specific region of the intestine (Figure 1.2) (Doran and Augustine, 1973). For example, infection with *E. tenella* usually produces indistinct lesions in chicken caecum, whereas *E. acervulina* damages the duodenum. The mechanisms involved in site specificity are not completely understood. An understanding of this mechanism can be valuable either to prevent the disease without using chemical compounds or to increase the binding of sporozoites of *Eimeria* to host cells in order to stimulate a strong immunity in chickens by vaccination.

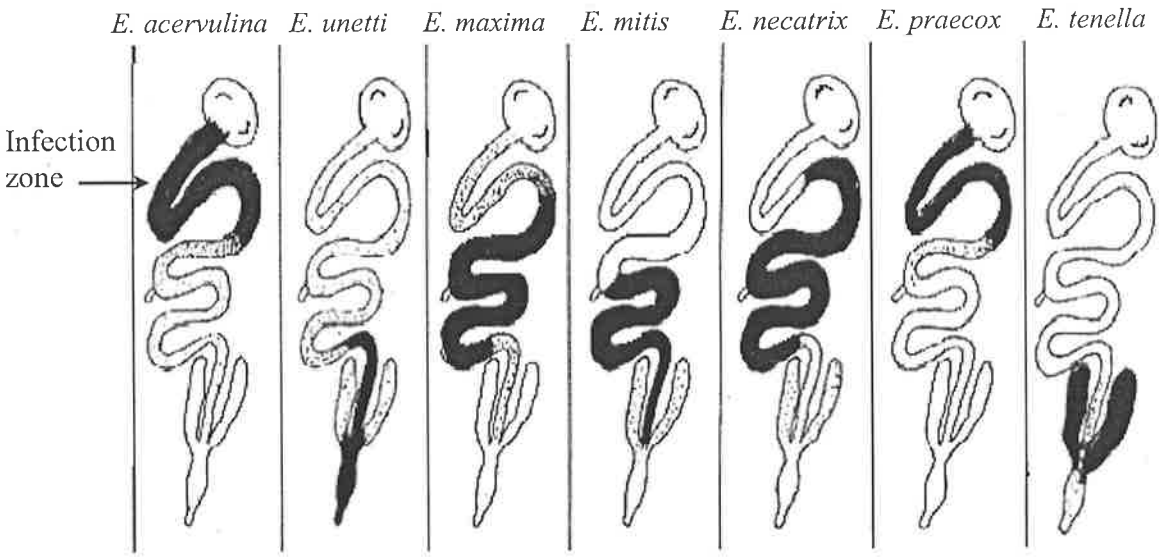


**Figure 1.2** Specific sites of binding and damage in the gastrointestinal tract of chickens when challenged with the seven species of *Eimeria* (Hungerford, 1969).

## 1.4 Immunity against coccidiosis

Immunity against *Eimeria* species is complex as a result of the multi-phase nature of the *Eimeria* life cycle, including intracellular, extracellular, asexual and sexual stages (Lillehoj and Choi, 1998). When chickens are exposed to *Eimeria* species, a number of immunological responses are stimulated to produce an overall anticoccidial effect. The cell-mediated immunity has a more important role against species of *Eimeria* than that by humoral immunity during infection and vaccination ( Wallach, 1997; Yuna *et al.*, 2000; Du *et al.*, 2005).

The immune reaction against *Eimeria* is rapid in chickens. This immunity is species specific. When chickens are infected or vaccinated with one species of *Eimeria*, they remain susceptible to infection by other species of this parasite. This lack of cross-protection between species causes a problem in the control of the disease by vaccination of chickens if only one species is used (Shirley *et al.*, 2005). Coccidiosis vaccines are composed of live *E.* oocysts and stimulate an immune response by causing a sub-clinical infection, a process that is similar to the virulent disease (Suo *et al.*, 2006). These processes show that attachment and penetration of sporozoites to host cells play an important role to stimulate the immune reaction. The determination of which factors influence the *Eimeria*-host cell attachment can lead to control measures that modify binding either positively to increase *Eimeria* sporozoite binding during vaccination or negatively to decrease *Eimeria* sporozoite binding to prevent the disease.

## 1.5 Control of coccidiosis

### 1.5.1 Prophylactic control

Anticoccidial drugs were introduced in the late 1940s (Chapman, 1993). Until recently, different anticoccidial drugs, including zoalene, diclazuril, roxarsone, sulfaquinoxaline, and amprolium have been important compounds administered in chicken feed to control coccidiosis (Chapman, 1989; Peeters *et al.*, 1994). The use of these chemoprophylaxes played a major role in the control of coccidiosis in the poultry industry in the past. For example, the activities of anticoccidials against *Eimeria tenella* showed that the drugs could control coccidiosis in chickens and improve their body weight in comparison with un-medicated controls (Ramadan *et al.*, 1997).

The mode of action of anticoccidial drugs in different stages of the life cycle of *Eimeria* is listed in Table 1.1.

**Table 1.1** The mode of action of anticoccidial drug in chickens (Vermeulen *et al.*, 2001).

| Class | Common anticoccidial drugs | The mechanism of effect |
|---|---|---|
| **Thiamine mimics** | Amprolium | Competitively inhibits active transport of thiamine in first generation schizont. |
| **Sulphonamides** | Sulfachloropyrazine<br>Sulfadimethoxine<br>Sulfamethazine<br>Sulfanitran<br>Sulfaquinoxaline | Inhibit folic acid synthesis by competitively inhibiting the enzyme that catalyzes formation of dihydropteroate from PABA more effective against second and later schizonts. |
| **Quinolones** | Decoquinate | Block DNA synthesis by inhibiting DNA gyrase in sporozoites. |
| **Ionophores** | Monensin<br>Lasolocid<br>Salinomycin<br>Narasin | These facilitates Na+ transport across the cell membrane Na+ influx, then $Cl^-$, water, cellular swelling, inhibition of mitochondria function and ATP hydrolysis in sporozoite and trophozoite. |

### 1.5.2 Vaccination

In 1965 *Coccivac,* and in 1985 *Immucox*, both live and unattentuated anticoccidial vaccines for use in poultry were introduced for prophylaxis of coccidiosis in chickens. These vaccines contained live oocysts from wild type *Eimeria* (Shirley *et al.*, 1995; Lillehoj and Lillehoj, 2000;

Dalloul and Lillehoj, 2006). Current live attenuated vaccines containing different species of *Eimeria* have been used to protect broilers from disease (Shirley *et al.*, 1995). The objective of live vaccines in chickens is to stimulate an adequate immune reaction that occurs naturally in infected birds. This immunity enables chicken to resist challenge from virulent species of *Eimeria*.

It is reported that over 2 billion doses of anticoccidial vaccine had been used in the field with no reports of adverse effects such as weight loss (Allen and Fetterer, 2002b). Currently, attenuated and non-attenuated live vaccines are used widely to prevent coccidiosis in breeder broiler and layer poultry (Williams, 2002). Vaccination is the method of choice to prevent coccidiosis, particularly for species of *Eimeria*, which are resistant to anticoccidial drugs.

#### 1.5.2.1 Attenuated vaccines

Attenuated vaccines are produced by passaging *Eimeria* sporozoites through chicken embryos, treatment with gamma irradiation, or selection for precocity (Table 1.2). These vaccines have low reproductive potential in comparison with wild species of *Eimeria*. However, they have a strong immunogenicity in chickens (Shirley *et al.*, 1995; Shirley, 1997). The use of attenuated vaccines resulted in increasing local populations of oocysts of *Eimeria* that are less pathogenic, and more drug sensitive and potentially out-competing their wild-type relatives (Chapman, 1993; Chapman *et al.*, 2002).

#### 1.5.2.2 Non-attenuated vaccines

The non-attenuated vaccines contain virulent sporulated oocysts of *Eimeria* (Table 1.2). The use of such vaccines in chickens can result in high numbers of pathogenic oocysts that contaminate the litter (Shirley and Bedrnik, 1997).

**Table 1.2** Attenuated and non-attenuated vaccines for broiler chickens exposed to *Eimeria* species ( Shirley and Bedrnik, 1997; Rojs *et al.*, 2002; Williams, 2002)

| Trade name | *Eimeria* species in vaccine | Vaccine type | Administration |
|---|---|---|---|
| **Livacox D** | *Ea, Et.* | Attenuated | Water |
| **Livacox T** | $E_a$, $E_{max}$, $E_t$. | | Water/ocular |
| **Livacox Q** | $E_a$, $E_b$, $E_{max}$, $E_t$. | | Water/ocular |
| **DM®** | $E_t$. | | |
| **Paracox®** | $E_a$, $E_b$, $E_{max} \times 2$, $E_{mit}$, $E_n$, $E_p$, $E_t$. | | Water/feed |
| **Paracox-5** | $E_a$, $E_{max} \times 2$, $E_t$, $E_{mit}$. | | Feed spray |
| **Eimeriavax® 4m** | $E_a$, $E_{max}$, *En*, $E_t$ | | Eye-drop or oral |
| **MF Cocciva®** | $E_a$, $E_b$, $E_t$ | Non- | Hatchery spray/ |
| **Cocciva-B** | $E_a$, $E_{max}$, $E_t$, $E_{miv}$. | attenuated | Water/feed |
| **Coccivac-D** | $E_a$, $E_b$, $E_{max}$, *Eh* $E_{miv}$, *En*, $E_p$, $E_t$. | | |
| **Coxine®** | $E_a$, $E_{max}$, $E_n$, $E_t$. | | |
| **Immucox -C1** | $E_a$, $E_{max}$, $E_n$, $E_t$. | | Water/oral gel |
| **Immucox C2** | $E_a$, $E_b$, $E_{max}$, $E_{mit}$, *En*, $E_p$, $E_t$. | | Water/spray |
| **Nobilis COX ATM** | $E_a$, $E_{max} \times 2$, $E_t$. | | Hatchery spray/water, |
| **NObiCox®** | $E_a$, $E_{max}$, $E_n$, $E_t$. | | Feed |
| **VAC M®** | $E_{max}$. | | Water/oral gel |
| **Supercox (R)** | $E_a$, $E_{max}$, $E_t$. | | Water/oral gel |

$E_{max} \times 2$ = two strains of *E. maxima*, *Ea* = *E. acervulina*, $E_b$ = *E. brunetti*, $E_{max}$ = *E. maxima*, $E_{mit}$ = *E. mitis*, $E_{miv}$ = *E. mivati*, *En* = *E. necatrix*, *Ep* = *E. praecox*, $E_t$ = *E. tenella*

### 1.5.3 Problems of coccidiosis control

Until the advance of oocyst vaccine, different anticoccidial drugs were used to control coccidiosis. However, the misuse of these drugs, including inadequate length of consumption and incorrect concentrations of these substances has led to resistant *Eimeria* species (Allen and Fetterer, 2002b).

In the study of effective anticoccidial drugs against coccidiosis in broiler chickens, isolated *E. tenella* from medicated chickens could infect the chickens treated with twice dose of salinomycin, narasin, maduramicin and lasalocid (Zhu and McDougald, 1992). In addition, *E.* species resistances to monensin, narasin, salinomycin, and maduramicin were observed in samples collected from broilers (Peeters *et al.*, 1994). In a similar study, the sensitivity of *Eimeria acervulina, E. maxima* and *E. tenella* to anticoccidial drugs, maxiban, maduramicin, monensin, salinomycin, robenidine and lasalocid showed that *E. acervulina* was resistant to maduramicin, *E. maxima* was sensitive only to robenidine, and *E. tenella* was resistant to all of these anticoccidial drugs (Naciri *et al.*, 2003). This evidence shows that there are serious concerns about the effect of anticoccidial drugs in different species of *Eimeria* for the chemoprophylaxci control of coccidiosis in chickens.

Chemical modification of anticoccidial compounds can prevent drug resistance in species of *Eimeria*. These strategies have had suitable results in the past. However, the continued usefulness of this approach is questionable. Until now, drug tolerance of parasites is the most serious limitation for anticoccidial treatment (McDougald *et al.*, 1996). New drugs are being developed and some have been introduced into the market. These could overcome the problem of resistance in the short-term. However the high cost of developing such drug is reflected in their price and, given the low financial margins in the poultry industry, may limit their widespread adoption. It seems likely that medium to long-term use of new drugs will only produce additional drug resistance.

*Eimeria* vaccines are given to the chickens in the water, feed or sprayed into the chickens' eyes soon after hatch (Chapman, 1996; Williams, 2002). The dose of oocysts given to the chicken plays an important role in stimulation of immunity against coccidiosis. There are serious concerns that even the use of correct methods of administration result in varied doses being given to different chickens, resulting in different levels of immunity, leading to inconsistent responses in poultry. That is, some chickens exhibit some signs of coccidiosis, whereas others show no immunity against disease and remain susceptible to coccidiosis. If coccidiosis occurs in vaccinated chickens, the assumption that birds have been vaccinated against the disease can make diagnosis difficult. As well as the method of administration of vaccines, the viability of sporozoites inside the oocysts is another factor that affects the immune response in chickens. The present *in vitro*

method to determine the viability of oocysts is the extraction of sporozoites. Although simpler and quicker than bioassays in chickens, such extraction methods have technical problems that limit their usefulness. The development of a fast and simple method to determine the change of the oocyst viability and quality control of vaccines to ensure no release of the sporozoites would be a major step forward for both the overall quality of vaccines and epidemiological studies of coccidiosis.

## 1.6 Intestinal barrier

The intestinal barrier is a large interface between the lymph-blood system and external environment in the digestive tract that protects the body against pathogenic micro-organisms and toxic compounds. The intestinal barrier comprises an unstirred water layer, mucus, glycocalyx and the intestinal epithelial cells (Crissinger *et al.*, 1990; Cunningham, 1997).

Epithelial cells are the main part of a continuous physical barrier to protect the internal body against the entry of pathogens (Hoerr, 2001). This is both a physical and physiological barrier, and represents part of the innate immunological defences (Kasper and Buzoni-Gatel, 2001). These cells play an important function in the life cycle and infection of poultry by *Eimeria* species. The binding of *Eimeria* sporozoites to intestinal epithelial cells is the onset of coccidiosis. In addition, this binding is an important step in stimulation of the immune reaction in chicken. Determining the function of epithelial cells in *Eimeria*-host cell binding might lead to alternative strategies, including dietary and probiotic approaches, to control the disease without chemoprophylaxis.

The intestinal mucus is secreted by goblet cells that are located between the intestinal enterocytes. This layer covers the surface of the intestinal epithelial cells. At the brush border surface of enterocytes, the mucus contains 95% water and 5% mucins. The mucin includes a protein core that is 20% by dry weight, with the remaining 80% of the mucin being carbohydrate. The oligosaccharide units of mucus are bound to N-acetylgalactosamine and diagnosis radiate out like the bristles of a bottle-brush (Cunningham, 1997). The dominant sugars of mucus are N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid (Cunningham, 1997). The structural components of the intestinal barrier system are shown in Figure 1.3.

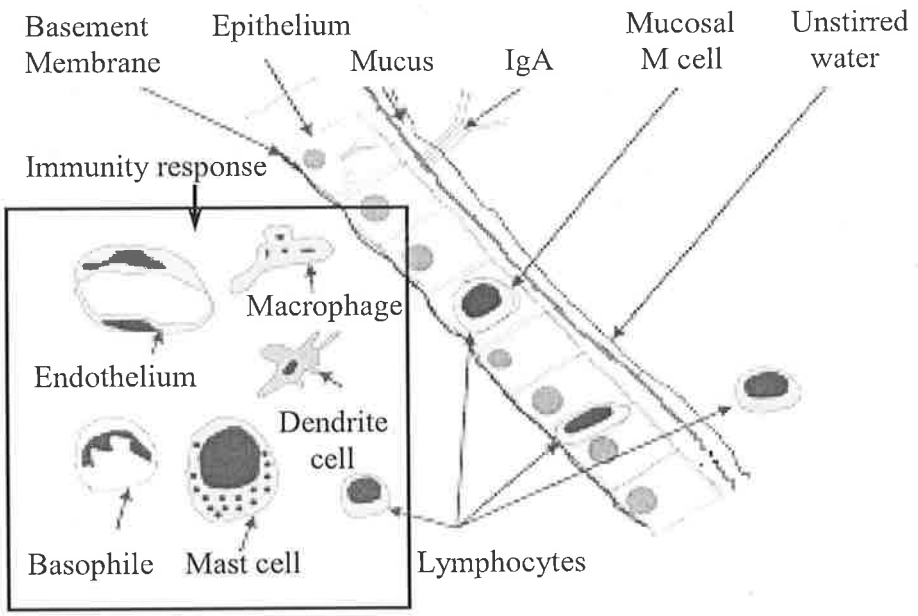


**Figure 1.3** Structural components of the intestinal barrier Structural components of the intestinal barrier include epithelium cells, mucus, and unstirred water layer (Farhadi *et al.*, 2003).

The intestinal barrier has two vital functions. The first is to act as a filter with selective permeability that moves nutrients from the intestinal lumen into the internal environment. This is achieved through both a physical diffusion barrier and carrier-mediated transporters of the regulated physiological barrier (Soderholm and Perdue, 2001). The second is an immunological barrier to prevent the entry of harmful micro-organisms, including bacteria and protozoa, into the body (Farhadi *et al.*, 2003).

There are other factors in the intestinal tract that can function as non-specific defence. They are gastric secretion, including lysozyme and bile salts, commensal microbial flora, and endogenous cationic peptides acting as defensins that have anti-microbial functions (Zhao *et al.*, 2001). Nitric oxide plays a crucial role in mucosal integrity and barrier function (Cunningham, 1997; Wallace and Miller, 2000).

The mucus is the first line of protection against pathogenic invasion by bacteria or parasites. For bacteria to interact with the host cells they first need to pass through the mucus layer. This situation may be also true for pathogenic protozoan organisms such as in the *Apicomplexa* (Fioramonti *et al.*, 2003). Any change in the mucus content and structure will

compromise the mucosal defence barrier function. Therefore, the ability to bind to and degrade mucus is considered to be one of the important indicators of the pathogenicity of bacteria (Fioramonti *et al.*, 2003). Specific carbohydrate epitopes found in mucins have been previously implicated as potential receptors for *Cryptosporidium parvum* adhesion to epithelial cells (Joe, 1994, 1998; Cevallos, 2000). Given that the composition of mucus changes along the intestinal tract, with age and in response to diet (Sharma *et al.*, 1997; Smirnov *et al.*, 2004), such variations in the complex layer of mucus that coats the intestinal epithelium may be the signals that coccidia use to find an appropriate site to infect.

## 1.7 The invasion process of *Eimeria*

Studies of *Eimeria* sporozoite attachment *in vivo* and *in vitro* showed that different molecules of sporozoites and enterocytes are involved in the interaction and attachment process (Blackman and Bannister, 2001; Labbe *et al.*, 2005). Thus, interaction studies are needed to determine which interacting factors of the enterocyte and the *E.* sporozoite play an important role for the identification and adhesion to host cells.

An early contact between sporozoites and recognized host cells must trigger a recognition event that starts the entire process, which is sequential according to Augustine (2001a). Invasion of the host-cell is initiated by contact between the apex of the parasite and the cell surface. This is directly followed by progressive internalization at the site of contact, proceeding from anterior to posterior, ultimately closing the vacuole behind the parasite (Labbe *et al.*, 2005). The entry stage of the sporozoites of *Eimeria* usually takes about 5-10 seconds (Saffer *et al.*, 1992; El Hajj *et al.*, 2006).

As invasion is fully driven by the parasite, it is completely dependent on the gliding motility of the sporozoite. This motility is known to be dependent on actin and myosin present in the pellicle (Heintzelman and Schwartzman, 1997). Inhibition of the sporozoite motility can block the invasion process (Schubert *et al.*, 2005).

### 1.7.1 *Eimeria* sporozoite motility

The study of *Eimeria* sporozoites by Russell and Sinden (1981) showed that *Eimeria tenella* and *E. acervulina* sporozoites move by gliding when they are in contact with the substratum. The cell membrane produces a cap, which is shed from the posterior of the sporozoite

during motility. This capping reaction takes place only during sporozoite locomotion. The mechanism of adhering to the host cell is by the use of surface ligands. These ligands or substratum complex are capped along the fixed spiral of the sporozoite body by a microfilament-based contractile system (Russell and Sinden, 1981).

Gliding movement causes sporozoites of *Eimeria* to move quickly on a solid substrate. This kind of movement causes the rapid migration and invasion of cells. Sporozoites, when placed near cultured cells, become active and move rapidly to penetrate host cells. *Eimeria* sporozoites can move at rates of 1 to 10μm per second (Upton and Tilley, 1992). These speeds of sporozoite motility are compatible with the observed invasion time of 5 seconds as well as the movement between epithelial cells, with the latter movement being between up to 10 cells within 3 minutes without the sporozoites returning to the lumen (Chobotar *et al.*, 1993).

The level of sporozoite motility and their ability to find binding sites on the surface of host cells is directly related to their viability. Thus, the sporozoite viability, and hence their ability to move, is the first limiting factor affecting the reliability and reproducibility of binding assays. As such, sporozoite viability needs to be assessed in each assay to avoid error.

### 1.7.2 The role of carbohydrates in *Eimeria* attachment

Glycoconjugates may have a role as potential host cell receptors for invasion. It has been proposed, that the attachment is mediated by lectin–ligand binding, which is a common feature for many different protozoan and host cell types. Strout *et al.* (1994) indicated that lectins were part of the biochemical make-up of *E. tenella, E. acervulina* and *E. maxima*. The lectin of each *Eimeria* species had a different sugar affinity and was found on the surface of sporozoites. Furthermore, there were marked correlations among the sporozoite lectins, the carbohydrate moieties of the intestinal cell surface, and the area of the intestine that was invaded by each species. Compelling arguments supporting the proposal that lectins aid in the site-selection by the *Eimeria,* including (1) the hemagglutination reaction produced by the *E. tenella* lectin was neutralised by fucose which is found in large quantities in the caecum; (2) fetuin, which also inhibited erythrocyte agglutination by *E. tenella* lectin, significantly inhibited invasion of cultured cells by *E. tenella* sporozoites (Strout *et al.*, 1994).

Augustine (1985) showed that several lectins bound to the surface of cultured primary turkey cells, but except for wheat germ agglutinin which is a lectin that binds N-acetylglucosamine,

all failed to inhibit invasion by several species of *Eimeria*. The action of wheat germ agglutinin was subsequently ascribed to binding of the lectin to anionic moieties of the host cell rather than to its specific sugar ligand, N-acetyl glucosamine. There are marked differences in the distribution of carbohydrate residues on the luminal surface of the intestine and caecal epithelium of chickens (Pohlmeyer *et al.* 2005). In addition, membrane carbohydrates may play a role in site specificity for invasion by *Eimeria* sporozoites similar to their role previously reported for the attachment of *Cryptosporidium* (Joe *et al.*, 1994; Joe *et al.*, 1998; Chen and LaRusso, 2000; Hashim *et al.*, 2006), and *Entamoeba* sporozoites (Adler *et al.*, 1995; Yi *et al.*, 1998; Boettner *et al.*, 2002) to host cells.

### 1.7.3 The role of *Eimeria* micronemes in invasion

Micronemes are secreted by *Apicomplexan* sporozoites in order to link with and invade host cells. There are similar microneme proteins in *Apicomplexan* families, including *Toxoplasma gondii*, *Plasmodium* species, and *Eimeria tenella*. It has been shown that microneme antigen was present on the surface of cells more than 60 minutes post-infection in *Sarcocystis muris* (Entzeroth *et al.*,1998). Similar study of *Toxoplasma* showed that a microneme protein found in *T. gondii*, contained a duplicated receptor-like domain that allocated to the host cell surface (Fourmaux *et al.*, 1996).

Micronemes have roles in host-cell recognition, binding, and possibly motility (Dubremetz *et al.*, 1998). In addition, it has been suggested that *Toxoplasma gondii* microneme protein plays an essential role in the invasion of host cells by direct host-parasite interactions (Bumstead *et al.*, 2000). This kind of attachment was produced by mediation through extra-cellular matrix proteins (Bumstead and Tomley, 2000). Host cells infected by *Toxoplasma gondii* showed that the invasion process would require multiple ligand-receptor for the interaction (Wan *et al.*, 1997). In another family of *Apicompelexa*, a particular protein plays an important role in connection and invasion of host cells by *Neospora caninum* (Lovett *et al.*, 2000).

In the genus *Eimeria,* micronemes have a similar role to that of other A*picomplexan* genera. It has been reported that the *E. tenella* micronemes support the attachment of sporozoites to host cells by recognition, binding, and motility (Kawazoe *et al.*, 1992; Tomley *et al.*, 1996). The investigated sequences of some microneme proteins of *E. tenella* are very similar to those of other *Apicomplexan* species, implying that a common function of these proteins is in the process of attachment (Entzeroth *et al.*, 1998).

## 1.8 Site specificity of *Eimeria in vivo*

*Eimeria* sporozoites have a high degree of host and site specificity during invasion and infection in chickens (Calnek *et al.*, 1997). This site specificity was observed when *E.* tenella sporozoites were inoculated intraperitoneally in chickens (Long and Millard, 1976). When foreign hosts are infected with other species of *Eimeria*, the site specificity is maintained when compared with the natural host (Table 1.3).

**Table 1.3** The site specificity of species of *Eimeria* in chicken and turkey hosts (Augustine and Danforth, 1990).

| ***Eimeria* species** | **Natural host** | **Secific site** | **Site specificity in** | |
|---|---|---|---|---|
| | | | Chicken | turkey |
| *E. tenella* | Chicken | Ceacum | + | + |
| *E. adenoids* | Turkey | Ceacum | + | + |
| *E. acervulina* | Chicken | Duodenum | + | + |
| *E. meleagrimitis* | Turkey | Duodenum | + | + |

The mechanism of site-specificity of each species of *Eimeria* is still unknown. However, Augustine (2001a) suggested some possibilities for the cause of the site-specificity. The first possibility is that differences in the times required for the release of sporozoite from the oocysts of the various species caused the site specificity of *Eimeria*. According to this hypothesis reported by Doran and Farr (1962), *E. tenella* sporozoites would require the longest time for release in order to invade the lowest sites in the intestinal chickens in comparison with *E. acervulina* sporozoites that invade the upper site of chicken intestine. By contrast, study of the distribution of oocysts and sporocysts of *E. tenella* and *E. maxima* in the digestive tract of chicken showed that a large number of *E. tenella* sporozoites were detected in the chicken caecum before sporozoites of *E. maxima* were observed in the duodenum. These results showed that differences in the time of sporozoite release may not be the reason for the site specificity of *Eimeria* species (Shiotani *et al.*, 1992).

The second possibility for site-specificity involves properties of the membrane glycoconjugate, mucus and cell metabolites (Augustine, 2001a). These factors may cause sporozoites of *Eimeria* to be attracted to specific sites of chicken intestine. Two studies support the

involvement of cell metabolic products in the site specificity of the invasion process. In the first study, it has been proposed that the release of sporozoite molecules to be involved in the attachment of *E. tenella* to host cells was induced by conditioned medium from uninfected MDBK cell cultures. The release of these molecules was induced by the binding of host cell molecules to the surface of the parasite, presumably via specific receptors (Tomley, 1997). In the second study, conditioned medium from cultures of the intestinal and caecal cells of both chickens and turkeys enhanced invasion of cells by sporozoites of turkey *Eimeria*. However, similar enhancement was not realized with conditioned medium from cultures of several other avian and mammalian primary and immortalised cells (Augustine and Jenkins, 1998). Thus, soluble products of host cells may be acting upon the cell itself, the sporozoite, or both, increasing either the attraction between the participants, or the permissiveness of the cells for invasion. There is no mention of chemotactic attraction in either experiment.

The last possibility, suggested by Augustine (2001b), proposes that a special receptor or molecule is important for recognition and attachment to the host cell surface by *Eimeria* sporozoites.

### 1.8.1 Host cell receptor

*Eimeria* sporozoites can attach to different types of cells in *in vitro* culture (Augustine, 2001b). Kidney cell culture is a common method to study the *Eimeria*-host cell interaction. It has been reported that kidney cells from chickens, guinea fowl, partridges, pheasants, quail, and turkeys were utilized to study *E. tenella* invasion (Doran and Augustine, 1973). In addition, *E. tenella* and *E. adenoeides* sporozoites significantly invade baby hamster kidney cells and chicken caecal cells more readily than primary cultures of kidneys cells derived from either chickens or turkeys (Augustine, 1994). The invasion of varying cell types by *Eimeria* sporozoites *in vitro* is most likely an artifact of cell culture, because *Eimeria* species have been shown to invade only a specific host cell type and site *in vivo*.

Attachment between sporozoites of *Eimeria* and enterocytes may require a special epitope or receptor. The study of molecular interactions of cultured turkey kidney cells with *E. adenoeides* sporozoites showed that the antigens of *E. adenoeides* sporozoites bound to primary cultured turkey kidney cells (Augustine, 1989). Indeed, Augustine (1991) demonstrated that monoclonal antibodies (MAbs) against these antigens derived from sporozoites of *E. adenoeides* could block

the invasion of sporozoites into host cells. Vervelde *et al.* (1993) reported that common epitopes were present on both the surface membranes of *E. tenella* sporozoites and their target site (caecal epithelium) as demonstrated by a monoclonal antibody. In a similar study, Uchida *et al.* (1997) showed that monoclonal antibodies against *E. tenella* sporozoites reacted with a polypeptide band of *Eimeria* sporozoite antigens and these MAbs prevented *Eimeria* invasion of the host cells. A similar phenomenon was observed by using MAbs against refractile body antigens of *E. acervulina*, *E. tenella* and *E. adenoeides* sporozoites. Therefore, it is clear that there are molecules on the surfaces of *Eimeria* sporozoites and cultured cell lines that are important for the interaction and invasion, some of which are unique to either the host or parasite whereas others may be common to both sides.

Collectively, these data indicate that the presence of a host cell receptor or recognition site on the surface of a host cell membrane determines site specificity for *Eimeria* invasion. However, whether these putative receptor molecules are the cause of site recognition and invasion *in vivo* is still to be resolved. To overcome this gap, an experimental system that maintains the *in vivo*, organisation of the tissue, which can be rigorously controlled, as is the case with cell culture systems, is needed. The development of such a method, which does not compromise the membrane composition, can assist to detect spatial distribution of the receptors and their role in the binding of sporozoites to host cells of the chicken intestine. Development of a system and its application to determine the relative importance of environmental factors that influence the binding of *Eimeria* to a host is a major focus of the work reported in this thesis.

# CHAPTER 2: GENERAL MATERIALS AND METHODS

## 2.1 Introduction

This chapter describes the common methods and materials for preparing tissue samples, *Eimeria* oocysts, sporocysts and sporozoites that are utilized in experiments reported in subsequent chapters.

## 2.2 Biological materials

### 2.2.1 Hatching and killing chickens

Newly hatched chickens (Cobb 500 broiler breed) were purchased from a commercial hatchery (Baiada Hatchery, Willaston, South Australia). The chickens were maintained in floor pens for a maximum of 12 hours at 35°C in a temperature-regulated room until used. Water was offered *ad libitum* to the chickens.

Tissues from three-week-old chickens were prepared from birds purchased for a related project investigating the metabolic activity of chicken enterocytes. These chickens were not vaccinated against *Eimeria* and were fed broiler starter crumbles purchased from Ridley AgriProducts Ltd (Australia).

### 2.2.2 Collection of tissues

For each experiment, newly hatched or three-week-old chickens were killed by cervical dislocation. The intestine was completely removed and put into ice-cold phosphate buffered saline (PBS, pH 7.2, Sigma, Australia). The duodenum from the gizzard outlet to the ligament of Treitz and the whole caecum beyond the ileo-caecal junction were isolated. The duodenum or caecum was excised and opened longitudinally. Specimens were flushed with ice-cold PBS to remove intestinal contents. Cleaned tissue samples were then used for organ cultures, isolation of epithelial cells or preparation of frozen tissue blocks.

### 2.2.3 Preparation of frozen segments

Tissue segments of intestine were obtained from the chicken intestine (section 2.2.2). The segments were then embedded in Tissue-Tek Compound (ProSciTech, Australia) and snap-frozen in liquid nitrogen as described by Korhonen *et al.* (1990) and Edelman *et al.* (2003). Frozen samples were stored at –80°C until used.

## 2.3 Histology

This section describes all the necessary steps for the processing of tissue for the preparation of either paraffin embedded or frozen tissue sections.

### 2.3.1 Fixation

In this study, tissues collected for each binding assay (section 4.2) were fixed to protect the structural integrity from autolysis by inactivating lysosomal enzymes and inhibiting the growth of bacteria and fungi according to the method of Liu and Giambrone (1997), and Yu *et al.* (2004). Samples were fixed by 10% neutral buffered formalin (NBF) in the range of 20 volumes of fixative to one volume tissue over 24 hours for normal histology.

### 2.3.2 Preparation of samples for paraffin imbedding

Fixed tissues as described in section 2.3.1 were placed in 70% ethanol for 12 hours. A 3 mm thick tissue was cut and placed in a tissue cassette (Bayer Diagnostics, Australia). The tissues were processed for 16.5 hours in an automatic tissue processor (Shandon, Pittsburgh, USA). Processing started with serial dehydrations with different dilutions of ethanol (Sigma, Australia), including 70, 90 and 100%. The dehydration was completed by placing the samples in histolene (Bayer Diagnostics, Australia). Samples were transferred into paraffin wax at 58°C twice each one for 2 hours. Each individual sample was placed in an embedding mold and paraffin wax at 58°C was added to create the tissue block. After solidifying the wax, blocks were removed from molds and stored at 4°C until used

Sections (5μm) from each tissue block were cut on a Leitz microtome (Ernst, Leitz Westlar GmBH, Austria) and placed in a 50°C water bath. Three sections were mounted from each tissue block on a glass slide and the section orientation was examined under a light microscope (Olympus BH-2, Japan).

### 2.3.3 Frozen sectioning

Frozen segments (section 2.2.3) were placed in a Leica cryostat (Cambridge Instruments, GmBH, Germany) at –22°C for one hour prior to sectioning as described by Edelman *et al.* (2002 and 2003). A section (5-14 μm, depending on experimental requirements) was cut from each tissue

block and placed on a glass slide. The orientation of each section was examined under a light microscope (Olympus BH-2, Japan).

### 2.3.4 Deparaffination of sections

The paraffin wax was removed from sections by putting them into a 60°C incubator for 10 minutes after which they were immediately immersed in histolene (Bayer Diagnostics, Australia) for 15 minutes. Sections were then re-hydrated before staining using serial washes in 100%, 80%, 30% ethanol and distilled water each for 2 minutes.

### 2.3.5 Staining methods

This section describes all necessary procedures for the staining of tissue sections.

#### 2.3.5.1 Hematoxylin and eosin staining

The prepared sections (sections 2.3.2 and 2.3.4) were rinsed with distilled water for 2 minutes and placed in Mayer's hematoxylin (Sigma, Australia) for 5 minutes. The sections were subsequently dipped for 10 seconds in 70% ethanol containing 1% HCl and washed for 10 minutes under running top water. The slides were immersed in 1% eosin (Sigma, Australia) for 30 seconds and subsequently washed with 80% ethanol for 20 seconds. Sections were dehydrated in absolute ethanol for 2 minutes and put in histoline for 5 minutes. They were mounted in DePex medium (Bayer Diagnostics, Australia) and coverred with a coverslip.

#### 2.3.5.2 Periodic-acid-Schiff staining

The tissue sections (sections 2.3.2 and 2.3.4) were stained using the method described by Bancroft *et al.* (1984) and Tierney *et al.* (2004). The sections were rinsed with distilled water for 2 minutes and oxidized in 1% (v/v) periodic acid solution (Sigma, Australia) for 5 minutes. Slides were washed under running tap water for 5 minutes, and bathed in Schiff reagent (Sigma, Australia) for 15 minutes. Sections were placed in tap water for 10 minutes, subsequently stained with Mayer's hematoxylin solution (Sigma, Australia) for 5 minutes, and rinsed under tap water for 5 minutes. Sections were differentiated with sulfurous acid for 2 minutes. This procedure was repeated twice. These were then washed with running water for 5 minutes and dehydrated in

absolute ethanol for 2 minutes. Sections were put in histoline for 5 minutes and then mounted in DePex medium (Bayer Diagnostics, Australia) and covered with a coverslip.

#### 2.3.5.3 Periodic-acid-Schiff and aniline blue-orange G staining

The tissue sections (sections 2.3.2 and 2.3.4) were stained with the method reported by Hofmann and Raether (1990). Sections were rinsed in tap water for 2 minutes and placed in a 1% (v/v) periodic acid solution (Sigma, Australia) for 10 minutes. Slides were washed in distilled water for 2 minutes and placed in Schiff reagent (Sigma, Australia) for 10 minutes. They were placed twice in a 5% solution of sodium disulphide for 3 minutes and washed in tap water for 10 minutes. Slides were counterstained with a solution containing 0.5% aniline blue and 2% orange G (sigma, Australia) for 5 minutes. Sections were rinsed in distilled water and dehydrated in absolute ethanol for 5 minutes. They were placed in histolene (Sigma, Australia) for 10 minutes, mounted with DePex (Bayer Diagnostics, Australia), and covered with a coverslip

## 2.4 *Eimeria* oocysts

*Eimeria tenella* and *E. acervulina* oocysts were provided by *Eimeria* Pty Ltd (Australia).

### 2.4.1 Purification of oocysts

*Eimeria* oocysts were purified using the method described by Tomley (1997). Oocysts were put in a 50 ml centrifuge tube and centrifuged at 350 x g for 10 minutes. The supernatant was discarded and oocysts were re-suspended in a saturated sodium chloride solution (Sigma, Australia) to within 4 cm of the top of the tube and then overlaid with distilled water. The tube was centrifuged at 400 x g for 10 minutes. Purified oocysts accumulated at the interface between the saturated sodium chloride solution and distilled water. Oocysts were washed twice with distilled water and resuspended in 5ml of PBS and stored at 4°C for a maximum of 1 hour.

The purified oocysts were cleaned of bacteria contamination as described by Wagenbach *et al.* (1966). Oocysts were put in 5% sodium hypochlorite for 20 minutes at room temperature. They were washed twice with distilled water, and stored in PBS.

### 2.4.2 Counting oocysts

The number of purified oocysts in each suspension was counted three times with a haemocytometer under a light microscope (Olympus BH-2, Japan). The mean number of oocysts was calculated for each experiment.

### 2.4.3 Determination of sporulated oocysts

A drop of buffer containing oocysts was placed on a glass slide and examined using light microscopy at a magnification of 40 x. The percentage of sporulated oocysts was determined by examination of oocyst morphology as described by Tomley (1997). Oocysts containing four sporocysts were counted as sporulated oocysts, and oocysts without visible sporocysts were counted as unsporulated oocysts.

### 2.4.4 Preparation of sporocysts

Purified oocysts (section 2.4.1) were put in a 50 ml centrifuge tube and centrifuged at 350 x g for 10 minutes. The supernatant was gently removed with a sterile pipette. Oocysts were then broken using a variety of methods as described by Tomley (1997) and Augustine *et al.* (1999).

### 2.4.5 Preparation of sporozoites

The following procedures were used to prepare pure *Eimeria* sporozoites from sporocysts.

#### 2.4.5.1 Extraction of sporozoites

*Eimeria* sporocysts were incubated in 10 ml of PBS (pH 7.2, Sigma, Australia) containing 0.25% (w/v) porcine trypsin (Sigma, Australia) and 1% (w/v) taurodeoxycholinic sodium salt (Sigma, Australia) in a 50 ml centrifuge tube. The suspension was mixed by gentle inversion and incubated in a 41°C water bath (Adelab, Australia). The incubation was continued until most sporozoites have been released as considered by microscopic examination.

#### 2.4.5.2 Purification of sporozoites

A combination filter was made using both nylon wool (Sigma, Australia) and preswollen microgranular anion dimethylaminoethyl cellulose (DE-52, Sigma, Australia) to purify sporozoites from debris as per the protocol described by Schmatz *et al.* (1984).

#### 2.4.5.2.1 DE-52 cellulose

Two grams of dimethylaminoethyl cellulose (DE-52, Sigma, Australia) was suspended in 100 ml buffer containing 2 parts 2xPBS (26.96 g/L $NaHPO_4$; 1.5 g/L $NaH_2PO_4$; 8.5 g/L NaCl; 20 g D-glucose) plus 6 parts distilled water adjusted to pH 8 using 5% orthophosphoric acid (Sigma, Australia). After the sedimentation of the cellulose, the supernatant containing the debris was discarded. This washing was repeated twice with the above buffer (pH 8) (Schmatz *et al.*, 1984; Hofmann and Raether, 1990). The cellulose was then transferred to 50 ml buffer (pH 8) described above.

#### 2.4.5.2.2 Preparation of DE-52 and nylon wool column

A 10 ml pipette was filled to a height of 2 cm with nylon wool. The pipette was wetted with PBS and loaded with 15 ml of the sterile cellulose suspension to make a 3 cm height of dimethylaminoethyl cellulose above the nylon wool and was autoclaved at 110°C (0.7 kilogram/centimetre$^2$) for 10 minutes (Schmatz *et al.*, 1984). The column was washed with PBS containing 1% glucose for a maximum of 10 minutes before use.

### 2.4.5.3 Identification of the sporozoite viability

Propidium iodide (PI, Molecular Probes, USA) was used to determine the viability of *Eimeria* sporozoites (Fuller and McDougald, 2001). A dilution of 5 mg PI in 100 ml of PBS was prepared. The sporozoite viability was identified by adding 10 µl of PI solution to 90 µl of sporozoites in a 0.5 ml Eppendorf tube (Fuller *et al.*, 1995). The tube was gently mixed and incubated for 5 minutes at 20°C. A drop of the suspension was placed on a glass slide and examined under fluorescent microscopy (Olympus BX 60, Japan) by an U-MWG filter exciting at 510-550 nm and emitting at 590 nm. The viability of *Eimeria* sporozoites was determined with the method described by Khater *et al.* (2004). Positive PI-stained nuclei (red nuclei) were counted as nonviable cells, and the negative PI-stained nucleus was scored as a viable sporozoite. In each batch isolated for experimentation, one hundred sporozoites were counted to determine the ratio of viable to non-viable sporozoites. The viable and nonviable *Eimeria* sporozoites are shown in Plate 2.1.

### 2.4.5.4 Staining *Eimeria* sporozoites

*Eimeria* sporozoites were stained with a PKH-67 kit (Sigma, Australia) according to the method of Fuller and McDougald (2001). A maximum 1 x $10^{7}$ sporozoites in 100 µl PBS (Sigma, pH 7.2, Australia) was placed in a 2 ml Eppendorf tube and centrifuged at 650 x g for 5 minutes. The supernatant was discarded and 1 ml of diluent C, provided in the kit (Sigma, Australia), was added to sporozoites. A further 1 ml aliquot of diluent C (Sigma, Australia) that contained 4 x $10^{-6}$ M PKH67 GL dye was added to sporozoites and incubated for 4 minutes at 25°C. The tubes were periodically inverted to obtain consistent staining of sporozoites.

To stop the staining reaction, an equal volume of heat-inactivated fetal bovine serum (FBS, Sigma, Australia) was added and incubated in a 25°C water bath (Adelab, Australia) for 1 minute. Sporozoites were centrifuged at 650 x g for 10 minutes and washed three times with Minimum Essential Medium (MEM, pH 7.2, Sigma, Australia). The sporozoites were examined under fluorescence microscopy with an U-MWB filter exciting at 450-480 nm and emitting at 515 nm. The stained sporozoites are shown in Plate 2.1.

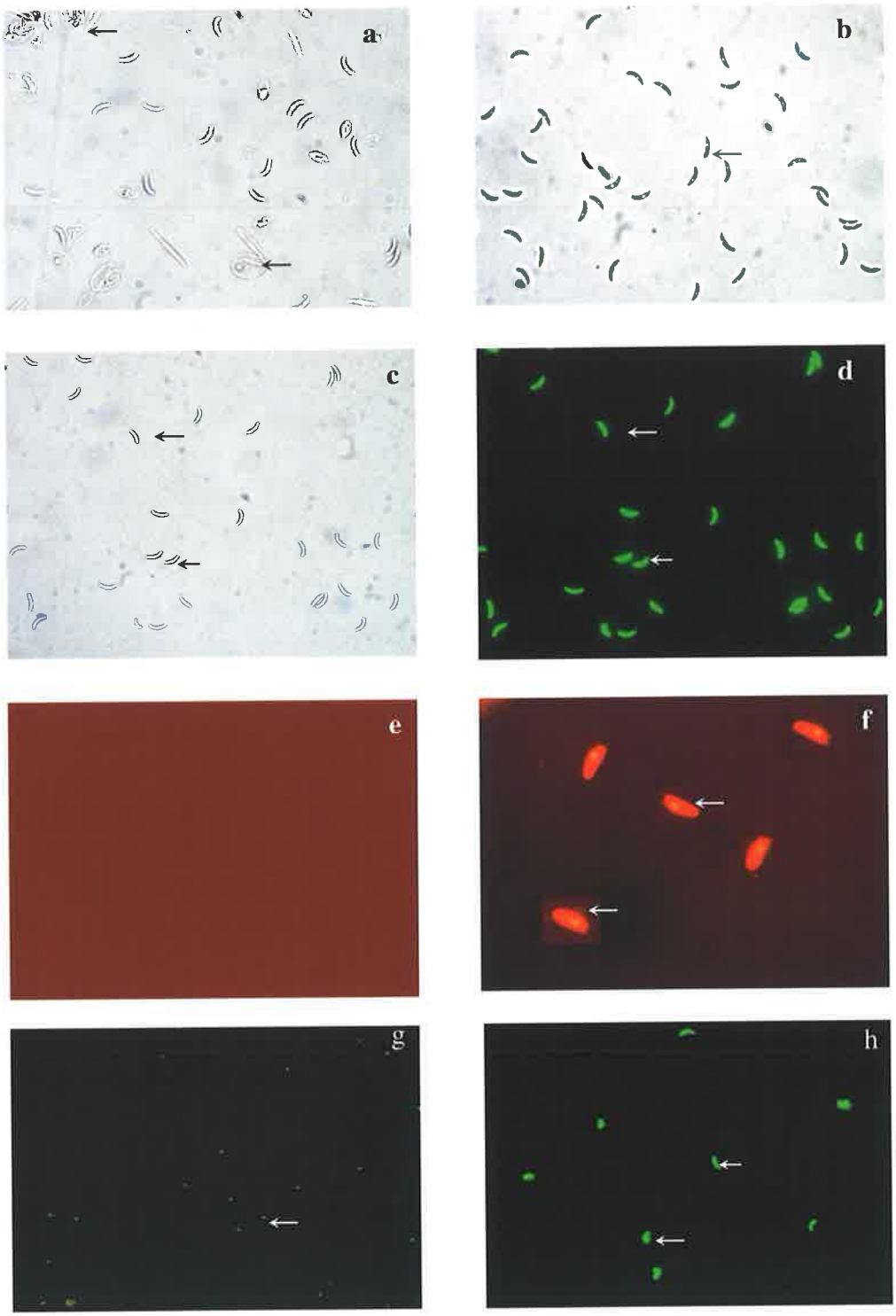


**Plate 2.1** *Eimeria* sporozoites extracted from sporocysts and staining with PKH-67 fluorescent dye. a) The released *E. tenella* sporozoites from sporocysts (arrow indicates cell wall debris), bright field view b) The purified sporozoites (arrow) by filtration, c) Stained *E. tenella* sporozoites with PKH-67 (arrow) bright field view, d) Stained *E. tenella* sporozoites with PKH-67 (arrow) under fluorescent light, e) The identification of viable sporozoites using the PI fluorescent dye method (no staining of sporozoites indicating viable cells), f) Non-viable sporozoites identified by propidium iodide (arrows), g and h) Stained *E. acervulina* sporozoites with PKH-67 with magnification of x20 and x40, respectively.

# CHAPTER 3: DEVELOPMENT OF METHODS FOR PREPARING PARASITES

# 3 Introduction

The experimental procedures described in this chapter are new methodologies or significant modifications of existing methods. The first studies were to establish new protocols to identify viable *Eimeria* oocysts and sporocysts. The final studies were conducted to develop procedures to release sporozoites and sporocysts from oocysts. These methods produced optimal conditions for the high quality sporozoites that were required to perform *in vitro* studies on *Eimeria*-host cell interactions.

## 3.1 Determination of viable sporulated oocysts

### 3.1.1 Introduction

The ability to determine oocyst viability is essential for the production of suitable sporozoites for *in vitro* interaction studies or live oocysts for chicken vaccination. Propidium iodide is a nucleic acid fluorescent dye usually used as a counter stain in multicolor fluorescent techniques. When the dye is bound to nucleic acids, its fluorescence is enhanced by 20–30 fold (Unal Cevik and Dalkara, 2003).

Propidium iodide is utilized routinely to estimate the cell viability in *in vitro* studies. In addition, this dye was used to determine the viability of *Cryptosporidium parvum* oocysts and *Giardia muris* cysts based on the inclusion or exclusion of dye observed using microscopy (Adams *et al.*, 1994; Khater *et al.*, 2004).

The cell walls of *Eimeria* oocysts and sporocysts have important roles in protecting sporozoites from harmful compounds during the exogenous phase of their life cycles (Bedrnik, 1968; Long, 1970; Tomley, 1997). Damage to the oocyst cell wall results in the rapid death of enclosed sporozoites (Ajayi, 1976; Ryley and Ryley, 1978). If this occurs, PI can pass through the oocyst cell walls and stain the DNA of the sporozoites. To my knowledge, there are no published studies, which describe a protocol to assess the viability of *Eimeria* oocysts before they are broken open. The objective of the study reported here was to evaluate the potential of PI to determine sporozoite viability inside intac oocysts.

### 3.1.2 Method

#### 3.1.2.1 Staining oocysts to assess viability

A dilution of 500 µg PI (Molecular Probes, USA) per ml PBS (pH 7.2 Sigma, Australia) was prepared (section 2.4.5.3). A 10 µl aliquot of this PI solution was added to 90 µl PBS containing purified oocysts (section 2.4.1) in a 0.5 ml Eppendorf tube. The suspension was mixed and incubated for 10 minutes at room temperature (19-21°C).

#### 3.1.2.2 Determination of *Eimeria* oocyst viability

A drop of the above suspension was placed in a heamocytometer. The oocyst morphology was examined to determine the percentage of sporulated oocysts (section 2.4.3). Oocysts were killed by freezing twice in liquid nitrogen to demonstrate the effectiveness of PI in staining nonviable sporozoites inside the oocysts and used as a positive control. The viability of oocysts was determined under a fluorescent microscope (Olympus BX-60, Japan) fitted with a red green filter block, wavelength 488 nm and 536 nm. For each viability test five runs, each with four replicates, were performed.

### 3.1.3 Results

Two types of oocysts could be observed under fluorescent microscopy after staining with PI: a) Oocysts with nuclei that are not stained were classified as viable oocysts (Plate 3.1). b) Oocysts with positive PI-stained nuclei (red) were scored as being nonviable (Plate 3.1) (Khater *et al.*, 2004). All oocysts without the PI staining were considered viable when compared with the positive control of killed oocysts (data not shown).

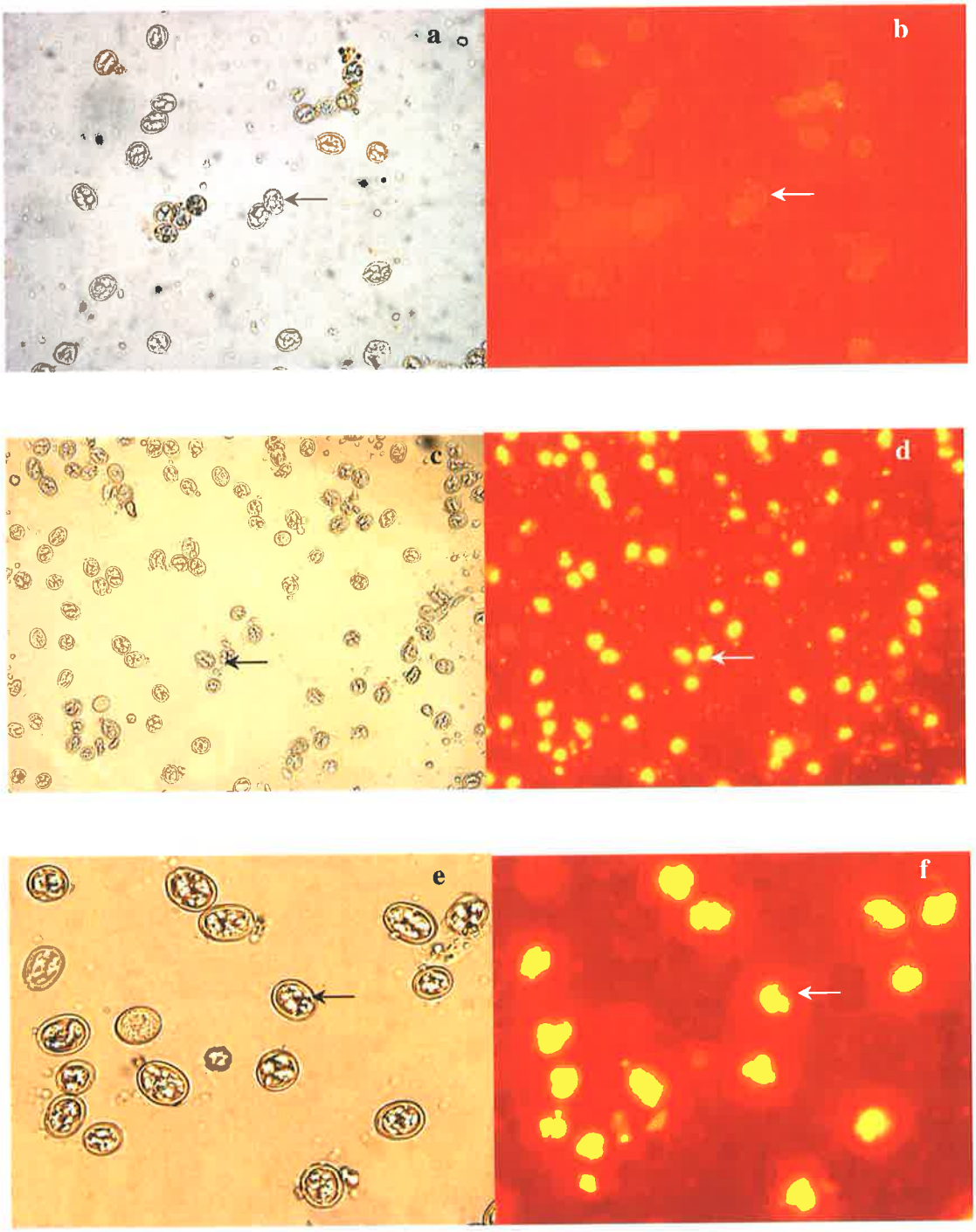


**Plate 3.1** Detection of *Eimeria tenella* oocyst viability using the propidium iodide method. a) Oocysts stained with PI (bright field at x 20), b) Viable oocysts (arrow) did not stain with PI (fluorescent microscope image), c) Oocysts killed by a freeze thaw process (repeated twice) and stained with PI (bright field), d) Represents the fluorescent image of oocysts shown in plate (c) The non-viable oocysts were labeled with the PI (arrow). Plates e and f are digital enlargements of plates c and d, respectively. These enlargements were performed to demonstrate the structure of oocysts after the freeze-thaw procedure.

### 3.1.4 Discussion

This finding (section 3.1.3) demonstrates that the exclusion of propidium iodide after staining oocysts is a possible indicator tool to determine the viability of *Eimeria* sporozoites inside oocysts *in vitro*. The main impediment to the use of PI is the integrity of oocyst cell walls (which is important for the long-term protection of the enclosed sporozoites). The difference between the PI method and the traditional methods of establishing viability, reported by Long (1970) and Tomley (1997), is that the test using PI involves intact *Eimeria* oocysts and hence lower cost in both time and reagents. Similar results have been obtained using the PI method for *Cryptosporidium* oocysts and *Giardia* cysts: reported by Schupp and Erlandsen (1987), Schupp *et al.* (1988), Smith and Smith (1989), Adams *et al.* (1994), and Khater *et al.* (2004).

## 3.2 Preparation of *Eimeria* sporocysts

### 3.2.1 Introduction

*Eimeria* sporocysts can be released from oocysts using a variety of mechanical methods (Hofmann and Raether, 1990; Michalski *et al.*, 1994; Tomley, 1997). Sporocyst preparations have been used for studying *Eimeria* interaction, *Eimeria* life cycle and chicken embryo vaccination. A percentage of sporocysts are lost during these mechanical procedures due to their rupture and disintegration. However, there are few articles (Hofmann and Raether, 1990; Michalski *et al.*, 1994; Tomley, 1997) published about the evaluation of sporocyst extraction methods. The objective of the present experiment was to determine the most efficient method for releasing *Eimeria* sporocysts.

### 3.2.2 Methods

#### 3.2.2.1 Purification of oocysts

The viability of purified oocysts (section 2.4.1) was determined (sections 3.1.2.2 and 3.1.2.3). Oocysts were suspended in PBS, placed in a 50 ml sterile tube and centrifuged at 400 x g for 10 minutes. The supernatant was discarded, and the oocysts were suspended in 2 ml PBS for preparing sporocysts with different mechanical procedures.

#### 3.2.2.2 Preparation of sporocysts by sonication

A 2 ml aliquot of oocysts in PBS was intermittently sonicated for 3 x 10 second intervals using a MSE Soniprep (Jepson Bolton and Co Ltd, Australia) set to 80% power output. Samples were maintained in an ice-bath. Sonication was continued for two additional 5-second bursts with samples collected after each burst. Samples were assessed for oocyst breakage in a haemocytometer and counted under a light microscope (Olympus BH-2, Japan) to obtain the ratio between intact oocysts and the released sporocysts (Michalski *et al.*, 1994).

#### 3.2.2.3 Preparation of sporocysts using glass beads with three breakage methods

A 2 ml aliquot of glass beads (1mm diameter, Sigma, Australia) was added to 2 ml of buffer containing oocysts in a 50 ml tube. Oocysts were broken in the solution using three methods: a) Vortex, b) Wrist shaker (Schmatz *et al.*, 1986), c) Micro tube shaker. A drop of treated solution was removed with a sterile pipette at different times and placed in a haemocytometer. The numbers of intact oocysts and separated sporocysts were counted under a light microscope (Olympus BH-2, Japan) at x 40. For each method three repeats, each with three replicates, were performed.

#### 3.2.2.4 Statistical analysis

The analysis of data was performed using the program S-plus 6.2 (Insightful Inc., New York City, USA) with SAMM (Spatial Analysis of Mixed Models). All comparisons between runs were tested with Wald tests (Cox and Hinkley, 1974).

### 3.2.3 Results

#### 3.2.3.1 Sonication method

After the onset of sonication, the numbers of *Eimeria* sporocysts released from oocysts increased with increasing time up to 40 seconds (Figure 3.1a). The mean percentage (± SE) of extracted sporocysts was 78.89 ± 7.3 and the maximum yield of this procedure was 3.15 sporocysts per oocyst.

### 3.2.3.2 Breaking oocysts using a vortex shaker

Yields of sporocysts increased with time up to 40 seconds of mechanical rupturing with a vortex (Figure 3.1b). The mean percentage (± SE) of extracted sporocysts was 44.8 ± 6.08 and the highest yield of this procedure was 1.8 intact sporocysts per oocyst.

### 3.2.3.3 Breaking oocysts using a wrist shaker

The mean numbers of released sporocysts obtained, and intact oocysts remaining, after using a wrist shaker initially increased with time (Figure 3.1.c). The maximum yield of sporocysts was gained after two minutes, and the yield then reduced with continued shaking. The mean percentage of extracted sporocysts was 33.4 ± 12.6 and the maximum yield was 1.16 ± 0.17 *Eimeria* sporocysts per oocyst.

### 3.2.3.4 Breaking oocysts using a micro-tube shaker

The mean numbers of released sporocysts obtained and intact oocysts remaining after using a micro-tube shaker increased only slightly with time (Figure 3.1.d). The mean percentage of extracted sporocysts was 13.3 ± 4.1 and this procedure produced only 0.52 ± 0.06 sporocysts per oocyst.

The results obtained with each method were compared, and the P-values for the comparisons confirmed that there were significant differences ($P < 0.01$) between them. The most efficient method of obtaining the maximum number of sporocysts was sonication, whereas the least efficient method was the micro-tube shaker method.

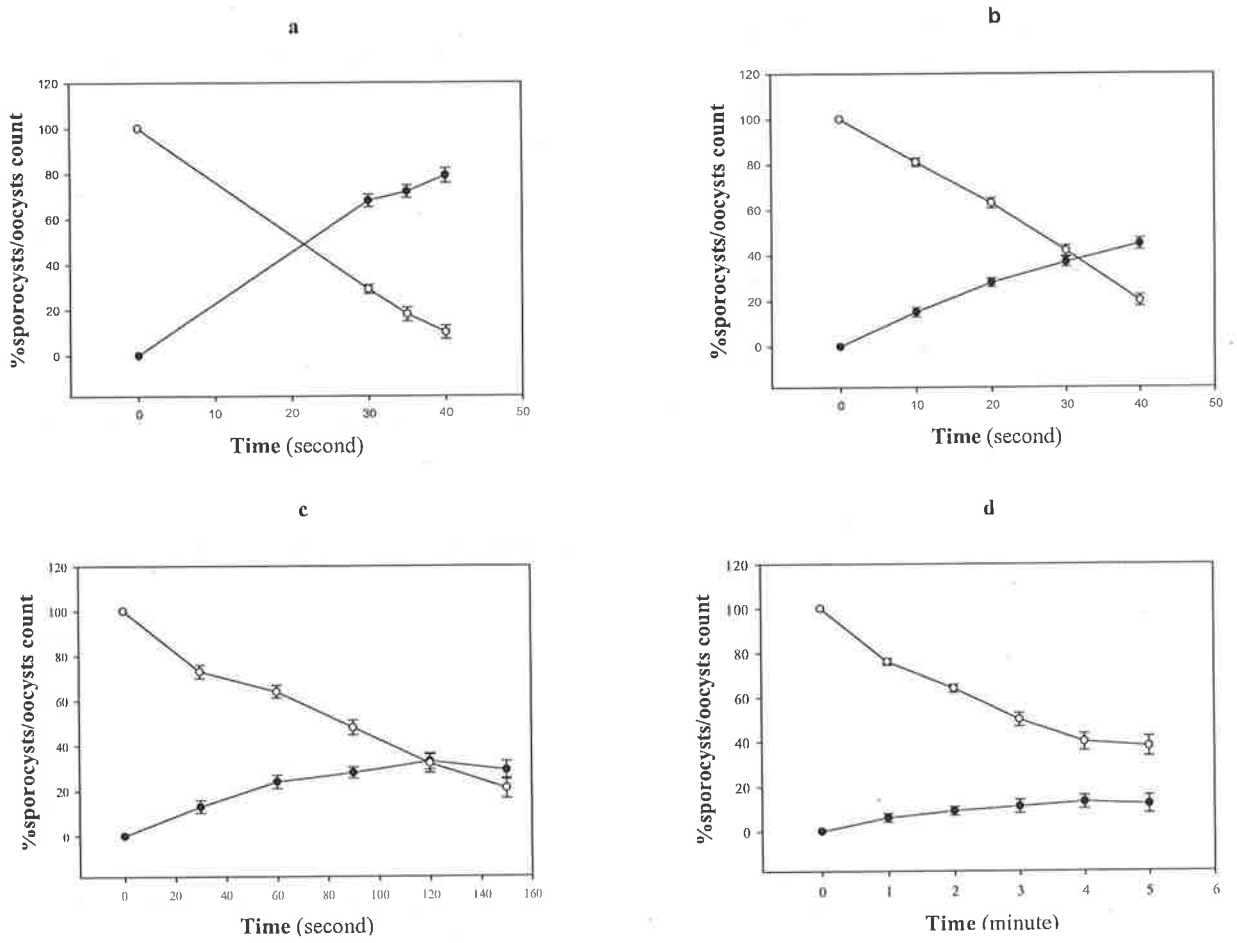


**Figure 3.1** The mean percentage of released sporocysts (●) and intact oocysts (○) using different procedures and times. a) Sonication after 0-40 seconds, b) Using 1mm glass beads with a vortex after 0-40 seconds, c) Using 1mm glass beads with a wrist shaker after 0-150 seconds, d) Using 1mm glass beads with a micro-tube shaker after 1-5 minutes.

### 3.2.4 Discussion

The results obtained (section 3.1.3) clearly showed that the sonication and vortex shaker procedures yielded higher numbers of sporocysts than those using the wrist shaker and micro-tube shaker procedures. The yields of sporocysts with sonication and vortex procedures reported here were greater by 2 and 7.5 percentage points than those reported by Long *et al.* (1978). Importantly, this result reported here showed that sporocysts were released in a shorter time than those reported by Long *et al.* (1978): for example, sonication of 40 seconds compared with 2 minutes.

## 3.3 The viability of *Eimeria* sporocysts

### 3.3.1 Introduction

The extraction method can greatly affect the yield of sporozoites obtained from sporocysts. The routine method for identifying sporocyst viability through releasing *Eimeria* sporozoites is costly in both time and reagents. However, the PI method of identifying oocyst viability can also be applied to determine sporocyst viability and therefore remove the need to release sporozoites. The objective of the experiments reported here was to determine the viability of sporozoites using PI before releasing them from sporocyst.

### 3.3.2 Materials and Methods

#### 3.3.2.1 Preparation *Eimeria* sporocysts

Purified *E. tenella* oocysts (section 2.4.1) were used to prepare sporocysts using the vortex procedure (section 3.2.2.3). *Eimeria* sporocysts were stored using three methods: a) Sporocysts were put in MEM containing 10% fetal calf serum (Sigma, Australia) and 8% dimethyl-sulfoxide (DMSO, Sigma, Australia) and then frozen in liquid nitrogen. b) Sporocysts were centrifuged at 400 x g, the supernatant was discarded and dry sporocysts stored at 4°C for 4 weeks. c) Sporocysts were stored in PBS at 4°C for 4 weeks. For each protocol three repeats, each with three replicates, were performed.

#### 3.3.2.2 Identification of viability

A dilution of 500 µg PI per ml in PBS was prepared (section 2.4.5.3). A 10 µl aliquot of this PI solution was added to 90 µl of PBS containing sporocysts in a 0.5 ml Eppendorf tube. The suspension was mixed and incubated for 10 minutes at room temperature (20°C). Sporocysts were killed by freezing twice in liquid nitrogen as a negative control. The numbers of viable and nonviable sporocysts were counted in a similar fashion to oocysts (section 3.1).

#### 3.3.2.3 Statistical analysis

The analysis of the data was performed as described in section 3.2.2.4.

### 3.3.4 Results

The viability of sporocysts stored in liquid nitrogen, with and without PBS buffer is shown (Figure 3.2) as mean percentages (± SD) of live sporocysts compared with the negative control of killed sporozoites enclosed in sporocysts. Sporocyst viabilities for control, stored in, liquid nitrogen, dry storage and PBS were respectively 1.3 ± 1.5, 95 ± 3.1, 18.3 ± 6.8 and 94.6 ± 2.3.

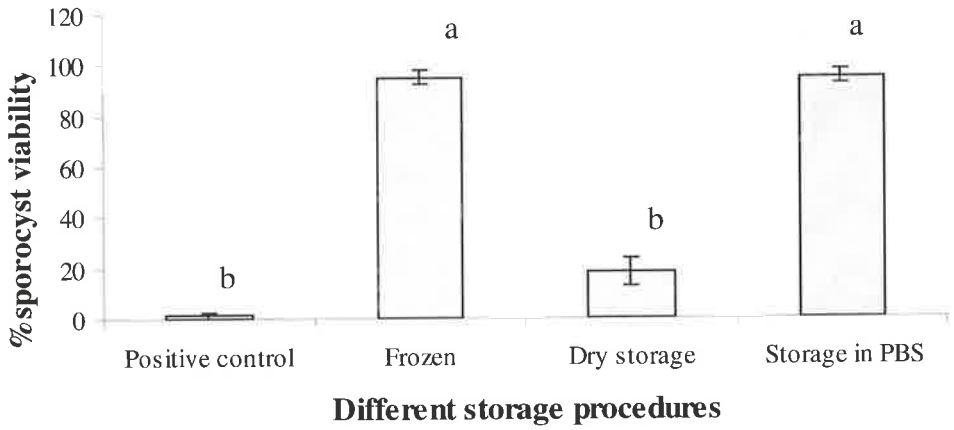


**Figure 3.1** The mean percentage of the viability of *Eimeria* sporocysts stored with and without PBS buffer at 4°C and frozen in liquid nitrogen by the PI method. Mean values with unlike superscripts (a, b, c) are significantly different between storage procedures.

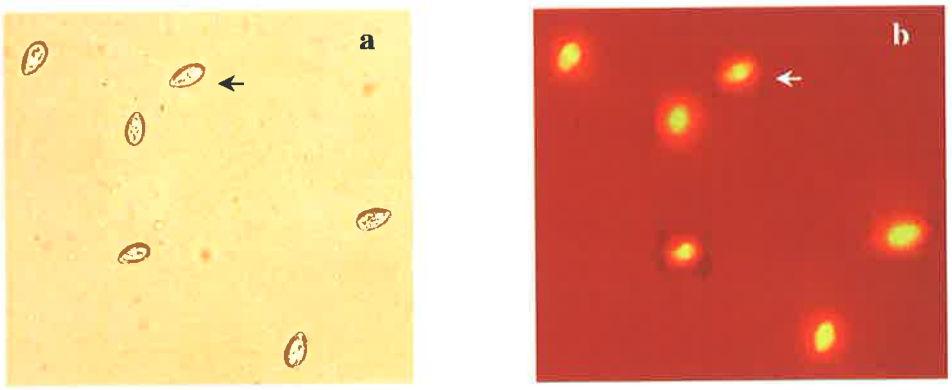


**Plate 3.2** The assessment of *Eimeria* sporocyst viability by the propidium iodide method. a) A light microscopy view of stained sporocysts, b) Non-viable sporocysts viewed under fluorescent microscopy. Arrows indicate sporocysts.

### 3.3.4 Discussion

Data reported here showed that proidium iodide was a sensitive and simple method to identify the viability of *Eimeria* sporocysts when compared with the method described by Hungerford *et al.* (1969). This result and that for oocyst viability (section 3.1) supported the hupothesis that exclusion of PI is a suitable procedure for determination of *Eimeria* viability at all stages of extraction.

## 3.4 Extraction of *Eimeria tenella* sporozoites

### 3.4.1 Introduction

The extraction of *E. tenella* sporozoites has been previously reported by Hofmann and Raether (1990) and Tomley (1997). These methods involve the incubation of sporocysts with trypsin enzyme and different combinations of bile salts at 41°C. The main difference between these methods is the buffer system used for extraction of the sporozoites. However, there are no published reports on the effects of buffers on the release of sporozoites. The objective of the experiment reported here was to determine the best buffer system to give the maximum yield of sporozoites.

#### 3.4.2.1 Preparation of sporocysts

Purified *E. tenella* oocysts (section 2.4.1) were used to prepare sporocysts using the vortex procedure (section 3.2.2.3).

#### 3.4.2.2 The incubation buffers

*Eimeria* sporozoites were released from the sporocysts using three buffer systems: 1) Hanks' Balanced Salt solution (HBSS, Sigma, Australia) containing 0.14 g/L $CaCl_2$ and 0.1 g/L $MgSO_4$, 2) PBS with 0.2 g/L $MgCl_2$ and (Sigma Australia), 3) PBS without $Mg^{2+}$ and $Ca^{2+}$ ions.

#### 3.4.2.3 The extraction of *Eimeria* sporozoites

Sporocysts were incubated in 20 ml of each buffer system, each of which also contained 0.25% (w/v) porcine trypsin (Sigma, Australia) and 1% (w/v) taurodeoxycholinic sodium salt (Sigma, Australia). The suspension was mixed 10 times by gentle inversion and incubated in a

41°C water bath for up to 120 minutes. The sporocyst suspension was gently mixed by inversion every 10 minutes and 20 µl of solution containing sporocysts was removed with a sterile pipette and placed in a haemocytometer. The numbers of intact sporocysts and released sporozoites were counted under a light microscope (Olympus BH-2, Japan). The incubation of sporocysts was continued until 75-80% of sporozoites had been released. The sporozoite extraction was stopped by adding 10 ml of the relevant buffer containing 1% FBS without enzyme and bile salt.

Sporozoites were isolated from incubation buffer by centrifugation (650 x g) for 10 minutes. The supernatant was discarded, and sporozoites were washed three times to eliminate enzyme and bile salt. The sporozoites were stored in 2 ml buffer containing 50 IU/ml penicillin (Sigma, Australia) and 50 mg/ml streptomycin (Sigma, Australia) at 4°C for a maximum of 1 hour (Tomley, 1997).

As the number of initial sporocysts varied for each replicate, the percentage of released sporozoites was determined. This was calculated from the number of sporozoites in each sample at a given time interval divided by two times sporocysts in the sample at time zero. The latter is necessary to adjust for the number of sporozoites per sporocyst. For each buffer system three repeats, each with three replicates, were performed.

#### 3.4.2.4 Statistical analysis

The data analysis was performed using S-plus 6.2 as described in (section 3.2.2.4).

### 3.4.3 Results

Yields of *Eimeria tenella* sporozoites released for the three buffer systems showed mean percentages (± SD) of released sporozoites as 88 ± 4.8 (65 minutes), 85 ± 4.1 (65 minutes), and 71 ± 4.7 (120 minutes) after incubation in 1) HBSS, 2) PBS with $MgCl_2$, and 3) PBS without $Mg^{2+}$ and $Ca^{2+}$ ions, respectively (Figure 3.3).

The results obtained with each method were compared; the data analysis showed that there was no significant difference between the mean proportions of sporozoites obtained from the buffer solution containing 1) HBSS compared with 2) PBS with $MgCl_2$. In contrast, there was a significant ($P < 0.01$) increase in the proportion of sporozoites obtained with a buffer system containing either 1) HBSS or 2) PBS with $MgCl_2$ in comparison to a buffer solution containing 3) PBS without $Mg^{2+}$ and $Ca^{2+}$ (Figure 3.3).

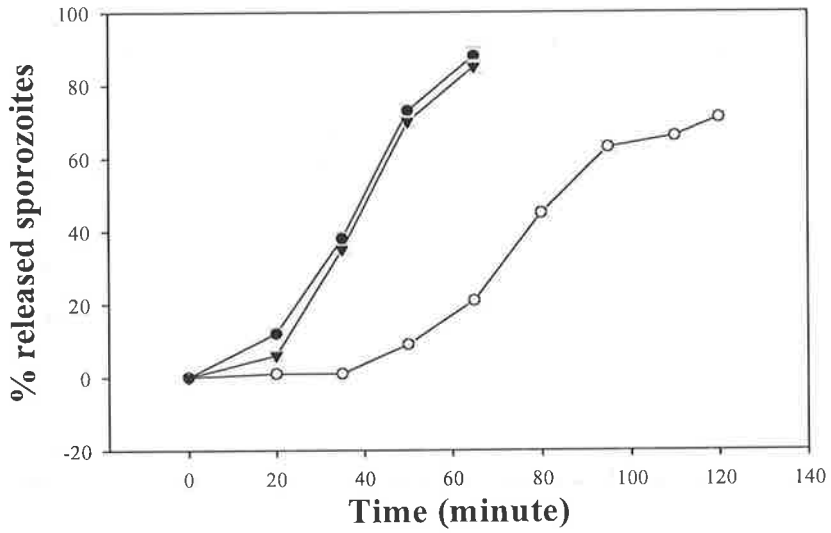


**Figure 3.3** The mean number of extracted *Eimeria tenella* sporozoites after incubation 1) HBSS (●), 2) PBS with $MgCl_2$ (►), 3) PBS without $Mg^{2+}$ and $Ca^{2+}$ (○), after 0-120 minutes at 41°C. The standard error was less than 5% of the mean; thus error bars are not shown for clarity.

### 3.4.4 Discussion

A more rapid release of *E. tenella* sporozoites was observed with the HBSS and PBS with $MgCl_2$ buffers when compared with PBS buffer. The extraction times reported here were similar to those reported by Long and Millard (1976) and Tomley (1997), who showed that an extraction time of 2 hours at 41°C was required to release *E. tenella* sporozoites. The PBS with $MgCl_2$ result reported here was similar to that reported by Dulski et al. (1988), who demonstrated that an extraction time of 45 minutes at 42°C was required, using a similar buffer, to release *E. tenella* sporozoites. Sporozoite extraction was slightly higher in the HBSS buffer system compared with PBS containing $MgCl_2$, which may be due to the existence of $Ca^{+2}$ ions in the HBSS buffer that can effectively increase the sporozoites motility and help their exit from sporocysts. The effect of $Ca^{2+}$ ions causes increasing the motility of *E. tenella* sporozoites has been reported by Schubert *et al.* (2005).

### 3.4.5 Conclusion

According to these results, a buffer containing $Mg^{2+}$ ions facilitates the extraction of sporozoites from sporocysts yielding higher number in a shorter time when compared with a buffer without $Mg^{2+}$ ions.

## 3.5 Extraction of *Eimeria acervulina* sporozoites

### 3.5.1 Introduction

Trypsin and bile salt are necessary for the extraction of *Eimeria tenella* sporozoites from sporocysts. However, applying the method for extraction of *E. tenella* sporozoites to *E. acervulina* sporozoites caused the latter to rupture following their release from sporocysts. In an attempt to overcome this sensitivity of *E. acervulina*, the sporocysts were stored in buffers with different osmolarities, which were adjusted using various monosaccharides. These manipulations failed to protect the *E. acervulina* sporocysts (data not shown). Thus, it seemed that less aggressive extraction procedures were required for *E. acervulina* sporozoites. The objective of the experiment reported here was to modify the extraction procedure to make it more suitable for *E. acervulina*.

### 3.5.2 Method

*E. acervulina* sporocysts ($10^6$) were put in a 1.5 ml Eppendorf tube containing MEM, (5 mM/L $MgCl_2$, 0.01% (w/v) porcine trypsin and 4% (w/v) taurodeoxycholinic sodium salt. The suspension was mixed 10 times by gentle inversion and incubated in a 36°C water bath. A 20 µl sample of solution containing sporocysts was removed with a sterile pipette every 5 minutes and placed in a haemocytometer. The number of intact sporocysts and released sporozoites were counted under a light microscope (Olympus BH-2, Japan). The incubation was continued until 85% of sporozoites had been released. They were then washed four times with MEM to remove enzyme and taurodeoxycholinic sodium salt. The sporozoites were counted and stored in MEM at 4°C until used.

### 3.5.3 Result

Ninety percent of *Eimeria acervulina* sporozoites were extracted from sporocysts 20 minutes after the onset of the incubation. The modification of reduced trypsin concentration and incubation temperature, along with increased bile salt concentration resulted in the minimal loss of *E. acervulina* sporozoites compared with the large losses incurred when using the method used for *E. tenella.*

### 3.5.4 Discussion

*Eimeria acervulina* sporozoites were extracted effectively by using this modified procedure. The main characteristics of this method were a lower concentration of trypsin and higher concentration of taurodeoxycholinic sodium salt in a smaller volume of MEM than those for the extraction of *E. tenella* sporozoites. This result indicates that trypsin has limited importance in the release of *E. acervulina* sporozoites from sporocysts. This may be due to the composition of the sporocyst walls being predominantly lipids.

### 3.5.5 Conclusion

The modified method, reported here for the first time, is suitable for extraction of *E. acervulina* sporozoites.

# CHAPTER 4: INTERACTION BETWEEN *EIMERIA TENELLA* SPOROZOITES AND CAECAL ENTEROCYTES

# 4 Introduction

*Eimeria tenella* is a pathogenic coccidian with worldwide occurrence in poultry (Lobago *et al.*, 2005) in past, because it invades a specific site of the caecum. The attachment of sporozoites of *E. tenella* to caecal epithelial cells is the first step in the onset of coccidiosis in broiler chickens. An understanding of this interaction may lead to better ways to protect commercial poultry against coccidiosis.

The lack of *in vitro* methods using specific host cells has limited the understanding of this important phase of the disease. The aim of the work reported in this chapter was to develop a method using specific host cells to study the attachment of *E. tenella in vitro*.

## 4.1 Determination of intestinal tissue viability in organ culture

### 4.1.1 Introduction

Maintaining epithelial cell viability and integrity is important when using organ culture for *in vitro* studies. In general, tissue viability and integrity are measured by the transport of marker molecules such as $^{14}$C-leucine (Dickinson *et al.*, 1984), mannitol, inulin, $^{51}$Cr-EDTA (Artursson *et al.*, 1993) or the presence of enzymes such as, lactate dehydrogenase (LDH) (Oberle *et al.*, 1995; Polentarutti *et al.*, 1999), lactase-phlorizin hydrolase and maltase (Boudry *et al.*, 2004). Another measurement was electrical properties of tissue reflecting ion transport and membrane integrity (Polentarutti *et al.*, 1999). In addition, previous studies showed that histological examination can demonstrate changes in intestinal epithelial cells earlier than chemical methods that monitor membrane structure and function (Oberle *et al.*, 1995; Polentarutti *et al.*, 1999). These studies support the use of histological methods to identify the integrity of epithelial cells, but they failed to recognize early loss of viability and metabolic deterioration of cells.

Propidium iodide exclusion is a routine method used to identify cell viability (section 2.4.5.3). It is utilized here for the first time, to my knowledge, to identify epithelial cell viability in tissues in organ culture during the period of an *in vitro* experiment. The objectives of the experiments reported here were to determine the integrity and viability of caecal epithelial cells in organ culture by measuring histological parameters with a propidium iodide-haematoxylin method for the

detection of non-viable epithelial cells. In addition, the life span of tissue under such experimental conditions was measured.

### 4.1.2 Materials and methods

#### 4.1.2.1 Selecting animals

This experiment was repeated three times. In each repeat, four newly hatched chickens were killed and the caeca were immediately removed (section 2.2.1).

#### 4.1.2.2 Tissue segments

The caeca were opened using sterile scissors and washed three times with ice-cold PBS (pH 7.2, Sigma, Australia). A 0.5 $cm^2$ (1 cm x 0.5 cm) segment of caecal tissue was fixed in 10% neutral buffered formalin (NBF) for each replicate as a control.

#### 4.1.2.3 Incubation of caecal tissue

Caecal segments with a 1 $cm^2$ surface area were stretched and pinned out on 4 mm thick paraffin wax (Sigma, Australia) in a Petri dish containing 3 ml of MEM (Sigma, Australia) with 5% fetal bovine serum (FBS, Sigma, Australia) at pH 7.2 and continuously aerated with a 95% $O_2$ and 5% $CO_2$ gas mixture. Tissue samples were incubated for 120 minutes in a 41°C water bath. One sample was cut from each segment every 30 minutes.

Half of each sample was fixed in 10% NBF for 24 hours prior to paraffin imbedding (section 2.3.2). The other half of each sample was stained with PI for 10 minutes (section 3.1) and washed twice with PBS such samples were embedded in Tissue-Tek Compound (O.C.T, PreSciThec, Australia) and snap-frozen in liquid nitrogen. Frozen samples were stored at –80°C until used.

#### 4.1.2.4 Evaluation of epithelial cell viability

In order to determine the viability of the epithelial cells two methods were performed: a) Basic histological assessment, b) Propidium iodide and haematoxylin staining procedure.

#### 4.1.2.4.1 Basic assessment of histology

The tissue samples were processed for normal histology (section 2.3.2) and three 5 μm sections from each tissue block were cut at intervals of 100 μm on a microtome. The sections were stained with Meyer's hematoxylin (Sigma, Australia) for 5 minutes (section 2.3.5.1).

All slides were observed at a magnification of x 40 using a light microscope. The tissues were evaluated for morphological changes using a selection of parameters, including nucleo-apical distance, villous index and morphological scoring as previously described by Polentarutti *et al.* (1999).

**Nucleo-apical distance**

The distance between the tip of the nucleus and the apical membrane (brush border membrane) in enterocytes was measured with an image analyser (Video Pro. 32 software, Australia). Five villi were measured on each slide with five measurements taken from each villous. The villi were randomly chosen and measurements were performed at the tips of the villi.

**Villous index**

The height and width of five villi selected randomly on each slide were measured using an image analyser (Video Pro. 32 software, Australia). The height was divided by the width to yield the villous index.

**Estimation of enterocyte numbers**

Calculations of the number of enterocytes per mm of caecal epithelial section length were based on light microcopy. The diameter of the enterocytes was determined to be 8.5 ± 0.41 μm (± SD) by image analysis (Video Pro. 32 software, Australia). The number of enterocytes per mm of epithelial cell length was determined using Equation 4.1. The number of epithelial cells per mm length was thus determined to be 117.9 ± 5.7.

**Equation 4.1** The number of enterocytes per mm length of epithelial section.

ANE = 1 /ED

ANE = The number of enterocytes, ED = Enterocyte diameter.

**Morphological scoring**

The impact of organ culture on tissue condition was assessed using the damage score described in Table 4.1.

**Table 4.1** Morphological scoring of caecal tissue in organ culture system adapted from Polentarutti *et al.* (1999).

| Damage | Description of damage | |
|---|---|---|
| | **Epithelial cells** | **Villi** |
| 0 | No damage | No damage |
| 1 | Some individual cells extruded | Slight villous height reduction |
| 2 | Group of cells extruded/ thin epithelium | Villous height decreased |
| 3 | Sheets of cells extruded/ thin epithelium | Villous height greatly reduced |
| 4 | Total loss of epithelium | Villous structure flattened |

#### 4.1.2.4.2 Propidium iodide-hematoxylin method

Frozen segments (section 4.1.2.3) were placed in a Leica cryostat (Cambridge Instruments, GmBH, Germany) for one hour prior to sectioning. The chamber temperature of the cryostat was set at –24°C. From each tissue block three 5 μm sections were cut and placed on a glass slide. The numbers of viable cells in tissue sections were counted under a fluorescent microscope (U-MWG filter exciting with green, 510-550 nm and emitting red at 590 nm). The red nuclei were counted as nonviable cells and their position noted with an England Finder slide (NEFS, Sigma, Australia). The sections were then stained with Meyer's haematoxylin for two minutes.

The number of epithelial cells per millimetre of section length was counted in the same position for each section located at the recorded New England Finder slide under bright-field illumination microscope. The percentage of viable cells was determined by calculating the ratio of labelled cells to total cells per millimetre of caecal epithelium using Equation 4.2.

**Equation 4.2** Number of viable epithelial cells per mm.

NV = 100 - 100 x (NNVC / NTC)

NV = Number of viable cells, NNVC = Number of non-viable cells, NTC = Total number of cells.

#### 4.1.2.5 Statistical analysis

The statistical analysis was undertaken using SAS Proc Mixed. The model was used to explain variation in cell viability per mm of caecal tissue, cell viability as a random effect with time, total cells, villi height, villi depth, villi height by depth ratio, and cells per mm caecal length; all treated as fixed covariates. All quadratic terms and first order interactions were tested. Non-significant ($P > 0.05$) effects and interactions were removed one at a time by backward elimination leaving only significant interactions and main effects. The model was developed while ensuring that all the marginality requirements were met. These marginality requirements state that where an interaction is included in the model, both contributing main effects must also be included irrespective of the level of significance associated with the main effects. Similarly if a quadratic term of a covariate is to be kept in the model the linear term of the covariate must also be kept in the model (Nelder, 1994).

### 4.1.3 Results

The caeca were morphologically intact with well-defined villi and epithelial surfaces (Figure 4.1). There were no changes in the height, width and index of the villi for each tissue in organ culture up to 120 minutes.

The nucleo-apical distances, between the nuclear and cell membranes, were decreased when the experimental time was longer than 30 minutes in organ culture systems. However, the changes in nucleo-apical distance changes were not significantly different ($P > 0.05$) from the start to the end of the experiments.

The damage scores of caecal tissues were 1 (Table 1) after 30 to 120 minutes incubation in the organ culture system.

The propidium iodide-hematoxylin method showed that the numbers of viable epithelial cells in tissue samples in organ culture decreased significantly ($P < 0.01$) at 30, 60, 90 and 120 minutes; in comparison with the time zero as a control (Figure 4.1). Example images for each stage of the propidium iodide-hematoxylin method are shown in Plate 4.1.

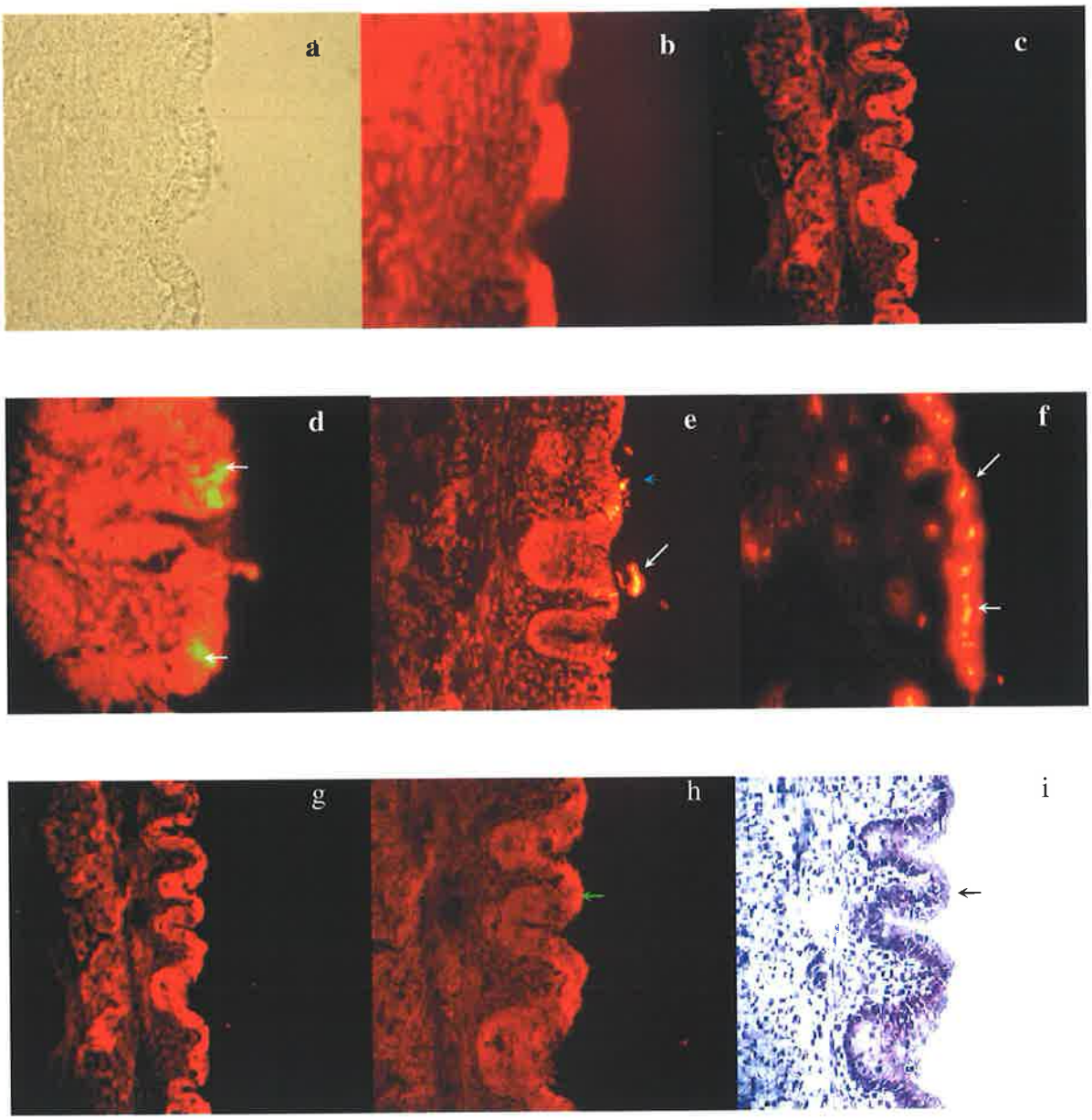


**Plate 4.1** The viability of caecal tissue maintained in organ culture systems at 41°C from 0 to 120 minutes as assessed by the propidium iodide-haematoxylin method. a) The light microscopy image of caecal section at time zero, b) The same section viewed under fluorescent microscopy. Plates c to f represent sections of caecal tissue after incubating in organ culture for 30, 60, 90, 120 minutes, respectively. Plates g (x 10) and h (x 20) represent a typical section from a routine experiment stained with PI to determine enterocyte viability. Arrow on plate h marks the feature used to confirm the New England finder coordinate for this section. Plate (i) is a microscopy image of the section shown in (h) after staining with Meyer's haematoxylin and relocated using the New England finder coordinates. Such images were used to count the non-viable cells (PI stain) and total number of cells per mm of epithelia (Meyer's haematoxylin stain).

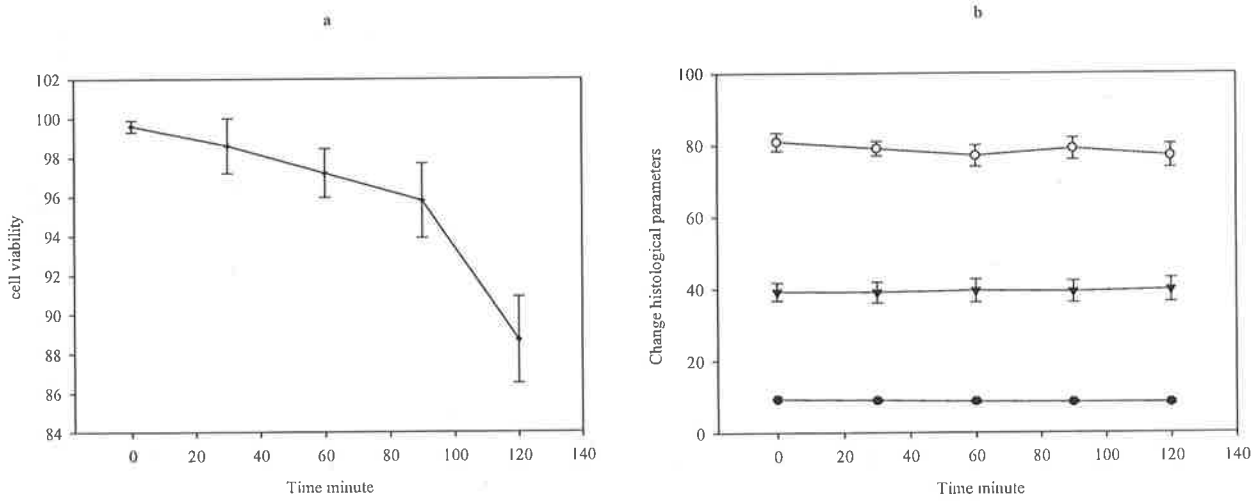


**Figure 4.3** The viability of caecal tissue in the organ culture system using a propidium iodide-hematoxylin and histology method at different times at 41°C. a) Changes in the cell viability, b) changes in villi height (○), willi width (▼) and nucleo-apical distance (●).

### 4.1.4 Discussion

Ensuring epithelial cells are viable is essential for interpretation of *Eimeria* binding experiments *in vitro*. The present results showed that chicken caecal tissue could be maintained successfully in organ culture. The viability of these tissues depended on the length of experimental time, with only minor changes being observed in tissue viability up to 90 minutes in a 41°C culture system. A similar result was reported for the viability of the ileal tissue derived from humans (Soderholm *et al.*, 1998) and intestinal segments obtained from rats (Polentarutti *et al.*, 1999).

Propidium iodide has been injected intravenously to identify necrotic cells in neural tissues of rats (Unal Cevik and Dalkara, 2003). Furthermore, in metabolically active cells, PI was excluded from entering the cells (Smith and Smith, 1989). Cells that are positive to PI were considered to be metabolically deficient. PI in organ culture as reported in the present study showed that incubation of caecal tissue with PI for 10 minutes after the experimental period could identify metabolically stressed cells (section 4.1.2.4.2).

There is a relationship between the thickness of the tissue and oxygen diffusion gradients that may result in tissue hypoxia. This is a limiting factor for maintaining the tissue viability during experimentation, as reported by Haglund and Park (1991), Soderholm *et al.* (1998), and Nejdfors *et al.* (2000). In the present study, the thickness of newly hatched chicken caecum was less than 1mm, but gassing during incubation with 95% $O_2$ and 5% $CO_2$ mixture should have

significantly reduced the risk of tissue hypoxia as indicated by the minimal changes in tissue indices reported here.

In addition, combining the PI method with histology allowed the recognition of both the necrotic process and the changes in morphology associated with tissue damage. Thus, PI with histology should be used to demonstrate that the tissue is both structurally and metabolically viable.

### 4.1.5 Conclusion

Based on the tissue viability results presented here, it is suggested that caecal organ culture is a suitable tool to study the initial binding and invasion of enterocytes by *Eimeria* sporozoites, if experimental conditions do not exceed 90 minutes at 41°C. Also, the propidium iodide-hematoxylin method can be used routinely to confirm tissue viability in such an experiment.

## 4.2 Investigating *Eimeria*-enterocyte interactions using caecal tissue in organ culture

### 4.2.1 Introduction

Organ culture has been used successfully by many researchers to investigate epithelial cell functions (Isolauri *et al.*, 1993; Budhoo and Kellum, 1994; Guarino *et al.*, 1994; Bijlsma *et al.*, 1995; Corbett *et al.*, 1977). This type of *in vitro* experiment has been previously described for iron and protein transport across epithelial cells. More recently, the method of organ culture has been adapted to study the interaction of bacteria with epithelial cells; such as, *Escherichia coli and Salmonella typhimurium (*Knutton *et al.*, 1987; Hicks *et al.*, 1996; Girard *et al.*, 2005*)*.

To date, there are no published studies investigating protozoan parasite attachment with epithelial tissue in organ culture. The results reported in section 4.1 showed that chicken caecal tissue could be maintained in organ culture in a condition similar to that *in vivo*. Thus, this method has potential for the study of the *in vitro* interaction between *Eimeria* sporozoites and host cells. The aim of the experiments reported here was to demonstrate that *Eimeria*-host cell interaction could be studied using organ culture.

### 4.2.2 Materials and methods

#### 4.2.2.1 Selecting animals

In each of three repeats, four newly hatched chickens were killed (section 2.2.1) and the caecal segments were collected (sections 2.2.2 and 4.1.2.2).

#### 4.2.2.2 Estimation of enterocyte numbers

The number of exposed enterocytes was calculated using a light microscopy method (Haglund and Park, 1991; Soderholm *et al.*, 1998). The surface area of tissue was calculated from the length and width of each sample. The average surface area was 1 $cm^2$.

As villi are cylindrical in shape, the mean surface area of a villous was calculated from measurement of height and width of ten villi. The mean number (± SE) of villi per $mm^2$ was determined to be 66 ± 4.7. Estimation of the actual surface area of one mm caecal tissue was determined by calculation (average villous surface area x number of villi). The mean diameter of the enterocytes was then determined (section 4.1.2.5). The total mean (± SE) number of enterocytes on a 1 $cm^2$ surface area was calculated to be 2.2 ± 0.24 x $10^7$.

#### 4.2.2.3 Estimation of sporozoite numbers

The ratio between the number of enterocytes and *Eimeria* sporozoites is important to demonstrate for quantify in sporozoite binding. Therefore, a minimum of 1 sporozoite to 44 enterocytes is required for any given binding assay. Based on the total number of enterocytes (2.2 ± 0.24 x $10^7$) in a 1 $cm^2$ surface area, 5 x $10^5$ sporozoites are needed for each assay per 1 $cm^2$ of caecal surface area.

#### 4.2.2.4 Preparation of sporozoites

*Eimeria tenella* sporocysts were prepared (section 3.2.2.3) from purified oocysts (section 2.4.1). Sporozoites were extracted and purified (section 2.4.5.2). The number of sporozoites was counted by haemacytometer and the volume of medium adjusted to yield a final density of sporozoites of $10^6$ per ml MEM. The *E. tenella* sporozoites were kept in MEM (pH 7.2) at 4°C until used in assays.

#### 4.2.2.5 Binding assay

Each experiment was started by adding 5 x $10^5$ *E. tenella* sporozoites to tissue maintained in MEM gassed with 5% $CO_2$ and 95% $O_2$ gas mixture (section 4.1.2.3). The organ cultures were incubated for 90 minutes in a 41°C water bath. In each replicate, samples were collected at 0 (as a control), 30, 60 and 90 minutes post onset of incubation. Samples were fixed in 10% NBF for histological processing.

#### 4.2.2.6 Histology

The fixed tissues were processed (section 2.3.2) for normal histology to identify the attachment of *Eimeria tenella* sporozoites to epithelial cells. Three 5µm sections from each tissue block were cut at intervals of 100 µm on a microtome.

#### 4.2.2.7 Staining of sections

Three series of sections were processed from the same samples (section 2.3.4). The first series of sections were used for staining with haematoxylin and eosin (section 2.3.5.1). The second series of sections were stained by periodic-acid-Schiff (section 2.3.5.2). The last series of sections were used for staining with periodic-acid-Schiff and aniline blue-orange G as a specific staining method for sporozoites (section 2.3.5.3). Stained sections were examined under a light microscope.

#### 4.2.2.8 Number of attached sporozoites

The mean length (± SE) of ten villi in each tissue was measured (section 4.1.2.4.1) to be 175 ± 7.8 µm. Then, the numbers of *E. tenella* sporozoites attached to epithelial cells were counted in 40 fields of view at x 40. This was equal to a 7 mm length of caecal epithelial tissue.

### 4.2.3 Results

**Identifying *Eimeria tenella* sporozoites by hematoxylin and eosin**

Sections stained with hematoxylin and eosin, were examined (section 4.2.2.9). No adherence of *Eimeria tenella* sporozoites to caecal enterocytes was observed in any section.

**Identifying *Eimeria tenella* sporozoites by periodic-acid-Schiff**

Results showed that the mucus was stained by periodic-acid-Schiff, but no *E. tenella* sporozoites could be seen on the tissue sections. These results were similar to those for hematoxylin and eosin staining.

**Identification of *E. tenella* sporozoites by periodic-acid-Schiff and AG**

In the last step, sections from the same tissue blocks, as described above, were stained by the periodic-acid-Schiff-aniline blue and orange G method (section 2.3.5.3), and examined (section 4.2.2.9). The results were negative, as for hematoxylin and eosin and periodic-acid-Schiff.

### 4.2.4 Discussion

The hematoxylin and eosin staining method has been used routinely in microscopic examination on slides for diagnosis of coccidiosis by recognizing the stage of the *Eimeria* life cycle, including the schizonts, macrogametocytes, microgametocytes and oocysts (Molloy *et al.*, 1998; Johnson and Reid, 1970). The present results showed that the *Eimeria* sporozoites were not detected with this staining method in any sections. Given that 5 $x10^5$ sporozoites were added to the tissue in organ culture the absence of sporozoites is puzzling. Two possibilities could explain the obtained results: 1) The hematoxylin and eosin staining was inadequately specific to identify *Eimeria* sporozoites in the tissue sections; 2) *Eimeria* sporozoites were not detected due to the low numbers of sporozoites relative to the number of epithelial cells in the tissue samples.

To test the first possibility, the stains with stronger sensitivity, periodic-acid-Schiff and periodic-acid-Schiff and aniline blue-orange G, were applied to sections from the same samples that were used for hematoxylin and eosin staining. All three staining methods produced the same results. This was in spite of the fact that these methods have been used routinely in histological examination and lesion scoring for diagnosing coccidiosis in chickens (Johnson and Reids, 1970; Hofmann and Raether, 1990; Molloy, 1998). Given that the staining methods selected are appropriate for identifying *Eimeria*, the observed negative result is most probably an error in sampling rather then the lack of binding. The surface area of the three sections examined per sample is minimal when compared to the surface area of tissue available for the binding and invasion by *Eimeria tenella* sporozoites in organ culture.

The second possibility is the low ratio between numbers of sporozoites and enterocytes. This is the more likely reason for the results reported in this binding assay. To overcome this limitation, a large number of caecal sections would need to be examined. However, this is

impractical for routine analysis. Alternatively, *Eimeria* sporozoites could be identified in an organ culture method, if the number of sporozoites were increased in each replication. However, it is not clear how many sporozoites would be needed in each replication, and preparing large numbers of sporozoites is not practical, because of the large number of *Eimeria* oocysts, which would be needed.

### 4.2.5 Conclusion

It would appear that sectioning tissue from organ culture experiments with conditions recorded here is not a practical method to investigate *Eimeria*-epithelial cell attachment. For the effective use of organ culture in this type of binding assay, alternative methods to determine the quantity of sporozoite bound are required.

## 4.3 *Eimeria tenella* sporozoite interaction with, and entry into, isolated enterocytes

### 4.3.1 Introduction

*Eimeria* sporozoites initially have to adhere to and enter into caecal epithelial cells to complete their life cycle. While the use of an organ culture method to study these phenomena *in vitro* was not successful (section 4.2), isolated cells systems have been used successfully to study cell-cell interaction or nutrient transport (Abud *et al.*, 1989; Dopido *et al.*, 2004). Such preparations have been adapted for preparing isolated enterocytes that retain functionality from the intestine of chickens (Kimmich *et al.*, 1970). Thus, it seems that isolated enterocytes might be used for the study of the interaction of sporozoites with target cells and may be a suitable alternative to change the ratio between host cells and sporozoites.

Previously, preparations of isolated single cells have been used successfully to study the adherence of *Esherchia coli* to enterocytes derived from human tissue (Knutton *et al.*, 1987) and of *Salmonella typhimurium* to rat intestinal tissue (Abud *et al.*, 1989). Recently, trypsinized Madin Darby Bovine Kidney (MDBK) cells, cultured repeatedly before final isolation, were used to investigate the interaction mechanism of the cells and *Cryptosporidium parvum,* a coccidian protozoan parasite with a similar life cycle to that of the *Eimeria* genus (Johnson *et al.*, 2004).

Taking these previously published results into consideration, it may be possible to adapt this method to study the *Eimeria*-chicken enterocyte interaction. No study has been published to date on the interaction between *Eimeria* and chicken enterocytes *in vitro*. The objectives of the experiment reported here were to isolate the epithelial cells of chicken caeca, to assess their viability and to develop an *in vitro* model suitable for the study of *Eimeria*-host cell interactions.

### 4.3.2 Materials and methods

#### 4.3.2.1 Selecting animals

This experiment was repeated three times. In each repeat, three newly hatched chickens were killed and caeca were immediately removed (sections 2.2.1 and 2.2.2).

#### 4.3.2.2 Isolation of enterocytes

Caecal enterocytes were isolated using a modification of the method described by Ferrer *et al.* (1986); the main modification of their method being the use of PBS instead of HBSS and Krebs' salt solution. This modification was found to result in a greater number of viable cells.

Caecal segments were incubated in 30 ml of a medium containing PBS (pH 7.2) with 0.5 mg/L hyaluronidase, 0.5 g/L dithiothreitol (DTT, Sigma, Australia), 0.1% bovine serum albumin (BSA, Sigma, Australia), 4% mannitol, and 100 IU/ml penicillin and 100 mg/ml streptomycin (Sigma, Australia) in a 37°C shaking water bath for 30 minutes. The suspension was filtered through nylon stocking material with a pore size of 150-200 μm to separate the isolated intestinal cells from tissue debris. The cells were re-suspended in fresh MEM with 4% mannitol and 0.1% bovine serum albumin and centrifuged at 20 x g for 5 minutes. Supernatant was discarded and the cell pellets were washed three times as above to remove any remaining enzymes and debris (Ferrer *et al.*, 1986).

#### 4.3.2.3 Identification of enterocyte viability

The viability of freshly isolated cells was identified using the method described by Tierney *et al.* (2004). In summary, a 0.1 ml of 0.4% trypan blue (Sigma, Australia) was added to 0.1 ml cells at room temperature for 3 minutes. A small drop of the suspension was then put on a glass slide and examined using a light microscopy (Olympus BH-2, Japan) for exclusion of the stain: the

uncolored cells were counted as viable cells and the colored cells (blue) were counted as nonviable cells.

#### 4.3.2.4 Preparation of sporozoites

*Eimeria tenella* sporocysts were prepared (sections 2.4.1 and 3.2.2.3). Sporozoites were extracted and purified (sections 3.4 and 2.4.5.2). The numbers of sporozoites were counted using a haemocytometer and the volume adjusted to produce a fluid with a sporozoite density of $10^6$ per ml. The viability of *E. tenella* sporozoites was assessed with PI (section 2.4.5.3).

#### 4.3.2.5 Staining of sporozoites

A suspension of 5 x $10^6$ purified sporozoites were stained fluorescently using a PKH-67 kit (Sigma, Australia) as described in Section 2.4.5.4. Sporozoites were counted and adjusted to $10^6$ sporozoites/ml MEM containing 5% FBS and 3 mM L-glutamine, and stored at 4°C for 2 hours.

#### 4.3.2.6 Binding assay

Each replicate was started by adding 1 x $10^5$ *E. tenella* sporozoites to 2 ml MEM containing 5 x $10^5$ isolated epithelial cells in a tube (0.5 cm diameter). The cells were incubated in a 5% $CO_2$ incubator at 41°C for up to 14 hours. Samples of cell suspensions (0.2 ml) were collected at 0.5, 1, 2, 4 and 14 hours post onset of incubation. To stop the reaction, samples were fixed with 10x volume of 10% NBF and maintained in this condition until examined.

#### 4.3.2.7 Quantification of invasion

The collected sample from each replicate (section 4.3.2.2) was put in a heamocytometer. The number of *Eimeria tenella* sporozoites penetrated into or attached to epithelial cells was counted in 40 fields of view with x 40.

### 4.3.3 Results

When trypan blue was used to determine the viability of enterocytes isolated from caecal tissue, the mean (± SE) of viable cells in the experimental repeats (section 4.3.2.1) was 90% ± 4.5%, 96% ± 2 % and 93% ± 3.6% respectively.

No *E. tenella* sporozoites were observed to attach or penetrate into the isolated enterocytes after 0.5 hour. A similar result was obtained at 1, 2, 4 and 14 hours after the commencement of the sporozoite binding assay with isolated epithelial cells.

Under observation with a light microscope, some samples showed that *Eimeria* sporozoites could abut to the surface of isolated caecal epithelial cells for a short time, but could not attach to and penetrate into the cells (Plate 4.2).

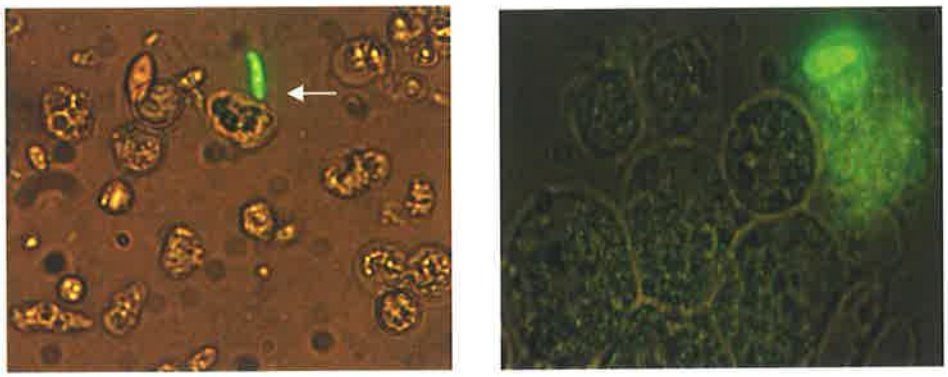

**Plate 4.2** The observed interaction of *E. tenella* sporozoites (arrows) with differing preparations of isolated caecal enterocytes viewed with fluorescent microscopy.

### 4.3.4 Discussion

Recognizing and adhering to caecal enterocytes is the first step for *E. tenella* sporozoites to enter host cells. Attachment is a pre-requisite in order to develop an *in vitro* model suitable for studying the interaction of original host cells and sporozoites. Adding *E. tenella* sporozoites to isolated enterocytes in the present study showed that they could not attach to and penetrate isolated enterocytes. The difference between the expected and obtained results in these experiments demonstrated that isolated enterocytes are probably inappropriate for use in this type of research with *E. tenella*.

Johnson (2004) reported that *Cryptosporidium* sporozoites could attach to and penetrate isolated Madin Darby Bovine Kidney (MDBK) cells. The main difference between the *Cryptosporidium* study and the work presented in this chapter is host cells. The MDBK cells

were isolated after many passages in a cell culture system using a trypsinization technique. The present study used freshly-isolated caecal enterocytes derived from chicken caecal tissue. It was reported previously by Augustine (2001b), who used primary cells isolated from chicken or turkey kidneys; maintaining and converting such cells to long term cell culture resulted in greater *Eimeria* sporozoite invasion. A similar result was observed in the interaction between *Salmonella enteritidis* and primary chicken enterocytes in an *in vitro* study (Van Immerseel *et al.*, 2004).

The viability of isolated epithelial cells could be an important factor for their interaction with *E. tenella* sporozoites. In this study, using a trypan blue exclusion test, the mean epithelial cell viability (± SD) after isolation was 93% ± 4%. These results were in agreement with those described by others studying enterocyte function (Kimmich, 1970), transfer of folic acid (Eilam *et al.*, 1981) and $Na^+$ transfer (Sepulveda *et al.*, 1982). This result showed that the viability and membrane integrity of isolated enterocytes were appropriate for the interaction by sporozoites.

Staining *E. tenella* sporozoites with PKH-67 GL fluorescent dye was another factor that might affect the recognition and attachment of sporozoites to isolated enterocytes. It was previously reported that PKH-67 GL was used successfully to stain *E. tenella* sporozoites for a study of *Eimeria*-host invasion in established culture cells *in vitro* (Fuller and McDougald, 2001). In addition, observation of *Eimeria*-enterocyte binding under a phase microscope, without staining the sporozoites with PKH-67 GL, showed that no sporozoites were attached to isolated enterocytes (data not shown). Thus, staining of sporozoites with PKH-67 GL did not affect the potential for invasion of sporozoites.

Data from the present study along with those reported by others indicates that a period in culture, or passage through culture, is necessary to restore the vulnerability of the cells to invasion by coccidian protozoa. Interestingly, the use of trypsin during the establishment of cell culture line did not reduce invasion (Augustine, 2001b; Tierney and Mulcahy, 2003; Johnson *et al.*, 2004). However, there are no data published about the effect of hyaluronidase enzyme (section 4.3.2.2) in *Eimeria* invasion to cells. The problem encountered with freshly isolated enterocytes, as reported here, can be due to either the procedure or enzyme used to isolate cells.

### 4.3.5 Conclusion

According to the present results, changes to the enterocyte membrane during the isolation procedure cause *E. tenella* sporozoites to be unable to attach to and enter into host cells. The use

of hyaluronidase enzyme may be the factor that inhibits this interaction by *Eimeria tenella* sporozoites. To continue using isolated epithelial cells, alternative isolation methods are needed.

## 4.4 Impact of cell isolation methodology on the interaction between sporozoites and enterocytes

### 4.4.1 Introduction

The experiment described in section 4.3 showed that using hyaluronidase enzyme to isolate enterocytes may be the cause of the negative results in the *Eimeria* sporozoites binding to isolated enterocytes. In addition, broiler chickens are more susceptible to coccidiosis from the age of 3 to 6 weeks (Calnek *et al.*, 1997). Isolated enterocytes from susceptible chickens are an alternative that may affect the outcome of the *Eimeria* interaction in comparison with those in newly hatched chickens.

There are no published data as to whether or not hyaluronidase enzyme changes the enterocyte membrane, thus influencing potential binding sites for *Eimeria*. The study described here investigates factors that could affect *Eimeria* binding to isolated enterocytes. The study was conducted to determine the role of the isolation procedure in the interaction mechanism.

### 4.4.2 Materials and methods

#### 4.4.2.1 Selecting animals

This experiment was repeated three times. In each repeat, four newly hatched or three-week-old chickens were killed. The caeca and kidneys were immediately removed (section 2.2.1). The chicken kidney cells were isolated to use as a negative control in *Eimeria* binding assay. Caecal samples were opened and washed (section 2.2.2).

#### 4.4.2.2 Preparing and staining of sporozoites

The *Eimeria tenella* sporocysts and sporozoites were prepared (sections 2.4.1 and 3.2.2.3). The purified sporozoites (section 2.4.5.2) were stained using a PKH-67 GL kit (section 2.4.5.4).

#### 4.4.2.3 Isolation of enterocytes

The caecal segments of newly hatched and three-week-old chickens (section 2.2.2) were separately placed in 50 ml centrifuge tubes containing PBS. Caecal epithelial cells were then isolated using the following protocols.

**Protocol A (hyaluronidase)**

Caecal epithelial cells were isolated using the modified method reported by Ferrer *et al.* (1986) (section 4.3.2.3).

**Protocol B (trypsin)**

Caecal segments were placed in 30ml of medium containing PBS, 0.25% trypsin (2.5 g trypsin, 1:250 enzymatic activity, Sigma, Australia), 0.5 mmol/L dithiothreitol (DTT, Sigma, Australia), 0.1% bovine serum albumin (BSA, Sigma, Australia), 4% mannitol and 100 IU/ml penicillin (Sigma, Australia), and 100 mg/ml streptomycin (Sigma, Australia). Tissues were incubated and agitated in a 37°C shaking water bath (100 cycles/minute) for 30 minutes. The suspension was filtered and washed (section 4.3.2.3) and stored in MEM at 4°C for 1 hour.

**Protocol C (mechanical)**

This procedure was performed using a combination of the methods described by Whitehead *et al.* (1987) and Grossmann *et al.* (1998). The caecal tissues were cut into 1 $cm^2$ pieces using a sterile scalpel and placed in PBS containing 0.5 mmol/L dithiothreitol, 0.1% BSA, 4% mannitol, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Tissues were incubated in a 20°C shaking water bath (100 cycles/minute) for 1.5 hours. Caecal epithelial cells were then liberated into 10ml of PBS by vigorous agitation using a wrist shaker set at 100 cycles/minute for 3 minutes. This process was repeated five times. The suspension was filtered after each isolation stage, and enterocytes were finally purified as described in Protocol B.

#### 4.4.2.4 Use of kidney cells

The chicken kidney tissues were isolated from newly hatched and three-week-old chickens and placed in cold PBS containing 100 IU/ml penicillin and 100 mg/ml streptomycin in a Petri dish. Connective tissues and blood clots were removed, and the kidney tissues were cut with sterile scissors into small pieces and washed twice with PBS as described above.

The kidney cells were isolated according the method described by Hofmann and Raether (1990). Briefly, kidney pieces were suspended in 30 ml of a preheated PBS (37°C) containing

0.25% trypsin for 10 minutes. After sedimentation of the tissue, the supernatant was discarded and 5 ml of BSA were added to stop the trypsin activity. The suspension was incubated for another 15 minutes, with stirring, using a sterile magnetic stirrer at 37°C. The suspension was filtered through a nylon stocking material with a pore size 150-200 μm to separate the isolated kidney cells from tissue debris.

The cells were re-suspended in fresh MEM with 4% mannitol and 0.1% BSA and centrifuged at 20 x g for 5 minutes. The supernatant was discarded and the cell pellets were washed three times, as above, to remove any remaining enzymes and debris. $10^5$ cells/ml in medium were prepared for the binding assay (Hofmann and Raether, 1990).

#### 4.4.2.5 The viability of cells

The viability of caecal enterocytes and kidney epithelial cells was determined using 0.04% trypan blue in an exclusion test (Section 4.3.2.4).

#### 4.4.2.6 Binding assay

Each replicate was started by adding 1 x $10^5$ *E. tenella* sporozoites to 2 ml MEM containing 5 x $10^5$ isolated epithelial cells. The cells were incubated in a 41°C incubator with an internal atmosphere of 5% $CO_2$ and 95% medical air for up to 14 hours. In each trial, 0.2 ml samples were collected at 0.5, 1, 2, 4 and 14 hours post onset of incubation. To stop the reaction, samples were fixed with x10 volume of 10% neutral buffered formalin and maintained in this condition until examined.

#### 4.4.2.7 Quantification of binding and invasion

The collected sample from each binding assay was put in a haemocytometer and the number of *E. tenella* sporozoites penetrated into, or attached to, epithelial cells was counted (section 4.3.2.7).

### 4.4.3 Results

#### 4.4.3.1 Cell viability

The viability of caecal enterocytes and kidney epithelial cells isolated by all methods was determined immediately after isolation from tissue using trypan blue exclusion. The mean viability (± SD) of enterocytes isolated by hyaluronidase, trypsin and mechanical protocol was 94% ± 3.3%, 92.5% ± 3.7% and 95% ± 2.5% respectively, and the mean viability of kidney epithelial cells was 98% ± 1.9% (Figure 4.2). The viability of isolated cells irrespective of protocol or cell type decreased significantly ($P < 0.001$) after 24 hours maintained in MEM at 4°C. The mean viability of isolated enterocytes and kidney cells of three-week-old chickens was not significantly different from the isolated cells of one-day-old chickens.

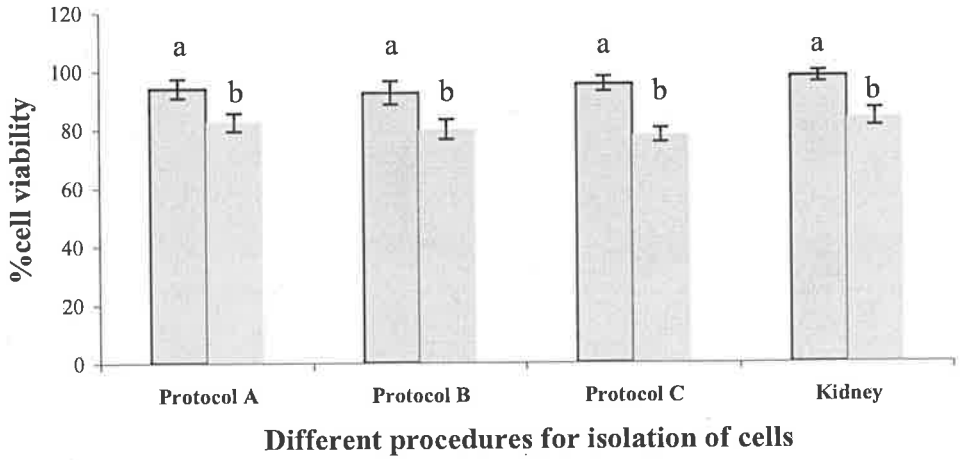


**Figure 4.4** The viability of isolated cells after 10 minutes ( ▩ ) and 24 hours ( ▩ ) by trypan blue exclusion. Mean values with unlike superscripts (a, b) are significantly different between different protocols.

#### 4.4.3.2 Binding of *E. tenella* sporozoites to cells

Irrespective of isolation protocol or cell type, the results for the *Eimeria tenella* binding assay were the same. *Eimeria* sporozoites could neither attach to and nor enter into the isolated enterocytes and kidney epithelial cells at 0.5 hours. These results were similar at 1, 2, 4 and 14 hours post incubation.

Observing a suspension of *E. tenella* sporozoites and isolated enterocyte and kidney cells under a light microscope revealed that sporozoites came into close proximity with the surface of cells for a short time, but they could not attach to and penetrate into caecal epithelial cells.

### 4.4.4 Discussion

The different isolation protocols were shown not to affect the attachment or penetration of *E. tenella* sporozoites into the isolated enterocytes. The differences between expected and obtained results in these studies showed that hyaluronidase enzyme did not inhibit the attachment of *E. tenella* sporozoites to isolated enterocytes. This inability to attach or penetrate contrasts with the observation that sporozoites routinely invade and develop in enterocytes in primary culture and enterocytes of caecum *in vivo* (Long and Millard, 1976; Hofmann and Raether, 1990; Shirley *et al.*, 2005).

As broiler chickens are more susceptible to coccidiosis from the age of 3 to 6 weeks (Calnek *et al.*, 1997), age may be an important factor in the *Eimeria* interaction. In these experiments, preparing isolated enterocytes and kidney cells from chickens at different stages of development showed that the age of the chickens did not influence the attachment of *Eimeria* to enterocytes or kidney cells.

Together these data showed that the lack of observed binding to isolated enterocytes was not related to either the age of the chicken from which the tissue was harvested, or the enzyme used in the cell isolation protocol. The question remained why *Eimeria* could not bind to enterocytes in *in vitro* conditions. To determine whether this negative result related to cell type, further experiments using kidney cells were conducted. Kidney cells were selected because both primary and established kidney cells lines have been used to study invasion by *Eimeria* species (Augustine and Danforth, 1985; Doran and Augustine, 1973; Hofmann and Raether, 1990). The interaction of *E. tenella* sporozoites with fresh isolated kidney cells in this study was negative. This result was similar to that of inoculating chickens intraperitoneally with *Eimeria* sporozoites (Long and Millard, 1976; Fernando *et al.*, 1983).

The difference between the results reported in this study and those obtained by others using primary and established kidney cell lines showed that changes occur as a result of cell culture, allowing *Eimeria* sporozoites to attach to and invade the cell culture. In addition, invasion of sporozoites and binding in established cell lines was greater than that observed into primary kidney

cells (Tierney and Mulcahy, 2003; Zhang *et al.*, 1997). It can be concluded that changes on the surface of cell membrane as a result of tissue culture caused the expression of suitable sites for *Eimeria* binding and invasion.

The above conclusion is supported by further lines of argument. The inhibition of *Apicomplexan* parasite invasion into epithelial cell cultures by substances that selectively alter the host cell membrane has suggested the existence of receptor molecules that facilitate parasite invasion. These substances include ferritin and neuraminidase (Augustine and Danforth, 1984), lectin (Augustine, 1985), and protease enzyme (Fuller and McDougald, 1990). The inhibition of *Eimeria*-host cell invasion by monoclonal antibodies against cell membrane proteins (Augustine and Danforth, 1986; Augustine, 1999, 2001b), also provided more specific evidence for the presence of receptors (Augustine, 2001a).

### 4.4.4 Conclusion

Based on the present negative findings, the enterocytes need to exhibit special characteristics or receptors as a prerequisite for attachment and invasion by *Eimeria* sporozoites.

## 4.5 *Eimeria tenella* sporozoite interaction with, and entry into, frozen sections

### 4.5.1 Introduction

Different cell lines have been used for studying the *Eimeria*-host cell attachment and invasion by various species of *Eimeria*. Such *in vitro* methods cannot provide evidence related to the prerequisite factors of the primary target cells in the *Eimeria* binding. In addition, the attempts to overcome this limitation through the use of organ culture and isolated enterocyte methodologies were not successful in the study of *Eimeria* sporozoites interaction (sections 4.2, 4.3 and 4.4) and hamper research into the role of intestinal enterocytes in the site specificity of *Eimeria* sporozoite binding. Furthermore, the work presented in this chapter indicates that two essential criteria may need to be met for an *in vitro* method to be successful. These are: a) A sporozoite to enterocyte ratio that approaches unity, and b) Tissue to be maintained in a condition close to that observed *in vivo*. Thus, an alternative method is required to allow both of these criteria to be met and thus provide an effective tool to study the *Eimeria*-host cell interaction.

Frozen sections have been previously utilized in order to study the adhesion sites of *Escherichia coli* to epithelial cells derived from human kidney tissues (Nowicki *et al.*, 1986 Nowicki *et al.*, 1987). This adhesion assay has been efficiently applied for the tissue tropism of various bacteria to human intestinal sections (Korhonen *et al.*, 1990) as well as chicken respiratory tissue samples (Dozois *et al.*, 1995). Recently, the frozen section method was adapted for the adhesion of *Lactobacillus* and *Escherichia coli* in epithelial cells of the chicken intestinal tissues (Edelman *et al.*, 2002; Edelman *et al.*, 2003). Taking these published results into consideration, the frozen section method, if appropriately adapted, could be an alternative for studying the *Eimeria*-host cell attachment.

The main characteristic of frozen sections is that limited changes occur to the surface of the cell membrane during the freezing process because no fixative, alcohol or high temperature are needed (Deplancke and Gaskins, 2001). In addition, in this method *Eimeria* sporozoites can be applied to sections at high density in a small volume. However, there are no published studies that report the use of frozen sections to study the protozoan-host cell interaction *in vitro*. The objective of the study reported here was the development of a frozen section method that might allow meaningful study *ex vivo* of *Eimeria*-enterocyte interaction.

### 4.5.2 Materials and methods

#### 4.5.2.1 Selecting animals

Newly hatched chickens were purchased and killed (section 2.2.1) and the caecal segments were collected (section 2.2.2).

#### 4.5.2.2 Preparation and staining of sporozoites

The purified *Eimeria tenella* oocysts (section 2.4.1) were used to prepare sporocysts (section 3.2.2.3) and sporozoites (section 2.4.5.2). The purified sporozoites were stained using a PKH-67 kit (section 2.4.5.4). The viability of sporozoites was assessed with propidium iodide (section 2.4.5.3) and kept at 4°C for a maximum of 1 hour.

#### 4.5.2.3 Frozen segments

Caecal samples were washed twice with PBS (section 2.2.2). Segments were then embedded in Tissue-Tek Compound (OCT, ProsciTech, Australia) and snap-frozen in liquid nitrogen. Frozen samples were stored at –80°C.

#### 4.5.2.4 Frozen sectioning

Frozen segments were placed in a Leica cryostat (Cambridge Instruments, GmBH, Germany) at –20°C for one hour prior to sectioning. Different caecal sections (6-14 μm) were cut from each tissue block derived from each chicken and placed on glass slides. A 10 μm section was cut and stained with 4% trypan blue for 5 minutes to test the epithelial cell viability in each frozen tissue block.

#### 4.5.2.5 Preparation of chamber

A chamber with a 0.5 $cm^2$ surface area was made to enclose each frozen section by gluing together four pieces of glass slides using paraffin wax.

#### 4.5.2.6 Binding assays

Three sections were placed onto a single glass slide; each section cut from a different caecal block. Multiple slides were prepared in this manner. Sections were incubated in MEM containing 10% FBS for 15 minutes to prevent non-specific attachments between *E.* sporozoites and host cells. The binding assay was started by adding a suspension of *E. tenella* sporozoites to the chamber enclosing the three sections on any given slide. Sporozoite densities of 0, 1 x $10^4$, 2 x $10^4$ and 4 x $10^4$ per ml were used to find optimum binding conditions, and all sections were incubated for 60 minutes in a $CO_2$ incubator at 41°C. Assays were stopped by fixing samples for 1 hour with 10% neutral buffered formalin, and the slides were stored at 4°C for a maximum 12 hours (Tierney *et al.*, 2004).

#### 4.5.2.7 Quantification of sporozoite binding

The average length of epithelium per field of view in each section was determined from ten individual fields of view (section 4.1.2.4.1). The number of *E. tenella* sporozoites attached to

epithelial cells was counted over 40 fields of view at x 40. The number of sporozoites per mm epithelial length was calculated (Augustine *et al.*, 1997; Tierney *et al.*, 2004).

#### 4.5.2.8 Statistical analysis

The statistical analysis was undertaken using SAS Proc GLM (SAS Institute Inc, 2006). The model fitted included replicate, organ and binding, all considered to be fixed categorical effects. All first order interactions were tested, except that only one bird was present in one replicate and so it was not sensible to test this interaction. Non-significant ($P > 0.05$) effects and interaction were removed one at a time by backward elimination leaving only significant interactions and main effects. This model was undertaken to ensure the marginality requirements as described in section 4.1.2.6 were obtained.

### 4.5.3 Results

Results of four experimental runs, each with three repeats, showed that *E. tenella* sporozoites could attach to and penetrate into caecal epithelial cells (Figure 4.3). The thickness of tissue section needed to be 14 μm to provide enough surface area for the attachment of *E. tenella* sporozoites to enterocytes. The use of trypan blue to assess the viability of epithelial cells in the frozen sections showed that there were no viable cells in sections prepared from each tissue block.

Compared with the negative control, the sections exposed to *E. tenella* sporozoites had significant ($P < 0.01$) numbers of sporozoites binding to the epithelial cell layer. Furthermore, the observed binding was dose-dependent ($P < 0.01$) up to $2 \times 10^4$ cells per ml. In contrast, the binding between $2 \times 10^4$ and $4 \times 10^4$ *E. tenella* sporozoites was not significant. Therefore, the best number of sporozoites for each replicate was $2 \times 10^4$ for the attachment study (Figure 4.3).

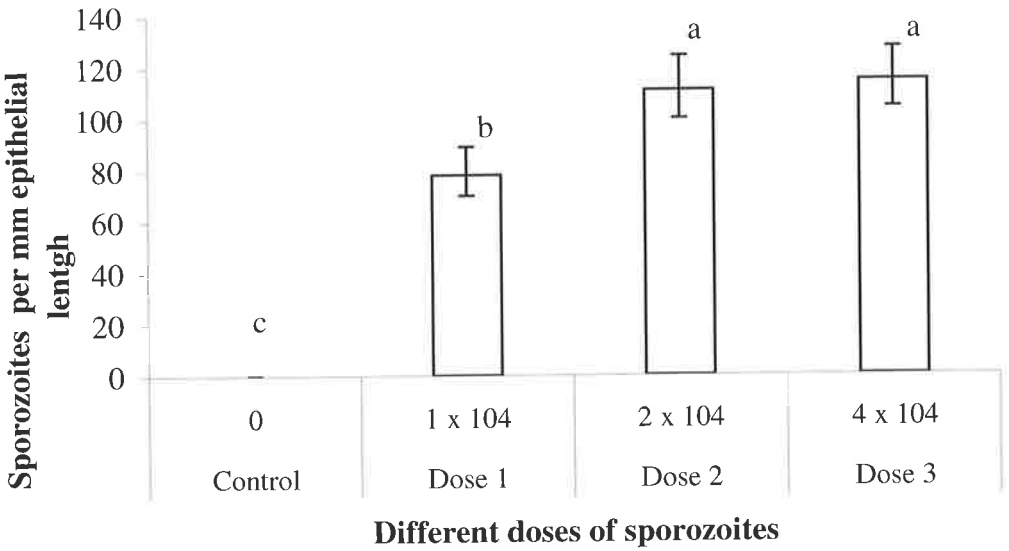


**Figure 4.1** The dose dependence of *E. tenella* sporozoites attaching to each mm length of epithelium of frozen caecal sections. Mean values with unlike superscripts (a, b, c) are significantly different from control group.

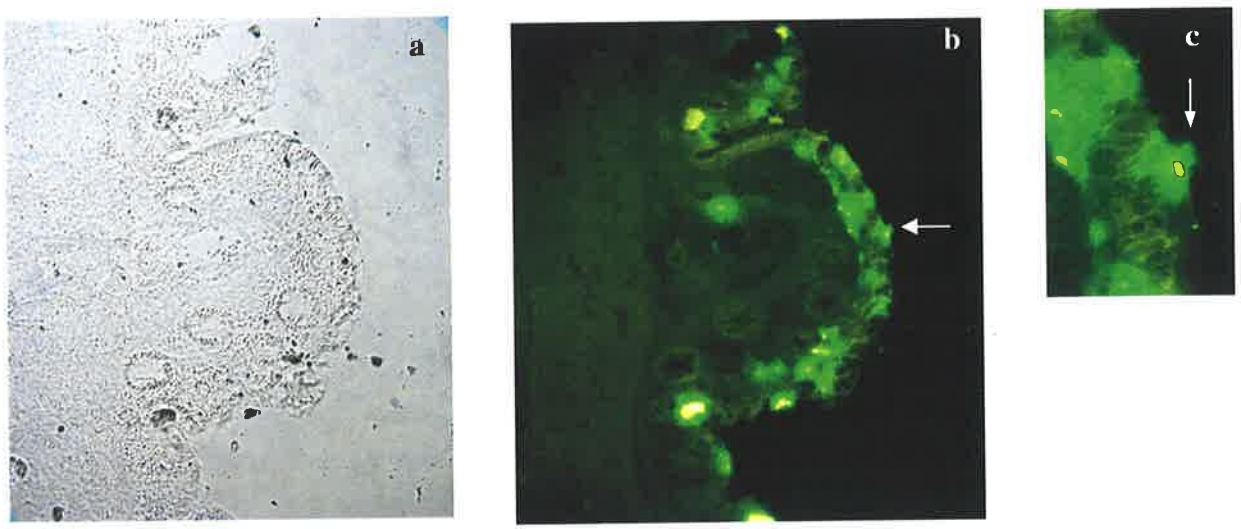


**Plate 4.3** The attachment of *Eimeria tenella* sporozoites to caecal epithelium in frozen section. a) Phase contrast view of a typical frozen section, b) The same section as viewed under fluorescent light (x 20), c) A digital enlargement of the area marked by the arrow in plate b.

### 4.5.4 Discussion

This is the first reported use of frozen sections to study *Eimeria* binding to enterocytes because the use of the target host cells previously was not possible due to the lack of a suitable methodology. Clearly, the use of frozen sections removes this impediment, allowing the study of the *Eimeria*-host cell attachment *in vitro* under similar conditions to those observed *in vivo*. The assay conditions reported here for protozoan parasites extend the use of frozen sections for the study of parasite-host interaction, as previously reported for bacteria ( Korhonen *et al.*, 1990; Edelman *et al.*, 2002; Edelman *et al.*, 2003).

The viability and active metabolic capacity of host cells do not appear to be the main factors that determine the interaction between *Eimeria* sporozoites and the epithelium. Related results, with the use of non-viable cells, were reported by Joe *et al.* (1998), and Chen and LaRusso (2000), who demonstrated that *Cryptosporidium* sporozoites could attach to cultured cells fixed by 4% paraformaldehyde. The result of these studies, taken together with those reported by Nowicki *et al.* (1986), Dozois *et al.* (1995) and Edelman *et al.* (2002 and 2003), all indicated that host cell viability and subsequently cell metabolites are not involved in the attachment of micro-organisms to host cells.

The major modifications reported in this study, as compared with the assay conditions used by Nowicki *et al.* (1986), Korhonen *et al.* (1990) and Edelman (2003), were: section thickness, incubation temperature (41°C) and elimination of a pre-fixative. In this study, the increase of the tissue section thickness was necessary to provide enough surface area for the attachment of *E. tenella* sporozoites to enterocytes (data not shown). Sporozoites require a temperature of 41°C to maintain activity and attachment to host cells, as previously reported by Tierney and Mulcahy (2003). The final modification was the elimination of any pre-fixative. This was necessary because the use of pre-fixative prevented the binding of *E. tenella* sporozoites to enterocytes.

The chicken intestine *in vivo* is a complex environment and includes digested food, bile salts, microflora and mucus that confound the study of individual factors. Clearly, frozen sections provide a tool for studying each of these complex compounds from the chicken intestine without interference.

### 4.5.5 Conclusion

The frozen section method is an effective tool for studying *Eimeria*-enterocyte interactions *in vitro*. This method more accurately reflects *in vivo* conditions as compared to those necessary for cell culture systems.

# CHAPTER 5: FACTORS INFLUENCING THE ATTACHMENT OF *EIMERIA TENELLA* TO SPECIFIC SITES OF THE INTESTINE

# 5 Introduction

The invasion of *Eimeria* into intestinal enterocytes occurs in a complex environment, including gastric secretion, bile salt, microbial flora, digested food and mucus layer. The mucus layer is present along the luminal surface of the chicken intestine. Its composition and thickness are variable in different parts of the intestine. This layer plays an important barrier role in the non-specific defence, preventing pathogenic micro-organisms entering the lymph-blood system (Farhadi *et al.*, 2003). *Eimeria* sporozoites have to cross this complex environment to invade intestinal epithelial cells that are the preferred host for their infection and subsequent life cycle.

Different established cell lines, including Madin Darby Bovine Kidney (MDBK) cells, chicken kidney cells and chicken enterocytes have been previously utilized for studying how *Eimeria* sporozoites interact with host cells (Augustine, 2001b; Tierney and Mulcahy, 2003). Interestingly, all species of *Eimeria* can invade these cell lines. For example, *E. tenella* and *E. acervulina* sporozoites can attach to and penetrate cultured enterocytes derived from the chicken small intestine. In addition, the results from the experiments 4.3 and 4.4, and those reported by others (Zhang *et al.*, 1997; Tierney and Mulcahy, 2003; Van Immerseel *et al.*, 2004), indicates that either a period in, or passage through, cell culture is necessary to restore the cell vulnerability to invasion by coccidian protozoa. Thus, the use of the cultured cells cannot explain the reason for the site specificity of the *Eimeria* attachment in chickens. Alternatively, the complex environment of the chicken intestine *in vivo* does not allow determination of which component of the intestinal environment is responsible for the site specificity of *Eimeria* species infection.

To date, the lack of a suitable *in vitro* method that uses chicken intestinal tissue without affecting its characteristics and chemical composition has been a major obstacle in determining what role mucus and cell membranes play in the *Eimeria* interaction. The development of the frozen section method described in section 4.5 also allows for the first time, a meaningful assessment of the *Eimeria*-enterocyte interaction/mucus *ex vivo*. The objectives of this chapter were to determine the respective influences of intestinal enterocytes and mucus in the *Eimeria*-host cell interaction using the frozen section method.

# Experiment 5.1 Attachment of *Eimeria tenella* sporozoites to their preferred and non-preferred sites of infection

## 5.1.1 Introduction

*Eimeria* species are highly host-specific and complete their life cycle in one host (Long and Millard, 1976). In addition to host specificity, each *Eimeria* species is found in a particular region of the chicken intestine (Augustine and Danforth, 1986; Augustine and Danforth, 1990; McDougald *et al.*, 1996). For example, *E. tenella* invades caecum, and *E. acervulina* infects duodenum (Chapter 1). This site specificity of *Eimeria* species indicates that each species has a unique set of requirements for successful attachment and invasion of its host cell.

In contrast to the situation *in vivo*, the site and host specificity in *in vitro* studies is ambiguous. Sporozoites of different *Eimeria* species can invade either one type of cell culture or a variety of cell cultures derived from different organs and animals (Augustine, 1994, 1996, 2001b; Tierney and Mulcahy, 2003; Dimier-Poisson *et al.*, 2004). This indicates that the sporozoites of each *Eimeria* species have a lower degree of the site and host specificity. Sporozoites of *Eimeria* species differ significantly in their ability to invade various cell cultures derived from different tissue sources (Augustine, 2001b; Tierney and Mulcahy, 2003). Importantly, the differences of invasion had been observed in different strains of *Eimeria* species. For example, the ability of three different isolated strains of *E. tenella* expressed different capabilities to invade baby hamster kidney (BHK) cell cultures (Augustine, 2001b).

These variations between *in vitro* studies showed that the use of the different types of cell cultures could not provide a complete answer to the role of enterocyte membranes in the attachment of *Eimeria*. Together these studies indicated that the attachment and the invasion of *Eimeria* sporozoites to host cells differ between *in vivo* and *in vitro* studies and complicate our understanding of the mechanisms of *Eimeria*-host cell attachment.

The existence of receptor molecule(s) on the surface of host cells for invasion by *Eimeria* can be inferred by the inhibition of *Eimeria* attachment and invasion to cell cultures by different substances, including cationized ferritin and neuraminidase (Augustine and Danforth, 1984), monoclonal antibody (Augustine, 1991; Augustine, 1999; Constantinoiu *et al.*, 2003; Matsubayashi *et al.*, 2005a) and parasite homogenate (Johnson *et al.*, 2004). In addition, a monoclonal antibody

(11M-2) against *E. tenella* sporozoites was utilized to find their epitopes of attachment to different regions of the chicken intestine *in vivo* by Vervelde *et al.* (1993) who reported that these epitopes exist only on caecal epithelial cells to act as recognition molecules. Interestingly, when sporozoites were injected intravenously, intramuscularly and intraperitoneally, they produce infection in the same area of the intestine as expected via the oral ingestion of oocysts (Long and Millard, 1976). These studies suggest the role of receptor(s) in the *Eimeria* site specificity.

The objective of the present study was to investigate the possible role of the receptor in the interaction of *E tenella* to caecal enterocytes as the preferred site and duodenal enterocytes as a non-preferred site. This study elucidated why different *Eimeria* species choose different regions of chicken intestine for attachment and infection.

### 5.1.2 Materials and methods

#### 5.1.2.1 Selecting animals

This experiment was repeated three times. In each repeat, three newly-hatched chickens were killed and caeca and duodenum were immediately removed (section 2.2.1). The intestinal segments were opened and washed with PBS (section 2.2.2). Segments were embedded in OCT and snap-frozen in liquid nitrogen (section 4.5.2.3).

#### 5.1.2.2 Preparing and staining of sporozoites

The purified *Eimeria tenella* sporozoites (section 2.4.5.2) were stained using a PKH-67 kit and the viability of sporozoites was assessed with propidium iodide (section 4.5.2.2).

#### 5.1.2.3 Frozen sections

A 14 μm section was cut from each frozen tissue block derived from each chicken and placed on glass slides (sections 4.5.2.4 and 4.5.2.5). For each experiment three sections from three chickens were used for the binding assay. .

#### 5.1.2.4 Binding assays

The binding assay was started by adding a suspension containing 6 x $10^4$ *E. tenella* sporozoites to the chamber enclosing the three caecal or duodenal sections. The caecal and

duodenal sections with no added *E. tenella* sporozoites were used as a negative control. The sections were incubated in a 5% $CO_2$ incubator for 60 minutes at 41°C. The assays were stopped by fixing samples in 10% NBF (section 4.5.2.6).

#### 5.1.2.5 Quantification of binding

The number of sporozoites attached to each mm length of enterocytes in intestinal sections was counted (section 4.5.2.11).

#### 5.1.2.6 Statistical analysis

The statistical analysis was undertaken using SAS Proc GLM (SAS Institute Inc, 2006). The data were transformed by log natural to account for a scaling effect. The model fitted included replicate, organ and binding, all considered to be fixed categorical effects. All first order interactions were tested, except that only one bird was present in one replicate and so it was not sensible to test this interaction. Non-significant ($P > 0.05$) effects and interactions were removed one at a time by backward elimination leaving only significant interactions and main effects as described in section 4.1.2.6.

### 5.1.3 Result

The results showed that a large number of *E. tenella* sporozoites attached to the caecal enterocytes (110.3 ± 8.1, mean ± SE, per mm length of epithelium) in comparison with the limited number of sporozoites attached to duodenal enterocytes (2.1 ± 0.15 per mm length of epithelium) in the sections (Figure 5.1). The data analysis showed that the attachment of *E. tenella* sporozoites to caecal tissue sections was highly different ($P < 0.00001$) in comparison with its control groups. The attachment of *E. tenella* sporozoites to duodenal sections was different ($P < 0.01$) in comparison with the control groups. As the number of sporozoites was constant for all assays, the data analysis showed that there was a significant difference ($P < 0.0001$) between the attachment of *E. tenella* sporozoites to duodenal and caecal enterocytes (Figure 5.1).

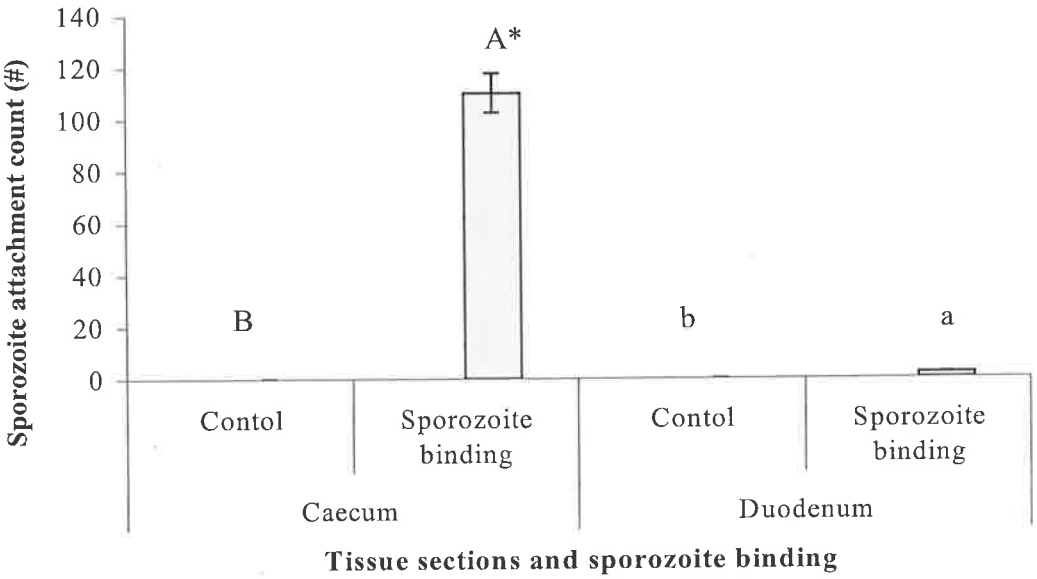


**Figure 5.1** The mean number of *Eimeria tenella* sporozoites attached to duodenal and caecal enterocytes per mm length of epithelial cells in frozen sections. Different capital letters (A, B) indicate significantly differences, in the caecal intestinal region, Different lower case letters (a, b) indicate significantly differences in the duodenal intestinal region from control group. The mean value for sporozoite binding to caecum (*) is significantly different from binding to duodenum.

### 5.1.4 Discussion

Based on the highly significant attachment of *Eimeria tenella* sporozoites to caecal enterocytes in comparison with duodenal enterocytes, the frozen section method in this study replicated the site specificity for *Eimeria tenella* attachment to caecal epithelial cells observed *in vivo* (Long, 1967; Long and Millard, 1976). This reinforces the idea that receptors on the cell membranes of caecal enterocytes could be the main factor causing *E. tenella* sporozoites to attach and penetrate caecal enterocytes. The role of a receptor in the *Eimeria* binding was reported by Augustine (1999 and 2001b) who showed inhibition of *Eimeria* attachment and invasion in treated cell cultures using a monoclonal antibody against the *Eimeria* refractile body. This monoclonal antibody reacted with discrete foci of baby hamster kidney cells. A similar role of an intestinal epithelial cell receptor (protein/lipid-like) in the binding of *Cryptosporidium parvum* to Caco-2 cells supported the present receptor for *Eimeria* attachment (Langer *et al.*, 2001; Johnson *et al.*, 2004). Alternatively, the lack of this receptor in other regions of the chicken intestine may be the

primary determinant of the resistance to attachment and hence infection. The existence of different receptors on the enterocyte membranes in each part of the chicken intestine is consistent with variations between membrane composition of enterocytes, including enzyme, residue carbohydrate and charged moieties (Smith and Lee, 1986; Mathis and McDougald, 1987; Pohlmeyer *et al.*, 2005). Therefore, the enterocyte composition of membranes is potentially important in *Eimeria* recognizing their preferred site of infection. Collectively, the present results and the *in vitro* and *in vivo* studies reported by others demonstrated that a receptor(s) on the surface of host cells might play an important role in recognition and attachment of *E. tenella* sporozoites. The receptor(s) may be the reason for the site specificity in the *Eimeria* infection.

The site specificity of *Eimeria tenella* in the chicken caecum was reported Shiotani *et al.* (1992). Other studies on the site specificity of infection with *E. praecox, E. maxima* and *E. acervulina* showed that no infection of chicken caeca was detected when sporozoites were inoculated directly into the chicken caeca (Long, 1967; Long and Millard, 1976). However, all of these studies could not determine which factor of the intestinal environment causes the site specificity. In addition, sporozoites of *Eimeria* species invade markedly different cell types *in vitro*. For example, the invasion by *Eimeria tenella* and *E. adenoeides* was greater in baby hamster kidney (BHK) and chicken caecal cell (CC) cultures than in primary chicken (PCK) or turkey kidney (PTK) cell cultures (Augustine, 2001b). Both *in vivo* and *in vitro* studies cannot discriminate the role of factors influencing the attachment of each *Eimeria* species in a specific region of the chicken intestine. In contrast, the frozen section method, that preserves the preferred and non-preferred sites without changes in their enterocyte composition, suggested that the receptor(s) are involved in the attachment of *Eimeria* to specific sites of the chicken intestine.

The limited attachment of *Eimeria* sporozoites to non-preferred locations reflects a non-specific binding, reported for the first time in this study. This finding is supported by work of Long and Millard (1976) and Augustine (2001a) who inoculated different numbers of motile sporozoites of *Eimeria acervulina* into the chicken caecum, and observed that a high number of sporozoites produced only a light infection in this region of the intestine that is not usually associated with that species of *Eimeria* (Long and Millard, 1976). The infection of *E. tenella* and *E. acervulina* in the turkey as a foreign host is similar to that observed in chicken intestine (Augustine and Danforth, 1990). In addition to the presence of receptors in host cells for *Eimeria*,

the complex environment of the chicken intestine, including mucus, pH and intestinal peristaltic movement, may affect the binding of *E. tenella* sporozoites to non-preferred sites. A similar effect of these factors was reported for the attachment of *Cryptosporidium* sporozoites to host cells *in vitro* (Hamer *et al.*, 1994; Chen and LaRusso, 2000). Furthermore, this unusual interaction of sporozoites to non-preferred sites may partly be due to the sporozoite motility (Bumstead and Tomley, 2000), the existence of non-specific binding sites and expression of low numbers of their preferred receptor.

### 5.1.5 Conclusion

According to these results, the reason for the site specificity of *Eimeria* species in chickens is the existence of a specific receptor on the surface of enterocyte membrane exposed to the luminal contents of the digestive tract.

## Experiment 5.2 Role of D-galactose and mucus in the *Eimeria*-enterocyte interaction

### 5.2.1 Introduction

The luminal surface of the gastrointestinal tract is coated with a thin layer of mucus (Samet and Cheng, 1994). This layer, depending upon intestinal region, differs in mucin glyco-proteins and the glycosylated and nonglycosylated domains (Deplancke and Gaskins, 2001) and has been reported to play an important role in the protection of epithelial cell and host defence (Zhao *et al.*, 2001; Nava *et al.*, 2005).

Many pathogenic bacteria can bind to intestinal mucus. The fate of pathogenic bacteria that bind to mucus can include removal with mucus flow, colonization within the mucus layer, and penetration of the mucus and adherence to underlying epithelial cells (Gusils *et al.*, 2004; Smirnov *et al.*, 2005). Binding sites on mucus may compare with receptors on epithelial cells, thereby retarding access of micro-organisms to the mucosal surface and thus favouring their removal. A similar function of the mucus has been observed for the attachment of *Cryptosporidium parvum* sporozoites to the epithelial cells of the chicken intestine (Joe *et al.*, 1994; Johnson *et al.*, 2004). In contrast to the substantial body of information that exists regarding the interaction of bacteria and

*Cryptosporidium* with intestinal mucus, relatively little is known about the function of the intestinal mucus in the attachment between *Eimeria* sporozoites and host cells. It is reasonable to assume that the mucus may affect the attachment of *Eimeria* sporozoite to host cells.

In addition, the sugar residues on the surface of host cells have important functions in the adhesion of protozoan and bacteria to host cells (Baba, 1993). Besides this role of mucus, Baba *et al.* (1996) reported that, from eight monosaccharides, only D-galactose could inhibit the penetration of *E. tenella* sporozoites into treated kidney cell cultures. However, there is no such assessment of the effect of D-galactose in the attachment of sporozoites to their intestinal target cells and also the biological characteristics of this sugar with enterocytes have not been described. The objectives of the present study were to investigate whether intestinal mucus along with D-galactose influence the attachment of *E. tenella* sporozoites to caecal enterocytes.

### 5.2.2 Materials and methods

#### 5.2.2.1 Selecting animals

This experiment was repeated three times. In each repeat, three newly hatched chickens were killed and caeca were immediately removed (section 2.2.1). Intestinal segments were frozen in liquid nitrogen (section 5.1.2.1).

#### 5.2.2.2 Frozen segments and sectioning

A 14 μm section was cut from each frozen tissue block derived from each chicken and placed on glass slides (section 4.5.2.4). For each experiment three sections from three chickens were used to study the binding assay. A chamber with a 0.5 $cm^2$ surface area was made to enclose each frozen section by gluing together four pieces of glass slides.

#### 5.2.2.3 Preparing and staining of sporozoites

*Eimeria* sporozoites were purified (section 2.4.5.3) and stained by PKH-67 (section 2.4.5.4). The sporozoite viability was determined (section 2.4.5.3).

#### 5.2.2.4 Preparation of intestinal mucus

The mucus sample was collected from six-week-old chickens that were free from coccidiosis, had not been vaccinated with any coccidia vaccine, and had anticoccidial drugs withheld for at least two week before killing. The luminal surface of chicken caecum and duodenum were scraped using glass slides. The mucus was washed twice with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-HBSS buffer (HEPES, pH 7.2, Sigma, Australia) and centrifuged at 6000 x g for 10 minutes to remove the cells from the mucus components. The glycoprotein of the mucus was quantified by measuring the protein concentration using a modified Bradford assay (Edelman *et al.*, 2003). The assay utilized the red form of Coomassie Brilliant Blue G-250 (CBB), which turns blue on binding to protein. The reaction was started by adding 1 ml of CBB to 25 ul of diluted sample (1/100 v/v) and reading at 595 nm after 5 minutes, with mucus-associated protein reported in mg protein/ml mucus.

#### 5.2.2.5 Binding assays

Three caecal sections derived from different chickens from different caecal blocks were placed onto a single glass slide for each replicate. Multiple slides were prepared in this manner. For each treatment and control, four different experimental repeats were performed. The experiment considered the binding of *E. tenella* sporozoites to sections of caecal tissue that were pre-treated 6 ways: with 1 mg caecal mucus (CM), 5 mg CM, 1 mg duodenal mucus (DM), 5 mg DM, 0.7% D-galactose and no pre-treatment (only HBSS buffer) as a control, all at the same pH (7) for 10 minutes at 20°C. The binding assay was started by adding 2 x $10^4$ *E. tenella* sporozoites to each treatment and control caecal section. These were incubated in a 5% $CO_2$ incubator at 41°C for 60 minutes. Assays were stopped by fixing samples (section 4.5.2.10). All treatments were compared with control assays under similar conditions.

#### 5.2.2.6 Quantification of *Eimeria* binding

The number of sporozoites attached to or penetrated into epithelial cells was counted (section 4.5.2.11).

### 5.2.2.7 Statistical analysis

For this experiment SAS Proc GLM (SAS Institute Inc, 2006) was used for analysis, as described in section 5.1.2.13.

## 5.2.3 Results

### 5.2.3.1 The effect of D-galactose on sporozoite attachment

The treatment of caecal sections with 0.7% D-galactose for 10 minutes before the onset of *E. tenella* sporozoites inhibited significantly ($P < 0.01$) the attachment of *E. tenella* sporozoites to caecal enterocytes in comparison with the control group (Figure 5.2).

### 5.2.3.2 The effect of mucus on sporozoite attachment

The differing doses (1 and 5 mg) of both duodenal and caecal mucus inhibited significantly ($P < 0.01$) the attachment of *E. tenella* sporozoites to caecal enterocytes in comparison with control group (Figure 5.2).

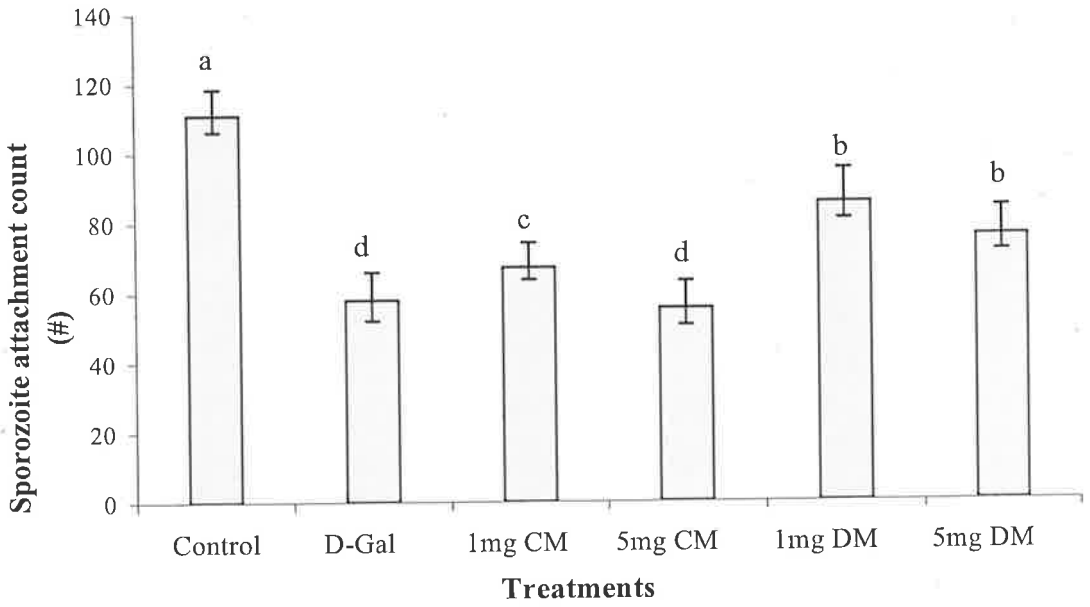


**Figure 5.2** The mean number of *E. tenella* sporozoite attached per mm length of enterocytes in caecal sections after treatment with 0.7% D-galactose, 1 mg CM, 5 mg CM, 1 mg DM, 5 mg DM, and in control group. Mean values with unlike superscripts (a, b, c and d) are significantly different between treatments.

### 5.2.4 Discussion

The significantly reduced attachment of *E. tenella* sporozoites to caecal enterocytes treated with D-galactose demonstrated that D-galactose residues on the surface of *E. tenella* sporozoites are involved in the attachment process to target cells. The reduction of attachment observed when the caecal tissues were pretreated with D-galactose indicates that D-galactose-binding sites are present on the surface of enterocytes. This result is similar to the attachment of *E. tenella* sporozoites to treated chicken kidney cells with D-galactose reported by Baba *et al.* (1996). In addition, such findings are consistent with the study by John *et al.* (1999) who demonstrated that L-fucose residues on the outer surface of *E. stiedai* sporozoites was important in their invasion of rabbit intestinal tissue. Such data indicate that the binding of such sugar residues on sporozoites to the binding sites on the surface of the host cells leads to attachment and penetration.

The incomplete inhibition (48%) of *Eimeria tenella* binding to treated caecal enterocytes with 0.7% D-galactose could be due to several reasons, including concentration of D-galactose and incubation time for tissue treatment. Additional experimentation is required to assess the dose and time for D-galactose to block the binding of sporozoites to caecal enterocytes.

The present findings taken together with those reported by Baba *et al.* (1996) and John *et al.* (1999) demonstrated the involvement of a sugar residue on the surface of *Eimeria* sporozoites for recognition of binding sites on the host cell. Specific carbohydrate receptors have also been reported to be involved in the adherence of *Plasmodium falciparum* (Friedman *et al.*, 1985; Rogerson *et al.*, 1995; Gaffar *et al.*, 2003; Winograd *et al.*, 2005) and *Trypanosoma cruzi* (Friedman *et al.*, 1985; Ming *et al.*, 1993; Agusti *et al.*, 2004) and bacteria (Baba *et al.*, 1992; Baba *et al.*, 1993) to their host cells. It can be concluded that D-galactose may be important in mediating binding of *Eimeria* sporozoites. In the case of *Eimeria tenella,* free D-galactose in the chicken caecum can result in reduction of attachments of *E.tenella* sporozoites to epithelial cells and consequently a reduction of its pathogenicity for the control of the disease. The free D-galactose could potentially be increased using a diet containing high D-galactose and/or microflora producing D-galactose from non-digestible compounds of the diet.

This study showed that the composition of caecal and duodenal mucus has no specific role in blocking the attachment of *E. tenella* sporozoites to the caecal enterocytes. If the caecal mucus plays a role in the *E. tenella* attachment to caecal enterocytes, adding the caecal mucus to caecal

sections should have significantly increase the binding of *E. tenella* to the caecal enterocytes in contrast to the duodenal mucus. However, the results obtained were not in agreement with the expected results as different concentrations of both caecal and duodenal mucus caused a significant reduction in the attachment of *E. tenella* sporozoites to host cells (Figure 5.2). Thus, the similar results of the reduced attachment of sporozoites to host cells by mucus demonstrated that mucus, irrespective of its origin, serves as a physical barrier in the non-specific defense against coccidiosis. This physical block complements the physiological barrier functions of the mucus reported by Deplancke *et al.* (2001), Soderholm and Perdue (2001), and Farhadi *et al.* (2003). These results are not in agreement with the role of the intestinal mucus as a receptor for adhering *Cryptosporidium parvum* sporozoites to the intestinal epithelial cells, as reported by Joe *et al.* (1994) Cevallos *et al.* (2000), Johnson *et al.* (2004), and Hashim *et al.* (2006).

### 5.2.5 Conclusion

Based on these results, D-galactose residues on the surface of *E. tenella* sporozoites may have an important roles for the attachment of *E. tenella* sporozoites to caecal enterocytes. The D-galactose is potentially a suitable way to prevent coccidiosis by *E. tenella* without using anticoccidial drugs and vaccines. In addition, the mucus is not a requirement for attachment of *E. tenella* sporozoites to their preferred region of intestine. That is, mucus acts merely as a physical barrier to *E. tenella* sporozoites.

# CHAPTER 6: SPECIFIC IDENTIFICATION OF *EIMERIA* SPOROZOITES IN SAMPLES BY PCR

## 6.1 Introduction

Being able to detect *Eimeria* during different stages in their life cycle would greatly assist the study of *Eimeria*-host cell interactions. Such studies are important for the development of methods to protect chickens against coccidiosis as well as preventing the spread of the disease. Three traditional methods of *Eimeria* detection have been used routinely for diagnosing coccidiosis using tissues and faecal samples: lesion scoring, microscopic examination, and histological examination.

Lesion scoring has been the most common method for identifying *Eimeria* infections. At post mortem, observed lesions in the intestine are scored according to the thickness of the intestinal wall, bleeding or the presence of red or white spots as well as the size of the region that has been affected. Each *Eimeria* species has characteristic lesions at specific sites in the intestinal tract. When a typical lesion of each *Eimeria* species is observed, the diagnosis of coccidiosis is simple with the lesion scoring method (Idris *et al.*, 1997b). Microscopic examination of oocysts is another method for diagnosis of coccidiosis. *Eimeria* at various stages of the life cycle are sieved from chicken faeces or obtained through mucosal scrapings (Johnson and Reid, 1970). A histological slide of *Eimeria* from the sample is examined microscopically to determine the morphological differences in the oocysts, schizonts, macrogametocytes or microgametocytes (Molloy *et al.*, 1998). Few species of *Eimeria* have been easily identified with this method. Histological examination is performed on intestinal tissue samples taken from birds with suspected coccidiosis. This requires the samples to be fixed, embedded, sectioned and then stained with the periodic-acid-Schiff reaction (Johnson and Reid, 1970). Sections are viewed under a light microscope to study the different stages of the *Eimeria* life cycle.

These conventional methods have limitations in terms of being time consuming and not capable of differentiating between *Eimeria* species. This is because there is overlap between the different *Eimeria* species in their morphological and pathological features, their predilection sites, the shape of the lesions and the characteristics of the endogenous stages in the infected tissue (Long and Joyner, 1984). Under the microscope, for most stages of the life cycle, it is impossible to identify a single *Eimeria* species due to the morphological similarities in the histological sections (Idris *et al.*, 1997a). These problems become more complicated when chickens are infected with multiple species of *Eimeria*.

The histological examination (section 4.2) could not identify the *Eimeria* sporozoites in the intestinal tissue even in an organ culture system. The results demonstrate that the identification of low numbers of *Eimeria* sporozoites in tissue requires the examination of many sections microscopically. Thus, a new and improved method with high sensitivity for *Eimeria* detection is needed to identify *Eimeria* species in intestinal tissues.

Molecular approaches to overcome the limitations of traditional methods have been examined (Woods *et al.*, 2000). One suitable method to identify *Eimeria* species has been to analyse enzyme variation through gel electrophoresis (Johnston and Fernando, 1997). However, this technique uses a limited number of variable enzymes. The low level of polymorphism in the enzymes makes the identification of *Eimeria* species problematic, and as such, is not a suitable method for the routine application to a large number of samples (Fernandez *et al.*, 2003a).

Consequently, polymerase chain reaction (PCR) has become the method of choice for *Eimeria* species identification. PCR is a technique for amplifying a specified region from a genome with the help of specific flanking primers and a DNA polymerase, under stringent temperature conditions. PCR has been used widely as a sensitive tool for diagnosis of many prokaryotic and eukaryotic pathogens ( Marzouk *et al.*, 1989; Rodgers *et al.*, 1990; Laxer *et al.*, 1991; Schnitzler *et al.*, 1999). Initially, the random amplified polymorphic DNA (RAPD) method based on the amplification of anonymous DNA targets was proposed as an alterative method to enzyme variation (Welsh and McClelland, 1990; Williams *et al.*, 1990). This method generates a fingerprint of multiple bands. RAPD-derived markers have been successfully developed for the molecular diagnosis of *Cryptosporidium parvum* (Morgan *et al.*, 1996) and *E. media* (Cere *et al.*, 1996). However, this approach has limited discrimination for the identification of different species of protozoan parasites due to the low specificity of the polymerase chain reaction (PCR) (Fernandez *et al.*, 2003a). To overcome this problem, Fernandez *et al.* (2003) prepared sequence-characterized amplified region (SCAR) markers, derived from RAPD fragmants and demonstrated that these markers can be used to identify the seven species of *Eimeria*. The SCAR primers are less sensitive to reaction conditions, allowing high reliability and reproducibility in different laboratories with different brands of reagents and equipment (Fernandez *et al.*, 2003a).

Several reports describe an alternate approach based on PCR assays that target different regions of the ribosomal cistrons, including the 5S rRNA (Stucki *et al.*, 1993), the small subunit

rRNA (Tsuji *et al.*, 1997), the ribosomal internal transcribed spacer 1 (ITS-1) (Schnitzler *et al.*, 1999), and spacer 2 (ITS-2) (Woods *et al.*, 2000; Gasser *et al.*, 2001). PCR with primers specific for the second internal transcribed spacer (ITS-2) of ribosomal DNA (rDNA) has been successfully used to differentiate between three species of *Eimeria; E. tenella*, *E. acervulina* and *E. maxima* (Lien *et al.*, 2005). In addition, a capillary electrophoretic approach was used to identify the seven species of *Eimeria* in faecal samples with a single primer set (Gasser *et al.*, 2005). Thus, PCR-based identification approaches have become increasingly utilized to distinguish coccidial parasites at the level of the species. PCR can be also utilized as a tool for the quality control of *Eimeria* vaccines, for determining maximal parasite reproduction after infection in chicken tissue, for the control of coccidiosis outbreaks and for high-throughput analysis of oocysts samples for epidemiological surveys. The next step is the application of this PCR technology to detect *Eimeria* species in infected tissue or mucus to overcome the limitations of the traditional methods. The objective of this study was the use of PCR to detect two *Eimeria* species (*Eimeria tenella* and *E. acervulina*) bound to intestinal mucus and tissue. PCR titration assays were performed to evaluate the minimum amount of parasite DNA that could be detected in these samples.

## 6.2 Materials and methods

### 6.2.1 Selecting animals

Three newly hatched chickens were infected orally with oocysts of *E. tenella* ($5 \times 10^3$) or *E. acervulina* ($5 \times 10^3$). Chickens were killed by cervical dislocation 12 hours after oocyst inucolation as a certain point post infection due to asexual replications of *E.* sporozoites. The caeca and duodenum were immediately removed and placed in cold PBS. Each intestinal segment was opened and washed twice with PBS (section 2.2.2). The samples were stored at –20°C for three weeks until the extraction of the DNA. Three uninfected newly hatched chickens were also killed as a negative control. The samples were collected and stored as described for infected chickens.

### 6.2.2 Preparation of sporozoites

*Eimeria* oocysts were purified (section 2.4.1). The sporocysts were prepared with the sonication method (section 3.2.3.1). Sporozoites were extracted from sporocysts using HBSS (section 3.4.2.3).

### 6.2.3 Binding sporozoites to intestinal mucus

A chamber with a 0.5 $cm^2$ (1 x 0.5 cm) surface area was made to enclose either caecal or duodenal chicken mucus by gluing four pieces of a glass slide to a slide with paraffin wax. Caecal or duodenal mucus was placed in the chamber and stored in a 20°C oven for 16 hours to concentrate the mucus. A 0.2 ml aliquot of MEM, containing $10^5$ *E. tenella* or *E. acervulina* sporozoites, was added to each chamber containing caecal or duodenal mucus and incubated in a $CO_2$ incubator at 41°C for 30 minutes. The mucus without *E.* sporozoites was used as a negative control. The surface of the mucus was washed gently twice with PBS and the mucus was collected to extract the *Eimeria* DNA.

### 6.2.4 DNA extraction

A QIAmp® DNA mini kit (Qiagen DNeasy, Australia) was used to extract the DNA from pure *Eimeria* oocysts, mucus (caecal or duodenal mucus with *E. tenella* sporozoites), infected tissue (chicken caecum and duodenum), and uninfected tissue as a negative control (chicken caecum and duodenum). DNA from $10^5$ *Eimeria* sporozoites and 35 mg of tissue derived from infected or uninfected chickens was prepared in parallel using the following procedure (Su *et al.*, 2003).

Samples were homogenised with an Ultra Turrax T25 homogeniser (Janke IKA, Australia) for 1 minute and sonicated with an ultrasound sonicator (Misonix, USA) at 17 watt (output) for 60 seconds. Each sample, 35 mg, was placed in a 1.5 ml Eppendorf tube and 180 µl of ATL buffer (Qiagen DNeasy, Australia) was added to the tissue and mixed by vortexing for 15 seconds. A 20 µl aliquot of Proteinase K (Qiagen DNeasy, Australia) was added to each sample and mixed for 15 seconds. The suspension was incubated in a 55°C water bath for two hours. The sample was vortexed for 15 seconds and 200 µl AL buffer (Qiagen DNeasy, Australia) was added. The sample was incubated in a 70°C water bath for 15 minutes. A 200 µl ethanol (100%) aliquot was added to each sample and vortexed for 15 seconds. The samples were placed on DNAeasy mini columns, which were supported by 2 ml collection tubes, and centrifuged at 5000 xg for 1 minute. The mini columns were placed in new collection tubes and 500 µl AW1 buffer (Qiagen DNeasy, Australia) was added to the column and centrifuged at 5000 xg for 1 minute. Following this, a 500 µl aliquot of the AW2 buffer (Qiagen DNeasy, Australia) was added to each sample and the samples

centrifuged at 15000 xg for 3 minutes. The aliquot of DNA was collected by adding 200 µl AE buffer (Qiagen DNeasy, Australia) to the mini column and centrifuging at 5000 xg for 1 minute. The DNA was stored at 4°C until assayed.

### 6.2.5 DNA concentration and quality

The concentration and quality of DNA were measured with a Shimadzu UV-260 spectrophotometer in 10 mm path-length 0.5 ml quartz glass cuvettes. The optical density (OD) of extracted DNA samples was measured at 260 nm and 280 nm against the AE buffer blank. When the OD ratio (260 nm: 280 nm) was between 1.2 and 1.6, the DNA was regarded of sufficient quality for PCR amplification.

### 6.2.6 Oligonucleotides

Specific primers for *E. tenella* and *E. acervulina* were obtained from published research (Table 6.1) (Fernandez *et al.*, 2003a). Positive control primers were designed for the chicken growth factor gene to identify the presence of chicken DNA in the samples. Primers were designed using the BioManager software (Primer 3 program) (Table 6.1). The primers were manufactured by GeneWorks Ltd (South Australia).

**Table 6.1** Oligonucleotide sequences of *E. tenella*, *E. acervulina* and chicken growth factor primers.

| Species | Primers Sequences | Amplicon size |
|---|---|---|
| ***E. tenella*** | F: 5'-AGTCAGCCACAGCGGGGATG-3'<br>R: 5'-AGTCAGCCACGTACTATCTAAACCAACCA-3' | 525 |
| ***E. acervulina*** | F: 5'-AGTCAGCCACACAATAATGGCAAACATG-3'<br>R: 5'-AGTCAGCCACAGCGAAAGACGTATGTG-3' | 750 |
| **Chicken growth factor** | F: 5'-GCCAGCCATGACAACTACAA-3'<br>R: 5'-TGGGACAGGCATTTCCATAC-3' | 750 |

### 6.2.7 Optimisation of PCR conditions

PCR conditions for the above-mentioned *Eimeria* primers were optimised in order to amplify a single product. PCR was performed at different DNA concentrations in a 96-well PCR

plate (Sigma, Australia) in a standard 25 µl reaction of 1.25 mM dNTPs (Promega, USA), 2.5 pmol forward primers, 2.5 pmol reverse primers, 2.5 mM $MgCl_2$ (Promega, USA), 80 ng DNA template, and 0.5 IU Ampli Taq Gold DNA polymerase enzyme (Roche, USA). The reactions were overlaid with mineral oil (Sigma, Australia) and amplified using an Omnigene HBTR3CM thermal cycler (Cambridge Scientific Products, UK) using the following PCR program: Step 1: one cycle: 94 °C for 7 minutes, Step 2: 35 cycles: 94°C for 1 minute, annealing temperature 60°C for 1 minute, and 72°C for 1 minute (the annealing temperature was decreased from 60°C by 2°C per cycle until final annealing temperature was 50°C) and Step 3: one cycle: 72 °C for 10 minutes.

Different $MgCl_2$ concentrations were used (1.5, 2, 2.5, 3 and 3.5 mM) in combination with different primers concentrations (25, 12.5 5, 2.5, 1.5 and 1 pmol) in order to obtain a single PCR product.

### 6.2.8 Electrophoresis and staining of PCR reactions

A 2% agarose gel (Progen, Australia) in 60 ml Tris Acetate-EDTA buffer (TAE, Sigma, Australia) was used for electrophoresis. A 1 µl aliquot of blue/orange tracking loading dye (Promega, Australia) was added to 10 µl of each PCR. One µl pGEM® diluted DNA Markers (Promega, Australia) was used as a standard to measure the size of the PCR products. The gels were run in 1 x TAE buffer at 110 volts for 50 minutes. The gels were stained with 0.5 µg/ml ethidium bromide (Molecular Probes, USA) for 20 minutes. The DNA bands were visualised with a Gel Doc System (image analyses software Quant1 4.1.0, Bio-RAD).

## 6.3 Results

### 6.3.1 Optimisation of PCR conditions

*Eimeria tenella*, *E. acervulina* and chicken intestinal tissue DNA were amplified and produced a single band in different PCR reactions with different concentrations of $MgCl_2$ and the specific primers (Table 6.2). Primers of both *Eimeria* species were tested for detecting *Eimeria* DNA in the tissue-*Eimeria* mix, infected tissue and mucus containing *Eimeria*. PCR results for *E. tenella* in such assays were successful at 2.5 mM $MgCl_2$ and 2.5 pM primer concentrations. A concentration of 2.5 mM $MgCl_2$ and 1.5 pM *E. acervulina* primers were required for detecting pure *E. acervulina* DNA. However, these conditions did not amplify the *Eimeria* DNA in an *Eimeria*-

duodenum tissue mix and infected tissue. To determine which primer concentrations can amplify the *E. acervulina* in the tissue, different primers concentrations (1, 1.5, 2 and 2.5 pM) were used in combination with 2.5 mM $MgCl_2$. The reaction results showed that 2.5 mM $MgCl_2$ and 1 pM of primer were optimal for amlifying *E. acervulina* DNA in such samples. Thus, the optimal amplification conditions for PCR of *E. tenella* and chicken growth factor gene were 2.5 mM $MgCl_2$ and 2.5 pM primers and for *E. acervulina* were 2.5 mM $MgCl_2$ and 1 pM primer concentration.

**Table 6.2** Determination of optimal PCR conditions for *Eimeria tenella*, *E. acervulina* and chicken growth factor gene primers.

| **Primers** | **Mg concentration (mM)** | | | | | **Primer concentration (pM)** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1.5 | 2 | 2.5 | 3 | 3.5 | 1 | 1.5 | 2.5 | 5 | 12.5 | 25 |
| ***E. tenella*** | - | - | + | + | - | - | + | + | - | - | - |
| ***E. acervulina*** | - | - | + | + | - | + | + | + | - | - | - |
| **Chicken** | - | - | + | + | - | - | - | + | - | - | - |

+ = Reaction was positive, - = Reaction was negative.

To determine the specificity of each primer set, PCR was performed using *E. tenella, E. acervulina* and chicken growth factor gene primers alone and with non-target DNA (*E. tenella, E. acervulina* and chicken intestine DNA). These results showed that there is no cross-reaction between the non-target DNA with each primer set or contamination with each primer set alone (lane 1-9, Figure 6.1). In contrast, the amplification with each primer set and its specific target DNA produced a single band of the expected size (lanes 10-12, Figure 6.1).

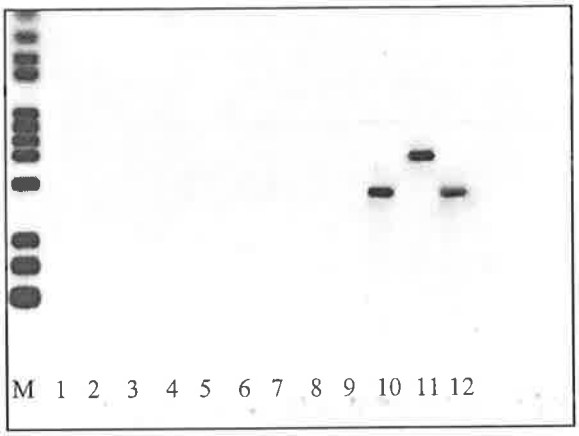


**Figure 6.1** Specificity of *Eimeria tenella* and *E. acervulina* primers with *E. tenella* and *E. acervulina* DNA. Lane M: DNA size markers, lane 1: *E. tenella* primers without DNA, lane 2: *E. acervulina* primers without DNA, lane 3: chicken growth factor gene primers without DNA, lane 4: *E. tenella* DNA with *E. acervulina* primers, lane 5: *E. acervulina* DNA with *E. tenella* primers, lane 6: uninfected chicken DNA with *E. tenella* primers, lane 7: uninfected chicken DNA with *E. acervulina* primers, lane 8: *E. tenella* DNA with chicken growth factor gene primers, lane 9: *E. acervulina* DNA with chicken growth factor gene primers, lane 10: chicken intestinal DNA with chicken growth factor gene primers, lane 11: DNA from chicken infected with *E. tenella* and *E. tenella* primers, lane 12: DNA from chicken infected with *E. acervulina* and E. *acervulina* primers.

### 6.3.2 Sensitivity of PCR for purified *Eimeria* DNA

*Eimeria tenella* and *E. acervulina* DNA was amplified with their respective specific primers at different DNA concentrations of 10, 5, 2.5, 1, 0.5, 0.2, 0.1 and 0.05 ng/µl (lane 7-12 in Figure 6.2, lane 6-11 in Figure 6.3 and Table 6.3). The reaction was negative for concentrations of less than 0.05 ng/µl DNA for both *Eimeria* species (lanes 5-6 in Figure 6.2, lane 4-5 in Figure 6.3 and Table 6.3). If the concentration of extracted *Eimeria* DNA is divided by the number of sporozoites, the mean concentration of DNA is 80.5 fg per sporozoite. Thus, the detection limit for both *Eimeria* species was approximately 62 oocysts in each sample.

### 6.3.3 Identification of *Eimeria* species from infected tissue DNA

. DNA extracted from the caecum of chickens infected with *E. tenella* and from the duodenum of chickens infected with *E. acervulina* was amplified with the specific primers and the individual single products of the expected size were obtained (lanes 11-12 in Figure 6.1, lane 15 in Figure 6.2, lane 14 in Figure 6.3 and Table 6.3). DNA derived from caecum and duodenum of uninfected chickens as a negative control did not amplify with the *E. tenella* and *E. acervulina*

primers (lanes 6-7 in Figure 6.1). The DNA from both infected and non-infected chickens did amplify with the chicken growth hormone primers (for example, lane 15 in Figure 6.2).

**Table 6.3** Identification of *Eimeria tenella* and *E. acervulina* in purified oocysts, tissue and mucus by PCR.

| Exp | DNA | DNA concentration (ng) | | | | | | | | | | Primers |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 | 5 | 2.5 | 1 | 0.5 | 0.2 | 0.1 | 0.05 | 0.025 | 0.01 | |
| 1 | *E. t* | + | + | + | + | + | + | + | + | - | - | *E. t* |
| 2 | *E.a* | + | + | + | + | + | + | + | + | - | - | *E. a* |
| 3 | M. *Et*.T | + | + | + | + | + | ND | ND | ND | ND | ND | *E. t* |
| 4 | M.*Ea*.T | + | + | + | + | ND | ND | ND | ND | ND | ND | *E. a* |
| 5 | CI*Et* | + | + | ND | ND | ND | ND | ND | ND | ND | ND | *E. t* |
| 6 | DI*Ea* | + | + | ND | ND | ND | ND | ND | ND | ND | ND | *E. a* |

Exp = experiment number, *E. t = Eimeria tenella,* *E.a = Eimeria acervulina,* M.*E.t*.T = mixed *E. tenella* and chicken DNA, M. *Ea*. T = mixed *E. acervulina* and tissue DNA, CI*E*.t = caecum of infected chicken with *E. tenella,* DI*E.a* = duodenum of infected chicken with *E. acervulina,* + = single PCR band observed, - = Reaction was negative, ND = Not determined.

### 6.3.4 Identification of *Eimeria* sporozoites in mucus

DNA extracted from the caecal and duodenal mucus after binding with either *E. tenella* or *E. acervulina* sporozoites amplified with the respective specific primers and produced a single product of the expected size (lanes 13-14 in Figure 6.2, lanes 12-13 in Figure 6.3 and Table 6.3).

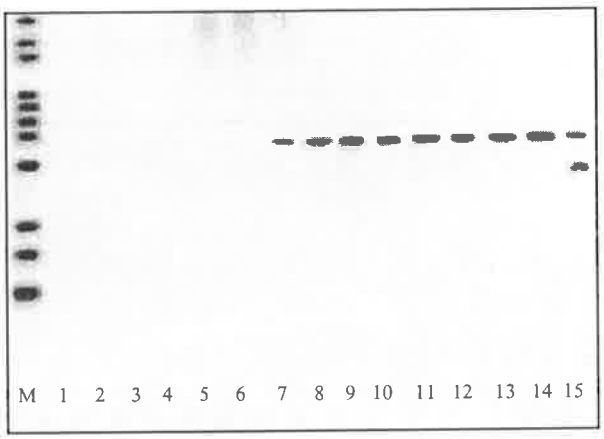


**Figure 6.2** Detection of *Eimeria tenella* (*E.t*) in purified oocysts, duodenal and caecum mucus and chicken caecum. Lane M: DNA marker, lane 1: Chicken growth factor gene primers without DNA, lane 2: *E. tenella* primers without DNA, lane 3: Infected chicken caecal DNA without primers, lane 4: 0 ng *E. t* DNA and *E. tenella* primers, lane 5: 0.01 ng *E. t* DNA and *E.t* primers, lane 6: 0.025 ng *E.t* DNA and *E.t* primers, lane 7: 0.05 ng *E.t* DNA and *E.t* primers, lane 8: 0.1 ng *E. t* DNA and *E.t* primers, lane 9: 0.25 ng *E. t* DNA and *E.t* primers, lane 10: 0.5 ng *E. t* DNA and *E.t* primers, lane 11: 1 ng *E. t* DNA and *E.t* primers, lane 12: 2.5 ng *E. t* DNA and *E.t* primers, lane 13: *E. t* in caecal mucus and *E.t* primers, lane 14: *E. tenella* sporozoites in duodenal mucus and *E.t* primers, lane 15: DNA from chicken infected with *E. tenella* using *E.t* primers and chicken growth factor gene primers.

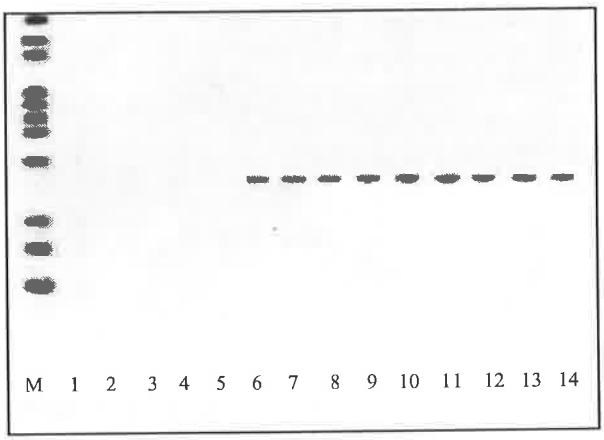


**Figure 6.3** Detection of *Eimeria acervulina* (*E.a*) in purified oocysts, duodenal and caecal mucus and chicken duodenum. Lane M: DNA marker, lane 1: *E. acervulina* primers without DNA, lane 2: Chicken duodenal DNA without primers, lane 3: 0 ng *E.a* DNA and *E.a* primers, lane 4: 0.01 ng *E.a* DNA and *E.a* primers, lane 5: 0.025 ng *E.a* DNA and *E.a* primers, lane 6: 0.05 ng *E.a* DNA and *E.a* primers, lane 7: 0.1 ng *E.a* DNA and *E.a* primers, lane 8: 0.25 ng *E.a* DNA and *E.a* primers, lane 9: 0.5 ng *E.a* DNA and *E.a* primers, lane 10: 1 ng *E.a* DNA and *E.a* primers, lane 11: 2.5 ng *E.a* DNA and *E.a* primers, lane 12: *E.a* in caecal mucus and *E.a* primers, lane 13: *E.a* in duodenal mucus and *E.a* primers, lane 14: DNA extracted from infected chicken with *E. acervulina* and *E.a* primers.

## 6.4 Discussion

The results from this study showed that PCR assays are a feasible option for early diagnosis of *Eimeria* infections and this is the first time that the application of SCAR markers for diagnosis *Eimeria* species in chicken intestinal tissue and mucus has been reported. Similar results with these *Eimeria* primers were obtained using DNA from oocysts of the seven species of *Eimeria* in individual reactions or multiplex reactions with all *Eimeria* primers and the DNA of seven species in a single-tube (Fernandez *et al.*, 2003a and b). In the present study, the *Eimeria* primer sets were successfully adapted to detect *Eimeria* species in chicken tissue and mucus. While the histological examinations (irrespective of the staining method used as described in section 4.2) were not successful in identifying the sporozoite-enterocyte interaction in the organ culture system, PCR can be utilized to identify *Eimeria tenella* and *E. acervulina* interactions with the intestinal epithelial cells using as little as 35 mg of tissue. This sample size represents more than a thousand histological sections. Thus, PCR can solve the problems of the histological methods and allow studies of the attachment of *Eimeria* species to different regions of the chicken intestine and the competition between different *Eimeria* species within a given intestinal region (for example, *E. acervulina* and *E. praecox* in the duodenum or *E. maxima* and *E. necatrix* in the jejunum). In addition, PCR can be performed to detect *Eimeria* sporozoites in intestinal mucus.

The sensitivity of assay was 0.05 ng/µl (62 oocysts) for detection of *Eimeria* DNA in samples. In contrast, the sensitivity of assay for detection of *Eimeria* species in the work reported by Fernandez *et al.* (2003b) was 8 oocysts in the sample. This difference is most likely due to the source of the DNA. The amount of DNA extracted from sporozoites in tissue could be less than expected, as the extracted DNA is mostly from chicken.

These findings demonstrate that PCR is potentially a suitable tool for diagnosis of chicken coccidiosis in different stages of the disease and determining the success of both chicken vaccination programs and chemoprophylaxis. PCR can be performed by collecting samples from chicken faeces or small sections of different intestinal regions of chicken tissue at post mortem. Importantly, collecting samples from chicken faeces is a non-invasive test to diagnosis of sub-clinical infections and the resistance of *Eimeria* species to anticoccidial drugs. Isolating parasites from chicken faeces and the subsequent assay by PCR has been utilized to detect *Eimeria* and *Cryptosporidium* species (Fernandez *et al.*, 2003a; Matsubayashi *et al.*, 2005b; Trotz-Williams *et*

*al.*, 2005). If this could be simplified to eliminate the need to isolate parasites before DNA extraction, then a relatively simple, yet specific, field test could be developed to facilitate early preventive measures in order to reduce mortality and stress associated with coccidiosis.

Collectively, the results from the PCR-based diagnostic assays for the detection of *Eimeria* species described here and elsewhere (Fernandez *et al.*, 2003 a, b) suggest that SCAR markers could potentially be converted to quantitative assays by the use of fluorescent probes in real-time PCR. Such quantitative assays could be utilized for not only identifying which species are present but also the number of sporozoites in the samples. Another PCR-based test using capillary electrophoresis was recently reported by Gasser *et al.* (2001, 2005) for an ITS-2 target in rRNA of the *Eimeria* genus. The advantage of this assay is the use of a single set of primers that produces different amplicon sizes for each *Eimeria* species allowing the detection of the seven *Eimeria* species in a single-tube reaction of PCR. However, the capillary electrophoresis produces two bands for six species of *Eimeria* (except *E. tenella*) and requires an expensive DNA sequencer. Amplification of a single amplicon for individual *Eimeria* species generated by the method reported here and by Fernandez *et al.* (2003 a, b) is required if the assay is to be adapted for real-time PCR quantification of sporozoites numbers.

# CHAPTER 7: GENERAL DISCUSION

## 7.1 Characteristics of *Eimeria* sporozoites and host cells in the attachment process

The characteristics of both *Eimeria* sporozoites and host cells play important roles in their attachment to each other. These characteristics are: 1) the presence of D-galactose residues on the surface of *Eimeria tenella* sporozoites and 2) receptors on the surface of enterocytes.

The role of D-galactose in *Eimeria* binding, as observed in the present study, is in agreement with reports where chicken kidney cells were used to study the *E. tenella* attachment (Baba *et al.*, 1996). The difference between these studies is the cell type used in the methodology. The use of caecal enterocytes in this study confirms the role of the D-galactose in the binding of *Eimeria tenella* sporozoites to caecal enterocytes *ex vivo*.

It is known that carbohydrates on the surface of host cells have important roles for recognition of receptor sites and hence adhesion of *Cryptosporidium* (Joe *et al.*, 1998; Joe *et al.*, 1994; Chen and LaRusso, 2000; Hashim *et al.*, 2006), *Plasmodium* (Breuer *et al.*, 1983; Gaffar *et al.*, 2003), *Trypanosoma* (Ming *et al.*, 1993; Agusti *et al.*, 2004) and *Entamoeba* (Adler *et al.*, 1995; Boettner *et al.*, 2002; Yi *et al.*, 1998). A similar phenomenon has been observed in bacterial invasion to host cells (Jones and Richardson, 1981; Baba *et al.*, 1993; Baumler *et al.*, 1996). In contrast, the findings in the present study, and that of Baba *et al.* (1996), suggest that the distribution of the sugar moieties and their receptors is reversed for *Eimeria tenella* and host cell interaction. That is, the sugar moiety is on *Eimeria* rather than on the host cell. This is dissimilar to other a*picompelexan* parasites and bacteria, indicating that the attachment mechanism in *Eimeria* differs from the use of other micro-organsisms. It can be concluded that the D-galactose may be a part of a glycoprotein, which adheres to a receptor (perhaps a lectin-like receptor) on the surface of caecal enterocytes. The role of D-galactose in the cell-cell attachment makes clear the existence of a carbohydrate moiety on the surface of *Eimeria* for attachment to enterocyte. Data from the present study (Experiments 5.1 and 5.2) and from other studies which report the inhibition by monoclonal antibodies of *Eimeria* species into host cell (Augustine and Danforth, 1985; Augustine, 1991; Vervelde *et al.*, 1993; Uchida *et al.*, 1997; Augustine, 1999, 2001b), also by cationized

ferritine and neuraminidase (Augustine and Danforth, 1984), all indicate that *Eimeria* sporozoites need to recognize a receptor on the surface of host cells for the attachment.

In general, free D-galactose in the chicken caecum may affect the attachment of *Eimeria* to epithelial cells. Indeed, this is consistent with the work of Harp (1999) who suggested that a high dose of sucrose or isomaltose administered orally in infant mice, either 24 hours before, or at the time of inoculation, with *Cryptosporidium* oocysts reduced Cryptosporidiosis (Harp, 1999). It is known that chickens fed with 5% menhaden oil, 15% flaxseed (Allen *et al.*, 1997) or sugar cane extracts (SCE) (El-Abasy *et al.*, 2003) have reduced *E. tenella* pathogenicity. It is likely that the composition of the diet can influence the *Eimeria* infection and activity of intestinal microflora (Djouzi and Andrieux, 1997; Smirnov *et al.*, 2005). Therefore, dietary fibre or non starch polysaccharides (NSP) (Cummings and Stephen, 1980; Cummings, 1981) and short chain oligosaccharides (Pomare *et al.*, 1985), that are not digested in the upper gastrointestinal tract, can be digested by hindgut microflora to liberate free monosaccharides such as D-galactose (Kay, 1982). It can be concluded that a dietary supplement of suitable carbohydrate composition could promote microfloral activity that releases galactose into the milieu of chicken caecum and thus reduce the attachment of the *Eimeria* sporozoite to the epithelial cell. A result of this should be a reduction in the incidence of disease produced by this organism.

## 7.2 The role of receptors in site specificity

It can be presumed that a receptor on the surface of caecal enterocytes is responsible for the site specificity of *E. tenella* infection in chickens. The development of the frozen tissue method that preserves both the integrity of the membrane as well as the tissue structure has allowed, for the first time, the distribution of binding sites to be elucidated. Results presented here, demonstrate the significant attachment of *E. tenella* sporozoites to caecal enterocytes in comparison with those to duodenal enterocytes (Experiment 5.1) and that the binding is blocked by D-galactose (Experiment 5.2). *Eimeria* sporozoites show a high degree of host specificity during invasion and infection in chickens. *Eimeria* species have been shown to infect a single host, selective organ, and type of cell (Vetterling, 1976; Calnek *et al.*, 1997; Augustine, 2001a). Indeed, the direct inoculation of chicken caeca or an intraperitoneal injection with sporozoites of *Eimeria* from species other than *E. tenella* failed to produce clinical disease in caecal tissue (Long, 1967; Long and Millard, 1976).

This is further supported by the observed differences in the penetration of cultured cells derived from various tissue types by *E. tenella* sporozoites (Augustine, 2001b; Tierney and Mulcahy, 2003). However, it is difficult to differentiate between environmental / luminal factors and presence of receptors on the cell surface as the primary determinant that controls *E. tenella* binding. These limitations were overcome by the frozen section method reported here, since the media and *Eimeria* species can be modified in a highly controlled manner and applied directly to tissue samples in which the membranes and structure are preserved.

The metabolic products of host cells, and the time taken to release sporozoites from the oocysts, have been implicated in determining the site specificity of sporozoite attachment in chicken intestine (Farr and Doran, 1962; Bumstead and Tomley, 1997; Tomley *et al.*, 1997). However, the binding of *Eimeria tenella* sporozoites to frozen sections, the cells of which were shown to be non viable (Experiment 4.5), demonstrate that an active metabolic capacity is not necessary for the interaction of *Eimeria* sporozoites with the enterocytes. In addition, the repeated washing of sections, along with the use of defined incubation media, indicates that the attachment site is a membrane-anchored system rather than a labile receptor. This finding showed that cell viability or a chemotactic agent released by the enterocytes is not a significant factor in the attachment of sporozoites to host cells. A similar result was observed in using different conditioned media derived from primary and immortalized cell cultures of avian and mammalian origin (Augustine and Jenkins, 1998).

The use of the frozen section method has also suggested the idea of a differential release of sporozoites from *Eimeria* species being the determinant of regional attachment to be tested. In this study, isolated *E. tenella* sporozoites were applied directly to either duodenal or caecal tissue sections, with incubation times being the same for both tissues. The overall results were an observed binding pattern that is the same in tissue sections as the distribution of lesions observed *in vivo*. Indeed, this supports the work of Shiotani *et al.* (1992) who demonstrated that after simultaneous administration of *E. tenella* and *E. maxima* oocysts to any given chicken, *E. tenella* sporozoites can be isolated from caecal contents one hour before sporozoites of *E. maxima* appear in the luminal contents of the jejunum.

In summary, different sites for *Eimeria* infection and clinical disease are determined primarily by the ability of the sporozoites to bind to the enterocytes rather than luminal factors or the fragility of the oocyst membrane.

## 7.3 The role of intestinal mucus in the attachment

The intestinal mucus does not appear to act as a receptor for *Eimeria* sporozoite adhesion in contrast with the specific binding site observed for *Cryptosporidium* (Thea *et al.*, 1992, Joe *et al.*, 1994, Barnes *et al.*, 1998, Cevallos *et al.*, 2000; Johnson *et al* 2004), and some bacterial species (Gusils *et al.*, 2004; Smirnov *et al.*, 2005). This conclusion on the role of mucus in *Eimeria* species infections is based on the marked differences between expected and obtained results reported for Experiment 5.2. The action of mucus in reducing the binding of *E. tenella* sporozoites was not affected by the origin of the mucus. Both caecal and duodenal mucus inhibited the *Eimeria* attachment by 40-50% compared with controls. Indeed, if mucus was the determinant for *Eimeria* binding and site specificity then application of caecal mucus to caecal tissue should have resulted in a significant enhancement in the adhesion of *E. tenella* sporozoites, which was not observed. Data from the present study, when considered with those reported by others, indicate that the role of mucus in the intestinal barrier varies from a simple physical block to specific binding sites relating to chemical composition of the glycoprotein(s) that make up the mucus.

Oligosaccharide units comprise approximately 80% of the mucin glycoproteins, which account for about 5% of the total weight of mucus. The carbohydrates are bound to N-acetylgalactosamine and radiate out like the bristles of a bottle-brush with the dominant sugars of mucus being N-acetylgalactosamine, N-acetylglucosamine, galactose, fucose and sialic acid (Cunningham, 1997). The inhibitory effect of mucus may be due to the D-galactose content since this sugar, when applied in solution to sections, reduces *E. tenella* binding. However, the D-galactose associated with mucin is held in complex structures that effectively reduce its availability and hence potential to bind to sites on the enterocytes. This argument is further supported by the fact that *E. tenella* binding is blocked by low concentration of mucus (1 mg) when applied to tissue sections.

In the case of the pathogen, *E. tenella*, it is more likely that mucus is a physical barrier to infection. The robustness of this barrier will depend on its physiochemical properties that are

known to vary with both the age and diet of chickens (Sharma *et al.*, 1997; Deplancke and Gaskins, 2001; Soderholm and Perdue, 2001; Smirnov *et al.*, 2004; Pohlmeyer *et al.*, 2005). An appropriate physical barrier would limit the access of the *E. tenella* to the underlying epithelium and may be a critical factor in determining an adequate signal to stimulate the mucosal immune system. Understanding how to manipulate the mucus component of the intestinal barrier may help in both the passive protection against disease as well as facilitation of oral vaccination procedures.

## 7.4 The effect of cell type on the attachment

The methods of preparation of host cells that are used for *in vitro* models of sporozoite attachment have to be selected with great care. This is because the mechanism of interaction will differ between cell types and thus affect results from binding assays. This is highlighted by the difference between the attachment of *E. tenella* sporozoites to caecal and duodenal enterocytes derived from chicken intestines. Enterocytes that have been separated from the tissue lose their ability to attach *E. tenella* sporozoites (Experiments 4.3 and 4.3) when compared with those maintained *in situ* in frozen sections (Experiment 4.5). Further to this, selection of the *Eimeria* species results in different binding patterns with cells *in vitro*. For example, *E. tenella* binds to caecal tissue (Experiments 4.5 and 5.1) but not duodenal cells (Experiment 5.1) when the cells retain the positional identity within a tissue section. In comparison, freshly isolated caecal enterocytes lose their ability to bind *E. tenella* sporozoites (Experiments 4.3 and 4.41) when compared to intact tissue (Experiment 5.1). This loss of function appears to be reversed after an extended time in primary culture or repeated passages of host cells through a cell culture system (Augustine, 2001b; Tierney and Mulcahy, 2003; Van Immerseel *et al.*, 2004). Furthermore, any given *Eimeria* species can bind and invade established cell-lines derived from different organs and animals (Chai *et al.*, 1989; Hofmann and Raether, 1990; Constantinoiu *et al.*, 2003; Tierney and Mulcahy, 2003; Tierney *et al.*, 2004). However, sporozoites of *Eimeria* species can attach to and penetrate into similar cell lines, including kidney and enterocyte cell cultures derived from like and unlike animals. This indicates that the determination of site specificity is lost when cell culture lines are used. This is contrasted with the observation of binding to frozen sections, as presented in this study (Experiment 5.1). In conclusion, the use of an *in vitro* method *ex vivo*, in this case frozen sections, can provide more biologically relevant data than that of a model based on cultured

cell lines. These observations for *Eimeria* species are similar to those reported for *Cryptosporidium parvum* where the selection of the *in vitro* system influences the binding of the pathogens and penetration of the host cell (Hashim *et al.*, 2006).

## 7.5 Determination of oocyst viability

The age of *Eimeria* oocysts is important to obtain viable sporozoites that are necessary to obtain optimal results from invasion studies as well as vaccination protocols. The viability of oocysts is reduced during storage. This reduction is directly related to the temperature and time of the storage (Tomley, 1997). The propidium iodide (PI) method is a simple method to determine the viability of *Eimeria* oocysts and sporocysts. The method is based on the microscopic observation that PI is excluded from living cells and, as such, is a direct measurement tool to identify viable sporozoites inside both the oocysts and sporocysts. The change of oocyst and sporocyst walls by necrotic processes allows PI to cross the membrane of both the oocysts and sporocysts. Similar results have been effectively obtained in the use of the PI for identifying the viability and infectivity of oocysts in *Cryptosporidium* (Adams *et al.*, 1994; Bukhari *et al.*, 2000; Al-Adhami *et al.*, 2006; Castro-Hermida *et al.*, 2006) and *Giardia muris* cysts (Schupp and Erlandsen, 1987; Smith and Smith, 1989; Adams *et al.*, 1994; Khater *et al.*, 2004). The use of PI for identifying the viability of *Giardia* cysts showed that there is a strong correlation between cyst morphology, animal infectivity and exclusion of the dye (Schupp and Erlandsen, 1987; Schupp *et al.*, 1988).

The *in vitro* evaluation of the *Eimeria* oocyst viability after mechanical and chemical extraction of sporozoites from oocysts is costly in both time and reagents. In addition, the routine method for extraction of sporocysts damages the released sporocysts and rapidly kills their enclosed sporozoites. The loss of oocysts during the preparation of sporocysts with different procedures can vary between 21–86%, depending on the method (Experiment 3.3). This loss of oocysts make it almost impossible to determine the true viability of a given oocyst preparation and its potential to release the infectious sporozoites. On the other hand, the assessment of oocyst viability *in vivo* is costly and difficult because of the need to use either chickens (Tomley, 1997) or chicken embryos (Long, 1970) that are free of anticoccidial drugs. This is further compounded by the need to collect enough oocysts for inoculation, and the preparation of samples for histological

procedures. In contrast, the propidium iodide method can determine the viability of each oocyst quickly and accurately. This method can be done with a fluorescent microscope with visual scoring or automated to a flow cytometry protocol. The method could be used in the identification of pools of infectious oocysts within production facilities, evaluation of anticoccidial products, and in quality control method for live *Eimeria* vaccines.

The PI method has great potential in the epidemiological studies of coccidiosis with the spread of *Eimeria* oocysts being via water, food and litter in poultry industries. However, determining which factor is the primary pool for a given environment is difficult. The use of the PI method would allow quick screening of environments, thereby identifying which of these harbour viable oocysts and therefore are the greatest risk to chickens. The identification of infectious sources can lead to reduction of the incidence of coccidiosis in the poultry industry by applying changes in management of facilities without the need of anticoccidial drugs and vaccines.

Another application of the PI method would be in the evaluation of commercial disinfectants designed to reduce spread of *Eimeria* oocysts in the environment. In this case, the PI method could successfully determine the effectiveness of anticoccidial detergents after cleaning poultry houses and after treating chickens with anticoccidial drugs. Proof-of-concept is provided by the studies reported by Castro-Hermida *et al.* (2006) who demonstrated the potential biocidal activity of two commercial disinfectants against *Cryptosporidium parvum* oocysts (Castro-Hermida *et al.*, 2006). In addition, this method is suitable for identifying the *Eimeria* resistant to anticoccidial detergents as well as development of new commercial disinfectants, all being performed *in vitro,* reducing cost and time.

An immediate use of the methodology would be as a quality assurance and control tool for the production of coccidian vaccines that contain live *Eimeria* oocysts. The numbers of which are important to ensure the release of an adequate number of sporozoites to induce immunity. The PI method can determine the viability of oocysts in such vaccines. Until the development of the PI method, as reported here, there was no *in vitro* method to determine the viability of oocysts without the need to release sporozoites.

## 7.6 Identification of *Eimeria* in samples

Another progress in this study was the specific diagnosis of *Eimeria* infection in intestinal tissue by the polymerase chain reaction (PCR). PCR can be used to identify coccidiosis in different stages of the *Eimeria* life cycle, including sporozoites in the tissue derived from infected chickens, chicken intestinal mucus and purified oocysts from faeces.

The diagnosis of disease is the key to the prevention, and control of coccidiosis. The conventional diagnosis has relied on detection of *Eimeria* oocysts excreted in the faeces from infected chickens and the assessment of their site and degree of the pathological lesions in the intestinal tract (Long and Joyner, 1984). However, this diagnostic approach is limited in terms of the identification of the seven *Eimeria* species, especially when *Eimeria* overlap in their morphological and pathological features, their predilection sites, shape of lesions and the characteristics of the endogenous stages in the infected tissue (Long and Joyner, 1984). A molecular approach to overcome these limitations of traditional methods has resolved the issue of identifying multiple species of *Eimeria* from infected chickens (Marzouk *et al.*, 1989; Rodgers *et al.*, 1990; Laxer *et al.*, 1991; Schnitzler *et al.*, 1999). This is further advanced by the work presented here in which PCR was used to identify *Eimeria* in a matrix that contains intestinal tissue and/or mucus. This advance may overcome the limitation encounterd with organ culture experiments (Experiment 4.2) that relied on the histological identification of *Eimeria* sporozoites. Organ culture, in combination with the frozen section method, would allow *in vitro* determination of *Eimeria* binding in systems that retain structural integrity as well as metabolic activity.

The development of the PCR method could solve the problem of the diagnosis of *Eimeria* sporozoites in tissue derived from infected chickens. This is of particular importance in the differential diagnosis from *Cryptosporidium* that is a protozoan parasite that infects the intestine of broiler chickens as well as *Eimeria*, but is not host specific. For example *Cryptosporidium meleagridis* infects broiler chickens and five mammalian species (Darabus and Olariu, 2003; Kimura *et al.*, 2004), which has been reported in many countries (Papadopoulou *et al.*, 1988; Hajdusek *et al.*, 2004; Cardozo *et al.*, 2005). *Cryptosporidosis* is a common disease in poultry but little is known of its importance (McDougald, 1998). Different species of *Eimeria* and *Cryptosporidium* have a very similar life cycle and oocyst morphology, which cause difficulty in

distinguishing between the lesions produced by *Eimeria* and *Cryptosporidium*. PCR is a specific diagnosis tool to distinguish these two genera and the multiple species of *Eimeria* that infect chickens. This method would also be important for identifation of *Eimeria* which have survived the action of anticoccidial drugs. Together the results of this study, and others, indicate that PCR is an economical and fast tool for diagnosis of infection, epidemiological study, and quality control of chicken vaccination.

## 7.7 Improvement of sporozoite extraction

The results presented in this study (Experiments 3.2 and 3.4) show that using the sonication procedure for breaking oocysts with the addition of $MgCl_2$ to buffer systems containing sporocysts, produced a yield of 5.5 *Eimeria* sporozoites per oocyst. This represents a 38% increase in release of sporozoites as compared to other methods. The higher yield of sporozoite numbers per oocyst is of economic value either for producing attenuated vaccines by inoculating chicken embryos (Chapman *et al.*, 2002) or for studying the cell-cell interaction with host as well as the biology of *Eimeria*.

## 7.8 Problems of *Eimeria acervulina* in binding assays

*Eimeria acervulina* sporozoites released from sporocysts were ruptured shortly after extraction by the method as described for *E. tenella*. The use of buffer systems with various osmolarities, and different concentration of trypsin and bile salt, resulted in defining a modified method to extract *E. acervulina,* successfully. The differences between the modified method in this study and those reported by Tomley, (1997) and Hofmann and Raether, (1990) are the use of a low concentration of trypsin, a high concentration of bile salt in MEM, and incubating at 36°C. The results suggest that concentration of trypsin and osmolarity of buffer system have an important role in the integrity of sporozoites. The purification of *E. acervulina* sporozoites was a further problem for using *E. acervulina* in binding assays. DE-52 and paper filters could not separate sporozoites from the oocysts and sporocysts (section 2.4.5.2). This purification is necessary to prevent oocyst and sporocyst fragments interfering with the assay, through toxicity and loss of membrane integrity in host cells, potentially causing the inhibition of sporozoite invasion to host cells. Similar results

of the effects of oocyst and sporocyst fragments in the interaction of sporozoites with cells have been reported by Doran (1970), Hofmann and Raether (1990), and Tomley (1997). The inability to obtain a clean preparation of *E. acervulina* limited the study of this species *in vitro*. However, the improvements in extraction of *E. acervulina* sporozoites, and the first demonstration of their labeling with the non-toxic dye (PKH-67), are significant steps to overcoming such limitations.

## 7.9 Direction of future studies

The frozen section method developed in the work reported here will allow the design of experiments within which the conditions relating to *Eimeria* binding can be rigorously investigated. Such work could address the role of mucus from vaccinated and non-vaccinated chickens, different compositions of diets, anticoccidial drugs, identification of carbohydrates that recognize receptor(s) and the specific receptors in different regions of the chicken intestine in the attachment of different species of *Eimeria* sporozoites. This method could be used to study the attachment and competition between *Eimeria* species and other protozoan parasites, and micro-organism in animals and humans.

This study led to the development of the propidium iodide method, which could be used as a quality assurance tool in production of *Eimeria* oocysts for vaccines and the effect of anticoccidial detergents on oocyst viability. This method could also be used for epidemiological studies to determine the infectious sources of *Eimeria* oocysts in food and water supplies on poultry farms as well as the efficacy of new anticocciadial agents *in vitro* and subsequent application in the field. The sensitivity of the PI method can be determined by an *in vivo* test, which identify non-viable oocysts as a consequence of aging and then inoculate into chickens.

PCR could be used to identify which *Eimeria* species infect chickens in a variety of sample types and reveal whether the infection was caused by multiple species or just one species. It helps to solve the problems associated with the traditional method of diagnosis using histology. The next stage would be the development of RT-PCR based on the primers used in this study. Once developed, a RT-PCR tool can be used objectively to measure the rate of infection in tissue.

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