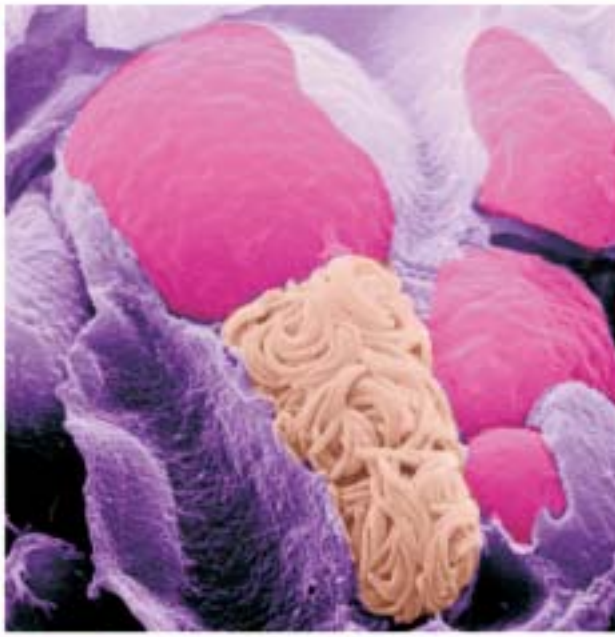
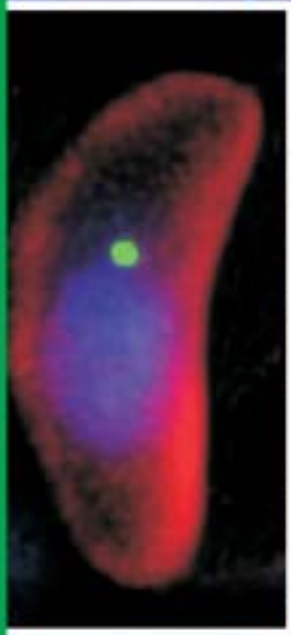
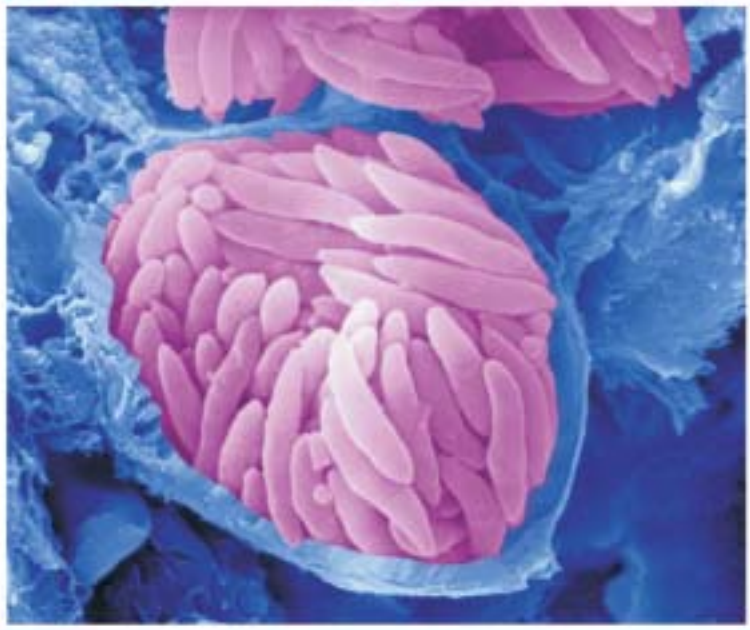
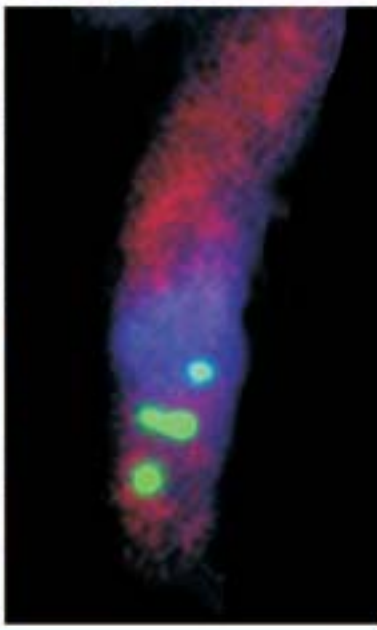
Proceedings of

The IXth International Coccidiosis Conference

Mabu Thermas & Resort, Foz do Iguassu, Parana, Brazil - September 19-23, 2005

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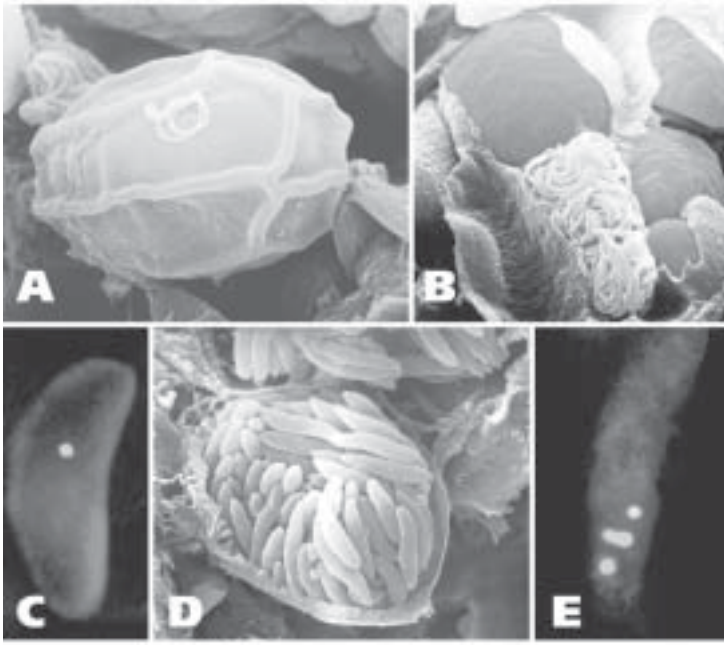

Front cover figure legends:

A. Scanning electron micrograph (SEM) of an oocyst in the gut lumen showing partial loss of the veil and with a microgamete adhering to the outer layer of the oocyst wall.

B. SEM of a fracture through a caecum infected with E. tenella showing a microgametocyte with numerous mature microgametes in a cell adjacent to a macrogamete.

C and E. Tachyzoite of T. gondii (C) and merozoite of E. tenella (E) and labelled with anti-enoyl reductase showing the peri-nuclear location of the single or multiple apicoplasts (green). .

D. SEM of a mature schizont of Eimeria tenella showing the individual merozoites.

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Introduction

It is a great honor to host the IXth International Coccidiosis Conference (IXth ICC) and welcome the delegates at the Mabu Thermas & Resort, in the city of Foz do Iguassu, Brazil. At its ninth edition, and being held each four years, since 1973, the Conference has become the most traditional meeting in coccidiosis research. This meeting has aggregated academic and private company researchers, product managers, and field professionals, providing an opportunity for information interchange of both basic and applied sciences.

We have decided to hold the IXth ICC in the city of Foz do Iguassu. World widely known for its famous Iguassu Falls, Foz do Iguassu represents Brazil's one of the most important touristic sites. A wonderful nature, including two National Parks, in Brazilian and Argentinean sides, ecological tours, and a very diverse wild life, composes a rich scenario for this meeting. We hope all delegates enjoy the conference and also have a pleasant stay in this natural paradise.

In this ninth edition of the Conference, we tried to maintain the spirit that has characterized the former events. Traditional approaches for coccidiosis control and diagnosis will be presented side-by-side with highly innovative technologies. We received contributed papers of many different topics, including vaccines, chemotherapy, basic immunology, molecular and cell biology, pathology, genomics and post-genomics. Taking into account the increasing importance of vaccines in coccidiosis control, we have prepared specific sessions to discuss new approaches and technologies for vaccination. We have also invited a group of selected academic researchers for the plenary lectures, to present the state-of-art of research and development. A new age has arisen since large scale genome sequencing became a mainstream approach to unravel the complexity of living organisms. For the first time in history, we now have access to the complete genome information of some coccidian parasites like *Toxoplasma gondii*, *Eimeria tenella* and two species of *Cryptosporidium*. Where is this information going to lead us? How will it contribute for new control measures? This is an issue that will also be discussed and, we hope, will open up new trends for the disease control. Finally, the increasing importance of new regulations on the use of anticoccidial drugs and vaccines stimulated us to include a symposium on current and future perspectives of legislation. This symposium will be followed by an organized public debate, where the major trends of this important issue will be openly discussed.

Social activities were also programmed to complement the scientific programme, allowing for delegates to know each other and create an environment of professional and personal integration.

We wish you a pleasant stay in Foz do Iguassu, and a very fruitful Conference.

Arthur Gruber
Conference Organizer

Ariel Antonio Mendes
President of FACTA

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The IXth International Coccidiosis Conference is dedicated to Dr. Peter Long

Professor Peter L. Long: an Appreciation

M. W. Shirley
Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berks. RG 20 7NN, UK, shirley@bbsrc.ac.uk

If asked to think of the most influential coccidiologist of the past four decades, the chances are that the name of Peter Long will feature at, or near, the top of most people's lists. If asked to think of the most influential coccidiologist of the past four decades who is also the most approachable and sociable, the likelihood is that Peter will be at the top of everyone's list! Peter has the rare gift of scientific excellence combined with a warm demeanour and fondness for *bon homie* that has endeared him to all who have had the pleasure of his acquaintance.

I first became a colleague in 1967 when I started work at the Houghton Poultry Research Station (HPRS) where Peter was a principal scientist looking to recruit a new member of his team. At that time he had not yet gained his PhD but he was established comfortably as a coccidiologist of increasing international renown and was a natural successor to Clifford Horton-Smith who had also previously graced HPRS with distinction and charisma. A symmetry in the three generations was to emerge - Clifford had been a mentor to Peter as Peter was to become to me. Moreover, there was to be further symmetry in the way that the academic career of Peter developed subsequently with how mine was to shape up under his guidance.

Edward Tyzzer was working on his *Magnum Opus* to be published in 1929 when Peter was born in London in 1928. Peter developed into a good sportsman with a special love of cricket – a game at which he was especially skilled and which he played on-and-off for several decades. He supplemented his cricket with both hockey and squash and his competitive nature at sports was mirrored by his determination to succeed professionally.

He began his professional life working on poultry parasites at the Central Veterinary Laboratory at Weybridge, Surrey as a laboratory technician with Dr Clifford Horton-Smith. Only four years later, however, he was conscripted into the Royal Army Medical Corps and served in Egypt, Malta and Palestine.

In 1949, following demobilisation, Peter and Clifford were reunited at the Ashford Hospital, Middlesex and then a more durable union followed with their relocation to the HPRS: Peter becoming the research technician for Horton-Smith within the Parasitology department working on a programme of research directed principally towards coccidiosis of poultry. Without any formal academic qualifications, Peter studied part-time to become a Member of the Institute of Medical Laboratory Technology in 1952 and obtained Fellow status by thesis in 1956.

A steady stream of publications was beginning to appear and a strong desire to publish his data was to define his entire working life. Some early publications included a very useful treatise on *E. maxima* [Long, P. L. (1959). A study of *Eimeria maxima* Tyzzer, 1929, A coccidium of the fowl *(Gallus gallus). Annals of Tropical Medicine and Parasitology*, 53, 325–333] that remains one of the few definitive studies on this major avian species. By now Peter was progressing through the ranks from his original grading of "Assistant Experimental Officer" such that by 1963 he was able to spend a sabbatical year at Cornell University in the USA working with the eminent poultry pathologist, Professor P. P. Levine. This very positive scientific experience left an indelible mark on his subsequent career and much later he was to return to the USA to the University of Georgia where he spent the final 10 years of his active working life.

Peter's taste for working abroad was strengthened in 1963 and 1964 with two visits to the Lebanon, where he worked on poultry coccidiosis and taught parasitology at the Institut de Recherches Agronomiques.

In 1964, and through further part time studies, Peter became a member of the Institute of Biology in the UK and in 1971 Peter was awarded a PhD by Brunel University for his work on the avian coccidia and in 1977 was awarded a DSc. His association with Brunel University led to a strong friendship with Professor Roland J. Terry, who subsequently paid a professional visit to Peter when he was at Athens.

Throughout the 1960's and 1970's Peter maintained a high output of publications from the parasitologists at HPRS with an impressive list of personal papers that laid much of the groundwork for later developments in the control and study of the avian coccidia including:

- two of the few papers on *Eimeria* to be published in the journal Nature [Long, P. L. (1965). Development of *Eimeria tenella* in avian embryos. *Nature* **208**, 509-10 and Long, P. L. (1970c). Development (schizogony) of *Eimeria tenella* in the liver of chickens treated with corticosteroid. *Nature* **225,** 290-1]
- pioneering studies on the growth of different species of *Eimeria* in avian embryos that would eventually help in the development of live attenuated vaccines [Long, P. L. (1966). The growth and attenuation of some species of *Eimeria* in avian embryos. *Parasitology* **56,** 575-81 and Long, P. L. (1972b). *Eimeria tenella*: reproduction, pathogenicity and immunogenicity of a strain maintained in chick embryos by serial passage. *J Comp Pathol* **82,** 429-37]
- the isolation and identification of different species [e.g. Long, P. L. (1973c). Studies on the relationship between *Eimeria acervulina* and *Eimeria mivati*. *Parasitology* **67,** 143-55 and Long, P. L. (1974). Experimental infection of chickens with two species of *Eimeria* isolated from the Malaysian jungle fowl. *Parasitology* **69,** 337-47]
- studies on immunity, often with Elaine Rose [Long, P. L., and Rose, M. E. (1972). Immunity to coccidiosis: effect of serum antibodies on cell invasion by sporozoites of *Eimeria in vitro*. *Parasitology* **65,** 437-45
- studies on chemotherapy [Long, P. L., and Millard, B. J. (1968). *Eimeria*: effect of meticlorpindol and methyl benzoquate on endogenous stages in the chicken. *Exp Parasitol* **23,** 331-8]
- the first studies to provide quantitative data on the incidence of *Eimeria* species in poultry houses [Long, P. L., Tompkins, R. V., and Millard, B. J. (1975). Coccidiosis in broilers: evaluation of infection by the examination of broiler house litter for oocysts. *Avian Pathology* **4,** 287-294].

This was indeed a golden period for Peter and his acclaim grew within HPRS, where he was appointed successor to Dr Donald L. Lee as Head of Parasitology in 1972; nationally, for example, he was awarded the Tom Newman Medal for Poultry Research from the British Poultry Breeders and Hatcheries Association in 1971 and made an honorary member of the British Veterinary Poultry Association in 1972; and internationally where he consolidated his position as arguably the leading scientist working on the biology of avian coccidia.

In 1973 Peter worked with Datus M. Hammond to edit "*The coccidia*", a book that was to become the first in a series of volumes on the biology of the coccidia and coccidiosis. These books, most especially the 1982 volume entitled "*The biology of the coccidia*" have stood the test of time and continue to be some of the most used reference texts. The most recent volume, now over 15 years old and entitled "*Coccidiosis in man and domestic animals*", is a further reflection of Peter's commitment and ability to work with others to effectively deliver knowledge of the avian coccidia into the public domain.

Within the UK Peter helped to establish the forum that is now known as the European Coccidiosis Discussion Group and he was one of the founding members of what was to become the British Society for Parasitology, which later made him an honorary member.

In 1979, Peter left the HPRS to succeed Professor W. Malcolm Reid as Professor of Parasitology and Chairman of the Faculty of Parasitology at the University of Georgia. In 1983, Peter was further recognised

by the University by the title, which he held until 1990 when he retired back to the UK, of D.W. Brooks Distinguished Professor of Poultry Science.

During his years at HPRS, Peter was most ably supported scientifically and technically by Brian Millard and, if there were an equivalent anywhere else in the world, he most definitely found that person in Joyce Johnson. Peter and Joyce developed a wonderful working rapport and the transition of his science from HPRS to the University of Georgia seemed almost seamless with virtually no interruption to the stream of papers.

Peter's later contributions to coccidiosis research continued to reflect his desire to improve and understand the control of disease and his early work in Georgia was pivotal to the first understandings of the relationship between pathological changes in the intestines and body weight changes of vaccinated or naive chickens after challenge, findings that were to become important considerations in the evaluation of vaccines [e.g. Long, P. L., Johnson, J., and Wyatt, R. D. (1980). *Eimeria tenella*: clinical effects in partially immune and susceptible chickens. *Poult Sci* **59,** 2221-4]. In addition he pursued work in the USA on the development of precocious, attenuated lines of *Eimeria* as well as addressing issues of disease control more generally.

In total, Peter published more than 200 scientific papers, countless popular articles and very many book chapters. He also helped stage the Coccidiosis Conference in Georgia in 1985.

The scientific excellence of Peter and his contribution to research on coccidia and coccidiosis has been recognised many times and, in addition to the Tom Newman Award, he was the first recipient of the Gordon Memorial Medal (1983); the Merck Award for Research from the American Poultry Science Association (1984); and the Creative Research Medal from the University of Georgia, Athens.

Throughout his career Peter influenced and mentored many colleagues including several PhD students (about whom he still speaks with great warmth and pride) as well as many more established researchers across the globe. He enjoyed interacting with, or working alongside, such luminaries as Alan Pierce, Akira Arakawa, Stan Ball, Peter Bedrnik, David Chapman, Aggie Fernando, Datus Hammond, Thomas Jeffers, Joyce Johnson, Len Joyner, P. P. Levine Lazlo Pellerdy, Larry McDougald, Malcolm Reid, Elaine Rose, Erich Scholtyseck and Ray Williams, to name just a few.

When David Chapman and I published a recent history of the Houghton strain, a strain that is now so firmly embedded within research into Eimeria spp. as the parasite used for the derivation of a genome sequence, we dedicated the paper to both Peter Long and Elaine Rose. Without the direct inputs of Peter on both the science and the people, the current landscape of coccidiosis research would almost certainly be very different from what we have now and probably less at the cutting-edge of microbiological research. Many of us working on Eimeria parasites might care to reflect upon the words of Isaac Newton to Robert Hooke on 5th. February 1676, that "If I have seen further, it is by standing on the shoulders of giants". Peter Long is a giant in the history of coccidiosis research.

A SELECTION OF OTHER PUBLICATIONS BY PETER LONG THAT FURTHER ILLUSTRATES HIS VERY SIGNIFICANT CONTRIBUTION TO STUDIES ON THE AVIAN COCCIDIA

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Long, P. L., and Millard, B. J. (1967). The effect of Meticlorpindol on *Eimeria* infections of the fowl. *Vet Rec* **81,** 11-5.

Long, P. L. (1968). The effect of breed of chickens on resistance to *Eimeria* infections. *Br Poult Sci* **9,** 71-8.

Long, P. L. (1968). The pathogenic effects of *Eimeria praecox* and *E. acervulina* in the chicken. *Parasitology* **58,** 691-700.

Long, P. L., and Horton-Smith, C. (1968). Coccidia and coccidiosis in the domestic fowl. *Adv Parasitol* **6,** 313-25.

Long, P. L. (1969). Observations on the growth of *Eimeria tenella* in cultured cells from the parasitized chorioallantoic membranes of the developing chick embryo. *Parasitology* **59,** 757-65.

Long, P. L. (1970). Coccidiosis: development of new techniques in coccidiostat evaluation. *Exp Parasitol* **28,** 151-5.

Long, P. L. (1970). *Eimeria tenella*: chemotherapeutic studies in chick embryos with a description of a new method (chorioallantoic membrane foci counts) for evaluating infections. *Z Parasitenkd* **33,** 329-38.

Long, P. L. (1970). Some factors affecting the severity of infection with *Eimeria tenella* in chicken embryos. *Parasitology* **60,** 435-47.

Long, P. L. (1970). Studies on the viability of sporozoites of *Eimeria tenella*. *Z Parasitenkd* **35,** 1-6.

Long, P. L., and Rose, M. E. (1970). Extended schizogony of *Eimeria mivati* in betamethasone-treated chickens. *Parasitology* **60,** 147-55.

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Long, P. L. (1972). Observations on the oocyst production and viability of *Eimeria mivati* and *E. tenella* in the chorioallantois of chicken embryos incubated at different temperatures. *Z Parasitenkd* **39,** 27-37.

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Long, P. L. (1973). Endogenous stages of a 'chick embryo-adapted' strain of *Eimeria tenella*. *Parasitology* **66,** 55-62.

Long, P. L. (1973). The growth of avian coccidia in the developing chick embryo and in cultured cells . *Folia Vet Lat* **3,** 89-109.

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Long, P. L., and Rowell, J. G. (1975). Sampling broiler house litter for coccidial oocysts. *Br Poult Sci* **16,** 583-92.

Long, P. L., Millard, B. J., Joyner, L. P., and Norton, C. C. (1976). A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. *Folia Vet Lat* **6,** 201-17.

Long, P. L., and Millard, B. J. (1976). The detection of occult coccidial infections by inoculating chickens with corticosteroid drugs. *Z Parasitenkd* **48,** 287-90.

Long, P. L., and Millard, B. J. (1976). Studies on site finding and site specificity of *Eimeria praecox*, *Eimeria maxima* and *Eimeria acervulina* in chickens. *Parasitology* **73,** 327-36.

Long, P. L., and Rose, M. E. (1976). Growth of *Eimeria tenella in vitro* in macrophages from chicken peritoneal exudates. *Z Parasitenkd* **48,** 291-4.

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Long, P. L., and Jeffers, T. K. (1982). Studies on the stage of action of ionophorous antibiotics against *Eimeria*. *J Parasitol* **68,** 363-71.

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Long, P. L. (1984). Gordon memorial lecture. Coccidiosis control: past, present and future. *Br Poult Sci* **25,** 3-18.

Long, P. L., and Joyner, L. P. (1984). Problems in the identification of species of *Eimeria*. *J Protozool* **31,** 535-41.

Long, P. L., Johnson, J., and Baxter, S. (1985). *Eimeria tenella*: relative survival of drug-resistant and drug-sensitive populations in floor pen chickens. *Poult Sci* **64,** 2403-5.

Long, P. L., Johnson, J., and McKenzie, M. E. (1988). Anticoccidial activity of combinations of narasin and nicarbazin. *Poult Sci* **67,** 248-52.

Plenary Lectures

Eight decades of research on *Eimeria* in poultry

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OVERVIEW

For around 80 years, the amount of knowledge gathered on the avian coccidial parasites and the disease of coccidiosis has been impressive and research remains at the cutting-edge of what is being undertaken in Microbiology more generally. Research in to avian coccidiosis has been exceptionally well served and the starting point for knowledge gathering of the disease and the parasites of poultry stems from pioneering studies conducted in the 1920's and 1930's by investigators who, with limited experimental facilities, were able to establish many basic principles, such as life cycles, epidemiology and control of the *Eimeria* parasite, that are still relevant today.

In the years that followed, research was conducted at institutions in the academic, government, and private sectors with the specialized facilities, especially access to buildings that prevented chances of extraneous infections of the hosts. Much of the work in the early period focused on the obvious practical control of *Eimeria* spp. and was supplemented by the acquisition of much basic information on the biology of the parasites, including host-parasite relationships, genetics, immunology, and pathology and some of this information was to lay the foundation for the introduction of a series of strategies to control the parasites by vaccination of the hosts.

Building on the early discoveries, the control of coccidiosis by chemotherapy was introduced and, by eliminating mass outbreaks of disease, it became a critical lynchpin in the expansion of the international poultry industry. Today, chemotherapy remains the dominant method of control but the use of live vaccines has a similar long history through the early work of Allen Edgar and others. The dramatic attenuation of the life cycle of *E. tenella,* as first described by Thomas Jeffers in the mid 1970's, was to lead to the selection of "precocious" lines of all species and these parasites, with their abbreviated life cycles and marked attenuation of virulence, proved to be a significant development as they led to a new generation of safe coccidiosis vaccines. Towards the end of the 20th century much interest was being directed towards the greater use of vaccination for the control of coccidiosis and, in contrast to an opposite trend in anticoccidial drugs – especially within Europe - many new products were introduced.

Some of the research conducted in recent years has helped to maintain work on *Eimeria* at the cutting edge of microbiological science and is providing a wealth of knowledge on the biology of the genome(s) of the parasite. A highlight has been a project to determine a whole genome sequence for *Eimeria tenella* and this work is currently nearing completion and being supplemented by many complementary studies focusing on gene expression. With access to a genome sequence, future researchers are now offered tremendous opportunities to investigate the biology of eimerian parasites and, for example, seek those elusive protective antigens that will enable new vaccines and new drugs to be developed.

This short account of coccidiosis research during the past 80 years is intended to provide only a superficial skim and the names of most of the scientists who contributed to a large body of knowledge on the parasites and the diseases they cause will not be given. We will also omit the names of very many other coccidiologists who, whilst primarily studying other coccidia such as *Eimeria* species from other hosts such as *Toxoplasma*, etc., nonetheless have contributed much to the body of relevant science on the avian coccidia. Apologies to them all.

SOLID BEGINNINGS

Present knowledge of the disease coccidiosis of poultry is founded firmly on pioneering studies carried out by Walter Johnson at the Oregon State University Experiment Station and Ernest Tyzzer at the Harvard Medical School in the 1920's (Johnson, 1923; 1923/1924; Tyzzer, 1929; Tyzzer et al., 1932). Prior to that time coccidiosis was thought to be caused by just one species, *Eimeria avium*, that could infect many hosts (avian and mammal); clinical effects were confused with those caused by other organisms such as *Histomonas* and intestinal bacteria, and a range of effects were thought possible, ranging from leg problems to "white diarrhea" (see Chapman, 2003; 2004). Between them, Johnson and Tyzzer described most of the now recognized species, *viz*. *E. acervulina*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella* and correctly identified their specific lesions. They also considered the phenomena of site and host specificity; the self-limiting nature of the eimerian life cycle; the relationship between magnitude of dose and severity of infection; the phenomenon of acquired immunity, and the epizootiology of coccidiosis infections. Although both Johnson and Tyzzer emphasized the importance of sanitation and hygiene in reducing environmental contamination with the infective stage of the parasites, they realized that eradication was an unrealistic goal for the control of coccidiosis and advocated exposure of the host to low numbers of oocysts to allow them to acquire protective immunity. Indeed Johnson took this further and at the end of his life was working on the possibility of immunizing birds by intentionally introducing controlled numbers of oocysts in the feed. In order to undertake critical studies on pure parasites, both Johnson and Tyzzer appreciated the importance of isolating species from single oocysts and of using sterilized cages sterility for the propagation of *Eimeria* infections (Tyzzer, 1932) and some of their methodologies form the foundation of modern procedures for working with these parasites (Chapman and Shirley, 2003).

Anyone with an interest in the biology of the avian coccidia is most definitely encouraged to read the two seminal texts by Tyzzer [Tyzzer (1929) Coccidiosis in gallinaceous birds. American Journal of Hygiene 10: 269-383, and Tyzzer *et al*. (1932) Coccidiosis in gallinaceous birds II. A comparative study of species of *Eimeria* of the chicken. American Journal of Hygiene 15: 319-393] because they contain wonderful descriptions of the parasites, exquisite drawings and data that are relevant today.

CONSOLIDATION

The efforts of Johnson and Tyzzer had put an understanding of coccidiosis on a solid footing, and in the years that followed their findings were widely communicated to the developing poultry industry. At this time the industry was progressing from small backyard flocks of a few dozen chickens to poultry farms with houses holding several hundred birds. The introduction of such large scale husbandry practices began to tip the balance in favour of the highly efficient transmission of coccidia between hosts and a rising incidence of disease was the spur for numerous, initially unsuccessful, studies to investigate possible remedies. Nutritional experiments were carried out at the Bureau of Animal Industry in Washington and elsewhere and pathological/physiological investigations were done at the University of Wisconsin. P. P. Levine at Cornell University made several important contributions, including the first description of *E. brunetti*, but it was his report of the activity of the compound sulphanilamide that was to determine a primary direction of coccidiosis research for the next few decades (Levine, 1939). Interestingly, Levine believed that use of drugs should be an adjunct to management and that medication should not take the place of proper husbandry (Levine, 1945), a viewpoint that is still relevant today. Levine's work was the first of many studies in several countries in the 1940's to define optimal conditions for the use of sulphonamides. The most significant study, however, that had the greatest impact on future control methods, was the demonstration by Delaplane and colleagues at the Rhode Island Agricultural Experiment Station that sulphaquinoxaline, administered at low concentrations in the feed, gave an effective control of coccidiosis (Delaplane et al., 1947).

This era of early research culminated in 1949 in the first conference entirely devoted to coccidiosis. It was organized by the New York Academy of Sciences (Anon, 1949) and leading researchers from government, university and industry in the USA attended, including one international delegate from the UK, Clifford Horton-Smith. The significance of the new era of chemotherapy was evident since half (11 of 22) presentations on poultry coccidiosis were concerned with drugs.

THE 1950S-1970S

Increasing recognition of the significance of coccidiosis and its deleterious consequences for the developing poultry industry required a more detailed and better basic understanding of the nature of the parasites and the disease. In retrospect, it is remarkable how much was accomplished prior to the 1950s, since animal facilities at that time were limited and, although the chicken remains inexpensive and relatively easy to maintain, the ubiquity of coccidial oocysts and the consequent difficulty of raising birds in the absence infection introduced a major constraint upon research.

Many of the laboratories that contributed to the very early work on the avian coccidia had by now relinquished their interest in this field and the mantle for research was passed to those institutions (government, university, and private) with the resources and specialized facilities necessary for this type of research. Such laboratories included the Houghton Poultry Research Station (HPRS) and the Central Veterinary Laboratory at Weybridge in the UK, the Parasite Research Laboratory at Beltsville and the Department of Poultry Science at the University of Georgia in the USA

Some of the leading established scientists of the day were Peter Long, Elaine Rose, John Ryley and Len Joyner in the UK; David Doran, Michael Ruff, Chin Chung Wang, Larry McDougald and W. Malcolm Reid in the USA; Aggie Fernando in Canada; Peter Bedrnik in the Czech Republic; Tamara Beyer and Theresa Shibalova in the USSR; other luminaries they interacted with included Datus Hammond, Clarence Speer, JP Dubey, Ron Fayer and Erich Scholtyseck (to name only a few). A new generation of coccidiologists was also entering the scene in the 1970's and included Pat Allen, Patricia Augustine, Harry Danforth, Thomas Jeffers, Dennis Schmatz and Ray Williams.

A broad range of scientific activities was undertaken at all the academic institutions and they included a fuller understanding of the life cycles (including descriptions of prepatent and patent times; endogenous stages), growth of parasites *in vitro* and in embryonating eggs, genetic recombination between strains, the nature of host immune responses and the immunizing abilities of the different parasite life cycle stages, pathology, chemotherapy, epidemiology and diagnosis of *Eimeria* infections. For many of the more fundamental studies, *E. tenella* became the most popular species for a number of reasons including its importance in the field, ease of propagation, high virulence and distinctive lesions, ability to grow cell culture and in the chorioallantoic membranes of embryonating eggs, robustness of the sporozoites for biochemical analyses, etc. A number of reference strains were introduced in to common usage from the 1950's and it was the well characterized Houghton strain of *E. tenella*, derived from oocysts recovered from a chicken that was sent to the HPRS for *post mortem* investigation in 1949, that was chosen for the ongoing internationally-coordinated genome sequencing project (Shirley et al., 2004).

A defining characteristic of research on *Eimeria* most especially during the 1950's to 1970's was the duality of programmes in the government and university settings with those in the commercial sector. An obvious broad distinction between the two research environments was that academic scientists had a stronger focus on the biology of the parasites, with some long-term studies on immunity and the potential for developing new vaccines and those in the commercial sector were working towards the development of better anticoccidials. The introduction of modern broad spectrum anticoccidial drugs from research done within companies such as Merck and Eli Lilly was to have a profound effect on the control of coccidiosis and the recognition by Eli Lilly of the anticoccidial efficacy of the ionophores was to dramatically

change prospects for the control of coccidiosis – an impact that remains to day. This period also saw some of the first attempts to introduce comprehensive biochemical studies of the coccidia, both in the academic and commercial sectors from which an understanding of the mode of action of the ionophores was to emerge. Sadly, the basis of how most other drugs exerted their lethal effects was not addressed and/or resolved to the same level of detail. Moreover, although relatively little biochemistry of the avian coccidia was to be done after the 1970's, the availability of an annotated genome sequence for *E. tenella* now provides an enhanced opportunity for studies on metabolism, etc.

The 1960's heralded the first work that examined the ability of *Eimeria* species to develop in cell culture and in the chorioallantoic membranes (CAM) of the embryonating egg. The findings that the life cycles of *E. tenella* and a few other species of avian *Eimeria* could be completed within the CAM following injection of sporozoites in to the allantoic cavity led to an evaluation of the effects of serial passage and, from that work, the emergence of the first attenuated lines that had potential as the basis of live attenuated vaccines.

Despite the success of a complete life cycle of *E. tenella* and some other species within the CAM, attempts to maintain the parasites in cell culture foundered and that specific challenge still remains today.

Whilst control of avian coccidiosis was being achieved through the introduction of a series of prophylactic anticoccidial drugs, the work of Allen Edgar had led to the introduction of the first commercial vaccine, *viz*. "Coccivac" in the 1950's, a history reviewed comprehensively by Williams (2002). In comparison to the use of anticoccidials, Coccivac® was (and still remains) a relatively minor product for control of disease in broilers, but along with a similar product, Immucox®, it represented a victory for an immunological approach and the prior acceptance of Coccivac® by the poultry industry was significant many years later when a new generation of attenuated vaccines was introduced. Their entry in to the marketplace stemmed from a truly significant piece of work around the middle of the 1970's by Thomas Jeffers (Jeffers 1975) who investigated the genetic stability of the prepatent time of *E. tenella*. His efforts to serially select the first oocysts to be produced during an infection were rewarded with the first ever "precocious line" and he thus laid the basis for the subsequent, and very comprehensive, work done elsewhere that led, around the end of the 1980's, to the introduction of the first live attenuated vaccines against the avian coccidia.

THE MODERN ERA

The 'politics' around in-feed medication of livestock have continued to evolve (especially within Europe) and an overall more negative view on the use of prophylactic chemotherapy is proving to be a spur for an increasing interest in the immunological control of avian coccidiosis. Not surprisingly, since Paracox® and Livacox® vaccines were developed in the early 1980's and introduced commercially towards the end of the 1980's, a slew of other live attenuated vaccines has been introduced worldwide (e.g. ADVENT®, Eimerivac®, Eimeriavax; Gelcox®, Inovocox® Nobilis® CoxATM, etc.). The new vaccines make use of different combinations of the seven recognized species and different methods of administration and this range of products is now supplemented with the first subunit vaccine (CoxAbic) that was developed from the efforts and insights of Michael Wallach.

In contrast to the enhanced research activities towards the development of vaccines, there is now significantly less research interest in new anticoccidials. A number of factors contribute to this and, in addition to the poorer political climate for the use of in-feed antibiotics and other drugs, new drugs are increasingly expensive to get to market and drug-resistance remains an inevitable threat to the financial investment. Thus whilst the marketplace for anticoccidials is still dominated by the use of drugs, the greatest financial investment (albeit limited) for future products within the commercial sector lay not now

with the large multinationals but with smaller companies (and some might be described as "bijoux") that are interested in live, and other, vaccines.

One noteworthy aspect of the current use of many live vaccines is that research has shown that introduction of drug-sensitive vaccinal strains (most were isolated before the onset of global chemotherapy) in to the field leads to a decrease in the numbers of drug-resistant parasites (e.g. Chapman, 1994). Thus ability of coccidiologists to help restore drug-sensitivity in the field through the tactical deployment of drug-sensitive strains is a considerable benefit arising from the use of some vaccines and may be unique in the field of microbiology.

The study of host immune responses to coccidial infection has also continued to run as a significant strand of international research from the 1970's to the present day and the highly productive efforts of Elaine Rose and Hyun Lillehoj are especially noteworthy.

The so-called modern era might also be thought of as the "molecular" era and an understanding of the biology of the parasites has been extended to new levels of detail and technical sophistication. Examples of areas where progress has, and is still being, made include a better understanding of parasite motility; the mechanisms of host cell invasion allied to the function of sub-cellular organelles, especially rhoptries and micronemes; the basis of virulence; host specificity and the process of differentiation as the life cycle progresses through the different asexual and sexual stages.

Diagnosis of infections and the definitive identification of the different species was once limited by consideration of the appearance of gross lesions, etc., but has now been transformed (at least in the laboratory setting) through the availability of DNA markers that permit rapid and unequivocal discrimination, not only between different species but also between some strains.

A highly significant outcome of current research is the close integration of many laboratories worldwide to form an "*Eimeria* Genome Consortium" that is working with a major genome sequencing facility in the UK (The Sanger Institute) to derive a sequence for the 55 million units (bases) of DNA that make up the 14 chromosomes within the nucleus of *E. tenella*. This global consortium is reflective both of the way in which large research projects are undertaken and funded and of the changing scientific grouping whereby smaller research groups around the world with distinctive and, sometimes, unique, expertise are able to tackle specific scientific problems. In the context of the *Eimeria* genome Consortium it is worth recording that membership comprises three laboratories in the UK; one in the USA; one in Brazil, one in Malaysia and one in China. The inputs of the group at the Institute for Animal Health in the UK (Martin Shirley and Fiona Tomley), Arthur Gruber at the University of Sao Paolo, Brazil and Wan Kiew-Lian at the Universiti Kebangsaan, Malaysia were at the core of the project to derive and annotate a whole genome sequence through funding of more than £1M (~$1.8M) from the Biotechnology and Biological Sciences Research Council (BBSRC) in the UK where the work was done at the Sanger Institute near Cambridge (http://www.sanger.ac.uk/Projects/E_tenella/), coordinated by Al Ivens and Matt Berriman. Complementing to this whole genome sequencing and annotation work are more detailed efforts on specific chromosomes (Malaysia), studies on expressed sequence tags (Brazil; Kate **Miska** and colleagues at the USDA, Beltsville and Jianping Cai, Guangdong Academy of Agricultural Sciences, China) and physical (HAPPY) mapping of chromosomes (Paul Dear, laboratory of Molecular Biology, Cambridge, UK). The sum total of this activity is that critical resources are now in place to assist the next generation of scientists address further important questions. Even after 80 years some fundamental questions remain to be answered. For example, "what are the molecules within the parasite that induce protective immune responses within the host?", "how do the avian species locate their preferred sites of development within the intestine?", "why does asexual reproduction end and sexual reproduction begin?", "what are metabolic pathways that the parasites use throughout their life cycles, both inside and outside of the chicken?". Answers to these questions might be needed to make the leap to the next generation of control measures.

Perhaps one of the real surprises from work on the genomes of *Eimeria* spp. and related parasites was the finding that they contain three genomes; *viz*. nuclear, mitochondrial, and a plastid (vestigial plant-like genome). A chance now to look at the roles that the other genomes play in facilitating the life style of parasitism (especially the plastid) offers further hopes for better control.

Finally, the ability to genetically engineer *Eimeria* parasites is now being achieved with some marked success. This considerable technical development by Fiona Tomley and colleagues (this meeting) potentially paves the way forward for the development of live vaccines in which it might be envisaged that one species of *Eimeria* could be engineered to deliver protective antigens of a number of species.

HAS EIGHT DECADES OF RESEARCH ON *EIMERIA* IN POULTRY MADE A DIFFERENCE?

It might be argued with some confidence that research on *Eimeria* has been one of the success stories associated with livestock production.

- The challenge of bringing under good control seven genetically complex species of *Eimeria* has been met through chemotherapy and vaccination. Control strategies have been developed both through knowledge gained empirically and that accumulated from studies on the basic biology of the parasites.
- Both the commercial and academic sectors have played a significant part in improving control of coccidiosis
- The two sectors have worked synergistically together through collaborative scientific and technical projects; meetings on coccidiosis arranged jointly; scientific exchanges between laboratories – both information and personal visits
- A steady stream of complementary publications has been delivered from both sectors in to the more popular press to ensure that the poultry industry is well informed
- Government funding in the UK and USA has provided a long term commitment to research on avian coccidiosis
- A constant stream of effective scientists in to the field has ensured that work on *Eimeria* spp. remains near to the cutting-edge of microbiological sciences

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Genome sequence of the avian coccidiosis-causing agent, *Eimeria tenella*

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Eimeria spp are major pathogens of intensely reared livestock. Drug resistance in the parasite is increasing and no new drugs have been introduced for more than 10 years. Live vaccination is effective but difficult to implement due to the large number of chicken required in the manufacture. Recombinant vaccines are desirable but their discovery will be preceded by a detailed molecular knowledge of the parasite.

Against this backdrop, a project to sequence the entire genome of *Eimeria tenella* was started in 2002. Whole genome shotgun sequencing has produced contiguous sequences totalling 58 Mb in length. Predicting the genes is the next challenge. Although distantly related to other Apicomplexan parasites that have been subjected to genome sequencing, *Eimeria* is the most complex yet. The base composition of the genome is unusual and the gene structures are complex. Gene predictions can be browsed, searched or downloaded via the GeneDB database (http://www.genedb.org/Eimeria), allowing scientists working on *Eimeria* to quickly access genes of interest to their own research, such as enzymes, ligands, transporters or surface proteins. Detailed annotation of the genes will allow aspects of the organism's biology to be reconstructed *in silico*, for instance to gain insights into pathways that would take years to characterise *in vitro* or *in vivo*.

The genome sequence provides a framework to underpin scientific research, not only accelerating hypothesis driven research but also generating hypotheses for further investigation.

Proteomics of *Eimeria* : a focus on host cell invasion

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Apicomplexan parasites are serious pathogens of man and domestic livestock for which there is an urgent need to develop novel, sustainable therapies based on new drugs or vaccines. The genomes of many species have been sequenced, or are nearing completion, including those of *Plasmodium falciparum*, *Cryptosporidium parvum*, *Theileria parva*, *Theileria annulata*, *Toxoplasma gondii* and *Eimeria tenella*. Detailed analysis of these genomes will be invaluable for developing an understanding of the biology and biochemistry of the Apicomplexa and for guiding the selection of novel, effective targets for drug and vaccine design. However, whilst genome sequences give good indications of the genes that are present within each parasite, they provide no clues about the expression or localisation of gene products, for example whether they are expressed at all, targeted to specific sub-cellular location, switched on or off at particular points during the parasite lifecycles or whether their level of expression is modulated by certain conditions. It is precisely these types of questions that proteomics, defined as the study of the full protein content of an organism, or an organelle, is designed to answer.

For obligate intracellular parasites such as the Apicomplexa, the molecular interactions between parasite and host-cell surfaces define uniquely each host-parasite relationship. The essential nature of host cell recognition, attachment and invasion, the repeated rounds of invasion that occur during the course of infections and the accessibility of the extracellular parasite make these interactions priority targets for intervention. The process of invasion is more or less conserved between most apicomplexans and consists broadly of (1) contact of parasite with host cell (2) reorientation of the parasite to make apical contact (3) tight attachment of the parasite apex to the host cell plasma membrane (PM) (4) rapid invasion of the parasite accompanied by deformation of the host PM to form a parasitophorous vacuole (5) pinching off of the PV from the PM. The process is driven by a parasite actinomyosin contractile motor and because host cell surface proteins are excluded from the PV membrane, parasites within the PV remain isolated from the host cell endocytic pathway. In recent years, a working hypothesis of apicomplexan invasion has evolved in which four classes of parasite proteins, in addition to the motor proteins, are implicated. These are GPI-linked surface antigens and a variety of soluble and membrane-bound proteins derived from the microneme (MIC), rhoptry (ROP) and dense granule (GRA) secretory organelles. However, as with the process of invasion itself there are many paradoxes that remain to be explored concerning apicomplexan secretory organelles. Chief amongst these is the fact that the number and type of organelles varies enormously between parasites, and between different developmental stages of the same parasite, and may even be absent. Thus micronemes range from a handful (*Plasmodium* merozoites and sporozoites) through to many hundred (*Eimeria* merozoites) per cell, rhoptries from zero (*Plasmodium* ookinetes) or one (*Cryptosporidium* sporozoites) to around a dozen (*Eimeria* sporozoites) and dense granules from possibly zero (*Eimeria* sporozoites) to around a dozen or more (*Toxoplasma* tachyzoites).

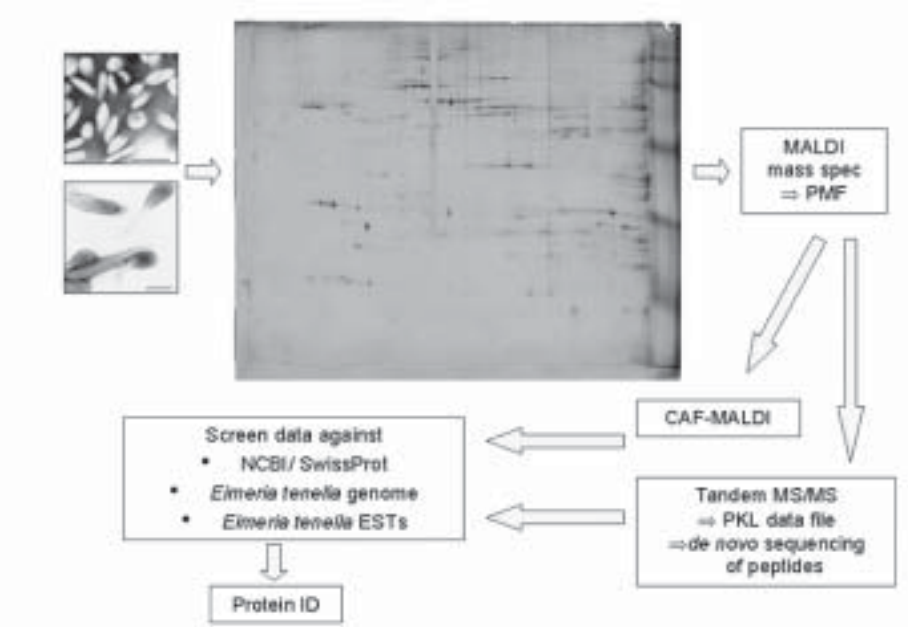
We have focused attention in our laboratory on defining the proteomes of the microneme and rhoptry organelles of *Eimeria tenella* with a view to understanding the precise function of these organelles. Using sequences of 36 characterised microneme, rhoptry and dense granule proteins from *T. gondii* and 21 from *Plasmodium* spp., we initially screened *E. tenella* genome and EST databases *in silico* to look for orthologues and homologues. Whilst we found some degree of conservation between the microneme proteins of the three species, largely due to the possession of common adhesive domains, there was little evidence for conservation of either rhoptry or dense granule proteins (for example, see Table below).

COMPARISON OF ORGANELLE PROTEINS BETWEEN *T. GONDII* AND *E. TENELLA*

Micronemes		Rhoptries		Dense Granules	
T. gondii	*E. tenella*	*T. gondii*	*E. tenella*	*T. gondii*	*E. tenella*
TgMIC1	EtMIC3	TgROP1	-	TgGRA1	-
TgMIC2	EtMIC1	TgROP2	?	TgGRA2	-
TgM2AP	EtMIC2	TgROP3	-	TgGRA3	-
TgMIC3	?	TgROP4	?	TgGRA3-like	-
TgMIC4	EtMIC5	TgROP5	-	TgGRA4	-
TgMIC5	-	TgROP6	-	TgGRA5	-
TgMIC6	?	TgROP7	-	TgGRA6	?
TgMIC7	?	TgROP8	?	TgGRA7	-
TgMIC8	?	TgROP9	EtROP9	TgGRA8	-
TgMIC9	?	TgSUB2	EtSUB	TgNTPase	EtNTPase
TgMIC10	-			TgCyclophilin	EtCyclophilin
TgMIC11	EtMIC11				
TgMIC12	EtMIC4				
TgAMA1	EtAMA1 EtAMA2				

Using proteomics tools we have now characterised the protein content of gradient-purified preparations of micronemes and rhoptries from *E.tenella* sporozoites. The methods for purifying these organelles is long-established in our laboratory (Kawazoe et al, 1992, *Parasitology* **104**, 1-9) and whilst there is some debate about whether dense granules are present in these stages of *Eimeria*, these organelles would most likely be isolated in the same fraction as the rhoptries. Organellar proteins were separated by two dimensional gel electrophoresis, which fractionates proteins on the basis of both their pI and molecular mass. After staining with a modified silver stain, protein spots were excised, digested with trypsin and analysed by various methods including matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), and tandem electrospray MS. Interpretation of the mass spectra and comparison of these data with *in silico* protein predictions derived from the *Eimeria* genome and other databases using Mascot software resulted in identification of a large proportion of the protein spots.

Pipeline: Microneme and rhoptry proteomes of *E. tenella*



Whilst 2D gels have the advantage over 1D gels of allowing differentiation between several proteins of the same molecular mass, they are acknowledged to be unsuitable for the separation of certain types of protein, including very large or low abundance proteins, hydrophobic proteins and proteins of extreme pI. Each large format 2D gel was loaded with parasite organelle protein purified from 5-10 infected chickens. However even when the technique was fully optimised with help from 2D gel experts it was clear that the amounts of proteins visible on the 2D gels did not reflect the quantities of protein loaded, particularly for the rhoptry fractions. Therefore we also separated batches rhoptry proteins by 1D SDS PAGE and found that many more of the larger proteins were separated so these were also analysed by Tandem MS.

The proteins identified using these approaches fell into three major groups. In the first group were exogenous non-*Eimeria* contaminants, such as trypsin inhibitor, that were introduced during subcellular fractionation. Second were *Eimeria* proteins that from their homologies to other known proteins are not expected to localise to the apical organelles and were most likely structural contaminants. The third group consisted of *Eimeria* proteins that most likely encode novel organellar proteins. This final group included 8 putative novel microneme proteins and 28 putative novel rhoptry proteins. We have selected a number of these novel proteins for further study, as demonstrated by some examples outlined below.

ROPF is a protein which shares some limited homology to hypothetical proteins from other apicomplexan parasites so may represent a novel class of organellar proteins from these parasites. ROPF is an 200kDa protein excised from a SDS PAGE gel and the sequences of tryptic peptides from this band all matched to a single contiguous genome sequence and to hypothetical proteins from two species of *Plasmodium*. Interestingly, peptides from proteins migrating above and below ROPF on SDS PAGE in the range 180-220kDa were also found to hit the same region of the genome Due to the overlap of the peptides from different proteins along the length of the gene we have concluded that these proteins are differently modified forms of expressed gene product. Sequencing the full-length cDNA of ROPF has revealed that it occupies >9kb of the genome and is organised over 22 exons. We have found evidence of extensive alternative splicing, which accounts for several of the isoforms and are currently sequencing clones from a ROPF mini-cDNA library to determine the exact sequence differences between the isoforms.

ROPJ is a putative rhoptry protein that shares homology with another set of *Plasmodium* hypothetical proteins, as well as scoring hits onto the genome and/ or ESTs of *T. gondii*, *N. caninum* and *T. annulata*, suggesting that this protein belongs to a well-conserved family of rhoptry proteins from across the whole apicomplexan phylum. We have sequenced the full-length cDNA of ROPJ, revealing that the gene is spread over 5kb of the genome, over 12 exons one of which is alternatively spliced between different cDNAs. ROPJ contains multiple predicted transmembrane domains and a putative C terminal tyrosine-based rhoptry sorting signal.

EtSUB is a subtlisin, a type of serine protease named after its similarity to a protease which was found to be secreted by the bacterium *Bacillus subtilis*. The active site of subtilisins consists of a characteristic catalytic triad of residues: aspartic acid, histidine and serine. The catalytic region of EtSUB has homology with *T. gondii* subtilisin TgSUB2, which itself is homologous to the *P. falciparum* subtilisin PfSUB2. The apicomplexan subtilisins are of interest to us as they are variously localised to all three apical organelles: the microneme (TgSUB1), the rhoptry (TgSUB2) and the dense granules (PfSUB1 and PfSUB2). Whilst EtSUB appears to be most similar to the rhoptry enzyme, TgSUB2 we have not yet definitively localised the enzyme to these organelles. We have sequenced the full length cDNA of EtSUB, which has revealed a transcript of approximately 1.1kb encoding an enzyme of ~114kDa. Again the gene is complex, being organised over 16 exons with one region of alternative splicing.

These three examples of putative rhoptry proteins highlight some of the characteristic features of *E. tenella* genes. They are typically complex, being organised over multiple exons and employing alternative

splicing as a mechanism for gene expression (see http://www.tigr.org/tigr-scripts/tgi/splnotes.pl?species=e_tenella for other examples of alternative splicing).

In conclusion, many novel proteins of *E. tenella* have been identified through proteomic analyses of subcellular fractions of parasites enriched for either microneme or rhoptry secretory organelles. The relatively complex nature of these organelles is readily apparent and the analysis of organelle-specific proteomes of one parasite can lead to the identification of previously unknown homologues in other parasites. It is clear that an understanding of the molecular structure, processing, cellular context and precise interactions of target molecules will be critical to the rational development of effective intervention strategies against apicomplexan parasites. Also, significant insights into the molecular evolution of this complex and important group of pathogens can be obtained by comparative analysis of their organelle-specific proteomes. In collaboration with experts on *Plasmodium* and *Toxoplasma* we are now expanding work in this area with the intention of (i) using our expertise in cellular fractionation to purify organelles from other parasites where organelles are less abundant (ii) generating organellar proteomes from important apicomplexan parasites to build a comprehensive picture of the evolution, diversity and function of secretory organelles across the phylum and (iii) exploiting the more tractable reverse-genetic systems of *Toxoplasma gondii* and *Plasmodium berghei* to begin functional analysis of organellar proteins that are conserved between different genera, focusing initially on the genes that we have already identified in the current work. We are also currently undertaking a much more comprehensive, high-throughput proteomics study in which we aim to define whole cell proteomes for several different developmental stages of *E. tenella* using both 2DGE and liquid-chromatography separations, a process that will not only help to build up knowledge regarding stage-specific gene regulation in this parasite but will also served to help and support the mammoth task of accurate and comprehensive genome annotation.

We would like to thank the large number of people who have contributed to this work including Rich Oakes, Pierre Rivailler and others from the parasitology group at IAH; Mike Dunn and colleagues at Proteome Sciences, London; Jonathan Wastling and colleagues from University of Liverpool, Bob Sinden and colleagues from Imperial College and by no means least the entire *Eimeria* genome consortium for providing the essential sequence data that underpins the work.

The role of morphology and microscopy in coccidian research in this genomic/proteomic age

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The history of the Coccidia is intimately involved with microscopy. The parasites are below the resolution of the human eye (with the exception of giant tissue cysts of certain *Sarcocystis* species) therefore microscopy is required for visualisation and identification of the parasite. The Coccidia belong to the phylum Apicomplexa, a widespread and successful group of protozoan parasites which includes many of medical and veterinary importance. The family Coccidia is characterised by a life cycle involving faecal/oral transmission with asexual and sexual development within the alimentary tract of the definitive host. To survive the rigors of the external environment the parasites have evolved a cystic stage (oocyst) that is released with the faeces and can survive in the external environment. The appearance and size of these oocysts are characteristic of a given species and microscopy of faecal samples has been used for many years to diagnose infections and to identify the species involved. This single host species life cycle is typical of those parasites of the genus *Eimeria* responsible for coccidiosis in chickens. However, a sub-group of the Coccidia (including the genera *Toxoplasma, Neospora, Hammondia, Besnotia* and *Sarcocystis*) has evolved a more elaborate life cycle involving intermediate hosts where tissue cysts are formed in the muscle or brain. In these cyst forming Coccidia, infection can be transmitted to the definitive host when ingested with the tissue of the intermediate host. There are obvious advantages for this complex life cycle for coccidian parasites of carnivores.

The vital role of microscopy, especially electron microscopy, can be inferred from the name of the phylum – the Apicomplexa. This name is derived from the ultrastructural identification of a characteristic group of organelles at the apical end of the infectious stages. Through the 1970s and 80s straightforward electron microscopy added greatly to knowledge and understanding of the Coccidia and the developmental processes involved in their life cycle. Unfortunately many of the papers were published before the modern electronic age and therefore are not readily accessible. More recently there has been a renaissance of both light and electron microscopy with the development of new molecular techniques. A major advance was the application of immunocytochemistry in which antibodies can be used to identify and localise specific molecules during the parasite life cycle. Now light and electron microscopy, in conjunction with modern genomic and proteomic techniques, plays a vital part in a multidisciplinary approach to improve our understanding of Coccidian parasites. A few areas where microscopy and immunocytochemistry have added to our knowledge of the Coccidia will be illustrated.

APICAL COMPLEX

The specific group of organelles found in the anterior of the infectious stages that give rise to the name Apicomplexa consist of three distinct structures; the micronemes (small cigar-shaped structures), the rhoptries (bulbous structures with ducts running to the anterior) and the dense granules (spherical structures) that can also be found in other regions of the parasite (Fig 1a). In the Coccidia, the infectious stages also possess an additional organelle, the conoid, which is a truncated cone comprised of microtubules (Fig 1a). The relative and absolute numbers of the various organelles can vary between species and even between the infectious stages in a single species. For example the merozoites of *Eimeria tenella* have large numbers of micronemes but few dense granules in contrast to the tachyzoites *Toxoplasma gondii* that have numerous dense granules but few micronemes. Within the Coccidia, this group of organelles are believed to play a co-ordinated role in the infection of new host cells. The contents of the micronemes are released first and are involved identification and adherence to suitable host cells. The content of the rhoptries are released during the invasion process and some proteins are incorporated into the parasitophorous vacuole. Finally the dense granules are secreted into and are believed to modify the

parasitophorous vacuole to facilitate parasite development (reviewed by Carruthers 2002, Mercier et al 2005). Using molecular techniques, numerous new proteins are continually being identified (Zhou et al 2005, Bradley et al 2005) and by using immuno-light and electron microscopy it has been possible to identify the location of certain of these molecules to one or other of the apical organelles. This can assist in understanding their biological function. By immuno-light microscopy, molecules can be localised to the apical region (Fig 1b) and using immuno-electron microscopy it is possible to identify the exact organelle they are located in (Fig 1c). It is possible to localise unknown apical proteins to the micronemes (MIC proteins), the rhoptries (ROP proteins) or the dense granules (GRA proteins). It is also possible using double or triple labelling, to show that different proteins are located within the same or different organelles. For example it can be confirmed that GRA2, 4 and 6 are located within the same dense granule (Fig 1d) and it is also possible to differentiate between organelles by their protein content with rhoptries labelled with anti-ROP2 and dense granules by anti-NTPase, a dense granule protein (Fig 1e). In elegant studies using *T. gondii*, which is most amenable to molecular manipulation, it has been possible to transfect parasites with fluorescent (GFP) proteins and identify the factors which specifically targets proteins to each of the various organelles (Gubbels and Striepen, 2004).

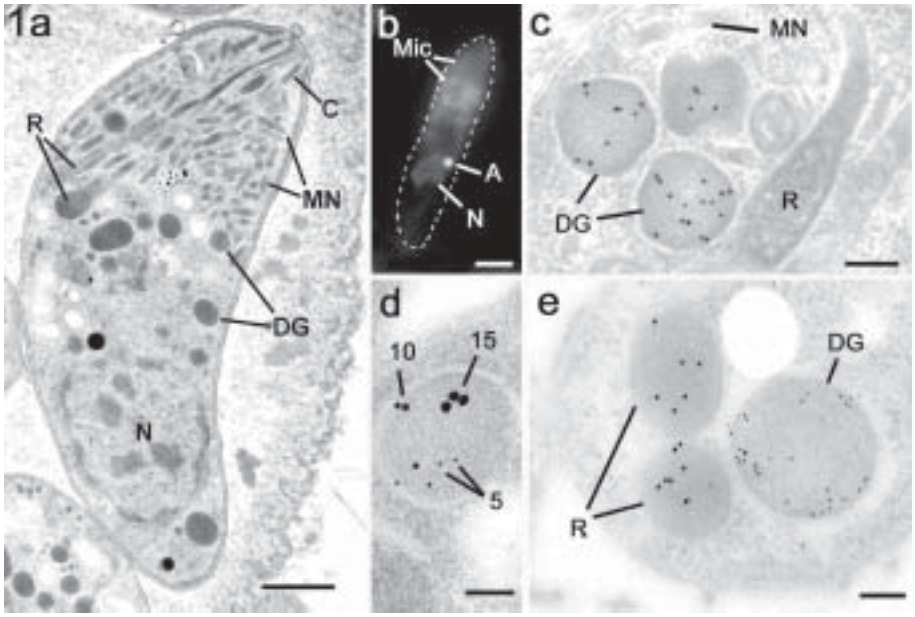

Figure 1

Figure 1a. Electron micrograph of a bradyzoite of *T. gondii* showing the characteristic compliment of apical organelles consisting of the conoid (C), micronemes (MN), rhoptries (R) and dense granules. N – nucleus. Bar is 1µm.

b. Immuno-fluorescent image of a double labelled merozoite of *E. tenella* showing labelling of the anterior with anti-MIC2 (Mic; red) and the small peri-nuclear apicoplast labelled with anti-enoyl reductase (A; green). Nucleus labelled with DAPI (N; blue). Bar is 1µm.

c. Immuno-electron micrograph of a tachyzoite of *T. gondii* labelled with anti-GRA6 showing the gold particles are limited to the dense granules (DG) while the micronemes (MN) and rhoptries (R) and negative. Bar is 100 nm.

d. A triple labelled section using anti-GRA2 (5 nm gold), anti-GRA4 (10 nm gold) and anti-GRA6 (15 nm gold) showing a dense granules labelled with all three markers. Bar is 100 nm.

e. Cross section through the anterior of a merozoite of *T. gondii* double labelled with anti-ROP2 (10 nm gold) and anti-NTPase (5 nm gold) showing the specific labelling of the rhoptries (R) and dense granules (DG). Bar is 100nm

APICOPLAST

More recently, the apicomplexan parasites have also been found to possess a second unique feature. They contain a non-photosynthetic plastid, which has been termed the apicoplast (reviewed by Wilson 2002, Waller and McFadden 2005). This residual plastid is believed to be derived from an ancient secondary endosymbiotic acquisition from a red alga (Wilson 2002). The plastid genome was first identified in *Plasmodium* sp. but has subsequently been shown to be present in all members of the Apicomplexa with the exception of *Cryptosporidium sp.* (Xu et al 2004). It has been shown that the apicoplast is essential for parasite survival and parasites without an apicoplast are non-viable. In the absence of a plastid, parasites displayed an unusual delayed death phenotype (He et al 2001). That is, the parasites without a plastid could continue to divide within the host cell vacuole as long as one plastid containing organism was present. Indeed they were able to escape and invade new host cells but were unable to undergo proliferation within the new host cell resulting in death of the parasite (He et al, 2001). These findings are extremely exciting since the prokaryotic nature of this organelle could represent new and unique targets for drug intervention. The role of the apicoplast is still incompletely understood but it is known to be involved in type II fatty acid biosynthesis and isoprenoid biosynthesis (reviewed Wilson 2002, Waller and McFadden 2005). By electron microscopy it was shown that this genome was located within an organelle characterised by being limited by multiple membranes (McFadden et al 1996, Kohler et al 1997) (Fig 2c). This organelle was first identified by electron microscopy in the 1960s (Sheffield and Melton 1968) although its function remained unknown. The exact number of membranes is still controversial (Kohler 2005) but the presence of multiple (3/4) membranes allows identification of the organelle and differentiates it from the other genome containing organelles (the nucleus and the mitochondrion) that are limited by two membranes (Fig 2c, e). It has been found that many of the plastid specific proteins are encoded by nuclear genes (lateral gene transfer) and these proteins have to be targeted back to the apicoplast using a specific bi-partite targeting sequence. Initial morphological studies of the apicoplast examined, in vitro, asexual development of parasites transfected with the green fluorescent protein targeted to the apicoplast (Striepen et al 2000, Vaishnava et al 2005). More recently, in vivo studies using antibodies to an apicoplast specific protein (enoyl reductase) have examined the changes in the apicoplast during both asexual and sexual development in *T. gondii* (Ferguson et al 2005) and *E. tenella*. In the infectious (sporozoite, merozoite, tachyzoite and bradyzoite) stages the apicoplast is normally present as a single small spherical structure adjacent to the nucleus (Fig 1b, 2a) although multiple plastids have been observed in the merozoites of *E. tenella* (Fig 2b). Asexual proliferation (schizogony) results in the simultaneous formation of numerous daughters. To ensure their viability, each merozoite must receive a nucleus and at least one apicoplast and mitochondrion. This requires a tightly co-ordinated process of nuclear, apicoplast and mitochondrial division and segregation. The possible mechanisms are still under investigation. There are conflicting observations with certain studies showing a close relationship between nuclear and apicoplast division (Striepen et al 2000, Vaishnava et al 2005) while others show evidence that nuclear and apicoplast division are independent events (Ferguson et al 2005) (Fig 2d). However, organelle segregation to the daughters may involve the nuclear pole/centriolar complex in all cases. When sexual development was examined, there was found to be little change in the apicoplast during microgametogony and the microgametes lacked an apicoplast. In contrast there is a marked increase in the size and activity of the apicoplast during macrogametogony perhaps reflecting the increased metabolic activity (Fig 2f). This means that there will be maternal inheritance of the apicoplast with the sporozoites, formed within the oocyst, receiving their apicoplast from the macrogamete (Ferguson et al 2005).

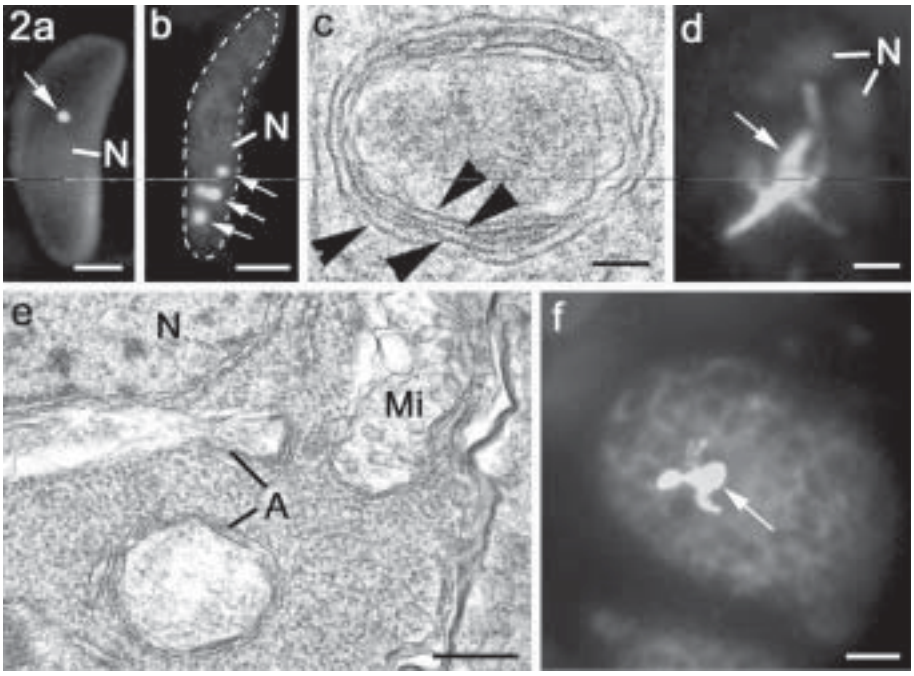

Figure 2

Figure 2 a and b. Tachyzoite of *T. gondii* (**a**) and merozoite of *E. tenella* (**b**) labelled with anti-enoyl reductase showing the peri-nuclear location of the single or multiple apicoplasts (arrows; green). Bar is 1μm.

c. High power electron micrograph of an apicoplast in *E. tenella* showing the four limiting membranes (arrowheads). Bar is 100 nm.

d. Immuno-fluorescent image of multinucleate schizont of *T. gondii* in the cat intestine showing a large elongated and branched apicoplast (arrows). Bar is 1μm.

e. Electron micrograph of part of a schizont similar to that in **d** showing the enlarged multimembraned apicoplast (A) and the nucleus (N) and mitochondrion Mi) limited by two membranes. Bar is 100 nm

f. Immuno-fluorescent image of macrogamete of *T. gondii* in the cat intestine showing a large elongated and branched apicoplast (arrows). Bar is 1μm.

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ASEXUAL DEVELOPMENT.

Asexual development within the Coccidia is often considered as a single process, which has been termed schizogony, only varying in the number of daughters formed. However when the process was examined in different species by electron microscopy, subtle differences could be identified showing at least four variations in the process exhibited by different parasites. There is classical schizogony which is undergone by the vast majority of coccidian species including the genera *Eimeria*. It can be summarised as involving a proliferative phase in which there are repeated cycles of DNA replication and nuclear division giving rise to a multinucleate stage followed by a differentiation phase where daughter formation is initiated at the parasite surface and the nuclei and other organelles segregate to the daughters as they bud into the parasitophorous vacuole (Fig 3). A variation of this process is undergone by the coccidian stages of *T. gondii* in the cat gut where there is an identical proliferative phase but during the differentiation phase daughter formation is initiated and completed within the mother cell cytoplasm (Ferguson et al 1974, 2005) and this process has been termed endopolygeny (Fig 3). A third variation is that undergone by certain asexual stages of *Sarcocystis spp.* where there are repeated cycles of DNA replication but with no nuclear division. In this case the initiation of multiple daughter formation occurs within the mother cell cytoplasm and coincides with fragmentation of the polyploid nucleus into a number of haploid nuclei each of which enters a developing daughter in a similar manner to that seen in endopolygeny (Speer and

Dubey 1999, 2001, Vaishnava et al 2005). The fourth variation is the one that has been most extensively studied since it is that undergone by the exo-enteric (tachyzoites and bradyzoites) forms of *T. gondii* and has been termed endodyogeny. In this case only two daughters are formed with the initiation of daughter formation within the mother cell cytoplasm occurring before completion of nuclear division (Fig 3). The process of daughter formation is similar to that observed during endopolygeny but only two daughters are formed (Sheffield and Melton1968, Striepen et al 2000). These three variations from classical schizogony are the exception and it may be significant that they are found within the sub-group of cyst forming Coccidia, which have had to adapt to development in intermediate hosts. Although these subtle variations can only be identified by electron microscopy, an appreciation of these differences can help to reconcile certain apparent conflicting results in relation to the division and segregation of the nuclei and apicoplasts during daughter formation in *T. gondii, Sarcocystis sp.,* and *E. tenella* (Striepen et al 2000, Vaishnava et al 2005, Ferguson et al 2005)

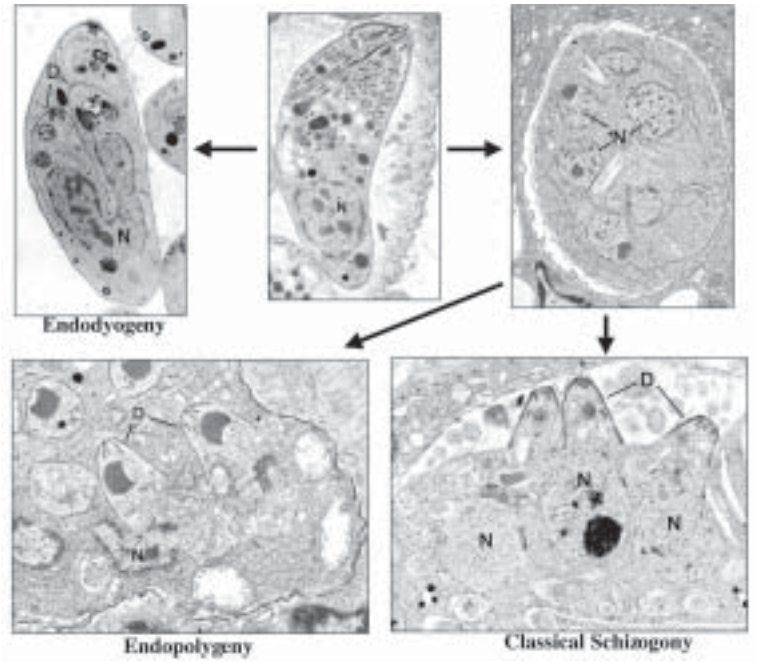

Figure 3

Figure 3a. A montage of asexual development illustrating certain of the ultrastructural differences associate with the process of endodyogeny, endopolygeny and classical schizogony within the Coccidia.

SEXUAL DEVELOPMENT

Microgamete formation

Within the Coccidia, microgametogony results in the formation of relatively low numbers of microgametes. The relative number of microgametocytes varies between species. In the case of *T. gondii,* where there are relatively few microgametocytes to the number of macrogametes, it has been questioned if there are sufficient microgametes to ensure that efficient fertilisation can take place (Ferguson 2003). By electron microscopy it is interesting to note just how similar microgametes are to mammalian sperm. They consist of a compact, electron dense nucleus, a mitochondrion and are powered by two large flagella (Fig 4a, b).

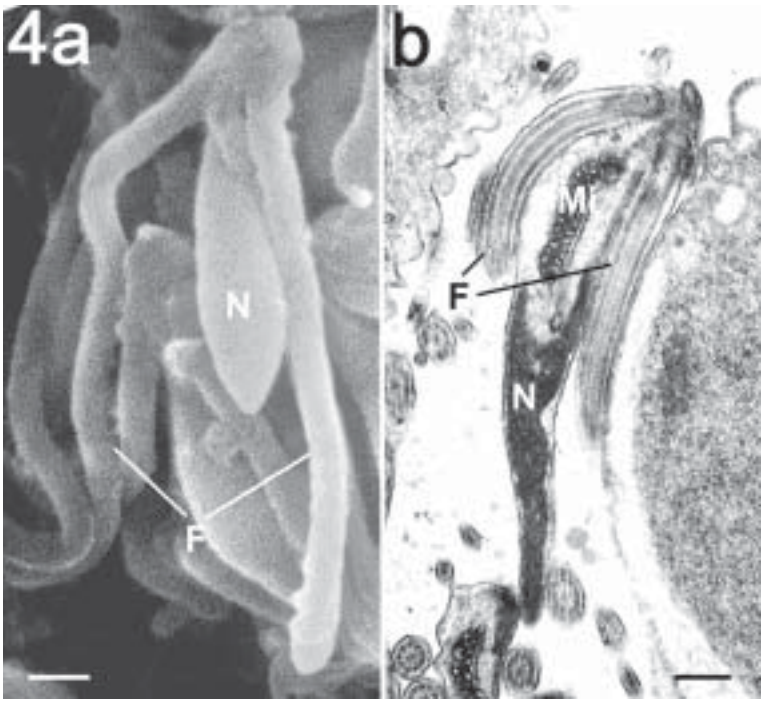

Figure 4

Figure 4. Scanning (**a**) and transmission electron microscopy (**b**) of the mature microgamete of *T. gondii* showing the nucleus (N), mitochondrion (Mi) and two flagella (F). Bar is 100 μm.

MACROGAMETE AND OOCYST FORMATION

The survival of this group of parasites depends on the ability of the oocysts formed from the macrogametes to survive for extended periods in the external environment. Not only that, but the oocyst have to undergo sporulation to form eight infectious sporozoites, which is the only example of extra-cellular development by coccidian parasites. This places two essential requirements on the developing macrogamete. The first is that it must acquire and store all the ingredients necessary to allow sporulation while cut off from additional nutrients in the hostile external environment. Thus the macrogamete contains large amounts of storage material in the form of polysaccharide granules and lipid droplets (Fig 5a). The second is for the macrogametocyte to synthesise and store the material required to form the oocyst wall. The oocyst wall is a complex structure consisting of a loose outer veil, which is lost during excretion, and a wall consisting of two distinct layers, a thick electron dense outer layer and a thinner more electron lucent inner layer (Fig 5g). The structure is pretty consistent across the Coccidia (Ferguson et al, 1975, 1977, 2003). The report of a thin (10 nm thick) outer layer and a thicker (90nm) inner layer observed for oocysts of *E. tenella* isolated from faeces (Stotish et al 1978) may represent an example of where part of the wall has been lost during processing. Any disruption in the process of oocyst wall formation would have serious implications for parasite transmission. To understand this process, genes for certain components of the oocyst wall have been identified (Belli et al 2002a, b, 2003, Wallach, 2002). Using antibodies to these and to other proteins it has been possible to start to dissect the changes occurring during macrogamete maturation and oocyst wall formation by combining electron microscopy and immunocytochemistry (Mouafo et al. 2002, Ferguson et al, 2003). An accidental and unexpected finding was that an antibody to the specific apple domains of the MIC4 protein of *T. gondii* (TgMIC4, Brecht et al 2001) cross reacted with a subset of granules in the early macrogametocyte of *T. gondii* (Ferguson et al, 2000) but also with small granules in the early macrogametocytes of *E. maxima* (Ferguson et al, 2003) and *E. tenella*. In all three cases, the contents of these granules were secreted into the parasitophorous vacuole and appeared to associate with the formation of the loose outer veil (Fig 6d). These previously unidentified granules have

been termed the veil forming bodies (VFB) (Ferguson et al 2003). It is interesting to speculate that the veil contains a protein with apple domains and, since these domains are associated with protein-protein or protein-carbohydrate interactions, they could be involved in microgamete recognition. The second type of granule is the large electron dense membrane bound spherical structures, which have been termed the wall forming bodies type 1 (WFB1) (Fig 5a, g). In addition, there is a third structure consisting of irregular shaped electron dense deposits located with the rough endoplasmic reticulum that have been identified as the wall forming bodies type 2 (WFB2) (Fig 5a, g). Using immuno-light and electron microscopy is was possible to identify and follow the changes in these structures during oocyst wall formation. Antibodies to affinity purified gametocyte antigens (anti-APGA, Belli et al 2002a, b) stained the WFB1 strongly and the WFB2 weakly (Fig 5c) and it was possible to follow the secretion of the WFB1 to form the outer layer of the oocyst wall (Fig 5e, f). In contrast, the anti-gam56 (Belli et al 2002b) and anti-gam82 (Belli et al, 2003a) antibodies only stained WFB2 (Fig 5d), the contents of which were released to form the inner layer of the oocyst wall (Fig 5f). The question of how material located in the endoplasmic reticulum could be secreted remained. However, by immuno-EM, it was observed that, only after the release of the contents of WFB1, the WFB2 material retained in the rER was transferred to the surface via the Golgi body (Ferguson et al, 2003). This sophisticated control mechanism allows independent and sequential secretion of the three components thus allowing efficient formation of the stratified oocyst wall. These results were obtained using antibodies to *Eimeria maxima* proteins but there is likely to be close homology between the genes within the genus *Eimeria* and indeed it was found that these antibodies cross reacted with the homologous proteins of *E. tenella* and gave a similar staining pattern.

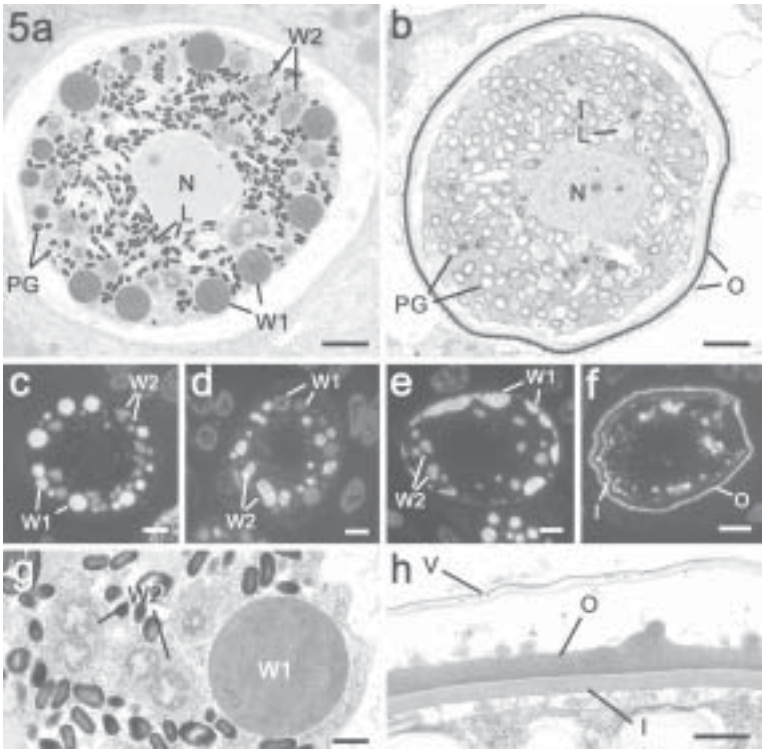

Figure 5

Figure 5a. Electron micrograph through a mature macrogamete of *E. maxima* showing the central nucleus and the cytoplasm packed with numerous polysaccharide granules (PG) and lipid droplet (L) in addition to the WFB1 (W1) and the WFB2 (W2). Bar is 1μm.

b. Sections through an early oocyst with a fully formed oocyst wall. Note that while the polysaccharide granules (PG) and lipid droplets (L) are still present but both types of WFB had disappeared. Bar is 1μm.

c. Immuno-fluorescent images through a mature macrogamete stained with anti-APGA showing strong labelling of the WFB1 (W1) and lighter staining of the WFB2 (W2). Bar is 1μm.

d. Similar organism to that in **c** stained with anti-gam56 showing that only the WBF2 (W2) are stained. The WFB1 (W1) were visualised by Evans blue staining (red). Bar is 1μm.

e. Early stage of oocyst wall formation showing secretion of the WFB1 to initiate formation of the outer layer of the oocyst wall (W1). Stained with anti-APGA. W2 – WFB2. Bar is 1μm.

f. Late stage in oocyst wall formation in which both the inner (I) and outer (O) layers are formed. Stained with anti-APGA. Bar is 1μm.

g. Detail from the macrogamete in **a** showing the large membrane bound WFB1 (W1) and the smaller irregular clumps of material representing the WBF2 (W2) located within the rER. Bar is 0.5 μm.

h. Detail of the fully formed oocyst wall consisting of the loosely attached outer veil (V) along with the electron dense outer layer (O) and the thinner and less electron dense inner layer (I). Bar is 0.5 μm.

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The process of fertilisation is yet to be observed in detail but it would appear that oocyst wall formation occurs prior to release from the host cell (Fig 6b). An extensive search failed to show microgametes associated with the macrogametes even when located in adjacent cells (Fig 6a). However, a high proportion of oocysts in the lumen were found to have attached microgametes (Fig 6c). These were located beneath the veil adjacent to the outer layer of the wall (Fig 6d). It is still not clear when and how the microgamete completes fertilisation.

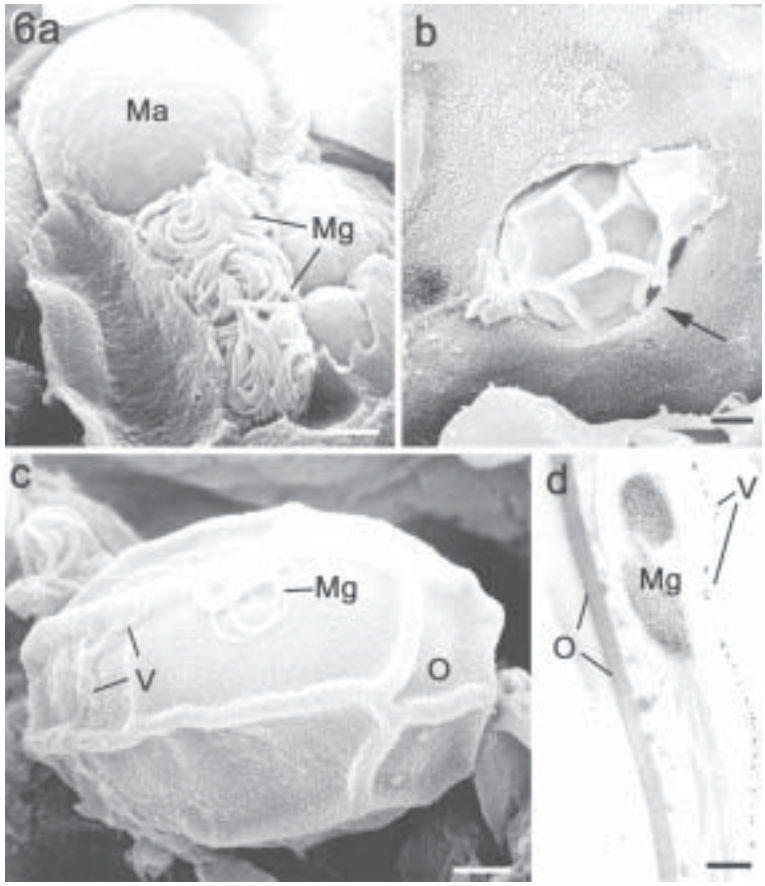

Figure 6

Figure 6a. Scanning electron micrograph (SEM) of a fracture through a caecum infected with *E. tenella* showing a microgametocyte with numerous mature microgametes (Mg) in a cell adjacent to a macrogamete (Ma). Bar is1 μm.

b. SEM of the luminal surface of a crypt showing an oocyst (characterised by the surface folds) escaping from and epithelial cell (arrow). Bar is 1 μm.

c. SEM of an oocyst in the gut lumen showing partial loss of the veil (V) and with a microgamete (Mg) adhering to the outer layer of the oocyst wall (O). Bar is1 μm.

d. Transmission electron micrograph through the surface of an oocyst showing the microgamete (Mg) located between the veil (labelled with anti-TgMIC5) (V) and the outer layer (O) of the oocyst wall. Bar is 100μm.

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This summarises a few examples where microscopy, combined with immunocytochemistry, has helped in our understanding of the Coccidia. There are still many areas where further research, using molecular techniques and microscopy, will provide new insights and perhaps identify areas of weakness in this important group of parasites.

ACKNOWLEDGEMENTS.

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New roles for proteases in the infection biology of coccidian parasites

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SUMMARY

Coccidian parasites including *Toxoplasma gondii* cause widespread infections and disease in humans and animals. The need for new therapeutic agents along with the fascinating biology of these parasites has fueled a keen interest in understanding how key steps in the life cycle are regulated. Proteolysis is intimately associated with cell and tissue invasion by these obligate intracellular parasites and recent studies have begun to identify the proteases involved in these processes. Based on clues from inhibitor experiments and cleave site mapping studies, we and others are using emerging genome information and molecular genetics to identify and validate proteases that regulate secretory organelle biogenesis and invasion protein activity. These studies are revealing roles for a variety of proteases including cathepsins, subtilases, and rhomboids in cell and tissue invasion. The identification of highly selective inhibitors for these proteases has the potential to not only further dissect their role in infection but also to potentially prevent or ameliorate disease.

1. INTRODUCTION

Coccidian parasites are responsible for several important animal and human diseases. *Neospora caninum* is a leading cause of spontaneous abortion in bovines, leading to significant economic losses in the dairy industry (1). *Eimeria* infection of commercial livestock remains an important source of production losses (2), particularly in the poultry industry where high density facilities provide an ideal environment for rampant spread of disease. *Toxoplasma gondii* commonly infects animals and humans, causing birth defects, ocular disease, and neurological complications, particularly in immunocompromised individuals (3). Collectively these parasitic infections have multi-billion dollar yearly economic impact due to production losses and the medical care and institutionalization of afflicted people.

Although a variety of antibiotics are currently used to treat coccidian infections, these anti-coccidial compounds remain hampered by limitations. *Eimeria* drug resistance or tolerance is an ongoing problem in poultry production facilities. Although drug resistance in *Toxoplasma* infections is uncommon, front line treatments such as antifolate combination therapy (e.g., pyrimethamine and sulfadiazine) are often poorly tolerated or cause severe allergic reactions in patients. These limitations, combined with the fascinating properties of coccidian parasites, have accelerated interest in investigating mechanisms governing the infection biology of these pathogens.

Most of the attention has focused on unique aspects of coccidian parasites that are distinct from their host or other eukaryotes because these exceptional features and properties represent both potential targets for therapeutic intervention and opportunities to expand the boundaries of biological insight. Cell invasion is a unique and essential property of coccidian parasites that has received particularly intense attention because of its dramatic features. Typically complete within 10-20 seconds, coccidian zoites power their way into susceptible host cells by using a unique form of locomotion, gliding motility. Molecular interrogations of gliding motility and host cell invasion have recently revealed that proteases play central

roles several distinct aspects of these processes. This article is intended to highlight new and emerging insight into the function of motility and invasion proteases in coccidian parasites.

2. INVASION ORGANELLES AND PROTEINS

Because of its experimental tractability, *Toxoplasma* has been most extensively investigated coccidian parasite for invasion studies. Like other coccidians, *Toxoplasma* actively invades host cells in a process that is largely parasite driven and distinct from the facilitated endocytosis mechanisms used by many intracellular bacteria and viruses. The parasite uses gliding motility to approach a target host cell and slide along its surface. Gliding motility is driven by the parasite's intrapellicular actin-myosin motor system (4), which connects both with the inner membrane complex (5) and transmembrane adhesive proteins secreted from micronemes (6). Micronemes are small cigar-shaped secretory organelles that discharge their contents by fusion at the extreme apical end of the parasite (7). Basal secretion of micronemes occurs continuously as the parasite glides over surfaces by treadmilling microneme adhesive proteins from the apical tip to posterior pole. Upon reaching a suitable site for invasion, the parasite further relies of sequential discharge of secretory organelles to mediate entry (8). In the first event, microneme contents accumulate on the parasite apical surface where they bind host receptors during a step that coincides with the formation of the moving junction, a tight apposition of the parasite and host plasma membranes. Immediately thereafter, the contents of the club-shaped rhoptries are extruded through an apical duct where they translocate into the host cell. Underneath the site of invasion, rhoptry proteins and lipids reconfigure into small vesicles (evacuoles) that fuse with the parasitophorous vacuole (PV) as the parasite plunges into the cell (9). Similar to gliding motility, entry is powered by the actin-myosin depend translocation of microneme adhesins backward across the parasite surface to the posterior end where they accumulate in a "cap-like" structure before being shed during the last few seconds of penetration (10). Although the PV is created by pushing in the host plasma membrane, the moving junction excludes most of the host surface components and this is thought to be a key survival strategy for avoiding acidification and fusion with the endocytic or lysosomal systems. Within a few minutes of entry, a third class of secretory organelles called dense granules (DG) are released by fusion with the apical membrane. DG proteins extensively modify the PV and are thought to participate in nutrient acquisition. Although DGs resemble regulated dense core secretory granules of higher eukaryotes, their secretion is not as tightly controlled as micronemes and rhoptries. This, along with evidence that DGs are a "default" pathway through which proteins traffic in the absence of specific forward targeting signals (11), suggests that DGs are not classic regulated secretory organelles. Interestingly, among the three classes of secretory proteins associated with invasion, DG proteins are the only group that is not proteolytically processed. Thus, proteolysis is tightly associated with micronemes and rhoptries, the two pathways that are most intimately coupled with parasite invasion.

3. UNDERSTANDING THE ROLE OF PROTEASES IN CELL AND TISSUE INVASION: FROM PROTEOLYTIC EVENTS AND INHIBITORS TO CANDIDATE ENZYMES

Two main tacks have been used to uncover evidence of proteases involvement in coccidian invasion. One approach has been to identify proteolytically processed invasion proteins and map their cleavage sites. Although usually not definitive, cleavage site information can often provide clues about the type of protease involved based on the known recognition specificities of different classes of proteases. A second approach is the use of class specific protease inhibitors. For example, the serine protease inhibitors 3,4 dichloroisocumarin (3,4 DCI) and 4-(2-Aminoethyl) benzenesulphonyl fluoride (AEBSF) have been reported to block *Toxoplasma* invasion of human fibroblast cells (12). Other serine protease inhibitors similarly block *Eimeria* invasion (13,14). Cysteine proteases have also been implicated in invasion based on findings that cysteine protease inhibitor (PRT2253F) with activity against cathepsin B inhibits *Toxoplasma* cell entry (15). Although, limited by the

possibility of non-specific "off target" effects, these studies at least provided initial evidence for the role of proteolysis in coccidian invasion. The identification and analysis of proteolytic substrates and the use of protease inhibitors have revealed that proteases function in several distinct steps of invasion (Fig. 1). This section will discuss how these approaches, along with the recent completion of the *Toxoplasma* genome, have recently led to the identification of candidate enzymes involved in each step.

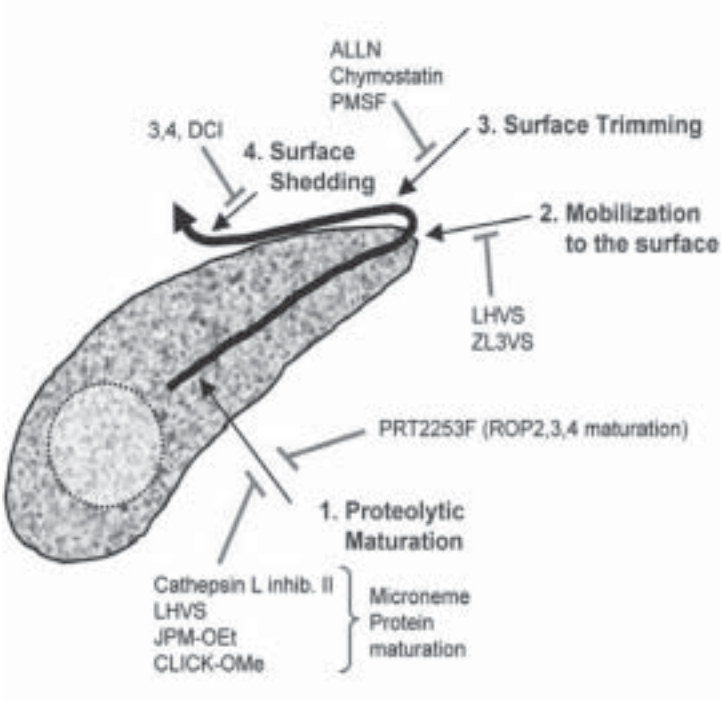

Figure 1. Sites of invasion protein proteolysis

Microneme and rhoptry invasion proteins are subjected to a series of proteolytic processing steps including: (1) Proteolytic maturation en route to secretory organelles; (2) Mobilization to the parasite surface during parasite attachment; (3) Primary processing that trims microneme proteins while they are expressed on the parasite surface; and (4) Shedding by which the microneme products are eventually released from the parasite surface after they translocate toward the posterior end. Protease inhibitors that block each of these steps are listed.

3.1 PROTEOLYTIC MATURATION OF INVASION PROTEINS

While performing pulse-chase metabolic labeling experiments, Achbarou (16) and Soldati (17) noted that nascent microneme (TgMIC3) and rhoptry (TgROP1) proteins are proteolytically processed within minutes of their initial translation. It is now appreciated that proteolytic maturation is a widespread phenomenon associated with most invasion proteins destined for secretion via the micronemes or rhoptries (Fig. 2). Such proteins are initially synthesized as preproproteins. The "pre" segment is the signal peptide, which is removed cotranslationally by signal peptidase during import into the endoplasmic reticulum. The "pro" peptide is subsequently removed as the protein transits through the secretory pathway. Rhoptry proteins appear to undergo processing in the nascent rhoptries during parasite cell division by endodyogeny (17,18). To investigate the sub-cellular site of microneme processing, we generated antibodies to the propeptides of TgMIC5 and TgM2AP. Immunofluorescent staining of extracellular or intracellular parasites revealed that these precursors occupy the trans-Golgi network and an early endosome compartment defined by co-staining with Rab51. No staining of mature micronemes was seen, however, some parasites showed partial localization of TgMIC5 within the DGs suggesting this may be an alternative route for secretion of immature microneme proteins. Collectively, our findings imply that microneme protein maturation occurs within or just beyond the early endosome, and not within in mature micronemes.

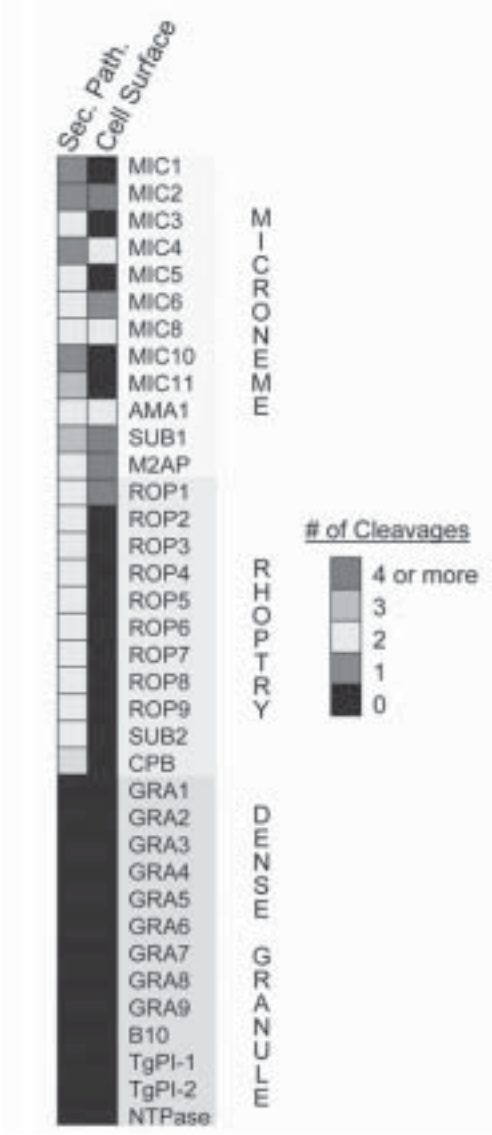

Figure 2. Selective proteolysis of *Toxoplasma* invasion proteins.

Microneme and rhoptry proteins are often multiply processed both within the secretory pathway and on the parasite surface whereas DG proteins are not subjected to postranslational processing.

What roles do propeptides play in the biogenesis of secretory organelles? Although this remains an incompletely resolved question, recent evidence suggests that propeptides assist in the trafficking and regulation of their cognate proteins. For example, the TgROP1 propeptide and a segment containing the TgROP4 propeptide both supported the trafficking of heterologous proteins to the rhoptries (19,20). It should be noted, however, that TgROP1 can also use alternative sorting signals since a propeptide deletion mutant was still correctly targeted to the rhoptries (17). Also, based on analysis of a non-cleavable site directed mutant, processing of TgROP1 is not necessary for its trafficking or for rhoptry biogenesis (21). However, since ROP1 is not an essential protein (22) and only one of many that are trafficked to the rhoptries, it may not be ideal for assessing a general role of propeptides in organellar biogenesis. On the other hand, protease inhibitors are capable of interfering with the processing of multiple substrates cleaved by a single protease and therefore are expected to have more profound effects. Consistent with this notion, Shaw et al. (23) showed that subtilisin inhibitor III and cathepsin inhibitor III block parasite replication

and cause marked abnormalities of secretory compartments including the rhoptries. Based in part on this study, Miller and coworkers (24) investigated a role for TgSUB2 in propeptide processing. TgSUB2 is a member of the subtilase family of serine proteases. Although *Toxoplasma* harbors at least 12 subtilase genes, most of these have not been investigated in any detail. TgSUB2 undergoes autocatalytic processing en route to the rhoptries and mutation of the natural autocleavage site revealed a key role for and acidic residue at the P1 position (cleavage site residues are designated P4-P3-P2-P1 / P1'-P2'-P3'-P4', where / indicates the scissile site). Interestingly, an acidic P1 residue is also required for ROP1 processing and TgSUB2 co-immunoprecipitates with ROP1. Also, ROP2, 4, and 8 have similar putative cleavage sites, suggesting they are candidate substrates for TgSUB2. However, a cysteine protease of the cathepsin family, TgCPB (also called TgCP1 or Toxopain 1), has also been implicated in rhoptry protein processing based on its trafficking to the rhoptries and on the observations that a cathepsin B inhibitor partially blocks ROP2,3,4 processing, disrupts rhoptry biogenesis, and interferes with parasite invasion and infection (15,25). Thus, it appears that *Toxoplasma* expresses at least two distinct rhoptry maturases that participate in the processing of rhoptry proteins and in rhoptry biogenesis.

Propeptides appear to also play a central role in the trafficking and regulation of microneme proteins. TgMIC3 uses a short N-terminal propeptide to mask the carbohydrate binding activity of its lectin-like domain (26). This presumably prevents inappropriate binding to parasite glycoproteins within the secretory pathway. A propeptide deletion mutant (Δpro) of TgMIC3 also fails to reach the micronemes and is instead retained within the secretory pathway along with its partner protein TgMIC8 (M. Lebrun, personal communication). Similarly, we have shown that ΔproTgMIC5 and ΔproTgM2AP are retained within or near the early endosome, in addition to other sites. These findings indicate that microneme propeptides function in the trafficking of their cognate proteins, possibly by binding to cargo receptors. Interestingly, TgM2AP's partner protein TgMIC2 also contains sorting signals in its C-terminal cytosolic domain (27) suggesting that multiple forward targeting elements are required for correct sorting to the micronemes. These signals may work at distinct sites, however, since deletion of TgMIC2 does not cause retention of TgM2AP but instead results in the misdirection of TgM2AP to the parasitophorous vacuole. Such findings are not universal though because deletion of the TgMIC6 propeptide has no effect on sorting of TgMIC6 or its partner proteins (TgMIC1 and TgMIC4) to the micronemes (28). To determine the type of protease involved in the proteolytic maturation of microneme proteins, we tested a series of protease inhibitors using the processing of nascent microneme proteins as an indicator of activity. Whereas serine or aspartyl protease inhibitors had no effect, cysteine protease inhibitors delayed the processing of TgM2AP and TgMIC3. Because cathepsin L inhibitor II was among the most effective inhibitors, we hypothesized that microneme proteins are processed by a cathepsin L-like enzyme. Analysis of the *Toxoplasma* genome database revealed that this parasite likely only has a single cathepsin L-like gene, termed *TgCPL*. TgCPL features a 30 kDa catalytic domain preceded by a proregion that includes a transmembrane anchor, which is a unique feature of apicomplexan cathepsin L proteases including the falcipain family in *Plasmodium* (29). After purification, recombinant proTgCPL undergoes autoactivation in vitro under mildly acidic conditions. Although definitive evidence is still forthcoming, several findings suggest a link between TgCPL and microneme protein maturation. First, the results of immunofluorescence and immunoelectron microscopy experiments suggest that TgCPL occupies a novel apical compartment intermediately positioned between the trans-Golgi network and the mature micronemes. Second, recombinant TgCPL can cleave recombinant proTgM2AP at or very near the correct cleavage site. Third, based on microarray screening of synthetic peptide substrate libraries, recombinant TgCPL shows a preference for leucine and structurally similar residues in the P2 position, which is consistent with the cleavage sites of TgM2AP, TgMIC3, TgMIC6, and TgAMA1, a microneme protein recently shown to be necessary for *Toxoplasma* invasion (30). Moreover, mutation of the TgM2AP P2 leucine residue to aspartic acid results in markedly less efficient cleavage and partial use of an alternative cleavage site 2 amino acids upstream of the normal

cut site. Additional studies to further test the role of TgCPL in microneme protein maturation are underway. A parallel may also exist in *Sarcocystis muris* since this parasite expresses both a cathepsin L like enzyme (SmTP1; (31)) and a microneme protein (16/17 kDa antigen; (32)) that undergoes proteolytic maturation at a cleavage site with a P2 leucine.

3.2 MOBILIZATION OF INVASION PROTEINS

While screening a small library of cysteine protease inhibitors in an invasion assay, we noted that two compounds substantially impaired invasion while simultaneously disrupting microneme protein release. These compounds LHVS and ZL3VS both feature a vinyl sulfone warhead for electrophilic attack of the active site cysteine of a thiolprotease. LHVS and ZL3VS showed low micromolar dose-dependent inhibition of parasite entry in two different invasion assays, and had similar potency for blocking microneme protein release. We focused on LHVS for subsequent experiments because it was slightly more potent than ZL3VS. Using fluorescent differential 2-dimensional gel electrophoresis (2D-DIGE), we showed that LHVS impaired the release of a variety of microneme proteins but did not affect secretion of DG proteins or proteins released from other internal sites. LHVS also disrupted gliding motility since treated parasites fewer and shorter trails compared to solvent or control compound treated parasites. We initially reasoned that these compounds may block gliding and invasion by preventing the proteolytic shedding of microneme proteins from the parasite surface. However, the recent evidence that serine proteases of the rhomboid family are responsible for microneme protein shedding (see below) is inconsistent with this notion. Also, unlike the serine protease inhibitor 3,4 DCI, LHVS treatment did not result in the accumulation of microneme proteins such as TgMIC2 and TgM2AP on the parasite surface after stimulating secretion with a calcium agonist. Based on these findings, we conclude that a cysteine protease is required at an earlier step, perhaps facilitating the mobilization of microneme contents to the parasite surface. Interestingly, a fluorescent derivative of LHVS (Bodipy-LHVS) covalently labels a single 30 kDa band that immunoprecipitates with TgCPL antibodies. Labeling was block by pretreatment with LHVS, suggesting that the recognition is highly specific. Moreover, bodypy-LHVS illuminates the same apical compartment occupied by TgCPL. Whether TgCPL's role in the mobilization of microneme proteins is related to its putative function as a maturase remains under investigation.

3.3 SURFACE TRIMMING (PRIMARY PROCESSING)

After being discharged from the micronemes, several invasion proteins are subjected to proteolytic cleavages that do not affect their association with the parasite surface. These events are termed primary processing or "trimming" because they occur before microneme proteins are proteolytically liberated from the surface (see below). Amino or carboxy terminal peptides of low structural complexity are often the targets of trimming, which often occurs at multiple sites in the same substrate. For example, an amino-terminal peptide extending from the globular A-domain of TgMIC2 is removed with with at least three endoproteolytic cleavages by microneme protein protease 2 (MPP2) activity (33). This processing was recently shown to activate the TgMIC2 A-domain for binding to ICAM1, which the parasite uses in part to traverse cell barriers to reach deep tissues where it replicates (34). Therefore, disrupting MPP2 activity is predicted to interfere with the pathogenesis of infection. MPP2 also cleaves TgMIC4 near its carboxy terminus and it trims off the carboxy terminal "coiled" domain from TgM2AP, along with another putative proteolytic activity MPP3 (35). Although all of the MPP2 cleavage sites for TgMIC2 and TgM2AP have been defined, this information was not particularly revealing, apart from suggesting that MPP2 prefers small to medium sized uncharged amino acids in the P1-P4 sites. Whereas MPP3 activity is resistant to all of the inhibitors tested, MPP2 activity is blocked by the tripeptide aldehyde compounds ALLN and ALLM and by the serine protease inhibitors chymostatin and PMSF (35). Although it was recently proposed (36) that surface trimming is responsible for activating adhesive proteins for tight binding to host receptors,

little evidence exists to support this idea. Critical to testing this hypothesis is the identification and characterization of the protease(s) responsible for MPP2 and MPP3 activities. Intriguing new findings from collaborative studies in between our labs (Carruthers and Kim) suggest a potential breakthrough in this quest.

While investigating phenotypic changes stemming from the targeted deletion of the *TgSUB1* gene, we noted from western blots that the trimming of TgMIC2, TgM2AP, and TgMIC4 was markedly diminished in the TgSUB1 knockout (KO) parasites compared to a control parasite line. This microneme derived subtilase uses a glycosylphosphatidyl inositol (GPI) anchor to transiently occupy the parasite surface before being proteolytically shed into the culture supernatant where it becomes a major component of the excreted/secreted antigen (ESA) fraction (37). To more widely examine changes in the processing of microneme proteins, we performed 2D-DIGE on ESA fractions collected from control parasites and TgSUB1KO. This analysis vividly showed the near complete absence of TgMIC2, TgM2AP, and TgMIC4 processing products, with the corresponding accumulation of precursor species. Since this pattern closely resembles that of ESAs collected from ALLN treated parasites (35), we tentatively conclude that TgSUB1 is MPP2. Although ALLN was initially reported to be a selective inhibitor of calpains (calcium dependent cysteine proteases), this compound also has activity against some serine proteases including the proteosome. Also, chymostatin and PMSF inhibition of MPP2 is also consistent with it being TgSUB1. Since subtilases are often activated by high calcium concentrations, this may be how TgSUB1 is regulated upon reaching the parasite surface and the extracellular environment. Intriguingly, preliminary mouse infection experiments suggest that TgSUB1KO parasites are moderately attenuated in virulence, with some mice surviving a normally lethal infectious dose. Further studies will be necessary to determine whether this is due to an effect on cell entry, tissue invasion, or both. Regardless, these studies may have wider implications since orthologous subtilases are expressed a variety of apicomplexans including *Neospora* (38,39) and *Plasmodium* (40).

3.4 SURFACE SHEDDING (SECONDARY PROCESSING)

In contrast to surface antigens (SAGs) which continuously occupy the parasite surface, invasion proteins derived from the microneme are only transiently associated with the parasite plasma membrane. Consequently, steady state levels of microneme proteins on extracellular tachyzoites are generally low. Microneme proteins are most readily detected on the parasite surface during invasion, where they can be seen accumulating on the extracellular portion of the parasite as they translocate backwards driven by the actin-myosin motor system. Our early studies on TgMIC2 indicated that this protein was shed into the culture supernatant as a smaller species that was devoid of its carboxy-terminal cytosolic domain and transmembrane anchor. This finding coupled with the observation that TgMIC2 is not seen on intracellular parasites suggested that microneme proteins are proteolytically released from the parasite surface during invasion. MPP1, the hypothetical protease responsible for shedding is unaffected by a wide range of protease inhibitors (33), suggesting it may be an unusual enzyme. This notion was subsequently corroborated when Dominique Soldati's group demonstrated that TgMIC6 is cleaved within its transmembrane anchor near the extracellular interface. Although this suggested the existence of an intramembranous protease, David Sibley's group simultaneously reported that mutation of two lysine residues outside the transmembrane anchor abolished shedding and disrupted invasion (41). To address this apparent discrepancy, Zhou et al. (35) used mass spectroscopy to determine that TgMIC2 is also cleaved intramembranously at a site precisely corresponding to that of TgMIC6. Consolidating these findings, it appears likely that while cleavage occurs within the transmembrane anchor, sequences outside of the anchor are required for protease recognition or for supporting a favorable structural configuration for cleavage.

Intramembrane proteolysis is a recently described phenomenon performed by integral membrane proteases that usually do not cleave until another protease has processed the substrate at another site (42). However, inhibition of MPP2 processing of TgMIC2 had no effect on its shedding, implying that MPP1 does not

require a primary processing event. This clue helped focus attention on the rhomboid family of intramembrane serine proteases, which can cleave their substrates without prior processing. Also, rhomboids typically cleave near the extracellular portion of the transmembrane anchor in a region populated by small, helix breaking amino acids such as alanine and glycine (43), properties that are consistent with the TgMIC2 and TgMIC6 cleavage sites. Similar highly conserved putative cleavage sites are seen a multitude of transmembrane microneme proteins expressed by *Neospora*, *Eimeria*, and *Plasmodium*, among other apicomplexans (44). Rhomboids are widely present throughout the phylum (44). Heterologous expression of fusion proteins with microneme transmembrane anchors showed susceptibility to cleavage by human and drosophila rhomboids (43). Analysis of the *Toxoplasma* genome revealed the presence of six rhomboid-like genes (ROM1, 2, 3, 4, 5, and 6) (44-46). Since ROM6 is highly homologous to a mitochondrial rhomboid involved in organellar fusion, this was eliminated as a candidate for MPP1. Also, ROM3 is not expressed in tachyzoites, reducing the likelihood that it encodes MPP1. Localization studies of the remaining candidates revealed that ROM1 is expressed in the micronemes, ROM2 is in the Golgi, ROM4 occupies the entire parasite surface, while ROM5 is most abundant on the posterior surface (45,46). Since MPP1 is constitutively active on the parasite surface (33,47), ROM4 and ROM5 are currently the best candidates. While ROM5 is the only ROM capable of cleaving a full length fusion protein of TgMIC2 in a heterologous expression system (45), this protease is not as well conserved among the Apicomplexa as is ROM4 (46). Determining precisely which ROM is MPP1 will probably require conditional expression experiments.

4. CONCLUSIONS

Building on data from protease inhibitor and cleavage site mapping, we and others are using the effectively complete genome sequences of *Toxoplasma* and other related parasites to begin matching proteolytic events with the associated enzymes. Emerging insight from these developments suggest that proteases play distinct roles in the trafficking, mobilization, and regulation of invasion proteins. Cysteine proteases (TgCPB and TgCPL) along with the subtilase TgSUB2 appear to participate in the proteolytic maturation of microneme and rhoptry substrates during the biogenesis of these organelles. Additionally, TgSUB1 and integral membrane protease of the rhomboid family function on the parasite surface where they regulate proteins involved in cell entry and tissue invasion. Future challenges not only include uncovering a deeper understanding of the biological roles of coccidian proteases but also the identification of selective inhibitors designed to interfere with their function for therapeutic gain.

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Horizontally transferred genes as new targets for drug development in *Cryptosporidium parvum*

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Cryptosporidium parvum has emerged as one of the most troublesome waterborne infections in the industrialized world. A number of large outbreaks have occurred in the U.S. with the largest in Milwaukee causing 403,000 cases of acute gastrointestinal diseases. Cryptosporidiosis is also an important factor in severe diarrheal disease in children around the world who lack access to contamination free drinking water. Crytosporidiosis is a gastrointestinal disease, characterized by watery diarrhea, abdominal cramps, nausea and fever. The disease is self-limiting with symptoms usually subsiding after 2 to 3 weeks. In contrast, immunosuppressed patients suffer from prolonged chronic disease resulting in severe dehydration and weight loss which can become live-threatening. It is these patients for which effective antimicrobial therapy is most urgently needed.

A wide variety of antimicrobial agents has been tested *in vitro*, in animal models and in clinical trials. Despite considerable effort no fully effective therapy has been established yet. However, two drugs have emerged which show a consistent albeit modest benefit in placebo controlled studies: paromomycin and even more promising nitazoxaninde, which has now received FDA approval for the treatment of immunocompetent children. The resistance of *C. parvum* against drugs, which are highly effective against related apicomplexan parasites has puzzled and frustrated researchers and clinicians alike. Two general models can be developed to explain this resistance. The first model is the extracytoplasmatic model. It argues that the peculiar extracytoplasmatic subcellular localization of the parasite within its host cell severely limits access of drugs to the parasite. In addition efflux pumps could further rid the parasite of toxic compounds protecting susceptible target enzymes. The second model, the metabolism model, proposes that the metabolism of *Cryptosporidium* differs from other Apicomlexa much more than initially appreciated and that drugs active against other apicomplexa fail because their targets are absent or divergent in *C. parvum*. Obviously, these models are not mutually exclusive and both mechanisms could act synergistically.

The metabolic hypothesis put forward in this talk explains *C. parvum's* resistance to typical anti-apicomplexan drugs as a reflection of its phylogenetic and metabolic uniqueness. One of the most striking differences between *C. parvum* and other apicomplexan pathogens is the absence of fully functional mitochondria and plastids. An experimental study by Zhu and colleagues had predicted the absence of an apicoplast in *C. parvum* based on PCR and hybridization experiments, which failed to detect the presence of sequences conserved among all plastid genomes. Indeed the completed and now published *C. parvum* and hominis genome sequences do neither contain the organellar genome nor the extensive set of nuclear encoded plastid targeted genes described for *P. falciparum* and *T. gondii*. The secondary endosymbiosis that let to the presence of the apicoplast is generally viewed as and early event in the evolution of Apicomplexa and Alveolata. The observation that several genes in the *C. parvum* genome show strong phylogentic relationships to plants and algae support this view, and suggest that *C. parvum* is derived from a lineage which once harbored an algal endosymbiont which was lost later. The lack of a plastid has important metabolic and pharmacological consequences as it e.g. explains *C. parvum's* resistance to macrolide antibiotics like clindamycin which specifically target protein synthesis in the plastid and which are quite effective in *T. gondii*.

C. parvum is a challenging experimental system, and the lack of continuous culture models and transfection technology has posed limitations on the molecular analysis of this pathogen. However, *C. parvum* has one of the Apicomplexa's most accessible genome. The genome is small and introns are rare making gene prediction straightforward. The analysis of the genome sequence has just begun, but it already uncovered a surprising number of metabolic differences between *C. parvum* and other apicomplexans.

Our work has been focused on nucleotide biosynthesis. Nucleotide biosynthesis has been a main stay of antiprotozoal treatment. Antifolates are highly active against *T. gondii* and *P. falciparum*. *C. parvum* on the other hand is resistant to pyrimethamine/sulfadiazine. DHFR-TS is one of the few potential *C. parvum* drug targets that has been studied in detail. Structural as well as kinetic analysis recently published by other investigators suggests that the *Cryptosporidium* enzyme is quite different from previously characterized fused enzymes from kinetoplastids and apicomplexans. In addition to differences in DHRF-TS the general pattern of pyrimidine nucleotide synthesis and salvage could equally modulate the efficiency of antifolates. Recent genomic and experimental work has uncovered a surprising diversity of pyrimidine biosynthetic pathways within the Apicomplexa. *P. falciparum* is entirely dependent on *de novo* synthesis of pyrimidines making DHRF-TS an essential enzyme. *T. gondii* posses the ability to salvage uracil using uracil-phosphoribosyltransferase. However as recently reported by Fox and Bzik, this salvage pathway is not sufficient to sustain the parasite in the absence of *de novo* synthesis . *C. parvum* finally, has lost all six genes for the enzymes in this pathway indicating that it is unable to synthesize pyrimidines *de novo*. The parasite depends entirely on salvage, and three pyrimidine salvage enzymes have been identified, two of them uridine kinase-uracil phosphoribosyltransferase (UK-UPRT) and thymidine kinase (TK) are not found in any other apicomplexan. Phylogenetic analysis suggests that both enzymes were obtained from other organisms by horizontal gene transfer, UK-UPRT from an algal endosymbiont which has since been lost, and TK from a proteobacterium. The presence of TK in *C. parvum* provides an additional and potentially alternative source of *d*TMP for this parasite and could reduce its sensitivity to inhibition of DHFR and the subsequent starvation of the thymidylate synthase reaction. Equally the finding of UK-UPRT explains the difference in susceptibility to cytosine arabinoside between *C. parvum* and *T. gondii*. While *T. gondii* is highly resistant to this prodrug, which has to be activated by cytosine or uridine kinase, *C. parvum* was surprisingly susceptible in a large drug screen conducted by Woods and Upton.

Both UK-UPRT and TK could be new targets to pursue for *C. parvum*. Especially TK might hold promise based on the successful exploitation of this target in the therapy of Herpes viruses. A large variety of compounds subverting the viral TK has been generated, and the relationship between enzyme structure and drug sensitivity and resistance is well understood. The divergent phylogenetic origin of the parasite enzyme from a proteobacterium might allow for the identification of compounds with selective specificity for the parasite versus the human enzyme. But will nucleoside analogs known to subvert this enzyme be able to reach their target within the parasites? Experiments using 5-deoxybromo-uridine in *C. parvum* infected tissue cultures indeed suggest that this class of compounds gains access to the parasite.

Horizontal gene transfers into the *C. parvum* nucleotide metabolism are not limited to the pyrimidine pathway. *C. parvum* has obtained its inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme central to the purine salvage pathway, from an e-proteobacterium whereas *P. falciparum* and *T. gondii* harbor enzymes of clear eukaryotic phylogeny. As TK, IMPDH is a well established target of antiviral and immunosuppressive therapy. Furthermore prokaryotic and eukaryotic IMPDH differ in structure and mechanism which should facilitate the identification of *C. parvum* specific inhibitors. Kinetic analysis of recombinant *C. parvum* IMPDH has shown pronounced differences between parasite and human enzyme, most importantly a 1000 fold difference in susceptibility to mycophenolic acid. This protein has also been crystallized recently and the results from the ongoing structural study should greatly enhance the ability to design parasite specific inhibitors.

Genomic and experimental studies show a highly streamlined salvage pathway for *C. parvum* which relies on adenosine as sole source of purine (earlier biochemical studies on crude parasite lysates had also predicted adenine, hypoxanthine, xanthine and guanine salvage, however the genes for these enzymes seem not to be present in the genome. IMPDH is at the center of this streamlined pathway and an essential enzyme of the multi-step conversion of AMP to GMP. Treatment of infected tissue cultures with the IMPDH inhibitors mycophenolic acid and ribavirin consequently results in dose dependent inhibition of *C. parvum* development.

One of these drugs, ribavirin, has also been tested in a neonatal mouse model of cryptosporidiosis and treatment with 50 mg/kg for one week resulted in a 90% reduction of parasite load when compared to untreated controls. Interestingly, in these experiments the drug was injected into the peritoneum, rather than given orally suggesting that uptake from the intestinal lumen through the apical membrane of the host cell might not be necessary for this compound. Ribavirin, like the aforementioned drug targeting TK, is a nucleoside analog. This class of drugs does not freely diffuse across membranes, but subverts the nucleoside transporters of their target cell to get access. But where is the parasite nucleoside transporter localized? Several nucleoside transporters have been characterized in related intracellular parasites and most seem to localized over the entire surface of the parasite. A potential nucleoside transporter with similarity to the *T. gondii* adenosine transporter has been identified in *C. parvum* and could provide an important molecular reagent to further address nutrient and drug transport in *C. parvum*.

A series of recent genomic, biochemical and cell biological studies has produced considerable support for the metabolism hypothesis for *C. parvum's* drug resistance. *C. parvum's* metabolism differs dramatically from its better studied cousins *P. falciparum* and *T. gondii*. In all cases where the target of a widely used anti-apicomplexan drug has been characterized in molecular detail, it was either absent in *C. parvum* (e.g. clindamycin and atovaquone) or the enzyme was highly divergent and resistant (pyrimethamine). Limited drug access might still remain as an important challenge to treatment, but the data warrants a fresh look at a new and metabolically more appropriate set of drugs and targets. The divergence of *C. parvum* from the generic eukaryotic metabolism might after all present an Achilles heel, and the presence of numerous bacterial enzymes provides an exciting set of candidate targets for parasite specific inhibition. Transgenic models might provide urgently needed assay systems to validate targets identified by genome mining and screen compounds.

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Immune response to Coccidia

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SUMMARY

In view of rapid development of new biotechnologies in veterinary science, novel control strategies using genomics, molecular biology and immunology will offer an alternative way to prevent the spread of coccidiosis in the future. Increasing understanding of the protective role of local intestinal immune responses and the identification of various effector molecules against coccidia provide optimism that novel means to control coccidiosis will be feasible in the near future. The aim of this presentation is to review the current progress in our understanding of the host immune response to *Eimeria* and to discuss potential strategies which are currently being developed for coccidiosis control. Due to complexities of the host immune system and the parasite life cycle, comprehensive understanding of host-parasite interactions that lead to protective immunity should precede before we can develop successful prevention and disease control strategies. Recent progress in *Eimeria* and poultry genome sequencing and rapidly developing functional genomics technology is facilitating the identification and characterization of host and parasite genes which are involved in immunoprotection and immunopathology in avian coccidiosis. Recent studies have provided much evidence that molecular and immunological-based strategies such as recombinant vaccines and dietary immunomodulation enhance gut immunity. Thus, successful application of new knowledge on host-parasite immunobiology, gut immunity and genomics in commercial settings will lead to the development of novel disease prevention strategies against coccidiosis in the near future.

INTRODUCTION

Avian coccidiosis is the major parasitic disease of poultry with a substantial economic burden estimated to cost the industry greater than $800 million in annual losses (Williams, 1998). In-feed medication for prevention and treatment contributes a major portion of these losses in addition to mortality, malabsorption, inefficient feed utilization and impaired growth rate in broilers, and a temporary reduction of egg production in layers. *Eimeria* spp. possess a complex life cycle comprising of both sexual and asexual stages, and their pathogenicity varies in birds of different genetic backgrounds (Lillehoj, 1988). In the natural host, immunity is species-specific, such that chickens immune to one species of *Eimeria* are susceptible to others. Additionally, *Eimeria* spp. exhibits different tissue and organ specificity in the infected host. Understanding the interplay between the host and the parasites in the intestine is crucial for the design of novel control approaches against coccidiosis.

While natural infection with *Eimeria* spp. induces immunity, vaccination procedures on a commercial scale have shown limited effectiveness and current disease control remains largely dependent on routine use of anti-coccidial drugs in most countries (Dalloul and Lillehoj, 2005). Recently several different live vaccines have been commercially developed and these are mostly composed of either virulent or attenuated parasite strains. Major disadvantages of live parasite vaccines are labor-intensive production and high cost due to inclusion of multiple parasite species in the vaccine. Although live oocyst vaccines represent a limited but useful alternative to anticoccidial drugs, a recombinant vaccine composed of parasite antigens/

antigen-encoding genes that elicit coccidia-specific immunity would be eminently preferable. While it would be cost-effective to produce recombinant vaccines (proteins or DNA), the difficulty remains to identify the antigens or genes which are responsible for eliciting protective immunity and to devise the most efficient delivery method for these recombinant vaccines to be delivered and presented to the bird's immune system. Also, such subunit vaccines would eliminate the danger of emerging resistant strains encountered with live vaccines, but unfortunately until efficient vaccines become commercially available, the poultry industry is forced to rely upon prophylactic chemotherapy to control coccidiosis. Further, the introduction of alternative prevention/treatment measures such as non-chemical feed supplements that effectively enhance productivity and non-specific immunity may help limit the use of anticoccidials. However, the lack of efficient vaccines and the increasing incidence of drug resistant strains and escalating public anxiety over chemical residues in meat and eggs mandate the development of alternative control methods. For developing a successful recombinant vaccine strategy, we need to better understand the chicken immune system and the means to elicit effective immunity against coccidia.

PATHOGENESIS AND IMMUNOGENICITY OF *EIMERIA*

The pathogenicity of coccidia depends largely on the successful replication of developing parasites inside the host. Theoretical estimates indicate that a single oocyst of a virulent species such as *E. tenella* could yield 2,520,000 invasive parasites after the 2nd merogony stage (Levine, 1982). *E. maxima* is thought to undergo a minimum of four generations of schizogony (McDonald *et al*., 1986). Most major enteric protozoa including coccidia invade the intestinal mucosa and induce a certain degree of epithelial cell damage, inflammation and villous atrophy (Pout, 1967). The signs of coccidiosis depend on the degree of the damage and inflammation and include watery, whitish diarrhea (*E. acervulina*) or hemorrhagic diarrhea (*E. tenella*), petechial hemorrhages and the marked production of mucus (*E. maxima*), dehydration, weight loss, rectal prolapse and dysentery. The profuse bleeding in the ceca is a characteristic feature of *E. tenella* infection due to its extensive destruction of the mucosa with histological lesions (Witlock *et al.,* 1975).

In general, young animals are more susceptible to coccidiosis and more readily display signs of disease, whereas older chickens are relatively resistant to infection (Lillehoj, 1998). Young animals which recover from coccidiosis may later be able to partly compensate for the loss of body growth, but their growth potential remains severely compromised. The magnitude of clinical signs resulting from *Eimeria* infection is significantly influenced by host genetic factors. In two genetically divergent strains of inbred chicken lines, SC (B^2B^2) and TK ($B^{15}B^{21}$), the different degrees of disease pathogenesis depended upon the genetics of host when they are infected with *E. tenella* or *E. acervulina* (Lillehoj, 1998). In general, SC chickens are more resistant than TK chickens to coccidiosis. Because of their genetic differences in disease susceptibility to coccidiosis, extensive research has been carried out regarding the underlying immunological mechanisms controlling intestinal cell-mediated immune responses. More detailed information concerning the genetic control of immune response to *Eimeria* can be found in previous reviews (Lillehoj, 1991 and 1998; Lillehoj and Lillehoj, 2000, Lillehoj et al., 2004).

Infection with *Eimeria* induces protective immunity that is long-lasting and exquisitely specific to that particular *Eimeria* species. While a large number of oocysts is generally required to generate a good immune response against *Eimeria*, some exceptions have been noted, e.g. *E. maxima* is highly immunogenic and requires only a small number of oocysts to induce almost complete immunity. The early endogenous stages of *Eimeria* life cycle are considered to be more immunogenic than the later sexual stages (Rose and Hesketh, 1976; Rose *et al*., 1984) although gamete antigens of *E. maxima* were shown to be immunogenic and induce protection against a challenge infection with the live parasites (Wallach *et al.* 1990 and 1995). However, activation of immune T cells released IFN-γ which inhibited the intracellular development of coccidia (Lillehoj and Choi, 1998) indicating that host protective immunity is

against the exponential growth phase of the parasite life cycle. Because of the complexity of host-parasite immunobiology which involve many different cell types and soluble factors, further studies are necessary to obtain insights on host immunity eliciting complete protection.

CHICKEN IMMUNE SYSTEM

Chickens have evolved sophisticated immune system much like mammals. Major defense mechanisms include a non-specific immune response which is activated immediately following exposure to potential pathogens. Non-specific immunity is mediated by macrophages, granulocytes, natural killer (NK) cells, serum proteins and soluble factors and precedes the development of antigen-specific memory immune response mediated by lymphocytes. Lymphocytes are generated in the primary lymphoid organs such as thymus and bursa of Fabricius where they acquire functional identity whereas it is in the secondary lymphoid organs such as lymph nodes, spleen and mucosal associated lymphoid tissues where they differentiate into effector cells upon encounter with antigens and potential pathogens. Lymphoid organs are organized into different compartments where lymphocytes and non-lymphoid cells form a microenvironment suitable for effective immune responses. In summary the major cellular components of the avian immune system include thymus-derived T lymphocytes, bursa-derived B lymphocytes, macrophages and NK cells.

B lymphocytes: B lymphocytes play an important role in host defense against many infectious diseases by producing antibodies which are specific for the eliciting antigen. Once produced, antibodies becomes effector molecules which can either block the invasion of host cells by pathogens, neutralize toxins or kill extracellular pathogens through antibody-dependent cell-mediated cytotoxicity (ADCC). Unlike other animals, chicken B cells develop in the bursa of Fabricius, a gut associated primary lymphoid tissue located near the cloaca. During embryonic development, pre-bursal stem cells enter the bursal rudiment in a single wave (Houssaint et al., 1976) where they undergo a maturation process which involves generation of antibody diversity by gene conversion and somatic diversification to generate different classes of immunoglobulins (IgM, IgG and IgA.) Bursal cells migrate out of bursa to the periphery a few days prior to hatch. Chickens generate strong antibody responses to both T cell-dependent and independent antigens. After an initial encounter with an antigen, B cells secrete the IgM isotype of antibody which later switches to IgG or IgA upon the secondary exposure. As in mammals, the secondary response is characterized not only by isotype switching but also an increase in magnitude compared with the initial response (Ratcliff, 1989). The activation of naïve B cells in vivo requires a direct interaction with helper T cells typically expressing CD4 and this interaction is restricted by antigen recognition in the context of class II genes of the major histocompatibility complex (MHC).

T lymphocytes: Thymus-derived lymphocytes in chickens are divided into 3 separate subpopulations on the basis of their cell surface antigen expression and biological function. Unlike mammals that possess two different types of antigen-recognizing receptors (TCR$\alpha\beta$ and TCR$\gamma\delta$), chicken T cells express 3 distinct T cell receptors, TCR1, TCR2 and TCR3. The TCR on a given T lymphocyte subset can be a heterodimer consisting either of a γ and δ chain (TCR1), an α and Vβ1 chain (TCR2) or an α and Vβ2 chain (TCR3) (Cooper et al., 1991; Gobel, 1996). As in mammals, immature T lymphocytes undergo differentiation in the thymus in chickens: CD4-CD8- thymocytes give rise to CD4+CD8+ which develop into CD4+CD8- or CD4-CD8+ T-cells. CD4 and CD8 T subsets express all 3 types of TCRs (Davidson and Boyd, 1992). Mature CD4 or CD8 single-positive T-cells leave the thymus to populate secondary immune organs, and also travel in the circulatory and lymphatic systems. In the blood, CD4-CD8-TCR1+ T cells, as well as CD4+CD8- and CD4-CD8+ T cells, expressing either TCR2 or TCR3 have been identified. The same T cell populations are found in the spleen where CD4-CD8+TCR1+ T cells also exist (Sowder et al., 1988; Gobel, 1996). With differentiation, functionally distinct T cell subsets express certain cell surface proteins. T cells expressing both CD4 and CD8 molecules are considered immature T cells and constitute the

majority of cells in the thymus. Single-positive T cells, expressing either CD4 or CD8 are mature T cells. Most CD4+CD8- cells are helper or inflammatory T cells responding to exogenous antigen in association with MHC class II molecules, whereas CD4-CD8+ cells represent cytotoxic cells which respond to endogenous antigen in association with MHC class I molecules. The αβ TCR (TCR2 and TCR3) are known to mediate MHC-restricted antigen recognition by single-positive T cells, whereas the physiological role of T cells expressing TCR1 is not well defined (Gobel, 1996).

Macrophages: Macrophages and dendritic cells represent components of the mononuclear phagocyte system (van Furth et al., 1972) and are involved in processing and presenting antigens to lymphocytes. Macrophages are highly heterogeneous cells present in primary and secondary lymphoid tissues and are important cells involved in host defense. Their functions are primarily phagocytosis, cytotoxic activity against tumors and production of chemokines and cytokines which mediate inflammatory responses. In chickens, interdigitating cells (IDC) which are found in situ in all T cell areas of all lymphoid tissues are characterized by dendritic extensions and express high level of MHC class II antigens and are probably the main antigen presenting cells to T helper cells *in vivo* during primary immune responses (Jeurissen et al., 1994). Macrophages express many cell surface antigens that have been detected by mouse monoclonal antibodies including K1 (Lillehoj et al., 1993) and KUL01 (Mast and Goddeeris,1995). Recent advances in our understanding of how these cells recognize diverse antigens led to the discovery of highly conserved pattern recognition receptors (PRRs) which are involved in recognition of highly conserved molecular structures on microbial components called pathogen-associated molecular patterns (PAMPs). The Toll-like receptor (TLR) family is membrane-bound PRRs that play critical roles in activating the innate immune response and phagocytosis (Underhill and Ozinsky, 2002). Activation of TLR by binding to a particular ligand leads to activation of the NF-κB signal transduction pathway inducing a wide variety of host genes involved in innate immunity, such as antimicrobial peptides, cytokines, chemokines and nitric oxide synthase (Barton and Medzhitov, 2002). Although in humans more than ten TLRs have been identified, only a limited number of homologues have been characterized in chickens. Thus, it appears that TLRs have been conserved through evolution and expressed in various immune related tissues and cell lines (Iqbal et al., 2005). Similar to the mammalian gene products, the secondary protein structure of chicken TLR1, 3, 5, 7, and 10 consist of several leucine rich domains, a transmembrane domain, and Toll/interleukin-1 receptor domains (Yilmaz et al., 2005). Understanding of how TLRs regulate immune response to pathogens in poultry will be important for future development of new strategies for disease control.

NK cells: NK cells are non-lymphoid, heterogeneous and nonphagocytic cells which mediate immediate response against infection. Along with macrophages, they are important in defense against pathogens and tumors in unimmunized hosts. NK cells in chickens have been identified from freshly obtained spleen of SPF chickens (Schat et al., 1986) and have been identified in many tissues including blood and the intestine (Lillehoj and Chai, 1988). In mammals, NK cells express the ζ chain of CD3, the IL-2 receptor, CD2, and CD16. Chicken NK cells have been identified using different monoclonal antibodies such as K108 (Chung and Lillehoj, 1991) and 28-4 and they do not express CD3, CD4 or TCR (Gobel et al. 1996). Rather, NK cells have been classified as TCR0 cells since they do not express TCRαβ or TCRγδ. NK cells have been implicated in resistance against MDV-induced tumors (Lam and Linna, 1979), and in intestinal defense against coccidia (Lillehoj, 1989) and rotavirus (Myers and Schat, 1990).

INNATE AND AQUIRED IMMUNE RESPONSE TO *EIMERIA*

Because the life cycle of *Eimeria* parasites is complex and comprised of intracellular, extracellular, asexual, and sexual stages, host immune responses are quite diverse and complex. After invasion of the host intestine, *Eimeria* elicit both nonspecific and specific immune responses which involve many facets of cellular and humoral immunity (Lillehoj, 1991; Lillehoj 1998; Lillehoj and Lillehoj, 2000; Dalloul and Lillehoj, 2005) Nonspecific factors include physical barriers, phagocytes, leukocytes, chemokines and

complement components. Antigen-specific immunity is mediated by antibodies, lymphocytes, and cytokines. Due to the specific invasion and intracellular development of coccidia in the intestine, understanding of the gut-associated lymphoid tissues (GALT) is important. The GALT serve three main functions in host defense against enteric pathogens: processing and presentation of antigens, production of intestinal antibodies, and activation of cell-mediated immunity (CMI). In the naïve host, coccidia activate local dendritic cells and macrophages eliciting various chemokines and cytokines (Lillehoj, 1998). In immune hosts, parasites enter the gut early after infection, but are prevented from further development, indicating that acquired immunity to coccidiosis may involve mechanisms that inhibit the natural progression of parasite development (Rose et al., 1984; Trout and Lillehoj, 1996; Lillehoj and Choi, 1998; Yun et al., 2000a). Recent studies demonstrated the role of several cytokines produced locally during coccidiosis (Yun et al., 2000b; Min et al., 2003; Lillehoj et al., 2004; Dalloul and Lillehoj, 2005) which are responsible for enhancing protective immunity against *Eimeria* (Lillehoj et al., 1998; Yun et al., 2000c).

Antibody responses: Following coccidiosis, both circulating and secretory antibodies specific for coccidia parasites are detected in serum, bile and intestine (Lillehoj and Ruff, 1987; Lillehoj, 1988; Yun et al., 2000c). However, antibody titers in serum and intestine do not correlate with the level of protection after oral infection with coccidia (Dalloul et al., 20003; Lillehoj and Ruff, 1987). Convincing evidence on the minimum involvement of humoral antibody to limit coccidian infection came from agammaglobulinemic chicken models where it was observed that chickens Bursectomized by hormonal and chemical means were resistant to reinfection with coccidia (Rose and Long, 1970; Lillehoj, 1987). Three isotypes of antibodies are recognized in birds, IgM, IgA, and IgY. IgY is considered the orthologue of the mammalian IgG (Leslie at al., 1969), even though the cDNA encoding the IgY heavy chain shows similarity to mammalian IgE (Parvari et al., 1998). The presence of other antibody classes such as IgD or IgE in chickens has not yet been documented. The role of parasite specific antibodies both in serum and mucosal secretions has been extensively studied in coccidiosis (Girard et al., 1997; Lillehoj and Ruff, 1987). Maternal IgY is concentrated in the yolk sac of the egg (Rose et al., 1974) where it is transported to the embryo during late development by a mechanism similar to that found in mammals (West el al., 2004), and is thus considered to be of some relevance in maternal passive immunity (Wallach et al., 1992). When hens were hyperimmunized with gametocyte surface antigens of *E. maxima*, passively transferred antibodies in young birds protected against challenge with sporulated *E. maxima* oocysts by reducing fecal oocyst production (Wallach et al., 1992). The efficiency of maternally transferred antibodies in protection against field infections needs to be verified using large scale trials. Considering the short life span of maternal antibodies in young chicks, it may not be feasible to maintain extremely high levels of antibodies in birds for a long time.

Lately, immunotherapy using whole antibody molecules or single chain fragments of the variable region (ScFv) with antigen binding activity has been gaining interest as a potential immunotherapy against infectious agents. Currently available immunological control strategies consist of sub-acute infection with virulent or live attenuated parasites. The main obstacle to the development of an antibody-based strategy against avian coccidiosis however, is the existence of many different *Eimeria* species. With recent progress in molecular biology and sequence information on chicken immunoglobulin genes, it is now possible to generate recombinant chicken antibodies (Min et al., 2001; Park et al., 2004). There are potentially two different approaches using antibodies against coccidiosis. One is to produce hyperimmune serum against major immunogenic proteins of coccidia and passively administer it to 18 day-old embryos or to feed orally to young chicks at hatch. In a previous study, a surface protein 3-1E, which was identified from the merozoites of *E. acervulina*, was used as a potential subunit vaccine for avian coccidiosis and found to be protective against challenge infection with the homologous *Eimeria* (Lillehoj et al., 2000). Moreover, a DNA vaccine prepared from the gene coding for the protein was partially protective against challenge with *E. acervulina* (Lillehoj et al., 2000). These results prompted us to investigate the potential use of

chicken antibodies against 3-1E in protection against coccidiosis. In a recent report (Ngyen et al., 2003), we tested the protective effect of chicken egg antibody (IgY) powder which was prepared from hens hyperimmunized with purified 3-1E recombinant protein in a challenge model with *E. acervulina* and *E. tenella*. Chickens which were fed standard diet with IgY powder containing antibodies against 3-1E (3-1E/IgY) were better protected against oral challenge with *E. tenella* or *E. acervulina* oocysts compared with those fed with standard diet supplemented with IgY-containing powder only. These results clearly indicated that 3-1E represents an important target antigen for coccidiosis prevention and that passive immunization of chickens with antigen-specific IgY powder is a promising method to confer protection against coccidiosis.

Another approach to generate therapeutic antibody is to develop recombinant antibodies against protective epitopes. Using the chicken B cell line R27H4, we previously developed several hybridomas producing coccidia-specific antibodies (Lillehoj et al., 1994). One of them, 6D-12-G10, was reactive with an *Eimeria* protein suggested to be involved in binding to a host cell receptor (Sasai *et al*. 1996). Unfortunately, the amount of antibody secreted by this hybridoma into culture medium was insufficient for further biochemical and physiological characterization of the antigen. To circumvent the problems associated with low yield, we produced an scFv fragment derived from the V_H and V_L genes encoding the 6D-12-G10 antibody. The single chain Fv antibody was expressed in *E. coli* and the recombinant gene product bound whole parasites (Min et al., 2002) by immunoblot, immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA). Chickens fed recombinant scFv antibodies showed reduced fecal oocysts upon challenge infection with live coccidia (unpublished data). Using similar approaches, we also generated other scFv antibodies detecting coccidia proteins (Park et al., 2004). Like the native monoclonal antibodies from which they were derived, these recombinant antibodies showed binding activity against *Eimeria* antigens and were secreted at 5 mg/L into culture medium, indicating that soluble, stable and functional chicken ScFv can be produced in large volume. Thus, recombinant antibody technology has advantages over hybridomas, which generally produce low quantities of antibodies (<0.5 mg/L), easily lose antibody activity and are not able to make high titer ascites.

Although the role of antibodies produced during natural infection is debatable, antibodies which are generated against specific epitope of coccidian parasites can be used to reduce parasite invasion and have been shown to be beneficial against coccidiosis infection (Walach et al., 1992; Ngyen et al., 2003). Antibody-based therapies can be useful, for example, to prevent *Eimeria* infections, where antibodies are known to play a role in protection against the parasite. The results presented here demonstrate an example of recombinant chicken antibodies useful to reduce parasites in the field. ScFv antibody fragments may offer advantages for *in vivo* applications as diagnostic and therapeutic reagents also. For example, because the scFv antibody is approximately 33 kiloDalton in size, representing 20% of an intact IgG molecule, it may penetrate tissues easily, an important consideration given the invasive nature of *Eimeria* parasites. The ability to generate unlimited amount of soluble and functional recombinant scFv antibodies will facilitate the investigation of their potential therapeutic value in passive immunotherapy against avian coccidiosis. Meanwhile evidence that antibodies in dietary supplements could protect against oral coccidiosis infection opens a new door for novel immunotherapy strategies against coccidiosis. Furthermore, given the limited information concerning the nature of protective antigens of *Eimeria*, these antibodies will be an important tool for affinity isolation of potential *Eimeria* subunit vaccines.

Cell-mediated immunity: The evidence that the removal of the bursa by chemical or hormonal means (Rose and Long, 1970; Lillehoj, 1987) did not interfere with the development of protective immunity against *Eimeria* indicated the importance of cell-mediated immunity in coccidiosis. The role of T cells in the protection against coccidiosis has also been studied in immunosuppressed chickens using T cell-specific drugs that selectively abrogate or severely impair T cell function. These treatments included thymectomy (Rose and Long, 1970), cyclosporin A (Lillehoj, 1987), betamethasone, dexamethasone

(Isobe and Lillehoj, 1993), and cell depletion using mouse monoclonal antibodies against CD8+ or αβTCR-expressing cells (Trout and Lillehoj, 1996). In all of these studies, the abrogation of T cell function impaired host protective immunity against coccidiosis. Additional evidence for the protective role of T cells came from adoptive transfer studies where peripheral blood lymphocytes (PBL) and spleen cells from *E. maxima*-immune chickens protected syngeneic recipients against a live parasite challenge infection (Rose and Hesketh, 1982). Lillehoj and Choi (1998) and Miller et al. (1994), using an *in vitro* culture, showed that splenocytes from *E. tenella*-immune chickens inhibited the intracellular development of *E. tenella* in kidney cells. The nature of these cells was not determined, but may be NK cells since they did not show any MHC restriction in their action. Direct evidence for the presence of *Eimeria*-specific T cells was demonstrated by an *in vitro* antigen-driven lymphoproliferation assay (Rose and Hesketh, 1984; Lillehoj, 1986; Vervelde et al.,1996).

T lymphocytes: In the gut, intraepithelial lymphocytes (IEL) represent an important component of the GALT (Guy-Grand et al., 1974). A unique feature of IEL is that γδ T cells are predominant, whereas the vast majority of mature T lymphocytes in the peripheral blood and lymphoid organs use the CD3-associated αβ TCR heterodimer for antigen recognition (Goodman and Lefrancois, 1988; Bonneville et al., 1988). Following primary and secondary infections with *E. acervulina*, an increased percentage of intraepithelial γδ T cells was observed in the duodenum (Choi and Lillehoj, 2000). The percentage of γδ T cells was significantly elevated by day 8 following primary infection with *E. acervulina* in SC chicken whereas a significant increase was seen as early as day 4 in TK chickens (Choi et al., 1999). Concurrent with the increase of γδ T cells, a significant enhancement of IL-2 mRNA transcripts was found (Choi and Lillehoj, 2000). The percentage of αβ T cells was elevated in IEL by day 4 after primary infection with *E. acervulina* in SC chickens whereas a significant increase of αβ T cells was not seen until 6 d post secondary infection in SC chickens (Choi et al., 1999).

The importance of CD8+ T cells has been shown in many intracellular parasitic infections including toxoplasmosis (Hakim et al., 1991) and malaria (Weiss et al., 1990). In avian coccidiosis, the selective elimination of CD8+ cells by anti-CD8+ monoclonal antibody resulted in exacerbation of the disease, as evidenced by increased oocyst shedding after infection with *E. tenella* or *E. acervulina* (Trout and Lillehoj, 1996). Significant increase of T cells expressing CD8+ molecules was noted in the intestinal IEL population following challenge infections of chicken with *E. acervulina* (Lillehoj and Bacon, 1991). Two-color immunofluorescence staining revealed that the majority of CD8+ cells in the duodenum intraepithelium of immune chickens co-expressed the αβ TCR. In both SC and TK chickens, the ratio of CD8+ to CD4+ T lymphocytes in IEL was elevated by day 4 following primary and secondary infections with *E. acervulina*. These cells continued to increase in SC chickens but showed a marked decrease in TK chickens following the secondary infection (Choi et al., 1999). When two MHC congenic chickens with a different disease susceptibility to coccidiosis were compared, the higher increase of ab TCR+CD8+ and gd TCR+CD8+ cells was associated with B2B2 chickens which are less susceptible. Similarly, Bessay et al. (1996) observed a significant increase in the proportion of CD4+, CD8+ and TCR γδ cells in duodenal IEL from day 4 to day 8 post-infection with *E. acervulina*. In contrast, the proportion of CD8+ cells decreased significantly in the blood and spleen on days 4 and 6 post-infection. After *E. tenella* infection, the proportion of CD4+ cells increased on day 8 post-infection and CD8+ cells on days 6 and 8 post-infection in cecal IEL. At the same time, the proportion of CD4+ cells decreased in the spleen on day 8 post-infection and CD8+ cells decreased in the blood on day 6. In chickens infected with *E. mivati*, the percentages of splenic lymphocytes bearing CD8+, γδ TCR, class II MHC, or surface IgM antigens, were decreased in the dexamethasone-treated chickens when compared to the normal chickens (Isobe and Lillehoj, 1993). Significantly higher numbers of total oocyst output in the dexamethasone-treated chickens following primary and secondary infections with *E. mivati* indicated the significance of CD8+ cells in primary as well as secondary immune responses.

In the peripheral blood, a transient but sharp increase in the proportion of CD8-expressing T cells was found in White Leghorn chickens at 8 days after a primary infection with *E. tenella* (Breed et al., 1996; 1997a,b). This increase was found to be concurrent with a marked increase in IFN-γ as well as nitric oxide (NO) production upon *in vitro* stimulation of PBL by T cell mitogen and *E. tenella* sporozoite antigen (Breed et al., 1997a). In *E. maxima* infection, both CD4+ and CD8+ cells were seen in the small intestine of Light Sussex chickens, but the proportion of CD8+ cells was higher (Rothwell et al., 1995). CD4+ cells represent a minor population of the IEL. During *E. acervulina* infection, CD4+ cells increased at day 7 after primary and day 14 after secondary infection (Lillehoj, 1994). Bessay et al. (1996) examined the T-lymphocyte subsets in the intestine following *E. tenella* and *E. acervulina* infections. Following *E. acervulina* infection, a significant increase in the proportion of CD4+ was observed in duodenal IEL from day 4 to day 8, and in the blood and spleen on day 8 post-infection. In *E. tenella* infection, CD4+ cells increased on day 8 post-infection in the cecal IEL but the proportion of CD4+ cells dropped in the spleen on day 8 post-infection. In the ceca, the number of CD4+ cell increased significantly at day 2 after *E. tenella* infection and in immune chickens, mainly CD4+ and CD8+ T cells infiltrated the lamina propria (Vervelde et al., 1996). A significantly higher number of sporozoites were found within or next to CD3+, CD8+, and αβ TCR+ cells in immune chickens. In a study aimed at elucidating the immunologic differences between resistant SC (B2B2) and susceptible TK (B15B21) chickens, duodenal CD4+ T lymphocytes increased significantly and rapidly at day 4 after primary and secondary infections with *E. acervulina* in SC as compared to TK chickens (Choi et al., 1999). The role of CD4+ T cells in coccidiosis may involve the production of soluble cytokines such as IFN-γ (Yun et al., 2000a,b,c). Using a quantitative RT-PCR, increased IFN-γ mRNA expression was observed in the cecal tonsil lymphocytes in *E. tenella*-infected SC chickens, and the selective depletion of CD4+ cells, but not CD8+ cells, reduced IFN-γ production.

Non-T Cells: The role of NK-cells in parasitic diseases has been well documented (Lillehoj et al., 2004). The chicken gut IEL are known to contain subpopulations of cells that can mediate NK cell activities as demonstrated in 4 hr ^{51}Cr release assays using different avian tumor cell targets (Chai and Lillehoj, 1988). The NK cell activity was higher in the jejunum and ileum than in the duodenum and cecum. Following infection with *Eimeria* parasites, the NK cell activities of both splenic and intestinal IEL decreased to a subnormal level during the early phase of infection (Lillehoj, 1989). NK cell activity returned to normal or slightly higher than normal levels about 1 week after the primary inoculation. Significant increases in the splenic and intestinal IEL NK cell activities were seen during the early phase of secondary infection. This increase in the IEL NK cell activity shortly after secondary infection was accompanied by a substantial increase in the number of IEL expressing the asialo-GM1 antigen, a NK marker (Lillehoj, 1989). In a recent study, we have identified a major effector molecule from IEL which shows lytic activity against sporozoites (unpublished observation). Chicken NK cells, defined phenotypically as CD8+ cells lacking T- or B lineage specific markers, constitute approximately 30% of CD8+ intestinal IEL, but < 1% of splenocytes or PBL (Gobel et al., 2001). Using the 28-4 monoclonal antibody, specific for CD8+CD3- IEL and an antibody for CD3, IEL were separated into CD3+ IEL T cells and the 28-4+ cells, both co-expressing the CD8 antigen. The 28-4+ IEL were able to lyse the NK-sensitive target cells. These results define the two major phenotypically and functionally distinct IEL subpopulations, and imply an important role of NK cells in the mucosal immune system (Gobel et al., 2001). Using mouse antibodies K-14 and K-108, Chung and Lillehoj (1991) identified NK cells which stain 6 to 17% of splenic lymphocytes, 11 to 14% of PBL, and fewer than 5% of thymic and bursal lymphocytes.

Chicken macrophages identified using the monoclonal antibody K1, express MHC class II antigens (Kaspers et al., 1993) and are involved in different phases of the host immune response to coccidia (Lillehoj et al., 2004). In *E. tenella*-immune chickens, more leukocytes were present in the lamina propria and leukocytes infiltrated the ceca more rapidly than in the naive chickens (Vervelde et al., 1996). By immunocytochemical staining, most infiltrating leukocytes were macrophages and T cells. Macrophages pretreated with the

culture supernatants of Con A-stimulated spleen cells or T cells exerted cytostatic effects on the growth of *E. tenella* sporozoites (Lillehoj et al., 1989; Dimier et al., 1998). Pretreatment of macrophages with culture supernatants of Con A-stimulated spleen cells induced NO synthesis, and the addition of NG monomethyl-L-arginine, a NO synthase inhibitor, also overcame the inhibition of *E. tenella* replication in macrophage cultures suggesting possible involvement of NO or toxic oxygen intermediates in inhibiting *E. tenella* growth (Dimier-Poisson et al., 1999).

Cytokine and chemokine responses: Extensive experimental evidence supports the notion that immunity mediated by lymphocytes and their secreted products such as cytokines mediate antigen specific protection against challenge infection with *Eimeria* (Lillehoj and Lillehoj, 2000; Lillehoj et al., 2004). For example, correlation of disease resistance and enhanced proliferation of T lymphocytes and recruitment of intestinal T cells into the duodenum following primary and secondary *E. acervulina* infections have been documented (Lillehoj, 1989 and 1998; Lillehoj et al., 2004). In the intestine, lymphocytes, macrophages, dendritic cells and other effector cells act in harmony to secrete cytokines and proinflammatory molecules which direct the appropriate immune responses to eliminate the invading parasite and to induce the development of memory responses. Recently, using T lymphocyte and macrophage cDNA microarrays, host genes related to the immune response in the gut have been identified and their role in protection against coccidiosis is being investigated (Min et al., 2003 and 2005; Dalloul et al., in preparation). Although the importance of cytokines in mediating innate and acquired immunity against coccidiosis has been suggested and documented, the nature of many chicken cytokines has not been well characterized due to slow progress in characterizing chicken cytokine genes (Lillehoj et al., 2003). In contrast to the plethora of mammalian cytokines, only a few chicken homologues have been described, the main ones being IFN-γ, TGF, TNF, IL-1, IL-2, IL-6, IL-8 and IL-15 (Lillehoj, 2004). Of late, a series of new chicken cytokines have been described including IL-17 (Min and Lillehoj, 2002), IL-18 (Gobel et al., 2003), IL-16 (Min and Lillehoj, 2003), IL-12(Degen et al., 2004), and Th2 type cytokines such as IL-3, IL-4, IL-13 and GM-CSF (Avery et al., 2004), IL-10 (Rothwell et al., 2004) and IL-5 (Koskela et al., 2004).

Many different types of chemokines and cytokines are produced following primary and secondary infection with *Eimeria* (Lillehoj et al., 2003). Interferons have been shown to have various immunomodulating effects on a wide variety of tissues. Interferons are classified into type I (IFN-α,-β, -ω, and -τ) and type II (IFN-γ). The chicken gene encoding IFN-γ has been cloned and its biological function studied by many laboratories (Digby and Lowenthal, 1995; Song et al., 1997). IFN-γ production during coccidiosis was examined using a quantitative RT-PCR (Choi et al., 1999; Yun et al., 2000c), and recently using gene expression profiling (Min et al., 2003). After *E. acervulina* infection, IFN-γ mRNA expression was detected in the cecal tonsils and spleen but not in the duodenum of SC chickens (Choi et al., 1999). In *E. tenella*-infected chickens, IFN-γ transcripts were detected in the spleens, cecal tonsils, and IEL following the primary and the secondary infections with *E. tenella*. The marked increase in the transcripts of IFN-γ was shown at day 6 after primary infection in the cecal tonsils. Laurent et al. (2001) recently showed that IFN-γ expression in the cecum and jejunum of White Leghorn (PA12) chickens increased over 200-fold above the control at day 7 after primary infection with *E. tenella* and *E. maxima*. The effects of pretreatment of chicken macrophages or fibroblasts with crude culture supernatants containing IFN-g on *E. tenella* sporozoites were examined in various *in vitro* systems (Lillehoj et al., 1989; Lillehoj and Choi, 1998; Dimier et al., 1998). Multiple intramuscular injections (three times) of the supernatant of recombinant chicken IFN-γ at one day prior to, and two and four days after infection with *E. acervulina*,, conferred significant protection as measured by body weight loss and oocyst shedding in both SC and TK strains (Lillehoj and Choi, 1998). Furthermore, *E. tenella* sporozoites were inhibited to undergo intracellular development in a chicken cell line stably transfected with the chicken IFN-γ gene. Treatment of chicken cells with recombinant IFN-γ inhibited the intracellular development of *E. tenella* without affecting sporozoite invasion of host cells (Lillehoj and Choi, 1998). These results provide the first direct evidence that IFN-γ

exerts an inhibitory effect against *Eimeria* and provides a rational basis for the use of this cytokine as a vaccine adjuvant against coccidiosis.

Interleukin-2 (IL-2) is a potent growth factor for a variety of cell types including T cell differentiation, B cell development and NK cell activation (Lillehoj et al., 1992; Farner et al., 1997). The chicken IL-2 gene has been cloned (Sundick and Gill-Dixon, 1997) and its biological function characterized (Choi and Lillehoj, 2000; Lillehoj et al., 2001). After primary and secondary infections with *E. acervulina*, a significant enhancement of IL-2 mRNA transcripts was observed in the spleen and intestine (Choi and Lillehoj, 2000). The protective effect of IL-2 on vaccination of chickens with the recombinant 3-1E coccidia gene was recently demonstrated by DNA vaccination (Lillehoj et al., 2000; Min et al., 2001). Co-injection of the IL-2 gene with the 3-1E or MIC2 antigen or gene enhanced the host response to the vaccination procedure (Ding et al., 2005a,b; Lillehoj et al., 2004, 2005).

IL-16 was originally described as a lymphocyte chemoattractant factor synthesized by CD8+ and CD4+ T cells and released in response to antigens, mitogens, histamine or serotonin (Cruikshank et al., 2000). Further analysis indicated that IL-16 is generated by B cells, mast cells, epithelial cells, macrophages, fibroblasts, and eosinophils (Cruikshank et al., 2000). Initially, IL-16 is produced as a 67 kDa pro-IL-16 (Baier et al., 1997) that subsequently is cleaved by caspase-3 producing a 17 kDa secreted form of the chemokine that aggregates to form biogically active homotetramers (Zhang et al., 1998). IL-16 is chemoattractive for CD4+ T cells, eosinophils, and monocytes through a mechanism involving binding to CD4 (Zhang et al., 1998), although recent data suggest that CD4 is not the only receptor for IL-16 function (Mathy et al., 2000). In addition to its chemotactic function, IL-16 induces the expression of the IL-2 receptor alpha chain and MHC class II molecules (Cruikshank et al., 1987). Recently, a cDNA from an expressed sequence tags (EST) cDNA library, prepared from intestinal IEL of *Eimeria*-infected chickens and containing a full-length open reading frame (ORF) of pro-IL-16, was characterized (Min and Lillehoj, 2004). The encoded protein, predicted to consist of 607 amino acids, showed 86% sequence homology to duck pro-IL-16 and 49-52% homology to various mammalian homologues. By Northen blot analysis, IL-16 transcripts were identified in chicken lymphoid tissues, but not in the non-lymphoid tissues examined. A recombinant protein containing the COOH-terminal 149 amino acids of pro-IL-16 when expressed in COS-7 cells showed chemoattractant activity for splenic lymphocytes.

IL-17 was cloned originally from an activated T cell hybridoma produced by the fusion of a mouse cytotoxic T cell clone with a rat T cell hybridoma, and referred to as CTLA-8 (cytotoxic T lymphocyte-associated antigen 8)(Rouvier et al., 1993). IL-17 was produced in a mixture of glycosylated (22 kDa) and non-glycosylated (15 kDa) forms and secreted by activated CD4+ T cells as covalently bound homodimers (Fossiez et al., 1996). Whereas IL-17 transcripts were restricted to activated T cells, their receptors were found to be expressed ubiquitously in a variety of mammalian tissues and cell lines (Yao et al., 1995,1997). Functional studies indicated that IL-17 is involved in a broad range of cellular activities. For example, IL-17 stimulated osteoclastogenesis (Kotake et al., 1999), granulopoiesis (Schwarzenberger, et al., 1998), and T cell proliferation by suboptimal concentrations of phytohemagglutinin (Yao et al., 1995). Chicken IL-17 was cloned from an EST cDNA library prepared from intestinal IEL of *Eimeria*-infected chickens (Min and Lillehoj, 2002). It contained a 507 bp ORF predicted to encode a protein of 169 amino acids with a molecular mass of 18.9 kDa, a 27 residue NH2-terminal signal peptide, a single potential N-linked glycosylation site, and 6 cysteine residues conserved with mammalian IL-17s. Chicken IL-17 shared 37-46% amino acid sequence identity with the previously described mammalian homologues and also was homologous to ORF 13 of Herpes virus saimiri (HVS 13). By Northen blot analysis, IL-17 transcripts were identified in a reticuloendotheliosis virus-transformed chicken lymphoblast cell line (CU205) and Con A-stimulated splenic lymphocytes, but not other chicken cell lines or normal tissues. Conditioned medium from COS-7 cells transfected with the chicken IL-17 cDNA induced IL-6 production by chicken embryonic fibroblasts suggesting a functional role for the cytokine in avian immunity.

Tumor necrosis factor (TNF)-α or β have not been well characterized in poultry at present. However, macrophages obtained during and immediately following an infection with *E. maxima* or *E. tenella* produced a TNF-like activity in a biphasic fashion, whereby the first peak was associated with the pathogenesis of disease and the second peak with the development of a protective immunity (Byrnes et al., 1993). The production of significantly greater amounts of TNF during day 3-6 after inoculation correlated with the appearance of the most characteristic local and systemic pathophysiological changes induced by coccidia (Byrnes et al., 1993). Zhang et al. (1995a,b) investigated the effect of a TNF-like activity on the pathogenesis of coccidiosis in inbred chickens. The TNF-like factor was produced by peripheral blood macrophages in time- and dose-dependent manners following primary, but not secondary, *E. tenella* infection. Treatment of chickens with antibody against TNF resulted in a partial abrogation of *E. tenella* induced body weight loss in SC chickens.

Transforming growth factor (TGF)-β is a pleiotropic anti-inflammatory cytokine that stimulates the repair of damaged mucosal epithelial integrity following injury (**Robinson et al., 2000**). Lymphocytes that secrete TGF–β downregulate host immune and inflammatory responses, especially in the intestinal mucosa (Strober et al., 1997). The expression of TGF–β2, 3, and 4 was investigated using cDNA probes and antibodies specific for the different TGF–β isoforms in chickens (Jakowlew et al., 1997). After infection with *E. acervulina*, expression of TGF–β4 mRNA which is equivalent to TGF–β1 in mammals, increased 5- to 8-fold in intestinal IEL and 2.5-fold in spleen cells, whereas the expression of mRNA for TGF–β2 and TGF–β3 remained constant in these cells. Administration of TGF–β to *T. gondii*-infected severe combined immunodeficiency (SCID) mice resulted in an earlier mortality and shortening of the survival time of mice given exogenous IL-12. Administration of anti-TGF–β to SCID mice beginning 4 hr prior to infection and every 2 days thereafter prolonged the survival time significantly. These data demonstrated the ability of TGF–β to antagonize IL-12-induced IFN-g production by SCID mice and suggested a role for TGF–β in the regulation of T cell-independent resistance mechanism to *T. gondii* (Hunter et al., 1995).

IL-6 is a pleotropic lymphokine originally described as a T cell-derived lymphokine that induced the maturation of B cells into antibody-producing plasma cells (Narazaki and Kishimoto, 1994). Chicken IL-6 shows about 35% sequence identity to human IL-6 (Schneider et al., 2001). Bacterially expressed chicken IL-6 carrying a histidine tag in place of the signal peptide was biologically active and induced the proliferation of the IL-6-dependent murine hybridoma cell line 7TD1 (Schneider et al., 2001). Production of chicken IL-6-like factor activity was detected by a murine IL-6 7TD1 bioassay in serum taken from chickens infected with *E. tenella* during the course of a primary infection (Lynagh et al., 2000). IL-6 activity was detected during the first few hours post-infection indicating a possible role of this cytokine in the development of acquired immunity.

In vitro production of IL-1 by macrophages obtained from *Eimeria*-infected chickens was observed during and immediately following infection with *E. maxima* or *E. tenella* (Byrnes et al., 1993). Lymphocytes from *Eimeria*-infected chickens produced a higher level of IL-1 following stimulation than cells from non-infected birds. RT-PCR measurement of IL-1 production demonstrated a 27- to 80-fold increase in the IL-1b transcript levels at day 7 after infection with *E. tenella* and *E. maxima* (Laurent et al., 2001). The precise role of IL-1 in the development of resistance against coccidiosis needs to be better characterized in view of its documented role in various infections. Chemokines are important mediators of cell migration during inflammation and in normal leukocyte trafficking. These proteins are generally active at the nanomolar concentration and are produced by a wide variety of cell types in response to exogeneous irritants and endogeneous mediators such as IL-1, TNF, PDGF, and IFN-g (Oppenheim, 1991). Chemokines are grouped into four structural families characterized by the position of their amino-terminal cysteine residues (Zlotnik and Yoshie, 2000). The CXC class, which has one amino acid separated by two cysteine residues, and the CC class, which possess two consecutive cysteine residues, is the most common chemokines. IL-8 and K60 are CXC chemokines (Kaiser et al., 1999; Sick et al., 2000) and K203 is a CC

chemokine recently cloned from chickens (Sick et al., 2000). The K203 cDNA cloned from the chicken macrophage cell line HD-11 stimulated with LPS, revealed 50% sequence identity to the mammalian macrophage inflammatory protein 1b (MIP-1b) (Sick et al., 2000). Laurent et al. (2001) showed that mRNA levels of the CC chemokines K203 and MIP-1β were upregulated 200- and 80-fold, respectively, in the cecum in response to *E. tenella* infection, and 100- and 5-fold in the jejunum in response to *E. maxima* infection. Interestingly, no discernible changes were observed in the mRNA levels of the CXC chemokines IL-8, and K60.

The role of various cytokines and chemokines needs to be better studied to understand how these different factors interact to eliminate parasites from the host and to develop memory responses against later infections. To accomplish this, we generated an EST cDNA library from IEL of *Eimeria*-infected chickens (Min et al., 2005) and have identified two new chicken cytokines, IL-16 (Min and Lillehoj, 2003) and IL-17 (Min and Lillehoj 2002). Both cytokines were elevated in *Eimeria*-infected tissues, they may be involved in regulating local immune response to coccidia. Analysis of 30 different cytokines and chemokines during coccidia infection indicated that cytokines invloved in mediating Th1 responses seem to be dominant during early times after coccidiosis (unpublished data) as best manifested by the proven involvement of IFN-γ (Lillehoj and Choi, 1998; Lillehoj et al., 2000; Lillehoj et al., 2004; Lowenthal et al., 1997). The role of Th2 type cytokines should also be investigated in coccidiosis in order to obtain better insights on protective immunity. In toxoplasma infection, mice defective in IL-10, an anti-inflammatory cytokine, showed enhanced susceptibility to disease suggesting the role of IL-10 in downregulating inflammatory response to prevent host immunopathology (Gazzinelli et al., 1996). Recent evidence indicated that IL-10 is produced during coccidiosis (Rothwell et al., 2004), but its role in disease pathogenesis has not been investigated. Future studies to delineate cytokine regulation of local immunity to coccidia will lead to better understanding of host-parasite immunobiology and novel control strategies against coccidiosis.

FUTURE DIRECTIONS IN STUDYING HOST IMMUNE RESPONSE GENES CONTROLLING COCCIDIOSIS RESISTANCE USING DNA MICROARRAY

With increased information on poultry genomics and the availability of several tissue-specific cDNA EST libraries, high throughput gene expression analysis is possible to study host immune response to *Eimeria* (Min et al. 2005). DNA microarray is a revolutionary tool for genomic study of interested traits in a high throughput manner. By immobilizing thousands of DNA sequences in individual spots on a solid phase, DNA microarray allows simultaneous analysis of a large number of genes in a single step, thereby identifying genes whose expression levels are altered during natural biological processes or experimental treatments, or vary due to genetic differences (Eisen and Brown, 1999). In one approach, the sample of interest, such as mRNA isolated from a certain tissue, is used to synthesize cDNA labeled with a fluorescent dyes. The labeled cDNA probe is then hybridized to the array and a post-hybridization image is developed. The color density of individual nucleic acid species reflects the relative amount of labeled cDNA hybridized to the DNA immobilized at the known position on the array. By comparing different samples tested in well-controlled conditions, changes in expression levels of individual genes can be detected. Once genes of interest are identified, Northern blotting or RT-PCR can be used to confirm genes with differential expression. The genes with significant differences can be used as potential candidate genes influencing disease susceptibility traits.

Development of chicken intestinal cDNA microarray to investigate host immunity against coccidia:

We have established a normalized chicken intestinal cDNA library using pooled intestinal tissues from coccidia-infected chickens (Min et al., 2005). The library was prepared from intestinal epithelial cells and lymphocytes at 0, 1, 2, 3, and 4 days post-infection with *Eimeria*. According to the normalization control, the redundancy in this library has been reduced by 37-fold. Individual clones (n=34,078) were randomly picked and sequenced, generating 14,409 chicken-specific ESTs that could be grouped into 9,446 unique

contigs. The majority of contigs (7,595; 80.4%) consisted of single ESTs and the remaining 1,851 contigs were composed of clusters of 2 or more overlapping/identical ESTs (average of 3.7 ESTs per cluster contig). Most cluster contigs (1,567; 84.7%) contained 2-4 ESTs comprising 30.6% (4,418) of the total number of ESTs. The contig average readable sequence length was 418 bp with almost half (45.8%) falling within the range of 400-600 bp and the average sequence length per contig was 712 bp and 71.2% (1,318) of all contigs ranged from 0.5-1.0 kb.

Using the Basic Local Alignment Search Tool (BLAST) program to perform sequence-similarity searches against the GenBank nucleic acid sequence database, we analyzed the 18 cluster contigs containing 15 or more ESTs, which could be regarded as abundant transcripts and therefore were most likely to match previously described genes. This group of contigs constituted 1.0% of the total number of clusters and 3.6% of the total ESTs. Ten sequences were highly homologous to previously described chicken genes. They included NK-lysin, apolipoprotein AIV, fatty acid binding protein, acid ribosomal phosphoprotein, a-tublin, GAPDH, and ferritin heavy chain. Comparison of our intestine cDNA sequence data with chicken DNA sequences in GenBank identified 125 clones which encoded novel genes. Of these, 110 genes were unknown and the remaining genes showed weak homologies to CC chemokine receptor type 8, cell adhesion receptor CD36, unc-51-like kinase 1, death-associated protein kinase 2, molybdenum cofactor sulfurase, lysosomal alpha-glucosidase precursor, FYVE and coiled-coil domain containing 1, NADH-ubiquinone oxidoreductase 23 kDa subunit, cytochrome b, proline rich protein 2, XE7 protein, glypican-5 precursor, nuclear receptor ROR gamma, and LPS-induced TNF-á factor. These EST sequences from *Eimeria*-stimulated intestinal IEL transcripts will be used to study global gene expression profiling and to identify novel immune-related genes during avian coccidiosis and in other enteric diseases of poultry.

Analysis of cell-mediated immune response to *Eimeria* using T lymphocyte cDNA microarray:

In view of importance of T cell-mediated immunity in coccidiosis resistance, we initially selected 450 clones encoding immune response associated genes from a cDNA library prepared from mitogen-activated T lymphocytes (Min et al., 2003). To assess the changes in intestinal gene expression of chickens infected with *E. acervulina* or *E. maxima,* IEL were collected from the duodenum or ileum at 1, 2, 3, and 4 days following primary or secondary infection. In general*, E. acervulina* primary and secondary infection resulted in up- or down-regulation of more transcripts compared with *E. maxima* infection and primary infection by either parasite induced changes in a greater number of transcripts compared with secondary infection (Min et al., 2003). Specifically, *E. acervulina* and *E. maxima* infection affected the levels of 99 and 51 gene transcripts respectively following primary infection and 46 and 25 transcripts following secondary infection. Conversely, *E. acervulina* and *E. maxima* decreased the levels 88 and 56 gene transcripts respectively following primary infection and 22 and 37 transcripts following secondary infection. When considering all time points examined following primary or secondary infection with *E. acervulina* or *E. maxima*, the quantities of 5 gene transcripts were commonly induced (CMRF35 leukocyte immunoglobulin-like receptor, zinc finger gene, PmbA homolog, granulysin precursor, cyclophilin A) and 8 were repressed (alpha-actinin, hypothetical protein F39H12.5, spleen mitotic checkpoint BUB3, interferon-induced granylate-binding protein 2, transcription factor NF-YC subunit, transport associated protein 3, alpha-adaptin, homobox protein HOX-D8).

Since the changes in cytokine genes following *Eimeria* infection is an indication of local cell-mediated immunity (Lillehoj et al., 2003), we included in our microarray analysis 12 cytokine genes to monitor changes in their corresponding transcripts subsequent to *Eimeria* infection. The transcript levels for IL-8, IL-15, and lymphotactin genes were increased at all time points examined following primary or secondary infection with *E. acervulina* or *E. maxima*, whereas IL-18 and osteopontin gene transcripts were repressed (Min et al., 2003). With the exception of TGF-b4, changes in the levels of all cytokine transcripts examined were similar when comparing primary infection of *E. acervulina* with *E. maxima*. Similarly, after secondary infection, all transcript levels

except those for IL-6 and TGF-β4 showed comparable changes when comparing *E. acervulina* with *E. maxima.*

CONCLUDING REMARKS

In view of increasing consumer's concern about drug residues in the food supply and impending regulations on the use of growth promoting drugs in poultry production, the industry will eventually look for alternative method for coccidiosis control. Problems associated with antigenic variation of field strains and the cost of producing multiple-species live vaccines pose limits on the current vaccination approaches. Thus, novel strategies to control coccidiosis are needed, but this will only be realized after a systematic and detailed analysis of host-parasite interactions at the molecular and cellular levels are completed. In particular, fundamental knowledge on the basic immunobiology from initial parasite invasion to intracellular development and ultimate elimination from the host is very limited. Increasing evidence shows the magnitude of complexity involved in host immune responses to *Eimeria.* Additional basic research is needed to ascertain the detailed immunological and physiological processes mediating protective immunity. The need to continue seeking more effective ways to minimize the impact of poultry coccidiosis is undisputable, but critical resources are severely lacking making it difficult to effect timely progress. Encouraging results obtained from recent molecular and immunological studies that show the ability of dietary modulation on intestinal immunity and enhanced disease resistance against enteric pathogens of economic importance need to be further explored. One encouraging finding is the feasibility to induce protective immunity against live parasites using recombinant vaccines delivered in ovo. The performance of these novel vaccines will have to be verified in field evaluations in commercial settings.

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Protective immune responses involved in host resistance to Brazilian isolates of *Toxoplasma gondii*: implications on vaccine development employing recombinant viral vectors

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Toxoplasma gondii is an intracellular protozoan parasite that is widespread in nature, with a high prevalence in a variety of warm-blooded animals, including humans. It is estimated that toxoplasmosis exists in a chronic, asymptomatic form in between five hundred million to one billion people (1). Toxoplasmosis establishes itself as a lifelong, chronic infection, with transmission occurring in two ways: (i) consumption or handling of uncooked meat bearing tissue cysts; and (ii) ingestion of food or water contaminated with oocysts shed in cat feces (1). Although infections are usually benign, high morbidity and even mortality can be seen in immunocompromised patients, particularly in cancer and transplant patients under immunosuppressive therapy or HIV infected individuals (2). In addition, infection with *T. gondii* is also a major cause of economical loss in veterinary medicine (3,4).

In humans, toxoplasmosis is a common cause of infectious retinitis in both immunocompetent and immunosupressed persons, accounting for 30-50% of all cases of posterior uveitis (5). Toxoplasmic retinochoroiditis is believed to result mainly from periodic reactivation of latent cysts associated with congenital infection. However, recent studies in France (6), Canada (7), Brazil (8-10) and the United States (11) indicate that ocular disease after postnatal acquired infection is also prevalent in healthy adults. Typical features of retinochoroiditis include unilateral focal retinitis at the border of a pre-existing pigmented retinochoroid lesion and an overlying vitritis. In some cases, the lesions may be atypical, consisting of large areas of retinal necrosis or retinochoroiditis without a preexisting scar (12).

Approximately, 50% of the adult population in Brazil is chronically infected with the parasite, this prevalence is similar to other countries in Latin America (8,10,13-15). However, in some areas of the country the prevalence is significantly higher, reaching up 95% of the population, e.g. Erechim, Rio Grande do Sul State (8,9). In regard to the ocular disease in the seropositive individuals, a high frequency has been described in different areas of Brazil. Studies performed in Erechim (8,9) show 17.7% of the infected population developed ocular lesions, whereas in Melquiades, Minas Gerais State, where 49% of prevalence was observed, 12.5% of the seropositive individuals presented signs of ocular disease (10). We believe that the high frequency of ocular toxoplasmosis in Brazilian populations may reflect the unique combination of infective parasite strains, host genetic background, and/or epidemiological aspects such as infection early in life as well as continuous exposure to infective parasites.

The main objectives of the studies performed in our laboratories are: (i) to define the genetic structure of *T. gondii* strains isolated in the Brazilian territory; (ii) to define the immunological components involved in resistance of the intermediary host to infection with Brazilian *T. gondii* isolates; (iii) to elaborate subunit vaccines that protect the intermediary host against infection with Brazilian *T. gondii* strains. Ultimately, we expect that this information may contribute to the rational design of an effective vaccine to be used in prophylaxis of *T. gondii* in humans and other intermediary hosts.

I – GENETIC ANALYSIS OF BRAZILIAN T. GONDII ISOLATES BY MULTILOCUS PCR-RFLP

T. gondii strains can be divided in three main lineages based on various genetic markers (16). Studies in mice have shown that infection with each of the three lineages of *T. gondii* results in different outcomes: Type I strains are highly virulent, whereas types II and III strains are relatively avirulent (16). Type I differ genetically by 1% or less from type II and type III (17). However, the main determinants that dramatically affect the virulence of different *T. gondii* strains in the host and pathogenesis of toxoplasmosis are poorly understood. Type II strains of *T. gondii* appear to be dominant in the US territory and are frequently isolated from AIDS patients with toxoplasmic encephalitis (17). Interestingly, different studies suggest the involvement of Type I and Type I/III strains in the development of ocular disease. Consistently, Type I strain was identified as responsible for a toxoplasmosis epidemic outbreak associated with a high rate of development of acquired ocular disease (18). In addition, a recent study indicates a high frequency of Type I as well as Type I/III recombinant isolates from ocular lesions in patients from US (19).

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) at eight independent loci was used to determine the type (I, II or III) lineage of 20 *Toxoplasma gondii* strains isolated from humans and animals in Brazil. RH (type I, highly virulent), ME49 (type II, avirulent) and VEG (type III, avirulent) were used as reference strains. Differently from expected frequencies, all Brazilian strains showed to have recombinant genotypes, with typical alleles of types I, II or III at almost all loci assessed. The *cB21-4* locus, a microsatellite marker, showed a higher allelic polymorphism with seven alleles among strains under analysis. Data have also shown that many Brazilian *T. gondii* strains presented a new haplotype at the *L363* locus. When results of the eight loci were combined, 14 schizodemes were characterized out of the 20 *T. gondii* strains isolated in Brazil. The phenogram representing PCR-RFLP data separated Brazilian strains into two distinct genetic groups associated with murine virulence phenotype, termed group I-A and group I-B. Strains from group I-A (AS28, BV and N) that were highly virulent in BALB/c mice, were clustered with RH reference strain. Only those strains presented the haplotype I at the *L363* locus, suggesting that this could be a possible marker of highly virulent strains. Strains from group I-B (cystogenic strains) showed a more heterogeneous behavior regarding virulence: a few of them (EGS, RAR, SAF, D5 and D6) were virulent, others (C4, P and D8) avirulent and most of them (D1, D2, D3, D4, D7, EFP, CH1, CH2 and CH3) intermediate virulent in mice. A significant linkage disequilibrium was observed in the population surveyed. However, the role of sexual recombination in the population structure of *T. gondii* in Brazil seems to be more central than in Europe and North America, where most studies have been performed.

The fact that all Brazilian *T. gondii* strains are more closely related to the type I lineage is noteworthy. From the studied strains, 85% showed a certain degree of virulence (highly virulent, virulent and intermediate virulence) and only 15% of the strains were avirulent (20). These findings contrasts with studies performed in the US and Europe, where most strains are avirulent of type II or type III (17). Although speculative at this point, we would like to suggest that the high frequency of virulent strains closely related to type I lineage may be in part responsible for the high frequency of acquire ocular toxoplasmosis commonly found in Brazil (8-10).

It seems that sexual reproduction may plays a central role in the population structure of *T. gondii* in Brazil, although we have detected a significant correlation between PCR-RFLP, RAPD-PCR and SSR-PCR genetic distances, suggesting linkage disequilibrium in this population. It is also possible that the epidemiology of toxoplasmosis may be different in Brazil, with ingestion of oocysts being the main mechanism of *T. gondii* transmission in our country, which could explain the high percentage (100%) of recombinant strains observed in our study. Circumstantial evidence of the potential importance of oocyst transmission in Brazil was the finding that the consumption of untreated or unfiltered water was implicated as a source of *T. gondii* infection for human populations in north Rio de Janeiro State, a disease endemic area (15). Moreover,

an outbreak of clinical toxoplasmosis in humans was epidemiologically linked to drinking water from a municipal water reservoir in Santa Isabel do Ivai, Paraná State (21). Whether asexual or sexual stage transmission is responsible for the majority of *Toxoplasma* infections, it should be possible to identify an oocyst-specific immunogenic peptide or whole protein that would identify the stage of the parasite responsible for causing infection. The identification of factors that contribute to the high percentage of genetic recombination in Brazilian *T. gondii* population would be of clinical and epidemiological importance and should be explored in the future.

II – ROLE OF CYTOKINES AND MHC HAPLOTYPE IN MOUSE RESISTANCE TO INFECTION WITH NATURAL RECOMBINANT (TYPE I/III) STRAINS OF T. GONDII.

Different studies performed in murine experimental show the important role of cytokines such as IL-12, TNF-α and IFN-γ and generation of reactive nitrogen intermediates (RNI) as mediators of host resistance to early *T. gondii* infection (22). Thus, animals deficient in IL-12, IFN-γ, inducible nitric oxide synthase (iNOS), treated with neutralizing antibodies anti-cytokines or specific inhibitors of iNOS are more susceptible to infection with *T. gondii* (23-27). Acquired immunity to *T. gondii* is associated with a Th1-type response (24). During chronic infection, neutralization of either IFN-γ or TNF-α results in the reactivation of disease and the development of toxoplasmic encephalitis (TE) (28,29). Further, other host genetic factors, including MHC alleles are important determinants of host resistance and susceptibility to early infection, as well as controlling cyst numbers and encephalitis at later stages of infection with *T. gondii* in mice (30,31). Consistently, both CD4+ T as well as CD8+ T lymphocytes are important components in host resistance to this parasite (28,30).

Considering the immunological studies described above, we decided to characterize the importance of such immunological mechanisms in host resistance to three natural recombinant *T. gondii* strains (Type I/III) isolated in Brazil, named P-Br (32), D8 and C4, which are cystogenic in mice. The IL-12/IFN-γ axis and iNOS were defined as main determinants of resistance during the acute infection with the Brazilian strains. Different from the Type II strain of *T. gondii* (ME-49), per-oral infection with the Type I/III strains led only to a light inflammatory infiltrate and no major lesions in the intestine of the C57BL/6 mice. In addition, the BALB/c (resistant to ME-49) and C57BL/6 (susceptible to ME-49) mice were shown, respectively, to be more susceptible and resistant to cyst formation and toxoplasmic encephalitis, when infected with Type I/III strains. Consistently, a congenic BALB/c strain containing MHC haplotype "b" was highly susceptible to ME-49 (Type II strain), but not to the recombinant Type I/III strains. Together, our results indicate that MHC haplotype "b" is a major determinant of susceptibility to cyst formation and toxoplasmic encephalitis induced during infection with Type II, but not with Type I/III strains of *T. gondii*.

III – PROTECTION AGAINST CHALLENGE WITH A NATURAL RECOMBINANT (TYPE I/III) STRAIN OF T. GONDII IN MICE VACCINATED WITH RECOMBINANT VIRUSES CODING THE MAIN TACHYZOITE SURFACE ANTIGENS.

Vaccine development is based on the observation that exposure to *T. gondii* can elicit a life-long immune response, capable of protecting the host against a secondary challenge with the parasite (22). In early stages of infection, this response is characterized by activation of innate mechanisms mediated by macrophages, that internalize free tachyzoites and produce IL-12, and by NK cells, which secrete IFN-γ (22). This last cytokine controls the replication of the parasite and, in conjunction with IL-12, drives the differentiation of $CD4^+$ T lymphocytes specific for parasite antigens to a Th1 cytokine profile (24). In addition, $CD8^+$ T cells are crucial to control parasite replication in the chronic phase of the disease, preventing reactivation of infection. Both $CD4^+$ and $CD8^+$ T cells secrete IFN-γ, and $CD8^+$ T cells develop cytotoxic activity against infected cells (30,31).

Initial attempts to induce protection against toxoplasmosis involved the use of live attenuated tachyzoites from mutant strains of *T. gondii*, like the heat sensitive ts-4 (33). Despite the high efficiency in terms of

activation of $CD4^+$ and $CD8^+$ T cells and the efficacy of protection, vaccines based on live tachyzoites are not applicable to humans, due to the risk of pathogenic side effects. Thus, most recent protocols focus on development of recombinant vaccines. As target antigens, there is a great interest in tachyzoite surface proteins, particularly SAG1 (34-38). In addition, a few studies have also been performed with SAG2 and SAG3 (39). The SAG proteins, the most abundant on the tachyzoite surface, are anchored to the parasite membrane by glicosylphosphatidylinositol (GPI) structures and are believed to be involved in the process of host cell invasion. Further, their sequences are highly conserved among different strains of *T. gondii*, sharing high degree of homology between type I (pathogenic and lethal to mice) and type II/III (cystogenic) strains (32,40).

Genes coding for *T. gondii* surface antigens SAG1 and SAG2 have been cloned and expressed in recombinant procariotic and eucaryotic systems, and both recombinant protein and plasmid have been used to immunize mice and other species. It was observed that recombinant proteins generally induce high titers of IgG1 antibodies, which have poor effect against challenge with pathogenic strains of *T. gondii* (38), and that improvement of the response depends on combination with proper adjuvants. On the other hand, vaccines based on plasmids coding for SAG1 and SAG2 were able in many cases, depending on administration route and formulation, to induce cellular immune response with Th1 profile, inducing better protection (34).

In this context, viral vectors such as adenoviruses can improve the immune responses in comparison to naked plasmid vaccines, since they are more efficient in transferring the genetic sequences and inducing protein expression (41). Adenoviruses infect a great variety of cells, including key immune cells, like dendritic cells, leading to an efficient activation of $CD4^+$ and $CD8^+$ T cells (42) and are capable of driving the immune response to a desired Th1 type (43). We generated recombinant adenoviruses encoding three genetically modified surface antigens (SAG) of the parasite *Toxoplasma gondii,* i.e. AdSAG1, AdSAG2 and AdSAG3 (44). Modifications included the removal of their GPI-anchoring motifs and, in some cases, the exchange of the native signal peptide by influenza virus haemaglutinin signal sequence (HASS). Adenovirus immunization of BALB/c mice elicited potent antibody responses against each protein, displaying a characteristic bias to a Th1 profile when the IgG2a/IgG1 antibody ratio was determined. Furthermore, the presence of parasite-specific IFN-γ producing $CD8^+$ T cells was analyzed in the same animals by ELISPOT. Splenocytes from immunized mice secreted IFN-γ after recognition of antigen-presenting cells infected with each recombinant adenovirus. However, only AdSAG2 was able to induce *in vivo* sufficient numbers of antigen-specific lymphocytes to be readily distinguished from the control animals vaccinated with an irrelevant virus. We finally tested the capacity of the immune responses detected to protect mice against a challenge with live *T. gondii* parasites. Although no major protection was observed against tachyzoites of the highly virulent RH strain, a significant reduction in brain cyst loads was observed in animals challenged with cysts of the P-Br strain. Thus, up to 80% of the parasitic forms were eliminated from animals vaccinated with a mix of the three recombinant viruses. Since adenoviruses seemed capable of inducing broad Th1-biased protective immune responses against *T. gondii* antigens, other parasite antigens should be tested alone or in combination with the ones described here to further develop a protective vaccine against toxoplasmosis.

Considering that the regular route of infection with *T. gondii* is through the mucosa of the digestive tract, we decided to direct our studies towards a vaccine that induces a strong mucosal immunity. For that we are considering the use a heterologous prime-boost protocol with SAG2 encoding adenovirus and influenza virus. It is noteworthy that both viruses infect cells from the digestive tract and can be use to elicit mucosal immunity. Thus, we have also constructed influenza vector (45) expressing the *T. gondii* surface antigens, and the experiments with heterologous prime-boost with recombinant virus are being performed.

IV – CONCLUSIONS

We conclude that the great majority of the *T. gondii* Brazilian isolates are natural recombinants generated from lineages Type I and III. Importantly, the majority of these isolates are virulent in mice. Whereas the IL-12/IFN-γ and iNOS were critical for host resistance to these isolates, we found that the mouse MHC haplotype "b" is not a determinant of susceptibility to infection, as previously determined by parasites of Type II lineage. Finally, adenovirus coding modified genes of SAG1, SAG2 and SAG3 led to induction of protective immunity, as indicated by 80% reduction of cyst numbers in mice challenged with a natural recombinant Type I/III Brazilian isolate.

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Immune responses to *Neospora caninum* and prospects for vaccination

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Reproductive failure in cattle is of major economic and welfare concern to producers worldwide. There are many different causes of reproductive loss, including infectious disease and an accurate diagnosis of the condition may be problematic. *Neospora caninum* is a recently recognised protozoan parasite that has been linked with causing bovine abortion in many countries worldwide (Dubey, 2003). The parasite is closely related to *Toxoplasma gondii*, an important zoonotic pathogen, also known to cause congenital disease (Buxton, 1990). In this paper we will discuss the disease in cattle with emphasis on understanding the host-parasite relationship leading to devising strategies to control bovine neosporosis.

THE DISEASE

Epidemiological studies in several countries have shown that cattle infected with *Neospora caninum* are three to seven times more likely to have an abortion compared with uninfected cattle, with the highest risk during a first pregnancy (Thurmond and Hietala 1997a; Moen *et al*, 1998; Wouda *et al*, 1998). Adult cattle rarely show clinical symptoms following infection and disease manifests in the placenta and developing foetus (Innes *et al* 2002; Buxton *et al* 2002). Clinical consequences of infection include abortion of the foetus, birth of a weak calf sometimes showing neurological symptoms or birth of a clinically normal but persistently infected calf (Dubey and Lindsay, 1996). The clinical outcome is likely to be related to the timing of infection during pregnancy (Innes *et al*, 2002). Economic losses associated with the disease include costs associated with loss of calf, fertility problems and increased calving interval, reduced milk production, reduced value of stock and increased likelihood of culling (Thurmond and Hietala, 1997b; Trees *et al* 1999; Dubey, 2003).

TRANSMISSION AND LIFE-CYCLE STAGES

Neospora caninum may be transmitted to cattle via consumption of feed or water contaminated with the oocyst stage of the parasite or by vertical transmission of the tachyzoite stage from dam to foetus during pregnancy (Dubey, 2003). Dogs have recently been identified as a definitive host of the parasite (McAllister *et al* 1998; Basso *et al* 2001). Oocysts may be shed in the faeces of acutely infected dogs that may acquire the infection through the consumption of infected bovine placentas (Dijkstra *et al* 2001). The oocyst stage of the parasite is thought to persist in the environment but currently little is known about the environmental conditions that may favour oocyst survival or the frequency of oocyst shedding by dogs (Dubey *et al* 2003). Following infection, the tachyzoite stage of the parasite actively invades host cells and multiplies by a process called endodyogeny resulting in many tachyzoites which burst from the cell ready to invade new cells and resume rapid multiplication (Dubey and Lindsay, 1996). Using this process the parasite can disseminate via the circulation throughout the host (Okeoma *et al* 2004). The parasite can only multiply within host cells and it is thought that under pressure from the immune response of the host, the parasite differentiates into the slower multiplying bradyzoite stage. Bradyzoites are usually observed within tissue cysts in neural tissues (brain and spinal cord) and this is thought to be how the parasite may cause persistent infection in cattle (Dubey and Lindsay, 1996). Vertical transmission from dam to foetus may occur following an exogenous challenge during pregnancy or may result following recrudescence of an existing persistent infection. A characteristic of bovine neosporosis is the high rate of

vertical transmission estimated at between 78-95% (Pare *et al* 1996; Davison *et al* 1999). Interestingly, transplacental transmission can occur over consecutive pregnancies and congenitally infected heifers can transmit the parasite to their own offspring (Bjorkman *et al* 1996).

DO CATTLE DEVELOP NATURAL IMMUNITY?

Cattle which have experienced an abortion due to neosporosis have a significantly decreased chance of having a repeat abortion due to the same infectious agent (Anderson *et al* 1995; Wouda *et al* 1998), implying that cattle can develop a degree of protective immunity against the parasite. Further evidence for this came from an investigation of a point source outbreak showing that those cattle which had evidence of prior exposure to *N. caninum* were less likely to abort compared with those undergoing a primary infection (McAllister *et al* 2000). While cattle may be able to develop some protective immunity to help prevent abortion, this immunity does not protect so well against vertical transmission as evidenced by the high rates of repeated vertical transmission seen in natural infection. Therefore a vaccination strategy to prevent/reduce abortion may a more feasible goal than to prevent vertical transmission.

In any host-parasite relationship a vast array of different immune responses are induced against the various life-cycle stages of the parasite. Some of these immune responses will be protective to the host, others protective to the parasite, some may cause pathology in the host and others may be largely irrelevant. In the following sections we will discuss the different roles of the host immune response and how this contributes to our understanding of the host-parasite relationship, disease pathogenesis and immunological strategies to control the disease.

HOST PROTECTIVE IMMUNE RESPONSES

The tachyzoite stage of *N. caninum* actively invades and multiplies within various cells of the host (Hemphill, 1999). The intracellular location of the parasite suggests that cell-mediated immune responses are likely to play a significant role in protective immunity (Marks *et al* 1998). Interferon gamma (IFNg) and tumour necrosis factor alpha (TNFa) are known to significantly inhibit intracellular multiplication of *N. caninum* (Innes *et al* 1995; Yamane *et al* 2000). The cytokines IFNg and interleukin 12 (IL-12) were shown to be important components of protective immunity using mouse models of infection (Khan *et al* 1997; Bazler *et al* 1999) and IFNg knockout mice showed a significantly increased vulnerability to *N. caninum* infection (Dubey *et al* 1998). The importance of CD4+ T-cells in protective immunity was highlighted in a study where mice were treated *in vivo* with antibodies to deplete CD4+ or CD8+ T-cells prior to challenge with *N. caninum* (Tanaka *et al* 2000). In the group of mice where CD4+T-cells were depleted, all mice died within 30 days of the challenge, in contrast no mice died within this time period in the control group or the group where CD8+T-cells had been depleted (Tanaka *et al* 2000). In addition, *N. caninum*-specific CD4+ T-cells, from infected cattle, were able to directly lyse parasite infected autologous target cells *in-vitro* (Staska *et al* 2003).

While we still know comparatively little concerning induction, function and regulation of protective immune mechanisms against *N. caninum* parasites in cattle current data would support an important role for CD4+T-cells and pro-inflammatory cytokines such as IFNg.

CHANGES TO THE HOST-PARASITE RELATIONSHIP AT DIFFERENT STAGES OF PREGNANCY

Neosporosis is a disease that manifests during pregnancy where the developing foetus is particularly vulnerable. Various changes occur in the maternal immune response to enable the dam to support the pregnancy and prevent immunological rejection of the semi-allogeneic foetus (Raghupathy, 1997**).** These natural changes in the immune system may favour the parasite and help to explain disease pathogenesis in pregnancy. Relevant to our understanding of bovine neosporosis are studies examining cytokine regulation in pregnancy, in particular at the materno-foetal interface (Innes *et al* 2002). The pro-inflammatory cytokines

such as IFNγ and IL-12 are involved in the generation of Th1-type immune responses that may be damaging to the pregnancy (Tangri *et al* 1993; Wegman *et al* 1993; Entrican, 2002). The cytokine environment of the placenta favours more regulatory Th2-type cytokines such as IL-10, IL-4 and transforming growth factor beta (TGF-β) whose role is to counteract the inflammatory responses induced by the Th1-type cytokines (Entrican *et al* 2002).

Therefore the natural immuno-modulation occurring in the pregnant dam resulting in a bias towards Th2-type immune responses may limit her ability to control *N. caninum* multiplication and the Th1-type immune responses, known to protect against *N. caninum* may be detrimental to the pregnancy. A similar example of pregnancy related changes to the immune system affecting the host-parasite relationship is seen with *Leishmania major* infection in mice where the protective immune response is also associated with a Th1-type immune response. During pregnancy there was a reduction in the IFNγ response and an increase in production of the more regulatory cytokines IL-4 and IL-10 that resulted in the pregnant mice being less able to control the infection compared to non-pregnant controls **(**Krishnan *et al* 1996).

A study examining cell-mediated immune responses in pregnant cattle infected with *N. caninum* noted that there was a significant reduction in the antigen-specific cell-proliferation and IFNγ response around mid-gestation compared to pre-pregnancy or early gestation (Innes *et al* 2001). Levels of progesterone in pregnant cattle are also known to increase steadily from early to mid-gestation (Pope *et al* 1969) and progesterone in known to bias a T-cell response towards a Th2 phenotype (Kalinski *et al* 1997). These studies indicate the changing dynamics of the maternal immune response as gestation progresses that may influence the response of the parasite within the host. Epidemiological studies have suggested that most recorded cases of Neospora-associated abortion occur between 4-6 months of gestation (Anderson *et al* 1991; Thurmond and Hietala, 1997; Moen *et al* 1998; Gonzales *et al* 1999). The changes in the maternal immune response around this time may influence recrudescence of a persistent infection or the ability of the dam to control a new infection. Recrudescence of *T. gondii* infection is known to occur in HIV infected patients when the T-cell and IFNγ response are diminished (Luft *et al* 1984).

Several studies using controlled experimental infections with *N. caninum* have shown that the timing of placental and foetal infection is important in determining the outcome, in general the earlier in gestation this occurs the more severe the consequences for the foetus (Barr *et al,* 1994; Buxton *et al* 1998, Williams *et al* 2000; Maley *et al* 2003; Macaldowie *et al* 2004). In the study outlined previously examining temporal changes to the maternal immune system during pregnancy (Innes *et al* 2001) the dams showed significantly higher antigen specific cell-proliferation and IFNg responses in early compared to mid-pregnancy. Therefore an infection occurring at this stage of pregnancy may invoke a strong Th1-type immune response that may in itself prove detrimental to the pregnancy. Recent data examining lesions in the placenta of cattle experimentally infected with *N. caninum* in early gestation has shown a strong maternal inflammatory response in those dams where foetal death had occurred (Macaldowie *et al* 2004). Further examination of the placental tissues has shown the presence of NK cells, CD4+, CD8+ and δγ T-cells and IFNγ associated with foetal death, as these responses were not seen in those infected cattle carrying live foetuses or in the uninfected control cattle (Maley *et al*, manuscript in preparation). It is known from other studies that direct administration of IFNγ can induce spontaneous abortion in pregnant mice (Chaout *et al* 1990).

Therefore while we know that Th1-type immune responses may be protective to the dam against *N. caninum* infection, this type of immune response induced in placental tissue may be highly detrimental to the foetus.These observations highlight how immune cytokines may have both a beneficial and detrimental effect on the host depending on their concentration and tissue location.

DEVELOPMENT OF FOETAL IMMUNITY

A further important influence determining the outcome of infection is the relative immunocompetence of the foetus at the time of challenge. The immune system of the foetus matures progressively throughout gestation (Osburn *et al* 1982). Studies examining foetal immune responses in cattle infected with *N. caninum* in early gestation have shown mitogenic responses in foetal spleen and thymus cells around day 100 of gestation but there was no evidence of antigen specific cellular or humoral immune responses at this stage (Innes *et al*, manuscript in preparation). Evidence of specific cell-mediated and humoral immune responses occurs around 4-7 months of gestation (Andrianarivo *et al* 2001; Almeria *et al* 2003; Bartley *et al* 2004). The increasing immunocompetence of the foetus as pregnancy progresses will enable the foetus to better control the parasite infection resulting in reduced disease severity.

Therefore the dynamics of the host-parasite relationship changes throughout pregnancy. Important factors influencing severity of disease in bovine neosporosis include the timing of the infection during pregnancy, the relative immunocompetence of the foetus and the various consequences of the maternal immune response being host protective, parasite protective and in causing immunopathology.

CONTROL STRATEGIES

As dogs are known to play an important role in the transmission of the parasite and oocysts may persist for some time in the environment it is important to introduce farm management procedures to prevent or minimise faecal contamination of feedstuffs and water (Dubey, 2003). Efficient disposal of infected placentas, foetuses or still born calves will also help to minimise sources of contamination. Testing of animals prior to introducing them to the herd and culling of infected cattle may be an option depending on the level of infection within the herd. Various pharmaceutical agents have been tested *in vitro* and *in-vivo* and have shown some efficacy against the tachyzoite stage of the parasite (Lindsay *et al* 1994; Gottstein *et al* 2001). However, there are no drugs available that are effective in curing cattle of *N. caninum* infection and there may be problems arising from drug residues in milk from lactating cows (Dubey, 2003).

There is currently much interest in developing a control strategy against bovine neosporosis based on vaccination. The targets for such a control strategy would include prevention of Neospora-associated abortion and ideally prevention of vertical transmission of the parasite.

INDUCTION OF PROTECTIVE IMMUNITY

Encouraging studies in this area have shown that experimental infection of naïve animals prior to mating induced protective immunity against both abortion and vertical transmission of the parasite following challenge during pregnancy (Innes *et al* 2001, Buxton *et al* 2001). In addition, persistently infected cattle were protected against a challenge that induced foetopathy in naïve control animals (Williams *et al* 2003).

A live vaccine preparation is likely to stimulate appropriate CMI responses against intracellular pathogens as it more closely mimics what is happening during natural infection and the parasite antigens are presented to the immune system in the correct context. There is interest in developing attenuated strains of the parasite that may be useful as vaccine preparations (Lindsay *et al* 1999). A highly successful commercially available vaccine to prevent toxoplasmosis in sheep utilises a live attenuated strain of *T. gondii* (Buxton and Innes, 1995). Drawbacks of live vaccines include a limited shelf-life and safety concerns therefore attention has also focussed on development of killed vaccines. The major challenges in designing an effective killed vaccine against an intracellular pathogen are to select relevant antigens and to deliver these antigens to the host to stimulate appropriate and long-lasting protective immune responses.

SELECTION OF RELEVANT ANTIGENS

Understanding protective host immune responses may be helpful in selection of relevant antigens. Antigens recognised by immune sera and also immune T-cells may prove to be useful vaccine candidates (Marks *et al* 1998; Hemphill, 1999; Staska *et al* 2005; Tuo *et al* 2005). In addition parasite antigens known to be involved in host cell invasion and survival are likely to be important (Hemphill 1999). Due to the complex interaction of the parasite and the bovine host involving different life-cycle stages a killed vaccine may have to comprise a cocktail of different antigens (Innes *et al* 2002).

ANTIGEN DELIVERY STRATEGIES

Live antigen delivery systems have been used to elicit immune responses against a wide range of pathogens. Recombinant virus vectors have been shown to stimulate specific CMI responses against other intracellular protozoan parasites (Honda *et al* 1998; Schneider *et al* 1998; Oliveira-Ferreira *et al* 2000).

Recombinant vaccinia viruses constructed to express the antigens Nc-SRS2 or NcSAG1 were able to induce protective immunity against acute *N. caninum* infection in non-pregnant mice (Nishikawa *et al* 2001a) and were also able to induce protection against abortion in a pregnant mouse model (Nishikawa *et al* 2001b). In both cases the best protection was achieved using the recombinant vaccinia virus expressing the NcSRS2 antigen.

Crude lysate antigen prepared from *N. caninum* tachyzoites has been tested using different adjuvant preparations in attempts to induce protective immunity in mice. The use of non-ionic surfactant vesicles as an adjuvant exacerbated encephalitis and clinical neurological disease in immunised mice (Bazler *et al* 2000) and administration of antigen with Quil A or ISCOMs resulted in enhanced protection (Lunden *et al* 2002). Administration of a crude tachyzoite lysate with ImmuMAXSRä adjuvant protected against vertical transmission of *N. caninum* in a pregnant mouse model (Liddell *et al* 1999). Protective immunity was also induced in mice using specific recombinant antigens, NcSRS2 incorporated into ISCOMs (Pinitkiatisakul *et al* 2005) and NcMIC3 antigen with the Ribi adjuvant system (Cannas *et al* 2003a).

DNA VACCINATION

With DNA vaccines the host is injected with DNA incorporated into a plasmid containing sequences encoding the antigens of interest. An advantage of DNA vaccination is the way that the plasmid is taken up and processed by antigen presenting cells resulting in the induction of both cell-mediated and humoral immune responses (Reyes-Sandoval and Ertl, 2001). This is of particular importance when trying to design vaccines against intracellular pathogens. Cytokines and immunostimulatory DNA sequences can be co-expressed to help modulate the type of immune response required (Sakai *et al* 2003).

Mice vaccinated intramuscularly (*im*) with a eukaryotic expression plasmid containing NcSRS2 or NcSAG1 cDNA inserts and then boosted using the recombinant antigens were better protected against *N. caninum* challenge than those mice receiving only recombinant antigen (Cannas *et al* 2003b). A further study showed direct immunisation of Balb/c mice with plasmid DNA encoding NcGRA7 or NcsHSP33 protected against congenital infection with *N. caninum* (Liddell *et a*l 2003).

CpGs (oligodinucleotides) are known to activate Th1 type immune responses and pro-inflammatory cytokines and are thought to be useful adjuvants to enhance the immune response to vaccines against intracellular infections (Klinman, 2003, Mutwiri *et al* 2003). Addition of the CpG adjuvant to the vaccination of mice with plasmid DNA expressing NcGRA7 significantly improved protection (Jenkins *et al* 2004).

KILLED VACCINE TRIALS IN CATTLE

A killed *N. caninum* preparation combined with a POLYGEN™ adjuvant was used to vaccinate heifers at 35 and 63 days of gestation (Andrianarivo *et al* 2000). The cattle were challenged with a combined *i.v/ i.m* inoculation of live *N. caninum* tachyzoites four weeks after the second inoculation. Following vaccination, the cattle developed specific humoral and cell-mediated immune responses and after challenge there was a boost to the antibody response but not to the cell-mediated immune response. All of the challenged heifers, either vaccinates or controls had infected foetuses indicating that under the challenge conditions used in this study the vaccine preparation had not successfully protected the cattle (Andrianarivo *et al* 2000).

A commercial vaccine, Bovilis ® Neoguard, Intervet comprising a killed Neospora tachyzoite preparation formulated with an adjuvant, SPUR® is currently commercially available in certain countries. The vaccine is administered sc on two occasions, 3-4 weeks apart in the first trimester of pregnancy. Data on the efficacy of the vaccine under field trial conditions showed that the vaccine had some protective effect against abortions occurring at 5-6 months of gestation in cattle in Costa Rica whereas, a similar study in dairy cattle from New Zealand resulted in no definite conclusions on the ability of the vaccine to protect cattle (Schetters *et al* 2004).

CONCLUDING REMARKS

Recent data from controlled experimental infections of pregnant cattle is helping us to understand the complex dynamics of the host-parasite relationship in bovine neosporosis and to determine why some cattle abort their foetuses while others produce clinically healthy, albeit congenitally infected calves. Additional studies looking at induction of protective immune responses has given encouragement to the possibility of controlling the disease by vaccination. However there are still several challenges to overcome. It is important that the vaccine is designed in such way as to induce protective immune responses without exacerbating pathology. In addition, further work needs to be done to determine the immunological implications of cattle becoming infected with the parasite *in-utero* when their immune systems are still developing and being born persistently infected with the parasite. Does this somehow compromise their ability to develop effective immunity against *N. caninum* later in life and does this in part explain the high rates of repeated vertical transmission observed in natural infection? This would have important implications in devising a vaccination strategy as it may prove to be more efficacious to target the vaccine to naïve cattle and cull out those that are congenitally infected.

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Perspectives for the control of coccidiosis in poultry by chemotherapy and vaccination

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INTRODUCTION

Despite the introduction of live vaccines, in most countries prophylactic chemotherapy is still the preferred method for the control of coccidiosis (Chapman, 2000). Significant improvements in the performance of commercially reared poultry have been made during the last half of the twentieth century and a recent study has shown that in the USA these improvements have continued, and are measurable even for the relatively short time period of 1995-2001 (Chapman et al., 2003). It has often been assumed that these improvements would not have been possible without the introduction of a succession of ever more effective anticoccidial agents to control coccidiosis. In recent years, however, few new drugs have been introduced. The most widely used compounds, ionophorous antibiotics, have been utilized with apparent success for more than thirty years; these drugs have been used extensively in commercial broilers and are also employed to some degree during the rearing of broiler breeder and replacement layer stock (Chapman, 1993; 2001). Synthetic drugs (chemicals) have been used to a lesser extent; in the USA nicarbazin was introduced in 1955 and is still used in broiler production (Chapman, 1994a)! In the 1970s many other highly efficacious synthetic drugs were introduced only to be withdrawn because of the development of drug resistance.

At first it was thought that resistance would not develop to the ionophores but many recent studies have shown this not to be the case. For example, Mathis (1999) examined the sensitivity of recent isolates of *E. acervulina*, *E. maxima*, and *E. tenella* to monensin, salinomycin, narasin and lasalocid and found that these drugs were only marginally or poorly effective; similar conclusions have been made by others (e.g. Chapman and Hacker, 1994). Anecdotal information provided to the author by those involved in rearing poultry indicates that coccidiosis is not generally considered to be a major problem. How can this be true if the principal drugs used are no longer as effective as in the past? Possible reasons are discussed in this article.

ACQUIRED DRUG RESISTANCE

Conventional methods for evaluating drug efficacy involve challenging birds with isolates of *Eimeria* obtained from the field using a sufficiently large dose of oocysts to induce a depression of weight gain and/or lesions in susceptible birds (Chapman, 1998; Holdsworth et al., 2004). Such studies can indicate whether a drug is able to control an infection capable of causing clinical disease and if not the isolates may be classified as showing various degrees of "resistance" to the drug. Some authors have questioned whether current experimental methods for determining resistance are appropriate (Watkins, 1997). Experiments with isolates considered "resistant" to ionophores showed lack of control by these drugs whether a large or small dose of oocysts was administered, and weight gain or oocyst production in the feces respectively used to assess efficacy (Chapman and Shirley, 1989). An explanation for the efficacy of ionophores under commercial conditions where ionophore resistant strains are present must be sought elsewhere. A possibility is that these drugs do not prevent the acquisition of immunity and that this may develop before birds are exposed to potentially pathogenic numbers of oocysts (Chapman, 1999a). Indeed, reliance upon immunity development in flocks medicated with ionophores is a major strategy used to control coccidiosis by poultry producers in the USA (see below). Whether resistance will translate into

drug failure in the field will depend upon the extent of exposure to infective oocysts in the commercial poultry house. Such exposure will in turn depend upon many environmental and management factors.

Our knowledge of the extent to which resistance is present in the field would be increased if methods were available for identifying resistant strains; such information may be forthcoming in the future (Sangster et al., 2002).

MANAGEMENT AND ENVIRONMENT

The pioneering studies of Johnson and Tyzzer in the 1920s laid the groundwork for our understanding of coccidiosis and the important management and environmental factors that affect the incidence and epizootiology of this disease (Chapman, 2003). Of the many improvements in husbandry and housing that have occurred over the years, one of the most significant may have been the comparatively recent introduction of nipple type drinking systems. These systems require careful management but have the potential to reduce litter wetness that is known to be a major factor in the occurrence of clinical coccidiosis. The introduction of efficient ventilation systems and enclosed housing has facilitated better environmental control and may also have helped reduce the likelihood of coccidiosis. An example of the effect of management upon the effectiveness of coccidiosis control programs was given by Williams; higher parasite numbers were produced by vaccinated birds where "clean-out" between flocks was demonstrably inadequate (Chapman et al., 2002).

OTHER DISEASES AND PATHOGENS

There is some published evidence that coccidiosis is more prevalent in flocks where other diseases, bacterial and viral, are present. The introduction of modern vaccination programs for a variety of such diseases may therefore have contributed to the decreased occurrence of clinical coccidiosis. Many antibiotics have been introduced to improve the performance of commercial poultry and are generally thought to work by suppressing the growth of pathogenic bacteria; these are often used along with anticoccidial drugs. A recent study has clearly demonstrated improved performance of flocks where growth promoters are included in the feed in combination with anticoccidial drugs (Chapman and Johnson, 2002).

HOST GENETICS

It is known that different breeds of chicken may vary in their susceptibility to infection with *Eimeria* species. Although breeding programs to select for resistance to coccidiosis have not been undertaken by the commercial poultry industry, selection for "fitness" will include resistance to common environmental pathogens of which *Eimeria* is one component. It would be of interest to establish whether the modern broiler is less susceptible to coccidiosis than its antecedents.

IONOPHORES AND IMMUNITY

Recent studies have shown that a factor in the efficacy of ionophores has been their lack of interference with the development of immunity (Chapman, 1999b). The poultry industry has taken advantage of this by increasing the withdrawal period of medication prior to slaughter with considerable savings in the costs of medication. This can, however, result in poorer performance, particularly in situations where birds may be exposed to heavy infections late in life (Chapman et al., 2004).

VACCINES

It seems logical that if immunity is desired then this should be achieved by use of live vaccines. Guidelines to assist those interested in designing studies to evaluate the efficacy of new vaccines have recently been published (Chapman, et al., 2005b). Vaccination has become more practical with the development of

new methods for vaccinating birds in the hatchery. It has recently been shown however that even with highly immunogenic species of *Eimeria,* such as *E. maxima*, reinfection is necessary for the establishment of solid immunity at 4 weeks of age when birds are given a small dose of oocysts following hatch (Chapman et al., 2005a). Exposure to large numbers of oocysts prior to 4 weeks could result in clinical coccidiosis before birds acquire protective immunity. Thus with vaccines as well as drugs success of vaccination is likely to depend upon environmental and management factors that affect numbers of infective oocysts in commercial poultry houses.

In recent years several new methods of vaccinating chickens have been introduced but in few cases has the route of infection been clearly demonstrated and the proportion of birds infected established; the latter is relatively easily determined (Chapman and Cherry, 1997).

RESTORATION OF DRUG SENSITIVITY

Use of live vaccines comprising drug sensitive strains results in the restoration of sensitivity to ionophores such as monensin (Chapman, 1994b); similar conclusions were reached for salinomycin and diclazuril (e.g. Mathis, 2003). It is difficult to demonstrate that restoration of sensitivity has resulted in long term improvements in flock performance, nevertheless programs involving the rotation of vaccines with traditional chemotherapy have been advocated (Chapman et al., 2002) and are used by the poultry industry.

CURRENT COCCIDIOSIS CONTROL IN THE USA

In the USA accurate information on the use of feed additives, including anticoccidial drugs, in the feed of poultry is available from a commercial database that covers almost the entire broiler industry (Agri. Stats Inc.). Data from 1995-1999 indicate drug usage had a characteristic cyclical annual pattern (Chapman, 2001). Programs comprising a single or two different ionophores were used extensively (80-90% of broiler complexes) in the summer and fall (June-November) followed by a decrease (70-50% of complexes) in winter and spring (December-May). During the latter period there was an increase in the use of shuttle programs (20-50% of complexes) in which a synthetic drug and ionophore were employed. Overall, more than 95% of broilers were given an anticoccidial drug in the feed for some part of their life.

Data from 2004/2005 indicate a different pattern of drug usage. Shuttle programs in which a synthetic drug was employed during winter and spring were still widespread (20-60% of complexes). However, during the summer and fall the use of a single or two ionophores had declined to approximately 50-60%. The remaining complexes (10-30%) used no anticoccidial medication during this period and it is speculated that the reason was the adoption of coccidiosis vaccines. In the USA, vaccines are primarily used in the summer months; it will be interesting to see if this pattern persists in the future.

THE FUTURE

Crystal ball gazing is not without risk as the premature anticipation of recombinant DNA vaccines illustrates. The dearth of new drugs to control coccidiosis makes it tempting to speculate that the "age of chemotherapy", having been extremely successful and lasting almost sixty years, (since the demonstration in 1947 that sulphonamides can be used prophylactically) may be nearing its end. As already indicated, in the USA where accurate data are available, the use of drugs is still extensive; in Brazil, a country with a broiler industry similar in size to that of the USA, approximately 92-95 % of broilers receive an anticoccidial drug in the feed (Viana, personal communication). Although coccidiosis vaccines have been available since the 1950s their use in the USA is limited although as indicated above this situation may be changing (attenuated vaccines that are apparently widely used in Europe and elsewhere are not presently available). It may be premature therefore to consider that the 'age of vaccination" is upon us. An unknown factor is the extent to which national authorities, in response to perceived consumer pressure, may impose restrictions on the use of anticoccidial drugs in poultry feeds. Vaccination is now considered a more realistic proposition

than in the past but this does not mean that it is without problems and many questions remain to be answered. In the case of attenuated vaccines, not the least will be the logistics of providing sufficient oocysts to vaccinate billions of broilers worldwide. A likely scenario in the coming decades is a combination of approaches in which chemotherapy and vaccination are integrated in programs designed to achieve sustainable coccidiosis control for the foreseeable future.

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Anticoccidial drug discovery: Approaches towards the identification of novel chemotherapeutic agents

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New chemotherapeutic agents that efficiently control avian coccidiosis have not been introduced to the poultry industry for three decades. Despite the creative use of shuttle programs, the biological cycling of *Eimeria* parasites and the high density housing that is typical for poultry operations today have predisposed the industry to drug resistance. The identification of novel antiparasitic agents has become essential for continued chemotherapeutic control of avian coccidiosis. A new anticoccidial agent must have the following features: (i) novel molecular target to minimize the potential for cross-resistance in field isolates, (ii) minimal resistance induction potential, (iii) potent spectrum of activity against all *Eimeria spp.* commonly encountered in the field, (iv) an acceptable therapeutic index without genotoxicity, (v) no tissue residue issues, and (vi) simple chemistry to be able to provide sufficient bulk material while meeting the stringent economic requirements imposed by the industry.

The list of potential drug targets promises to grow as a result of the availability and comparative analysis of apicomplexan parasite genomes, including that of *E. tenella*. Until this time, our discovery efforts have been based in empiric screens, scoring for inhibition of parasite growth in cell culture. The ability to conduct "clinical" efficacy studies early in the program using a minimal amount of a compound with empiric whole cell in vitro activity is an enormous advantage for anticoccidial discovery. Conventional biochemical tools are used to help identify potential targets or pathways responsible for the antiparasitic activity of empiric hits. Critically important to the discovery process, and in conjunction with chemical validation, is genetic validation of the molecular target. Using *T. gondii* as a model parasite, demonstration that a potential gene target is essential rather than functionally redundant or dispensable in the parasite life cycle is required to warrant medicinal chemistry involvement. Synthetic modification of the primary hit to develop suitable pharmacokinetic and safety properties, with a focus on simple inexpensive chemistry then follows. In this presentation the identification of parasite cGMP-dependent protein kinase (PKG) as a molecular target and optimization of PKG inhibitors will be used to illustrate the discovery process.

Vaccination against coccidial parasites. The method of choice?

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INTRODUCTION

The family of Coccidian parasites comprising genera such as *Eimeria, Toxoplasma, Neospora* and *Sarcocystis* is more or less particular, because to date effective vaccines have been developed against each of its members. Having vaccines available might suggest that problems are solved and other diseases can be tackled; however, this is an oversimplification of the complex nature of immunity and disease control in different animal species and against different members of each genus.

Present vaccines may be improved with respect to better control of parasitemia and easier and more cost-effective production and distribution.

A major disadvantage of an immunological approach is that for each species to be controlled a separate and specific vaccine needs to be developed. This may seem obvious, but it is a drawback when compared to the development of a drug.

Most anti-coccidial drugs developed in the second half of the 20th century are active against multiple species and even genera and can act in different, albeit not all, hosts (examples are ionophores and anti-folates).

The occurrence of resistance makes it still necessary to develop new drugs, preferentially targeting common pathways of Coccidia or even Apicomplexa, of which some are even absent in vertebrate hosts. Coombs (1) reviews such targets exemplified as the functional apicoplast, the shikimate pathway, mannitol cycle and the polyamine metabolism. Recently developed organotypic cultures of neural tissue may be of benefit in the selection of active compounds (2). It is to be foreseen that although more and better vaccines become available, several animal species suffering from the parasites mentioned above, will remain dependent on drug therapy because vaccines can't handle the many different species involved (10 species of *Eimeria* are attacking young lambs) (3).

Reduction of the use of drugs in consumption animals however also drives the generation of biological solutions (vaccines).

EIMERIA VACCINES

Eimeriosis, often designated as coccidiosis, is the disease caused by *Eimeria* parasites resulting in severe mucosal damage, weight loss and sometimes even death. The disease is widespread and many species occur in poultry, livestock and small animals such as rabbits. Especially the intensification in poultry production (broiler farms) could only be achieved by the concurrent development and application of effective drugs (4).

Since chickens readily develop immunity from natural infection, vaccines were developed based on virulent strains of the most frequently occurring species *E. tenella, E. acervulina* and *E. maxima.* These vaccines were shown to be effective when administered at low dose, early in life.

Attenuation of strains could be achieved by adaptation to development in fertilized eggs. Passage of strains through eggs rendered them less pathogenic, although for instance *E.acervulina* could not be adapted to the egg (5). Livacox ® contains an embryo-adapted *E. tenella* line. Precociousness is a

characteristic of most *Eimeria* referring to a naturally occurring population of parasites that complete their lifecycle from sporozoite to oocyst 20-30 hrs faster than their fellow parasites from the same parent. This is a selectable trait and sometimes this is accompanied with a decrease in proliferative capacity and pathogenicity. Paracox® was the first line of vaccines that utilized this feature for live vaccines with a better safety profile (6). Recent publications report on newly developed precocious vaccines in different parts of the world (4,7,8). Kawazoe *et al.* (7) reported that as a result of the selection for precociousness one strain appeared less pathogenic, whereas the other did not differ from the parent line in that respect. This emphasizes the fact that the trait precociousness is not unambiguously linked to the aspect of low pathogenicity.

Strains selected for naturally occurring low pathogenicity were included in NobilisCox ATM® (9). Whereas most vaccines allegedly contain sensitive strains, the latter comprises strains with a defined tolerance for specific drugs. Thereby it allows the concomitant use of certain ionophores until immunity is fully developed. The strains are, on the other hand, fully susceptible to chemical drugs such as diclazuril and toltrazuril, which allows removal if preferred. Li *et al.* (8) recently selected ionophore-tolerant, precocious strains for similar purpose, and Kawazoe *et al*. (7) demonstrated that a certain degree of ionophore tolerance is a natural feature of different *Eimeria* strains, even if they did not have contact with these drugs before.

Antigenic variability becomes more and more evident, especially when live *E. maxima* vaccine strains are applied (10). Only Paracox® and NobilisCox ATM® have included antigenically different strains. The two strains in the latter vaccine appear to act synergistically (4).

Vaccine failures were often attributed to highly virulent strains. However, with improved administration of live oocysts, chickens develop immunity more readily as a consequence of early cycling of the parasites.

Vaccine spray on day-old birds is nowadays widely considered as the best method to convey the infection to the chicks. *In ovo* application of live coccidial vaccines (such as Inovocox®, Embrex) may also turn out to be effective. It at least ensures that all birds receive the same dose. Now that more and more vaccines of similar kind enter the scene, objective criteria need to be set for these products as presently there are no studies regarding safety and efficacy requirements.

Being realistic, we estimate that the vaccination of forty billion broilers each year will be very difficult if using live oocysts produced by chickens. Thus, there is a great need for mass production and mass application of effective vaccines. Subunit vaccines are generally not suited for such purpose, although a maternal vaccine Coxabic® was licensed. This vaccine is based on *E. maxima* gametocyte-derived antigens gam56 and gam82, which should protect the offspring by the action of maternal antibodies present in the yolk. An Emax250kD antigen was recently cloned, and predominantly recognized by maternal sera infected with *E. maxima* (11). It is not clear what the relation between these approaches is, but they could work in concert.

Recombinant vaccines should be the only long term solution for the problem in the future, especially since more drugs will be banned and requirements for live vaccines will become stricter.

It was shown that target antigens are involved in essential parasite functions. Surface-expressed and apical complex-associated proteins are prime targets for antibody attack (12). Effective protection using invasion-related proteins has not been demonstrated so far. In our lab, we have extensively tested EtMic1 and EtMic5; protein or DNA-based antigens EtMic2 and EtMic4 have been tested and showed some protective effect (13,14). Presentation is of essential importance and may influence the level of the effect observed.

The discovery of an array of 23 different genes (variants), encoding a SAG family of GPI-anchored proteins (no homology to *Toxoplasma* this time.) expressed on the surface of *E. tenella* merozoites, cannot be

currently be evaluated in relation to vaccine development, but may underline the complexity of our quest to find a vaccine based on only few components covering the major species of *Eimeria* in chickens (15).

Immunogenic soluble/cytoplasmic proteins, such as LDH and enzymes from the anti-oxidant pool (SOD and 1Cys-peroxidoxin), have been shown to be promising candidates, since they are recognized by T-cells, CD4+, and CD8+, producing IFN-γ. Partial protection was elicited in vaccinated chickens using *Salmonella typhimurium* expressed genes (16).

As said above, the presentation of all candidate antigens to the host immune system is decisive for inducing good levels of protection.

Lillehoj and coworkers have concentrated their work on this issue as reviewed in (13). A series of cytokine genes were reported to enhance the effect of the *E.acervulina* 3-1E or EtMic2 vaccination, although there was apparently no consensus cytokine that promoted protective effects for both antigens.

DNA application is more frequently reported. Song *et al* (17) and Min *et al.* (18) used pcDNA3-1E plasmid and co-injected it with cytokine genes in day-old chicks; however, protection was minimal (<30% oocyst reduction). Wu *et al.* (19) found Et1A and TA4 genes effective due to improved weight gain and >60% reduction in oocyst output.

DNA plasmid deposition was applied using *Salmonella typhimurium* bacteria (14), achieving a 50% oocyst reduction after challenge with a pcDNA5401 plasmid.

Scientifically, these approaches will stimulate our thinking about immune mechanisms involved, the role of cytokines in maturation of an adaptive response, and antigen processing. Practical cost-effective solutions for a mass application of *Eimeria* vaccines will most probably not come from these approaches. Viral vector vaccines are, in my opinion, the best solution, but still a number of obstacles have to be overcome. Herpes and Pox viruses are able to harbor the insert sizes needed to express multiple genes, which are necessary to control the multiple species involved. Fowl pox and Herpes virus of turkeys (HVT) are possible candidates (4,20). We have designed cassettes containing three *Eimeria* genes, each under the control of its own promoter in HVT, whereby each of the genes was expressed efficiently.

This indicates that we have the tools to construct vector vaccines for multiple antigen expression. Proof of principle to find the effective combination needs further study.

TOXOPLASMA GONDII VACCINES

In contrast to the vast experience with vaccination against *Eimeria* in chickens, only limited data are published on the induction of protection against *Toxoplasma*-related disease in mammals.

Toxoplasma infections occur in nearly all warm-blooded animals, including man. Apart from felids that can act as definitive host (sexual cycle producing oocysts) and intermediate host, the remaining hosts are only intermediate hosts acquiring the infection by ingestion of oocysts or tissue cysts contained in food (muscle, brain or organs of chronically infected animals). The tachyzoites are the pathogenic stage of the parasite causing high fever, strong inflammatory reactions, and inducing abortion or birth of the offspring with neurological symptoms by the passage from the pregnant host to the fetus. Due to the innate immunity response of the host, the tachyzoite stage converts into bradyzoites embedded in tissue cysts, after which a life long immunity is established.

Immunedepression may cause recrudescence, but in man this is only seen in HIV patients or in patients under post-transplantation treatments, and this seems to be caused by vast impairment of macrophage function in the brain (21). There is vertical transmission of the parasite, but in sheep, for instance, solid immunity is induced after the primary infection and subsequent pregnancies do not induce recrudescence (22). Some conflicting papers (23) detected high percentage of familial abortion in sheep, but no association to *Toxoplasma* was evidenced.

When a primary infection is acquired during pregnancy, the embryo is seriously at risk due to a very efficient infection of placenta and fetal tissue.

In humans it is generally believed that most ocular toxoplasmosis is caused by congenital infection, but recent studies have shown that ocular lesions are seen mostly in postnatally acquired infection (24,25).

In animals, the main health problems are abortions in sheep and goats (26). In UK and Spain, the prevalence of *Toxoplasma* in sheep is high and it is responsible for 25% of abortions (26,28). This is probably true in most sheep-producing countries. Cattle seem to be less efficiently infected by *Toxoplasma gondii* (27), and cats usually do not develop clinical symptoms. Prevalence in pigs is very variable, and very much dependent on management and facility sanitation. In Argentina, indoor reared pigs only had 4% seroprevalence, whereas outdoor reared sows were 100% positive (29).

It is generally accepted that the main source for human infection is eating infected pork or lamb. Since indoor farming started to be practiced in Europe, the prevalence in pigs and humans has dramatically decreased, with only a population of young women at risk, since most are seronegative up to child-bearing age (30).

A risk factor for acquiring *Toxoplasma* infection also seems to be pregnancy itself. Dramatic changes in the CD4/CD8 ratio and reduced functionality of macrophages and NK cells especially in the third trimester of gestation render a state of high susceptibility for infections such as *T. gondii* (31). In a recent multicenter study in Europe 62% of women seroconverted during pregnancy, with 18.5% of the children acquired congenital toxoplasmosis (32).

Vaccine research for farm animals has focused on the control of abortions in sheep and goats, and the only commercial vaccines available are live vaccines comprising the S48 strain of *T. gondii* (Ovilis®Toxovax, Intervet) (Toxovax, Agvax New Zealand) (33). This strain is peculiar, as it does no longer produce tissue cysts ,and it is regarded as a deficient strain, that can be safely used as a live vaccine for livestock. The product is registered for use in sheep, and reduces the risk of abortion due to *T. gondii* infection (33). It is applied either intramuscularly or subcutaneously no later than three weeks prior to mating. In a challenge study it was shown that the duration of immunity was of at least 18 months (59). This could indicate that the parasite deposited antigenic materials at remote sites, which are able to sustain the immunity. The S48 strain was attenuated by serial passages for over 30 yrs. Mutagenesis has also been used and has resulted in temperature-sensitive variants, which optimal proliferation temperature is 28-32 C. These strains (TS4 mutant) (34) could be used as a vaccines, but have not been developed commercially.

A bradyzoite-based vaccine was developed using a mutant T263, deficient in sexual replication. This product could be used as a vaccine to reduce oocyst production in cats. It needed storage in liquid nitrogen and was applied orally to cats by a straw.

Vaccines for humans probably will not be based on live attenuated parasites. Recombinant approaches could result in defined products of sustainable quality. In this respect, progress can be made along the lines of immunology and the functional role of proteins in the life cycle of the parasite.

The completion of the *Toxoplasma* genome, the availability of extensive EST databases, and further detailed studies of stage conversion mechanisms have elucidated different host-parasite interactions that could be of value for the future development of vaccines based on individual proteins, genes or combinations of these.

Some success is reported from latest studies using DNA-encoding granule-dense antigens in conjunction with SAG1 DNA-plasmids (35). Almost 90% reduction of mortality after a lethal challenge was achieved in mice, and a significant reduction of tissue cyst establishment was produced. Similarly, the use of SAG1 DNA-plasmids reduced cysts numbers, but no effect was detected on vertical transmission (36). The latter indicates that even few tissue cysts can be responsible for the generation of fetus infection.

Although such studies are performed in animal models, this is certainly encouraging for future development of vaccines for women during their reproductive phase of live. However, the progress mentioned in molecular systems driving stage conversion, such as the role of Hsp90, especially in the conversion of tissue cysts to tachyzoites, may also provide alternative ways to fight the disease. Also, drugs that could interfere in this process could be developed. The main challenge will be how to cross the blood-brain barrier (37).

NEOSPORA CANINUM VACCINES

Neospora caninum has been recognized as the most commonly diagnosed cause of abortion in cattle, after its discovery in 1984 (38). Its life cycle is very similar to that of *T. gondii,* with asexual multiplication through tachyzoites and bradyzoites in different mammals – though mainly ruminants – and sexual multiplication in canids, such as the dog. It was demonstrated that coyotes are part of a sylvatic cycle (39). Although extensively studied, there is no indication that human infections occur on any significant scale. Very low seroprevalence is detected in man and no relation to any pregnancy problems could be found (38).

Cattle do mount an effective immune response protecting the fetus from aborting, although repeated abortions may occur in 5% of the animals. Previously infected animals have a greater chance of abortion than seronegative animals due to recrudescence of existing infection during pregnancy, whereas exogenous infections from dog-spread oocysts are relatively rare (40). The efficiency of transplacental transmission is over 90%, which makes this disease hard to control, since no drugs are available against the acute phase, and certainly not the tissue cyst stage.

The negative effects of *N. caninum* infection are not only abortion, but also embryo mortality, reduction in milk production, higher culling rate, birth of calves with congenital abnormalities, and decreased growth rate (60). The consequences of *N. caninum* infection depend on the time of gestation in which the parasitemia occurs (44). Infection during late gestation seldom results in abortion, but in the birth of congenitally infected calves or calves with congenital abnormalities.

Control measures are focused on reducing the chance of infection during gestation and on preventing the vertical spread of infection by not breeding infected cows. Intensive systems of screening of serum or milk tank antibody levels have been developed to identify farms at risk, but these systems require guidance and are very costly.

Vaccination therefore seems to provide the best tool for long lasting control of neosporosis.

As with the use of live vaccines against *Toxoplasma*-induced abortions, it was also shown that experimental infection of naïve animals prior to pregnancy can reduce or even prevent abortion if challenged at 10 weeks of gestation (41), and can even reduce vertical transmission if animals were challenged around 130 days of gestation (42). However, no effect was seen on the recrudescing infection during mid-gestation in already infected animals (41).

This indicates that vaccination with live tachyzoites is feasible in seronegative animals. However, since no attenuated strain of *Neospora caninum* that would not induce a chronic infection is available, no such vaccine was developed. Some naturally low pathogenicity strains may be used for this purpose, as reported for the Nowra strain in Australia (43). Moreover, the main target is to develop a vaccine that prevents vertical transmission of the parasite in previously infected animals and not only in naïve animals.

Since the disease is associated to immunological changes that occur during gestation, the main focus of research has been on the immunological responses of both the cow and the fetus during the whole gestational period in relation to the effects needed to control an active or reactivated infection of *N. caninum*. Innes *et al* (44) reviewed the state of the art in this subject, and concluded that the reduction of

maternal T-cell mediated immunity or the tilting of the balance towards the Th-2 type of response reduces the ability of the mother to generate IFN-gamma, TNF-alpha and other typical responses of the anti-inflammatory repertoire, which clears the way for the parasite to proliferate more or less uncontrolled and pass to the foetus.

The timing of this passage during gestation determines the outcome of infection. Early infection leads to high innate IFN-g response, resulting in abortion or mummification. Infection during mid-gestation is accompanied by a reduction in maternal response and the outcome is mainly abortion. When the infection occurs late in gestation, the immune system of the fetus is already able to cope with the infection and a healthy but infected calf is born (44).

Congenital acquired infection leads to life-long infection as a postnatal infection would, but the immune status of the animal may differ according to the timing of infection acquisition. There may be a point during which tolerance converts into responsiveness.

In Bovine Viral Diarrhoea (BVD), disease-tolerant calves are known to carry BVD virus when infected before gestational day 120; these calves do not produce antibodies, but shed virus during their entire life (45).

The immunological studies have been mainly carried out in rodent models for the availability of different cytokines, antibodies, and knockout strains. These models confirm the consensus hypothesis that the key cytokines during pregnancy are IL-4 and IL-10, which modulate the response towards a Th-2 bias (46).

Cattle were vaccinated with killed tachyzoites, and this was found to be effective in reducing the chance of abortion. The commercial vaccine resulting from these studies (Bovilis® Neoguard or in USA called NeoGuard, Intervet) consists of killed tachyzoites with Havlogen adjuvant in an oil-in-water emulsion given subcutaneously to 1-3 months pregnant cattle (47). Romero *et al.* (48) applied this vaccine in farms in Costa-Rica and found a 50% reduction in the risk of abortion in vaccinated animals (total n=876). Most abortions occurred during 5-6 months of gestation, which is consistent with earlier observations. Heuer *et al.* (47) used the same vaccine in New Zealand, and demonstrated similar efficacy. Interestingly, Heuer was able to detect a vaccination effect in seropositive cattle as well as seronegative cattle, although sample size was too small to be significant. Killed tachyzoite preparations were also efficacious in reducing transplacental transmission in pregnant mouse studies (49), whereas no data are reported that this is also true for cattle.

Rodent studies showed that recombinant antigens can be effective either as proteinaceous vaccine for SRS2 (50), for combinations of SAG1, SRS2 and DG1 and DG2 (51) or as plasmid-DNA for SAG1 and SRS2 (52), or presented by vaccinia virus for SRS2 (53). The extrapolation of such studies to the problems in cattle needs to be further studied.

In conclusion, *N. caninum* is an important cause of abortion and congenital infection of cattle. Although a killed vaccine is available, which reduces the chance of abortion, prevention of transplacental infection is the final challenge. Such vaccine could derive from defined antigens, which should be tested in the final host. A vaccine for dogs has limitations, since other canids or stray dogs can also transmit the infection, and are sources which are hard to control.

SARCOCYSTIS NEURONA VACCINE FOR HORSES

Sarcocystis neurona is the causative agent of a neurological disease in horses known as EPM (equine protozoal myeloencephalitis), observed especially in the Americas. The definitive host for this coccidian parasite is the opossum. Horses acquire the disease from sporocysts spread by roaming opossums. Although until recently parasites were detected only in horses that were severely immunocompromised (54), Rossano *et al* (55) described the culture of viable merozoites from the blood of an immunocompetent horse artificially infected with sporocysts obtained from opossum. The incidence of clinical EPM in the USA is

rather low (<0.15%), but over 40% of horses are seropositive in areas where the opossum is prevalent (56). It is suggested that stress factors induced by transportation or heat can elicit the clinical phase of the disease, but that has not been proven yet. Cutler *et al.* (57) showed that dexamethasone-treated horses developed neurological symptoms when infected, but the parasite could not be detected as the unambiguous cause of the diseased state.

Notwithstanding the low chance of clinical disease, a vaccine has been conditionally launched in USA consisting of killed merogonic stages.

The efficacy is not documented since no challenge model is established. Field serology seems to be interfered by vaccination titers (58).

So, in conclusion, coccidial parasites are highly immunogenic and, due to their role in causing disease in animals and man, have been target for development of vaccines based on either live or killed parasites. Due to their diversity in species and hosts, vaccination not always the first method of choice, and new drugs are needed. However, there has been progress in the development of the current solutions into more sustainable products for the future. The increasing knowledge of molecular processes by unraveling of the genomic organization of the first three genera will pave the path for the development of more defined therapeutics and vaccines.

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Symposium
“Legislation on drug and vaccine use”

A US perspective on the current and future regulation of anticoccidial drugs and vaccines

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ABSTRACT

The US broiler industry is currently thriving, with more than 8.5 billion broilers produced per year. This success is partly due to the availability of multiple anticoccidial drugs, both chemicals and polyether ionophores, and anticoccidial vaccines. In the US, the Center for Veterinary Medicine (CVM), Food and Drug Administration, is responsible for approval of new anticoccidial drugs, while the United States Department of Agriculture (USDA) is responsible for approval of anticoccidial vaccines. The CVM anticoccidial drug approval process is quite rigorous, requiring demonstration of manufacturing capability, efficacy, target animal safety, human food safety, and environmental safety. The USDA approval process for anticoccidial vaccines, which differs somewhat from the CVM approval process, also requires demonstration of manufacturing capability, efficacy and safety. Although many anticoccidial drugs are currently available (and expected to remain available), few, if any, new drugs are under development, due to the high cost and length of the approval process. Only a few anticoccidial vaccines are currently available, but new vaccines are under development. The US and worldwide broiler industry should continue to thrive as long as currently used anticoccidial drugs are used prudently, anticoccidial vaccines are used when and where effective, and necrotic enteritis and other diseases that exacerbate coccidiosis are controlled.

INTRODUCTION

The broiler industry in the US is currently a thriving industry. US broiler producers currently produce more than of 8.5 billion broilers per year. The industry relies on various feed additives, including growth promotants used to enhance productivity and maintain the health status of the animals, therapeutic antibiotics used for disease treatment, and anticoccidials. Without these products, all of which are approved by FDA after extensive testing to demonstrate efficacy and safety, it would be impossible for the industry to maintain the quality of its products and to survive in its current form.

In order to understand the current and future of the industry in general, and anticoccidials in particular, it is important to have a historical perspective.

HISTORY OF THE US BROILER INDUSTRY AND ANTICOCCIDIALS

In his campaign for the presidency in 1928, Herbert Hoover used a campaign slogan "a chicken in every pot and a car in every garage". Hoover's intent was to promise affluence to the US public. However, his slogan gives an important perspective regarding the broiler industry. In 1928, only the affluent, or farmers who grew their own, could afford to eat chicken, which was considered a "high priced" protein source. Although numbers for broiler production aren't available for 1928, they are available beginning in 1940. In 1940, 143 million broilers were raised for consumption in the US. This number grew to 631 million in 1950, 1.8 billion in 1960, 3 billion in 1970, 4 billion in 1980, 5.8 billion in 1990 and 8.3 billion in 2000, and reached over 8.8 billion in 2004. During this time period, average live weight of broilers increased from 3 pounds to over 5 pounds, feed conversion decreased from 4 pounds of feed per pound of broiler to under 2 pounds, mortality decreased from 10% to 5%, and market age decreased from 12 to less than

7 weeks. [Note that the production data listed above was obtained from various United States Department of Agriculture (USDA) publications. For more information on the US poultry industry, see the USDA web site at *http://www.ams.usda.gov*.]

The tremendous improvements in the broiler industry can be attributed to many factors. Intensive breeding programs have resulted in highly improved genetics. Management practices include knowledge of nutrient requirements and improved feedstuffs. Antibiotic feed additives, especially those that control necrotic enteritis, allow for intensive rearing conditions with reduced mortality, while growth promotants improve feed efficiency and growth rate.

Of course, without anticoccidial drugs and vaccines, the growth of the US broiler industry described above would have been impossible, as coccidia are ubiquitous and extremely deleterious to the growth and survival of broilers. Fortunately, introduction of the sulfur/sulfonimide drugs in the 1930s started an era of treating and preventing coccidiosis. Since that time, the emphasis has been on prevention, with the introduction of the chemical anticoccidial drugs in the 1940s through 1970s, introduction of the first polyether ionophores in the 1970s, and the more recent introduction of anticoccidial vaccines (although vaccines have been used in broiler breeders since the 1950s, formulations for broiler production are relatively new). Growth of the US broiler industry demonstrates a high correlation with the discovery of new anticoccidials.

THE CURRENT STATUS OF ANTICOCCIDIALS AND ANTICOCCIDIAL REGULATION IN THE US

Anticoccidial drugs and vaccines are regulated by two US agencies. The Center for Veterinary Medicine (CVM), Food and Drug Administration (FDA), regulates anticoccidial drugs, while the United States Department of Agriculure (USDA) regulates anticoccidial vaccines. As such, they will be discussed separately, below.

ANTICOCCIDIAL DRUGS

The US Food, Drug and Cosmetic Act mandates that a new animal drug may not be sold in interstate commerce unless it is the subject of a New Animal Drug Application (NADA). In order to obtain an NADA, a drug product sponsor must demonstrate that the drug is safe and effective, and can be manufactured in a manner that preserves its identity, strength, purity and quality. The Center for Veterinary Medicine (CVM), Food and Drug Administration (FDA) is responsible for the review and approval of NADAs. See U.S. Code: Title 21-Food and Drugs Part 514 – New Animal Drug Applications at *http://www.access.gpo.gov/nara/cfr/waisidx_98/21cfr514_98.html* for more information on the NADA process.

Twenty anticoccidial drugs/drug combinations are currently codified (approved) for use in broilers in the US. Table 1 lists the approved US anticoccidial drugs, their approved withdrawal time, and their tissue tolerances/safe concentrations.

Table 1. Anticoccidial drugs approved for use in broilers in the US.[a]

Drug	Class[b]	Withdrawal time (days)	Tolerance or Safe Concentration[c]
Aklomide	C	0	4.5 ppm liver; 4.5 ppm muscle; 3.0 ppm skin/fat
Amprolium	C	0	1 ppm liver; 1 ppm kidney; 0.5 ppm muscle
Clopidol	C	0	15 ppm liver; 15 ppm kidney; 5 ppm muscle
Decoquinate	C	0	1 ppm muscle; 2 ppm other edible tissue
Diclazuril	C	0	3 ppm liver; 0.5 ppm muscle; 1 ppm skin/fat
Halofuginone Hydrobromide	C	4	0.16 ppm liver 0.3 ppm liver; 0.1 ppm muscle; 0.2 ppm skin/fat
Lasalocid	I	0	1.2 ppm skin/fat; 0.4 ppm liver
Maduramicin Ammonium	I	5	0.38 ppm fat; 0.24 ppm muscle; 0.72 liver; 0.48 skin; 0.48 fat
Monensin	I	0	Not required
Narasin	I	0	480 ppb abdominal fat
Nequinate	C	0	0.1 ppm edible tissues
Narasin/Nicarbazin	I/C	5	See Narasin and Nicarbazin
Nicarbazin	C	4	4 ppm muscle, liver, skin, kidney
Nitromide + Sulfanitran	C	5	0 ppm either compound in edible tissues
Robenidine Hydrochloride	C	5	0.2 ppm in skin/fat; 0.1 ppm other edible tissue
Salinomycin	I	0	No tolerance set
Semduramicin	I	0	400 ppb liver; 130 ppb muscle
Sulfadimethoxine + Ormetoprim	C	5	0.1 ppm either compound in edible tissues
Sulfaquinoxaline	C	10	0.1 ppm in edible tissues
Zoalene	C	0	6 ppm liver; 6 ppm kidney; 3 ppm muscle; 2 ppm fat

[a] Data from United States Code of Federal regulations, 21 CFR Parts 556 (tolerances) and 558 (approvals and withdrawal times), 2005. For further information, see *http://www.gpoaccess.gov/cfr/index.html*.

[b] C = chemical; I = polyether ionophore.

[c] US FDA sets tissue tolerances and/or safe concentrations, which may differ from European MRLs. Tolerance refers to a concentration of a marker residue in the target tissue selected to monitor for total residues of the drug in the target animal, and safe concentrations refers to the concentrations of total residues considered safe in edible tissues. Tolerances are shown in normal font, while safe concentrations are shown in *italics.* In cases where no safe concentration is listed separately, the tolerance is the safe concentration.

Although all of the drugs listed in Table 1 are approved, many of them are no longer marketed. *The US CVM approval process for animal health drugs used in food animal species (including anticoccidial drugs) is quite rigorous and comprehensive. Submissions for approval can be phased (each "technical section" submitted separately) or complete (all "technical sections" submitted simultaneously). For further information on phased submissions, see Guidance for Industry (GFI) #132 located on the CVM website at* http://www.fda.gov/cvm/Documents/dguide132.doc*; for further information on complete submissions, see GFI #41* http://www.fda.gov/cvm/Guidance/Guideline41.htm*). Whichever submission format is chosen, the following technical sections are required:*

- Chemistry, Manufacturing, and Controls
- Effectiveness
- Target Animal Safety
- Human Food Safety
- Environmental Impact
- Labeling
- Freedom of Information Summary
- "All other information"

Each of these sections will be discussed briefly.

The Chemistry, Manufacturing, and Controls (CMC) section should contain complete information regarding the manufacture of the new animal drug active ingredient and the new animal drug product. It should contain information on personnel, facilities, components and composition, manufacturing procedures, analytical specifications and methods, control procedures, stability, containers and closures, Good Manufacturing Practice (GMP) compliance, and other aspects of the chemistry and manufacturing processes. CVM has published multiple GFIs that describe different aspects of the CMC section process, including those listed in Table 2.

Table 2. CVM Guidance on Chemistry, Manufacturing and Controls.[a]

GFI #	Title	Date Published
5	Stability Guidelines	12/1990
42	Series of four guidelines entitled "Animal Drug Manufacturing Guidelines"	1994
43	Draft Guideline for Generic Animal Drug Products Containing Fermentation-Derived Drug Substances	10/1995
57	Master Files: Guidance for Industry for the Preparation and Submission of Veterinary Master Files	1995
63	Guidance for Industry: Validation of Analytical Procedures: Definition and Terminology	7/1999
64	Guidance for Industry: Validation of Analytical Procedures: Methodology: Final Guidance	7/1999
73	Guidance For industry: Stability Testing Of New Veterinary Drug Substances And Medicinal Products VICH GL3: FINAL GUIDANCE	9/1999
74	Guidance for Industry: Stability Testing of New Veterinary Dosage Forms VICH GL4: FINAL GUIDANCE	9/1999
75	Guidance For Industry: Stability Testing: Photostability Testing of New Veterinary Drug Substances and Medicinal Products: Final GUIDANCE	9/1999
91	Guidance for Industry: International Cooperation on Harmonization of Technical Requirements for Approval of Veterinary Medicinal products (VICH); Final Guidance on Stability Testing for Medicated Premixes (VICH GL8); Availability	3/2000
92	Guidance for Industry: Impurities In New Veterinary Drug Substances	7/1999
93	Guidance for Industry: Impurities In New Veterinary Medical Products	7/1999
100	Guidance for Industry: Impurities: Residual Solvents in New Veterinary Medicinal Products, Active Substances and Excipients: VICH GL18, Final Guidance	5/2001
135	Guidance for Industry: Validation of Analytical Procedures for Type C Medicated Feeds - Draft Guidance	4/2004
169	Guidance for Industry: Drug Substance: Chemistry, Manufacturing, and Controls Information, Draft Guidance	1/2004
176	Guidance for Industry #176 - Specifications: Test Procedures and Acceptance Criteria for New Veterinary Drug Substances and New Medicinal Products: Chemical Substances - VICH GL-39, Draft Guidance - May 24, 2005	5/2005

[a] All CVM GFI can be found on the CVM website at *http://www.fda.gov/cvm/guidance/published.htm*.

The **Effectiveness** section must contain full reports of all studies that show whether or not the new animal drug is effective for its intended use. CVM has published Guidance for Industry (GFI) that specifically outlines the studies necessary to demonstrate effectiveness of anticoccidial drugs. Specific studies described in this GFI include battery studies (with both single *Eimeria* species and mixed cultures), floor pen challenge studies, and field trials. For further information, see GFI #40, at *http://www.fda.gov/cvm/Guidance/dguide40.pdf*.

The **Target Animal Safety** technical section must contain full reports of all studies that show whether or not the new animal drug is safe to the target species. In addition, any target animal safety issues that become apparent in efficacy studies must be reported in this section. Target animal safety study design for poultry anticoccidial drugs is specifically mentioned in section IX of GFI # 33, located at *http://www.fda.gov/cvm/Guidance/Guideline33.htm*.

The **Human Food Safety** technical section is the most comprehensive technical section, and must contain information on residue toxicology, residue chemistry, residue analytical methods, pharmacokinetics and bioavailability. CVM has recently (June 2005) updated GFI #3, General Principles for Evaluating the Safety of Compounds Used in Food-Producing Animals, including references to the following Guidelines: Guidance for Metabolism Studies and for Selection of Residues for Toxicological Testing; Guidance for Toxicological Testing; Guidance for Establishing a Safe Concentration; Guidance for Approval of a Method of Analysis for Residues; Guidance for Establishing a Withdrawal Period; Guidance for New Animal Drugs and Food Additives Derived from a Fermentation; and, Guidance for the Human Food Safety Evaluation of Bound Residues Derived from Carcinogenic New Animal Drugs. In addition, CVM has adopted various guidelines (GL) developed by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) regarding safety studies for residues, including those listed in Table 3.

Table 3. VICH safety study guidelines adopted by CVM.[a]

CVM GFI# and Date	VICH GL #	Title
115 January 3, 2002	22	Safety Studies for Veterinary Drug Residues in Human Food: Reproduction Toxicity Testing
116 January 3, 2002	23	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Genotoxicity Testing
141 May 24, 2004	28	Guidance for Industry: Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Carcinogenicity Testing, VICH GL28, Final Guidance
144 April 27, 2004	27	Guidance for Industry: Pre-Approval Information for Registration of New Veterinary Medicinal Products for Food-Producing Animals with Respect to Antimicrobial Resistance - VICH GL27, Final Guidance
147 November 12, 2003	31	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Repeat-Dose (90-Day) Toxicity Testing
148 March 16, 2004	32	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Developmental Toxicity Testing
149 May 18, 2004	33	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Testing
159 February 10,2005	36	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: General Approach to Establish a Microbiological ADI
160 February 4, 2005	37	Studies to Evaluate the Safety of Residues of Veterinary Drugs in Human Food: Repeat-Dose (Chronic) Toxicity Testing

[a] These guidelines are available on the CVM *http://www.fda.gov/cvm/guidance/published.htm*.

It should be noted that, for anticoccidial compounds that have anti-infective properties (such as the polyether ionophores), CVM has recently begun requiring studies on microbiology, including the effects of residues on human intestinal microflora (GFI #159) and evaluation of the safety with regard to their microbiological effects on bacteria of human health concern antimicrobial resistance (GFI #152). GFI #152, which describes a "qualitative" approach to antibacterial resistance risk assessment, may be superceded by a more "quantitative" approach. Although no examples of a quantitative risk assessment are available for the polyether ionophores, CVM has published a draft risk assessment on virginiamycin, a product used in poultry to control necrotic enteritis and improve growth rate and feed efficiency. The CVM document (http://www.fda.gov/cvm/Documents/SREF_RA_FinalDraft.pdf) demonstrates the CVM approach to risk assessment (and also demonstrates that the continued use of virginiamycin poses no significant risk to human health).

The **Environmental Impact** section must, by regulation, contain either an environmental assessment (EA), or a request for categorical exclusion. A claim of categorical exclusion must include a statement of compliance with the categorical exclusion criteria and must state that to the sponsor's knowledge, no extraordinary circumstances exist. "Environmental Impact Considerations" and directions for preparing an EA can be found in 21 CFR Part 25. In addition, CVM has adopted 2 VICH environmental guidelines, GL6 [CVM GFI #89 Guidance for Industry - Environmental Impact Assessments (EIA's) For Veterinary Medicinal Products (VMP's) - Phase I, *http://www.fda.gov/cvm/Guidance/guide89.doc*] and GL38 [GFI #166 Draft Guidance for Industry - Environmental Impact Assessments (EIA's) for Veterinary Medicinal Products (VMP's) - Phase II, *http://www.fda.gov/cvm/Guidance/dguide166.doc*].

The **Labeling** section should include facsimile copies of container labels, package inserts and any other labeling that will be used with the products. For medicated feeds, copies of representative labeling for the Type B and Type C medicated feeds, referred to as "Blue Bird" labeling, should also be included. Facsimile labeling is nearly final labeling that adequately reproduces the package size (actual or to scale); graphics; pictures; type size, font, and color of text; and, the substance of the text to demonstrate to the reviewing Division that the final printed labeling will be in compliance with applicable regulations. Labeling should address any user safety concerns identified during the review process. CVM has not published final guidance on labeling,; however, labeling requirements are codified in 21 CFR Part 201 (*http://www.access.gpo.gov/nara/cfr/waisidx_98/21cfr201_98.html*).

The completed **Freedom of Information Summary (FOI) Summary** should include the specific language relevant to a technical section that was agreed upon during the review of the individual technical section (e.g., the tolerance and withdrawal time for a new animal drug intended for use in food-producing animals) and should be accepted by the Division responsible for the evaluation of the target animal safety technical section. For further information on FOI summaries, see GFI # 16 (FOI Summary Guideline, *http://www.fda.gov/cvm/Guidance/Guideline16.htm*).

The **All Other Information** section must include all other information, not included in any of the other technical sections, that is pertinent to an evaluation of the safety or effectiveness of the new animal drug for which approval is sought. All other information includes, but is not limited to, any information derived from other marketing (domestic or foreign) and favorable and unfavorable reports in the scientific literature.

It should be noted that, in the US, anticoccidial drugs cannot be used in combination with any other drugs until the combination is approved by CVM (see CVM Guidelines for Drug Combinations for Use in Animals, GFI # 24, at http://www.fda.gov/cvm/Guidance/Guideline24.htm.

For further information on the animal drug approval process in the US, visit the CVM website and associated links at http://www.fda.gov/cvm/nadaappr.htm.

ANTICOCCIDIAL VACCINES

The U.S. Department of Agriculture (USDA) is authorized, under the 1913 Virus-Serum-Toxin Act (see http://www.aphis.usda.gov/vs/cvb/vsta.htm) as amended by the 1985 Food Security Act, to ensure that all veterinary biologics produced in, or imported into, the United States are not worthless, contaminated, dangerous, or harmful. Federal law prohibits the shipment of veterinary biologics unless they are manufactured in compliance with regulations contained in Title 9 of the Code of Federal Regulations, Parts 101 to 118. Veterinary biologics for commercial use must be produced at a USDA-approved establishment, and be demonstrated to be pure, safe, potent, and efficacious.

Three anticoccidial vaccines (Table 4) are currently USDA approved for use in broilers in the US.

Table 4. Anticoccidial vaccines approved for use in broilers in the US.[a]

Vaccine	Type	*Eimeria* species	Delivery Method
Advent	Wild type live oocycts	*E. acervulina, E. mivati, E. maxima, E. tenella*	Spray cabinet or feed
Coccivac-B	Wild type live oocycts	*E. acervulina, E. maxima, E. tenella*	Spray cabinet or feed
Immucox	Wild type live oocycts	*E. acervulina, E. maxima, E. necatrix, E. tenella*	Water or gel

Note in the above table that all currently approved vaccines contain wild type live oocysts, but vary in the *Eimeria* species they contain and their delivery method.

The USDA vaccine approval process is outlined as follows (see *http://www.aphis.usda.gov/vs/cvb/lpd/lpdfaqs.htm*):

In order to manufacture and sell veterinary biologics in the US, domestic manufacturers are required to possess a valid U.S. Veterinary Biologics Establishment License and an individual U.S. Veterinary Biologics Product License for each product produced for sale.

Foreign manufacturers of veterinary biologics may export veterinary biologics to the United States, provided that the manufacturer's legal representative (permittee) residing in the United States possesses a valid U.S. Veterinary Biological Product Permit to import these products for general distribution and sale.

The following documentation must be submitted to USDA for a biologics establishment license:

- Application for United States Veterinary Biologics Establishment License
- Articles of Incorporation for applicant and any subsidiaries if a corporation
- Water quality statement
- An application for at least one United States Veterinary Biological Product License
- Personnel Qualifications Statement for supervisory personnel
- Blueprints, plot plans, and legends

The following documentation must be submitted to USDA for a biologics product license:

- Application for a United States Veterinary Biological Product License
- Outline of Production and, if applicable, Special Outlines
- Master Seed data, including test results for purity, safety, identity, and genetic characterization
- Master Cell Stock data, including test results for purity and identity
- Product safety data from laboratory animal and contained host animal studies
- Host animal immunogenicity/efficacy data
- Field safety data
- Labels for all containers, cartons, and enclosures (circulars)

Potency test validation data, including correlation with host animal efficacy 3 consecutively produced pre-license serials that have tested satisfactorily for purity, safety, and potency Guidelines (Veterinary Services Memorandums) on preparation and submission of the above information can be found at the USDA website, *http://www.aphis.usda.gov/vs/cvb/vsmemos.htm*.

In addition to the approved vaccines listed in Table 2, vaccines may be produced and used without USDA approval, provided that:

- The product was manufactured by veterinarians and intended solely for use with their clients' animals under a veterinarian-client-patient relationship.
- The product was manufactured by individuals or companies for use only in their own animals.
- The product was manufactured in States with USDA-approved veterinary biologics regulatory programs, for sale only in those States.

THE FUTURE OF ANTICOCCIDIALS AND ANTICOCCIDIAL REGULATION IN THE US

The Animal Health Institute (AHI) has recently published a news release entitled "Antibiotic Use in Animals Rises in 2004" (see *http://www.ahi.org/mediaCenter/documents/Antibioticuse2004.pdf*) that shows that ionophore/arsenical (grouped to abide by disclosure agreements) sales in the US increased from 8,644,638 pounds in 2003 to 9,444,107 pounds in 2004. At first glance, this appears to be good news for the animal health industry,; however, these data demonstrate increased reliance on existing compounds, as no new compounds have been approved in the past few years. In fact, the last new anticoccidial drug approval in the US occurred in 1999, when diclazuril was approved. Prior to diclazuril, semduramicin was approved in 1994. The approval of new anticoccidial drugs has come to a standstill due to the high cost and length of time it takes for approval with the current approval requirements: estimates are that it currently costs more than $25 million and at least 10 years to obtain CVM approval of a new anticoccidial drug (information based on AHI sponsor input). Industry information suggests that no new anticoccidial drugs are under development. The good news is that currently marketed anticoccidial drugs will remain on the market, as there is no indication that CVM has any concerns with these compounds.

If no new anticoccidial drugs will become available, what will happen to the US and worldwide broiler industry? It appears that new anticoccidial vaccine technology may fill some of the void. Dalloul and Lillehoj (2005) have recently published a paper describing functional genomic technology and recent advances in live and recombinant vaccine development that may result in broiler vaccination strategies that work better than current vaccines. Bedrnik (2004) reviews information on multiple anticoccidial vaccines that have been or are being developed worldwide. MacDougald et al. (2005) have recently patented vaccine for coccidiosis in chickens prepared from four attenuated *Eimeria* species: *E. acervulina, E. maxima, E. mitis* and *E. tenella* (see *http://www.uspto.gov/* patent number 6,908,620). Industry sources suggest that at least 4 new anticoccidial vaccines are currently under development for the US market.

However, anticoccidial vaccines are not the complete answer as they are not always effective and do nothing to control necrotic enteritis, and anticoccidial drugs must continue to be used. The broiler industry must use these products prudently to avoid over use and possible resistance issues. Shuttle programs must be adopted to "rest" popular anticoccidial drugs. Both chemical and polyether ionophore anticoccidial drugs should be used as seasonal conditions and coccidial challenge allow. Efforts should be made to control necrotic enteritis and other disease conditions that may exacerbate coccidiosis.

The US and worldwide broiler industry should continue to thrive as long as currently used anticoccidial drugs are used prudently, anticoccidial vaccines are used when and where effective, and necrotic enteritis and other diseases that exacerbate coccidiosis are controlled. The anticoccidial drugs are amongst the

most important tools that allow the broiler producer to produce healthy birds, because they are the safest and most effective method to control coccidiosis. In turn, the consumer benefits through low-priced, high-quality animal protein, produced using drugs that have been proven safe and effective in rigorous scientific studies.

REFERENCES

[Note: much of the information presented in this paper was gathered from official US government websites, which have been referenced throughout this document. Key websites used, and referenced publications, are listed below.]

Websites

United States Department of Agriculture, Agriculture Marketing Service at *http://www.ams.usda.gov*

United States Code of Federal regulations, 21 CFR Parts 556 (tolerances) and 558 (approvals and withdrawal times), 2005 at *http://www.gpoaccess.gov/cfr/index.html*

United States Food and Drug Administration, Center for Veterinary Medicine guidance documents at *http://www.fda.gov/cvm/guidance/published.htm*

United States Department of Agriculture, Center for Veterinary Biologics, Veterinary Services Memorandums (guidance documents) at *http://www.aphis.usda.gov/vs/cvb/vsmemos.htm*

United States Patent and Trademark Office at *http://www.uspto.gov/*

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Feed Safety: Evolution in Legislation, Implementation and Harmonisation

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As a result of several contaminations, especially in feed ingredients destined for the compound feed industry, various initiatives were undertaken. On the one hand authorities published new legislation with additional requirements. On the other hand the private sector invested a lot in a pro-active approach assuring feed ingredients at source being the most cost effective action for the feed chain as a whole.

First, the General Food Law was published. Following principles were established:

1. the responsibility of the producer
2. the obliged notification to the authorities concerning withdrawal and recall
3. traceability.

The legislation on feed additives also had to be revised thoroughly. The principle of the positive list is maintained. All additives on this positive list are divided into 5 categories. For each additive a procedure for approval has to be sublitted. In case of zootechnical additives (coccidiostatica included), the legislator states explicitly that there has to be a "Brand Specific Approval" (BSA). The state of the art for the coccodiostatica : a transitional period is ongoing.

The regulation on Feed Hygiene, will come into force on January1st of 2006. All actors worldwide in the feed/food chain, will at least be obliged to be registered. All actors of the feed/food chain will have to implement a self-control system which has the HACCP-principles built in (except for the primary production). In addition, certain minimum conditions concerning the obliged notification and the traceability, have to be fulfilled. The necessity for financial guarantees is still under examination.

The private sector accelerated investing in a pro-active feed chain approach from the year 2000 on. In several member countries (the Netherlands, the UK, Belgium, Germany,…) projects were started, with the intention to write out and implement a quality system for control on food safety in the whole feed chain. Most of these projects were guided by the feed industry under severe pressure of the retailers. All actors of the feed chain (feed ingredient producers, traders, premix manufacturers, feed manufacturers, transport,…). Each EU-member country anticipated on the political and social bottlenecks. The standards (codes of good practices) were elaborated and introduced for accreditation per individual member country at the level of the national accreditation boards. Independent certification bodies could certify the individual companies and therefore sustain the credibility of the quality system .

These individual, national initiatives resulted in the fact that the importers all over the world were confronted with different quality systems applied by the different countries. The lack of communication was apparent . Furthermore, even the countries with a quality system, approved by an accreditation body, had problems with the export of their certified commodities/services.

Several member countries took the initiative to strive for bilateral agreements between quality systems. These latter were compared through well developed comparison tables and were put through a benchmarking procedure. That way, the biggest bottlenecks for the incoming and outgoing fluxes with

the neighbouring countries were eliminated. Still, the suppliers from countries spread all over the world kept complaining about the several quality-requirements : they were still confronted with different quality systems and -demands.

Four countries, Belgium, Germany, the Netherlands and the UK, already involved in bilateral agreements, decided to establish an international platform with the main purpose of harmonising the quality systems worldwide. Therefore, a legal entity was created, IFSA (International feed Safety Alliance). IFSA wrote a completely new standard (IFIS), meant in the first place for the processed feed ingredients . The standard contains four important chapters: Quality management System, resources and good hygienic practices, transport and product safety management. This horizontal standard is a convenient heading to classify several "sector notes" under. The purpose of these sector notes is to lay the emphasis on some sector specific requirements. The risk analysis, which is sector specific, is translated into the registration of the risks and the determination and implementation of the necessary monitoring.

Of course, beside the standard, some other certification rules need to be figured out. The rules of certification contain:

- The sort of certification (ISO guide 65: EN45011)
- The job description for the auditors
- The necessary training for the auditors
- The checklists
- The audit conditions
- The fees that go with the auditing and the fees for tha maintenance of the quality system.

Like already mentioned IFSA's standard (IFIS) covers in the first place aims the processed feed ingredients. This is the scope to start with. The producers of these processed feed ingredients were closely involved with the development of the standard and especially the sector notes. It is expected that all suppliers worldwide are certified against the IFIS-standard towards 01/01/2007.

Other stakeholders have requested the extension of the IFSA initiative to activities such as trade, transport, additives, premixes, …IFSA is working on the evaluation and implementation of these extensions.

Finally, in several member countries, there was a fuss about the notion of 'zero-tolerance'. Methods of analysis made tremendous progress which resulted in LOD (Limits of Detection) at ppb-level. LOD's of less than 5 ppb's are very common now. Production processes are not developed to provide a zero-cross-contamination. Food Safety Agencies, during their monitoring program, detected residues of additives and medicines in meat, milk, eggs as non-target product. This means, that for instance coccidiostatica used in the feed for target animals, can end up in the feed for non-target animals, because of the dragging (cross contamination), in spite of all necessary control measurements (flushing) within the quality system of the feed manufacturers.

The compound feed industry developed the necessary methods to measure the cross contamination in the compound feed production process. Only few countries in the EU, have developed methods: Belgium, the Netherlands, France en Germany. Apart from the cross contamination, the compound feed manufacturer has to know the legal norms to be respected at feed level. These norms have to guarantee that the norms on finished products (meat, milk and eggs) are not exceeded . So they postulate guarantees that the national health won't be put in danger. Therefore, threshold levels were developed based on scientific research (ADI as basis and if possible transfer studies (carry over) from feed to food, the ALARA principle and benchmark procedures on the ADI). This proposition has been transmitted to the European Commission and the EFSA.

Current and futures perspectives on the regulation of anticoccidial drugs and vaccines.

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Coccidiosis is one of the most relevant diseases concerning economic losses it causes on livestock production. The world losses attributable to avian coccidiosis were estimated in 1,5 billion dollar per year. In Brazil, a 30 million dollar damage per year was assigned to this disease. Many of those figures are based on grants spent with anticoccidials. In this respect, what about the future of anticoccidial drugs in EU?

According to the Regulation (EC) nº 1831/2003 of the European Parliament and of the Council on additives for use in animal nutrition, coccidiostats and histomonostats are feed additives (articles 5 & 6). Thus, their placement on the market and use within the Community and in other countries that export to EU must follow the conditions laid down in this Regulation. Regulation articles 10 & 11 state *"... with a view on the phasing out of the use of coccidiostats and histomonostats as feed additives by 31 December 2012, the Commission shall submit to the European Parliament and to the Council before 1 January 2008 a report on the use of these substances as feed additives and available alternatives, accompanied, where appropriate, by legislative proposals"*. As far as it is of our concern, no decisions have been taken on the coccidiostats yet. They require prescriptions in Sweden and it seems they are pushing for the rest of Europe to go down this route. However, taking into account the economic importance of coccidiosis, the most likely outcome by 2012 will be the transition of anticoccidials to a new veterinary product status; perhaps they will be classified by medicine legislation when reviewed. Anyway, the impact on both pharmaceutical companies and livestock production should be small as the requirements for feed additives of this type are now very similar to the medicines. It should not be forgotten, however, that vaccines against coccidiosis are becoming more and more sophisticated and also more consistent. For instance, it was recently observed that interleukins, such as INF-**γ**, IL-2 and IL-15, strengthened the antigenic effects of recombinant DNA vaccines. Thus, although anticoccidial use might be kept in Europe for the control of coccidiosis, the establishment of vaccines in this scenario should increase, becoming more relevant worldwide.

Symposium "*Histomonas meleagridis*: Update on Life Cycle, Control and Diagnostics"

Histomonas meleagridis: life cycle and epidemiology

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H. meleagridis is a member of the class *Sarcomastigophora*. This protozoal parasite of poultry bears characteristics both of both amoeba (*Sarcodina*) and flagellates (*Mastigophora*). *H. meleagridis* is a pleomorph organism, as morphology is adapted to the location in the final or intermediate host.

When present in caecal cavities or in cultures, one or two flagellae can be found. This form is also able to form pseudopodes, which are used for feeding with bacteria, cell debris and other small particles. Their size is more or less 10 to 20 µm. Reproduction is through simple binary division.

When present in tissues such as the caecal wall or the liver, only the intercellular amoebal form will be found. In the intermediate host *Heterakis gallinarum* another, smaller form is found. Fresh *Histomonas* is inactivated within hours when outside its final or intermediate hosts. Unlike *Eimeria* species for example, no cyst, that allows survival longer than approximately one day, is formed. The inability of *Histomonas*, whenever not protected by the eggs of the intermediate host *Heterakis gallinarum*, to pass the pH barrier of the stomach emphasizes the importance of *Heterakis* in the life-cycle of *Histomonas*.

Only when a turkey sits on the caecal dropping within hours after discharge, and cloacal drinking allows the re-introduction into a new bird's caeca, direct transmission can be successful.

Histomonas meleagridis is an obligatory parasite. When introduced in the caeca of its host, either by transmission through the intermediate host or directly through cloacal drinking, they start dividing in the lumen.

When *Heterakis* is present in the caecum, the adult worms can again ingest the histomonads. An active invasion of the ovum causes the inclusion in the intermediate host..

After loosing its flagellum the histomonads invade the caecal wall, using pseudopodia. In this phase, the invasive forms release proteolytic enzymes, which aid in phagocytosis of partly digested host tissue. The histomonads are transported towards the liver and other organs, most probably via the portal vein system.

Pathogenicity largely depends on whether the histomonads reach the liver or not. This depends more on host factors than on strain factors, although pathogenicity differences between strains are evident. The host factors include species, breed, strain, age, intestinal flora and concurrent infections such as coccidiosis.

The diagnostics of *Histomonas meleagridis*

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Histomoniasis is a parasitic disease mainly affecting Galliformes, most prominently turkeys (*Meleagris gallopavo*). Its causative agent is the protozoan *Histomonas meleagridis*, which may induce necrotic foci in liver and necrotic typhlitis. In severe cases other organs may also be affected. A presumptive diagnosis can be made relatively easy based on macroscopic lesions, especially when subacute to chronic liver lesions characterized by a circular or oval depressed area of necrosis of about one centimeter in diameter occur in combination with inflammation, ulceration and cecal necrosis. Because of the variable aspect of lesions, especially in early stages and/or severe infestations, confusion with other diseases is possible. Therefore, diagnosis should be confirmed by histopathology. Routine staining techniques such as H&E easily lead to identification of the tissue form of *H. meleagridis*. Individual or clustered histomonads may appear as round or ovoid eosinophilic bodies laying in lacunae at the periphery of lesions. Other stains like periodic acid-Shiff's and Grocott's stain may be useful to differentiate histomonads from fungi. Fluorescent labeled antibody techniques can be used to demonstrate *H. meleagridis* in tissue samples and culture. As a confirmation test histomonads may also be cultured in special media such as Dwyer's medium and modifications. A requirement for the culture is that samples of cecal contents are gathered from readily sacrificed birds before cooling. The medium, which consists of 85% medium 199 with Hank's salts, 10% heat-inactivated horse serum, 5% chicken embryo extract and 10 to 12 mg of rice powder per 12.5 ml medium, is inoculated and incubated at 40°C. Histomonads may also be demonstrated in cecal fluid and scrapings using a phase contrast microscope. Applying a warm stage will enable observation of typical 'rocking' movement of histomonads. More recently, a number of molecular techniques have further expanded above mentioned list of diagnostic possibilities. Several PCR techniques have been mentioned including nested and quantitative PCR. In all cases, primers were designed based on the nucleotide sequence of small subunit ribosomal RNAs. PCR techniques have been successful at detecting *H. meleagridis* in organs as well as faeces samples. Finally, *in situ* hybridization for *H. meleagridis* has been described. This technique, regarded as complementary to the histological analysis, enables detection of specific RNA sequences and at the same time histomorphological analysis of tissue samples.

Histomoniasis, an ancient disease threatening the European poultry production. What are the current and future options for control ?

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Histomoniasis is a disease with a (re)-increasing importance. The disease is caused by *Histomonas meleagridis*, a protozoan parasite that can infect different poultry-species.

Research had ceased more or less some thirty years ago, due to the fact that the disease was very well controlled (both preventative and curative) with products now banned.

Histomoniasis is jeopardising the liveability of the European turkey-production. At present in Europe, no products, either preventative nor therapeutic, to control *Histomonas meleagridis* in turkeys are available.

But also in breeders and in layers in newer, so called sustainable, housing systems, histomoniasis is a re-emerging disease for some years now in the EU.

There are some indications that in the USA and other parts of the world, the incidence and severity of the disease is increasing in breeders.

It is noteworthy that histomoniasis has become a huge problem not only because of the direct economic damage, but also animal welfare is seriously at stake.

Since the real overall impact of this disease remains hard to measure, there surely is a need for improved diagnosis tools. A lot of possible 'alternative' control means have been introduced, but until now without consistent satisfactory results. Hence the gap which apparently can be filled only with registered pharmaceutical products.

At least one pharmaceutical company is working on the registration of a FDA-approved product, while the possibility of vaccines is seriously considered by various research-groups. Several companies are believed to screen 'older' molecules for anti-histomoniasis activity.

Enteric Flagellated Protozoa in Turkeys

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Several types of protozoa are associated with enteric disease of turkeys. Protozoal enteritis can present with general signs, including dehydration, loss of appetite (off-feed), loose droppings and watery intestinal contents. Flagellated protozoa include Cochlosoma, Tetratrichomonas, Histomonas and Hexamita. Coccidia Cryptosporidia and *Eimeria* are non-flagellated protozoa of turkeys. Cochlosoma and Hexamita have recently been associated with enteritis, primarily in young turkeys, especially in the summer months. There are field reports of co-infections with Cochlosoma and Tetratrichomonas, or Cochlosoma and Hexamita, or flagellated protozoa and *Eimeria*.

Cochlosoma anatis is an enteric flagellate protozoan. It is about the same size as the turkey's red blood cell. Microscopically, it is will have a rapid, spiral movement. Cochlosoma organisms are found in the villi of the jejunum (upper small intestine), and attaches to the mucosa by means of a sucker-apparatus. C. anatis is associated with significant production losses in turkeys.

Hexamita meleagridis, now called Spironucleus meleagridis, is another enteric protozoa. Hexamita is smaller than a red blood cell. It has a characteristic torpedo, fast, straight movement and has a narrow, elongate appearance on the microscope slide. Hexamita is found in the crypts or lumen of the upper small intestine (duodenum, jejunum, and ileum).

Tetratrichomonas gallinarum, another common flagellate protozoa of turkeys, is larger than a red blood cell. Microscopically, Tetratrichomonas has a jerky motion and is found in the ceca. Trichomonads have an undulating membrane and are plump, pyriform in shape. Unless found in high numbers, Tetratrichomonas, commonly referred to as "Trich", probably does not cause clinical disease.

Blackhead (Histomonas) is caused by Histomonas meleagridis, a flagellated protozoan. The bird ingests embryonated ova of cecal worms (Heterakis gallinarum) that contain Histomonas.

Protozoan infections can be diagnosed in the laboratory from fresh intestinal mucosa scrapings; each organism has a distinctive microscopic appearance. Recent research has reported the negative effects Cochlosoma has on bird performance. Biosecurity and sanitation are essential steps in controlling and preventing flagellated protozoan infections. Chlortetracycline, oxytetracycline and tetracycline have FDA-approved label claims for the control of hexamitiasis. In the USA, both nitarsone and roxarsone have been used successfully in the field and research settings to control enteritis caused by flagellated protozoan.

Contributed Papers: Oral Presentations

GUIDELINES FOR EVALUATING THE EFFICACY AND SAFETY OF LIVE ANTICOCCIDIAL VACCINES, AND OBTAINING APPROVAL FOR THEIR USE IN CHICKENS AND TURKEYS

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Guidelines have recently been published in Avian Pathology that are intended as an aid in the design, implementation and interpretation of laboratory, floor-pen and field studies for the assessment of the efficacy and safety of live anticoccidial vaccines for immunisation of chickens and turkeys against *Eimeria* species. In addition to efficacy and safety requirements, manufacture, quality control, and licensing considerations are discussed. The guidelines do not address sub-unit vaccines, but many of the principles described will be relevant to such vaccines if they are developed in the future. Guidelines are available in some countries for avian vaccines of bacterial or viral origin, but specific standards for anticoccidial vaccines in poultry have not, as far as we know, been produced. Information is provided on general requirements of registration authorities (based upon regulations applicable in the European Union and the USA) for obtaining marketing authorisations for vaccines. These guidelines may assist poultry specialists in providing specific information for administrators involved in the decision-making process leading to registration of new vaccines, and are intended to facilitate the worldwide adoption of consistent, standard procedures.

SELECTION OF A PRECOCIOUS LINE OF THE RABBIT COCCIDIUM *EIMERIA PIRIFORMIS* AND CHARACTERIZATION OF THE LIFE CYCLE OF BOTH THE PARENT STRAIN AND THE PRECOCIOUS LINE

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The principal method used to attenuate coccidian strains is selection for early development of oocysts (precociousness). In rabbit coccidia, the precocious lines (PL) have already been obtained from five species (*Eimeria coecicola*, *E. flavescens*, *E. intestinalis*, *E. magna* and *E. media*), but not from *E. piriformis*. The aim of this work was to select PL of *E. piriformis* and to characterize endogenous development of both of original strain (OS) and PL. SPF rabbits were inoculated with OS oocysts and the first newly developed oocysts recovered from the intestine were used for the infection of other rabbits. The prepatent period (PP) was shortened after 12 passages from 194 to 170 hours and remained stable after 5 passages without any selection pressure. Histology and transmission electron microscopy were used to characterize the endogenous development and oocyst morphology of PL and OS. PL oocysts exhibited peculiar morphology. Besides refractile bodies (RB) within sporozoites, one huge RB merged with a residual body was present inside each sporocyst. The parasite developed in the proximal colon and in lesser extent in other parts of the large intestine. All stages were seen in the epithelium of crypts. In OS, four asexual generations preceded gamogony and, like in other rabbit coccidia, two types of meronts were observed: meronts of the type A that develop in polynucleate merozoites in which endomerogony takes place, and meronts of the type B that form uninucleate merozoites. The endogenous development of PL was identical with that of OS except the last merogony which was absent. This apparently resulted in earlier appearance of gamogony and shortening of PP. These observations of the life cycle of *E. piriformis* substantially correct its descriptions made 50-60 years ago, which have not been verified until recently.

This work was performed in cooperation with BIOPHARM, Research Institute of Biopharmacy and Veterinary Drugs, Czech Republic.

LONG TERM VIABILITY OF CRYOPRESERVED *EIMERIA* SPOROCYSTS

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As *Eimeria* oocysts have limited viability and cannot be effectively cryopreserved, sporocysts from individual *Eimeria* strains are the desired life stage for cryopreservation. However, little data are available in the literature indicating the effects of long-term frozen storage on the viability of different *Eimeria* strains. Our laboratory has used standard sporocyst preparation and cryopreservation techniques on several *Eimeria* strains including *E. acervulina, E. maxima*, and *E. tenella.* After freezing, sporocysts were held in the vapor phase of liquid nitrogen for long-term storage prior to testing. To determine viability of the frozen stock cultures, sporocysts were thawed, washed one time, and administered by oral gavage to broiler chickens at one of two doses. Fecal material from inoculated birds was collected during the appropriate patent period and oocyst output per bird determined. All species tested were viable after three years and oocyst output trended with sporocyst dosage used for gavage. Oocyst output data from sporocysts stored for three months in the vapor phase of liquid nitrogen were comparable to oocyst output from stocks frozen for three years. These data indicate frozen sporocyst stocks from *Eimeria acervulina*, *E. tenella*, and *E. maxima* can be maintained with acceptable viability for at least three years.

NON-INTERFERENCE OF INOVOCOX™, A LIVE COCCIDIAL OOCYST VACCINE, WITH MAREK'S DISEASE VACCINE OR BURSAPLEX®, A LIVE BURSAL DISEASE VACCINE, AFTER *IN OVO* ADMINISTRATION

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Studies were conducted to determine the compatibility of Inovocox™, a live coccidiosis vaccine, with other *in ovo* vaccines. Safety and efficacy of Inovocox and a commercial live Marek's Disease (MD) HVT-SB1 vaccine or Bursaplex®, a commercial live infectious bursal disease (IBD) vaccine, was determined after co-administration *in ovo*. Inovocox consists of four strains of coccidia including *Eimeria acervulina, E. tenella,* and two strains of *E. maxima.* For each study, *in ovo* treatments included sham-vaccination with diluent, Inovocox alone, MD or IBD vaccine alone, or Inovocox combined with MD or IBD vaccine. Each vaccine preparation was administered in 100 ul of Marek's diluent at 18 days of embryonation to broiler or SPF eggs. To test Inovocox efficacy, broilers were placed in floor pens and challenged with mixed *Eimeria* strains at 21 days of age. Efficacy was assessed by weight gain and intestinal lesion scores at six days post challenge. Viral vaccine efficacy was tested in SPF birds in isolation units. Groups receiving MD or IBD viral challenge were monitored for challenge-related mortality. Birds were also necropsied for relevant gross lesions at 49 days post challenge for MD and 3 days post challenge for IBD. There were no differences in percent hatch and no treatment related mortalities in any treatment group. Weight gain and lesion scores in the Inovocox vaccinated treatment groups demonstrated significant protection when Inovocox was administered alone or in combination with MD vaccine or Bursaplex. Both the MD and Bursaplex vaccines demonstrated significant protection when administered with Inovocox or alone. Together, these results demonstrate that co-administration of Inovocox with either MDV or Bursaplex is safe and does not interfere with vaccine efficacy.

UNIFORMITY OF INFECTION AFTER INOVOCOX™ DELIVERY AT E18 USING THE INOVOJECT® SYSTEM

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This study investigated the uniformity of infection among individual chicks vaccinated at 18 days of embryonation with a controlled dose of Inovocox™. Inovocox is a live oocyst vaccine consisting of four strains of coccidia including *Eimeria acervulina*, *E. tenella*, and two strains of *E. maxima*. A 50 ul dose of Inovocox in Marek's diluent was administered to Cobb x Cobb broiler embryos using the Inovoject® system. Percent hatch of vaccinates was normal. At hatch, 60 *in ovo* vaccinated chicks were placed in individual cages facilitating fecal collection from individual birds. Fecal collections were performed over days 4-9. Oocyst presence and relative oocyst sizes in feces were determined using Whitlock flotation chambers. The percentage of birds demonstrating infection was 90% with representative sizes of all vaccine strains present. These data indicate that *in ovo* administration of a live oocyst vaccine to broiler embryos results in a consistent, uniform infection during the first week of life when administered via this specific injection system.

IN OVO AND DIETARY MODULATION OF HOST INTESTINAL IMMUNITY AND ITS SUBSEQUENT EFFECTS ON COCCIDIOSIS

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Various agents of known influence on the mucosal immune system were evaluated as immunomodulators in poultry in response to coccidial infections. These included vitamin A, probiotics, and synthetic CpG oligodeoxynucleotides (ODNs). Vitamin A deficiency impaired the gut immune defenses of broiler chickens, which are best characterized by alteration in intraepithelial lymphocyte (IEL) subpopulations, mainly $CD4^+$ T cells, resulting in increased susceptibility to coccidiosis. Conversely, supplementing broiler diets with a *Lactobacillus*-based probiotic stimulated the local immune system and improved resistance to *Eimeria*, as shown in a 4-fold reduction in oocyst shedding. Further investigation on the probiotic effects demonstrated that probiotic-fed chickens shed fewer oocysts than chickens without probiotic even in vitamin A-deficient birds, thus confirming improved resistance to coccidiosis. Studies with other immunoprotective agents involved *in vitro, in vivo,* and *in ovo* testing of a number of CpG ODNs. Several CpG ODNs including D19 and 2006 induced strong IL-6 and nitric oxide secretion by HD11 cells. Intracellular killing of *Salmonella enteritidis* was also increased in ODN 2006-activated HD11 cells. Also, *in vivo* CpG treatment with ODN 2006 enhanced birds' resistance to coccidiosis in a normally susceptible chicken strain (TK), as shown by reduced oocyst shedding and improved weight gain during *E. acervulina* infection. Further, *in ovo* vaccination with CpG ODNs and an *Eimeria* recombinant microneme protein (MIC2), alone or in combination, showed positive effects on susceptibility to coccidiosis. *In ovo* injection of ODNs D19 and 2006 alone reduced oocyst shedding, but did not affect weight gain during *E. acervulina* infection. When co-administered with MIC2, both ODNs reduced oocyst shedding; however, only ODN D19 plus MIC2 consistently improved weight gain. Also, vaccinating with ODN 2006 or MIC2 protein curtailed oocyst shedding, but did not enhance weight gain in *E. tenella*-infected birds. Collectively, these agents demonstrated immunoenhancing and adjuvant effects during *Eimeria* infections. However, regulation of immune responses is extremely complex and complete knowledge of how the immune system responds to *Eimeria* infections requires more research.

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EFFECTS OF DIETARY SHORT CHAIN FATTY ACIDS ON EXPERIMENTALLY INDUCED COCCIDIOSIS AND NECROTIC ENTERITIS IN BROILERS VACCINATED AGAINST COCCIDIOSIS.

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Introduction. In the poultry industry, necrotic enteritis (NE) manifests itself when a large *Clostridium perfrigens* (CP) colonization coincides with significant damages to the gut epithelium, often caused by coccidia. Since antibiotics and coccidiostats are to be banned in the European Union next year, short chain fatty acids (SCFA) could replace them.

Objective. The aim of the present work was to verify the onset of experimentally induced NE in vaccinated chickens (Paracox-5, sprayed in the hatchery), either fed SCFA for 21 days only or avilamycin for 42 days.

Birds were orally challenged on day 21 with CP ($5x10^5$ CFU/ml) and *Eimeria acervulina*, maxima and tenella (10^4; 10^3 and $5x10^3$ oocysts, respectively). The groups (40 chickens each) were: 1) control; 2) avilamycin, 10 ppm; 3) butyric triglyceride (BT), 0.2%; 4) BT, 0.2% + caprylic and capric triglycerides, 0.15% and 5) BT, 0.2% + free caprylic and capric acids, 0.1%.

From day 28 until day 42, three birds *per* group were euthanised each week and their intestines examined for coccidia, CP, NE lesions, and number of oocysts for gram of faeces (OPG).

Main findings. All the examined birds presented NE lesions after *Eimeria* spp. and CP. The lesion score was higher in the first two weeks following infection, and no mortality was recorded. The most severe lesions were associated with a high degree of sub-epithelial/interstitial coccidian colonization. *Clostridia*, when largely present, were associated with necrotic material or into severely damaged crypts. OPG resulted highest with avilamycin at day 28.

Conclusions. Although vaccinated, all the birds developed gut coccidian lesions and NE. Avilamycin was linked with a high number of coccidian OPG. The birds fed diet 4 had better live performances.

THE EFFECT OF LIGHT INTENSITY DURING BROODING ON THE PRODUCTIVITY AND HEALTH OF PARACOX-5 VACCINATED BROILERS

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Producers often try to increase bird activity during brooding to stimulate water and feed intake, increase growth, reduce early mortality, and improve subsequent flock uniformity.

Some experimental studies show that in young chickens, high intensity light (>70 lux sqm.) significantly increases activity as compared to low lux regimens (<20 lux sqm). To further assess the effects of lighting, a trial was performed in Paracox-5 vaccinated chickens.

Broiler chicks (Ross 508, 20,000 males and 20,000 females) were placed in one of two experimental houses. From placement until 4 days of age, both houses received 23 light hours and 1 dark hour. Lighting in one house was 75 lux and, in the other, 20 lux. After 4 days of age and until slaughter, light intensity for both groups was maintained at 20 lux.

Birds were evaluated at 0, 4, and 7 days of life, and then weekly, until 49 days of life. Statistical analysis was conducted as a one- way ANOVA and, when significant, ($p<0.05$) means were compared by Duncan's Multiple Range Test.

Crop palpation to evaluate food and water intake showed that 8 hours after most birds were placed, 98.5% of those on 75 lux were filled compared to 83.0% on 20 lux. Early growth rate, liveability and flock uniformity were increased by higher light intensity, while overall mortality and the incidence of intestine bacterial overgrowth were significantly reduced. There were fewer deaths due to necrotic enteritis in the 75 lux group during the first 4 days of life. Both groups demonstrated standard oocyst shedding, but peaked on day 19 in the 75 lux group as compared to day 27 in the 20 lux group. No coccidiosis outbreaks occurred in either group.

This study provides compelling evidence that high intensity light regimens enhance activity in chicks, which in turn increases feed intake and early growth. Early complete feed consumption appears to enhance endogenous trypsin release, protein digestion, and vaccinal oocyst excystation, which may explain their earlier multiplication and peak shedding. Light manipulation during brooding also appears to minimize factors such as litter eating and dehydration that predispose to intestinal bacteria overgrowth, possibly necrotic enteritis and mortality. These results should be confirmed in additional trials before advising producers to manipulate light intensity.

EFFICACY OF GEL SPRAY AS A DELIVERY SYSTEM FOR TURKEY COCCIDIOSIS VACCINE CONSISTING OF *EIMERIA ADENOEIDES* AND *E. MELEAGRIMITES*

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The first coccidiosis vaccine launched in the market, based on the theory of uniform exposure (Lee, 1986; Shirley & Long, 1990), was a carrageenan-based vaccine, which objective is to increase water viscosity and to maintain an even suspension of oocysts. This procedure was later adopted to produce a commercial product in the form of edible gel placed in the chick tray (Dasgupta & Lee, 2000). The idea of uniformity was also tested in the spray cabinet (Danforth, 1997) as water spray. This became popular because of its convenience. However, oocyst distribution before and after vaccination remains an issue. There is no problem in uniformity during stirring before spraying, but uniformity after spraying and before ingestion may be questionable. Here we report a gel spray delivery system using a gel diluent that may solve this problem.

Experiment 1: A turkey coccidiosis vaccine, containing *Eimeria meleagrimitis* and *E. adenoeides,* was administered to poults by one of three routes: gel spray, edible gel, or drinking water. Positive- and negative- control groups were included in the study. Poults were challenged with a high dose of turkey coccidia on day 15. Edible gel vaccine delivery provided the best protection , as measured byweight gain, against the challenge with turkey coccidia as compared to drinkingwater or spray delivery.. However, all three delivery routes significantly ($p<0.03$) prevented weight reduction as compared to non-vaccinated and challenged controls. When efficacy was measured as lesion score, vaccine delivered by gel spray or in drinking water produced better results than edible gel. We concluded that gel spray is a more efficient anti-coccidial vaccine delivery route, as measured by weight gain and lesion score, then drinking water or edible gel..

Experiment 2: Poults, which were vaccinated with the two *Eimeria* species vaccine and were challenged, gained more weight than non-vaccinated and challenged poults This weight gain was statistically significant ($p<0.0093$ for *E. ad* challenge, and $p=0.0380$ for *E.me* challenge). Vaccinated and challenged poults had statistically significant lower ($p<0.001$) lesion scores as compared to non-vaccinated and challenged

poults. We again concluded that poult vaccination by gel spray prevents weight gain reduction and also decreases morbidity as compared to unprotected and challenged controls.

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COMPARISON OF PERFORMANCE OF ROSS BROILER BREEDER MALES TO FULL, HALF-DOSE AND 10X DOSE OF COCCIVAC®-D DAY-OF-AGE VACCINATION

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Coccivac®-D is commonly administered to commercial broiler breeder replacement pullet and cockerel flocks at a half-dose of the manufacturer's recommended dosage in the theory that the lower dosage results in improved flock livability and uniformity compared to full dose administration. This commercial practice of using the half-dose of Coccivac®-D is most prevalent in flocks of Ross broiler breeder males. The objective of this study was to determine if there are differences in the Ross 344 male broiler breeder in its response to varying doses of Coccivac-D day-of-age vaccination. Environmental conditions including floor, feeder and water space, temperature, lighting, vaccination and feeding programs were designed to replicate typical commercial broiler breeder production in the USA industry. Treatments were compared by 21 day gross coccidiosis scores, 28 and 70 day body weights, uniformity and feed conversion and 70 day livability results. The 21 day gross coccidiosis lesion scores resulted in significantly (P>.05) lower scores in the 10X dose treatment compared to the half-dose plus Amprol treatment. Body weights and the individual bird weight uniformity results were similar and showed no differences between treatments at 28 and 70 days. There was also no significant difference between treatments in 70 day livability results. This study concluded that there were no measurable performance improvements gained by administering a half-dose of Coccivac®-D to Ross 344 broiler breeder males compared to the manufacturer's recommended dosage. The investigation shows that multiple doses (10X) of the vaccine resulted in less 21 day coccidiosis lesions compared to the half-dose application. These results may indicate that the lower dosage practice may inhibit rapid and uniform flock coccidiosis immunity that could lead to rolling reactions and delayed flock immunity.

SAFETY AND EFFICACY OF ADVENT® COCCIDIOSIS VACCINE

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This paper describes a series of experiments to determine the safety and efficacy of a new coccidiosis vaccine. ADVENT® Coccidiosis Control contains sporulated viable oocysts from the three commercially relevant species in broilers, *E. acervulina* (strain VND-A10), *E. maxima* (strain VND-M27), and *E. tenella* (strain VND-T49). The strains have been selected to have the robust protection necessary for today's broilers without including any unnecessary species, they were also selected for broad immunogenicity and undiminished oocyst yield. A unique methodology has been developed that inactivates extraneous agents without the need to use any hazardous substances such as potassium dichromate which is used as preservative in other coccidiosis vaccines. Ability of the sterilent and the processing methodology to inactivate viruses was evaluated using two viruses; chicken anemia virus (CAV) and infectious bursal disease virus (IBDV), selected for their stability, resistance to disinfectants, and importance to the poultry industry. It was demonstrated that the sterilent and procedures used were sufficient in eliminating at least 4-5 $\log_{10}$ of IBDV and CAV. It was proven that the procedures also inactivate extraneous bacterial contaminants such as *Salmonella spp*. Extensive screening for anticoccidial drugs resistance confirmed sensitivity of the strains to ionophores and chemical drugs. An *in vitro* potency method – the VIACYST® Assay -- was used to determine the viability of sporocysts of each of the three *Eimeria* strains. Studies confirm that the *in vitro* viability assay correlates to the ability to confer resistance to a coccidiosis challenge and permits accurate and consistent vaccine formulation. Different studies confirmed the safety of the minimum immunization dose for each species as well as a dose approximately 10 times that for each species administered ether via spray cabinet or spray on the feed. Oral immunization with ADVENT results in resistance to significant challenges of oocysts: at least 300,000 viable sporulated oocysts of *E. acervulina*, and 40,000 viable sporulated oocysts of *E. maxima* or *E. tenella*. Doses resulting in immunity have been evaluated both on the basis of lesion score reduction and post challenge broiler performance. The special sanitation and purification process along with the exclusive viability assay used in the formulation of the ADVENT vaccine results in coccidiosis control that is consistent from lot to lot and vial to vial. The resulting vaccine provides protection without excess immune stimulation and is a consistent and pure source of contemporary coccidial oocysts.

PERFORMANCE OF BROILERS USING A LIVE VACCINE AGAINST AVIAN COCCIDIOSIS

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Avian Coccidiosis is a parasitic disease which affects the digestive tract in birds, causing considerably high economic losses. Anticoccidial drugs, such as chemicals and ionophores, are administered to prevent it, but there is a risk of resistance development by the different Emeria species, and residues are likely to be present in poultry meat for human consumption, in addition to many other disadvantages.

Vaccine prophylaxis is an alternative method to control this disease.

Farm trials were carried out in Argentina on a 500,000 broilers population vaccinated on their first day of age with Inmuner® Gel-Coc, by a specially designed massive application method called "Lluvia – Gel". This vaccine aims at the prevention of Avian Coccidiosis in broilers and is formulated with live-attenuated oocysts of *E. acervulina, E. brunetti, E. maxima,* and *E. tenella*.

The most important productive parameters were analysed from an economic point of view and compared to objective parameters, which were compared with the average performance indexes obtained during a production year without using vaccines.

At the same time, successive comparative trials focused on production were performed. Performance rates reached in broilers flocks vaccinated with Inmuner® Gel-Coc on the first day of age were compared to the ones obtained in medicated flocks which had received ionophores or chemical anticoccidial drugs through the meal.

Performance indexes measured were flock average weight, feed intake, daily weight gain, feed conversion ratio, and mortality. Each of them was weekly analyzed.

Simultaneously, systematic and periodic coccidiological monitoring was carried out in each of these flocks, as well as and histological studies in order to evaluate possible anatomic-physiological development and/or anatomic-pathological changes caused by the replication of different species of *Eimeria* in the intestine.

Production data obtained in each of these trials showed a better performance of the vaccinated groups as compared to the medicated ones.

Therefore, we conclude that this vaccine against Avian Coccidiosis does not interfere with the most important production parameters from an economic point of view. Moreover, the fact that no Coccidiosis clinical signs were found in the vaccinated flocks demonstrate the protective effect of the vaccine and its lack of harmful effects.

EFFICACY OF COCCIVAC AGAINST FIELD ISOLATES OF CHICKEN *EIMERIA*

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This report includes a series of tests to compare the antigenicity of coccidia isolated from recent field samples against those in Coccivac. Based on the data, the antigens in Coccivac provided good protection against field isolates.

The objective of the study was to determine the incidence of field strains of *Eimeria* that show differences in antigenic properties from those antigens in the Coccivac vaccine. Field isolates collected from 60 broiler and breeder pullet farms across the USA. The chickens (n=70/group) were inoculated *per os* with one dose of Coccivac-B or Coccivac-D during the first week of life. Inoculated birds were placed into floor pens on clean wood shavings, then grown to approximately 35 days of age. Following immunization, birds were tagged and placed into assigned treatment groups. Attempts were not made to purify the inocula, but to standardize the *E. maxima* level to 100,000 sporulated oocysts per bird. Birds from the immunized group and their un-immunized hatch-mates were challenged with inocula of field isolates. The un-immunized hatch-mates served as the positive controls. Birds kept in the challenge phase between 144 to 156 hr, the following processes were followed: euthanasia, necropsy and intestines examined grossly for coccidial lesions using a 0 to 4 scoring system. Mucosal scrapings taken from the duodenal loop, jejunum, ileum and ceca were then placed on microscope slides for microscopic evaluations. Microscopic evaluation (scored 0 to 4) was made using a compound light microscope. Severity of infection (0 = no parasite, 1 = 1 to 10 per field, 2 = 11 to 20 per field, 3 = 21 to 40 per field and 4 = > 50 per field for *E. maxima*).

Birds immunized with Coccivac and challenged with field isolates demonstrated substantial immunity as determined by the level of parasitism in the immunized birds vs the non-immunized groups for *E. acervulina, E. tenella* and *E. maxima.* The average level of protection provided by Coccivac against the three predominant species of field isolates of *Eimeria (E. acervulina, E. maxima* and *E. tenella*) were: 97%, 86% and 91%, respectively.

GROWOUT PERFORMANCE FOLLOWING VACCINATION WITH INOVOCOX™ AS COMPARED TO SALINOMYCIN

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Floor pen studies were conducted to assess the performance of broilers after *in ovo* vaccination with Inovocox™, a live oocyst vaccine containing *Eimeria acervulina*, *E. tenella,* and two strains of *E. maxima*. Broiler eggs were vaccinated with Inovocox *in ovo* or sham injected with diluent. Chicks were vent sexed and 25 males and 25 females placed in floor pens at 0.75 sq. ft. per bird. Vaccinated chicks were fed a diet without anticoccidials throughout the study. Non-vaccinated (sham injected) control chicks were fed the identical diet containing 66 ppm salinomycin through 35 days of age, and non-medicated finisher diet thereafter. In one study chicks were placed on clean litter, in the other on built up litter. Body weight and adjusted feed conversion were compared at 21, 35, 42, and 49 days of age. On clean litter at 49 days of age, salinomycin bird weights averaged 2.396 kg, compared with 2.403 kg for the vaccinated birds. Feed conversion was equivalent at all ages. On built up litter at 49 days of age, salinomycin birds weighed 2.241 kg, compared to 2.252 kg for the vaccinated birds. Again there was no difference in feed conversion at any age tested. Birds vaccinated with Inovocox demonstrated equivalent performance to birds fed salinomycin.

ANTICOCCIDIAL SENSITIVITY PROFILE OF COCCIDIAL STRAINS IN INOVOCOX™

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These studies were conducted to determine the anticoccidial sensitivity profiles of the vaccine strains comprising Inovocox™. This live vaccine is delivered *in ovo* to late stage embryos, and is composed of *Eimeria acervulina*, *E. tenella*, and two strains of *E. maxima.* Two studies were conducted. Broiler chicks were placed in cages and fed unmedicated diet until 12 days of age. Two cages were then assigned to each treatment group as follows: Non-medicated non-challenged, non-medicated challenged, salinomycin (SAL), monensin (MON), nicarbazin (NIC), or diclazuril (DIC). After two days on the medicated diets, chicks were challenged with oocysts. Intestinal lesions and body weight gain were assessed 6 days later. *E. acervulina* and *E. tenella* were sensitive to all anticoccidials tested, as demonstrated by both lesion scores and body weight gain. Mild lesions were evident in some birds medicated with DIC. This anticoccidial primarily affects the sexual development of *E. maxima* after the point of lesion development, so mild lesions are not unexpected in drug sensitive strains. Improved weight gain in the birds on DIC treatment indicated sensitivity of both *E. maxima* strains. The chicks medicated with SAL, MON, or NIC and challenged with either of the two *E. maxima* strains had no significant lesions. Both *E. maxima* strains were sensitive to all of the anticoccidials as determined by body weight gain. These studies demonstrate that all four vaccine strains comprising Inovocox are sensitive to the most commonly used anticoccidials.

MATERNAL PROTECTION AGAINST *EIMERIA* CHALLENGE OF COXABIC® VACCINATED CHICKENS

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CoxAbic®, the subunit vaccine was tested for its ability to protect young broiler chicks by way of Maternal Immunity. Broiler breeder pullets were twice vaccinated with the vaccine during rearing. Eggs collected from these and from unvaccinated hens were hatched and groups of the offspring chicks were raised on litter floor cages. On day 8 after hatch, a simulated natural exposure was applied by infecting orally 1 chick from every 13 chicks with 50 sporulated oocysts each of four species of coccidia – *E. maxima, acervulina, tenella* and *mitis.* Groups of chicks were kept on wire floored cages unexposed to coccidia and served as negative controls. On day 35 groups of the chicks were orally challenged with one of the four *Eimeria* species (80,000 oocysts of *E. acervulina*, 25,000 *maxima*, 120,000 *mitis* and 25,000 *tenella*). Oocysts were counted in the litter (OPG) after the exposure of the seeder chicks and after challenge. Body weights were recorded before and after challenge. OPG indicated that the simulated natural exposure to coccidia resulted in infection of the birds through cycling parasites. Chicks from vaccinated and unvaccinated dams became actively immune by way of the exposure as elicited by their resistance to the challenge at 35 days of age in comparison to the negative control chickens. The difference between the two groups was expressed in their body weights. There was a consistent gap of approximately 25% in favor of the CoxAbic ® chicks, which was recorded after the early exposure through rearing until the end of the trial at 55 days of age.

The trial showed that maternal immunity protects broiler chicks from the effects of early exposure to coccidian parasites. This early natural exposure to the ubiquitous parasite is the way that chicks become actively immune to coccidiosis, but in birds without the protection of maternal antibodies (or of coccidiostats in their feed), this early exposure results in a significant and economically important weight reduction, from which CoxAbic® chicks are spared.

USE OF AVIAN IMMUNOGLOBULINS IN THE CONTROL OF COCCIDIOSIS

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Coccidiosis research should be directed towards the control of the parasites through immunological methods, including the generation of new vaccines or other methods with special emphasis in avoiding detrimental lesions, in diminishing time to develop an effective immune response as well as in avoiding the drug-resistance problem derived from chemotherapy. Transfer of humoral immunity from hyperimmunized hens to susceptible chicken may be an attractive alternative. The aim of the present research was to demonstrate the usefulness of avian immunoglobulins in the control of coccidiosis.

MATERIAL AND METHODS: Two ml of specific liquid immunoglobulins against *Eimeria* spp. obtained from egg yolks of hyperimmunized SPF hens were administered by oral route to thirty chickens (2 groups of 15 each). A second group of thirty birds received the immunoglobulins in a dry form at a dose of 100g/T of feed, and a control group of thirty birds received food without any anticoccidial drug plus placebo. The treatments were administered during two weeks before challenge with 150,000 oocysts of *E acervulina* and *E maxima* by oral route. The treatments continued for one more week and the birds were sacrificed and submitted to necropsy. Weight gain, feed conversion, oocyst count in the gut, and lesion scores were determined. Birds were housed under controlled conditions along the whole assay.

RESULTS: The weight gain during the last week was of 273 and 220 g for the group which received immunoglobulins in the feed, whereas the group of liquid immunoglobulins had 281 and 310 g and the control group 193 g. Feed conversion was of 1.82 and 1.85 for the birds that received liquid treatment as compared with 1.66 and 1.74 in the feed plus immunoglobulins group. The control group showed feed conversion of 1.98. The average lesion score was 1.67 and 1.87 in the liquid treatment group, 2.06 and 2.13 in the feed plus immunoglobulin group and 2.8 in the control group. In both treated groups, a 126,000 oocyst count was recorded as compared to 192,000 oocysts in the control group.

DISCUSSION The liquid treatment group showed the best performance in weight gain as compared to feed plus immunoglobulin and control groups. Lesion scores of the liquid treatment and the feed plus immunoglobulin groups were lower as compared with the control group. A significant reduction (66%) in oocyst production was observed in the treated groups as compared to the control group. The specific antibodies against coccidia provided effective protection decreasing intestinal damage and oocyst production. Antibody therapy may be an innocuous tool in the control of coccidiosis in birds.

ACQUISITION OF IMMUNITY TO *EIMERIA MAXIMA* IN NEWLY HATCHED CHICKENS GIVEN 100 OOCYSTS.

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The acquisition of immunity to *E. maxima* by chicks infected 18 hr post-hatch with a single dose of 100 oocysts was investigated. In the first experiment, birds were moved each day to clean cages in order to prevent the possibility of secondary infection resulting from ingestion of oocysts passed in their feces. Immunity was measured at 4 wk of age by calculation of oocyst production following challenge with 500 oocysts or weight gain following challenge with 100,000 oocysts. Large numbers of oocysts were produced by infected birds following challenge although numbers were significantly less than those from birds that had been reared in the absence of infection (susceptible controls). The weight gain of infected birds following challenge was significantly greater than that of susceptible controls but less than that of unchallenged controls. Thus only partial protection had been acquired, whether parasite replication or bodyweight gain was used to assess the extent of immunity development. In a second experiment, acquisition of immunity at 4 wk by chicks infected 18 hr post-hatch with 100 oocysts of *E. maxima* and reared in floor-pens in contact with their droppings was investigated. Infected birds produced no oocysts following challenge and weight gains were not significantly different from the unchallenged controls indicating that full immunity had developed by 4 wk. It is concluded that if oocysts of *Eimeria* species are used to vaccinate day-old chicks, re-infection by oocysts present in the litter is necessary for the establishment of protective immunity.

INOVOCOX™ ONSET OF IMMUNITY AFTER IN OVO DELIVERY TO BROILERS

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Broiler eggs were vaccinated *in ovo* at 18 days of embryonation with Inovocox™, a coccidiosis vaccine consisting of live sporulated oocysts of *E. acervulina*, *E. tenella* and two strains of *E. maxima*. Vaccine safety was demonstrated by comparable percent hatch for vaccinates and non-vaccinated controls. At hatch, chicks were vent-sexed and male chicks were selected. Birds were placed in 5 floorpens per treatment with 45 birds per pen. To assess efficacy, a subset of birds (5 per pen; 25 per treatment group) were weighed and orally challenged with mixed *Eimeria sp* (homologous strains) on days 14, 21/22, 28, and 35. Six days post challenge, birds were individually weighed and lesions evaluated in the upper, mid, and cecal regions of the intestines. Results indicated a progression in development of immunity over time. Following challenge, statistical improvement in weight gain and some decrease in lesion formation was observed as early as day 14. By day 21/22, full immunity was achieved with improved weight gain and lower lesion scores for all *Eimeria* species demonstrated in vaccinated birds when compared with non-vaccinated birds. These data establish that *in ovo* vaccination with live oocysts is safe and efficacious in broiler chickens.

IMMUNE REACTIONS TO DIFFERENT DOSES OF *EIMERIA ACERVULINA* IN DAY-OLD BROILERS.

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T-cell responses are the dominant immune reaction in broilers infected with *Eimeria.* Different factors that may influence the kind of T-cell responses to an *Eimeria* infection are e.g.: species of *Eimeria,* age of the host, amount of parasite, infection history. In young chicks, the intestine is still developing. This process means not only an increase in length, but also that lymphocyte population in the gut develops and differentiates. In chicks infected at young age, the immune response may be different in quality as compared to the response in chicks in which the gut is fully developed. We investigated the (T-cell) immune responses of young broilers to a primary *E. acervulina* infection in relation to the number of parasites used for infection.

In our experiment, we infected day-old broilers with a low (500 oocysts) and a high (50.000 oocysts) dose of *E.acervulina*. We used a species-specific real-time PCR to quantify total amount of parasites in the intestine as the number of oocysts in faeces may not be representative for the exposure of the gut immune system. We characterized T-cell subsets in the duodenum by means of fluorescence antibody cell sorting (FACS), performed lymphocyte proliferation assays with spleen lymphocytes with *E. acervulina* antigens, and characterized the mRNA profiles of different cytokines (TGF-beta2, 4, IFN-gamma, IL2, 6, 8 and 18) in the duodenum by means of real-time PCR.

From day 5 p.i., broilers infected with a high dose of *E.acervulina* had significantly lower body weight than the control group. There was no increase in $CD4^+$ T-cells, but a strong increase in $CD8^+$ T-cells in the duodenum at day 7 and 9 p.i. was observed in broilers infected with a high dose *E. acervulina* at one day of age. Strong IL8 and IL18 mRNA responses were observed after infection with both doses, but no IFN-gamma and TGF-beta mRNA responses were found in the duodenum. The specific proliferative T-cell responses to a low infectious dose were not significantly different as compared to the control group. In conclusion, based on the kinetics of observed phenomena, a primary infection with a high dose of *E. acervulina* in day-old broilers seems to generate an immune response that shows a peak at the time of oocyst excretion, whereas the immune response to a low dose is less explicit.

HAPPY MAPPING – A TOOL FOR GENOME FINISHING

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The majority of small genome sequences, including those of most parasites, are assembled using a shotgun strategy. However, shotgun methods alone are scarcely ever capable of producing a complete and finished genome sequence: cloning biases, sequencing problems and repetitive regions leave many gaps and potential mis-assemblies. For these reasons, a genome that has only been shotgunned will be left in a large number of un-connected contigs.

A collection of contigs is sufficient for finding many of the genes in the organism, but is inadequate for other purposes. No reliable estimate of the degree of completeness of the sequence can be made, and many genes lying at (or beyond) contig ends will have been missed or mis-predicted. It becomes impossible to comprehensively catalogue the organism's genes, or to infer the absence of a specific orthologue simply from its absence in the contig set. Nor can any conclusions about genome organisation or long-range synteny be drawn. In short, a shotgun project tends to produce a pile of unbound pages rather than a genomic atlas.

Genome finishing (ordering and joining contigs to produce a complete sequence) is therefore one of the most challenging but important aspects of sequencing. It is usually frustrating because the resources used for finishing – additional sequencing templates and larger-insert clones – are essentially similar to those used in the shotgun, and tend to have the same limitations.

HAPPY mapping is rapid *in vitro* technique that can be used to make extremely accurate maps, which allow shotgun contigs to be located precisely in the genome. Once this has been done, it becomes far simpler to close the remaining sequence gaps. Even where gaps cannot be closed (for example, because a short region is unsequenceable), the map provides a framework within which the sizes and locations of the gaps are known.

HAPPY mapping works by the direct analysis of genomic DNA, and does not involve cloning or the creation of any prior resources. Its freedom from 'biological' steps means that it is applicable to any genome, and is not adversely influenced by 'difficult' sequences, repetitive regions or other peculiarities of the genome. The cost and time required to make an accurate HAPPY map of a genome is normally a fraction of that expended on shotgun sequencing, and enables rapid completion of the genome sequence.

The presentation will give details of HAPPY mapping, and of how it has been applied successfully to a range of genomes including *Dictyostelium*, *Cryptosporidium*, *Eimeria* and others.

SURVEY OF *EIMERIA* SPP. TRANSCRIPTS USING OPEN READING FRAME ESTS (ORESTES)

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EST sequencing of different developmental stages is a simple approach to unravel the transcriptome of a parasite. ORESTES (ORF ESTs) is a cDNA synthesis method involving low-stringency RT-PCR using arbitrary primers. Since this technique is based upon a high number of amplification cycles, even rare transcripts can be amplified. In addition, the cDNAs are biased towards the central part of the mRNAs, which represents the most informative region of the transcripts. In order to derive data complementary to the currently available *Eimeria tenella* sequences, we utilized ORESTES to generate cDNAs of different developmental stages of *E. tenella*, including sporozoites, second-generation merozoites, and unsporulated, sporulating and sporulated oocysts. In addition, for comparative purposes, we have also generated ORESTES reads for two additional and relevant species of chicken *Eimeria*: *E. acervulina* and *E. maxima*. All cDNA clones were sequenced in a 96-capillary DNA sequencer (ABI-3700) and submitted to an automated sequence processing pipeline (EGene system - Durham *et al. – Bioinformatics* **21**: 2812-3, 2005). We have generated a total of 14,025 reads of *E. tenella*, 16,151 reads of *E. acervulina* and 13,667 reads of *E. maxima.* Clustering was performed using CAP3 assembly program. A total of 3,755, 3,310, and 3,051 clusters were obtained for *E. tenella*, *E. acervulina* and *E. maxima*, respectively. The *E. tenella* ORESTES reads were also clustered with ESTs already available for *E. tenella*, totaling 42,288 sequences. A total of 7,296 clusters (3,196 contigs and 4,100 singlets) were obtained, a number which is possibly not far from reflecting the transcriptome size of the parasite. EST sequencing of different developmental stages would help to clarify this point. In order to investigate differential gene expression, cluster sequences of distinct developmental stages were submitted to similarity searches against one another using BlastN. Preliminary results show that only a small fraction of the transcripts of any stage are shared by other stages. This finding suggests that any stage expresses only a small set of transcripts that is switched to another small set upon differentiation. We intend to validate this hypothesis through an extensive SAGE analysis of different developmental stages of *E. tenella*.

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MOLECULAR CHARACTERIZATION AND COMPARATIVE ANALYSIS OF MITOCHONDRIAL GENOMES OF *EIMERIA* SPP.

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The mitochondrial genome of Apicomplexa has been characterized in *Plasmodium* spp. and *Theileria* spp. In the former genus, it corresponds to a linear molecule composed by 15-150 tandemly repeated copies of a 6 kb element, comprising three cytochrome genes (*cyt*b, *cox*I and *cox*III) and some stretches of rRNA genes. In order to characterize the mitochondrial genomes of *Eimeria* spp., our group has determined the complete mtDNA sequences of the seven *Eimeria* species that infect the domestic fowl, plus two species of domestic rabbit: *E. coecicola* and *E. flavescens*. In addition, the mitochondrial genomes of five distinct strains of *E. tenella* were also sequenced. All mtDNA sequences showed a size of circa 6 kb and contained the cytochrome genes reported for other apicomplexan parasites. A multiple sequence alignment, using sequences derived from the nine *Eimeria* species, revealed a cross-similarity of around 90%. A comparison of the mitochondrial genomes of *Eimeria* spp. and other apicomplexan parasites showed a conservation of the gene order and orientation among different species, but not across distinct genera. These "shuffling" events could be related to the replication mechanism of these genomes, primarily based on recombination events and rolling circle activity. Another interesting finding was the high AT content, of circa 65%, with a highly conserved codon usage, strongly biased towards the use of A/T bases at the third position. Regarding intra-specific variability, the five *E. tenella* strains revealed a high conservation, with only two consecutive thymines being deleted in strains MC (Brazilian) and Wisconsin (North American), when compared to strains H, TA and Wey (isolated in the UK). This high level of conservation has also been observed in mtDNAs of *P. falciparum* strains, and may reflect a very recent common origin of the strains. A phylogenetic reconstruction of the different *Eimeria* species, using both mitochondrial nucleotide sequences and 18S rRNA, showed a good agreement between these markers. Using cytochrome b sequences in a phylogenetic analysis of Apicomplexa, the Coccidia, Piroplasmida and Haemosporida classes were clearly grouped into distinct clades, as should be expected according to their taxonomy.

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MOLECULAR AND PHYLOGENETIC FEATURES OF TWO DISTINCT DSRNA VIRUS FAMILIES INFECTING *EIMERIA* SPP. OF DOMESTIC FOWL

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Virus-like particles (VLPs) classified in the Totiviridae family have been described in distinct protozoan hosts. Here we report the complete genome sequencing of five distinct viruses of *E. brunetti*, *E. maxima*, *E. mitis* and *E. praecox*. A comparative analysis permitted to classify them into two groups: Group 1, composed by Eb-RV1, Ep-RV1 and Emt-RV1, presenting a genome size of 5.3-6.2 kb; and group 2, composed by Ep-RV2 and Em-RV1, with a genome size of 4.2-4.3 kb. Cesium chloride gradients of parasite lysates were performed and fractions containing dsRNA bands were analyzed by electron microscopy (EM). Group 1 revealed icosaedric particles similar to the morphology of Totiviridae. Despite experimental evidences that Group 2 dsRNA genomes are encapsidated, we were not able to evidentiate viral particles by EM. Finally, 0.7 kb dsRNA bands were also observed in co-infection with two distinct Group 1 *Eimeria* viruses. These small elements are encapsidated and may correspond to satellite viruses. Similarity searches of Group 1 sequences showed significant hits to Totiviridade, whereas Group 2 sequences presented only a small similarity block to an unclassified virus of *Zygosaccharomyces bailii*. No similarity was observed between viruses of groups 1 and 2. Thus, Group 2 viruses may represent a novel family of protozoan viruses. Phylogenetic inference of Group 1 viruses confirmed that these viruses constitute a monophyletic group and are more closely related to fungal viruses than to other protozoan viruses. These results may suggest that a progenitor of these viruses might have been a non-infectious virus of a cell type that predated the differentiation of protozoa and fungi. However, the finding of two distinct and non-related virus groups, infecting the same protozoan host cells, may provide evidence that more than one single progenitor may have originated the viruses currently found in *Eimeria*.

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LONG SAGE (SERIAL ANALYSIS OF GENE EXPRESSION) IN *EIMERIA TENELLA* – A PRELIMINARY STUDY

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Eimeria tenella is one of the most relevant causing agent of poultry coccidiosis. Since this species is amenable to laboratory manipulation, including *in vitro* cell culture and transfection, it became the model species for coccidiosis research. Genome and EST sequencing projects are underway in several laboratories throughout the world, including ours. Identification of the complete set of transcripts expressed in a genome is essential for genome annotation and gene function studies. Another central topic to better understand the biology of the parasite is the transcriptome expression level and, most important, the differential gene expression. In order to address this point, we decided to perform serial analysis of gene expression (SAGE) on second-generation merozoites and sporozoites, both representing invasive developmental stages of the parasite. In this work, we employed the long SAGE method, a modified protocol that generates 21-bp tags, thus allowing a better tag mapping than the conventional SAGE. We have obtained so far a total of 8,096 tags (3,457 unique tags) from a merozoite-derived library. Analysis of individual tag abundance showed that 2,464 tags (30%) occurred only once, 425 tags occurred 2 times (10%), 206 tags 3 times (8%), 322 tags repeat from 4 to 20 times (30%), 29 tags from 21 to 36 times (10%) and 11 tags from 38 to 186 times (12%). These results suggest that a very small number of genes is highly expressed. In another preliminary analysis, the 3,457 unique tags were mapped onto the *E. tenella* genome sequence. From this set, 46.6% (1,612 tags) did not present any hit on the genome. A frequency distribution of the unique tags presenting genome hits revealed that 85% (1,561 tags) mapped to a single site, 12% (218 tags) to two sites, whereas 3% (66 tags) mapped to multiple sites. We intend to map the tags on EST clusters in order to improve the corresponding gene assignment. We are now constructing other SAGE libraries from sporozoite and merozoite mRNAs, whose tags will be incorporated into the study. Statistical modeling and analysis will be used to evaluate the differential gene expression.

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DNA MICROARRAY GENE EXPRESSION ANALYSES IN ATTENUATED AND VIRULENT *E. TENELLA*

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Eimeria tenella is one of the most pathogenic coccidian species of domestic poultry. Interactions of this parasite with the host cells and/or host immune system are predominantly mediated through membrane structures or secreted proteins. Abundant proteins synthesised during critical periods of the infection process could be potential vaccine candidates or important factors of pathogenicity. We have used microarrays, comprising 2,700 long oligos designed from the currently available EST/ORESTES dataset, to study differential transcriptional activity in a subset of the developmental stages of *E. tenella*. Several developmentally regulated genes were identified. Interestingly, virulent and attenuated *E. tenella* (strain M 98 and the embryo-adapted line CH-E-A, respectively) appeared to exhibit differing profiles of gene expression in some developmental stages. Changes in gene expression induced upon interaction with epithelial cells (MDBK cells or primary chicken kidney cells) were also analysed. The differential transcriptional activities of the selected developmentally regulated candidate genes in both the virulent and attenuated strains were confirmed using Northern blot analyses. We are thus confident the resources we have developed provide a useful tool for the study of differential gene expression in *Eimeria*.

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PROGRESS IN THE DEVELOPMENT OF STABLE TRANSFECTION IN *EIMERIA TENELLA*

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Many practical difficulties have to be overcome to develop transfection techniques for *Eimeria tenella* because these parasites do not invade and develop in vitro with sufficiently high efficiencies to allow the screening for or propagation of transfected parasites. Recently we have made significant progress in (1) the efficiency of electroporation and transient transfection of *E. tenella*; (2) the development of more reliable methods for infecting chickens with electroporated sporozoites; and (3) the administration of drugs to establish an effective selection barrier in vivo.

In addition, we have for the first time expressed successfully at a high level the fluorescent marker protein YFP within *E. tenella*. These advances mean that we can now dose chickens with significant numbers of potentially transfected parasites, select progeny oocysts with an effective drug barrier and FACs sort these parasites before re-propagating them in vivo. Using these approaches stable integration of YFP into the *E. tenella* genome has been achieved.

COMMON AND UNIQUE GENE EXPRESSION OF AVIAN MACROPHAGES IN RESPONSE TO THREE MAJOR *EIMERIA* SPECIES

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Host immune responses to *Eimeria* are very complex and involve both innate and adaptive immunities. The chicken intestine is an intricate and dynamic ecosystem where immune elements interact with antigenic components of pathogens as well as with those of nutrients, exogenous compounds and microflora. These immune responses are also influenced by the host genetic makeup and their interactions are essential in the defense against enteric pathogens like *Eimeria*. The outcomes of these complex interactions determine resistance to infection, and the development of genomic tools and techniques allows for analysis of such multiple and complex host responses. Macrophages and intraepithelial lymphocytes (IELs) are critical components of the intestinal immune system and have been shown to play significant roles in both the innate and acquired immune responses to *Eimeria*. In order to better understand the immunobiology of host-parasite interaction, avian macrophage and IEL cDNA microarrays were used. Using avian macrophage microarray containing 4,906 unique gene elements obtained from chicken macrophage cDNA library and spotted in triplicates, we identified important host genes which expression were enhanced following infection of macrophages with sporozoites of *E. tenella, E. acervulina*, and *E. maxima*. Genes which showed significant expression were confirmed using real-time quantitative PCR. Host genes, which expression changed significantly after infection with *Eimeria,* included many genes encoding cytokines and chemokines that are associated with inflammatory responses. Among key cytokine genes that increased shortly after infection IFN-γ, IL-2, IL-15, IL-16 IL-17, and IL-18 were included. Furthermore, we prepared a cDNA library from intestinal IELs of *Eimeria*-infected chickens and constructed a small intestinal microarray containing over 10,000 unique elements spotted in duplicates. Investigations are currently under way to study kinetic gene expression in mucosal scrapings of birds infected by one of the three *Eimeria* species. Further analysis of these expression patterns will contribute to a better understanding of the mechanisms underlying resistance and susceptibility to coccidiosis.

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MOLECULAR CLONING AND EXPRESSION PROFILES OF CHICKEN NK-LYSIN DURING *EIMERIA* INFECTIONS

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NK-lysin is known to have antibacterial and antitumor activities and is expressed by NK cells as well as T- lymphocytes. In a previous report, the contig 171 (NK-lysin- like sequence), composed of 87 ESTs, occurred with the highest prevalence in an *Eimeria*-induced intestinal cDNA library. This study was conducted to clone, express, and characterize contig 171 as the chicken NK-lysin, which consists of an 868 bp DNA sequence and an open reading frame of 140 amino acids with a predicted molecular mass of 15.2 kDa. Comparison of deduced amino acid sequences showed less than 20% identity to granulysin and other mammalian NK-lysins. When infected with *E. maxima*, chicken total intraepithelial lymphocytes (IELs), CD4+ and CD8+ IELs showed higher expression levels of NK-lysin mRNA than CD8- IELs as quantified by real-time PCR. Upon infections with three *Eimeria* species, chicken NK-lysin RNA expression level normalized to GAPDH exhibited differential and interesting patterns. Further, chicken NK-lysin was expressed into COS-7 cells and its antitumor activity was demonstrated in killing LSCC-RP9 macrophage transformed cells. Also, chicken NK-lysin showed sequence similarity to other mammalian cDNA with cytotoxic activity to tumor cells. In these studies, we cloned and characterized chicken NK-lysin and studied its expression patterns in mRNA of *Eimeria*-infected birds. As in other animals, chicken NK-lysin may play an important role in antimicrobial defenses against enteric pathogens such as *Eimeria*, especially at the early stages of such infections.

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DEVELOPMENT OF FUTURE STRATEGIES FOR VACCINATING CHICKENS AGAINST COCCIDIOSIS USING RECOMBINANT ANTIBODIES, GENES AND PEPTIDES.

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Avian coccidiosis is caused by several different species of the intestinal protozoan parasites, *Eimeria.* Ability to develop novel control strategies against coccidiosis will reduce major economic losses to the poultry industry worldwide. Using functional genomics and molecular biology tools, we have now better understanding of host innate and acquired immunity to *Eimeria*. This paper will discuss various new strategies that we have taken to induce protective immunity to avian coccidiosis. Furthermore, novel vaccine delivery technologies are being applied to poultry to induce protective immunity against coccidioisis using recombinant proteins, genes and immunomodulators. For example, we have demonstrated that recombinant coccidian protein or recombinant DNA carrying a coccidian gene, when injected either into 18-day-old embryos using a commercial in ovo injector, Intelliject, or injected intramuscularly using a gene gun, reduced oocyst shedding and enhanced body weight gain. Addition of chicken cytokines or CpG oligodinucleotides further enhanced the vaccine-induced protection against coccidiosis. These results indicated that the type and dose of DNA vaccine and adjuvant influence the quality of the local immune response to DNA vaccination against coccidiosis. Another method that we are developing utilize recombinant antibodies whose specificities are directed against apical complex proteins involved in host cell invasion. Recombinant antibodies directed against apical complex antigen of *Eimeria* blocked sporozoite invasion of host cells and reduced oocyst shedding when injected in ovo at 18-day of embryonic development. Taken together, we have demonstrated how new generation vaccines and immunomodulators could be used to enhance host resistance against avian coccidiosis.

GENETICS, IMMUNITY AND DNA FINGERPRINTING IN THE IDENTIFICATION OF PROTECTIVE ANTIGENS OF *EIMERIA MAXIMA*

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Many protozoan genomes are the subject of extensive sequencing projects, but the identification of genes that encode antigens capable of stimulating a protective immune response remains a demanding task. The rational identification of genuinely immunoprotective gene products encoded by unsequenced genomes is even more daunting. The majority of screens for vaccine candidates protective against protozoan infection have been largely empirical, based upon the induction of an immune response rather than protection. However, consideration of the genetics of immune escape by different strains of *Eimeria maxima* has raised the possibility of the targeted identification of a gene (or genes) under immune selection. Central to our strategy is the concept that the inheritance of genetic markers is influenced by their proximity within the genome to loci under deleterious selection. Initially multiple independent hybrid populations were created through the mating of two strains of *E. maxima* that each induce a lethal strain-specific protective immune response in the host and show a differential response to the anticoccidial drug robenidine. Escape by a sub-population within the progeny of this cross from a double-barrier consisting of immune and chemotherapeutic selection lethal to either parent proved that loci encoding molecules stimulating strain-specific protective immunity or resistance to robenidine segregate independently. Comparison of parasite replication in the presence of the selective double-barrier, either individual component of the barrier or no barrier, implicates a highly restricted number of key protective-antigen-encoding loci. The selection of recombinant parasites through the double-barrier imposed genetic-bottleneck was accompanied by the elimination of ~3% of the polymorphic DNA markers that defined the parent strain used to immunise the host. Hybridisation studies with Southern-blotted digested and undigested karyotypes resolved by PFGE and a BAC library derived from the parent strain under immune selection have revealed two clusters of these DNA markers correlated with selection that cover independent regions of ~220 Kb (13 markers) and ~400 Kb (8 markers) within the genome. Our identification of polymorphic DNA markers that associate with immune-mediated killing and are physically co-localised within the genome support our strategy to identify loci under selection in *Eimeria* spp. and should be applicable to other loci under selection in other Apicomplexa including *Plasmodium spp.*.

APPLICATION OF FUNCTIONAL GENOMICS, IMMUNOLOGY AND MOLECULAR BIOLOGY TOOLS TO EXPLORE HOST IMMUNE RESPONSE TO *EIMERIA*.

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Avian coccidiosis is caused by several different *Eimeria* species which infect different regions of the intestine inducing a specie-specific immunity. Coccidiosis usually stimulates a number of immunological defense mechanisms, namely antibody- and cell-mediated. Recent technological advance in molecular genomics is opening a novel way for the discovery of molecular/cell biological pathways associated with complex biological phenomenon and is facilitating the development of an alternative strategy to combat coccidiosis. Using high-throughput molecular genomics approaches, we have identified host genes involved in the disease process and resistance. Since intraepithelial lymphocytes (IELs) play a critical role in protective immune response to *Eimeria,* a list of genes expressed by intestinal IEL of *Eimeria*-infected chickens was compiled using the expressed sequence tag (EST) strategy. The 14,409 ESTs consisted of 1,851 clusters and 7,595 singletons, which revealed 9,446 unique genes in the data set. This EST library will be a valuable resource for profiling global gene expression in normal and pathogen-infected chickens for the identification of host immune-related genes. We have also carried out fine mapping of quantitative trait locus (QTL) which control coccidiosis resistance and identified a QTL on chromosome 1 that significantly affects *Eimeria* oocyst shedding and three QTLs that influence body weight of chickens during coccidiosis. These results provide the foundation for further investigation to validate the QTL. In addition, we have investigated local host immunity to *Eimeria* using tissue-specific cDNA microarrays. The results of these studies clearly indicate that the intricate and complex interactions of host local innate immune response and parasites determine the outcome of host response to coccidiosis.

PROTEINS FROM THE WALL- FORMING BODIES OF *EIMERIA MAXIMA:* FUNCTION AND IMMUNOGENICITY

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Wall- forming bodies type 1 of *Eimeria maxima* are large, electron- dense vacuoles that stain positively with antibodies in an enriched preparation of native forms of 56, 82 and 230 kDa gametocyte proteins (APGA). Wall- forming bodies type 2 also react positively with these antibodies, but are less electron- dense than wall- forming bodies type 1, have whorled or donut-shaped profiles and remain enclosed in the rough endoplasmic reticulum. At the initiation of oocyst wall formation, wall- forming bodies type 1 first align at the periphery of the macrogamete, then release their contents to form an electron- dense outer oocyst wall that reacts with anti-APGA antibodies. Wall- forming bodies type 2 subsequently give rise to an electron- lucent inner wall. Proteomic and Western blot analysis has revealed that the 56 and 82 kDa proteins are processed into smaller, tyrosine-rich proteins that can cross-link and harden to form the oocyst wall of *Eimeria maxima.* This observation helps to explain the molecular basis of action of the subunit vaccine, CoxAbic®, which includes native 56 and 82 kDa proteins and stimulates strong antibody responses to protect the hatchlings of vaccinated hens from coccidiosis. Genes encoding these two immunodominat components of CoxAbic® were cloned and the recombinant proteins expressed in *E. coli* and purified. Both recombinant proteins are immunogenic, eliciting dose-dependent antibody responses comparable to that provoked by CoxAbic® and able to recognise the native proteins in that vaccine. Furthermore, the recombinant proteins are able to inhibit the binding of anti-APGA antibodies to APGA, indicating that the recombinant versions have some promise as components of a second-generation subunit vaccine against poultry coccidiosis.

RESTORATION OF FIELD *EIMERIA* ANTICOCCIDIAL SENSITIVITY WITH COCCIVAC-B, A LIVE COCCIDIOSIS VACCINE

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Vaccination was examined as an alternative strategy to rotation of in-feed anticoccidials to maximize the sensitivity of *Eimeria spp.* to anticoccidial compounds. The study used anticoccidial sensitivity battery tests (AST's) to determine the baseline diclazuril sensitivity of field *Eimeria* isolates from seven broiler complexes that had recently used diclazuril. Based on weight gain and lesion scores, 25% or fewer of the isolates submitted by each complex demonstrated good sensitivity to diclazuril. Following the baseline sampling, four of the complexes rotated to non-diclazuril in-feed anticoccidial programs and three of the complexes rotated a Coccivac-B vaccination as the sole coccidiosis control program for two broiler grow-out cycles. *Eimeria* isolates were subsequently collected from the identical houses on all seven complexes and diclazuril AST results were compared to the baseline AST results. Following the two grow-out cycles, sensitivity on the four complexes that rotated to in-feed anticoccidials remained essentially unchanged. The three complexes that rotated to Coccivac-B vaccination demonstrated marked improvement in diclazuril sensitivity, with good sensitivity in 60 to 100% of the samples from each complex. Earlier examinations of reversal of sensitivity using Coccivac-B used the ionophore salinomycin as the baseline. Sensitivity is easier to determine with a chemical anticoccidial than an ionophorous anticoccidial. This study used a chemical anticoccidial and clearly demonstrated that vaccination with Coccivac-B has the potential to replace resistant coccidia with sensitive coccidia.

ROTATIONAL PROGRAMS COMBINING DRUGS AND COCCIDIOSIS VACCINE IMPROVE PERFORMANCE AND PROFITABILITY IN A COMMERCIAL BROILER FARM

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It is documented that production performance will improve after the use of coccidiosis vaccines due to the replacement of drug resistant oocysts with a more drug sensitive oocyst population. A study consisting of seven grow-outs was conducted on the SFASU Broiler Research Farm to determine the effect of ADVENT® Coccidiosis Control, a live oocyst coccidiosis vaccine, on performance, and to determine if there was an improvement in performance when going back on a coccidiostat program with a more drug sensitive population of coccidial oocyst.

ADVENT contains sporulated viable oocysts from the three commercially relevant species in broilers, *E. acervulina* (strain VND-A10), *E. maxima* (strain VND-M27), and *E. tenella* (strain VND-T49). Extensive screening for anticoccidial drugs resistance confirmed sensitivity of the strains to ionophores and chemical drugs.

Five consecutive broiler flocks were grown on a commercial broiler farm. The study consisted of comparing performance parameters on a four house solid-wall modern broiler farm. Houses 1 & 2 were deemed Farm 1 and houses 3 & 4 were deemed Farm 2 for comparison purposes. Farm 1 used a typical chemical/ionophore and 3-nitro coccidiostat program and farm 2 used a coccidiosis vaccine program. Both Farms employed the use of BMD-60. Averaging the adjusted feed conversion on five consecutive flocks, farm 1 had a 0.03 adjusted feed conversion advantage over farm 2.

For the sixth flock, both farm 1 and farm 2 were placed on the same anti-coccidial program. Farm 2 had a 0.11 advantage for adjusted feed conversion.

On the seventh flock, both farm 1 and farm 2 were placed on the same anti-coccidial program. Farm 2 had a 0.07 advantage for adjusted feed conversion.

Coccidial lesion scoring (Johnson and Reid Method) was done on five birds from each house on days 14, 21, 28, 35, and 42 through out all the grow-outs. The improvement in Adjusted Feed Conversion on Farm 2 for the 6th and 7th flock can be directly related to the decrease seen in coccidial lesion scores as compared to farm 1.

The improvement in performance in the subsequent two flocks after the five consecutive grow-outs using ADVENT coccidiosis vaccine in the same houses may be the result of shifting the oocyst population to a drug sensitive population from the vaccine.

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COMPARISON OF DRUG-SENSITIVITY AND SPECIES COMPOSITION OF *EIMERIA* ISOLATED FROM POULTRY FARMS UTILIZING EITHER ANTI-COCCIDIAL DRUGS OR A LIVE OOCYST VACCINE TO CONTROL AVIAN COCCIDIOSIS.

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The purpose of this study was to compare drug resistance and species composition of *Eimeria* isolated from two types of poultry operations that differed in the means of controlling avian coccidiosis (i.e. drugs vs. live oocyst vaccine). Poultry litter was collected from 10 different broiler operations; 5 utilizing anti-coccidial drugs, 5 utilizing a live oocyst vaccine. The number of species present in vaccine-control facilities was greater than in drug-control operations, with all 6 species present in the former, and 3-4 species present in the latter. The pathogenicity of oocysts propagated from the vaccine-using farms was greater than in the drug-utilizing farms, in that 3.5 times fewer oocysts were needed to cause similar levels of weight depression. *Eimeria* propagated from drug-utilizing operations displayed greater anti-coccidial resistance, as measured by lower weight gains, higher intestinal lesion scores, and poorer feed conversion ratios, compared to *Eimeria* recovered from farms using a live oocyst vaccine. Only one drug, Avatec, provided greater than 90% weight gain protection against coccidiosis in broilers challenged with oocysts recovered from drug-utilizing farms. In contrast, three drugs-Coban, Biocox, and Avatec, provided greater than 90% protection against challenge with oocysts recovered from vaccine farms. Quite unexpected was the partial resistance to two drugs, Clinicox and Monteban, observed in oocysts isolated from vaccine-using farms. These results suggest that conventional drug treatment leads to lower *Eimeria* species diversity coincident with slightly greater drug resistance. The mechanism by which non-vaccine drug-resistant species are introduced into vaccine-utilizing farms remains to be determined.

HIGHER INCIDENCE OF *EIMERIA* SPP. FIELD ISOLATES SENSITIVE TO DICLAZURIL AND MONENSIN AFTER LIVE COCCIDIOSIS VACCINATION WITH PARACOX™-5

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Twenty *Eimeria* spp. field isolates originating from Danish, German, Greek, Italian, Portuguese and Rumanian broiler farms were subjected to anticoccidial drug sensitivity testing. The anticoccidial drugs tested were diclazuril (Clinacox®) and monensin (Elancoban®) at a concentration of 1.1 mg/kg and 103 mg/kg respectively. The assay was performed in experimental battery cages. Broiler chickens fed with anticoccidial drugs were infected with a defined number of sporulated oocysts of the *Eimeria* spp. field isolates. An infected unmedicated control group was given the same inoculum and served as positive control while an uninfected unmedicated group was used as negative control. Coccidial lesion scores, oocysts shedding and weight gain were used as parameters. Based on the percentages of reduction of mean lesion scores of the infected medicated birds compared to the infected non-medicated chicks, the results show that resistance is common amongst European coccidiosis field isolates, especially *E. acervulina* (68 and 53% resistance for diclazuril and monensin, respectively). Resistance is less frequent amongst *E. maxima* (38 and 50% resistance to diclazuril and monensin, respectively) and *E. tenella* isolates (23 and 38% resistance to diclazuril and monensin, respectively). Statistical analysis by the Chi-Square test showed a highly significant influence of the coccidiosis prevention program (live coccidiosis vaccination with Paracox™-5 versus anticoccidial drugs in feed) on the sensitivity pattern of *Eimeria* spp. field isolates to both diclazuril (P = 0.000) and monensin (P = 0.001). When looking at coccidia species and anticoccidial drug level, a significant effect of vaccination on the sensitivity profile of *E. acervulina* to monensin (P = 0.018), *E. tenella* for diclazuril (P = 0.007) and *E. maxima* to diclazuril (P = 0.009) was found – thus, in half of the cases. The present study shows that sensitivity to the anticoccidial drugs diclazuril and monensin is more frequent in *Eimeria* spp. field isolates originating from broiler farms where a coccidiosis vaccination policy was followed.

COCCIDIOSIS IN TURKEYS: PATTERNS OF SENSITIVITY TO DICLAZURIL FOLLOWING VACCINATION WITH COCCIVAC-T VACCINE

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A series of trials were conducted to determine the efficacy of Clinacox (diclazuril) in turkey flocks against isolates collected from farms using different control programs for coccidiosis. Isolates of coccidia were collected from farms that:

(1) Used only Coccivac-T vaccine in the year prior to collection

(2) Used Clinacox for six months, followed by an ionophore drug prior to collection

(3) Used Clinacox for six months, followed by Coccivac-T prior to collection.

Three groups of poults did not receive medication and were challenged with sporulated oocysts coming from each of the three sources (infected, unmedicated controls, IUC). Three groups of poults received feed with diclazuril and were inoculated with oocysts coming from one of the above sources. Another group received diclazuril-free feed and was not challenged, serving as the unmedicated, unchallenged control (UUC).

Growth rate at the end of 6 days post-challenge was depressed by an average of 37% on the IUC groups as compared to the UUC group. The average gross lesion score of IUC birds was 2.0 and a microscopic parasite score of 3.27 (range 0 to 4, the higher the score the greater the severity) was assessed, compared to no lesions and zero parasite burden in the UUC birds.

Index scores were determined using growth, gross lesions, microscopic parasitism and livability; A higher index score indicates better efficacy. The average indexes for the isolates obtained from source 1 were 40 and 96, for IUC and diclazuril medicated birds, respectively. The average score for the samples collected from source 2 were 35 and 60, for IUC and diclazuril medicated birds, respectively. The average score for the samples collected from source 3 were 35 and 86, for IUC and diclazuril medicated birds, respectively.

The infectivity of the isolates was severe and similar on the IUC birds regardless of source. On the other hand, the response of the isolates to medication with diclazuril was different. Birds challenged with isolates from sources 1 and 3 (where Coccivac-T vaccine had been used) showed better response to medication than birds challenged with isolates from source 2, where diclazuril had been used, but no vaccination was applied.

Using Coccivac-T vaccine in rotation with diclazuril appeared to provide a sparing effect on the efficacy of the drug.

SEQUENCE ANALYSIS OF THE 18S RNA GENE OF *ISOSPORA BELLI* AND IDENTIFICATION OF *CARYOSPORA*-LIKE OOCYSTS

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Isospora belli is an important coccidian protozoan that parasitizes both immunocompetent and immunocompromised patients, especially HIV-1 infected patients with low CD4+ lymphocyte counts. During 2002-2004, 38 patients infected with *I. belli* were identified by examination of 78,743 stool specimens submitted to the parasitology laboratory of King Chulalongkorn Memorial Hospital in Bangkok. Isosporiasis patients comprised 35 Thai from diverse regions of the country and 3 recent immigrants from Cambodia, Laos and Pakistan. Out of these, 30 were HIV-positive, 3 received prolonged corticosteroid therapy for other diseases and 5 immunocompetent individuals. In order to verify if genetic heterogeneity or cryptic species occur in *Isospora* infecting humans, we determined the morphometry of oocysts from all patients and performed sequencing analysis of the small subunit ribosomal RNA gene (*SSU rDNA, 18S RNA*) spanning 1,680 base pairs from 26 isolates. Morphometric study of oocysts revealed that oocyst dimension in this study varied from 17 to 37 (28.3±3.0) mm in length, 8 to 21 (13.5±1.9) mm in width and the shape index (length by width) 1.3 to 3.3 (2.1±0.3). Although the oocysts exhibited shape and size variations both within and among isolates in this study, the shape indices of all oocysts observed were consistent with that of *I. belli* being more than 1.2 (range=1.3-3.3), which were distinct from those for *I. natanlensis* and other species infecting nonhuman mammals (<1.2). Additionally, we observed oocyst maturation after passage from intestine from 3 patients, who had not yet taken anti-coccidial drugs: 2 HIV-infected patients, one of them had relapse, and an immunocompetent patient presented with chronic diarrhea. In total 100 oocysts for each isolate were examined for sporulation every 6 hours under light microscope using 400x magnification for 20 days. Results revealed that 27% of oocysts (range=20-33%) underwent complete sporulation and the duration for generating 2 sporocysts each of which contained 4 sporozoites was variable ranging from 24 hours to 10 days (3.9±3.4 days)(n= 66). More interestingly, fully sporulated oocysts having a single sporocyst covering 8 sporozoites, designated *Caryospora*-like oocysts, were found in

all 3 isolates representing 9-29% of all mature oocysts (n=15). Meanwhile, the SSU rDNA sequences revealed minimal sequence variation containing 2 sequence types. Thus, no correlation between distinct *I. belli* strain and disease severity was observed. Phylogenetic tree showed that *I. belli* in this study was within the same clade as *I. ohioensis, I. suis, I. orlovi, I. felis, Toxoplasma gondii* and *Sarcocystis* sp. In conclusion, unlike *Cryptosporidium* infecting humans that comprises both zoonotic and anthroponotic species, our study demonstrated that human isosporiasis is caused by a single species belonging to *I. belli* based on morphometric and molecular evidences.

DETECTION AND IDENTIFICATION OF *NEOSPORA CANINUM* IN A NATURALLY INFECTED BUFFALO FETUS (*BUBALUS BUBALIS*) FROM BRAZIL

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The water buffalo (*Bubalus bubalis*) is an important natural host for *Neospora caninum*. In this study, we aimed at detecting and identifying *N. caninum* from fetuses obtained from pregnant water buffaloes sacrificed at slaughter house in order to confirm the congenital transmission of the parasite in this intermediate host. Specimens of brain and thoracic fluid from nine fetuses with gestational ages ranging from two to five months were obtained from females water buffaloes raised under grazing condition. The brain specimens of one fetus proved positive by two PCR assays, one of them directed to *Neospora*-specific Nc5 gene and the other to the common toxoplasmatiid ITS-1 sequence. The PCR products of gene 5 and ITS-1 were sequenced and revealed the identity of PCR products, which were confirmed as *N. caninum*. All fetal fluid samples examined by indirect fluorescent antibody test were negative for the presence of *N. caninum* antibodies (antibody titer $\leq$ 25). The absence of antibodies in the *N. caninum* positive fetus may be due to lack of fetal immunocompetence, or a short interval between infection and sample collection. In bovine fetuses younger than six months, lack of fetal immunocompetence may explain the low sensitivity of fetal serology for the detection of *N. caninum* infection. In conclusion, this is the first detection and molecular identification of *N. caninum* in fetuses of naturally infected water buffaloes and confirms the serological observations that many of these animals may already be infected at calving, and also that congenital transmission of *N. caninum* occurs in buffaloes. However, the actual role played by this protozoan as a causative agent of reproductive disorders in buffaloes remains to be elucidated.

MOLECULAR EPIDEMIOLOGY OF CRYPTOSPORIDIOSIS AMONG HUMAN IMMUNODEFICIENCY VIRUS-INFECTED PATIENTS IN THAILAND: ANALYSIS OF THE 18S RNA AND THE CPG60/45/15 *LOCI*

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Diarrheal disease caused by *Cryptosporidium* sp. is globally prevalent, affecting both immunocompetent and immunocompromised hosts. The consequences of cryptosporidiosis in human immunodeficiency virus (HIV)-infected patients can lead to more significant morbidity and mortality than in individuals with normal immune status. Because an effective anti-cryptosporidial agent is currently unavailable, prevention of disease transmission is of primary importance. Several epidemiological studies indicate that human cryptosporidiosis is caused by diverse species of *Cryptosporidium,* one of which deploys exclusive anthroponotic transmission cycle and others are considered as zoonosis. To examine the extent of species of *Cryptosporidium* infecting humans in Thailand, we recruited 67 isolates from HIV-infected patients who sought medical treatment at King Chulalongkorn Memorial Hospital in Bangkok during 1997-2003. Species determination was performed by analysis of partial sequences of the 18S RNA gene encompassing species-specific domain and compared with those in the GenBank database. The Cpg60/45/15 gene was used for subgenotypic analysis of *Cryptosporidium*. After PCR amplification and purification, sequences were determined directly from the PCR-amplified products and/or from subclones, which were done from both directions. Results revealed that the 18S RNA genes defined 8 sequence types belonging to *C. hominis* (38.6%), *C. meleagridis,* (28.6%), *C. parvum,* (12.9%), *C. canis,* (10%), *C. felis,* (2.8%), *C. muris,* (1.4%), *Cryptosporidium* Pig1 genotype (1.4%), and a novel sequence (4.3%). Mixed infections between *C. hominis* and *C. meleagridis* were observed in 3 isolates (4.3%). Phylogenetic construction has placed the novel sequence type of the 18S RNA gene close to that from an environmental isolate from water in USA. Meanwhile, PCR amplification targeting the Cpg65/45/15 gene of *Cryptosporidium* was successful for *C. hominis*, *C. parvum* and *C. meleagridis,* while the rest yielded negative results. The Cpg64/45/15 sequences obtained from *C. hominis* belonged to groups Ia, Id, and Ie, while those from *C. parvum* displayed a novel type II sequence and all *C. meleagridis* in this study possessed type IIIa sequence. Sequence comparison with those previously reported showed that the Cpg65/45/15 gene was highly polymorphic, rendering it an attractive marker for subgenotyping or strain differentiation of *C. hominis*, *C. parvum* and *C. meleagridis*. In addition, no significant correlation between species or subgenotypes of *Cryptosporidium* and clinical symptoms of the patients was found in this analysis. Hence, human acquisition of *Cryptosporidium* can plausibly be from diverse sources and the zoonotic transmission cycles of this coccidian protozoan in Thailand are common among HIV-infected patients.

MOLECULAR GENOTYPING OF *EIMERIA TENELLA* AND *E. ACERVULINA* THROUGH MICROSATELLITE MARKERS

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Chicken coccidiosis is caused by seven species of the genus *Eimeria*. Species identification can be accurately attained using either classical biological and morphological features, or molecular targets for PCR amplification. On the other hand, strain differentiation remains a complex task due the lack of good morphological/biological discriminative features. In addition, the only available molecular assays rely on DNA fingerprinting (Southern blotting) and RAPD. The former technique is tedious and time-consuming, and the latter presents low reliability and reproducibility across different laboratories. The aim of the present work was the development of reliable microsatellite markers for the intra-specific discrimination of two of the most relevant *Eimeria* species: *E. acervulina* and *E. tenella*. In order to select potential candidate *loci*, we employed genomic sequences of the *E. tenella* Genome Project (http://www.sanger.ac.uk/Projects/E_tenella/) and also *E. acervulina* open reading frame EST (ORESTES) sequences generated in our laboratory (http://www.lbm.fmvz.usp.br/eimeria/). The sequences were analyzed with the program Tandem Repeats Finder, and the best candidates were selected using TRAP, the Tandem Repeat Analysis Program (Sobreira, T.J.P.; Durham, A.M. & Gruber, A. – manuscript in preparation). TRAP was developed in our laboratory as a bioinformatic tool for selection, classification and quantification of tandemly repeated sequences. The best candidate markers were chosen and primers pairs flanking the microsatellite repeats were designed. From a total of 57 markers tested for *E. acervulina*, 16 presented both species-specificity and intra-specific polymorphism, with an average of 2.8 alleles per *locus*. For *E. tenella*, 79 markers were screened, yielding a total of 15 polymorphic *loci*, with an average of 2.1 alleles per *locus*. Taking these data together, the microsatellite markers obtained so far allow for the theoretical differentiation of more than 7 million haplotypes for *E. acervulina* and circa 70,000 for *E. tenella*. We intend to extend this microsatellite development to *E. maxima*, another relevant pathogenic species of the domestic fowl, and to reach a theoretical differentiation of at least 1 million haplotypes for each species. Among the possible applications of this technology, we can cite the detection of the unauthorized use of commercial strains, discrimination between field and vaccine strains, purity control of vaccine strains on production, and field monitoring of vaccine propagation and crossing with field strains.

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COUNTING COCCIDIA: A GENOMIC APPROACH

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Quantification of infection with the *Eimeria* spp. through monitoring pathological severity or magnitude of oocyst excretion has greatly informed our understanding of the eimerian life cycle. However, attempts to quantify fluctuations in parasite reproduction within the host have largely relied upon labour-intensive microscopic analysis, creating a 'grey box'. The development, validation and application of a quantitative real-time PCR assay has opened this biological 'box', permitting the sensitive and reproducible enumeration of intracellular parasite genomes present throughout the course of infection. Generic and species-specific quantitative PCR assays that target the conserved 5S ribosomal RNA coding sequence of nine avian and murine *Eimeria* species and the *E. maxima* MIC1 gene have been validated as capable of detecting >0.01 and >1 *E. maxima* genomes respectively. The generic 5S ribosomal RNA assay has been shown to enumerate the genomes of *E. acervulina*, *E. mitis*, *E. tenella*, *E. pragensis* and *E. vermiformis* with a comparable sensitivity and efficiency to that recorded for *E. maxima* over at least seven orders of magnitude. Preparation of samples collected *in vivo* with a commercial DNA extraction kit allowed the rapid enumeration of parasite genomes within an excised tissue section in just two working days. Parasite genomic DNA could first be detected within the host three days after infection with only 100 sporulated *E. maxima* oocysts.

Application of these complimentary generic and species-specific assays to study the dynamics of *E. maxima* replication within hosts representing 'susceptible' and 'resistant' genotypes indicate a brief period of differential killing four to five days after infection, suggesting that the third and fourth generation schizonts, but not the gametocytes, are targeted by the strain-specific host immune response. Other potential applications for the quantitative PCR assays described here include investigating the efficacy and point of action of existing and novel chemotherapeutic compounds and vaccine candidates, together with the continued characterisation of the eimerian life cycle.

DIGITAL IMAGE ANALYSIS IN THE DIAGNOSIS OF CHICKEN COCCIDIOSIS

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Coccidiosis of the domestic fowl is an enteric disease caused by seven distinct species of the genus *Eimeria*. Species discrimination is classically performed using morphological/pathological parameters like oocyst size and shape, aspect and location of the intestinal lesions, etc. However, accuracy of species assignment by visual inspection is severely restricted by the slight differences and overlap of characteristics among the different species. This work aimed at developing a non-subjective process for the morphological oocyst identification and classification through computational analysis of microscope digital images. Our methodology involves three steps: (i) image acquisition and preprocessing, (ii) image characterization, and (iii) image classification. Initially, digital oocyst images were obtained from pure strains of each *Eimeria* species, using a 4-megapixel CCD camera. Each image was preprocessed to define the parametric contour of the oocyst wall. In addition, the images were submitted to an automated feature extraction and classification system, using shape and textural characteristics (oocyst length and width, perimeter, area, curvature, texture and symmetry), and comprising a set of 13 distinct features [1]. For species discrimination, we used the multivariate normal density as the discriminative function for the multidimensional Bayesian learning classifier [1]. A total of 2,177 oocyst micrographs of the seven species were captured and 30% of the images were used as a training set for the generation of the classification model. The rest of the images, taking 100 randomly generated groups, were submitted to two iterations for Bayesian learning. The rate of correct species assignment varied from 64.9% (*E. necatrix*) to 97.7% (*E. brunetti*), with and overall rate of 86.8%. A standalone program was implemented in C^{++} programming language and installed on a web server. Thus, a remote user can upload an image and obtain a real-time electronic diagnosis of the *Eimeria* species through the internet. The approach proposed here may represent an auxiliary tool for the differential diagnosis of chicken coccidiosis, with the advantage of not requiring biological sample transportation between the poultry farm and the reference laboratory.

References

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THE MACROPHAGE INHIBITORY FACTOR OF *EIMERIA* SPECIES

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Development of drug resistance and the paucity of new chemotherapeutic agents have increased the need for new drugs as well as vaccine targets against poultry coccidiosis. One approach used in identifying novel targets is the random screening of transcripts expressed during the invasive stages of the parasite life cycle. While screening a cDNA library derived from merozoites of *Eimeria acervulina* a single full-length clone was isolated, and this shared between 35-38% amino acid identities with the Macrophage Inhibitory Factors (MIFs) of vertebrates. To further characterize *Eimeria* MIF the full-length *Eimeria tenella* cDNA was also cloned and sequenced. The amino acid identity between the two *Eimeria* MIFs is 64%. The mRNA expression pattern of *Eimeria* MIF was determined using quantitative RT-PCR on RNA collected from several stages of the parasite life cycle. MIF expression profiles in both *E. acervulina* and *E. tenella* were found to be almost identical, with high levels of transcripts present in merozoites, while developing oocysts and sporozoites expressed only small amounts of MIF mRNA. Using Western analysis, a 12 kDa protein, corresponding to the molecular weight of MIF, was shown to be highly expressed in merozoites, while expression in other stages were significantly lower. It also appears that MIF is secreted by *E. acervulina* merozoites. The amount of secreted MIF is increased proportionately with the temperature in which merozoites were incubated. Because *Eimeria* MIF is strongly associated with merozoites its potential as a vaccine candidate should be evaluated in the future.

THE DEVELOPMENTAL EXPRESSION OF *EIMERIA* HEAT SHOCK PROTEIN 90

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Heat shock protein 90 (Hsp90) represents one of the most abundant and evolutionarily conserved proteins. Because in most species studied Hsp90 was found to be essential for proper cell function, a study investigating *Eimeria* Hsp90 was initiated.The full-length *Eimeria acervulina* and *Eimeria maxima* Hsp90 cDNAs were isolated and sequenced. From evolutionary analysis and sequence identity, it is likely that the *Eimeria* Hsp90 sequences described so far encode the cytosolic versions of the protein. Although at nucleotide and amino acid level *Eimeria* Hsp90s are highly similar, their expression profiles differ considerably. The *E. acervulina* Hsp90 transcripts are developmentally regulated, with little or no expression in developing and in fully sporulated oocysts. The analysis of Hsp90 protein in *E. acervulina* corroborates with RT-PCR data, which show that the highest protein levels are expressed by sporozoites and merozoites, with little or no expression in oocysts. While *E. tenella* and *E. maxima* Hsp90 transcripts were present in every stage tested, the level of expression differed among stages. The mRNA expression profile was very similar to the protein expression, with the highest amounts of protein expressed in sporozoites and merozoites. We have also observed that *E. tenella* Hsp90 is not actively secreted by sporozoites or merozoites. The significance of differential Hsp90 expression profiles in these species is currently unknown; however, it is clear that the molecular mechanisms occurring during oocyst development may be quite different between *Eimeria*.

ACETYL CoA CARBOXYLASE IN AVIAN *EIMERIA*.

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Acetyl coA carboxylase, catalyzes the first committed step in the biosynthesis of fatty acids. In some apicomplexan parasites such as *Toxoplasma gondii* and *Plasmodium sp.,* a multi-domain form of this enzyme (ACC 1) has been identified as a nuclear-encoded protein that is transported to and apparently functions in the apicoplast organelle found in zoite stages of parasites life cycles. The unique genetic origin of this enzyme and its apparent requirement for parasite development suggests its potential as a target for control of apicomplexan parasites. We have therefore undertaken the characterization of ACC1 in avian *Eimeria.* BLAST searches of both nucleotide and protein data files from *T. gondii* ACC1 (tACC1) precursor against the Sanger Institute *Eimeria tenella* genome data base found a putative homolog *Eimeria* ACC1 (eACC1) gene to be contained in Contig 5754, and the carbamyl phosphate synthase, (5'end), biotin binding, and carboxyl transferase (3' end) domains were identified both through bioinformatics as well as RT-PCR. The low genetic complexity of the central portion of the eACC1 gene made its complete sequencing difficult. A 224 nt sequence that is 73 nt upstream from the first exon common to tACC1 and eACC1 was identified as a possible signal sequence for eACC1. However, no tripartite leader sequence, such as is found in tACC1 precursor that allows transport of the protein into an apicoplast, was defined. These observations, along with the lack of evidence for an *Eimerian* apicoplast in the ultrastructure literature suggest major differences between tACC1 and eACC1. Expression of eACC1 protein was documented through detection on polyacrylamide gels of a biotin-containing protein band of about 250 kd. This band appears most intense in merozoite extracts as compared to extracts of oocysts and sporozoites. Determinations of differential gene and protein expression of eACC1 among stages of development are ongoing through use of RT-PCR and Western blots of extracts.

THE SATELLITE DNA OF *EIMERIA TENELLA*: A QUANTITATIVE AND QUALITATIVE STUDY

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Eimeria tenella genome has a complexity of 58 Mb, distributed in 14 chromosomes ranging from 1 to more than 7 Mb. One of the most interesting features of this genome is the very high tandem repeat content, with the triplet (GCA) and heptamer (TTTAGGG) repeats constituting the most predominant repetitive units. The genome sequencing started in 2002 and a set of circa 800,000 shotgun reads was generated at the Wellcome Trust Sanger Institute, and made available on the internet at the address ftp://ftp.sanger.ac.uk/pub/pathogens/Eimeria/tenella. Aiming at characterizing and quantitating the whole satellite content of *E. tenella* genome, our group in Brazil developed TRAP, the Tandem Repeat Analysis Program (Sobreira, T.J.P.; Durham, A.M. & Gruber, A. – manuscript in preparation). TRAP is a companion tool for Tandem Repeats Finder (Benson, 1999), a popular worldwide used application for *ab initio* tandem repeat finding. The program provides a unified set of analyses for the selection, classification and quantification of tandemly repeated sequences. The *E. tenella* genome assembly file (version of May 24, 2005) was downloaded from the Sanger's FTP site and processed by TRF version 3.21. TRF output files were analyzed by TRAP, selecting repeat *loci* with at least two repeat units, a minimum repeat period of 2 bp and a maximum period of 1,000 bp. The repetitive content of the genome was calculated using different identity percentages (id%), where id% values represent percentages of matches between adjacent repeat units overall. The whole genome satellite content varied from 1.9% at 100% identity to 16.8% at 70% identity. From this latter result, 9.0% corresponded to microsatellite (repeat period size of 2-6 bp), 7.5% to minisatellite (period size of 7-100 bp) and 0.3% to satellite (period size longer than 100 bp) sequences. The five most prevalent repeat units were GCA, TTTAGGG, TAAA, GCTA and AAATT, with the two former units corresponding to 8.8% of the genome content. A complete catalogue of the repeat units and statistics will be publicly available on the internet upon publication of the corresponding paper. In the meantime, the authors can provide a username and password under request.

CHARACTERIZATION OF SERINE PROTEASES IN DEVELOPMENTAL STAGES OF *EIMERIA TENELLA.*

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Because of the importance of enzymes of the serine class as targets for novel controls of human diseases, the current study investigates the occurrence and function of serine proteases in *Eimeria tenella* developmental stages. Using gel electrophoresis with casein imbedded gels (zymograms), bands of proteases activity with relative molecular weights (Mr) of 15, 24, 40 and 90 kDa were observed in soluble extracts of oocysts following 0, 12, 24, 48 and 72 hr of sporulation. Inhibition of the activity by serine protease inhibitors suggests that the observed proteolysis was the result of a serine protease. Purification of the proteolytic activity from unsporulated (0 hr) oocysts by affinity and anion exchange chromatography yielded a protein with an Mr of 24 kDa. Assessment of proteolytic activity from *in vivo* derived merozoites (MZs) demonstrated a similar pattern of proteolytic activity as seen for oocysts. Excretory/secretory (ES) products obtained from MZ after 3hr in culture at 41 C were enriched 10 fold in serine protease activity compared to MZ extract. In contrast, proteolytic activity from soluble extracts of sporozoites (SZs) contained only a single band of serine proteases activity (24 kDa), but ES had little protease activity. The invasion of *E. tenella* SZs into cultured cells was inhibited by the irreversible serine protease inhibitor, AEBSF, in a dose- dependent manner. AEBSF (1mM) also inhibited the release of the microneme protein MIC2 by SZs into the cell culture media suggesting that AEBSF prevents cell invasion by inhibition of a serine protease involved in the release or processing of micronemes. Searches of the *E. tenella* genome for enzymes homologous to the serine protease class indicated that trypsin-like enzymes are absent, but enzymes related to bacterial subtilisins and a rhomboid protease are present in the genome. Consistent with this finding, analysis of transcripts expressed by *E. tenlla* oocysts using substractive hybridization methods indicate the presence of two serine protease, a subtilisin and a rhomboid protease. These findings together suggest that serine proteases are widely distributed in *E. tenella* developmental stages. The enzymes function during sporulation is unknown, but a serine protease may be important for sporozoite cell invasion.

PLASTID REPLICATION IN APICOMPLEXAN PARASITES

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Apicomplexan parasites harbor a secondary plastid which is essential for development and pathogenesis. Several plastid localized metabolic and housekeeping enzymes have emerged as promising drug targets in *Toxoplasma* and *Plasmodium*. We are studying the replication and segregation of this important organelle and its genome. Our genomic analysis reveals that Apicomplexa have lost all elements of the conserved chloroplast/cyanobacterial division machinery including FtsZ, and several Min and Arc genes. We hypothesize that in contrast to plants and algae the plastid in Apicomplexa, is segregated using a genuinely eukaryotic mechanism, association with the centrosomes of the mitotic spindle. We have tested this hypothesis in *T. gondii* and *S. neurona*, which we show to have highly organized linear plastids. We show tight association of the plastid and spindle poles throughout the cell cycle. Using pharmacological experiments ablating the parasite's microtubules we show that spindles are essential for plastid organization. Using several molecular markers we further show that fission of the single plastid occurs late and concurs with daughter cell formation. Using transgenic S. neurona and in-vivo microscopy and laser bleaching experiments we have further tested our model of organellar fission in living cells. FISH analysis employing probes specific for the 35 kb plastid genome suggest that the organellar genome might be positioned and segregated by centrosomes as well. In contrast to the fission machinery the genome replication apparatus of apicomplexans seems of prokaryotic origin. To develop robust molecular markers for the genome's position we have cloned the putative *T. gondii* plastid histone-like HU, PolA and helicase genes. We show that the *T. gondii* HU protein complements the respective mutant in *E. coli*. Antibodies raised against the recombinant protein show that HU is indeed localized to the plastid and associates with the plastid genome. Overexpression of a HU-YFP fusion protein in *T. gondii* has a pronounced dominant negative effect on organellar genome replication yielding plastids devoid of a genome.

IDENTIFICATION AND FUNCTION OF THE ETMIC4-ETMIC5 PROTEIN COMPLEX. A NOVEL MICRONEME COMPLEX OF *EIMERIA TENELLA*

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Eimeria tenella, like other members of the Apicomplexa, shares a conserved mechanism of motility and invasion which relies on the sequential exocytosis of adhesive proteins from secretory organelles such as micronemes. We have investigated the function of EtMIC4, a 240 kDa transmembrane microneme protein of *Eimeria tenella* that displays a striking multimodular organisation. EtMIC4 contains 16 TSP-1 like domains and 31 EGF-like domains, both of which are found in other soluble and cell surface eukaryotic proteins that mediate protein interactions.. Using non-denaturating anion exchange chromatography and size exclusion chromatography, we identified the presence of a high molecular weight protein complex formed between EtMIC4 and EtMIC5, another microneme protein which contains 11 Apple domains. Cell blot assays showed that EtMIC5 is responsible for binding the complex to cell lines. We have also used recombinant polypeptides expressed in *E. coli* to investigate structural properties of the calcium binding EGF-like domains in EtMIC4. Circular dichroism analysis and mass spectrometry we show that, in the presence of calcium, these EGFs mediate resistance to proteolytic degradation and suggest that this confers on the molecule an elongated shape. This suggests a potential role for the calcium binding EGF repeats in EtMIC4 in forming an extended stalk region within the molecule

CHARACTERISATION OF APICAL MEMBRANE ANTIGENS IN *EIMERIA TENELLA*

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Apical membrane antigen 1 (AMA-1) was first identified within the blood stages of *Plasmodium knowlesi* as a target of invasion inhibitory monoclonal antibodies and homologous proteins are conserved in other species of malaria parasites and in *Toxoplasma gondii.* AMA-1 is a transmembrane protein that is secreted from the microneme organelles during invasion onto the parasite surface from where it is proteolytically cleaved by a muti-functional membrane-bound serine proteinase that is also responsible for the shedding of other parasite surface molecules.

We have identified two *Eimeria tenella* homologues of AMA-1 with all the characteristic hallmarks of the AMA-1 family including N-terminal signal peptides, extracellular domains organised around 16 conserved cysteines and C-terminal transmembrane and cytoplasmic domains. EtAMA-1 is expressed exclusively within the sporozoite stage of the parasite and EtAMA-2 is expressed only within the merozoite stages. Further studies to examine the secretion and processing of these molecules are underway.

INVESTIGATION OF THE ROLE OF PROTEOGLYCANS DURING INVASION OF *EIMERIA TENELLA* SPOROZOITES

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The ability of *Toxoplasma, Plasmodium, Eimeria* and *Neospora* parasites to invade a wide range of epithelial cells *in vitro* suggests that these parasites recognise abundant or widely distributed molecules on the cell surface. A large array of glycosoaminoglycans (GAGs) are found on the surface of nearly all vertebrate cells and many pathogens including *Plasmodium* (Pancake *et al*., 1992 J. Cell Biol 117, 1351-1357)) and *Toxoplasma*, (Carruthers *et al.*, 2000, Inf. Immun. 68, 4005-4011; Ortega-Barria and Boothroyd, 1999, J. Biol Chem 274, 1267-1276) have evolved strategies to recognise them.

Experiments presented here show that GAGS are important receptors for attachment and invasion of *E. tenella* sporozoites in vitro. The most common GAG on the cell surface is heparan sulphate, (HS), and both HS and heparin inhibited the invasion of sporozoites in vitro. In contrast there was no difference in the numbers of sporozoites able to invade GAG-deficient Chinese Hamster Ovary (CHO) cells compared to the CHO parent line over a period of 1 hr. However, further experiments showed that significantly fewer sporozoites adhere to or invade the GAG-deficient lines when the time of incubation is reduced. In addition, sporozoites and cells are incubated with constant rocking, significantly fewer sporozoites adhere to the GAG-deficient cell lines compared to the CHO parent line.

These results suggest that GAGS could play a role in the initial contact of sporozoites with host cells and may be important for facilitating rapid parasite attachment, particularly under 'flow' conditions. They may be significant *in vivo* where the movement of the caecal contents could inhibit adhesion.

CHARACTERISTIC OF *CRYPTOSPORIDIUM* APICAL ANTIGENS USING CHICKEN MONOCLONAL ANTIBODIES AGAINST *EIMERIA*

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Cryptosporidium is a coccidian parasite that causes diarrhea in human and other animals. Although *Cryptosporidium* represents a pathogen of importance to the health of humans and animals, there are no effective drugs and vaccines. In the previous study, we have developed chicken monoclonal antibodies (mAbs) against *Eimeria acervulina* and demonstrated their use in the identification of potential vaccine antigens for avian coccidiosis. Furthermore, we found that these antibodies cross reacted with *Cryptosporidium* parasites. In the present study, we characterized the antigen of *Cryptosporidium,* which is was recognized by one of these chicken mAbs, 6D-12-G10. In indirect immunofluorescent analysis, the chicken mAb 6D-12-G10 showed intense staining on the apical region of *C. parvum* and *C. muris* sporozoites, and merozoites of *C. parvum*. In western blot analysis, 6D-12-G10 antibody identified a 48-kDa molecular weight band of *C. parvum* and *C. muris* soluble antigens. Furthermore, the mAb 6D-12-G10 significantly inhibited the invasion of *C. parvum* sporozoites into HCT-8 cells *in vitro*. Immunoelectron microscopy revealed that the antigen recognized by this mAb was located only at the apical surface membrane of the invasive stages of *C. parvum*. Additionally, we compared the antigen specificity of mAb 6D-12-G10 with several anti-cytoskeletal (actin, myosin, á-, ß-, ã-tubulin) antibodies, and the reactivity of mAb 6D-12-G10 was clearly different from those of anti-cytoskeletal antibodies. These results indicate that the target antigen recognized by mAb 6D-12-G10 is a good vaccine candidate for *Cryptosporidium* infection.

EFFECTS OF NATUSTAT™ SUPPLEMENTATION ON PERFORMANCE, FEED EFFICIENCY AND INTESTINAL LESION SCORES OF BROILER CHICKENS CHALLENGED WITH *EIMERIA ACERVULINA, EIMERIA MAXIMA* AND *EIMERIA TENELLA.*

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Intestinal parasitism is a major stress factor leading to malnutrition and lowering of performance and production efficiency of livestock. This is particularly true in the case of poultry. Coccidiosis is an intestinal infection caused by species of intracellular protozoan parasites belonging to the genus *Eimeria,* resulting in intestinal lesions, diarrhoea, enteritis, and death. The three most common species that affect the poultry industry are *E. tenella*, *E. maxima* and *E. acervulina*.

The effects of dietary supplementation of Natustat™, a propriety plant derived product (Alltech Inc., KY, USA) and Salinomycin, on performance, feed efficiency, and intestinal lesion scores were observed during two *Eimeria* challenge trials in broiler chickens. In the first trial, chickens were challenged with *Eimeria* sp. by infecting the litter with a known amount of *Eimeria* oocysts. In the second trial the source of the *Eimeria* challenge was the litter from the first trial and the same treatment groups were assigned to the same pens as in the initial trial.

Birds were housed seven pens per treatment, with 55 birds per pen. Performance parameters were recorded on days 21 and 42 during both trials. Intestinal lesion scores were assessed on days 14 and 21 during Trial 1 and on day 21 during Trial 2.

Average weight gain and feed conversion ratios significantly improved in the Natustat™ and Salinomycin treatment groups when compared to the non-supplemented infected group. Furthermore, lesion scores were lower on all sampling days in the Natustat™ and Salinomycin groups when compared to the non-supplemented group. However, only lesions associated with *E. tenella* were significantly lowered by Natustat™ and Salinomycin supplementation.

Natustat™ and Salinomycin were equivalent in alleviating the negative performance effects associated with coccidiosis challenge.

THE IMPACT OF THE CROWDING EFFECT OF CHICKEN *EIMERIA* ON THE EFFICACY OF ANTICOCCIDIALS: A HYPOTHESIS

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Chemicals, such as diclazuril (Clinacox[â]), are often used to reduce coccidial infection pressure (IP). Oocyst output (OO) and lesion scores (LS) are used to evaluate levels of IP. Remarkably, without indications of resistance, OO and/or LS are not always reduced when introducing an anticoccidial in a programme.

A crowding effect (CE) may explain certain parasite-host relationships. It can be defined as a lowered fecundity whenever the number of infective parasites exceeds a certain threshold, which may apply to a population of hosts or an individual. With *Eimeria,* CE reducing OO has been demonstrated by several authors.

Several factors may account for CE.

The most striking applies when a heavy infection causes death of the host before oocysts are produced.

Heavy oocyst doses may exceed host-cell availability. *In vivo*, sporozoites of apicomplexans such as *Eimeria* spp. do not invade enterocytes in all segments of the gut. Interaction between proteins on the parasite and on the host-cell surface may explain different infection sites of various species of *Eimeria*. Cell-age dependent expression of host-cell proteins may significantly reduce the number of specific cells available for invasion. Availability of host-cells might therefore be more limited than generally assumed. At a high IP, host-cells may be destroyed before development of the parasite is completed, reducing the number of oocysts produced. Furthermore, if a CE exerts an early influence on the life cycle, it may cause diminution of lesions or pathology, if these are caused by stages occurring later in the life-cycle.

Another factor may be the more rapid response of the immune system with higher infection levels.

Causes of CE may differ for various *Eimeria* species. The effects of CE (altered OO, LS or bird performance) will differ with the stage of the life-cycle impacted by CE.

Whatever cause or effects, reducing the infectious dose per chicken from levels above the CE threshold might cause a "reverse-CE", increasing OO and LS, or even decreasing performance of birds. Reverse-CE can explain why anticoccidials do not always reduce LS and OO when just introduced in a programme, while effectively reducing IP. A false impression of drug-resistance may result.

The relevance of CE for poultry producers has never been fully investigated. Further evaluation of the relation between CE and anticoccidial programmes is therefore required.

COCCIDIOSIS CONTROL IN POULTRY: IMPORTANCE OF THE QUALITY OF ANTICOCCIDIAL PREMIXES

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In commercial poultry production, coccidiosis control is mainly achieved by supplementing feed with chemical anticoccidials such as diclazuril (Clinacox[â]), and ionophore anticoccidials. Resistance is encountered when products (chemicals and ionophores) are overused. Producers carefully consider the balance in using the few anticoccidials available by implementation of rotation, full and/or shuttle programmes.

Because of a strong coccidiocidal effect against all sensitive parasites, chemicals like diclazuril reduce the coccidiosis infection pressure. Generally, stronger coccidiocidal effects result in faster resistance. Since many sensitive parasites escape the action of ionophores, resistance is induced more slowly.

On the other hand, ionophore mode-of-action (interference with ion pumps, essential cell organelles in both parasite and host) explains narrow safety margins for some ionophores. Depending on the field conditions, it is common to treat feed with lower than the most effective dose, optimizing the balance between cost, efficacy and toxicity.

There is no reason to lower diclazuril doses, since the safety margin is at least 20 times the recommended dose of 1 ppm. Moreover, it is crucial to maintain this 1 ppm of diclazuril: next to reduced coccidiosis control, suboptimal dosing has shown to induce diclazuril-resistance.

Additives are usually formulated as simple-mixtures or granulated premixes. Although concept and quality of any anticoccidial are crucial, when mixing at 1ppm diclazuril (the lowest anticoccidial dose on the market), a more sophisticated approach is required. A vacuum-coating production technique, combined with use of strong but porous particles as carrier, assures that diclazuril in Clinacox[â] remains firmly attached to the carrier. Clinacox[â] 0.5%, mixed in at 200 g/ton, therefore provides excellent mixing and minimal segregation, guaranteeing a maximal number of birds receiving the right dose.

A recent survey of 702 samples from Clinacox[â] treated feed from mills worldwide showed that 86.47% of the samples contained diclazuril levels over 0.8 ppm. Samples were sent for a routine check of the mixability of Clinacox[â]or when poor quality of mill infrastructure was suspected. Therefore, the results are likely biased towards lower inclusion levels than generally encountered. Anyhow, conclusions drawn from this survey are that

- a large majority of feed treated with Clinacox[â] is within standards, and
- the development of special vacuum-coated premix for anticoccidials, dosed at very low levels, is crucial in maintaining both good coccidial control and in avoiding resistance problems.

ANTICOCCIDIAL SENSITIVITY PROFILES OF RECENTLY OBTAINED DUTCH, GERMAN, AND SPANISH *EIMERIA* SPP. ISOLATES

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Anticoccidial sensitivity profiles of thirty nine *Eimeria* spp. field isolates collected from broiler farms between 1996 and 2002 were determined. The tested products were: diclazuril (Clinacox®: 1 mg/kg), halofuginone (Stenorol®: 3 mg/kg), lasalocid (Avatec®: 90 mg/kg) , maduramycin (Cygro®: 5 mg/kg), meticlorpindol/methylbenzoquate (Lerbek®: 100 mg/kg), monensin (Elancoban®: 100 mg/kg), narasin ((Monteban®: 70 mg/kg), narasin/nicarbazin (Maxiban®: 40/40 mg/kg), nicarbazin (Nicarb®: 125 mg/kg), robenidine (Robenz®: 33 mg/kg) and salinomycin (Sacox®: 60 mg/kg). The tests were performed in battery cages. The sensitivity profile of each *Eimeria* spp. present in the field isolates was based on the reduction of the mean lesion score of the infected medicated group as compared to the infected unmedicated birds. A reduction percentage of 0-30% indicates resistance, 31-49% reduction indicates reduced sensitivity, and 50% or more indicates full sensitivity. Most field isolates (n = 21) consisted of mixtures of two or three *Eimeria* spp., while eighteen isolates comprised a single species (*E. acervulina* n = 16; *E. maxima* and *E. tenella* in the other cases). Regarding the distribution of species, *E. acervulina* was most frequently found (n = 37) followed by *E. maxima* (n = 15) and *E. tenella* (n = 12). Almost all *Eimeria* spp. found in the field isolates showed resistance or decreased sensitivity against the selected anticoccidial drugs. Exceptions were some *Eimeria acervulina* strains, which were sensitive to diclazuril (2/30), meticlorpindol/methylbenzoquate (17/19), monensin (3/20), narasin (5/25), narasin/nicarbazin (1/16), nicarbazin (3/30), robenidine (3/6) and salinomycine (11/36). All *E. acervulina* isolates tested against halofuginone (n =16), lasalocid (n =19) and maduramycin (n = 4) showed resistance or decreased sensitivity. Some *E. maxima* strains showed sensitivity to diclazuril (1/11), halofuginone (4/5), meticlorpindol/methylbenzoquate (6/9), narasin (2/8), narasin/nicarbazine (5/8), nicarbazin (4/13), robenidine (1/1), and salinomycin (3/14). All *E. maxima* isolates tested for lasalocid (n = 3), maduramicin (n = 1) and monensin (n = 6) showed resistance or decreased sensitivity. A number of *E. tenella* strains were sensitive to diclazuril (4/11), halofuginone (3/3), lasalocid (2/8), maduramycin (1/1), meticlorpindol/methylbenzoquate (7/7), monensin (1/8), narasin (2/10), nicarbazin (2/11), robenidine (1/1), and salinomycin (1/12). All *E. tenella* isolates (n = 4) tested for narasin/nicarbazin showed resistance. The present study shows that resistance against most anticoccidial drugs, except meticlorpindol/methylbenzoquate, is fairly common amongst European coccidiosis field isolates.

TREATMENT OF MICE WITH THE ANTICOCCIDIAL DRUG TOLTRAZURIL DOES NOT INTERFERE WITH THE DEVELOPMENT OF A SPECIFIC CELLULAR INTESTINAL IMMUNE RESPONSE TO *EIMERIA FALCIFORMIS*

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Therapeutic in-water treatment with anticoccidial compounds is an important tool to minimize the damage caused by coccidiosis in animal husbandry. Toltrazuril (Baycox), a symmetrical triazinetrione, terminates ongoing infections with *Eimeria* parasites in avian and mammalian species. Immunity against eimerian infections is highly specific and depends on cell-mediated immunity. In order to investigate the influence of toltrazuril treatment on the development of intestinal cellular immune response, cells of mesenteric lymph nodes, Peyer´s patches, intraepithelial lymphocytes and spleen from infected and treated BALB/c mice were investigated. Infection of BALB/c mice with 1000 oocysts of *Eimeria falciformis* led to protection against a repeat challenge infection. Treatment with the anticoccidial toltrazuril terminated primary infections, but did not interfere with the establishment of protective immunity against challenge infections. Mesenteric lymph node cells of non-treated as well as treated BALB/c mice showed a similar rate of proliferation upon stimulation with parasite antigens. In contrast, neither cells of the Peyer´s patches, intraepithelial lymphocytes nor spleen cells responded to stimulation with parasite antigens at this time point. Cells from all compartments of all investigated groups proliferated and released the cytokines IFN-gamma and IL-4 in response to the mitogen concanavalin A. The number of cells releasing IFN-gamma or IL-4 were not dependent on the status of infection or previous treatment with toltrazuril. The IgG response against total sporozoite antigens in the sera of individual mice showed that a systemic humoral response developed in both infected groups, although the specific IgG antibody concentration was higher for non-treated mice. In conclusion, toltrazuril does not impair the parasite-specific intestinal cellular intestinal immune response and allows equal protection against challenge infection as in non-treated animals.

THE IMPACT OF COCCIDIAL DRUG-RESISTANCE ON THE COMMERCIAL PERFORMANCE OF BROILERS

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Coccidial drug-resistance is often said to be a serious problem to the chicken industry, but broiler performances indicate that resistant parasites are often tolerated by the host. How can this be? The present retrospective synthesis of laboratory and field studies of coccidial drug-resistance, population dynamics and epidemiology is instructive. Some synthetic or ionophorous anticoccidial drugs may control clinical coccidioses while allowing some oocyst "leakage". This phenomenon, when there has been no prior exposure of coccidia to a particular drug, reflects a fundamental drug-parasite interaction which Ryley (1980) termed "drug insensitivity". True drug-resistance involves selection.

Williams (1972) demonstrated pre-existing mutants in coccidial populations, selectable during a single life cycle in the presence of the synthetic quinolone, decoquinate. This was termed "inherent resistance", distinguished from "acquired resistance", a gradual physiological adaptation over several generations facilitating preferential survival of the least sensitive individuals, not necessarily mutants. Ionophores, which affect ion transport across cell membranes, are fundamentally different from chemicals active against cofactor synthesis or electron transport. Selection of physiological variants during a single passage in monensin-medicated birds results in far less reduction in sensitivity than selection of decoquinate-resistant mutants (Williams, 1998). Thus, ionophore-resistance is probably "acquired", and quinolone-resistance is "inherent". Hence, in all cases of drug insensitivity, inherent resistance or acquired resistance, oocysts are produced in the presence of drug under commercial conditions. Their effect is crucial.

Oocyst production by chickens first increases, until marked reductions occur towards the end of each crop, due to development of flock immunity, stimulated by early exposure to oocysts that have avoided drug action. Adverse litter conditions further reduce numbers of viable oocysts, but small numbers remain at the end. The pattern is similar with untreated birds and those receiving live anticoccidial vaccines. The often innocuous immunization occurs as a result of the initiation of strongly immunogenic trickle infections by between-crop carry-over of residual oocysts. The phenomenon is most marked if litter is replaced between crops.

Other factors moderating the impact of resistant populations include a physiological compensation that allows infected chicks to attain increased growth

rates during recovery from coccidiosis, and the effect of *E. acervulina* in evidently suppressing more pathogenic *Eimeria* species under certain conditions (Williams, 1973). Adverse effects of drug-resistant parasites might, therefore, be ameliorated by such natural events, supported by careful husbandry.

CHICKEN COCCIDIOSIS: A FRESH LOOK AT LESIONS

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Macroscopic coccidial lesions in chickens are usually assessed by the subjective scoring systems developed by Johnson & Reid (1970). Johnson & Reid's system was developed using naïve birds; the grades correlate negatively with weight gain or FCR. In immune birds (drug-treated or vaccinated) lesion scoring can be misleading because mild lesions may sometimes occur in birds with normal weight gain or FCR. Furthermore, any lesions in vaccinated birds that are clinically protected frequently contain few or no observable parasites, whereas those in naïve birds are packed with parasites.

Association of specific pathogens with a parasitic lesion is not, by definition, a necessity, but may help with lesion identification. The similarities in gross appearance of lesions and the differences between associated parasite burdens in naïve and immune birds suggest that coccidial lesions are due more to host responses than to the parasite itself. The lesions sometimes present in immune birds in the field are presumably the result of successful repulsion by the host of a parasite challenge. What, then, accounts for the gross appearance of lesions; and are there fundamental differences between those occurring in naïve and immune birds?

Perhaps in naïve birds, white plaques caused by *Eimeria acervulina* or *E. necatrix* are due to endogenous parasites (schizonts, gametocytes or oocysts), fibrin and/or invading leucocytes. Red lesions (*E. brunetti*, *E. maxima*, *E. necatrix*, *E. tenella*) may be due to haemorrhage resulting from rupture of capillaries or diapedesis. But in immune birds with lesions superficially similar to those in naïve birds, immunological responses alone may be responsible for the white lesions, and inflammation (vasodilatation, hyperaemia, congestion) may account for the red lesions. But why do *E. mitis* and *E. praecox* not cause gross lesions? Explanations might be provided by histological and immunological techniques.

A few commercially-vaccinated birds may develop lesions directly resulting from vaccination, but attenuated vaccines do not harm performance. Lesions may also result later from natural field challenge in some vaccinated birds, again without affecting their performance. Clearly, lesion scoring alone under-rates vaccine efficacy, and should not be used to judge the immune status of birds, but do lesions in vaccinated birds damage the gut epithelium? Direct microscopical examination under physiological saline of the fresh intestinal mucosa of affected birds facilitates immediate clinical assessment of their gut integrity, and might provide the answer.

THE STEALTH CHICKEN *EIMERIA*: *E. MIVATI* - A NEW PERSPECTIVE

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Summary: For several decades, the validity of *Eimeria mivati* had been challenged and some researchers have accepted this species to be invalid. Over the past decade or more, several field isolates that morphologically fit the description of *E. mivati*, have been secured. Three of the recent field isolates were purified and aliquots prepared for evaluations via PCR assays to determine speciation. Thirteen samples of *Eimeria* including three *E. mivati* were submitted blindly to an independent laboratory for PCR evaluations. The current primers are for *E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*. The results showed, the samples detected were *E. acervulina*, E*. maxima* and *E. tenella,* but the samples with *E. mivati* were undetected. Chickens not protected against *E. mivati* by either pharmaceuticals or immunization may suffer morbidity and even mortality.

During 1988 and 1990, Fitz-Coy (University of Maryland Eastern Shore), secured three isolates thought to be *E. mivati* from commercial broiler farms on the DelMarVa Peninsula (DMV). Immunization and cross-immunization trials with one of the isolates against E*. acervulina* showed that chickens immunized with *E. acervulina* and challenged with *E. mivati* were not protected. However, chickens immunized with *E. mivati* and challenged with *E. mivati* showed good protection. Between 1991 and 2001, Fitz-Coy isolated several field isolates of *Eimeria* fitting the description of *E. mivati.* These isolates were from broiler farms from the state of Georgia and the DMV, three of these were selected. The selected isolates were further studied, including species determined via polymerase chain reaction (PCR) assay. In 2004, 13 samples of coccidia, identified only by a numbering system were submitted to an independent laboratory for PCR assays. Most of the samples were single species, but a few were multiple species. The identities of the samples were unknown and blindly evaluated. Following evaluations, the results were compared to the key to match the results with codes. The only samples that were unidentified via the PCR assay were *E. mivati.*

E. mivati is moderately pathogenic for chickens and on some occasions, has caused mortality. In one such study, a mortality rate of 40% occurred in a group of naïve chickens; however, there was no pathology in the hyper-immunized hatch-mates.

THE ROLE OF *E. MITIS* IN CHICKEN COCCIDIOSIS: A STUDY OF PATHOGENICITY

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E. mitis is one of seven known *Eimeria* species that infects chickens. Its second Latin name, "*mitis*," means mild, because investigators of this pathogen have always considered it to be of minor significance in poultry.

E. mitis, however, is common in broilers and breeders. It affects the large intestine, and produces large amounts of small, almost round, oocysts, but it is hard to identify because it does not cause gross lesions. In addition, some studies indicate that *E. mitis* is more pathogenic when it occurs concomitantly with *E. acervulina*.

To further investigate *E. mitis*, a two-phase trial was conducted. In the first phase, three groups of 14-day- old Ross 508 chickens were infected either with *E. mitis*, *E. acervulina* or saline solution (negative control); on days 4 and 14 after challenge, oocyst shedding and bird performance were evaluated.

Oocyst counts demonstrated successful challenge with both *Eimeria* species; peak numbers were hundreds of thousands oocysts per gram (OPG) for *E. mitis* and millions of OPG for *E. acervulina*.

Compared to controls, chickens infected with *E. mitis* and *E. acervulina* had significantly lower daily weight gain, feed intake, and water consumption, higher feed conversion ratio, and lower final body weight. There was a numerical, but not statistically significant difference in water and feed intake between the two infected groups.

In the second trial, three additional groups of 14-day-old Ross 508 chickens were challenged with *E. mitis* and *E. acervulina*, and then injected with Pontamine Sky Blue dye, enabling evaluation of the intestinal mucosa. *Eimeria*-infected chickens exhibited colour differences in the mucosal surface as compared to controls. Permeability was determined by increased transfer of dye, which specifically binds to serum proteins and migrates outside blood capillaries. Dye leakage between 72 and 144 hours post-infection stained intestinal mucosa and gut contents, confirming cell damage and increased gut permeability due to multiplication of both *Eimeria* species.

Both *Eimeria* infections also caused a significant gut wall thickening as measured by stereoscopic microscope due to oedema and inflammation from 72 and 144 hours post-infection.

The results indicate that *E. mitis* can impair chicken performance and cause losses just like other, better known *Eimeria* species. Consequently, the name *E. mitis* does not, in fact, reflect the real pathogenicity of this *Eimeria* species, hence, *nomen non est omen*.

THE COMPARISON AND VALIDATION OF METHODS FOR MONITORING COCCIDIOSIS IN BROILERS

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Coccidiosis is an ubiquitous disease of poultry and of extreme economic importance. In an attempt to control the disease various methods have been used to monitor it.

The objective of this study was to determine the correlation of different monitoring methods in experimental coccidiosis. Six hundred and eight male chicks were raised in an environmentally controlled facility. The effect of various doses of *Eimeria maxima* and thus severity of infection was assessed by comparing microscopic lesion scoring (MLS), gross lesion scoring (GLS), faecal oocyst counts (FOC), litter oocyst counts (LOC), feed conversion ratio, and body weight gain at days 10, 14, 21, 28 and 42 of age. MLS was performed both manually and with an image analyzer.

A negative correlation was found between the median GLS and the log functions of FOC and LOC. A very strong positive correlation (0.81) was seen between the median MLS and the log functions of FOC and LOC. No significant differences were found between the FCRs of the chicks receiving various oocyst doses. With a correlation of 0.987, there was no statistically significant difference between the manual and image analyzer MLS methods.

COMPARISON OF *E. ACERVULINA* OOCYST COUNTS IN SINGLE DROPPINGS OF BROILERS AND IN DROPPINGS COLLECTED DURING 24 HOURS

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At present, coccidiosis in broilers is mainly controlled by anticoccidial drugs in the feed. Development and evaluation of new intervention strategies, e.g. vaccination, are necessary, due to the increasing resistance of *Eimeria* spp against anticoccidials, legislative restrictions, and consumer's objections against medicated feed.

Transmission experiments can play an important role in development and evaluation of these strategies. In future transmission experiments, both infected and susceptible birds will be housed together on litter to quantify the rate of transmission. Therefore determination of OPG in individual chicks is needed. This study was carried out to compare the OPG in droppings collected during 24 hours with the OPG of a single dropping, produced during one hour of individual housing in a cardboard box.

Thirty SPF broiler chicks, individually housed in wired floor cages, were orally inoculated at 6 days of age (D6) with 50, 500 or 50,000 sporulated *E. acervulina* oocysts or received trickle infection during 10 days with 50 oocysts. Droppings produced during 24 hours were collected from underneath the wired floor and were mixed thoroughly. Furthermore, each chick was individually housed in a cardboard box during one hour to produce a single dropping.

The OPG of 4 g of the 24h sample and of the single dropping was determined daily from D11-D27 using the McMaster counting technique.

The ^{10}Log(OPG+1) of the single dropping was significantly associated ($P<0.001$) with ^{10}Log(OPG+1) of 24-hour faeces for all inoculation doses during the entire course of the infection. This association is described by a linear regression line: $^{10}\log(OPG+1)24h = 0.1938 + 0.8779 \times {}^{10}\log(OPG+1)$single dropping.

Therefore we conclude that the OPG of a single dropping can be used to determine oocyst production in individual chicks, housed in the same litter pen, e.g. in transmission experiments.

REASONS FOR FIELD PROBLEMS WITH *EIMERIA MAXIMA*: *E. ACERVULINA* VERSUS *E. MAXIMA*

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The three most commonly occurring species of *Eimeria* infecting chickens are *Eimeria acervulina, E. tenella,* and *E. maxima*. Even though *E. maxima* is very immunogenic, lesions are often observed in the field late in a growout. A survey of 50 coccidial field isolates showed that 36 were predominately *E. acervulina*, 4 *E. maxima*, and 10 *E. tenella*. All of the *E. maxima* isolates came from farms where the broilers were over 28 days old. Most of the *E. acervulina* isolates were from broilers that were 18 to 28 days old . The daily oocyst shedding pattern for a commercial coccidial vaccine was examined in floorpen birds. Birds vaccinated for coccidiosis at the hatchery were placed into pens on new pine shavings. The shedding of *E. acervulina* type oocysts peaked around 18 days. A small peak of *E. maxima* was observed around 28 days. A battery cage study was conducted to examine whether *E. acervulina* could be interfering with *E. maxima* development. Birds were challenged at 14 days of age with *E. acervulina* and/ or *E. maxima*. The oocyst per bird challenge levels were none (Trt. 1), *E. acervulina* 100,000 (Trt. 2), *E. acervulina* 100,000 plus *E. maxima* 5,000 (Trt. 3), *E. acervulina* 50,000 plus *E. maxima* 5,000 (Trt. 4), *E. acervulina* 25,000 plus *E. maxima* 5,000 (Trt. 5), and *E. maxima* 5,000 (Trt. 6). Each treatment consisted of 3 replications in a complete randomized block design. *E. maxima* alone caused 21 % weight reduction and 2.75 lesion score. The 100,000 and 50,000 *E. acervulina* oocyst level reduced *E. maxima* lesions to 1.33. The 25,000 *E. acervulina* oocyst level only slightly reduced *E. maxima* lesions to 2.25. The *E. maxima* did not interfere with any of the *E. acervulina* infections. This study suggests that *E. acervulina* interferes with colonization or development of *E. maxima*. As birds become more immune to *E. acervulina,* then *E. maxima* has more of an opportunity to develop.

Contributed Papers: Posters

COMPARISON BETWEEN THE MODIFIED ZIEHL-NEELSEN AND FLUORESCENT ANTIBODY TESTS FOR THE DETECTION OF *CRYPTOSPORIDIUM* SPP. OOCYSTS IN FAECES.

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In both human and animal laboratories *Cryptosporidium* oocysts have for many years been identified in faeces using the modified Ziehl-Neelsen staining method (mZN). Collaborative work between the UK Health Protection Agency (HPA) and the Veterinary Laboratories Agency (VLA) has compared this traditional method with the Fluorescent Antibody Test (FAT), which is now considered by the OIE as the "Gold Standard". Faeces from experimentally *Cryptosporidium*- infected lambs were examined daily using both methods up to 21 days post infection (pi). Each sample was given a simple score ranging from 0 to 5 depending on the number of oocysts observed and results using both methods were compared. Results indicate that the FAT was more sensitive than the mZN and was able to detect the presence of oocysts 1–2 days pi earlier. Furthermore, it was also possible to detect *Cryptosporidium* oocysts deep in intestinal tissues of infected animals by the FAT, but not the mZN.

OCCURRENCE OF *TOXOPLAMA GONDII* ANTIBODIES IN FATTENING PIGS FROM THE STATE OF SÃO PAULO, BRAZIL

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Pigs are an important source of meat-borne *Toxoplasma gondii* infections in humans, but little is known about *T. gondii* infections in fattening pigs in Brazil. Antibodies against *T. gondii* were determined in the sera of 286 male and female fattening pigs of 6-8 months of age raised in 17 counties of the State of São Paulo, Brazil. Sera were tested for *T. gondii* antibodies by the modified agglutination test (MAT), which measures *T. gondii*-specific IgG antibodies. Antibodies (MAT $\geq$ 25) were found in 49 (17%) pigs with titers of 1:25 in 3, 1:50 in 3, 1:100 in 2, 1:200 in 8, 1:400 in 8, 1:1600 in 14, 1:3200 in 3, and 1:6400 in 3 pigs. Most (107 out of 286) of the pigs were from Areiópolis and 17% were seropositive. Antibodies were not found in 25 pigs from five counties (Itaguaí, Itararé, Lácio, Pereiras and Taboão da Serra). The results indicate 17% of fattening pigs from the Sate of São Paulo had contact with *T. gondii,* and are a potential source of infection to humans.

PREVALENCE OF COCCIDIA IN FRENCH CALVES: RESULTS OF SEVERAL SURVEYS

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Coccidia are parasites which have an underestimated economic importance in ruminants.

The aim of the surveys was to have a better knowledge of oocyst shedding in dairy calves during weaning and in sucking calves aged 4 to 7 weeks.

On farms where animals did not show clinical signs of coccidiosis, faecal samples were taken from 2 to 10 calves. The 4 labs, each of which participating in one of the 4 surveys, all used an iodomercurate flotation method. Identification of the different species of coccidia was carried out by examining the oocysts.

3 surveys were performed on milk calves.

In 1997, on a total of 338 calves tested on 66 farms all over France, 94 % of the calves and 61 % of the farms were positive, *Eimeria* Bovis being observed in 25 % of the cases, E.subspherica in 21 %, E.zuernii in 18 %, and finally auburnensis in12 %.

In 2001, on a total of 54 farms in the Massif Central region, 51 were positive, 47% of them with an oocyst-per-gram count between 100 and 1,000 and 6 % with one greater than 1,000.

In 2002, in a survey carried out in Brittany (western France) the 18 farms assessed were all positive (96 heifers) with 8 % of the calves shedding between 1,000 and 10,000 oocysts per gram. *Eimeria* bovis was found on 18 farms and on 55 % of the calves, followed by E.alabamensis, then subspherica and finally zuernii and auburnensis.

The survey on sucking calves was carried out in the Allier region (central France) using groups of 7 calves aged 4 to 7 weeks from 10 farms. There again all farms were positive and the oocyst count was significant : 14 calves out of 70 shed between 1,000 and 10,000 oocysts per gram of faeces and 5 calves out of 70 shed between 10,000 and 100,000.

The various species of coccidia were frequently associated on a same animal and on the farms. Thus, in the sucking calves survey, all farms hosted 3 different species (up to 5 in heifers in 2002).

These surveys carried out in different regions, by different laboratories, show that coccidia are present on the almost total of calves farms.

They provide a better understanding of growth improvements obtained on several farms with decoquinate (a synthetic molecule active on certain protozoa: coccidia, cryptosporidia, toxoplasma… and having no antibiotic action), and this, even in the absence of clinical signs of the disease.

STUDY ON THE ABILITY OF WATER SUPPLY TO CONTAMINATE POULTRY FLOCKS WITH COCCIDIA

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Coccidia belonging to the genus *Eimeria* are the most frequent parasites found in poultry. Their occurrence can be of great economic importance, and they must be controlled control is in order to maintain performance and profitability in poultry industry. One way to control the side effects of these parasites is to reduce the contact of oocysts with the birds. There are several routes of contamination of poultry flocks with *Eimeria* oocysts; some of them are known, but others remain to be investigated.

The objective of this study was to investigate the potential carrier role of drinking water in coccidiosis. This work was a first approach, focusing on farms supplied with forage water or surface water. We did not include farms supplied by the local network of treated water. Twenty-four farms were investigated. Filters were placed at water entrance in the building, allowing the capture of oocysts. The technique was set up and validated under laboratory conditions, using selected filters and different sizes of oocysts.

Fecal samples collected in the investigated farms showed that coccidia were present in 75% of the flocks. Four water samples were positive for coccidia, as shown by passage of the samples on coccidia-free birds. The species found in the water was *Eimeria acervulina*, which was also present in the litter of the considered farms.

These preliminary results suggest that water can be a potential carrier of coccidia for chickens. However, further investigations are needed to confirm this potential role and to evaluate the impact of climatic factors. Other forms of water supply could also be considered in future studies.

EVALUATION OF A RAPID SCREENING ASSAY FOR *CRYPTOSPORIDIUM* IDENTIFICATION (DOT-ELISA) USING MONOCLONAL ANTIBODY

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A rapid screening assay for *Cryptosporidium parvum* was evaluated using monoclonal antibody with the objective of standardizing a Dot Enzyme-Linked Immunosorbent Assay (Dot-ELISA) to detect antigens of fecal parasite samples with different concentrations of this parasite, and its efficacy.. The immunoenzymatic Dot-ELISA test, derived from the Dot-Immunobinding Assay with a modification of the Dot-hybridization to test monoclonal antibodies, offers extensive applicability in laboratory diagnosis. The efficiency of the Dot-ELISA was evaluated by using oocyst concentrations of 25, 50, 10^2,10^3,10^4 and 10^5 cysts. As a support for the technique, a nitrocellulose membrane 0.22μχ pore size was cut into 7 × 0.5 cm strips and carefully placed in the canals of an acrylic tray. Prior to this, areas of 0.5 × 0.5 cm were marked off on the strips and onto each of these squares, samples obtained from fecal material antigen or pure parasites were applied with a micropipette (1ml). The antigen was then fixed in oven at 37°C for 15 minutes. This was achieved by adding a blocking solution selected through pre-testing: (gelatin at 5% in Tris-buffered-saline, pH 7.5) for 60 minutes at 37°C under constant shaking, with mild movements on a stirring plate. The nitrocellulose strips in the acrylic tray were washed 3 times with Tris-buffered-saline (TBS pH 7.5) for 10 minutes under constant shaking. The diagnosis of intestinal parasites was performed by microscopic examination of the stool, which is recognized as the gold standard method. Confirmation of the presence of *C. parvum* in feces samples is labor-intensive, time-consuming, costly, and often difficult, highly dependent on training and expert knowledge in morphologic differentiation. The Dot-ELISA is simple and rapid to use, and offers a less subjective method than microscopy for detecting the protozoan in fecal samples submitted to a busy diagnostic laboratory. It is a highly sensitive and specific technique, and it is useful for screening large numbers of specimens in a short period of time. Also, it does not really need special microscopy skills. Overall,

sensitivity of the immunoenzymatic test was 100%, with a specificity of 98%. In conclusion, our study showed that Dot-ELISA is clearly a reliable and rapid method for the detection of Cryptosporidium in fecal samples.

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EIMERIA SPECIES IDENTIFICATION IN FAECES AND LITTER SAMPLES FROM CHICKENS

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Coccidial infections in chickens are caused by one or several *Eimeria spp*. The oocyst wall of *Eimeria* spp. is particularly rigid and resistant, which makes it difficult to achieve an effective DNA extraction. A classic but time-consuming method for *Eimeria* species identification is by first breaking the oocysts by glass bead grinding, followed by phenol DNA extraction and identification of species-specific genomic regions by PCR. This study aimed to find a fast, robust and efficient method for identifying chicken *Eimeria* spp. in field samples. The methods were evaluated according to inter-species variations in detection level, repeatability, hands-on-time and cost efficiency. Five methods for rupturing oocyst walls were tested, including sonication, microwaves, heating, pestle- and glass bead grinding. Two series of suspensions containing faecal debris and with known number of oocysts from *E. mitis*, *E. preacox*, *E. maxima* and *E. tenella* were grinded with glass beads and pestle, respectively. DNA was extracted from oocysts ruptured with pestle using either commercial systems (GeneReleaser, Qiagen Stoolkit and Prepman) or phenol extraction, and then compared to the classical method. Detection levels were evaluated by identifying species-specific ITS-1 regions using optimized single species PCRs. The StoolKit protocol showed poor reliability and high variability in detection levels, and is expensive and relatively time-consuming. Although the Prepman protocol requires minimal hands-on-time and it is cheap, detection levels were not consistent among the species. The detection level for the GeneReleaser protocol was very stable, both between species and within the method, detecting less than 2 oocysts of each species per PCR. The phenol DNA isolation method, using either method for oocyst rupture, showed similar results as the GeneReleaser protocol. Our results suggest that isolation of DNA using the GeneReleaser kit combined with a pestle grinder is a repeatable and cost-efficient method with limited inter-species variation in detection level. Importantly, it also provides minimal hands-on-time in the pre-PCR process.

DETERMINATION OF THE SENSITIVITY OF THE SERIAL SCRAPING METHOD OF INTESTINAL MUCOSA IN THE DIAGNOSIS OF SUBCLINICAL COCCIDIOSIS

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In industrial poultry production, serious clinical presentations of coccidiosis (degrees 2, 3 and 4) have opened the way to less intense levels of disease. Subclinical coccidiosis, had particular importance as the disease cannot be controlled, and new forms can develop due to the parasite's resistance to anticoccidials drugs. Its diagnostic cannot be performed by conventional methods due to the lack of symptoms and lesions, and to small amount of shed oocysts. These are not detected by the coproparasitological analysis. The objective of this work was to demonstrate the sensitivity of Serial Scraping Method of Intestinal Mucosa (SSMIM) in mild cases of the disease as compared to the coproparasitological count using MacMaster chamber. From 1991 to 2001, we performed systematic and weekly diagnosis of coccidiosis in chicken broiler farms in the province of Buenos Aires, Argentina. We collected 486 samples of 6 birds per house and farm. The research of coccidiosis was performed by SSMIM. This technique involved the serial scraping of the mucosa in 1 of every 6 birds of 4 sites in the duodenum, 4 in the jejunum-ileum, and 2 in the ceca. These scrapings were observed in optical microscope under 100-x magnification, which allowed not only the visualization of oocysts, but also other stages of parasite development. At the same time, a pool of fecal matter from 6 birds submitted to the coproparasitological count using a MacMaster chamber. The results showed that, MRSMI allowed the detection of 47 % of the subclinical cases of coccidiosis in the analyzed farms. On the other hand, using the method of parasitological count, only 10.4 % of the samples were positive. We conclude that SSMIM is the best tool to the diagnosis of subclinical coccidiosis due to its higher sensitivity, allowing not only the observation of oocysts, but also the detection of the intracellular development stages before the protozoan excretion starts. This provides an earlier diagnosis of this parasitic disease.

IDENTIFICATION OF COCCIDIA SPECIES IN BROILER CHICKENS IN ARGENTINA

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We need not to assert that coccidiosis is highly prevalent in poultry production. Therefore, it is important to determine the presence and the frequency of the species present in any region. The identification of species depends on the ability to use traditional diagnostic methods. The development of molecular techniques in different studies requires simple techniques for DNA extraction. The identification of coccidia in broiler chickens in the provinces of Buenos Aires and Entre Rios was made using conventional methods, and the presence of *Eimeria* sp. was confirmed using molecular biology. In both provinces, 63 samples of 74 farms were positive for presence of coccidia, whereas the remaining 11 were negative. All seven species were present in variable percentages. Characteristic lesions of *Eimeria acervulina* and ovoid oocysts were present in all 59 positive samples (93.65%). Typical ovoid oocysts of *Eimeria maxima* and typical lesions in the jejunum-ileum were present in 34 samples (53.97%). *Eimeria mitis* was present in 24 farms (38.10%), where it produced typical sub spherical oocysts before 93 hours post-inoculation. *Eimeria tenella* was confirmed in 15 samples based on oocyst size and typical lesions in the cecum (hemorrhages, coagulated blood in the lumen, and thickened cecal mucosa). *Eimeria praecox* was conclusively identified in 14 samples (22.22%) by the typical oocysts present in the feces 84 hours post-inoculation. Lesions in the lower small intestine of 5 samples (7.94%) were typical of *Eimeria brunetti,* and *Eimeria necatrix* was present in 5 farms (7,94%). Finally, the high prevalence of *Eimeria acervulina* and *Eimeria maxima* was related to the high incidence of mild clinical and subclinical cases. The conventional methods are not adequate for a definitive identification for the differentiation between *Eimeria acervulina* and *Eimeria mitis*. The low incidence of *Eimeria praecox, Eimeria brunetti,* and *Eimeria necatrix* warrants the reconsideration of the inclusion of these species in the specific immunogen for broiler vaccination. The presence of *Eimeria* DNA, generated by the amplification of regions ITS1 using primers EF1 and ER1, and ITS2 with primers WW2 and WW4r developed in the

University of Melbourne, confirm the presence of these species in our country. It is concluded that the methodology used may be the starting point for the identification of specific primers aiming at the development of vaccines based on native strains.

DEVELOPMENT OF A HISTOLOGICAL SCORING MODEL FOR USE IN INTERPRETING THE IMMUNE RESPONSE

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A number of the stages of *Eimeria* growth can be identified visually using gross lesions. These observations are the tried and true method of intestinal lesion scoring. Histology can identify lesions not visible to the eye and can visualise infections at an earlier stage of the life cycle. Some lesions uniquely identify the species. Therefore, it should be possible to identify mixed infections using histology. This hypothesis was investigated using a mixed infection of *Eimeria* with a high chance of being differentiated using histology. In addition, it was hypothesised that with the onset of an immune response, the number of microscopic lesions in tissues would decrease. EIMERIAVAX 4m® is a 4 species precocious coccidiosis vaccine of Australian origin and contains *Eimeria acervulina*, *E maxima*, *E necatrix*, and *E tenella*. This vaccine was used to validate the accuracy of histology. A trial was designed in 2 parts: the first inoculated ten (10) 7-day-old chickens with a times-10 dose of vaccine. At 5 and 7 days post- inoculation, tissues were fixed and stained with PAS or H&E. The number of chickens showing lesions was enumerated, and the species were identified. This trial was repeated with different batches of vaccine ten times. A second trial examined the relationship between the histological lesions present and the onset of immunity following eye-drop vaccination with EIMERIAVAX 4m. These birds were challenged with a times-80 dose of the same vaccine.

In trial 1, 100% of chickens were infected following an oral dose of vaccine. In trial 2, an immune response was developed which resulted in the dramatic reduction in the number of lesions detectable in immune birds. As the result of this data, a simplified scoring model was developed and is used for vaccine testing.

The results indicate that histology detects consistent infections, is a useful technique for examining multiple species infections, and has the potential to be used to examine the onset of an immune response. Unlike faecal oocyst counts, histological slides can be archived, which facilitates Good Laboratory Practice (GLP) compliance.

The conclusion is that histological lesion scores are repeatable. This paper also presents arguments in favour of the use of the homologous challenge models to simplify the traditional challenge models using wild *Eimeria* sp.

PROGRESS TOWARD THE DEVELOPMENT OF SEROLOGICAL TESTS FOR DIAGNOSIS OF COCCIDIOSIS IN CHICKENS

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Vaccines comprising attenuated strains of each of the seven species of *Eimeria* that cause coccidiosis in Australian chickens have been shown to be effective in preventing acute disease. Improved diagnostic tests are now required for monitoring vaccine efficacy, epidemiological studies and for the implementation of effective control strategies. As a first step toward developing species-specific serological tests we have investigated the chicken antibody response to infection. Birds infected with the *E. tenella* vaccine strain produced parasite-specific antibodies between 16 and 30 days post-infection. Experiments with parasite antigens prepared from heavily parasitized gut linings harvested from birds sacrificed at varying times post-infection suggested that the antibodies were directed exclusively against gametocyte antigens. Immunostaining infected gut sections confirmed this observation. Merozoites, gametocytes and oocysts were all present in the sections but antibodies bound only to gametocytes. Monoclonal antibodies that recognize *E. tenella* gametocytes epitopes have been developed and characterized by immunofluorescent and immunoperoxidase staining of parasitized tissue and purified gametocytes and in Western blots. Implications for the development and application of species-specific serological tests for coccidiosis are discussed.

CHARACTERIZATION OF CRYPTIC FIELD ISOLATES OF *EIMERIA* SPP. FROM CHICKENS.

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The identification of variants of *Eimeria* species has important implications in discovering emerging strains not covered by current vaccines. It was therefore important, in Australia, to determine if local strains reflected overseas trends in this regard.

We have been studying the species composition of coccidiosis outbreaks in Australian chickens. The technique we employ involves the isolation of DNA from faecal oocysts and a PCR/electrophoresis analysis of the ITS-2 region of the parasite genome. All previously analysed strains, encompassing the seven known species of chicken *Eimeria*, are present species-specific banding patterns, with only minor variation in band positions between strains.

From the analysis of field samples, we have obtained isolates that represent two novel banding patterns. Pure isolates of these two cryptic taxa were obtained first by passage of mixed-species oocysts through hyperimmunized birds, and then isolation and amplification of single oocysts. The isolates were then characterized by biometrics, macro- and microscopic gut lesions, prepatent period, reproductive index and genetic sequencing. This poster details the isolation, characterization, and taxonomic affiliation of these two taxa.

EXPERIMENTAL PRODUCTION OF NECROTIC ENTERITIS AND ITS USE FOR STUDIES ON THE RELATIONSHIPS BETWEEN NECROTIC ENTERITIS AND COCCIDIOSIS IN CHICKENS.

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Necrotic enteritis is a potentially fatal disease of poultry, and is both a welfare and economic problem. The disease is an enterotoxaemia caused by *Clostridium perfringens* types A and C, and manifests itself when the presence of high numbers of bacteria coincides with significant damage to the gut epithelium. Gut damage caused by various species of the coccidian genus *Eimeria* has been implicated. A new method for the experimental production of necrotic enteritis in chickens is described, and this was used to examine the relationship between clostridial and coccidial infections. Groups consisted of an uninfected control, a group which received *Cl. perfringens* only, another which received *Eimeria maxima* only, and a fourth which received *E. maxima* followed by *Cl. perfringens.* In all cases the *Cl. perfringens* was administered directly to the intestine using a catheter, and the disease monitored. Parameters measured were clinical symptoms, lesions and weight gain. Results: Coccidiosis caused by virulent *E. maxima* exacerbated lesions of necrotic enteritis and other clinical effects caused by a subsequent challenge of virulent *Cl. perfringens* type A. This group had the highest lesion score, and also a highly significant weight difference when compared to all other groups.

CLINICAL AND PATHOLOGICAL CHANGES IN CHICKENS (*GALLUS DOMESTICUS*) EXPERIMENTALLY INFECTED WITH *EIMERIA ACERVULINA* (TIZZER, 1929) OOCYSTS.

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In the present study, clinical signs and pathological changes were evaluated for thirty days in chickens experimentally infected with *E. acervulina*. One hundred and eighty Cobb male broiler often days of age were randomly distributed into three groups (A: inoculated with 1 x 10^6 oocysts; B: inoculated with 1 x 10^5 oocysts; C: inoculated with distilled water) of sixty birds. Iso-nutritional and iso-energetic diets without anticoccidial drugs were offered *ad libitum* to the chickens. Two chickens of each group were daily sacrificed. The pathological and clinical changes were identical in all groups, despite being more intense in group A. On the 1st DPI, both infected groups showed duodenum hyperemia, which developed as congestion on the 2nd DPI. The congestion remained present until the 30th DPI, and was associated to thickening of the gut mucous membrane. On the 2nd DPI, the two infected groups still presented petechial hemorrhages in the duodenum, which progressed to the jejunum and ileum. After the 5th DPI, a mucus exsudate was present in the lumen of the small intestine, especially in the duodenum. Other findings were food retention in proventriculus and gizzard, gall bladder full of liquid, pale yellow liver, and pale muscles. The clinical signs after the 4th DPI were: anorexia, apathy, and diarrhea. Other findings were digested blood in the feces, associated or not with the presence of small whitish streaks. Microscope examination showed immature oocysts attached to the intestinal mucous membrane. The results demonstrate that chickens experimentally infected with *E. acervulina* present progressive intestinal lesions of variable intensity, and that these abnormalities are the main cause of reduction of bird performance.

ASSOCIATION BETWEEN COCCIDIA AND INTESTINAL HELMINTS IN BROILER CHICKENS

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The highly intensive systems in which commercial birds are reared favors the simultaneous occurrence of many diseases. Coccidiosis has a largeconomical importance and it has been studied in combination with a great number of affections, such as salmonellosis, Marek´s disease, Clostridium, reovirus infections, tec.. Until now, the relationship between the clinical coccidiosis and other intestinal parasites was not studied. The objective of this study was to demonstrate by monitoring of broiler chickens, the relation between different clinical presentations of coccidiosis and intestinal helminths. Between 1991 and 2001, we performed systematic and weekly diagnosis of coccidiosis and helminthiasis in broiler farms in the province of Buenos Aires. We collected 486 samples of 6 birds per house and farm.. The presence of coccidia was investigated using the Serial Scraping Method of the Intestinal Mucosa (SSMIM), whereas macroscopic parasites were identified first. Out of the collected samples, 80 % were positive for coccidiosis, out of which47 % were subclinical,, 27 % clinical degree 1, and 60 % clinical degree 2 and 3. The ratios were maintained relatively constant during the study. In all analyzed samples, the duodenum was affected. The other intestinal sections were always also involved. Twenty five per cent of the farms were infected with variable proportions of *Ascaris*, *Heterakis,* and tapeworms during the 10 studied years, although the first half of the period there was a higher frequency of findings. The relationship between the presence of coccidia and helminths was similar either in negative coccidiosis cases or in any clinical presentation. Therefore, the presence or absence of helminths was independent from the diagnosis of coccidiosis. We concluded that, during the studied period, there was a high prevalence of coccidiosis, which was subclinical or mild (degree 1), being the latter more frequent. The duodenum was affected in 100 % of the chickens, in which we found possible predominance of *Eimeria acervulina*. On the other hand we did not detect any relationship between the macroscopic presentation of coccidiosis and intestinal parasites.

PHENOTYPIC CHARACTERIZATION OF CHICKEN SELECTION LINES INFECTED WITH *EIMERIA TENELLA*.

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The objective of this study was to characterize the susceptibility to coccidiosis of one broiler line (TT; 12 generations of selection) and two layer lines (CC, selected for egg production, and CCc, is CC control line randomly selected, both with 10 generations of selection). On hatching day, groups of chicks were allocated in cages (10 to 11/cage/line). On day seven, chicks were inoculated with 30,000 *E. tenella* oocysts and non- inoculated groups were kept as controls. Mortality was evaluated before and after inoculation, and fecal oocysts were counted for each cage (days 5 to 9 post- infection). From 7 to 25 days of age, post-infection mortality rate differed (different letters, $P<0.05$) as follows: TT = 35.0%a (35/100), CCc = 25.0%ab (24/100) and CC = 18.7%b (18/101). Different traits were evaluated 7 days after infection including feed consumption (FC), body weight at 14 days of age (BW14), weighted weight gain (WWG), average weighted weight gain (AWWG), average feed intake (AFI), weighted feed conversion (WFC). In infected chickens, the results for AWWG includes least square means and standard error are: TT = 131.97g ±6.41a, CCc = 24.08g ±3.42b and CC = 22.68g ±3.44b ($P<0.01$). In non-infected chickens, the results for AWWG are: TT = 274.7g ±8.45a, CCc = 63.19g ±3.42b and CC = 61.83g ±3.85b ($P<0.01$). In relation to the WFC, in infected chickens, the results are: TT = 1.90 ±0.26b, CCc = 3.49 ±0.33a, CC = 3.93 ±0.33a ($P<0.1$). In non-infected chickens, the results are: TT = 1.27 ±0.33b, CCc = 1.81 ±0.39.42a and CC = 1.96 ±0.37a ($P<0.1$). The level of infection used in the present study significantly decreased production performance by reducing FC, BW14, WWG, AWWG, and WFC (results not shown). The line with the highest mortality rate (TT) presented best performance in terms of AWWG and WFC, when compared to CC, the line least susceptible to mortality. The mortality rate shows a trend, indicating that selection to egg production may be correlated with lower

susceptibility to *E. tenella* infection; however, additional experimentation is required for verification. Summing up, significant differences in mortality and weight gain post-*E. tenella* infection were demonstrated for two lines.

NECROTIC ENTERITIS ASSOCIATION WITH *EIMERIA ACERVULINA* AND *E. MAXIMA*

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Necrotic enteritis is a common poultry disease caused by *Clostridium perfringens*. Reductions in feed efficiency, lower weight gain, and mortality are associated with this disease. *Clostridium perfringens* can rapidly grow when disturbances in the intestinal microflora or damage to the intestinal mucosa occur. An example of damage to the intestinal mucosa occurs with coccidiosis. Restrictions on in-feed anticoccidial usage and increasing usage of live coccidial vaccines, increases the potential for Necrotic Enteritis in commercial broiler chickens. The objective of this study was to examine association and experimental reproduction of Necrotic Enteritis (NE) in broiler chickens using *Eimeria acervulina*, *E. maxima*, or a combination of the two. The study consisted of rearing 10 male chickens/ cage from day of hatch until 22 days of age. The treatments were noninfected, *E. acervulina* (75,000 oocysts/ bird) challenged, *E. maxima* (10,000 oocysts/ bird) challenged, and a combination at the same dose levels of *E. acervulina* and *E. maxima*. Each treatment was replicated 4 times. Birds were coccidia challenged at 14 days of age and *Clostridium perfringens* challenged at 19, 20, and 21 days of age. The performance parameters measured were feed conversion, average live weight gain, NE mortality, coccidiosis lesion scores (Day 20) and NE lesion scores (Day 22). Birds infected with *E. maxima* alone were more significantly affected by NE than birds challenged with the combination of species or *E. acervulina* alone. The least affected birds were those inoculated with *E. acervulina* alone. This study showed that both *E. acervulina* and *E. maxima* can cause enough intestinal damage to allow *Clostridium perfringens* proliferation and NE development. Even though NE developed with *E. acervulina* alone, the primary *Eimeria* species causing NE was *E. maxima*.

INTERACTION OF *E. COLI* O157:H7 WITH *CRYPTOSPORIDIUM PARVUM* IN 6-WEEK-OLD LAMBS

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Enterohaemorraghic *Escherichia coli* (EHEC) O157:H7 carriage in ruminants has frequently been reported and deliberate inoculation studies have demonstrated that this pathogen can colonise and persist in cattle and sheep. A recent study from our laboratory has shown that *E. coli* O157:H7 can colonise 8-week-old conventionally reared goats for up to 29 days post inoculation. Furthermore, high levels of *E. coli* O157:H7 shedding was associated with a diffuse heavy infection of *Cryptosporidia*. Abundant, multifocal *E. coli* O157:H7 associated attaching and effacing (AE) lesions were observed in the distal colon, rectum and recto-anal-junction (RAJ). *Cryptosporidium* is isolated from farmed animals, including sheep. Since EHEC O157:H7 has been recovered from sheep, the presence of *Cryptosporidium* in the ovine host may modulate EHEC O157:H7 pathogenesis. Therefore, the purpose of the study to be reported here was to assess any interaction between *E. coli* O157:H7 and *Cryptosporidium parvum* in colostrum with-held and conventionally reared 6-week-old lambs. *Post mortem* (bacteriology/parasitology and immunohistochemistry) and persistence (faecal shedding) data will be discussed for *E. coli* O157:H7 and *Cryptosporidium parvum*.

APPLICATION OF *EIMERIA MAXIMA* TO A *CLOSTRIDIUM PERFRINGENS*-ASSOCIATED NECROTIC ENTERITIS MODEL FOR STUDYING BACTERIAL COMPETITIVE EXCLUSION IN POULTRY.

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C. perfringens-associated necrotic enteritis (NE) is an enteric bacterial disease of poultry. It may present as an acute clinical disease, leading to increased mortality, or a sub-clinical disease which impairs feed conversion. Recognised predisposing factors include environmental stress, diet (particularly when high in wheat, barley or fishmeal) and coccidiosis. To test *Bacillus subtilis* as a competitive exclusion (CE) agent for prophylactic control of NE, we used a recently developed, non-lethal challenged model. Chicks were fed a diet which was high in wheat and fishmeal content to predispose them to *C. perfringens* infection. Moreover, 2 x 10^4 *Eimeria maxima* oocyts were administered to each bird by oral gavage, at four weeks of age. *C. perfringens* challenge was then administered via the cloaca one week later. NE lesions were observed in all groups by histological examination of tissue samples *post mortem*. Interestingly, *C. perfringens* colonisation was reduced in one group of birds receiving *B. subtilis*. This work demonstrates the value of *E. maxima* administration in combination with diet content controls, for modelling *C. perfringens*-associated NE in poultry. Furthermore, our results show that certain strains of *B. subtilis* may be useful CE agents for prophylactic control of *C. perfringens* in poultry.

CRYPTOSPORIDIUM PARVUM CELLULAR RECRUITMENT DURING THE INFLAMMATORY RESPONSE IN MICE.

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C. parvum is a common opportunistic infection associated with HIV/AIDS. Infection by this organism triggers a complex array of innate and cell-mediated immune responses within the intestinal mucosa. T-helper type 1 cytokines, and particularly IFN-γ, have long been considered to be the main orchestrators of the immune responses to this infection, but recent studies suggest that T helper type 2 cytokines may also be involved. We have it was previously demonstrated that B10A mice (H-2^a) were more resistant to *C.parvum* infection and produced high level of oocysts shedding (20-fold higher) as compared to C57BL/6(H-2^b), BALB/c(H-2^d), and A/Sn (H-2^a) strains after (i.g.) infection. To better understand the immunoregulatory phenomena of infection of *C.parvum,* the type 2, IL-4, IL-5 and IL-10 cytokine profiles of mice were studied . Mice were infected with 1 dose of $5x10^6$ parasites (waterborne). On the 7th day, exudates were centrifuged, supernatants were removed for cytokines analysis, and cells pellets were re-suspended in 1.0 mL PBS and counted in Fuchs-Rosenthal double-ruling chamber. Two hundred thousand cells were centrifuged onto microscope slides at 500 rpm for 5 min using a cytospin centrifuge. The slides were stained by modified papanicolaou method, and the presence of the *C.parvum* was determined by modified Romanowsky stain. The percentages of macrophages, leucocytes, eosinophils, monocytes and lymphocytes were also determined. IFN-γ, L-4, IL-5, and IL-10 levels were measured by capture ELISA. The parasites induced IFN-γ in all analyzed strains. In contrast, no IL-4 response was detected. Taken together, these results demonstrated higher production of Th 1 cytokines in all analyzed strains, whereas IFN-g was higher in B10A.The best pattern of Th 2 cytokines was observed in the strains producing IL-5 (B10A), IL-10 (C57Bl/6), as compared to lower production of IFN-γ (BALB/c), IL-5 (C57BL/6) and IL10 (B10A). A monoclonal antibody against *C.parvum* using i.g. immunization was produced.

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THE USE OF A VIABILITY ASSAY TO FORMULATE A NEW COCCIDIOSIS VACCINE

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Determination of coccidial oocyst viability has traditionally been done using *in vivo* methods such as lesion score or oocyst shedding following the oral delivery of a dose determined by microscopic enumeration. The microscope is readily used to determine number of sporulated vs non-sporulated oocysts per ml, but the determination of % live oocysts is not possible using a microscope. This introduces a source of uncontrolled variability when multiple suspensions of sporulated oocysts are used to formulate a live coccidiosis vaccine. An *in vitro* assay (VIACYST® assay) has been developed to measure the viability of sporocysts of *E. tenella*, *E. maxima* and *E. acervulina.* The assay is based on the use of a non-vital stain, ethidium bromide. The assay requires the excystation of sporocysts and depends on the permeability of sporocysts from *Eimeria* or any other sporocyst-forming protozoan to this non-vital dye. Preliminary experiments confirmed that vaccine from a suspension of *E. tenella* oocysts assessed non-vital by ethidium bromide staining were also not capable of generating resistance to a coccidiosis challenge. As the proportion of oocysts from the non-vital suspension increased in the vaccinating dose, there was a concomitant decrease in the resistance of the bird to oral challenge as indicated by lesion scores. When the immunizing inoculum contained 1000 viable oocysts or less, lesion scores were not different than non-vaccinated controls. This study confirmed that the in vitro viability assay correlates to the ability to confer resistance to a coccidiosis challenge. A second experiment was done to test the use of the viability assay to formulate vaccines from suspensions of oocysts that ranged from 24 to 52 weeks old and 16 to 82% viability. Results indicated that suspensions of *E. acervulina* varying widely in viability and age were all capable of generating resistance to an oral challenge providing the vaccine was formulated on a viable oocyst basis.

Ethidium bromide assay detects non-viable sporocysts that reflect the original oocyst population and that formulation of the vaccine based on viable sporulated oocysts/bird will yield consistent efficacy across a wide range of age and viability.

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COMPARISON OF THE ONSET OF AN IMMUNE RESPONSE USING THREE OOCYST VACCINATION STRATEGIES

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EIMERIAVAX 4m® is a 4 species coccidiosis vaccine containing Australian precocious strains of *E acervulina*, *E maxima*, *E necatrix* and *E tenella* administered to 100% birds by individual eye-drop application from day-old. Aerosol spray is appealing because it is less time- consuming for mass application of oocysts. Unfortunately, aerosol is not as precise. There is no information on whether the alternative of eye drop vaccination of a percentage of chickens plus coarse aerosol spraying the remainder would overcome the reduced accuracy of aerosol. To investigate this hypothesis, three groups of broilers were vaccinated at day-old with a) 100% coarse aerosol, b) 15% individual eye-drop and 85% aerosol and c) 100% individual eye-drop. One group of birds was unvaccinated. Oocysts per gram of faeces (OPG) were enumerated for each group at 5, 6, 7, 14, 21, 28, 35, and 42 days of age. At 11, 20, and 29 days post-infection, birds were removed and challenged with live oocysts of the same species in the vaccine. At 6 days post challenge, intestinal tissues were fixed. Following histological preparation and staining, tissues were scored for lesions. Weight gain post challenge was collected.

At 5, 6 and 7 days post vaccination there was a spike in the OPG from the group vaccinated at day-old by eye-drop. Following the first challenge at 11 days of age, comparative analysis of the OPG showed eye-drop vaccinated chickens always showed the lowest OPG for each day. This trend was repeated with the histological analysis. Following the challenge at 20 and 29 days of age, OPG results indicated that the challenged groups had the highest oocyst output, the highest histological lesion scores and the lowest weight gain. There was no consistent difference between any vaccination regime.

The results show the immune response by 10 days post vaccination was sufficient to give the birds vaccinated by eye-drop some protection from challenge. There was no difference between any groups by 21 days. It is concluded there is a delay in the onset of immunity when administering live coccidiosis vaccines by aerosol spray. This paper also describes the success of the in field use of the aerosol and eye-drop technique.

QUANTIZATION OF PLASMA D-XYLOSE CONCENTRATION FOLLOWING OF VACCINATION AGAINST COCCIDIOSIS AND EXPERIMENTAL SUBCLINICAL COCCIDIOSIS INDUCED BY *EIMERIA MAXIMA* IN BROILERS

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Coccidiosis, particularly its subclinical form, which usually is not being promptly and correctly diagnosed, causes economic losses in broiler production. Each year, a significant amount of money is spent in its chemical prevention, but this is not always effective due to several causes, such as management problems, drug resistance, etc. The use of vaccination for the prevention of coccidiosis has been recently proposed, and different vaccines have been evaluated and compared. The intestinal D-xylose absorption test is a suitable method for studying intestinal mal-absorption in humans and animals, such as poultry. The main aim of this study was to evaluate the effects of vaccination against coccidiosis on the absorptive index by using the intestinal absorption test in broilers. A total number of 60 healthy Arbor Acres one-day-old broilers was purchased and equally divided into 6 experimental groups as follows: Group 1 –negative control with no vaccine and no challenge by *E. maxima*; Group 2 – 15-day-old birds were challenged with a suspension containing 50,000 sporulated oocysts of *E. maxima,* but no vaccinated; Group 3 – vaccinated at 4 days of age with **Iracocc** (a non-attenuated live vaccine) and not challenged; Group 4 – vaccinated at 4 days of age with **Livacox T** (a live attenuated vaccine) and not challenged; Group5 vaccinated with Livacox T and challenged with *E. maxima*; Group 6 – vaccinated with **Iracocc** and challenged with *E. maxima*. Seven days after challenge (at 21 days of age), 5 chicks were randomly chosen from each group, and after being submitted to 12 hour fasting, each chick received a solution of D-xylose at 5% by stomach tube. Blood samples were taken 30, 60, and 90 minutes after D-xylose administration, using heparinized microhematocrite tubes. Plasma D-xylose concentration was determined using the Modified

Ebert's Methods (Goodwin, 1984). The obtained results showed that the lowest plasma concentration of D-xylose belonged to the challenged group. However, no significant difference was observed between the negative control group and all vaccinated groups, including the group that was vaccinated and challenged with *E. maxima*. The results of this experiment indicate that concentration of plasma D-xylose in vaccinated broilers was not significantly reduced in comparison with normal broilers. On the other hand, it was shown that this concentration was significantly higher in all vaccinated groups as compared to the group that was challenged, but was not vaccinated. No significant difference was found between group 4 (vaccinated with Livacox and not challenged) and group 5 (vaccinated with Livacox and challenged) or group 2 (challenged and not vaccinated). The same results were obtained for the Iracocc-vaccinated groups. Therefore, it seems that both vaccines are not efficient, and that it is possible to use intestinal D-xylose absorption test to evaluate anticoccidial vaccines, in addition to parasitological and immunological evaluation. In fact, we recommend the use of these physiological experiments to better evaluate anti-coccidial vaccines, and we believe that this method can be used for evaluating broilers flocks in which subclinical coccidiosis is suspected.

RELATIONSHIP OF *E. ACERVULINA* IONOPHORE RESISTANCE TO FIELD PROBLEMS WITH *E. MAXIMA*

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An earlier study suggested that *E. acervulina* can interfere with colonization of *E. maxima*. The object of this study was to examine the relationship of E. *acervulina* sensitivity to Salinomycin and subsequent infection level with *E. maxima*. A battery cage study was conducted. Birds fed nonmedicated or Salinomycin 60 g/t feed were challenged with either a Salinomycin sensitive, resistant strain of *E. acervulina* and/or an E*. maxima* field isolate. The oocyst per bird challenge levels were none; *E. acervulina* (sensitive); 50,000, *E. acervulina* 50,000 (resistant); *E. acervulina* 50,000 (sensitive) plus *E. maxima* 5,000; *E. acervulina* 50,000 (resistant) plus *E. maxima* 5,000; or *E. maxima* 5,000. *E. maxima* alone caused 20 % weight reduction and 2.70 lesion score. Salinomycin controlled the sensitive strain with 5 % weight reduction and 1.25 lesion score. Salinomycin did not control the resistant strain, with 22 % weight reduction and 2.75 lesion score. The birds infected with *E. maxima* and sensitive *E. acervulina* had *E. maxima* lesion scores of 2.25. The birds infected with *E. maxima* and resistant *E. acervulina* had *E. maxima* lesion scores of 1.30. From the results it can be inferred that *E. acervulina* interfered with development of *E. maxima*. Higher anticoccidial resistance allows more *E. acervulina* colonization, which appears to interfere with colonization of *E. maxima*, and thus indirectly slows *E. maxima* immunity development. This increases chance for late problems with *E. maxima*, possibly explaining an increase in field reports of late *E. maxima* infections where Salinomycin has been extensively used.

EFFICACY OF DECOQUINATE AT DIFFERENT ADMINISTRATION STRATEGIES AGAINST CRYPTOSPORIDIOSIS IN NATURAL INFECTED CASHMERE GOAT KIDS

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This trial was carried out to evaluate the efficacy of decoquinate to prevent cryptosporidiosis in goat kids.

The experiment (3 groups) was conducted in a flock of Cashmere goats with a confirmed history of neonatal diarrhoea caused by Cryptosporidium sp.

- *Group A.*- 24 kids unmedicated,.
- *Group B.*- 25 kids that received orally from 3 days old 2.5 mg/kg/day of décoquinate for 21 days.
- *Group C.*- 15 kids that were not medicated, but born from goats treated during 21 days before the expected day of kidding with 2.5 mg/kg/day of décoquinate.

The animals included in the control group showed the most severe clinical signs such as significantly softer faecal consistency at 11 days after birth, and one kid died 9 days after birth Episodes of diarrhoea were only observed in some kids included in the unmedicated group between 11 and 13 days after birth. Kids in group B showed softer faeces than those included in group C at 15 and 17 days after birth. Abdominal pain was only observed in some kids showing diarrhoea in control group.

The mean weight gains during the experimental period for groups A, B and C were 2.61, 2.57 and 3.23 kg, respectively.

The pattern of oocyst shedding was similar in the three groups. However, oocyst shedding scores in groups B and C were notably lower than those observed in the control group over the experiment. In addition, the maximum oocyst shedding scores were reached sooner in the control group (11 days after birth) than in groups B (13 days) and C (15 days). The number of unmedicated Cashmere kids shedding Cryptosporidium sp. oocysts was significantly higher than those observed in kids of groups B and C. In general, the percentage of treated kids shedding oocysts was significantly lower than those kids born from medicated does.

The present study shows that decoquinate treatment at 2.5 mg/kg/day for 21 days delay the appearance of clinical cryptosporidiosis and reduces the severity of naturally acquired cryptosporidiosis in kids and prevented episodes of diarrhoea.

On the other hand, the kids born from mothers treated with decoquinate has similar results to those obtained in treated kids and notably better than those observed in the untreated ones. These kids showed a better final weight gain than medicated and control kids.

An explanation of this result may be the effect of decoquinate on the periparturient rise.

EFFICACY OF TOLTRAZURIL AND AMPROLIUM AGAINST TURKEY COCCIDIOSIS CAUSED BY A MIXED *E. ADENOEIDES* AND *E. MELEAGRIMITIS* INFECTION

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Ten-day-old turkeys, raised in floor-pens (2 replicate pens of 5 turkeys per experimental group, 14/24 h lighting), were gavage challenged with a mixed inoculum containing drug sensitive Weybridge strains of *E. adenoeides* (*Ead*) and *E. meleagrimitis* (*Eme*) at 3,000 and 10,000 oocysts/bird, respectively. Experimental groups consisted of positive controls (challenged, untreated), negative controls (unchallenged, untreated), and 4 groups that were challenged and subsequently treated in the drinking water with amprolium (Nemaprol 10.6 %) at 240 ppm for 5 days (114 mg/kg daily), or with toltrazuril (Baycox 2.5%) using either one of 3 dosage regimens, viz. 7 mg/kg daily for 2 days (label claim), 15 mg/kg for one day, and 20 mg/kg for one day. Six out of 10 challenge control turkeys died from coccidiosis whereas all treated turkeys survived. The oocyst count of faecal samples pooled at day 4-8 post infection (PI) amounted to 544,000/g for the challenge controls and was reduced in the amprolium treated group to 51,000/g. No oocysts were shed in any of the toltrazuril treated groups. Mean lesion scores (on a scale of 0-4) for *Ead/Eme* were 3.5/3.3 and 1.7/2.2 in the positive control and amprolium group, respectively. A mean score as low as 0.1 was observed in the toltrazuril 7mg-2days group, only for *Ead*, and in the toltrazuril 15mg-1day group, only for *Eme*. Also in the toltrazuril 20mg-1day group a very low score of 0.6 was observed, only for *Eme*. Negative controls gained 303 $\pm$46 g of weight from the day of infection until 7 days PI whereas weight gain was severely depressed in the surviving challenge control turkeys (161 $\pm$75 g) and the amprolium treated turkeys (162 $\pm$14 g). Growth was unaffected by coccidiosis challenge in all toltrazuril treated groups with weight gains of 320 $\pm$35 g (7mg-2days), 318 $\pm$12 g (15mg-1day) and 300 $\pm$49 g (20 mg-2days). In conclusion, toltrazuril cured turkey coccidiosis better than amprolium and limiting the duration of toltrazuril treatment to a single day, whilst maintaining the total therapeutic dose, did not diminish the efficacy.

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