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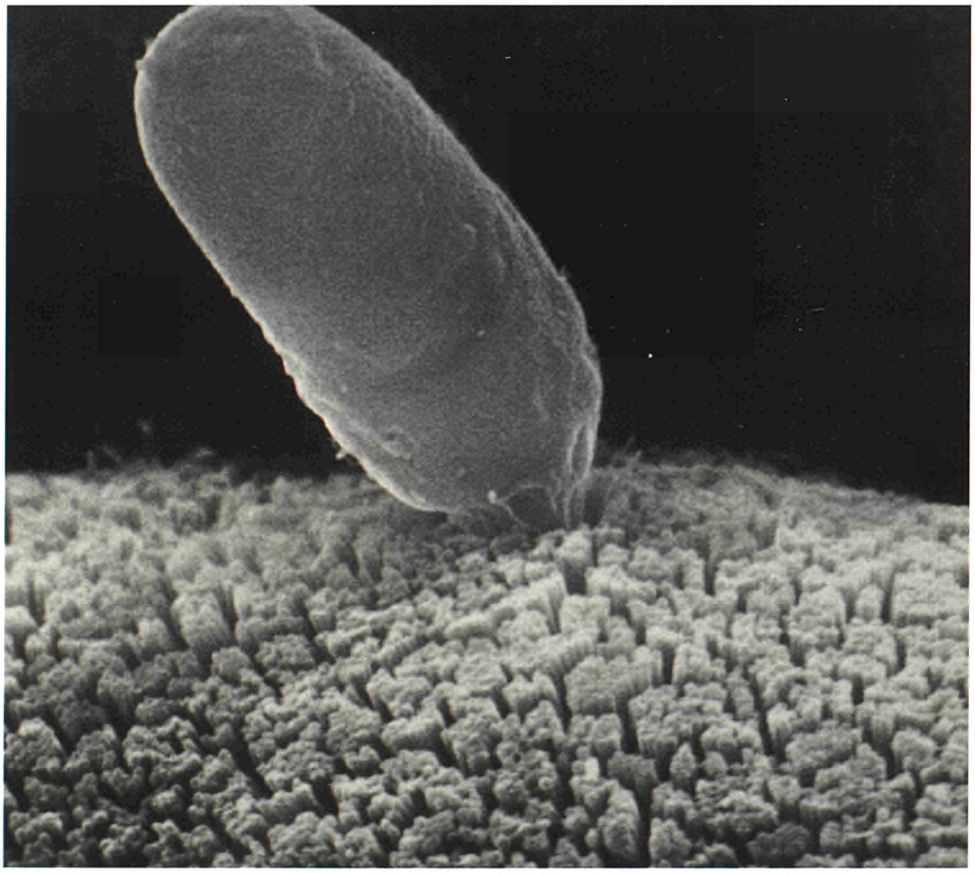
# COST 820

# Vaccines against animal coccidioses

## Annual report 1998



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European Commission

**Agriculture and biotechnology**

# COST 820

# Vaccines against animal coccidioses

## Annual report 1998

*Edited by*

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1999

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## Foreword

This is the fourth Annual report prepared under the auspices of COST820 and it serves to highlight some of our scientific activities and laboratory visits during the past 12 months. At one time I anticipated that this report would be the last annual description of our work but COST820 was granted an extra one year to contine with its work. Naturally the membership of COST820 are delighted that that our hard efforts have been recognised in Brussels. The COST820 action will now close in June 2000.

Looking back over the life of COST820, I think that we are proof for the success of the COST scheme. Certainly our model has been looked at enviously by our colleagues in, for example, the USA and as I pointed out in an introduction to the 1998 Annual Workshop -

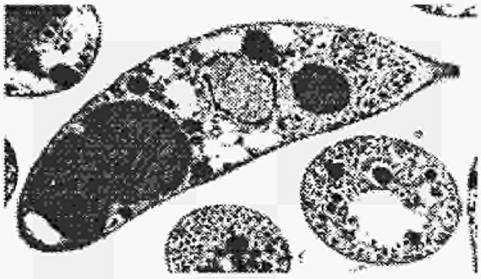
*At the end of September I participated in meeting in London that was organised by the UK Ministry of Agriculture Fisheries and Food and which considered the value of COST actions in agriculture and biotechnology to the UK scene. My task of both defending and promoting our COST820 action was incredibly easy as I was able to illustrate my presentation with many references to the substantial networking that now exists across most of Europe (and not just those countries within the EU). As an example, I used the links involving the UK scientists that have been established because of COST but, equally, I could have shown the links emanating from other countries. Any one network that has arisen because of our participation in COST820 is proof that the concept works but within COST820 a substantial number of new links have been forged since 1994.*

In the context of the 1998 Annual meeting, I would like to thank Professor Luis Ortega-Mora and his colleagues for their efforts in making the local arrangements and, with Dr Astrid Tenter, for preparing the scientific programme and liasing with all delegates. Also, I would again like to thank all those members of COST820 who have contributed towards the preparation of this document (with their accounts of Working Groups, scientific activities, etc.), Professor M. Rommel for editorial help and senior colleagues within COST820 who promote and direct the activities of our action.

Martin W. Shirley
Chairman
June, 1999

# COST-ACTION 820:
# VACCINES AGAINST ANIMAL COCCIDIOSES

**COMMISSION OF THE EUROPEAN UNION**
**EUROPEAN CO-OPERATION IN SCIENTIFIC AND TECHNICAL RESEARCH (COST)**



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## CHAIRMANS REPORT FOR JANUARY 1998 to DECEMBER 1998

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# 1. SUMMARY OF OBJECTIVES OF THE COST820 ACTION

COST820 Action began formally in July 1994 following the signing of a "Memorandum of Understanding" by at least seven countries. The main objectives are the:

- Evaluation of new vaccination strategies
- Development of new vaccines, with the emphasis on recombinant vaccines
- Study of innovative ways for vaccine production
- Preparation of recommendations for industrial production and efficacy and safety evaluations of vaccines
- Development of biomathematical models for evaluating the potential impact of immunisations on the epidemiology of infections with coccidia

For these objectives, it was anticipated that high priorities will be given to three areas loosely defined as:

**Basic Studies**: to include aspects of the molecular biology and immunology of the coccidial parasites that have potential relevance for vaccine development. Such studies might include the development of strategies to identify protective antigens, the isolation and characterisation of antigens and fundamental molecular analyses of the parasites, etc.

**Intermediate Studies**: to include evaluations of eucaryotic expression systems to produce modified parasite proteins, investigations of the abilities of different viral and bacterial vectors to present antigens in different sites, examinations of different adjuvants and the initial small-scale evaluation of potential vaccine candidates.

**Vaccination:** to include the more practical aspects of vaccinations and larger scale trials of any outstanding recombinant vaccine candidates will be required.

# 2. CURRENT STATUS OF COST820

The status of the action as at 1st January 1998, is that the following European countries are listed as participants in COST820. Austria*, Belgium, Czech Republic, Denmark, Germany, Hungary, Italy, Ireland, The Netherlands, Poland, Spain, Sweden, Switzerland and United Kingdom.
* Note that Austria has observer status in the Management Committee.

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# 4. WORKING GROUPS

To facilitate the objectives of the Action, six Working Groups were established at the outset of the COST820 Action to provide a focus for specific areas of interest:

WG1 Avian coccidiosis
WG2 Mammalian coccidiosis
WG3 Cryptosporidiosis and cyclosporosis
WG4 Sarcocystiosis, toxoplasmosis and neosporosis
WG5 Biomathematical modelling
WG6 Immunology and cell biology

# 5. REPORTS OF THE WORKING GROUPS

## 5.1 WG1 and WG6 Avian coccidiosis and immunology and cell biology

Co-ordinators: Drs Martin W. Shirley and Fiona M. Tomley

A highly successful meeting, entitled "Transfection and whole genome approaches to gene discovery and function: the way forward for vaccine research in coccidians?" was held jointly between the two Working Groups at Grottaferrata, Rome, Italy on 19$^{th}$ –21$^{st}$ June, 1998.
The scientific programme was organised by Drs Fiona M. Tomley and Martin W. Shirley with help from Dr Furio Spano.
Dr Furio Spano and Dr Andrea Crisanti were the local hosts and provided an excellent venue

The programme for the joint meeting and abstracts of the papers presented are included in the Appendix.

A meeting of WG1 was also held on 27th February 1998 at the Veterinary Laboratories Agency, Weybridge on "Identification of avian species of *Eimeria*, further experiments and the impact that the new work may have for vaccination strategies". The primary purpose of the meeting was to discuss the work of Dr Beate Schnitzler (Sweden) who had recently developed a PCR diagnostic test based on the amplification of the ITS1 region of the genome.

## 5.2 WG2 Mammalian coccidiosis

Co-ordinator: Janet Catchpole

A well attended and successful meeting was held on 27th February 1998 at the veterinary Laboratories Agency, Weybridge on the topic "Coccidiosis in Pigs". A copy of the proceedings is included in the Appendix

## 5.3 WG3 Cryptosporidiosis and cyclosporosis

Co-ordinators: Dirk C. de Graaf & Alain Bonnin

### Background

Organisms of the genus *Cryptosporidium* are Apicomplexan protozoans that infect several groups of vertebrates. Six *Cryptosporidium* species are currently considered valid: *C. parvum* and *C. muris* in mammals, *C. meleagridis* and *C. baileyi* in birds, *C. serpentis* in reptiles and *C. nasorum* in fish.

Among these species, *C. parvum*, that is infective for mammals, has the most important economic impact. In calves and sheep *C. parvum* has been identified as the most common infectious agent in outbreaks of diarrhoea. Large numbers of cryptosporidial oocysts produced during commercial calf production contaminate drinking water resources and contribute to human cryptosporidiosis. Only ozone is effective in disinfecting drinking water from *C. parvum*. Few drugs are active against the parasite, and even less are commercially available. Moreover, public opinion is more and more opposed against systematic chemoprevention of parasitic diseases. Therefore, immuno-prophylactics should be considered.

The prevention of cryptosporidiosis is difficult. Whereas most other species of coccidia are incapable of recycling within the host, *C. parvum* has two stages that initiate auto-infectivity : type I merozoites and sporozoites derived from thin-walled oocysts. These characteristics allow *C. parvum* to develop severe infections in hosts exposed to small numbers of thick-walled oocysts, and development of an effective immune response seems to be the only way to control infection.

Little is known about the immunobiology *C. parvum.* In immunocompetent hosts cryptosporidiosis is self-limiting. Calves that recover from infection are resistant to a second challenge with the same strain. So recovery depends on a specific, acquired immune response, although the exact mechanisms responsible for resistance have not been defined yet.

Colostrum plays a limited role. Although neonatal calves can be partially protected by hyperimmune colostrum, normal colostrum does not protect ruminants from infection. This may be due to the fact that the time of exposure of parasitic stages to colostral antibodies is too short to protect neonates. In humans passive lacteal immunity against C. parvum in breast-fed children has been described. No lactogenic immunity seems present in cattle, as most calves will experience cryptosporidiosis while they are nursing from immune cows.

The role of immunoglobulins in the elimination of the parasite is controversial. Some evidences support the involvement of antibodies in the clearance of the infection, such as i) the persistency of cryptosporidiosis in congenitally hypogammaglobulinemic individuals, ii) the good temporal association between *C. parvum* oocyst excretion and the amount of specific IgA in the faeces of calves and lambs and iii) the protective role of neutralising antibodies as demonstrated by their ability to inhibit invasion both in vivo and in vitro. On the other hand, there are observations arguing against the importance of the antibody response: i) oocyst shedding patterns do not differ between B-cell-depleted neonatal mice or bursectomised chickens and their controls, ii) challenged immune calves do not show a secondary mucosal antibody response and iii) AIDS patients with persistent cryptosporidiosis produce *C. parvum* specific serum and/or mucosal IgG, IgM and IgA.

Whitmire & Harp showed that peripheral lymphocytes recovered on the second day post-infection recognise antigens in a lysate of *C. parvum* oocysts. Natural Killer cells and tumour necrosis factor seem to have no influence on the progress of infection in BALB/c mice. This was confirmed by the fact that severe combined immunodeficient mice - who have natural killer cells but no T and B cells - develop a persistent infection and die within 12 weeks after infection. However, when their immune system is reconstituted by injection of spleen cells from normal donors they can reduce the infection level. This reconstitution seems to be $CD4^+$ T cell dependent, as was seen by depletion of different T cell subsets of the samples to be transferred. Similarly, administration of neutralising anti- $CD4^+$ T cells and anti-INFγ monoclonals prevented the recovery from infection after adoptive transfer with spleen cells. Interferon-gamma seems to be of major importance in the development of protection against *C. parvum* infection. Daily administration of INFγ to immunosuppressed rats reduced the intensity of subsequent ileal infection. And administration of IL12 to immunocompetent and immunosuppressed mice reduced the severity of infection by a INFγ dependent mechanism. Recently a specific INFγ response was found after *Cryptosporidium* infection of neonatal calves. The antigens that provoke this cellular response might be interesting vaccine compounds.

In chickens disease associated with *C. baileyi* manifests itself most often as a respiratory disease in immunosuppressed birds. Only occasionally the parasite causes intestinal or renal disease although combinations with tracheitis may be observed.

*Cyclospora* may cause diarrhoea and weight loss in humans. The infection is felt to be a waterborne disease. The economic impact in animals is unknown.

**Activities of WG 3 in 1998**

A Working Group 3 Meeting was held on Saturday, 24th October 1998, during the Annual Workshop in Toledo, Spain. This meeting was entitled: "A vaccine against cryptosporidiosis: reality or fantasy?".

The programme was as follows:

**D.C. de Graaf:** General introduction.

**D.C. de Graaf:** Is a vaccine necessary in veterinary medicine?

**A. Bonnin:** Is a vaccine necessary in human medicine?

**S. Sagodira:** The answers currently available.

**F. Petry:** Mechanisms of the immune response to cryptosporidiosis relevant to vaccination.

**F. Spano:** Possible targets offered by the molecular biology.

**A. Bonnin:** Priorities for the future and conclusion.

Based on this WG3 meeting a new list of **points of action for the near future** was derived:

**i)** a clinico-epidemiological analysis to confirm the impact of cryptosporidiosis on the nutritional status and development of young children

**ii)** a study of the consequences of combined antiviral chemotherapy on AIDS cryptosporidiosis

**iii)** the study of the immune effector mechanisms involved in the clearance of the infection

**iv)** the identification of *Cryptosporidium* T-cell antigens

**v)** the understanding of the mechanism involved in the passive immunisation of neonatal ruminants by colostral transfer

**vi)** the identification and complete characterisation of new targets offered by the molecular biology

**vii)** the confirmation of the protective potential of candidate vaccine antigens in vaccination trials

**viii)** the study of the genetic structure of *C. parvum* and the characterisation of the pathogenicity of different genotypes to ensure the choice of the most appropriate target.

Representation of WG 3 at the Annual Workshop:

At the 1998 Annual Workshop of the COST 820 Action in Toledo, the following oral presentations were given by WG 3 members:

**S. Sagodira, D. Buzoni-Gatel, S. Iochmann, M. Naciri and D. Bout:** Protection of neonates against cryptosporidiosis after genetic vaccination of dams.

**F. Petry and V. McDonald:** Further characterisation of recombinant antigens of *Cryptosporidium parvum*.

**A. Bonnin, J.F. Dubremetz, J. Lopez, S. Robine, C. Chaponnier and G. Gabbiani:** Detection of microvillous cytoskeletal components in the parasitophorous vacuole wall of *Cryptosporidium parvum*.

**M. Giles, K.A. Webster, J.A. Green, J. Catchpole, C. Dawson, S. Pedraza-Diaz and J. McLauchlin:** Host specificity, pathogenesis and molecular classification of *Cryptosporidium* species

**S. Patel, J. McLauchlin, S. Pedraza-Diaz and D. Casemore:** Seroepidemiology and molecular characterisation of cryptosporidiosis.

**D.C. de Graaf, E. Vanopdenbosch and J.E. Peeters:** Introduction to the debate on the epidemiology and economic importance of Cryptosporidium spp.

**C.A. Vergara-Castiblanco, S. Santos-Nunez, F. Freire-Santos and E. Ares-Mazas:** *Cryptosporidium parvum* in two Colombian populations: immunoblot analysis of specific serum response (IgA, IgG and IgM).

The following poster presentations were given by WG 3 members:

**F. Petry and J. R. Harris:** Ultrastructure and molecular analysis of the extracellular stages of **Cryptosporidium parvum.**

**S. Pedraza-Diaz, S. Patel and J. McLauchlin:** Molecular typing of *Cryptosporidium* from human water-borne outbreaks.

**D. Champliaud, P. Gobet, M. Naciri, O. Vagner, J. Lopez, Y. Varga, G. Harly, R. Mancassola and A. Bonnin:** Failure to differentiate *Cryptosporidium parvum* from *C. meleagridis* based on PCR amplification of eight DNA sequences

**F. Freire-Santos, C.A. Vergara-Castiblanco and E. Ares-Mazas:** An attempt of experimental infection by *Cryptosporidium parvum* in rainbow trout.

**H. Abbassi, F. Coudert, Y. Chérel, J. Brugère-Picoux and M. Naciri:** Effect of pathogenic virus of Infectious Bursal Disease (IBD) on the pathogenicity of *Cryptosporidium baileyi* and the development of acquired immunity to parasite.

**S. Hornok, Z. Széll, J. Nieuwenhuijs, M.G.B. Nieuwland, A.W.C.A. Cornelissen and I. Varga:** Immunogenicity of three oocyst extracts of *Cryptosporidium baileyi* in experimentally infected chickens.

**S. Hornok, Z. Bitay, Z. Széll and I. Varga:** Assessment of maternal immunity to *Cryptosporidium baileyi* in chickens.

Representation of WG 3 at the Joint Meeting of WG 1 and WG 6:

At the Joint Meeting of WG 1 and WG 6 in Rome, Italy, the following oral presentations were given by WG 3 members:

**M. Piper:** Happy mapping in *Cryptosporidium*.

**S. Caccio:** Establishment of the molecular karyotype of *Cryptosporidium parvum*.

**F. Spano:** Gene discovery and genetic variation in *Cryptosporidium* and *Toxoplasma*.

**D.C. de Graaf, P.T. Hoff, F. Petry and J.E. Peeters:** First attempts to isolate recombinant *Cryptosporidium* T cell antigens.

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### 5.4 WG 4, Sarcocystiosis, toxoplasmosis and neosporosis

Co-ordinators: Dr D. Buxton and Prof. M. Rommel

1998 has been a particularly productive year for COST 820 Working Group 4. Apart from numerous informal links between laboratories that it has created, more formal meetings funded by the EU have been very successful.

At the COST 820 meeting held to discuss "Vaccines Against Animal Coccidioses" in Toledo 22-25 October, members of Working Group 4 were seen to have been particularly active. Of 40 oral presentations 19 (47.5%) were by members of WG4.
Under the umbrella title "Recombinant antigens and other tools for the development of molecular-engineered vaccines against coccidial parasites" six papers from WG4 demonstrated the very significant advances in our understanding of protective immunity to *Toxoplasma gondii* and three were presented on the very much more recently recognised *Neospora caninum*.

Oral sessions.
Within the first session, "DNA Vaccines and Recombinant Antigens", one was on vaccinating mice with recombinant proteins derived from *Neospora caninum* (presented by Simone Eperon, **1 below**) and the other on the use of recombinant surface antigen,SAG1, to vaccinate mice against *Toxoplasma gondii* (presented by Henrik Vedel-Nielsen, **2 below**).

In the second session "Identification of New Vaccine Candidates" there was a further paper on the potential of SAG from *T. gondii* to induce protective immunity (presented by Furio Spano, **3 below**) and two on specific surface antigens of *N. caninum* (presented by Simone Sonda, **4**, and Nicole Fuchs, **5**).

In the "Transfection" session the three papers concerned with *T. gondii* presented data on how the parasite could now be engineered to meet specific experimental needs which in turn could lead to the production of a successful vaccine (Adrian Hehl, **6**). Jens Mattsson then spoke on the use of transfection to investigate and characterise the genetic background of certain phenotypes (**7**) and Martine Soete on the bradyzoite-specific surface antigen SAG4 in the temperature sensitive (Ts-4) vaccine strain (**8**). Bruno Gottstein then presented data from original research using knockout mice and wild-type mice as models for Neospora infection and showed the importance of both cellular and humoral immunity to this parasite (**9**).

These sessions were then followed by a very focussed and relevant examination of the "Epidemiology and Economic Impact of Coccidiosis In Animal Production" starting with a scientific session followed by a round table discussion.

In the scientific session speakers examined the incidence of protozoan cysts in bovine, ovine and caprine abortions in Italy (Gabriella Di Francesco, **10**). Five of the following six papers from members of WG4 then highlighted the very rapid progress being made in piecing together our understanding of bovine neosporosis. These covered its seroprevalence in England and Wales (Helen Davison, **11**) and Spain (Enrique Tabares, **12**) and then Jonathan Wastling emphasised the value of being able to accurately distinguish between different isolates of *N. canimum* by genetic typing to allow an accurate analysis of field data (**13**).

As well as molecular techniques it is crucial that we have, and understand, what serology can do to help. In this respect Peter Lind moved the discussion significantly further forward in his paper (**14**) and then Jo Marks, summarising her studies into bovine immunity to *N. caninum,* also demonstrated the importance of cell-mediated mechanisms and highlighted the role of interferon gamma (**15**). While these studies were in non-pregnant cattle the following paper by Catherine Guy examined immunological responses in pregnant cattle (**16**). The importance of cell-mediated immunity in protecting animals against the protozoa worked on by members of WG4 was further emphasised when the session ended with a paper on the role of immunological memory in *Sarcocystis* infection, by Thomas-Erich Jekel (**17**).

In the round table that followed papers, by David Buxton, Jonathan Wastling and Astrid Tenter attempted to estimate how much *T. gondii, N. canimum* and *Sarcocystis* spp. respectively cost the EU in terms of inefficiency and lost production (**18, 19, 20**).
The overarching conclusion from all three presentations was **a**) how important these parasites are in causing considerable losses in EU agriculture (as well as, in the case of *T. gondii*, causing human disease) and **b**) how these figures were essentially honest but crude estimates, as much work remains to be done in gathering more accurate data.

**Poster session.**
Eight of the 18 posters (44%) were presented by members of WG4.
While one addressed the problem of toxoplasmosis in pigs (G. Jungersen et al, **21**) the other 7 all presented research into neosporosis. Four tackled epidemiological aspects and incidence in cattle (A. Schock et al, **22**; W. Wouda et al, **23**; H.C. Slotved et al, **24**; A. Uggla et al, **25**) and one examined the potential role that foxes might play (S. Wright et al, **26**). Two others looked at immunity to

*Neospora* in mice (A. Lunden et al, **27**) and a comparison between bovine and canine *Neospora* isolates in a mouse model (R. Atkinson et al, **28**).

**46.5%** of this very successful conference was devoted specifically to Working Group 4 with many of the other presentations also being of very great relevance to the group.

A business meeting of COST 820, working group 4 was also held (at 21.00h on 22/9/98 at the Beatriz Hotel, Toledo Spain). **The object of the meeting was to take advantage of having "so many members of WG4 under the same roof at the same time" to discuss** the way forward.

Those present were:-

| | | |
|---|---|---|
| C. Björkman (S). | D. Buxton (UK) – in the chair. | F. J. Conraths (D). |
| B. Gottstein (CH). | P. Lind (DK). | A Lundén (S). |
| J. Mattsson (S). | N. Müller (CH). | L. M. Ortega Mora (Es). |
| J. Pereira Bueno (Es). | G. Savini (I). | S. Stenlund (S). |
| A. M. Tenter (D). | A. Vermeulen (N). | J. M. Wastling (UK). |
| W. Wouda (N). | | |

The meeting was convened to discuss two matters.

1. The proposal to run a joint meeting between COST 820 WG 4 (Sarcocystiosis, Toxoplasmosis and Neosporosis) and WG 5 (Epidemiology).
2. **2.** Future funding for WG 4.

**1.** Neoporosis is now recognised as being of very great importance in European and World agriculture. Very recent data from members of WG 4 and scientists in other countries in the world variously indicate levels of calf abortion attributable to *N. caninum* being at 10 to 40% (with some estimates as high as 50%). The full life cycle of the parasite was shown to take place in the dog in the summer of 1998.

In view of the importance of bovine neosporosis the meeting discussed the proposal that WG 4 hold a joint meeting with WG 5 and focus on epidemiological aspects of neosporosis in cattle. The recent

discovery that the dog is the definitive host for *N. caninum* would make such a meeting very timely. All those present felt that it was vital that such a meeting should be held. It was unanimously agreed that we would ask Professor A. W. C. A. Cornelissen of Utrecht University (WG 5) and Dr W. Wouda (WG 4) to explore the possibility of holding such a meeting in Amsterdam either in January 1999 or around the end of April 1999. It was felt that the latter date was more realistic.

**2.** The meeting then discussed the possibility of seeking funding to permit the activities of WG 4 to continue after the demise of COST 820. It was agreed unanimously that in view of the important and very exciting progress in *Neospora* research that we would work to this end. It was also universally recognised that a very considerable amount of research into the parasite as well as pathogenesis and immunity in the host and epidemiology was required if the real losses caused by neosporosis were to be tackled in a meaningful way. It was felt that a provisional working title of "The Economic Impact of Bovine Neosporosis" could be adopted for the time being.

**COST 820 funded visits between WG4 laboratories.**

Dr Anja Heckeroth from the Institute for Parasitology, Hannover Veterinary School, Germany, visited the Moredun Research Institute, Edinburgh, Scotland in the summer in an exchange which successfully transferred the technologies for the detection of *Sarcocystis* spp and *Neospora* by PCR between the two laboratories. In addition transfer serological methods for detecting infection by both parasites was also achieved. .

# 6 SHORT-TERM SCIENTIFIC MISSIONS

## 6.1 Anja Heckeroth

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**Visit to Moredun Research Institute, Edinburgh, United Kingdom, September 1998**

In September 1998, I spent 18 days at the Moredun Research Institute, Edinburgh, United Kingdom. The aim of the STSM was to standardise methods for *in vitro* cultivation and diagnosis of *Neospora caninum*, *Toxoplasma gondii*, *Sarcocystis tenella* and *Sarcocystis arieticanis* between the two laboratories.

Firstly, I was introduced to cell culture techniques for *N. caninum* and *T. gondii*. I learned different methods to test new cell culture reagents and to optimise the culture conditions. In addition, I learned a method for the isolation of *N. caninum* from infected tissue and the start of a cell culture using this material. After I learned the routine maintenance of Vero cells and parasites I expanded one strain of each parasite, *N. caninum* and *T. gondii*, and harvested the tachyzoites. Subsequently, the parasites were purified from Vero cells and other culture material. Most of the parasites were frozen and shipped to the Tierärztliche Hochschule Hannover where two-dimensional electrophoretic analysis and comparison of protein profiles will now be carried out. A portion of the parasites were used to prepare antigen for serological tests, the enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence antibody test (IFAT).

Secondly, I was trained in the methodology of an ELISA and IFAT for the diagnosis of *N. caninum* in cattle sera. Evaluation of the tests with the newly prepared antigen was intensively discussed. In addition, sera from a German dairy herd were examined with both tests. The results of the two tests were compared with those obtained by a different ELISA which was carried out in Hannover earlier. Furthermore, I established an ELISA for the diagnosis of *S. tenella* and *S. arieticanis* infections in sheep and trained the staff at the Moredun Research Institute in the methodology of this test. The antigen, reference sera and conjugate for this ELISA originated from the Tierärztliche Hochschule Hannover.

Thirdly, different PCR methods developed at both laboratories for the diagnosis of different cyst-forming coccidia were compared. I introduced a Nested-PCR for the species-specific diagnosis of *S. tenella* as well as *S. arieticanis* at the Moredun Research Institute. Primers for this tests were previously developed at the Tierärztliche Hochschule Hannover. In return, I learned the Nested-PCR methods for the diagnosis of *N. caninum* or *T. gondii* currently carried out at the Moredun Research Institute. These methods will now be established as diagnostic tools at the Tierärztliche Hochschule Hannover. In addition, blood samples from a German dairy herd and a German sheep herd as well as samples from tissue sections were examined by the PCRs.

I wish to thank Dr. David Buxton and all the members of his group at the Moredun Research Institute for their kindness and helpfulness during my stay in Edinburgh.

**6.2 Beate E. Schnitzler**
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**Visit to Animal Health in Compton, Newbury, United Kingdom, November/December, 1998**

From 16th November to 18th December 1998, I spent several weeks working at the Institute for Animal Health in Compton, Newbury, UK. During this period I learned some basic as well as some more advanced techniques applicable to research on Poultry *Eimeria*.

Aim of this Short Term Scientific Mission was to plan several different experiments which are part of a big 3-year collaborative project between the Swedish University of Agricultural Sciences in Uppsala, Sweden and the Institute for Animal Health in Compton, UK. These experiments had to be planed and divided between the two locations. Additionally I had the opportunity to learn and understand the basic techniques that should enable me to proceed with the part of the experiments taking place in Uppsala.

Basic techniques I learned and commonly used, at the Institute for Animal Health include large scale preparations of faecal samples, flotations, harvesting and counting of oocysts, preparation for DNA extractions and different methods for DNA extractions (extractions from oocysts and from sporocysts), different techniques for inoculations of chickens (single sporocyst inoculation).

Furthermore I was introduced to a new technique which implies the use of Amplified Fragment Length Polymorphism markers (AFLP's). This is a popular new technique based on a molecular marker system and helpful for the construction of a genetic linkage map.

I would like to thank Drs Fiona Tomley and Martin Shirley and all the members of the Parasitology Department at the Institute for Animal Health for their kindness and helpfulness during my stay in Compton. The introduction to the techniques will certainly help me not only with the experiments

planed in the near future which are based on a long cooperation between the two institutes but also contribute substantially to my general knowledge of Coccidiosis.

Furthermore I would like to thank COST 820 for the financial support that made this short scientific visit possible.

### 6.3 Enrique Tabares

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**Visit to Animal Health in Compton, Newbury, United Kingdom, November/December, 1998**

My visit to the Institute for Animal Health (IAH) took place from November 30th to December 18th 1998 (3 weeks). The aim of this visit was to construct a Cryptosporidium parvum cDNA library in order to identify parasite genes of interest. Contacts with Dr. Fiona Tomley and Dr. Martin W. Shirley at the IAH were established because of their expertise developing cDNA libraries for the close related protozoa *Eimeria tenella*.

During this three weeks, two different cDNA libraries were developed. The first was from a cattle isolate (passage in sheep) from Spain (CUCM-1) and the second from a cattle isolate from U.S.A. (CUSA-1). *C. parvum* mRNA was extracted from $10^{10}$ sheep oocysts or $2x10^9$ cattle oocysts using the Fast Track 2.0 Kit (Invitrogen). For the development of the cDNA libraries the l-ZAP cDNA Synthesis Kit (Stratagene) was used. Synthesis of the first and second cDNA strands was followed using label dNTPs. The cDNA obtained was fractionated in sepharose CL-2B columns and 1 and 3 high molecular weight fractions were recovered from the CUCM-1 and CUSA-1 isolates respectively. Approximately, 70 mg of each fraction were ligated into Uni-Zap XR vector and 1 ml of each ligation was packaged using the Gigapack III Gold Cloning Kit. Finally, phages were growth into XL1-BLUE cells.

Titres of primary cDNA libraries were around $2x10^6$ with a ratio of and recombinant and non recombinant colonies was greater than 100:1. The length of the inserted DNA fragments was determined in 9 random colonies for each library by PCR (Polymerase Chain Reaction) using the T3 and T7 promoter sequences as primers and was found to be between 800 and 4,000 base pairs. Primary libraries were amplified and number of the titres were between $2x10^9$ and $1.6x10^{10}$ pfu/ml.

This is the first l-ZAP C. parvum cDNA library developed in Europe and has been possible due to COST action 820. The expertise of Dr. Fiona Tomley was central to the successful end of the experiment and the work has opened the possibilities for future collaborations between our laboratories.

# 7. ACTIVITY REPORTS

## 7.1. DENMARK

REPORTERS

Peter Lind, Lene Jensen, Gregers Jungersen, Hans-Christian Slotved

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### *Toxoplasma gondii*

A 5-year study on identification of genetic markers for *T. gondii* subpopulations and virulence properties were completed in early1999. More than 40 isolates, collected from swine, sheep, cats and foxes in DK after 1992 have been typed using monoclonal antibodies. A more discriminatory typing system, based on a repetitive non-coding element was recently developed by the project partner at the Statens Serum Institute, Copenhagen, and will be used to study a possible clustering of the Danish isolates according to host species of origin and already established virulence characteristics after inoculations to mice, young pigs and pregnant mini-gilts.

### *Neospora caninum*

Studies on *N. caninum* infection in cattle herds have been a major activity during the last couple of years in cooperation with Swedish colleagues in Uppsala and the Danish Agricultural Advisory Centre. Longitudinal blood samplings of cows from moderately to heavily infected herds over an 18 mo. period have shown a reasonable constancy in serological status (specific IgG1 in ISCOM-ELISA) over the cows' gestation, with 90% maintaining either positive or negative status, 8% showing apparent seroconversion and 2.5% with fluctuating status. The IgG1/IgG2 ratio for Neospora-specific antibodies showed a drop at the time of calving/abortion, which may reflect changes in Th1/Th2 stimulation during gestation. A number of cows in two heavily infected dairy herds were subsequently sampled with monthly intervals during 1998 to study changes in IgG subclass profiles, phagocytosis and killing ability towards intracellular agents as well as cytokine

mRNA expression during gestation. The data is presently under analysis in order to find prognostic parameters for transplacental transmission of neosporosis in the individual cows.

A swine antiserum with specificity for *N. caninum*, obtained from experimentally infected gilts has been distributed as a positive control reagent to several partners within the COST 820 action. No indications of naturally prevailing infections with *N. caninum* in European pig populations have so far been reported.

**Publications**:


- Jensen, L., Jensen, T.K., Lind, P., Henriksen, S. A., Uggla, A. & Bille-Hansen, V. Experimental porcine neosporosis. APMIS, 106, 475-482, 1998.
- Jensen, L., Heegaard, P.M.H. & Lind, P.: A study of virulence parameters for *Toxoplasma gondii* infections in mice. Parasitology Research, 84, 382-87, 1998 .
- Jensen, L., Petersen, E., Henriksen, S.Aa., Dietz, H.H. & Lind, P. 1998: Monoclonal antibodies to *Toxoplasma gondii* strain 119 identify recently isolated Danish strains as one group. International Journal for Parasitology, 28, 1305-1313.
- Jensen, A.M., Björkman, C., Kjeldsen, A.M., Wedderkopp, A., Willadsen, C., Uggla, A. & Lind, P.: Sero-epidemiological aspects of *Neospora caninum* infection in Danish dairy herds. Preventive Veterinary Medicine (submitted).
- Petersen, E., Lebech, M., Jensen, L., Lind, P., Rask, M, Bagger, P., Björkman, C. & Uggla, A.: Infection with *Neospora caninum* is not a major cause of habitual abortion in humans. Emerging Infectious Diseases (accepted for publication).
- Slotved, H.C., Jensen, L. & Lind, P.: Antibody response in bovine fetuses with *Neospora caninum* infection. International Journal for Parasitology (submitted).
- Jungersen, G., Jensen, L., Riber, U., Heegaard, P., Petersen, E. & Lind, P.: Pathogenicity of selected *Toxoplasma gondii* isolates after intravenous inoculation in pigs. International Journal for Parasitology (submitted).
- Jungersen, G., Jensen, L., Rask, M.R. & Lind, P.: Differences in virulence of selected Danish *Toxoplasma gondii* isolates in mice. Parasitology Research (submitted).

## 7.2. FRANCE

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Following ongoing programs, our 1998 efforts focused on the molecular characterisation of *Cryptosporidium* isolates, and the study of interactions between *Cryptosporidium* life cycle stages and the apical region of enterocytes.

### 1. Genotyping *Cryptosporidium* isolates

**1a**. As part of a project aimed at understanding the population structure of *C.parvum*, we characterised a new polymorphic DNA region of the *C.parvum* genome, 8G26. This DNA region had been cloned following a previously described procedure (FEMS Microbiology Letters 1996, **137** : 207-211). Direct sequencing of PCR products obtained from the central region of this clone identified 6 polymorphic nucleotide positions that determined 2 distinct genotypes. This new typing system was applied to human, calf, and goat isolates of *Cryptosporidium*, and the distribution observed at the 8G26 locus was compared to the distribution of the same isolates obtained with the 5C12 PCR-RFLP assay we described previously (FEMS Microbiology Letters 1996, **137** : 207-211) (manuscript in preparation)

**1b**. We further characterised a bird isolate of *Cryptosporidium* that had been identified as *C. meleagridis* on the basis of its host specificity and site of infection, but gave positive reactions with PCR reactions aimed at identifying *C.parvum* (Applied and Environmental Microbiology 1998, **64**: 1454-1458; Parasitology Today 1999, **15**: 80-81).

## 2. Interactions between *C. parvum* and the apical region of enterocytes

In order to understand the molecular basis of the *Cryptosporidium*-enterocyte interaction, we analysed the organisation of cytoskeletal components of the microvilli during invasion and intracellular development of enterocytes by *C. parvum*. To do so, we used immunoelectron microscopy and antibodies to villin, ezrin and actin, 3 of the microvillus cytoskeleton components, to study the Localization of these molecules in *C. parvum* infected enterocytes. These experiments showed that the vacuolar wall surrounding *C. parvum* contains 2 microvillar derived components, villin and ezrin (manuscript in preparation).

REPORTER: Dr Pierre Pery

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## 1) Amplification of immune responses against recombinant proteins

In order to provoke and improve a local immune response against *Eimeria* recombinant proteins, the effect of cholera toxin (CT) was investigated.

CT alone was shown to be very immunogenic in chickens when given by the oral route and to evoke both a local and systemic antibody production. When a recombinant protein, 1pe1 was mixed with CT and given, by this same route, an increase of the anti 1pe1 IgA production was noted in the duodenum 3 weeks post immunisation together with an increase of specific IgG production both in duodenum and caecum.

This effect was however too moderate to be used in further trials, perhaps due to the paucity of Peyer's patches in the chicken intestine.

ISCOMs were then used with the same goal and since it was expressed, purified and renatured, Ea HSP 70 was the incorporated antigen. When tested by the oral route, these ISCOMs were unable to evoke an immune response either at the local level or in the serum although they were given several times to each birds.

On the contrary, when given by parenteral routes, this ISCOMs formulation was very immunogenic both in antibody production and spleen cell priming, whereas HSP 70 alone in the same conditions did not induce these responses at all.

A protection trial was then performed with these immunised chickens and a slight protection was obtained both at the oocyst output level (38% for the first day of oocyst secretion ) and at the body weight loss level. However, when the total oocyst number were compared this significant decrease was no longer observed. We did no find explanation for this delaying effect on the life cycle and such an experiment must be reproduced before we can conclude

The carrier effect of the HSP 70 from mycobacterium tuberculosis is well know and was studied with a peptide repeat from the circumsporozoite protein from *Plasmodium falciparum* and as fusion protein with protein from the HIV virus .
We are then trying to express fusion proteins with the Ea HSP70 in order to study this carrier effect. Ea aspartyl protease or peptides thereof were then tentatively fused at the plasmid level. Until now we were unable to obtain expression of such constructs and we are study other constructs and other expression conditions.

**2) Identification and characterisation of proteins from primary schizonts.**
this project continued through 1998 and certainly 1999 and after, being now our most important project. Several new genes were identified and their expression compared between sporozoites and primary schizonts. When isolated, the gene coding for such proteins are expressed, and antibodies obtained against the recombinant proteins. These antibodies will be used to localise the relevant antigens and to check an effect in inhibition of parasite development in *in vitro* culture of Eimeria tenella and finally to study and understand the cellular biology of the primary schizont.

**3)Aspartyl-proteinase**
This project has continued in collaboration with Fiona Tomley in Compton were L. Jean is performing her PhD on this topics. In Jouy, because we were unable to activate bacterial recombinant Ea prepro aspartyl proteinase after renaturation, all the genes related to the proteinases were successfully transferred in the yeast *Pichia pastoris* and expression after methanol induction is now under study

## 7.3. GERMANY

REPORTER: Professor H. Zahner

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**Changes of epithelial ion transport by intracellular coccidia stages (S. Kowalik et al.)**

Coccidia rely on their host cells for energy supply and nutrition and cause clinical signs as diarrhoea. Changes of ion transport systems in the host cells may be involved in these processes.

An in vitro system was therefore established to study the effects of coccidian parasites on ion transport systems in epithelial tissues. It used HT29/B6, a human colon carcinoma cell line, which builds a polarised monolayer like an epithelium. *Toxoplasma gondii* served as a model parasite as tachyzoites invade HT29/B6 and replicate rapidly. Mature schizonts are formed within 20 h and merozoites are released after 24 - 30 h. Ion transport was measured in a special perfusion chamber (ussing system). Parameters determined were: short circuit current and conductance to record the electrogenic ion transport, chloride fluxes with $^{36}Cl$ (mucoserosal, seromucosal) and the paracellular water transport with $^{3}H$-mannitol. Measurement was performed 5 h, 10 h and 15 h *post infection* using monolayers in which approximately 30% of the cells were parasitised. The infection had rapid effects on the conductance of infected monolayers , which was two to three times higher than that of uninfected HT29/B6 monolayers throughout the observation period. Chloride fluxes of both directions were also increased two to three times when compared to uninfected cells, independent of the time after infection. However, the chloride netto fluxes and short circuit currents were unaffected by the parasites. Data on the paracellular water transport will show whether the increased chloride fluxes and conductances are due to a higher chloride transport through the cells or through paracellular pathways.

**Immunity in rats against Eimeria separata, a coccidial parasite of the large intestine (M. Q. Shi et al. )**

The study aimed to characterise the local reactions in the caecum of naive rats and rats immune against *E. separata* to a primary and a challenge infection, respectively. Optimum immunity was induced by two infections with 5000 oocysts per animal each, given in a 10 days interval. The oocyst production of a challenge infection administered after further 10 days was almost completely abolished. Histopathological investigations performed up to 72 h after challenge showed, that predominantly the development of first generation schizonts were affected in immune rats.

Challenged animals showed significantly increased infiltrations with lymphocytes, plasma cells, macrophages, eosinophils and mast cells when compared with rats after primary infection. Immunohistological studies compared local tissue infiltrations in naive and immune rats over a period of 48 h after primary infection and challenge, respectively. There was no difference between the groups concerning $CD45R^+$ cells, whereas significantly more $CD3^+$ cells were found in the caecum wall of the immune rats. $CD4^+$cells predominated in animals after primary infection, whereas $CD8^+$ cells represented the predominant T cell subset in challenged rats. The proportion of $\gamma\delta^+$ T cells did not differ between the groups, whereas challenged rats showed significantly increased numbers of $\alpha\beta^+$ T cells in the caecum wall, when compared with animals after a primary

infection. These findings suggest that predominantly $CD4^+$ cells may be involved in the immune response after primary infection, and that immunity to a challenge infection may be mediated by $CD3^+$, $CD8^+$ and $\alpha\beta^+$ T cells.

**Development of effective in vitro methods for excystation of sporozoites (S. Kowalik et al.)**
In vitro studies with coccidia are sometimes hampered by a lack of suitable protocols for the isolation of viable sporozoites. Thus, in case of *Eimeria separata,* a parasite of the large intestine of rats, none of the techniques described for other coccidia was found sufficient. Therefore a method was developed particularly suitable for this parasite. The procedure uses conditions which resemble the *in vivo* environment. A first treatment of the oocysts in a 0.4 % pepsin/HC1 solution alters the oocyst wall, which becomes thinner. The second treatment in a 0.4 % trypsin/0.75 % taurocholate medium breaks the oocyst wall and sporocysts release. A third incubation of the oocyst-sporocyst mixture in a trypsin-free medium with 0.75 % taurocholate and an additive of $MgCl_2$ followed by a final incubation in medium RPMI with 1 % foetal calf serum yields an excystation of sporozoites up to 90 %.

**Phophocholine epitopes in *Eimeria bovis* (B. Heise et al.)**
Phosphocholine (pc) epitopes are well known in parasitic helminths and seem to be involved in immunoregulative processes. In parasitic protozoa there is no information on this posttranslational modification. Studies with TEP15, a myeloma protein which specifically recognises PC showed 3 reactive molecules of 66, 70 and 75 kDa in *E. bovis* merozoites I. PC epitopes are localised on the surface of merozoites and sporozoites in relative large amounts and can be removed by treatment with methanol or phospholipase C. Together with other data the results suggest that PC is linked to glycosphingolipids as they were described in *Ascaris spp.* Methanol treatment disclosed additional TEPC15-reactive material in an apical subpellicular, cap-like structure. Current experiment attempt to isolate PC-bearing molecules for further detailed characterisation.

**REPORTER**

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**1 Report on VIIth International Coccidiosis Conference/COST820 Annual Workshop 1997**

Together with Dr N. Smith, Professor H. D. Chapman and Dr Anna Lundén I wrote a conference report which was published in Parasitology Today 14, 215-218.

**2 COST820 Annual Workshop 1998, Toledo, Spain**

I contributed to the general scientific planning of the conference, provided data about the economic impact of bovine neosporosis in Germany (presented as part of a European overview by Dr J. Wastling) and acted as a chairperson in one of the scientific sessions.

**3 Management Committee (MC)**

As one of the German members, I attended two meetings of the MC held on 25 February, 1997, in Woking, United Kingdom, and on 22nd October 1998 in Toledo, Spain. Together with Dr Astrid M. Tenter, Dr Fiona Tomley, Professor B. Gottstein and Dr F. Spano I work on the design of networking activities in the field of coccidiosis research beyond COST820.

**4 Involvement in Working Group 4 "Toxoplasmosis, Sarcocystiosis, Neosporosis"**

As a result of the activities of Working Group 4, I co-ordinated the preparation of a research proposal on diagnostic and epidemiological aspects of bovine neosporosis which was submitted to the FAIR Programme.

**5 Exchange of expertise and material**

As a consequence of scientific contacts and discussions at COST820 meetings, expertise and materials were exchanged with several scientists participating in the COST820 action.

## 7.4. ITALY

REPORTER: Dr. Furio Spano

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**Progress report:**

a) Cloning of *Cryptosporidium parvum* proteins with adhesive properties.

In our search for *Cryptosporidium* proteins potentially involved in the invasion process we have

recently cloned a *C. parvum* cDNA encoding a polypeptide, named sporozoite cysteine-rich protein (SCRP), possessing the following structural features: i) a leader peptide, ii) three thrombospondin-like repeats, iii) one EGF-like domain and iv) a transmembrane sequence. SCRP is the second thrombospondin-related protein identified in *C. parvum* that adds to TRAP-C1 (Spano et al., *Mol. Biochem. Parasitol.* 92: 147-162, 1998), a micronemal protein recently cloned in our laboratory sharing structural similarity with Etp100 of *E. tenella* and MIC2 of *T. gondii*. The SCRP gene is highly expressed in *C. parvum* sporozoites, where the protein seems to accumulate in the apical portion of the parasite. Work is in progress to establish the exact sub-cellular location of SCRP and assess its possible involvement in sporozoite gliding motility and/or host cell invasion.

b) Analysis of genetic variation in *Cryptosporidium parvum.*

Our interest in *C. parvum* has recently extended to the study of the parasite genetic variation. Most of the *C. parvum* typing studies carried out to date are based on the analysis of single polymorphisms. A multilocus approach has the potential to better define the structure of the *C. parvum* population and assess the degree of genetic isolation of the H (genotype1) and C (genotype 2) subpopulations. With this in mind we genotyped 28 *C. parvum* isolates of various host and geographical origin by simultaneously analysing up to five polymorphic loci belonging to at least three distinct linkage groups. Isolates were from Europe, North and South America and Australia. Seventeen isolates originated directly from humans, 3 were derived from humans and propagated in calves, and 8 were from various animals. Human isolates were both from sporadic cases and from documented outbreaks. Two isolates were derived from AIDS patients. Animal samples included the widely used Moredun and GCH1 isolates. The five polymorphic loci selected for this study have been shown to consist of at least two alleles differentially associated with animal or human *C. parvum* isolates. Four of the genetic markers were analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays and corresponded to the protein-encoding genes *Cryptosporidium* Oocyst Wall Protein (COWP) (Spano et al., *FEMS Microbiol. Letters* 152: 209-217, 1997), polythreonine (polyT) (Carraway et al., *Infect. Immun.* 65: 3958-3960, 1997), thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) (Spano et al., *Mol. Biochem. Parasitol.* 92: 147-162, 1998) and ribonucleotide reductase (RNR) R1 subunit. The fifth polymorphism, residing within the internal transcribed spacer region 1 (ITS1) of the Type A ribosomal unit, was analysed by PCR employing genotype-specific primers (Carraway et al., *Appl. Environ. Microbiol.* 62: 712-716, 1996). Consistent with previous studies, isolates segregated into two groups, one comprising both human and animal isolates, the other only isolates

of human origin. No recombinant genotype was detected. This evidence, together with the broad geographical distribution of the multilocus genotypes identified, brings further support to the hypothesis that *C. parvum* reproduction is substantially clonal.

c) Molecular characterization of novel SAG1-related *Toxoplasma gondii* antigens.

The invasive stages of the apicomplexan parasite *Toxoplasma gondii* are known to express a set of surface antigens, known as the SAG proteins, sharing structural and functional characteristics. These molecules, which are anchored to the plasmamembrane *via* a GPI motif, are differentially expressed in sporozoites, tachyzoites and bradyzoites and are believed to play a role in the early steps of host cell invasion. The most extensively characterised among the SAG proteins are SAG1 (p30), which is expressed exclusively in the tachyzoites and represents the immunodominant antigen during *T. gondii* infection (Burg et al., *J. Immunol.* 141: 3584-3591, 1988) and SAG3 (p43), which is present in all invasive stages (Cesbron-Delauw et al., *J. Biol. Chem.* 269: 16217-16222, 1994). These two molecules share approximately 40% amino acid identity and the presence of two amino acid repeats each containing 6 cysteine residues at conserved positions. *T. gondii* lines defective for either SAG1 or SAG3 have been shown to possess a reduced invasive activity. Recently, a series of novel *T. gondii* proteins sharing close structural similarity with SAG1 and SAG3 have been identified: SRS1-4 and BSR4 by Boothroyd and co-workers (Hehl et al., *Mol. Biochem. Parasitol.* 89: 271-282, 1997) and SAG5 in our laboratory.

A portion of the SAG5 gene was accidentally amplified from the genomic DNA of *T. gondii* (RH strain) employing unrelated primers. The PCR product 12G/16, encoding a 212 amino acid-long polypeptide showing a significant homology to both SAG1 and SAG3, was used as a probe to screen a genomic *T. gondii* cosmid library. Southern blot analysis of the positive cosmid clones and of *T. gondii* genomic DNA employing the 12G/16 fragment as a probe yielded complex hybridisation patterns, suggesting the existence, in the genome of *T. gondii*, of a family of related genes. Extensive sequence analysis confirmed this hypothesis and revealed the presence of three tightly clustered genes encoding closely related proteins, denominated SAG5.1, SAG5.2 and SAG5.3. All three polypeptides show the hallmarks of the family of SAG1-related molecules, being characterised by a leader peptide, 12 cysteine residues whose distribution suggests a tandem duplication of an ancestral 6-cysteine motif and a C-terminal GPI-anchoring signal. SAG5.2 and SAG5.3 are 367 amino acid-long and differ from each other at 8 amino acid positions (98% identity), while SAG5.1, which consists of 362 amino acids, is virtually identical to the other two SAG5 proteins only within the C-terminal region (positions 162-362). Notably, in the N-terminal

half of the molecule the degree of amino acid identity to SAG5.2/SAG5.3 drops to approximately 40%, which represents the average homology shared by all members of the "SAG1" protein family. Northern blot and RT-PCR analysis of poly(A)+ RNA showed that all three SAG5 genes are expressed in tachyzoites. The transcriptional activity in the other invasive stages is under study. The Localization of the SAG5 proteins in *T. gondii* tachyzoites was investigated by immunofluorescence and immunoblot employing polyclonal antibodies raised against recombinant histidine-tagged fragments of SAG5.3 and SAG5.1 produced in *E. coli* and affinity purified by nickel chelate chromatography. SAG5.2 and SAG5.3 appear to be exposed on the parasite plasmamembrane, while the localisation of SAG5.1 will require further investigation.

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**COST820 Meetings**

- Participation to the annual COST820 general meeting: "Vaccines against animal coccidioses". Toledo, Spain, 22-25 October, 1998.
- Organisation of the COST820/WG1-WG6 joint meeting: "Transfection and whole genome approaches to gene discovery and function: the way forward for vaccine research in coccidians?". Grottaferrata (Rome), Italy, 19-21 June, 1998.

## 7.5 SWEDEN

**Participating departments and scientists**

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**Avian coccidiosis - Working Group 1**

PCR methods for species identification

A polymerase chain reaction (PCR) assay, based on the amplification of internal transcribed spacer 1 (ITS1) regions of ribosomal DNA, was developed for the chicken coccidian species *Eimeria maxima*, *E. mitis* and *E. praecox*. Thus, in combination with our previous work (5), a complete set of ITS1 based, species-specific primers for the detection and discrimination of all seven *Eimeria* species that infect the domestic fowl is now available.

ITS1 primers for each of these seven species of *Eimeria* were further tested and evaluated in field samples. Additionally, they could be used as capture probes in a colormetric paper chromatography assay (PACHA). The addition of PACHA to the PCR assay provided a faster, more simplified alternative to the visualisation of PCR fragments by ethidium bromide staining and can be a useful approach in epidemiological studies (6-12).

Alternatives to coccidiostats

Non-chemotherapeutic means to control coccidiosis in broiler chickens have been studied in a series of experiments (13-14). In Sweden antimicrobial and growth promoting feed additives are not routinely used. Since some anticoccidials have antibacterial as well as anticoccidial effects, we have also studied how such substances affect the bacterial flora of the gut (2-3).

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**Sarcocystiosis, toxoplasmosis and neosporosis - Working Group 4**

Serological surveys of *N. caninum* infections

An ELISA based on *N. caninum* antigens incorporated into iscoms (immune stimulating complexes) has previously been developed (1-2). It has been used for serological studies of *N. caninum* infections in dogs, cattle and water buffaloes.

In a study comprising 80 dogs from the Czech and Slovak Republics, the sera were analysed for antibodies to *N. caninum* by the iscom ELISA and the indirect fluorescent antibody test (IFAT). One (1.3%) of the sera tested positive in both assays. Retrospective studies of the records of this dog revealed that it had shown clinical symptoms suggestive of *N. caninum*-infection (7).

Serum samples from 200 dairy cattle and 200 beef water buffaloes were collected in southern Vietnam during May to September 1995. The sera were analysed for antibodies to *N. caninum* by the iscom ELISA and IFAT and for antibodies to *Toxoplasma gondii* by the direct agglutination test. Significant levels of *N. caninum* antibodies were detected in 5.5% of the cattle sera and in 1.5% of the water buffalo sera. 10.5% of the cattle sera and 3% of the water buffalo sera were found to contain *T. gondii* antibodies. Two of the cattle sera had both *T. gondii* and *N. caninum* antibodies. The present communication is the first to report serological evidence of *N. caninum* infection in the water buffalo (5).

In collaboration with scientists from Hungary, 97 sera from dairy cows that had aborted were analysed for antibodies to *N. caninum* and other infectious agents known to cause bovine abortion. Antibodies to *N. caninum* were found in 10% of the cows indicating that this parasite might be a cause of abortion in Hungarian cows (4).

As part of a study comprising 1439 Norwegian dairy goats in 22 herds experiencing abortion problems, sera from 80 goats which had lost their foetuses and 25 goats with normal pregnancies were analysed for antibodies to *N. caninum*. Twenty three foetal brains were examined histologically and immunohistochemically for *N. caninum*. No evidence of *N. caninum* infection was found by any of the methods (3).

Oral *N. caninum* infection in neonatal calves

Out of 4 calves dosed orally soon after birth with *N. caninum* tachyzoites mixed with colostrum, 2 became infected as shown by serological reactions and demonstration of *N. caninum* in their brains by PCR (12-14). The data suggest that oral infection of *N. caninum* via colostrum is a possible route of vertical transmission in newborn calves, in addition to transplacental infection.

Experimental *N. caninum* infection in pigs

At the Danish Veterinary Laboratory, experimental infection of 6 pregnant gilts with the bovine *N. caninum* isolate Nc-SweB1 was performed (6). All gilts showed evidence of generalized infection, and, in one of them, transplacental transmission of the parasite to three foetuses was demonstrated. The study showed that *N. caninum* readily establishes in the pig and has the potential to be transmitted transplacentally to the offspring of infected sows.

Immunisation of mice against neosporosis

The possibility of inducing protection against *N. caninum* infection by immunisation, was investigated using a BALB/c mouse model. It was found that immunisation of mice with live parasites or parasite antigens either mixed with the saponin Quil A or incorporated into iscoms resulted in at least partial immunity against experimental neosporosis. In contrast, immunisation with parasite antigen without any adjuvant appeared to increase the susceptibility to infection (8-9).

*Sarcocystis* species in water buffaloes in Vietnam

Studies on *Sarcocystis* species of the water buffalo in Vietnam have been continuosly performed by a veterinary PhD student at at SWEPAR. Previously, three species of the water buffalo have been recognised, namely *S. fusiformis, S. buffalonis* and *S. levinei.*. During 1998, a paper describing a fourth bubaline species, *S. dubeyi,* was accepted for publication in the Journal of Parasitology, due out in January 1999.

Recognition of a *Sarcocystis* species in otter *(Lutra lutra)*

A paper describing the finding of large numbers of sarcocysts in the skeletal muscles of an otter in Sweden constitutes the first report of *Sarcocystis* infection in this species (15). The sarcocysts were 0.3 - 2.3 mm long and 0.06 - 0.25 mm wide, and ultrastructurally they had a serrated surface without any visible projections. The otter, although mainly a predatory animal, may serve as

intermediate host for a *Sarcocystis* species that completes its life-cycle in a not yet identified carnivore, or it may in this case have acted as accidental host due to possible immunosuppression.

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14. Uggla, A., Stenlund, S., Holmdahl, O. J M., Jakubek, E.-B., Thebo, P., Kindahl, H., Schnitzler, B. E. and Björkman, C. (1998). PCR-based detection of *Neospora caninum* in newborn calves: galactogenic transmission as a possible route of infection. Proceedings of the 11th Japanese-German Symposium on Protozoan Diseases, Chiba, Japan, p. 46.
15. Wahlström, K., Nikkilä, T. and Uggla, A. (1998). *Sarcocystis* species in skeletal muscle of otter *(Lutra lutra).* Parasitology, 118, 59-62.

**Immunology and cell biology - Working Group 6**

Molecular biology of *T. gondii*

We have previously used an RNA display technique to identify genes that are down-regulated in an avirulent *T.gondii* mutant. One of these genes have now been characterised in detail. The gene encodes for a 36.2 kDa large surface protein that is expressed in tachyzoites (1). Sequence analysis suggests that this protein most likely carries a signal peptide that is of importance for its targetting to the surface, and Western blot data suggest that this signal peptide is cleaved off during maturation. Preliminary experiments with antibodies raised against a recombinant form of the protein localise the native protein to the apical end of the parasite. Studies are now underway to determine its importance for virulence.

In collaboration with Dr. J. Wastling, University of Glasgow, UK, a number of *T. gondii* mutants overexpressing a cell-cycle dependent kinase have been constructed (2). On a short term scientific mission within the COST 820 framework, during the summer of 1998, Dr. Wastling visited SWEPAR where he studied the integration of novel genes into the genome of *T. gondii.*

**Publications**

1. Mattsson, J.G., Ljunggren, E., Bergström, K. and Soldati, D. (1998). Identification of a novel surface protein of *Toxoplasma gondii* using RNA display. Proceedings of the COST 820 Annual Conference on Coccidiosis, Toledo, Spain.
2. Wastling, J.M., Kinnaird, J.H. and Mattsson, J.G. (1998). Investigating the functional significance of *Toxoplasma gondii* cdc2-related kinases. Proceedings of the COST 820 Meeting on Coccidiosis, Rome, Italy.

## 7.6. SWITZERLAND

REPORTERS:
Research group members: A. Hemphill, S. Eperon, S. Sonda, N. Fuchs, H. Sager, B. Gottstein (Institute of Parasitology, University of Berne)

**Research on *Neospora caninum* and neosporosis: 1998**

**Part 1. Identification and characterisation of cell surface-associated- and intracellular antigens in *N. caninum* tachyzoites**

***Results based on a polyclonal anti-N. caninum antiserum:*** The approach used in our laboratory to identify possible parasite molecules involved in host cell entry was based on the rationale that these molecules must, at some stage, interact with membrane components. Thus, to identify membrane-interacting proteins of *N. caninum* tachyzoites, purified tachyzoites were fractionated using the non-ionic detergent Triton-X-114. We were successful in the identification, characterisation and purification of three antigenically reactive bands of approximately 43, 36 and 33 kDa, as described earlier. Nc-P36 exhibits a striking amino acid sequence similarity (76.3% similarity with 51.3% identities) to P30 (SAG1) the major *T. gondii* tachyzoite surface protein 1 which has previously been shown to serve as attachment factor. The deduced amino acid sequence of Nc-P43 was also determined (Genbank accession No. U93870). Comparison of the Nc-P43 sequence with *Toxoplasma* surface antigens shows a high degree of similarity to the members of the family of SAG1-related antigens, with the highest similarity (44% identical amino acids) to the SAG1-related sequence 2 (SRS2) protein (GenBank accession No. AF012276). The degree of conservation is less than for the SAG1/Nc-P36 pair, but more careful analysis unambiguously shows the preservation of an overall architecture which is a hallmark for the members of this family

of GPI-anchored surface antigens. All 12 cysteine residues in the mature proteins after cleavage of the putative N-terminal signal sequence can be aligned without introduction of significant gaps. Further biochemical evidence with regard to GPI-anchoring of Nc-P43 and Nc-P36 was provided in a collaboration with PD Dr. Peter Büttikofer's group at the Institute of Biochemistry and Molecular Biology of the Medical Faculty at the University of Bern by performing *in vivo* metabolic labelling of *N. caninum* tachyzoites using $^{3}$H-ethanolamine (which incorporates almost uniquely into GPI-anchors) and subsequent immunoprecipitation of respective proteins using specific antibodies.

**Part 2. Murine model for *N. caninum* infections, immunology and the role of NO.**

A mouse model for *N. caninum* infections was established in our laboratory. It is comprised of B-cell-deficient antibody knock-out mice (C57BL/6 mice with a transgenic mutation in the transmembrane exon of the IgM μ chain gene; μMT mice) and the corresponding parental strain C57BL/6. Wild type (wt) mice exhibited no symptoms upon an infective dose of $10^5$ parasites / mouse, while the μMT mice succumbed to lethal infection within 29-31 days p.i. PCR-analyses showed that more organs with a significantly higher infection and multifocal necrotic lesion intensity were parasitised in μMT mice than in wt mice. In both wt and μMT mice infected with live parasites, spleen cells stimulated with Nc-antigen produced high levels of IFN-γ and IL-10 in comparison to mice immunised with dead parasites. μMT spleen cells produced less IL-10 than wt splenocytes, the phenomenon became more evident with increasing time of infection. No IL-4 production was detected in either mouse strain. Infected mice produced higher amounts of IL-2 than control mice inoculated with dead parasites, but no significant differences were seen between wt and μMT mice. Splenocyte proliferation was actively suppressed by live *N. caninum* infection. Expression of inducible nitric oxide synthase (iNOS) was monitored on brain sections using an anti-murine iNOS antibody detected by the peroxidase method. The sections were subsequently immunolabelled with an anti-*N. caninum* antibody detected by the alkaline phosphatase method. In wt mice, parasite tachyzoites were found only at extremely low numbers in the brain and no iNOS could be detected in any brain cell. Conversely, in the brain of μMT mice, multifocal lesions were seen: numerous parasites, often clustered, were present around a central necrotic lesion. In this heavily infected region and in the more peripheral region as well, numerous macrophage-like cells were iNOS-positive. Spleen cells obtained from mice infected for 10 and 24 days were *in vitro* cultivated during 72 h in presence of either crude extract antigen from *N. caninum* or from

*Toxoplasma gondii* or LPS. Cells from μMT mice infected for 10 days produced nitric oxide (NO), whereas those from infected wt mice did not produce any NO. These results suggest that *N. caninum* increases both expression of iNOS and production of NO in cells of brain and spleen. The lethal outcome of *N. caninum*-infection in B-cell deficient mice may be partially explained by an overproduction of NO.

**Part 3. Vaccination studies**

Two types of vaccination studies are currently being carried out, using the murine model established in our lab. In collaboration with Camilla Björkman's group in Uppsala, passive immunisation with anti-*Neospora*-ISCOMs mAbs in μMT antibody knock-out mice is investigated, while active immunisation trials with recombinant Nc-P43 and Nc-P36 are performed using the parental C57BL/6 mouse strain. Planning of a third vaccination trial employing DNA vaccination of C57BL/10 mice (with cDNA corresponding to Nc-P43 and Nc-P36) is in progress.

**Part 4. Bovine neosporosis and epidemiology**

*Neospora caninum* has been recognised as one of the major protozoal abortion-inducing parasites in Swiss cattle. Diagnostic tools were initially validated upon experimental infection protocols. PCR was positive predominantly for the foetal brain and, additionally, for the abomasal and amniotic fluid of foetuses derived from experimentally infected heifers. The heifers seroconverted between day 10 to 17 p.i., foetuses remained serologically negative in all cases. By using these validated diagnostic methods, we have recently determined first prevalence data for the live-stock production in Switzerland. Thus, *Neospora*-DNA was detected by PCR in 24 brains (29%) from 83 bovine abortions, many of these brains were simultaneously characterised by histopathological findings typical for a protozoal, cerebral parasitosis. In another study we obtained first data on the *Neospora*- and *Toxoplasma*-seroprevalence in Swiss cattle. We tested 1,689 sera obtained from 113 dairy farms: The seroprevalence was 11.5% for *Neospora*-SA-ELISA and 10.7% for *Toxoplasma*-P30-ELISA. From the same samples, 1.1%, less than statistically expected, were positive in both ELISAs. Within selected groups of beef cow-calf farms, the seroprevalence using the *Neospora*-SA-ELISA was 14% for dams and 15% for offspring calves. Seroprevalences determined by *Toxoplasma*-P30-ELISA were 8% for dams and 3% for calves, respectively. None of the sera were double-positive by both ELISAs. In order to investigate the epidemiological situation in Switzerland further, a case-control-study covering six regions of Switzerland is presently performed.

## 7.7. UK

**REPORTER:**Dr D. Buxton

Pentlands Science Park, Bush Loan, by Edinburgh EH26 0PZ, Scotland, UK.

Publications from the Moredun Research Institute, Pentlands Science Park, Bush Loan, by Edinburgh EH26 0PZ, Scotland, UK.

**Working Group 3 (Cryptosporidiosis and cyclosporosis).**

1. Spano F, Putignani l, Naitza S, Puri C, Wright S E, Crisanti A. (1998). Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family. *Molecular and Biochemical Parasitology*. **92** 147-162.
2. Coop R L, Wright S E, Casemore D P. (1998). Cryptosporidiosis. Ch.45. 563-578. Zoonoses. Biology, Clinical Practice and Public Health Control. Eds. Palmer, Soulsby and Simpson. Oxford University Press.

**Working Group 4 (Sarcocystiosis, Toxoplasmosis and Neosporosis).**

**Papers in refereed scientific journals.**

1. Buxton, D. (1998). Protozoan infections (*Toxoplasma gondii, Neospora caninum* and *Sarcocystis* spp.) in sheep and goats: recent advances. **In** *Veterinary Research* Ed M. Pepin, **29**, 289-310.
2. Osawa, T., Wastling, J., Maley, S., Buxton, D. and Innes, E.A. (1998). A multiple antigen ELISA to detect Neospora-specific antibodies in bovine sera, bovine foetal fluids, ovine and caprine sera. *Veterinary Parasitology*. **79**, 19-34.
3. Buxton, D., Maley, S.W., Wright, S., Thomson, K.M., Rae, A.G. and Innes, E.A. (1998). The pathogenesis of experimental neosporosis in pregnant sheep. *Journal of Comparative Pathology*. **118**, 267-279.

4. Harkins, D., Clements, D.N., Maley, S., Marks, J., Wright, S., Esteban, I., Innes, E.A. and Buxton, D. (1998). Western blot analysis of the IgG responses of ruminants infected with Neospora caninum and Toxoplasma gondii. *Journal of Comparative Pathology* **119**, 45-55.
5. Gonzalez, L., Buxton, D., Atxaerandio, R., Aduriz, G., Maley, S., Marco, J.C., Cuervo, L.A. (1999). Bovine abortion associated with *Neospora caninum* in Spain. *Veterinary Record* (in press).
6. Björkman, C., Näslund, K., Stenlund, S., Maley, S.W., Buxton, D. and Uggla, A. (1999). An IgG avidity ELISA for the estimation of recency in bovine Neospora caninum infection. *Journal of Veterinary Diagnosis and Investigation* (in press).
7. Marks, J, Lundén, A., Harkins, D. and Innes, E. (1998). Identification of *Neospora* antigens recognized by CD4+ T cells and immune sera from experimentally infected cattle. *Parasite Immunology*, **20**, 303-309.
8. Lundén, A., Marks, J., Maley, S.W. and Innes, E.A. (1998). Cellular immune responses in cattle experimentally infected with *Neospora caninum. Parasite Immunology*, **20**, 519-526.
9. Ferguson D P J, Wright S E. (1998). An ultrastructural study of the host/parasite relationship of *Toxoplasma gondii* during enteric development in the small intestine of the cat. Electron Microscopy. Volume IV 335-336

REPORTER: Dr J M Wastling

In August 1998 a Short Term Scientific Mission funded under the COST 820 programme enabled Dr Jonathan Wastling of the University of Glasgow, United Kingdom, to spend a week at the laboratory of Dr Jens Mattsson of the Swedish National Veterinary Institute, Uppsala, Sweden. This collaboration arose directly as a result of the COST820 programme. The objectives of this visit were:

1. To strengthen the collaborative link between the University of Glasgow, United Kingdom, and the Swedish National Veterinary Institute, Uppsala, Sweden, particularly in the area of research into cyclin-dependent kinases of *Toxoplasma gondii*.
2. To enable the application of the technique of stable transformation of *T.gondii* to investigate the functional role of *Toxoplasma* CDKs.
3. To investigate collaborative projects and funding opportunities that might arise from the collaboration between Glasgow University and the Swedish National Veterinary Institute.

4. For Dr Wastling to meet other members of the Swedish National Veterinary Institute as well as other academic members of Uppsala University.
5. For Dr Wastling to present a seminar to the Swedish National Veterinary Institute on current research in Glasgow with the objective of highlighting areas of mutual interest between the two institutions, especially those topics relevant to the COST820 programme.

The funds provided by the European Commission under the *Short Term Scientific Mission* enabled the above objectives to be fulfilled. A strong link encompassing a number of scientific projects has now been established between the two institutions. Future studies on the use of yeast-two hybrid technology for detecting molecules that interact with *T.gondii* cyclin-dependent kinases and on reverse genetic technologies for determining gene function were planned during the visit. This work is currently in progress in the respective laboratories.

Attendance at two subsequent WG meetings in Rome and Toledo was also made possible by funding under the COST820 programme. These subsequent meetings were valuable in sustaining the outcomes of the Short Term Scientific Mission.

**Publication List 1998**
**Working Group Rommel/Tenter,**
**Tierärztliche Hochschule Hannover, Germany**

**Papers**


- Carreno, R.A., Schnitzler, B.E., Jeffries, A.C., Tenter, A.M., Johnson, A.M., Barta, J.R.: Phylogenetic analysis of coccidia based on 18S rDNA sequence comparison indicates that *Isospora* is most closely related to *Toxoplasma* and *Neospora.* J. Euk. Microbiol. 45, 184-188, 1998
- Daugschies, A., Rupp, U., Rommel, M.Blood clothing disorders during experimental sarcocystiosis in calves. Int. J. Parasitol. 28, 1187-1194, 1998
- Daugschies, A., Gäßlein, U., Rommel, M. Comparative efficacy of anticoccidials under the conditions of commercial broiler production and in battery trial. Vet. Parasitol. 76, 163-171, 1998

Abstracts

**Heckeroth, A.R., Tenter, A.M.:**

PCRs für die artspezifische Diagnose akuter Sarkozystiosen beim Schaf. In: 18. Tagung der Deutschen Gesellschaft für Parasitologie, Dresden, 24.-28.3.1998, V 37

**Heckeroth, A.R., Tenter, A.M.:**

18S rDNA based PCR: The first test for in vivo diagnosis of acute sarcocystiosis in sheep. IXth International Congress of Parasitology, Makuhari Chiba, Japan, 24.-28.8.1998. In: Parasitology into the 21st Century, Parasitol. Int., 47, 233

**Heckeroth, A.R., Tenter, A.M.:**

Comparison of immunological and molecular methods for the diagnosis of infections with pathogenic *Sarcocystis* spp. in sheep. In: 11th Japanese-German Cooperative Sympsosium on Protozoan Diseases, Isehara, Japan, 29.-31.8.1998

**Mugridge, N.B., Morrison, D., Johnson, A.M., Votypka, J., Tenter, A.M.:**

Phylogeny of the family Sarcocystidae. In: Proc. Annual Scientific Meeting Australian Society for Parasitology, Melbourne, Australia, 27.9.-1.10.1998

**Tenter, A.M.:**

- Life cycles, taxonomy and genetic relationships of intestinal coccidia of pigs. In: Proc. COST 820 "Vaccines against coccidiosis", Meeting Working Group 2, Weybridge, UK, 27.2.1998
- Bedeutung von molekularen phylogenetischen Untersuchungen für die Klassifikation und Taxonomie von Kokzidien mit Oozysten vom *Isospora*-Typ. In: 18. Tagung der Deutschen Gesellschaft für Parasitologie, 24.-28.3.1998, Dresden, V 117
- Epidemiological importance of animals in the transmission of *Toxoplasma.* IXth International Congress of Parasitology, Makuhari Chiba, Japan, 24.-28.8.1998. In: Parasitology into the 21st Century, Parasitol. Int., 47, 82
- Impact of molecular phylogenetic analyses on classification and taxonomy of coccidia with isosporan-type oocysts. IXth International Congress of Parasitology, Makuhari Chiba, Japan, 24.-28.8.1998. In: Parasitology into the 21st Century, Parasitol. Int., 47, 139

- Current knowledge on the epidemiology of infections with *Toxoplasma*. In: 11th Japanese-German Cooperative Sympsosium on Protozoan Diseases, Isehara, Japan, 29.-31.8.1998
- Epidemiology and economic impact of *Sarcocystis* species. In: Proc. COST 820 "Vaccines Against Coccidiosis", Annual Workshop, Toledo, Spain, 22.-25.10.1998

Dr. med. vet.-Thesis

**Heckeroth, A. R.:**
Entwicklung einer PCR-Methode zum artspezifischen Nachweis von akuten Sarkozystiosen bei Schafen. Dissertation, Tierärztliche Hochschule Hannover, 1998

## 8. COST 820 ANNUAL MEETING, TOLEDO, SPAIN

This Workshop was organised superbly by Professor Luis Ortega-Mora and his colleagues, with help from Dr Astrid Tenter, for preparing the scientific programme and liasing with all delegates.

Details of the programme are given in the Appendix, and copies of the Proceedings are available from Professor Luis Ortega-Mora.

## 9. APPENDICES

# COST 820 ANNUAL MEETING
# TOLEDO, SPAIN
# PROGRAMME

***Thursday, 22nd October 1998***

Arrival and accomodation at Beatriz Hotel

15.00 - 18.30 Registration (Tavera Conference Room lobby)

14.00 - 16.30 COST-820 Management Committee Meeting (Castilla I Room)

17.00 - 18.00 OPENING LECTURE (Chairperson: M.W. Shirley)

Invited expert: M. Tibayrenc

**Molecular epidemiology and evolutionary genetics of pathogens: the *Leishmania* case; comparison with other pathogens**

19.00 Wellcome reception (buffet) (Social Room)

***Friday, 23rd October 1998***

7.30 - 9.00 Breakfast (La Romana Cafeteria)

**RECOMBINANT ANTIGENS AND OTHER TOOLS FOR THE DEVELOPMENT OF MOLECULAR-ENGINEERED VACCINES AGAINST COCCIDIAL PARASITES**

**DNA Vaccines and Recombinant Antigens** (Chairpersons: A.M. Tenter and B. Gottstein)

9.00 - 9.45 INVITED LECTURE: R.C. Kennedy and A.M. Watts
**DNA vaccination strategies and the induction of protective immunity**

9.45 - 10.00 D. Licois, K. Daveau, N. Cere, D. Rasschàert and F. Drouet-Viard
**First step in DNA vaccine against rabbit coccidiosis: expression library construction from *Eimeria intestinalis* sporozoites**

10.00 - 10.15 S. Sagodira, D. Buzoni-Gatel, S. Iochmann, M. Naciri and D. Bout
**Protection of neonates against cryptosporidiosis after genetic vaccination of dams**

10.15 - 10.45 INVITED LECTURE: A. Hemphill
**Antigen detection and characterization**

10.45 - 11.00 S. Eperon, N. Fuchs, S. Sonda, A. Hemphill and B. Gottstein
**Vaccination of mice with recombinant proteins against *Neospora caninum***

11.00 - 11.15 H. Vedel-Nielsen, L. Christiansen and E. Petersen

**Use of recombinant SAG1 in a subunit vaccine against infection with *Toxoplasma gondii* in mice**

11.15 - 11.30 F. Petry and V. McDonald
**Further characterisation of recombinant antigens of *Cryptosporidium parvum***

11.30 - 12.00 Coffee (Social Room)

**Identification of New Vaccine Candidates** (Chairpersons: A. Hemphill and P. Pery)

12.00 - 12.15 J.M. Bumstead and F.M. Tomley
**Secretion of microneme proteins during invasion of *Eimeria tenella***

12.15 - 12.30 P.J. Brown, K.J. Billington, J.M. Bumstead, J.D. Clark and F.M. Tomley
**A microneme protein of *Eimeria tenella* shows homology to the binding domains of factor XI and plasma pre-kallikrein**

12.30 - 12.45 A. Bonnin, J.F. Dubremetz, J. López, S. Robine, C. Chaponnier and G. Gabbiani
**Detection of microvillous cytoskeletal components in the parasitophorous vacuole wall of *Cryptosporidium parvum***

12.45 - 13.00 F. Spano, I. Ricci, M. Di Cristina, C. Puri, L. Putignani and A. Crisanti
**The growing family of SAG-1 related antigens of *Toxoplasma gondii*: molecular characterization and expression analysis of the SAG-5 gene cluster**

13.00 - 13.15 S. Sonda, N. Fuchs, B. Connolly, N. Müller and A. Hemphill (Switzerland)
**Molecular characterization of the *Neospora caninum* tachyzoite surface protein Nc-p36**

13.15 - 13.30 N. Fuchs, S. Sonda, B. Gottstein and A. Hemphill
**Differential expression of cell surface -and dense granule- associated *Neospora caninum proteins* in tachyzoites and bradyzoites**

13.30 - 15.00 Lunch (Beatriz Restaurant)

**Adjuvants and Vectors** (Chairpersons: D. Williams and F. Petry)

15.00 - 15.30 INVITED LECTURE: K. Lövgren Bengtsson
**Vaccine adjuvants**

15.30 - 15.45 F. Girard, P.Quéré, K. Lövgren, R. Mancanola and P. Péry
**Systemic immunoadjuvant activity of ISCOMs presentation for *Eimeria* recombinant HSP70 in chickens and protection against the parasite**

15.45 - 16.30 INVITED LECTURE: J.M. Escribano

**Antigen delivery estrategies for mucosal vaccines**

16.30 - 16.45 A. Vermeulen, D. Schaap, J. Dorrestein, G. Arts, N. Verhoeven, S. Vos, S. Steeghs, M. Cronenberg, C.v. Geffen and P. Sondermeyer
**Vector systems used to present *Eimeria* antigens to the immune system of the host**

16. 45- 17.00 S.H.M. Jeurissen, E.M. Janse, M. Roos and L.J. Wiley
**The use of an apathogenic *Eimeria* as a parasitic vector**

17.00 - 17.30 Coffee (Social Room)

**Transfection** (Chairpersons: A. Vermeulen and F. Spano)

17.30 - 18.00 INVITED LECTURE: A. Hehl
**Transfection in *Toxoplasma gondii***

18.00 - 18.15 J.G. Mattsson, E. Ljunggren, K. Bergström and D. Soldati
**Identification of a novel surface protein of *Toxoplasma gondii* using RNA display**

18.15 - 18.30 M. Soete, T. Meyer, D. Schlüter, D. Soldati
**Expression of the bradyzoite-specific surface antigen SAG-4 in the Ts-4 vaccine strain of *Toxoplasma gondii***

18.30 - 18.45 B. Gottstein, A. Hemphill and S. Eperon (Switzerland)
**μMT knockout and wild-type mouse model for immunological studies in experimental neosporosis**

19.15 Dinner (Beatriz Restaurant)

20.45 Guided Tour of Toledo (Assemble in the Hotel lobby)

*Saturday, 24th October 1998*

7.30 - 9.00 Breakfast (La Romana Cafeteria)

## EPIDEMIOLOGY AND ECONOMIC IMPACT OF COCCIDIOSIS IN ANIMAL PRODUCTION

**Morning Scientific Session** (Chairpersons: M. Rommel and J. Catchpole)

9.00 - 9.15 B.E. Schnitzler, P.L. Thebo, F.M. Tomley, A. Uggla, M.W. Shirley
**PCR and PACHA as tools to investigate coccidian epidemiology**

9.15 - 9.30 F. Drouet-Viard, V. Veneziano, L. Rinaldi, G. Cringoli, D. Licois and P. Coudert
**Use of precocious lines in rabbit farms. Preliminary field surveys in France and Italy**

9.30 - 9.45 M. Giles, K.A. Webster, J.A. Green, J. Catchpole, C. Dawson, S. Pedraza-Díaz and J. McLauchlin
**Host specificity, pathogenesis and molecular classification of *Cryptosporidium* species**

9.45 - 10.00 S. Patel, J. McLauchlin, S. Pedraza-Díaz and D. Casemore
**Seroepidemiology and molecular characterisation of cryptosporidiosis**

10.00 - 10.15 G. Di Francesco, A.R. D'Angelo, A. Petrini and G. Savini
**Protozoal infections associated with abortion in Italian livestock**

10.15 - 10.30 H.C. Davison, A. Otter, N.P. French and A.J. Trees
**The epidemiology and significance of bovine neosporosis in England and Wales: interim results of a collaborative study**

10.30 - 10.45 A. Quintanilla-Gozalo, J.M. Pereira Bueno, E. Tabarés, E.A. Innes, R. González-Paniello and L.M. Ortega-Mora
**Seroprevalence of *Neospora caninum* infection in dairy and beef cattle in northern Spain**

10.45 - 11.00 J.M. Wastling, A. Schock, E.A. Innes and Y. Yamane
**Genetic polymorphisms in laboratory isolates of *Neospora caninum***

11.00 - 11.30 Coffee (Social Room)

**Round Table** (Chairperson: M.W. Shirley)

11.30 - 11.50 R. B. Williams
**What is the cost of coccidiosis to the world's chicken production industry?**

11.50 - 12.10 L.A. Stubbings and J. Catchpole
**Epidemiology and economic impact of coccidiosis in sheep and goats**

12.10 - 12.30 D.C. de Graaf, E. Vanopdenbosch and J.E. Peeters
**Introduction to the debate on the epidemiology and economic importance of *Cryptosporidium* spp.**

12.30 - 12.50 D. Buxton
**Epidemiology and economic impact of toxoplasmosis in animal production**

12.50 - 13.10 J.M. Wastling and E.A. Innes
**The economic impact of neosporosis**

13.10 - 13.30 A. M. Tenter
**Epidemiology and economic impact of *Sarcocystis* species**

13.30 - 13.40 Overall Discussion

13.40 - 15.15 Lunch (Beatriz Restaurant)

**Afternoon Scientific Session** (Chairpersons: F. Conraths and D. Buxton)

15.15 - 15.30 M. Pakandl, F. S. Renaux and F. Drouet-Viard
**Penetration of sporozoites of the rabbit coccidium *Eimeria coecicola* into the intestine of immune and naive rabbits**

15.30 - 15.45 C.A. Vergara-Castiblanco, S. Santos-Nuñez, F. Freire-Santos and E. Ares-Mazás
***Cryptosporidium parvum* in two Colombian populations: immunoblot analysis of specific serum response (IgA, IgG and IgM)**

15.45 - 16.00 P. Lind, A.M. Jensen, A.M. Kjeldsen, H.C. Slotved, C. Björkman and A. Uggla
**Follow-up studies in *Neospora caninum* infected dairy herds using IgG-subclass ELISAs**

16.00 - 16.15 J. Marks, I. Esteban-Redondo, A. Lundén, S. Maley, A. McLean Tooke, T. Duggan and E.A. Innes
**Immunity to *Neospora caninum***

16.15 - 16.30 C.S. Guy, D.J.L. Williams, F. Guy, J.W. McGarry, K. MacEacheran, R. Smith, D.F. Kelly and A.J.Trees

**Immunological and clinical responses in cattle following experimental infection with *Neospora caninum* at different stages of gestation**

16.30 - 16.45 T. Jäkel, J. Sangchai and Y. Khoprasert

**The role of immunological memory in *Sarcocystis* infection**

**POSTER SESSION** (Social Room)

16.45 – 18.00

1. A. Gawel, M. Mazurkiewicz and J. Jurowski

   **Influence of selected chemoprophylaxis methods on immunity against coccidiosis in chickens**

2. F. Cerník and P. Bedrník

   ***Eimeria tenella*- monitoring of cross resistance between ionophorus anticoccidial drugs**

3. A. Ramisz, A. Balicka-Ramisz and B. Pilarczyk

   **Epidemiological and economic impact of coccidiosis in cattle production**

4. F. Petry and J.R. Harris

   **Ultrastructural and molecular analysis of the extracellular stages of *Cryptosporidium parvum***

5. S. Pedraza-Díaz, S. Patel and J. McLauchlin

   **Molecular typing of *Cryptosporidium* from human water-borne outbreaks**

6. D. Champliaud, P. Gobet, M. Naciri, O. Vagner, J. Lopez, Y. Varga, G. Harly, R. Mancassola and A. Bonnin

   **Failure to differentiate *Cryptosporidium parvum* from *C. meleagridis* based on PCR amplification of eight DNA sequences**

7. F. Freire-Santos, C.A. Vergara-Castiblanco and E. Ares-Mazas

   **An attempt of experimental infection by *Cryptosporidium parvum* in rainbow trout**

8. H. Abbassi, F. Coudert, Y. Chérel, J. Brugère-Picoux and M. Naciri

   **Effect of pathogenic virus of Infectious Bursal Disease (IBD) on the pathogenicity of *Cryptosporidium baileyi* and the development of acquired immunity to parasite**

9. S. Hornok, Z. Széll, J. Nieuwenhuijs, M.G.B. Nieuwland, A.W.C.A. Cornelissen and I. Varga

   **Immunogenicity of three oocyst extracts of *Cryptosporidium baileyi* in experimentally infected chickens**

10. S. Hornok, Z. Bitay, Z. Széll and I. Varga

    **Assessment of maternal immunity to *Cryptosporidium baileyi* in chickens**

11. A. Lundén, J.E. Allen, D. Buxton and S. Wright
**Immunisation of mice against neosporosis**

12. A. Schock, D. Buxton, J. Wastling, J.A. Spence, A. Rae, J. Yamane and J.C. Low
**Histopathological survey with special reference to *Neospora caninum* induced abortion and preliminary molecular biological characterisation of *N. caninum* isolates**

13. R. Atkinsons, P.A.W. Harper, C. Ryce, D.A. Morrison and J.T. Ellis
**Investigations on the biological characteristics of two isolates of *Neospora caninum***

14. S.Wright, C. Gortazar, D. Fernández de Luco and D. Buxton
**Seroepidemiology to *Neospora* antibody responses in red foxes (*Vulpes vulpes*) in Spain**

15 W. Wouda, C.J.M. Bartels, A.R. Moen and Y.H. Schukken
**Case control study of *Neospora caninum* associated abortion in dairy herds in The Netherlands**

16. H. C. Slotved, L. Jensen and P. Lind
**Antibody responses in bovine fetal abortion due to neosporosis**

17. A. Uggla, S. Stenlund, O.J.M. Holmdahl, E.B. Jakubek, P. Thebo, H. Kindahl and C. Björkman
***Neospora caninum*: is oral transmission a possible route of infection?**

18. G. Jungersen, L. Jensen, U. Riber, P. Heegaard, E. Petersen and P. Lind
**Pathogenicity of selected *Toxoplasma gondii* isolates after intravenous inoculation in pigs**

17.45 - 18.45 **MEETING OF WORKING GROUP 3** (Chairpersons: D.C. de Graaf and A. Bonnin)

**A vaccine against cryptosporidiosis: reality or fantasy ?**

- General introduction (D.C. de Graaf)
- Is a vaccine necessary in veterinary medicine ? (D.C. de Graaf)
- Is a vaccine necessary in human medicine ? (A. Bonnin)
- The answers currently available (S. Sagodira)
- Mechanisms of immune response to cryptosporidiosis relevant to vaccination (F. Petry)
- Possible targets offered by the molecular biology (F. Spano)
- Priorities for the future and conclusion (A. Bonnin)

20.00 Conference Banquet (Conde de Orgaz Parador)
(Assemble in the Hotel lobby)

***Sunday October 25th***

7.30 – 9.00 Breakfast (La Romana Cafeteria)

9.0- Bus transfer from Beatriz Hotel to Madrid airport
(Assemble in the Hotel lobby)

**COST820 : *Vaccines against Animal Coccidioses***

# *"Transfection and whole genome approaches to gene discovery and function: the way forward for vaccine research in coccidians?"*

*Joint meeting of Working Groups I and VI*
*June 19 - 21, 1998*
*Rome, Italy*

*INTRODUCTORY LECTURE*
***Friday 19th June***

*ROUGH GUIDE TO GENOMIC SEQUENCING :*
*FROM DOLLARS SPENT TO BASE PAIRS COVERED.*
Daniel Lawson
*The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 ISA*

The past decade has seen the movement of sequencing technology from the laboratory bench to the factory floor. The majority of large scale (Mb's) sequencing is undertaken at a handful of genome centres specifically founded for this purpose. The scale of these centres has meant a reduction in the cost of sequencing, and has underpinned the successful completion of numerous bacterial genomes and the Yeast *Saccharomyces cerevisiae*.

These triumphs have been achieved without a radical change in technology. Fluorescent labelling of Sanger dideoxy chain termination method remains the primary sequencing chemistry. The major changes in methodology have been an increase in the size of DNA fragment to be attacked; from small lambda clone (kb's), through cosmids (10's kb), BACs (100's kb) to entire chromosomes or bacterial genomes (Mb's).

Recently, The Institute for Genomic Research (TIGR) and Perkin-Elmer announced an initiative which, in theory, revolutionalizes genomic sequencing. The arrival of capillary technology may hold the key to cheaper, faster sequencing. It re-opens old debates and at the least requires a reappraisal of what is the most cost effective strategy for genomic sequencing.

*SESSION ONE*
***Saturday June 20th, 0900-1300.***

*LESSONS LEARNT FROM LEISHMANIA*
Al Ivens
*(Dept. of Biochemistry, Imperial College of Science, Technology and Medicine, Exhibition Road, London SW7 2AZ, UK.)*

As part of the UNDP/World Bank/WHO-TDR parasite genomes initiative, our laboratory has been involved in the construction of a physical map of the *Leishmania major* Friedlin reference strain. The map has been assembled by the combination of fingerprint analysis of a shuttle vector cosmid library and probe hybridisation. The integrated data obtained from the fingerprints and over 1000 probes have placed approximately 94% of the ~33.6Mb genome into contigs representative of the 36 chromosomes. Sequencing tile sets have been identified, and a number of sequencing projects are underway within the Leishmania Genome Network. The data obtained from the Leishmania genome project have been analysed for possible functional significance, the output of which will be described. Data from the genome project are publicly available on the WWW at: http://www.ebi.ac.uk/parasites/leish.html

*HAPPY MAPPING OF CRYPTOSPORIDIUM PARVUM.*
Michael B. Piper, Alan T. Bankier and Paul H. Dear.
*(MRC Laboratory of Molecular Biology, Protein and Nucleic Acid Chemistry Division, Hills Road, Cambridge CB2 2QH, England.)*

HAPPY mapping is a fast and accurate method for the physical mapping of genomes. Many parasite genomes are difficult to map by traditional methods due to their unusual biological characteristics, e.g. genomic regions with high AT contents can lead to instability of bacterial clones. HAPPY mapping is an entirely in-vitro method and hence mapping is unaffected by such biological phenomena. A HAPPY mapping panel is constructed by breaking genomic DNA at random and then taking samples, each containing less than one genomes worth of DNA, from this pool of random fragments. Breaks between closely linked markers are rare, and these markers tend to co-segregate on the same DNA fragments, and hence also co-segregate in the same samples. Breaks between widely spaced markers are more common and hence they co-segregate less frequently. The principle is similar to that used in genetic mapping, when recombination between closely linked markers during meiosis is rare. We have constructed a HAPPY map of the apicomplexan parasite *Cryptosporidium parvum*. It

consists of over 130 STS (sequence tagged site) markers, which have been screened against a HAPPY mapping panel by nested PCR. They fall into 10 contigs. Cryptosporidium is believed to have 8 chromosomes and a genome size of ~10.4Mb, meaning that there are only 2 gaps in the map and that on average there is a marker every 78kb. The map is expected to be accurate to about 30kb, and was constructed by a single person in under six months. In addition we have constructed a PAC (P1 artificial chromosome) library of Cryptosporidium with an average insert size of 38kb. The library is publicly available, and in conjunction with the map, should greatly facilitate genomic studies of this organism.

*ABOUT THE TINY NUCLEAR GENOME OF MICROSPORIDIA*

Christian Vivares
*(Universite Blaise Pascal, Aubieres, France)*

Microsporidia, obligate intracellular parasites, with a wide host spectrum, represent a large group of pathogens of veterinary and medical importance mainly in AIDS patients. They can form a very small spore, contain 70S ribosomes and are devoid of mitochondria.

The haploid genome size varies between 2.3 Mbp and 19.6 Mbp and the number of chromosomes between 8 and 16, according to species. Karyotype polymorphism has been shown in *Encephalitozoon cuniculi*. The variants discriminated on the basis of rDNA ITS structure may be heterogeneous as regard to the karyotype.

The analysis of the *E. cuniculi* genome was performed through the systematic sequencing of the smallest chromosome (217 kbp) and the screening of a partial genomic library. The repetitive DNA comprises short micro- and minisatellites and rDNA units dispersed over all the chromosomes. SSU and LSU rRNAs are very reduced as a result from the deletion of numerous variable regions.

Currently, concerning chromosome I, a set of 30 randomly arranged contigs represents a total length of 180 kbp with 30 genes including 21 ORFs for known proteins and 9 ORFs with unknown function. Some intergenic regions are very short (DHFR,TS,SHMT contig) and the intron frequency very low. The sequence alignment of promoter regions suggests 2 boxes which may be involved in the initiation of transcription. A downstream box complementary to a part of the decoding region of the 16S rRNA frequently occur and can play a role in traduction initiation. A typical polyadenylation signal is often present. The mitochondrial signature of an HSP70 gene supports that microsporida have secondarily lost mitochondria.

With respect to proteins specifically associated to the major part of the invasive apparatus (polar tube), two different genes, close together on the chromosome VI, are of potential interest for diagnosis and therapeutics.

*TOWARDS A PHYSICAL MAP OF BRUGIA MALAYI : NEW INSIGHTS INTO THE BIOLOGY OF FILARIAL NEMATODES*

D.B.Guiliano[1], J. Daub[1], S.J. Jones[2],and M.L.Blaxter[1]
*([1] I.C.A.P.B. University of Edinburgh, West Mains Road Edinburgh EH9 3JT, UK. [2] The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge, CB10 1SA, UK)*

The *Brugia malayi* genome project is an international collaborative effort between seven laboratories funded by the WHO and MRC. The goal of this project is the identification of thousands of new Brugia genes and their placement on a medium resolution physical map. Over 14,000 ESTs have been sequenced from stage-specific *B. malayi* cDNA libraries. When grouped by sequence identity these 14,000 ESTs form over 5,000 clusters. A consensus sequence for each cluster has been generated from a multiple alignment of the constituent ESTs and this has been used in subsequent database analyses.

The *B. malayi* physical map will be generated by hybridisation of several thousand Brugia genes identified during the EST sequencing phase of the project and BAC and YAC end probes selected using a sampling without replacement strategy. Generation of high quality genomic libraries has been complicated by AT bias (~70%) of Brugia genomic DNA and the presence of a bacterial endosymbiote which efficiently clones into cosmid and BAC vectors. However we are now entering into the preliminary mapping phase of the project with over two dozen Brugia genes mapped to our BAC filters and the initiation of several walking experiments.

A single BAC containing the genomic copy of Bm-MIF-1 has been selected and is being sequenced at the Sanger Center. Construction of a physical map and the sequencing of a large piece of

Brugia genomic DNA will allow several important questions to be answered about nematode genomic organisation including the presence of operons, transposons, and the possibility of synteny between Brugia and *C. elegans*.

*CHROMOSOME ANALYSIS AND DIPLOKARYON ULTRASTRUCTURE IN MICROSPORIDIAN PARASITE NOSEMA GRYLLI (MICROSPORIDA: NOSEMATIDAE).*

Elena Nassonova[1,2], Julia Sokolova[2], Irma Issi[2], Sergei Skarlato[3] and Rolf Entzeroth[1]
*([1]-Institute of Zoology - Spezielle Zoologie, Technical University Dresden, Germany. [2]-All-Russian Institute of Plant Protection, Russ. Acad. Agric. Sci., St. Petersburg, Russia. [3]-Institute of Cytology, Russ. Acad. Sci., St. Petersburg, Russia.)*

The life cycle of *Nosema grylli* consists of three phases: infective, proliferative and sporogonial. The nuclear division, which occurs during the proliferative phase, involves a sequence of mitosis in the meronts and sporonts. The nuclear apparatus or "diplokaryon", consists of two closely adjacent nuclei, each of which is surrounded by a typical nuclear envelope. Diplokaryon is a very characteristic peculiarity of microsporidian intracellular stages, which allows them to be easily recognised in samples by different kinds of DNA-specific stains, e.g. by DAPI-staining. In dyplokaryotic nuclei of meronts and sporonts, closed intranuclear pleuromitosis was described. In this type of mitosis, the nuclear envelope stays intact; the entire mitotic apparatus is localized into the nucleus and the spindle is organised by special microtubule-organizing centres (MTOCs), so-called "centriolar plaques". In the course of mitosis chromosomes are weakly condensed and do not form a metaphase plate. Thus, the exact determination of the chromosome number by TEM is difficult. To obtain the detailed information on the chromosome content and basic genome organisation of *N. grylli*, the transverse alternating field electrophoresis (TAFE) was used.

By TAFE, using a program for DNA separation within a wide size range (50-1000kb), a set of chromosomal DNAs of *N. grylli* with the sizes 95-450kb was revealed. To separate numerous DNA molecules of similar sizes, a program with discrete increasing of pulse time was elaborated. It revealed 14 chromosomal bands ranging in size from 95 to 440kb. The total genome size of *N. grylli*, estimated on the basis of chromosomal DNA sizes, is about 3460kb.

The method of chromosomal DNA purification from intracellular stages of *N. grylli* was adapted. The attempt to compare the molecular karyotype from spores and intracellular stages of *N. grylli* was done. There seems to be no difference between electrophoretical patterns of DNA from infective and proliferative stages of the *N. grylli* life cycle.

Our data were compared to earlier reports on chromosomal DNAs from other species of the *Nosema* genus. The size of chromosomes of other species ranged from 139 to 1810 kb. The *Nosema spp.* from Orthoptera have the smallest genome sizes within the genus and have no chromosomal DNAs greater than 1000kb.

The variations of chromosome and genome sizes within *Nosema spp.* confirms the heterogenity of this genus, which has been discussed by Sprague, Issi and other authors (Sprague et al., 1992; Issi, 1986). Recent studies on phylogenetic relationships among *Nosema spp.* based on ribosomal RNA sequence data confirmed that this genus is composed of several unrelated groups (Baker et al., 1994).

*ONGOING CONSTRUCTION OF AN RFLP LINKAGE MAP OF EIMERIA TENELLA: (USE OF RAPD-PCR AND AFLP MARKERS)*

M. W. Shirley
*Institute for Animal Health, Compton, Nr Newbury, Berks. RG20 7NN*

An RFLP linkage map is being constructed of the genome of *Eimeria tenella*. The map derives from analyses of the inheritance of polymorphic DNA markers in 22 cloned progeny of a genetic cross made between two parents characterised by complementary phenotypic traits. The map currently comprises just over 200 polymorphic markers, of which those defined by RAPD-PCR and, especially, AFLP are the most numerous.

The results of an experimental cross which used parents with phenotypes of precocious development (attenuation) and resistance to arprinocid are providing insights into the behaviour of *Eimeria* chromosomes during meiosis and reveal an association between two different chromosomes and resistance to arprinocid or precocious development.

Some findings include:

- The extent of crossing-over between homologous chromosomes varies considerably within the karyotype and, for example, is substantially greater for chromosomes 1 and 2 than for chromosome chromosomes 9, 10 and 14.
- The frequency of DNA markers identified varies for the different chromosomes. For example, chromosomes 9, 11 and 14 are substantially over-represented in comparison to chromosome 10. Most chromosomes are characterised by a combination of polymorphic AFLP, RAPD-PCR and RFLP markers but, perhaps surprisingly, no AFLP markers have been identified for some chromosomes, including chromosome 14 which, at more than about 7 Mbp, is the largest in *E. tenella*.
- Linkage is inferred between arprinocid resistance and chromosome 1 and precocious development and (putative) chromosome 9.

*ESTABLISHMENT OF THE CRYPTOSPORIDIUM PARVUM KARYOTYPE*

S. Cacciò, R. Camilli, G. La Rosa, E. Pozio
*Laboratory of Parasitology, Istituto Superiore Sanità, Rome, Italy*

In several protozoa, including parasitic species, the chromatin is poorlycondensed during mitosis and distinct chromosomes can not be visualized. A breakthrough in the study of the genomes of parasites and of other lower eukaryotes was the development of the Pulsed Field Gel Electrophoresis (PFGE) technique, which allows for the separation of intact chromosomes. The use of PFGE has been instrumental for describing karyotypes, determining the ploidy status and genome size, investigating chromosomal polymorphisms and mapping of genes. So far, such studies have been conducted on important parasites such as *Trypanosoma*, *Leishmania*, *Giardia*, *Plasmodium*, *Toxoplasma*, *Eimeria*, *Babesia* and *Theileria*. We are interested in the study of the chromosome organization in the coccidian parasite *Cryptosporidium parvum* and our goal is the development of a physical map of the genome. So far, PFGE analysis of the *C. parvum* chromosomes revealed the presence of five bands in the 1-1.5 Mb range. However, it has been suggested that at least two of these bands are composed of multiple chromosomes very similar in size, which comigrate during the electrophoresis. This situation has hampered the determination of the chromosome number and size and also complicates the assigment of genes and of other DNA sequences to individual chromosomes. Since resolution of these bands into individual components is difficult to achieve, we have used restriction enzymes to cut chromosomes into fragments which can be more easily resolved by PFGE. Considering the AT-richness of the *C. parvum* genome, several restriction enzymes recognizing GC-rich motifs have been used. On the basis of these initial experiments, NotI and SfiI enzymes were selected for this study. The optimal separation of the NotI and SfiI restriction fragments in the low, intermediate and high molecular size range was obtained with three different PFG electrophoretic conditions. These experiments showed that the karyotype is comprised of eight chromosomes, ranging in size from approximately 0.95 to 1.45 Mb; of the eight chromosomes, five were cut by the SfiI enzyme whereas only three were cut by the NotI enzyme, confirming the rarity of GC-rich motifs in the genome. The genome size of *C. parvum* was estimated to be 9.6 Mb in size, a figure very close to that reported for *Theileria parva* and *Babesia bovis* (about 10 Mb), but quite different from that of two other coccidians, *Toxoplasma gondii* and *Eimeria tenella*, which have larger genomes (50-80 Mb). To confirm the identity of the chromosomes and of their restriction fragments, Southern blots of the PFGE gels were probed with a series of 20 markers, representing 16 coding sequences, the ribosomal DNA, a pair of repetitive DNA sequences and a telomeric repeated sequence, (TTAGGG)n. All gene probes hybridized to individual chromosomes or to their restriction fragments, as expected form their single-copy nature, whereas hybridization with the ribosomal DNA probe revealed the presence of 5 copies that were scattered over four different chromosomes. Finally, all chromosomes and restriction fragment hybridized with a telomeric probe, indicating that no internal fragments were generated by either NotI or SfiI restriction. Recently, a general model of chromosome organization in parasites has been proposed. This model suggests that chromosomes are compartmentalized into conserved central domains and polymorphic chromosome ends. The central domain is highly transcribed and contains most of the structural genes, whereas the regions close to the telomeres contain few structural genes but several repetitive elements and have low transcriptional activity. The development of a physical map will help to clarify whether this particular organization is conserved in *C. parvum*.

## *GENE DISCOVERY AND GENETIC VARIATION IN CRYPTOSPORIDIUM AND TOXOPLASMA.*

F. Spano[1], L. Putignani[1], I. Ricci[1], M. Di Cristina[1], C. Puri[2], and A. Crisanti[1,3]

*[1] Istituto di Parassitologia e [2] Istituto di Istologia e Embriologia Generale, Università di Roma "La Sapienza", Rome, Italy; [3] Imperial College, Department of Biology, London, U.K.*

The invasive stages of the apicomplexan parasite *Toxoplasma gondii* are known to express a set of surface antigens, known as the SAG proteins, sharing structural and functional characteristics. These molecules, which are anchored to the plasmamembrane via a GPI motif, are differentially expressed in sporozoites, tachyzoites and bradyzoites and are believed to play a role in the early steps of host cell invasion. The most extensively characterized among the SAG proteins are SAG1 (p30), which is expressed exclusively in the tachyzoites and represents the immunodominant antigen during *T. gondii* infection (Burg et al., *J. Immunol.* 141: 3584-3591, 1988) and SAG3 (p43), which is present in all invasive stages (Cesbron-Delauw et al., *J. Biol. Chem.* 269: 16217-16222, 1994). These two molecules share approximately 40% amino acid identity and the presence of two amino acid repeats each containing 6 cysteine residues at conserved positions. *T. gondii* lines defective for either SAG1 or SAG3 have been shown to possess a reduced invasive activity. Recently, a series of novel *T. gondii* proteins sharing close structural similarity with SAG1 and SAG3 have been identified: SRS1-4 and BSR4 by Boothroyd and co-workers (Hehl et al., *Mol. Biochem. Parasitol.* 89: 271-282, 1997) and SAG5 in our laboratory.

A portion of the SAG5 gene was accidentally amplified from the genomic DNA of *T. gondii* (RH strain) employing unrelated primers. The PCR product 12G/16, encoding a 212 amino acid-long polypeptide showing a significant homology to both SAG1 and SAG3, was used as a probe to screen a genomic *T. gondii* cosmid library. Southern blot analysis of the positive cosmidic clones and of *T. gondii* genomic DNA employing the 12G/16 fragment as a probe yielded complex hybridization patterns, suggesting the existence, in the genome of *T. gondii*, of a family of related genes. Extensive sequence analysis confirmed this hypothesis and revealed the presence of three tightly clustered genes encoding closely related proteins, denominated SAG5.1, SAG5.2 and SAG5.3. All three polypeptides show the hallmarks of the family of SAG1-related molecules, being characterized by a leader peptide, 12 cysteine residues whose distribution suggests a tandem duplication of an ancestral 6-cysteine motif and a C-terminal GPI-anchoring signal. SAG5.2 and SAG5.3 are 367 amino acid-long and differ from each other at 8 amino acid positions (98% identity), while SAG5.1, which consists of 362 amino acids, is virtually identical to the other two SAG5 proteins only within the C-terminal region (positions 162-362). Notably, in the N-terminal half of the molecule the degree of amino acid identity to SAG5.2/SAG5.3 drops to approximately 40%, which represents the average homology shared by all members of the "SAG1" protein family. Northern blot and RT-PCR analysis of poly(A)+ RNA showed that all three SAG5 genes are expressed in tachyzoites. The transcriptional activity in the other invasive stages is under study. The localization of the SAG5 proteins in *T. gondii* tachyzoites was investigated employing polyclonal and monoclonal antibodies raised against a recombinant histidine-tagged fragment of SAG5.3 produced in *E. coli* and affinity purified by nickel chelate chromatography. In contrast with the other SAG1-related proteins, immunofluorescence analysis failed to show for SAG5 any significant staining of the tachyzoite plasmamembrane. Instead, anti-SAG5 antibodies specifically decorated the apical end of the tachyzoites. This evidence was confirmed by immunoelectron microscopy experiments in which anti-SAG5 antibodies were shown to label the cytoplasmic side of the tachyzoite pellicle and electrondense structures possibly associated with the microtubular components of the apical complex. In our search for *Cryptosporidium* proteins potentially involved in the invasion process we have recently cloned a *C. parvum* incomplete cDNA encoding a polypeptide, named cysteine-rich sporozoite protein (CRSP), possessing adhesive amino acid motifs. CRSP is a novel thrombospondin-related protein that adds to TRAP-C1, a micronemal protein recently cloned in our laboratory and sharing structural similarity to Etp100 of *E. tenella* and MIC2 of *T. gondii*. The CRSP gene is highly expressed in sporozoites. Work is in progress to clone the entire ORF encoding CRSP and to establish the exact sub-cellular location of this molecule.

Our interest in *C. parvum* has recently extended to the study of the parasite genetic variation. Most of the *C. parvum* typing studies carried out to date are based on the analysis of single polymorphisms. A multilocus approach has the potential to better define the structure of the *C. parvum*

population and assess the degree of genetic isolation of the H and C subpopulations. With this in mind we genotyped 28 *C. parvum* isolates of various host and geographical origin by simultaneously analysing up to five polymorphic loci belonging to at least three distinct linkage groups. Isolates were from Europe, North and South America and Australia. Seventeen isolates originated directly from humans, 3 were derived from humans and propagated in calves, and 8 were from various animals. Human isolates were both from sporadic cases and from documented outbreaks. Two isolates were derived from AIDS patients. Animal samples included the widely used Moredun and GCH1 isolates. The five polymorphic loci selected for this study have been shown to consist of at least two alleles differentially associated with animal or human *C. parvum* isolates. Four of the genetic markers were analysed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays and corresponded to the protein-encoding genes *Cryptosporidium* Oocyst Wall Protein (COWP) (Spano et al., *FEMS Microbiol. Letters* 152: 209-217, 1997), polythreonine (polyT) (Carraway et al., *Infect. Immun.* 65: 3958-3960, 1997), thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) (Spano et al., *Mol. Biochem. Parasitol.* 92: 147-162, 1998) and ribonucleotide reductase (RNR) R1 subunit. The fifth polymorphism, residing within the internal transcribed spacer region 1 (ITS1) of the Type A ribosomal unit, was analysed by PCR employing genotype-specific primers (Carraway et al., *Appl. Environ. Microbiol.* 62: 712-716, 1996). Consistent with previous studies, isolates segregated into two groups, one comprising both human and animal isolates, the other only isolates of human origin. No recombinant genotype was detected. This evidence, together with the broad geographical distribution of the multilocus genotypes identified, brings further support to the hypothesis that *C. parvum* reproduction is substantially clonal.

*FIRST ATTEMPTS TO ISOLATE RECOMBINANT CRYPTOSPORIDIUM T CELL ANTIGENS.*

de Graaf D.C.[1], Hoff P.T.[1], Petry, F.[2] & Peeters J.E.[1]

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When peripheral blood mononuclear cells from *Cryptosporidium parvum* infected neonatal calves are stimulated with crude *Cryptosporidium* oocyst extract they produce IFNγ. Depletion of different T-cell subsets revealed that CD4+ T cells are involved. A low molecular weight (LM) oocyst-derived antigen fraction that evoke this immuneresponse could be partially purified. This LM fraction consists of a simple protein pattern with bands below 50 kDa. However, efforts to identify the antigen(s) that was/were responsible for the stimulation of IFNγ production were hampered by the low quantities of antigen available. Therefore we have chosen to screen a *Cryptosporidium* cDNA library for recombinant antigens involved in this T cell response. In this presentation we present preliminary work done so far, showing the different strategic approaches that have been applied.

*LIVE RECOMBINANT SALMONELLA AS CARRIER FOR EIMERIA ANTIGENS*

Dick Schaap[1], Gerben Arts[1], Volker Spehr[2], Yan Zheng-Xin[2], Thomas F. Meyer[2] and Arno Vermeulen[1]

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*Eimeria* species cause intestinal coccidiosis, an enteritis which is of significant economic loss in the poultry industry. At present, coccidiosis is mainly controlled by the use of antibiotics. However, the emergence of drug resistant *Eimeria* strains, requires the development of alternative methods of control. We are presently analysing various *Eimeria* antigens for their protective immunity in subunit vaccines. For this purpose live recombinant *Salmonella* strains are used, expressing *Eimeria* gene products. Attenuated *Salmonella* strains, expressing heterologous antigens, are known to induce an immune response against other parasites, like *Plasmodium* and *Leishmania*. However, expression of toxic gene products or overexpression of the foreign antigens limits the survival of these recombinant *Salmonella* in the host. To circumvent this problem, an expression system was developed and optimised, in which foreign antigen expression is dependent on the orientation of an invertible promoter. The invertible promoter will be either in the "ON" or "OFF" orientation, resulting in a mixed *Salmonella* population. "OFF" *Salmonella* will not transcribe heterologous proteins and "ON" *Salmonella* will transcribe them.

The subpopulation in the "ON" orientation will be overexpressing the heterologous proteins, and as a result will be inhibited in its growth. The "OFF" subpopulation is not growth inhibited, and shall maintain the population, from which also new "ON" *Salmonella* will be generated, each time that inversion takes place.

We will present our expression system in *Salmonella typhimurium*. It utilises a genomically inserted T7 RNA polymerase driven by an invertible promoter. T7 RNA polymerase subsequently transcribes our heterologous antigens, located on an expression plasmid. Green fluorescent protein was initially expressed to show with FACS studies, that inversion indeed occurs. Furthermore, studies with broiler chickens will be presented to show the stability of our expression system *in vivo* and to demonstrate the induced protection against coccidiosis.

*CHARACTERISATION OF TWO MAJOR SURFACE PROTEINS OF NEOSPORA CANINUM TACHYZOITES*

Sabrina Sonda
*(Institute of Parasitology, University of Bern. Switzerland)*

*Neospora caninum* is structurally and biologically closely related to, but antigenically distinct from, *Toxoplasma gondii*. *N. caninum* is an obligatory intracellular parasite, with two stages, namely tachyzoites and bradyzoites, being identified to date. Molecules associated with the surface and with secretory organelles are likely to participate in the host cell entry process, and they could influence the pathogenesis of neosporosis. Therefore, our investigations have aimed towards the identification and characterization of surface-associated proteins *of N. caninum* tachyzoites. Two proteins namely Nc-p43 and Nc-p36 have been identified and characterized. The cDNA coding for these two proteins has been sequenced. Nc-p36 is highly homologous to p30 (SAG1) from *T. gondii*, while Nc-p43 exhibits a lesser degree of similarity to Toxoplasma surface proteins. Recombinant proteins were expressed *in E. coli*. Immunogold electronmicroscopy using antibodies affinity-purified on these recombinant proteins showed that Nc-p36 is found exclusively on the surface of *N. caninum* tachyzoites, while epitopes reacting with anti-Nc-p43 antibodies were found on the surface and within the parasite dense granules. Most likely both proteins are anchored into the surface membrane via a GPI-anchor. Nc-p36 is expressed exclusively in the tachyzoite stage, while Nc-p43 can be detected in both tachyzoites and bradyzoites. Further studies will be aimed towards the functional characterization of these two surface proteins and their immunological significance during infection.

*IMMUNOLOGICAL PROPERTIES OF SOME SOLUBLE AND MEMBRANE BOUND MICROSPORIDIAN ANTIGENS*

Julia Sokolova
*(All-Russian Institute of Plant Protection, Russ. Acad. Agric. Sci., St. Petersburg, Russia)*

Microsporidia (phylum Microspora) (M) is the group of vast distributed (from Protozoa to Primates including Humans) intracellular parasitic protists, baring very primitive and very specialized features simultaneously. RNA and DNA sequences
suggest they are probably the most ancient eukaryots. Information about structural, transport and regulatory proteins of M, as well as on their enzymes is scarce, though it may serve the basis for the understanding of M pathogenicity and indicate the possible sites of suppressive therapy. The experimental host-parasite system: *Gryllus bimaculatus - Nosema grylli* we are working with, has certain advantages to perform biochemical and immunological studies on parasite antigenes. The present study persuaded two main goals: (1) to examine different ways of antigen preparation
and to evaluate their influence on the resulted poly- and monoclonal antibodies; (2) to identify and localize new proteins with the help of obtained antibodies by means of immunofluorescent assay analysis, immune electron microscopy and Western Blots. Besides two types of highly specific immune sera, the following monoclinal antibodies antibodies were obtained: (1) 1BF3 recognized a protein of 55 kDa that is connected with the spore filament as it was clearly suggested by IEM and IFA; (2) 1BD9 recognized the 25, 34, 43 kDa proteins associated with spore walls; (3) 1BB9 reacted with 36, 45, 65 and 75 kDa proteins and gave weak fluorescence around spores; (4) 2BB3 recognized 44 and 55 kDa proteins; (5) 2BD4 - a single protein of 54 kDa. The obtained antibodies add to the existing microsporidian antibody bank and can be used for the further work of isolation, description and sequencing the microsporidian proteins to understand their functions.

*GUEST LECTURE*
***Saturday 20th June, 17.00-17.45***
*"THE ROLE OF SECRETORY ADHESINS IN MOTILITY AND INVASION BY TOXOPLASMA GONDII*
David Sibley, University of Washington, USA

***SESSION THREE***
***Sunday June 21st 0900-1300***

*TOOLS FOR THE GENETIC MANIPULATION OF TOXOPLASMA GONDII AND THEIR APPLICATION TO STUDY HOST CELL INVASION.*
S. Brecht, B. Frank, A. Geiter, C. Hettmann, M. Reiss and D. Soldati.
*(Zentrum für Molekulare Biologie der Universität Heidelberg, Germany.)*

The random cDNA sequencing project for *Toxoplasma.gondii* has generated over 10,000 ESTs resulting in an explosion of gene discovery. A significant number of sequences revealed homologies to genes phylogenetically restricted to the Apicomplexa which are potentially playing crucial role in the establishment of intracellular parasitism. The apicomplexan-specific proteins include apical antigens involved in recognition and adhesion of host cells, proteins associated with specific life cycle stages, and unique metabolic enzymes involved in energy production.
However a genome sequence is useless as long as the function of a gene can not be tested. *T. gondii* is particularly well-suited to laboratory study and its accessibility to genetic manipulation provides the opportunity to explore many questions relevant to the biology and pathogenicity of this organism and its closest relatives. The repertoire of molecular genetic tools currently available offers great potentials and some limits to study the function of both non-essential and essential genes. We have applied some of these tools to study proteins potentially implicated in the process of invasion.

Apicomplexan parasites rely on an unusual form of gliding motility for host cell invasion. The basic engine for gliding locomotion is the actin cytoskeleton and likely involves a myosin to generate the mechanochemical force along the actin filaments. We have cloned and characterised two *T. gondii* myosins by a PCR screen of parasite genomic DNA. The two myosins, MyoA 93kDa (recently described by Heintzelman and Schwartzman) and MyoD 91kDa belong to a novel phylogenetic and structural class of myosins (XIV). To identify which myosin might be involved in invasion, we have first determined the subcellular localisation of MyoA and MyoD using epitope tagging of their coding sequences and stable transformation into tachyzoites. Whereas MyoD is more diffusely found at the periphery of the cell, the sharply defined plasma membrane localisation of MyoA makes it a good candidate as motor of the gliding motility. Moreover, a parasite transmembrane protein is necessary to associate tightly with the host cell surface and to transfer the mechanical force across the plasma membrane. The C-terminal domain shall be establishing directly or indirectly a connection with the actin cytoskeleton and inducing gliding motility. The *Plasmodium berghei* TRAP protein appears to fulfil this double function in malarial sporozoites. Mic 2 has been recently described as the homologue of TRAP in *T. gondii*. and was the only known protein bearing a transmembrane domain. We have cloned and characterised a novel *T. gondii* microneme protein which carries EGF-like domains as well as the conserved C-terminal domain, including the transmembrane region common to the family of TRAP proteins. Mic 4 coding sequence predicts a protein of 34 kDa which appears to be present as a dimer in the parasite.

*THE CURRENT STATUS OF TRANSFECTION IN PLASMODIUM BERGHEI.*

T. de Koning-Ward
*(Department of Parasitology University of Leiden P.O. Box 9605 2300 RC Leiden The Netherlands.)*

Although genetic transfection in Plasmodium still requires further development, it is now possible using the currently available technology to express introduced transgenes in Plasmodium and disrupt malarial genes by site-specific homologous recombination. These recent advances in the transfection of Plasmodium provide not only a means to determine the necessity of specific malarial gene products but allow a more detailed analysis of many aspects of the parasite's cellular biology, including the mechanisms underlying drug resistance. In this presentation, we will describe the methods used to stably transfect the rodent malarial pathogen, *P. berghei* as well as the expression of the exogenous reporter, green fluorescent protein. We will also show the effect of disrupting two genes that are specific to the mosquito stage of the parasite's life cycle: (i) the S-type ribosomal RNA genes, and (ii) the Pbs21 gene which encodes an ookinete surface protein that has been implicated as a potential transmission blocking vaccine candidate. The ability to complement these disrupted genes in trans to help elucidate the function of these gene products in *P. berghei* is not yet feasible due to the lack of another suitable selectable marker. In this report we will also discuss the approaches currently taken towards finding additional selectable markers in *P. berghei* and what further tools are required to fully exploit transfection technology in this parasite.

*TRANSIENT TRANSFECTION OF E. TENELLA AND HETEROLOGOUS EXPRESSION OF MICRONEME PROTEINS IN T. GONDII*

Fiona Tomley,
*(Institute for Animal Health, Compton , Newbury, RG20 7NN UK)*

A transient transfection system was developed for *Eimeria tenella*, using β-galactosidase (βgal) as a reporter enzyme. Successfully expressed constructs contained transcriptional control sequences, derived from either the *E. tenella* microneme gene *Et*mic-1 or from the *E. tenella* actin gene, fused to the coding region of *lacZ*. Transfectants expressing βgal were able to invade host cells and proceed through part of the life-cycle, forming schizonts from which merozoites were released. Some merozoites which arose from transfected sporozoites were also found to express βgal. These results are encouraging for the development of a stable transfection system for *E. tenella*, using βgal as a reporter enzyme.

Micronemes are secretory organelles which are conserved across the phylum Apicomplexa and which are critical for the early stages of host cell invasion by these parasites. Microneme proteins (mics) are expressed late during the process of oocyst sporulation and again during the maturation of intracelllular schizonts and are assembled into the micronemes by an, as yet, uncharacterised process. To help understand how mics reach their target organelle, we have expressed two *E. tenella* mics in the related coccidian *Toxoplasma gondii*, generating stable lines of *T. gondii* which express either *Et*mic-1 or *Et*mic-2 in addition to the normal complement of *T. gondii* mics.. Both proteins were expressed in *T. gondii* but whereas *Et*mic-2 was targetted to the apical tip, the majority of *Et*mic-1 was not. Further recombinants are now being generated to examine targetting signals in more detail. The successful expression of two *E. tenella* mics in *T. gondii* demonstrates the value of this heterologous system for experimental analysis of *E. tenella* proteins.

*HOMOLOGOUS RECOMBINATION AT THE GRA2 LOCUS CAUSES PARTIAL ATTENUATION OF TOXOPLASMA VIRULENCE*

C. Mercier, D.K. Howe, D.G. Mordue, M. Lingnau and L.D. Sibley
*(Washington University School of Medicine, Department of Molecular Microbiology, Saint-Louis, MO, US.)*

Following invasion into the host cell, *Toxoplasma gondii* secretes a variety of proteins that modify the parasitophorous vacuole. Amongst them, the 28-kDa dense granule protein GRA2 is specifically targeted to the tubulo-vesicular network connected to the vacuolar membrane.To investigate the function of GRA2, a mutant T. gondii line was derived from the RH strain in which GRA2 was disrupted by replacement with the selectable marker Ble. The mutation does not affect the parasite

growth rate in vitro. However, the mutant was less virulent during acute infection in mice. In contrast to the wild-type strain which always leads to death in infected mice, 44% of infected mice survived acute infection with the knock-out line. Moreover, chronic infection was demonstrated by the presence of both parasites and cysts immunohistochemically detected in the brain of infected animals, six weeks post-infection. Thus, absence of GRA2 partially attenuates the virulence of *T. gondii* during the acute phase of infection by the otherwise highly virulent RH strain.

*STRUCTURE TO FUNCTION ANALYSIS OF THE ADHESIVE DOMAINS WITHIN TRAP (THROMBOSPONDIN RELATED ADHESIVE PROTEIN) OF P. FACLIPARUM*

Firman Ghouze and Andrea Crisanti.
*Department of Biology, Imperial College of Science, Technology and Medecine Prince Consort Road, London, SW7 2BB*

The malaria parasite *Plasmodium falciparum* expresses specifically at the sporozoite stage the thrombospondin related adhesive protein (TRAP). An increasing amount of evidence indicates that this protein plays an important role in hepatocyte invasion. TRAP contains several highly conserved amino acid sequences. One amino acid motif based around the sequence WSPCSTVCGKGXRXR, known as region II, is also present in the circumsporozoite (CS) protein of different *Plasmodium* species and other unrelated adhesive proteins. Studies have demonstrated that this region confers to the proteins the ability to bind to sulphated glycoconjugates and to hepatocytes. TRAP also contains, at its amino terminal region, a sequence of approximately 200 amino acids known as the A-domain. This domain is present in a number of integrins and mediates binding to various surface ligands. Additionally, TRAP of *P. falciparum* contains a RGD motif which has been demonstrated to be essential in the interaction of several extracellular glycoproteins with members of the integrin superfamily. In this study we have analysed the structure to function relationship of these adhesive domains within the TRAP molecule. For this purpose we have generated, expressed and purified a series of TRAP constructs carrying a series of substitutions in specific amino acid residues. Furthermore a construct with a deletion spanning the 15 amino acids of region II, named TRAP 1.0R2- was also generated. The binding activity of these constructs to both immobilised sulphatides and different tumour derived cell lines has been assessed. Data show that region II and the A domain but not the RGD motif play a role in binding. These findings further our understanding of the molecular interactions which occur during hepatocyte invasion.

*SPECIFIC TERMINAL DELETIONS CAN BE INDUCED ON PLASMODIUM BERGHEI CHROMOSOMES THROUGH TRANSFECTION TECHNIQUES.*

Marta Ponzi, Raffaella Scotti, Tomasino Pace.
*Laboratory of Cellular Biology, Istituto Superiore di Sanità, Rome, Italy*

Large subtelomeric rearrangements are often responsible for chromosome size polymorphisms often observed in *Plasmodium*. The instability of chromosome ends has been shown to be mostly associated with the loss of subtelomeric genes. Moreover, large deletions of terminal portions of chromosome 5 in *P. berghei* and chromosome 9 in *P. falciparum* correlate with the loss of gametocytogenesis in laboratory infections or in cultures. A linear vector containing a selectable marker and telomeric sequences was constructed and shown to be able to induce, in transfection experiments, specific chromosome deletions when provided with a short genomic region homologous to the target site. This new approach will allow to verify the phenotipic effect of controlled chromosome deletions and to study position effects exerted on functions located in the vicinity of the breakage point.

*COMPARISON OF THE HOST/PARASITE RELATIONSHIP IN THE ENTERIC AND EXOENTERIC FORMS OF TOXOPLASMA GONDII*

D.J.P. Ferguson
*Nuffield Department of Pathology, Oxford University, John Radcliffe Hospital, Oxford, UK.*

The host parasite relationship of *Toxoplasma gondii* during coccidian (asexual and sexual) development in cat and tachyzoite and bradyzoite the intermediate host was examined by electron microscopy and immunocytochemistry. *T. gondii* undergoes a unique form of asexual (endopolygony) and sexual development only within the enterocytes of the cat and no other species or cell type.

Ultrastructural examination of host parasite relationship shows that the enteric forms are located within a parasitophorous (PV) which is significantly different from that described for all other forms of the parasite. The various sexual and asexual stages are located within a tight fitting PV limited by thickened membrane with a laminated substructure. This membrane appear to be the result of the fusion of three unit membranes. A number of conical shaped indentations are located on the parasite side of the PV. There was no evidence of an intravacular network or the collecting of host cell mitochondria and rough endoplasmic reticulum around the PV which are characteristic of the tachyzoite PV. It Is possible that this unique host/parasite relationship and the coccidian devlopment of the parasite are interrelated. The biological nature of the PV formed in cat enterocytes is unclear but is appears to be triggered at the time of parasite penetration.

The expression and location of the dense granule proteins (GRA1-6 and NTPase) in the merozoite and during coccidian development of *T. gondii* in the small intestine of the cat (definitive host) has been examined by immuno-light and electron microscopy. This was compared with that of tachyzoites and bradyzoites present in the intermediate host. It was found that the merozoite contained the characteristic apical organelles plus a few large dense granules. By immunocytochemistry, these granules were negative for GRA proteins 1 to 6 in contrast to both tachyzoites and bradyzoites where dense granules were positive for all six proteins. The GRA proteins were associated with the parasitophorous vacuole (PV) during tachyzoite and bradyzoite development but were absent from the PV of the coccidian stages. However the dense granules were positive for NTPase which was similar to the tachyzoite but appears to be down regulated in the bradyzoite. The apparent release of the NTPase into the PV was also similar to that described for the tachyzoite. This may reflect the relative metabolic activity of the various stages. This study shows that eof the GRA proteins has a similar stage specfic expression which is independant of NTPase expression. These observations are consistent with *T. gondii* having a different host parasite relationship in the enteric forms which does not involve the GRA proteins.

*T. gondii* is a coccidian parasite with the cat as its definitive host but any warm blooded animal, including Man, can act as an intermediate host. In the intermediate host the parasite undergoes an acute phase where the tachyzoites reproduces rapidly followed by a chronic phase when the bradyzoites reproduce slowing giving rise to tissue cysts which are found predominantly in muscle and brain. These tissues cysts are believed to remain viable for long periods and can transmit the infection if eaten by other intermediate hosts or the cat. In the case of the cat, infection results in typical coccidian asexual and sexual development in the small intestine with the production of oocysts which are passed in cat faeces (Hutchison et al 1971, Frenkel and Dubey 1970).

*T. gondii* is an obligate intracellular parasite and can only proliferate within nucleated cells. Therefore the interaction with the host cell is vital for parasite survival. The tachyzoite and bradyzoite are ubiquitous in the host species and cell type within which development can occur. In contrast, the coccidian development is restricted to single host species and cell type and to date no *in vitro* model exists. Due to the ease of obtaining parasites, the host/parasite relationship has been extensively studied in the tachyzoite.

*MICRONEME SECRETION DURING INVASION EIMERIA*

J.M Bumstead and F.M. Tomley
*(Institute for Animal Health, Compton, Newbury, Berkshire, RG20 7NN).*

The invasion of *in vitro* cultured host cells by *Eimeria tenella* sporozoites involves the rapid relocalisation and secretion of microneme proteins. Microneme proteins are secreted from the apical end of the sporozoite and can be detected in the culture supernatant and also on the surface of host cells. Secretion is triggered by contact with cells and also by extracellular factors including serum and albumin. Microneme proteins can also be found in trails deposited by the sporozoite as it glides over the cell surface. If motility is inhibited with cytochalasins, secretion still occurs, but in this case microneme proteins are released apically and do not trail, presumably because the actin-myosin motor which is essential for motility does not engage. This suggests that microneme proteins may form a bridge between the motility motor, located in the sub-pellicular microfilaments and the surface of the host cell to which they bind *via* their many adhesive domains.

*TOWARDS A FUNCTIONAL ANALYSIS OF CYCLIN DEPENDENT PROTEIN KINASES IN THEILERIA ANNULATA.*

Jane H. Kinnaird, Mary Logan and Geoffrey Ingram
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Cyclin dependent kinases (CDKs) are highly conserved serine/threonine protein kinases which are intimately involved in regulating many essential cellular functions by phosphorylation of their target substrates. The activity of the kinase catalytic subunit is itself regulated by binding of a cyclin subunit and an inhibitor as well as by phosphorylation. Amongst the best characterised CDKs are those which are involved in regulating progression of the cell cycle although others have roles in cell cycle entry and in differentiation. In higher eukaryotes there is a large family of CDKs, whose activities are regulated by different cyclin partners. In lower eukaryotes there tend to be fewer CDKs, and notably in yeast, many elegant mutational studies have been carried out which demonstrate the essential role of these kinases. Amongst protozoan parasites, several genes encoding CDK related proteins have been isolated and none of these show the extremely high levels of conservation found in other eukaryotes. Two such genes have been characterised from *Theileria annulata*. The first, *ThaCRK2* encodes a 34 kDa polypeptide which is most closely related to the CDK1/2 groups which regulate G1-S and G2-M transitions of the cell cycle in other eukaryotes. *ThaCRK2* RNA and protein are detectable in all dividing life cycle stages, which is consistent with a constitutive role in parasite division. Using a specific antiserum generated against recombinant ThaCRK2 we have shown that this protein localises to the cytoplasm in the macroschizont. In *T. annulata* the piroplasm stage does not show extensive multiplication but both Northern and Western blotting show a high levels of ThaCRK2 RNA and protein in comparison to signals obtained for other markers of nuclear division. This points to an additional role in the life cycle. The more recently isolated *ThaCRK3* encodes a 45 kDa polypeptide with most significant homology to CDK7 which in combination with cyclin H is believed to activate some CDKs by phosphorylation. Although the relationship is not strong, a probable homologue, Pfmrk, has been identified in *Plasmodium falciparum*. The RNA expression pattern of *ThaCRK3* is distinct from *ThaCRK2* in the different life cycle stages.

p13 is a small protein originally identified in yeast and which has homologues in other eukaryotes. It is found complexed with some CDKs and has been extensively used in their purification. Recombinant histidine tagged p13 coupled to sepharose has been used to affinity purify kinases from native extracts of *T. annulata* piroplasms. At least two kinase activities have been identified in this way, one of which is attributable to ThaCRK2. Prior immunodepletion of ThaCRK2 showed that at least one other kinase activity remained which showed a bias towards α-casein as an *in vitro* substrate whereas extracts which were not depleted in ThaCRK2 showed more extensive phosphorylation of histone H1. These results confirm ThaCRK2 is active as a kinase in the piroplasm stage and also demonstrates that its substrate specificity is different from other kinases present in the extracts which can bind to yeast p13.

The ability to carry out *Theileria* transfection would greatly aid functional studies of these kinases in different life cycle stages. Preliminary studies have been carried out to establish transfection based on methods used for the intraerythrocytic stage of *Plasmodium* spps. We have designed constructs using chloramphenicol acetyl transferase as a reporter linked to 5′ and 3′ regulatory regions of the *T.annulata* β-tubulin gene. A construct has also been made again using a CAT reporter but under the control of the putative 5′ and 3′ regulatory regions of *Tams1*, a gene encoding a 30 kDa merozoite surface polypeptide, whose transcription is up-regulated during differentiation to the merozoite. Various parameters of electroporation and use of different life cycle stages have not produced any conclusive evidence for parasite specific transient expression of the reporter. However the constructs based on the parasite β-tubulin control sequences gave significant transient expression of CAT originating from the host cell, indicating that sequences within this particular parasite promotor region can be recognised and used by host proteins. Work is now proceeding, in collaboration with Roger Hall's group at York University, to develop a selectable marker for transfection using sporozoite and infected lymphocyte stages as the initial targets. Other methods of introducing DNA are also being investigated.

*INVESTIGATING THE FUNCTIONAL SIGNIFICANCE OF TOXOPLASMA GONDII CDC2-RELATED KINASES*

J.M. Wastling[1] , J.H. Kinnaird[2] and J.G. Mattsson[3]

*([1]Institute of Biomedical & Life Sciences, Division of Infection & Immunity, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK. [2]Wellcome Unit for Molecular Parasitology, University of Glasgow. [3]Department of Parasitology, National Veterinary Institute, Uppsala, Sweden.)*

Infection with *Toxoplasma gondii* is initially characterised by the rapid multiplication of tachyzoites followed by their differentiation into slowly-dividing bradyzoites and the formation of long-lasting tissue cysts. The transformation of tachyzoites into bradyzoites involves a dramatic change in the rate of cell division, implying a high degree of control over cell-cycle. Regulators of the *Toxoplasma* cell cycle have until recently received little attention. Cyclin-dependent kinases (CDKs), which include the cdc2 kinases, are evolutionary conserved molecules many of which regulate the eukaryotic cell cycle. cdc2 was first identified in yeast, where it regulates both the G1-S and G2-M phase cell cycle transitions, then subsequently in a wide range of eukaryotes. Using consensus primers we have identified a number of *T.gondii* cDNA and genomic clones which show close homology to eukaryotic CDKs. For one of the clones (designated *TgCRK2*) we have obtained a full length genomic sequence which encodes a predicted polypeptide of 33kDa that is most similar to the cdc2 type of CDK (< 55%). However, the closest homologies are with a *Theileria* cdc2-related kinase, ThCRK2 (72% identity) and with PfPK5 (72%), the CRK from *Plasmodium falciparum* most closely related to cdc2. TgCRK2 contains the characteristic PSTAIRE motif, implicated in the binding of activating cyclins of A, B, D and E types to CDKs. Transcripts of *TgCRK2* could be detected in *T.gondii* M3 strain bradyzoites using RT-PCR but appeared to be relatively more abundant in the rapidly dividing RH and S48 strain tachyzoites. Western blotting of *Toxoplasma* tachyzoites using an antiserum against the conserved PSTAIRE region detected a molecule of 32-33 kDa, consistent with the predicted size of TgCRK2, but also suggested the presence of other related molecules. We are currently using a number of approaches to analyse the functional significance of *Toxoplasma* CDKs including transient and stable transfection of *TgCRK2* constructs in tachyzoites, homologous gene targeting, complementation and identification of the corresponding cyclin binding partners of *Toxoplasma* CDKs.

# PROCEEDINGS

# COST 820 WG 2 Meeting

# COCCIDIOSIS IN PIGS

27th February 1998

Held at Central Veterinary Laboratory, Veterinary Laboratories Agency.

**Veterinary Laboratories Agency**

WEYBRIDGE

With the Financial Support of the Commission of the European Communities

European Cooperation in Scientific and Technical Research ( COST)

Veterinary Laboratories Agency

# Cooperation in the Field of Scientific and Technical Research

## COST 820 WORKING GROUP 2

### Central Veterinary Laboratory
**February 1998**

**PROGRAMME**

| | | |
|---|---|---|
| **9.30** | **Opening address - Aims of Meeting** | **Janet Catchpole**<br>***CVL, Weybridge*** |
| **9.40** | **Life cycles, taxonomy and genetic relationships of intestinal coccidia of pigs** | **Astrid Tenter**<br>***Institut fur Parasitologie Hanover*** |
| **10.00** | **Occurrence of *Isospora suis* on pig breeding farms in Germany** | **Dr Arwid Daugschies**<br>***Institut fur Parasitologie Hanover*** |
| **10.20** | *Coffee* | |
| **10.50** | ***Isospora suis* in suckling pigs- the role of histopathology in the diagnosis of field outbreaks.** | **Robert Higgins**<br>***VIC Thirsk*** |
| **11.20** | **Ideas on control and treatment** | **Bill Smith**<br>***SAC Aberdeen*** |
| **11.40** | **Discussion** | |
| **12.15** | *Lunch* | |
| **13.40** | **Review of field survey methods in Nordic countries** | **Kirsten Larsen**<br>***Bayer, Denmark*** |

# LIFE CYCLES, TAXONOMY AND GENETIC RELATIONSHIPS OF INTESTINAL COCCIDIA OF PIGS

Astrid Tenter

Institut fur Parasitologie, Hanover

Keywords: *Isospora*, *Toxoplasma*, *Neospora*, *Sarcocystis*, 18S rDNA; PCR; phylogenetic analysis

The phylogenetic relationships and taxonomic affinities of coccidia with isosporan-type oocysts have been unclear as overlapping characters, recently discovered life cycle features, and even recently discovered taxa, continue to be incorporated into biological classifications of the group. We determined the full 18S rRNA gene (rDNA) sequences of two mammalian *Isospora* spp., *Isospora felis* and *Isospora suis*, and a *Sarcocystis* spp. of a rattlesnake, and used these sequences for a phylogenetic analysis of the genus *Isospora* and the cyst-forming coccidia. Various alveolate 18S rDNA sequences were aligned and analyzed using maximum parsimony to obtain a phylogenetic hypothesis for the group. The two *Isospora* spp. were found to be most closely related to *Toxoplasma gondii* and *Neospora caninum*. This clade in turn formed the sister group to the *Sarcocystis* spp. included in the analysis. The results confirm that the genus *Isospora* does not belong to the family Eimeriidae, but should be classified together with the cyst-forming coccidia in the family Sarcocystidae. Furthermore, there appear to be two lineages within the Sarcocystidae. One lineage comprises *Isospora* and the *Toxoplasma/Neospora* clade which share the characters of having a proliferative phase of development preceding gamogony in the definitive host and an exogenous phase of sporogony. The other lineage comprises the *Sarcocystis* spp. which have no proliferative phase in the definitive host and an endogenous phase of sporogony. A likely evolutionary scenario for heteroxeny in the group of coccidia with isosporan-type oocysts is that heteroxenous life cycles are derived features that have arisen independently in the different lineages, and that homoxenous *Isospora* spp. have retained the ancestral life cycle of the *Isospora/Toxoplasma/Neospora* lineage.

# OCCURRENCE OF *ISOSPORA SUIS* IN PIG BREEDING FARMS AND SPECIALIZED PIGLET REARING FARMS IN GERMANY

Dr Arwid Daugschies

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Bunteweg 17, D-30559 Hannover, Germany

Key words:- Isospora, pig, prevalence, Germany

The prevalence of *Isospora suis* in breeding farms with 12 to 238 sows was investigated by fecal examination of litters in the 2nd, 3rd and 4th week after farrowing.

Two farms specialized in pig rearing with conventional weaning (CW) at an age of 4 to 5 weeks, or segregated early weaning (SEW) at an age of up to 21 days were also examined immediately after stalling up, 2 weeks later and at the end of the rearing period.

*I. suis* was diagnosed in all farms.
The prevalence increased during the suckling period from 17 to 19 % in the 2nd week to almost 40 % at the time of weaning. The cumulative prevalence in the litters was above 50 %.
Diarrhoea was significantly associated with *Isospora* prevalence.

In the farm managed according to the SEW system the prevalence decreased during rearing from 37.5 % to 19.3 % and 4.1 % and the occurrence of diarrhoea decreased simultaneously from 36.5 % to 11.0 % and 3.3 %.

In the farm in which CW was practiced the *Isospora* prevalence ranged from 17.2 % to 21.9 % during the rearing period and diarrhoea was seen in about 34 % of the piglets at the first and second visit and decreased at the end of the rearing period to 5.7 %.

Thus *I. suis* is a frequent endoparasite in piglet breeding farms irrespective of the size of the herd and infections are often associated with diarrhoea.

*Isospora* also occurs in piglet rearing farms, at least at the beginning of the rearing period.

# *ISOSPORA SUIS* IN SUCKLING PIGS - THE ROLE OF HISTOPATHOLOGY IN THE DIAGNOSIS OF FIELD OUTBREAKS.

Dr Robert Higgins,

Veterinary Investigation Centre,
Thirsk, UK

## DIAGNOSIS can be based on several criteria

1. Clinical signs

2. Poor response to antibiotics

3. Faecal examination for oocysts,
   parasite has a prepatency 5 days, patency 5-14 days

4. Post-mortem examination

   This requires the sacrifice of LIVE pigs in the acute stage and examination

   (a) Impression smears of mucosa
   (b) Histology

5. Prophylactic response to toltrazuril?

## CLINICAL SIGNS

Age range 6-15 days, peak 7-10 days

Diarrhoea - yellow pasty initially, profuse white watery later

Afebrile

Usually continue to suckle

Vomiting ±

Dehydration

High morbidity

Low mortality< 10% typically

Poor response to antibiotics

Weight loss ± scour

Uneven growth rate in litter

## EPIDEMIOLOGICAL FEATURES

1. Intensive indoor systems
2. Sucklers only
3. All parity litters
4. Passive immunity ineffective
5. Poor response to antibiotics
6. Hygiene status ?
7. Seasonality? submission bias

# FAECAL EXAMINATION

Glucose salt solution for flotation -fat content can cause technical problems

Oocyst ID - hazy bodies at margins or 2 cell sporoblast stage

Differential identification from Eimeria and cryptosporidia oocysts

There are several problems with faecal examinations

**For samples to be positive** the infection must be at patent stage

Multiple samples are often necessary from the same pig or from several pigs

Correlation with gut damage and numbers of oocysts is unknown

**False Negatives** may occur for several reasons

Prepatency stage rather than patent stage

There is thought to be a biphasic cycle of oocyst production, usually 9-11 days post infection, when no oocysts may be excreted

Severe pathology will interfere with oocyst production

Only 7% histologically confirmed pigs were oocyst excreters (Sandford 1983)

## MUCOSAL SMEARS

### Technique

1. Thin smear is essential.
2. Scrape mucosa, compress between two slides, separate.
3. Air dry
4. Stain with Giemsa or modified Wright (Diff-Quick).
5. Meronts can be seen that are binucleate or multinucleate.
   Merozoites in pairs or multiples

### Assessment of technique

1. Rapid, cheap, simple requires minimal laboratory equipment.
2. Reasonable correlation with histology. But may be unnecessary duplication?
3. May be more sensitive in severe cases?

## GROSS PATHOLOGY

Non-specific lesions

Thin walled intestines

Excess liquid contents

Mesenteric lymph nodes enlarged

Necrotic enteritis ± fibrin cast

No Haemorrhage

## HISTOLOGICAL DIAGNOSIS

**Rapid fixation after euthanasia** is essential by simple immersion in 10% NBF/FS

Sites - duodenum, jejunum*, ileum*, caecum, colon, 1-2 cm tubes

* preferred sites

Standard haematoxylin and eosin stain

| | |
|---|---|
| Lesions-. | villous atrophy<br>condensed villous cores<br>enterocyte metaplasia<br>microulceration ±<br>reactive crypt hyperplasia Special stains<br>Isospora suis asexual/sexual stages<br>parasitiphorous vacuoles in enterocyte (not LP)<br>necrotic enteritis ± secondary bacteria<br>Jejunum, ileum mainly - rarely LI |
| Special stains | PAS,<br>Giemsa - limited value |

## PATHOLOGY

Neonatal challenge

Age dependent effect - epithelial dynamics

Field cases correlate best with 200,000 oocyst dose.

Necrotic enteritis with 400,000 oocyst dose

Biphasic damage has been shown experimentally

> 4-6 days post infection see moderate villous atrophy, reactive CH.
> At 8-10 days post infection see severe necrosis and crypt damage.
> This can not be explained by reinfection.
> Is there a possible extra-intestinal stage returning to mucosa and causing damage?

Parasite difficult to find in later stages

Non-specific villous atrophy (early cases for diagnosis)

Other organs - granulomatous lymphadenitis seen

## HISTOLOGICAL DIAGNOSIS

**Advantages**

Comprehensive assessment of gut

Direct visualization of parasite in situ

Direct correlation with pathological change

Differential diagnosis

**Disadvantages**

Expensive

Requires laboratory facilities

May be slow

## EXTRA-INTESTINAL STAGE ?

Experimental evidence - need transmission studies

Possible sites for stage- Reticulo-endothelial system, liver, spleen, lymph nodes

Difficult to visualize - may need markers to identify?

## DIFFERENTIAL DIAGNOSIS

Clinical signs may be seen with several other conditions/infective agents

ROTAVIRUS principally.

Colibacillosis ETEC? - opportunistic

Clostridium perfringens type C

Salmonellosis - *S. typhimurium*

Transmissible gastroenteritis TGE

Porcine epidemic diarrhoea PED

Adenovirus

Cryptosporidia, *C.parvum*

Strongyloides ransomi (not in UK)

PRRS - immunosuppressive?

Adverse environmental conditions will increase mortality

## ECONOMIC EFFECTS

Reduced growth rates

Cost of treatment

Increased susceptibility to other diseases both preweaning and postweaning

## SOURCE OF INFECTION

ENIGMATIC

SOW - minimal faecal excretion of *I.suis*, not via milk or transplacental

Contaminated environment - previous litters in pen or other litters in house

**Appropriate** disinfection/sanitisation to kill oocysts; most standard farm disinfectants will not kill oocysts

# IDEAS ON CONTROL AND TREATMENT

**Dr Bill Smith**

**Scottish Agricultural Colleges,**
**Aberdeen, Scotland**

# SOME ASPECTS - LIFE CYCLE

| | T | H |
|---|---|---|
| Oocyst sporulation in 4 days | 30°C | 80-85% |

Sporulated oocyst ingestion

→ Clinical disease 3-4 days

→ Shedding oocysts in faeces

3-4 days after diarrhoea onset

# SPREAD OF DISEASE

Sow

Environment/Food trough/Water bowl

Flies

Other vectors

# PREVENTION

Sanitization

Preventive Treatment

Sow Faeces Removal Daily

Shift Farrow

# SANITIZATION

Flame Gun

Oo-cide (Antec Int)

Stalosan

Biosuper

# ORAL DRUGS - Piglets

Baycox

Midicel

Sulphonamides

Coccidiostats eg Deccox

# DRUGS IN FEED - Sow

Deccox (Decoquinate)

Monensin

Amprolium

Salocin

# REVIEW OF FIELD SURVEYS AND METHODS IN NORDIC COUNTRIES

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Review of the literature, 1995

# *Isospora suis.* Porcine neonatal coccidiosis.

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## Contents

# *Isospora suis.*
# Porcine neonatal coccidiosis.

By Kirsten Larsen


## Summary

Neonatal coccidiosis caused by *Isospora suis* has been described in many different countries throughout the world. The life-cycle follows three phases: sporogony, excystation and an endogenous phase. It is discussed whether there is also an extra-intestinal phase. The typical clinical symptoms are diarrhoea in suckling pigs in the second week of life. The diarrhoea will typically be yellow and pasty, the morbidity rate is high, whereas mortality is often low. Herd conditions and management will be very significant factors in the spread of the disease. Routes of transmission are constantly being discussed, but the most likely is pig to pig transmission via contamination of the surroundings. Strong immunity develops after infection with *I. suis*. The diagnosis is made on the basis of the clinical symptoms and detection of oocysts or other development stages, either by means of a modified McMaster flotation technique, or by microscopic examination of a smear from the mucous membrane of the intestine. There are no highly suitable therapeutic agents or coccidiostatics on the market at present, which is why it is important to institute thorough hygiene measures. Sulpha preparations are used prophylactically by injecting the suckling pigs several times during the first week of life. A new product, toltrazuril (Baycox® 5% suspension), is under development. Toltrazuril has been shown to be especially effective in preventing coccidiosis in suckling pigs after a single oral dose of 20 mg/kg bodyweight.


## Introduction

Diarrhoea in suckling piglets is a common disease in pig production in Denmark[1]. In particular, diarrhoea in the piglets' 2nd week of life does often occur. Most of these cases of diarrhoea are caused by the coccidia species *Isospora suis*. Thus *Isospora suis* was diagnosed in 20% of the faecal- or organ samples sent to the National Veterinary Laboratory in 1993 for parasitological examination.

Coccidiosis in suckling pigs is also reported from abroad to a constantly increasing degree.

This literature review updates the knowledge about *Isospora suis*: prevalence, life-cycle, pathogenesis, clinical symptoms, immunology, diagnosis and prevention and control.

## Coccidia in pigs

In all, identification of 13 species of *Eimeria* and 3 species of *Isospora*[2] has been reported in pigs. Many of these species are only known as the finding of oocysts in faeces, and some of these are probably not independent species. Some of the species found are thought to be only occasionally passing through the intestine [3]. Only *Isospora suis* is considered to cause a pathological problem in pigs, although *Eimeria* species, in rare cases, can cause mild transient diarrhoea[2].

*Isospora suis* has been shown to cause the same clinical picture in wild boars [4] and miniature swine[5].

## Prevalence

*Nordic countries*

In a joint Scandinavian project concerning parasitic infections in pigs[6], several different *Eimeria* species as well as *Isospora suis* were detected. The *Eimeria* species were not determined. In the prevalence study there was found a great variation in the different Nordic countries (Fig. 1). The largest prevalence was observed in Iceland, while in Norway and Finland there was a very low occurrence of coccidia. The occurrence in Denmark and Sweden was more or less on the same level. One have to note that in Fig. 1 no differentiation is made between *Isospora suis* and *Eimeria* species.
In 71% of the positive samples, identification of the species was successful (Fig. 2). Here, *Isospora suis* was confirmed in: 96% of the suckling piglets, 67% of the weaners, 57% of the prefatteners and in 40% of the fatteners respectively. 97% of the identified samples from the adult breeding animals were *Eimeria* spp. *Isospora suis* was only diagnosed in 3 pregnant sows and in 4 lactating sows, all from Iceland.

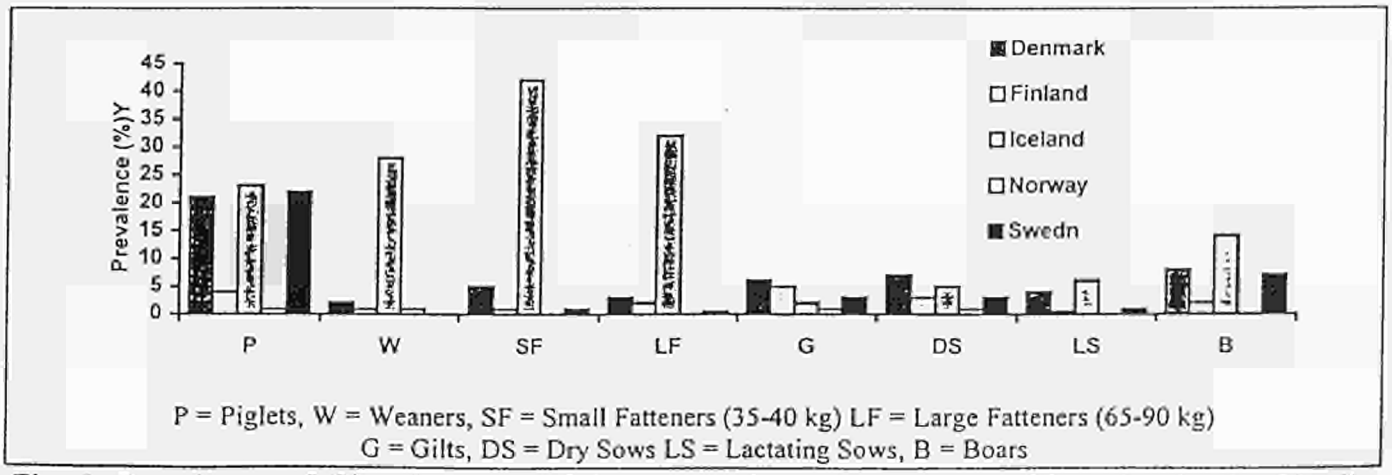



Fig. I. Prevalence of Eimeria spp. and Isospora suis oocyst-count positive pigs in the Nordic countries in 1986-1988 (random herds)[6].

*Types of herds*

In this study[6], the occurrence of *Isospora suis* correlated strongly with the size of the herd. Only 6.9% of the found positive samples from piglets were from herds with less than 50 sows, 21.5% from herds with 50-99 sows, and 34.3% from herds with more than 100 sows.

The occurrence of *Eimeria* species from the breeding animals was positively correlated with access to outdoor sties.

In Denmark it was also found that a high *Isospora* prevalence correlated strongly with a low weaning age, which may be explained by a rapid turnover of susceptible piglets in the farrowing pens. In addition, it was found that the SPF/MS system (declared Specific Pathogen Free) correlated with a low prevalence.
In addition, in intensively farmed herds, coccidiosis is said to occur far more frequently in herds where the farrowing pens have solid or partially solid floors[7].

The results from the joint Nordic study correspond to other prevalence studies. In 1993, Nielsson et al.[8] examined faecal samples from 34 Swedish sow herds. In the samples from the sows, they found that, *Eimeria spp.* were detected in 38.2% of the herds (17.9% of the sows examined), but *Isospora suis* was not detected in any of the herds. In the samples from the suckling piglets (1-4 weeks old), *I. suis* was detected in 25% of the herds, where there were problems with steatorrhoea (13% of the litters examined), and in 12.5% of the herds where no diarrhoea problems were recorded (8.3% of the litters examined). In another study[8], Nielsson et al. detected oocysts in faeces from one lactating sow. However, the piglets of this sow were all negative for *I. suis*.

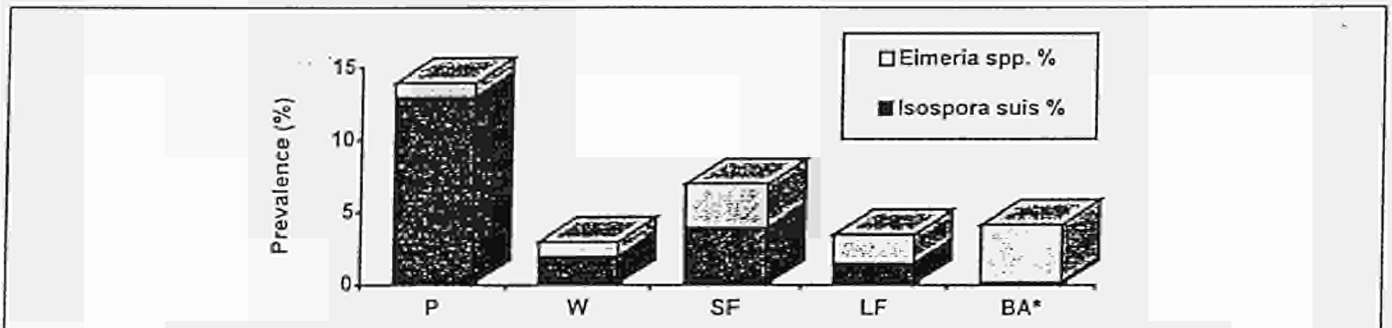



Fig. 2. Prevalence of the total *Eimeria* and *Isospora suis* oocyst-count in the Nordic countries in 1986-1988[6].

*Other countries*

Coccidiosis in piglets in the 2nd week of life is being described in many other countries. Apart from the Scandinavian countries, particularly USA[9] and Canada[10,11] are aware of the problems that *Isospora suis* can cause. From other overseas countries, *I. suis* coccidiosis has been described in Australia[12, 13, 14], Brazil[15], Venezuela[16] and China[17]. From the other countries in Europe, there have been only a few reports of the occurrence of coccidiosis in piglets, from, among others, Czechoslovakia[18] and Switzerland[19].

*Seasonal variation*

Robinson and Morin[11] showed in 1982, that coccidiosis was a problem throughout the year, but that there were clear seasonal variations. The highest prevalence was found in July - September. This seasonal variation was not demonstrated in studies from Denmark[20] and in Australia[14].

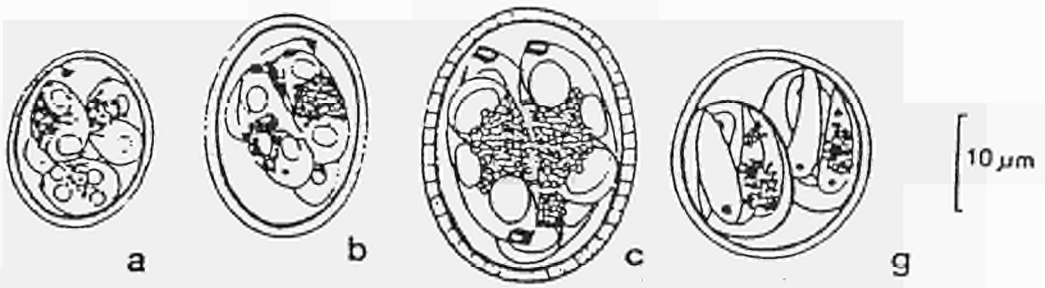
**Life-cycle**

Coccidia are protozoa, which are all obligatory parasites[2]. The classic coccidia belong to the family of Eimeriidae; this includes the genera *Eimeria* and *Isospora*. With just a few exceptions, they are parasites in the gastrointestinal tract. So far, 248 species of *Isospora* have been described[21].

The typical life-cycle for coccidia is in three phases: sporogony, excystation and endogenous development[2, 21, 22].

*Sporogony*

Sporogony is the exogenous phase of the life-cycle. Oocysts are excreted with faeces, and sporulation to the infective stage takes place outside the host (see Ill. 2).
The sporulation is dependent on humidity, temperature and oxygen. The sporulation of *Isospora suis* oocysts is apparently inhibited by temperatures >40°C or <20°C. At optimum temperatures (30-37°C), sporulation can take place in less than 16 hours[8].
When the *I. suis* oocyst is sporulated, it contains two sporocysts, each with four sporozoites. This is different from the *Eimeria* species, where the sporulated oocysts contain four sporocysts, each with two sporozoites[2, 4] (Ill. 1).




Ill. 1. Sporulated oocysts from a: Eimeria suis, b: Eimeria debliecki, c: Eimeria polita, g: Isospora suis. (after Löwenstein and Kutzer, 1989)[4].

*Excystation*

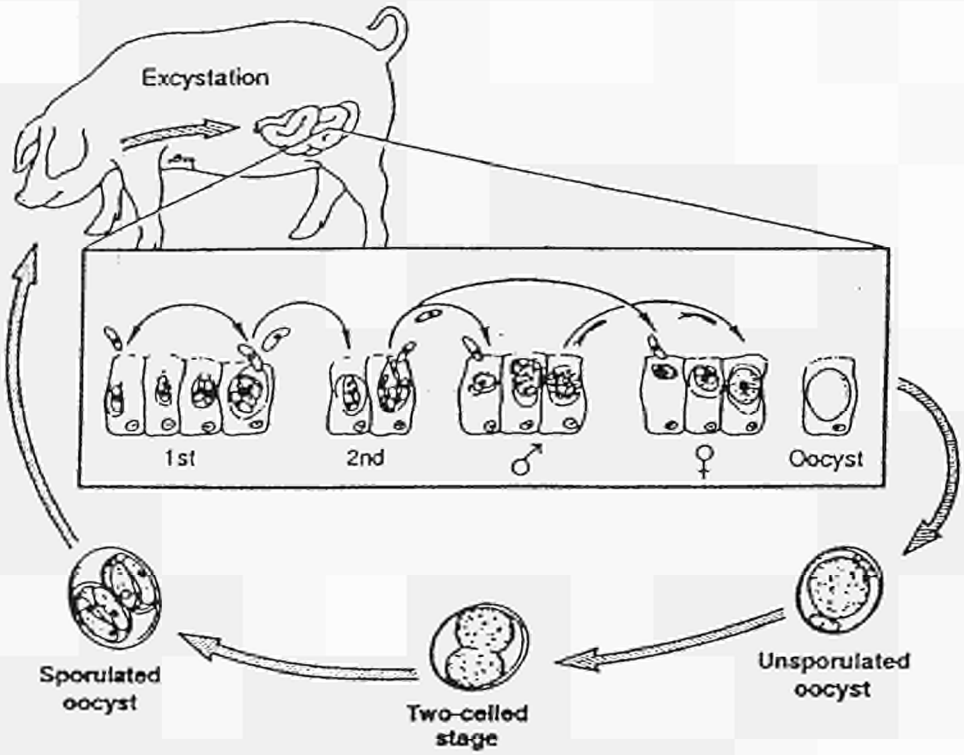
Excystation is the process in which sporozoites are released from the sporocyst/oocyst. The release of sporocysts from the oocyst takes place with assistance from the gastric acid and in the presence of $CO_2$[21] . Bile and pancreatic enzymes in the small intestine activate the sporozoites, which become mobile and cause the sporoblast/oocyst to burst. The sporozoites penetrate the villous epithelium, normally in the outermost 1/3 of the villi. The greatest concentration is seen in the middle part of the jejunum, but the ileum is also infected. In severe infections the colon can also be involved[22] .

*Endogenous development*

The endogenous development can be seen from Ill. 2. In the cells of the intestinal epithelium, the meronts are formed by asexual reproduction, and a number of merozoites are produced. With the release of the merozoites, the cells of the intestinal epithelium are destroyed. The merozoites now invade new intestinal cells, and the asexual reproduction is repeated. According to Tubbs 1986[22], the process involves 3 asexual reproductions, whereas others believe that the number of asexual reproductions can vary[21].

The last asexual stage begins to form the sexual stages in the life-cycle, the macrogametes (female) and the microgametes (male). The microgamete fertilises the macrogamete, and the hereby produced zygote develops cell walls and becomes an oocyst. The oocyst is released into the lumen of the intestine, and is shed with faeces.

The pre-patent period for *Isospora suis* is 5-7 days and the patent time is from 5 up to 16 days[23].




Ill. 2. *Isospora suis* life-cycle in pigs (after Lindsay and Blagburn, 1994)[21].

**Isospora suis. Porcine neonatal coccidiosis.**

## Extra-intestinal stages?

In the case of *Isospora* species in dogs and cats, extra-intestinal stages occur in the tissue of the host. Instead of going through the normal life-cycle in the intestinal tract, some sporozoites and merozoites leave the intestine and invade extra-intestinal sites in the host. The mesenteric lymph nodes are most frequently infected, but other organs can also be involved.
Whether such extra-intestinal stages occur with *I. suis* in pigs has not yet been clarified [22].
Henriksen et al.[24] showed in 1992, that piglets infected with low doses of *I. suis* oocysts (100 oocysts) shed oocysts in a cyclical pattern with a periodicity of 5 days. Up to three periods were documented, separated by sub-patent periods. This discovery supports the hypothesis of extra-intestinal stages for *Isospora suis*.

## Clinical symptoms

Clinical coccidiosis is seen most frequently in 5-14 days old piglets, but can occur in piglets up to 21 days old [3, 7, 8, 21, 22, 25]. The piglets develop diarrhoea, which initially is pasty, then becomes more liquid. The faeces are typically yellow and greasy/creamy, but can vary to brownish/greyish and be slightly frothy or watery. Typically, in the same herd there will be piglets with diarrhoea as well as piglets with normal faeces. From several places a typical picture has been described, where some piglets in the infected herd are constipated, with excretion of dry, pellet-like faeces ("sheep pellet faeces") [26, 27]. This is thought to be pathognomic for neonatal coccidiosis. The diarrhoea lasts from a few days up to 5-6 days. In occasional cases the diarrhoea can last until weaning. The majority of affected piglets will continue to suckle, but become thin and possibly dehydrated. The morbidity is high, but the mortality is low to moderate. In a survey among Danish pig veterinary surgeons carried out in the spring of 1990[7], it was judged that slightly more than half of the litters in a herd typically were affected, and that, on average, slightly less than half of the piglets in affected litters developed diarrhoea. However, a significant variation from one herd to another were seen. In the same survey, the disease was judged to have only a slight influence on mortality, but to reduce growth by 15% on average.

## Pathogenesis

The piglets become infected by absorbing sporulated oocysts.

### *Route of transmission*

It is extremely rare for *Isospora suis* oocysts to be detected in the faeces of sows [4, 5, 6, 8]. One example from a study from USA[9]: From herds with a known history of *Isospora* coccidiosis in the piglets, no sows were found to be positive for *I. suis;* in herds without clinical coccidiosis, *I. suis* was isolated from one out of 249 sows.

It must therefore be assumed that the piglets absorb oocysts that are present in the farrowing pen from the previous litter, or are brought in by the stall personnel, etc. Infected piglets shed 10-100 thousand oocysts per gram of faeces, and since coccidia oocysts are very resistant to disinfectants, it is difficult to take precautions against the transfer of coccidiaoocysts from one litter to the next.

## The significance of the infective dose

The degree of clinical coccidiosis is to some degree dependent on the number of oocysts absorbed[21, 22, 23]. Christensen and Henriksen investigated in 1994 which impact the infective dose of oocysts had on the clinical outbreak of coccidiosis in piglets[23]. With the infective doses of 3,000 to 50,000 oocysts, clear clinical symptoms were seen from 5-7 days after infection. 8 out of 32 infected piglets died or were destroyed on account of severe clinical symptoms of coccidiosis. The shedding of oocysts from the infected piglets followed a cyclic pattern with 2 sequences with an interval of approx. 5 days. Between the periods, very low-grade oocyst shedding was observed. The greatest shedding of oocysts was found 6-8 days after infection. However, an infective dose as low as 100 sporulated oocysts also caused clear shedding of oocysts and clinical manifestation of coccidiosis, although the highest oocyst shedding here was only recorded in the 2nd cycle 11-13 days after infection. In this group, a clear 3rd cycle was seen, with manifest peaks in the shedding of oocysts 16-19 days after infection.

## Interactions with other pathogens

Interactions between neonatal coccidiosis and other pathogens have been studied.

Vitovec et al.[28] studied the interaction between *Isospora suis* and rotavirus. 1-2 day-old piglets were experimentally infected orally with *Isospora suis* or rotavirus, either individually or with both agents, simultaneously or consecutively. After infection with *I. suis* alone, there was the typical picture with diarrhoea and biphasic shedding of oocysts. The changes in the intestine and the clinical symptoms were more severe after rotavirus infection alone than after *I. suis* infection alone. Synergistic effects of simultaneous infection with *I. suis* and rotavirus were manifest both clinically and pathologically, with watery, slightly yellowish diarrhoea and increased mortality as the result.

In 1993, Driesen et al.[14] studied the occurrence of *I. suis*, rotavirus and *E. coli* in faeces from 1054 piglets with diarrhoea. The piglets were 5-30 days old, and came from 151 different herds in Australia. More than 90% of the cases of diarrhoea were in the age group of 6-15-day old piglets. In 35.8% of the samples *I. suis* was found alone, in 10.7% *I. suis* was found together with *E. coli*, in 6.7% *I. suis* was found together with rotavirus, and in 0.6% of the samples *I. suis* was found together with both rotavirus and *E. coli*. Rotavirus and *E. coli* were found alone or together in 8.6%, 5.9% and 1.0% of the samples respectively. The study showed that by far the most frequent cause of diarrhoea in this age group was neonatal coccidiosis, and that mixed infections with *E. coli* and rotavirus were frequently seen.

## Pathological changes

On necropsy examination of piglets with coccidiosis, often only minor changes in the intestine are seen macroscopically [6, 9, 10, 11, 21, 28]. In severe clinical cases a green/grey, adherent, fibrinonecrotic pseudomembrane covering the mucous membranes in the jejunum and ileum is seen. The stomach is mostly filled with milk coagula.
Microscopically, villus atrophy, villus fusion, necrotic enteritis and hyperplasia of the crypt are seen. *I. suis* coccidia in different stages of development can be found in the cells of the intestinal epithelium in affected villi [2, 6, 10, 21, 28].

## Immunity

In general, infections with most species of coccidia will provide lifelong immunity[29]. This is also true for *Isospora suis*. Experiments have revealed a strong acquired resistance to re-infections[30]. Age-related resistance to *I. suis* has also been described[31]: The piglets were infected experimentally with *I. suis*. Piglets that were 2-4 weeks old at the time of infection had less pronounced signs of the disease than piglets that were 2-3 days old.

Attempts have been made to try to immunise the piglets via colostrum from the sows. Clinical trials[32], in which sows late in pregnancy were fed faeces from piglets suffering from diarrhoea have indicated that such an autogenic vaccination can reduce the occurrence of diarrhoea in the offspring. This was especially true in herds in which neonatal coccidiosis was a problem. However, later controlled studies[33] have showed, that it was impossible to transmit passive immunity to *Isospora suis* from the sows to the offspring. The piglets in the trial groups shed just as many oocysts as the piglets in the control groups.

The type of immunity to coccidia [*Eimeria* species] is an intestinal immunity. It is very complex, and still only partly understood[34]. The mechanism of immunity to *Isospora suis* has not so far been studied in depth.

## Diagnosis

The diagnosis of *Isospora suis*-related diarrhoea in piglets is based on the history of the herd, clinical findings, necropsy examination findings and detection of *I. suis* development stages in the epithelium of the small intestine and/or the finding of *I. suis* oocysts in faeces [2, 4, 6, 9, 18, 21, 22, 25, 26].

In the acute phase of neonatal coccidiosis in a litter, it is not possible to detect oocysts in the faeces[25, 26]. When the clinical symptoms of coccidiosis first appear, the *I. suis* oocysts still have to be developed. In these cases the diagnosis must be based on the finding of different development stages of *I. suis* in a smear from the small intestine or histopathological findings.

A little later in the course of coccidioses, or where one is trying to make a diagnosis of the herd, faecal samples are valuable tools[25, 35]. Here, it is important to remember that the shedding of oocysts varies greatly from one individual to another, and that shedding is intermittent in the individual piglet.

Henriksen et al.[35] has drawn attention to the importance of the procedure when collecting faecal samples for examination for the presence of oocysts, and they have shown that the used examination method has great influence on the result[36]. The following procedure should be followed:

1. *Faecal samples must be taken from piglets aged 10-14 days, preferably from animals with diarrhoea.*

   In a study from 1986-1998 carried out at the National Veterinary Serum Laboratory, Copenhagen[20], 5398 faecal samples (primarily from animals with diarrhoea) were examined for coccidia. In the age group 8-14 days, 33.4% were positive.
   Lindsay (1989)[25] mentions that samples from piglets with pasty faeces are more likely to contain large amounts of oocysts, compared with more watery faeces.

2. *The samples must be taken from at least 3-5 litters, from 3-5 pigs in each litter. The samples from the same litter can be collected as one pooled sample.*

   In the above-mentioned Danish study, a relatively marked correlation was found between the amount of samples investigated from a specific herd and the detection of coccidiosis in the herd. When 1-2 samples per herd were investigated, 32.3% of the herds were found to be infected with *Isospora suis*, with 3-5 samples per herd, 68.7% of the herds were positive, with 6-10 samples, 75.7% were positive, and when >10 faecal samples were investigated simultaneously, 90.2% of the herd was found to be infected with *Isospora suis*.
   This is consistent with Lindsay's observations[25]. He mentions that it is best to collect faecal samples from 10 litters where diarrhoea has been observed for 2-3 days.

3. *The examination of faecal samples must take place with a flotation test according to a modified McMaster technique.*

   Henriksen et al.[36] have shown for the *Isospora suis* oocysts' that, the ability to float, and the visual appearance in the microscope, depends on the flotation medium used. By comparing examining of the same faecal samples with the McMaster technique with a saturated NaCl solution (solution A) as the flotation medium, and the same NaCl solution with 500 g glucose per 1000 ml added (solution B), there was a marked difference: where solution B was used, the found OPG (oocysts per gram of faeces) values were 6-7 times higher than in group where solution A was used, and the oocysts appeared much more clearly.

4. *If the litters are clinically affected, the shedding of oocysts will often be of the order of magnitude $10^4$-$10^5$ OPG (oocysts per gram of faeces). However, there may be great individual variation.*

## Prophylaxis and control

*Hygiene measures*

The best way of preventing coccidiosis in suckling pigs is by reducing the number of oocysts in the farrowing pen before each farrowing. The oocysts are sensitive to heat (>70°C)[2], but apart from that they are very resistant to external influences. Tubbs (1987)[37] states, that the sow should be washed with a mild disinfectant, and that the farrowing pen must be thoroughly cleaned mechanically, and disinfected, before each farrowing. The majority of general disinfectants are ineffective against oocysts. Chlorine or ammonia in a 50% solution is said to be the most effective[37]. Cleaning with steam, high-pressure cleaning with warm water and direct heating with gas burners are also said to be effective. Thorough limewashing with a solution of slaked lime can replace the burning[38].

When the sow is installed in the farrowing pen, measures should be taken to reduce the risk of infection of the piglets: all faecal material should be removed daily, and care should be taken to prevent oocyst-containing faeces introduced from other pens.

## Coccidiostatics and therapy

Since the primary source of infection is primarily piglets in the farrowing pens, it is pointless to treat the sows prophylactically against the shedding of oocysts.

Several studies have been carried out in which the suckling pigs were treated prophylactically with various coccidiostatics.

Doré and Morin[39] from Canada have tested the active ingredient monensin in piglets which at 3 days of age were infected experimentally with 50,000 sporulated oocysts of *Isospora suis*. The piglets were given monensin orally every other day in a dose of 15 mg/kg from 3 days of age. No difference with regard to clinical symptoms was found between the treated and untreated litters, and the treated piglets shed considerably more oocysts than untreated animals. The conclusion was that piglets should not be treated with monensin in order to prevent *Isospora suis* coccidiosis.

Corresponding tests have been carried out with amprolium and furazolidone[40]. These coccidiostatics were given to piglets experimentally infected with *Isospora suis* oocysts as described above. Amprolium 9.6 % was given orally in a dose of 2 ml/piglet/day, starting on the day of infection. Furazolidone was given orally in a dose of 1 ml/piglet/day for 3 days from the day of infection, and thereafter 1 ml orally every other day for 6 days. All the treated piglets showed symptoms as in coccidiosis. A slight reduction in oocyst shedding was seen in piglets that were treated with amprolium; this was not observed in the group treated with furazolidone.

Tests with halofuginone and lasalocid[41], two coccidiostatics that are used in poultry feed, showed discouraging results.

Newer coccidiostatics have also been tested for the prevention of *Isospora suis* coccidiosis in suckling pigs. Decox® (declazuril) [42] given orally to the pigs (100 mg/kg b.w.) on the 3rd, 5th and

7th days of life, resulted in significantly better growth (days 3-21), lower mortality, and markedly lower oocyst shedding in treated pigs, compared with the placebo-group.

Sulpha preparations have an effect on coccidia. They are used prophylactically by being injected several times within the piglet's first week of life. In Denmark, several sulpha preparations are registered for prophylactic treatment against coccidiosis.

All the above-mentioned coccidia agents have either shown discouraging results in clinical trials, or are too time-consuming for use in practice. There is therefore a great need for more suitable products for the prevention of coccidiosis in suckling pigs.

A relatively new coccidia agent, toltrazuril (Baycox®), has been developed by Bayer AG for the treatment of coccidiosis in poultry[43]. Toltrazuril has proved to be extremely effective against a wide range of *Eimeria* and *Isospora* species, without influencing the development of immunity[44], [45]. Tests of toltrazuril as a preventive agent against neonatal coccidiosis caused by *Isospora suis* have shown that one oral treatment with 20 mg toltrazuril (Baycox 5% suspension) per kg bodyweight to 3-5 day-old piglets gives extremely satisfactory results [16, 18, 46, 47, 48, 49, 50]. Baycox in a 5% oral suspension is expected to be registered for pigs within the next few years.

## Literature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**Isospora suis. Porcine neonatal coccidiosis.**

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# TOPICS FOR DISCUSSION

Janet Catchpole

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# What are the important questions we need to ask about coccidiosis in pigs?

**What is:-**

- **the role of *Isospora suis* in pig health?**
- **the role of *Eimeria* species in pig health?**
- **the inter-relationship with other pathogens?**

# What are the important questions we need to ask about coccidiosis in pigs?

- **Are coccidia acting as**
  - primary pathogens?
  - opportunists?
  - precursors?

- **What is the route of transmission?**
  - from sow
  - from other piglets
  - environment/housing

# What are the important questions we need to ask about coccidiosis in pigs?

**What is:-**

- **Immunitity to *Isospora* and to *Eimeria*?**
- **Influence of management systems on disease?**
- **Life cycle**
  - particulary ideas about a possible extra-intestinal stage of *Isospora*

# What are the important questions we need to ask about coccidiosis in pigs?

- Does *Eimeria* play a role in pig health at any stage in it's growth?
- Do we need more information about the parasite or management system?
- What about interactions with other disease pathogens?
- Are coccidia important in their own right, are they opportunists or are they precursors to other problems?
- Control strategies??

# What are the important questions we need to ask about coccidiosis in pigs?

- Are coccidia in pigs sufficently important to cause concern in the pig industry?
- Do we need more information about the parasite or pigs?
- How do we get this information?

# Delegate List

| | |
|---|---|
| Dr Theodore. Alogninouva | Ecole Nationale Veterinaire de Lyon, France |
| Dr Alex Barlow | VLA, Bristol |
| Dr Petr Bedrnik | Biopharm, Prague |
| Dr Tony Carter | Hoechst UK |
| Miss Janet Catchpole | VLA Weybridge |
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| Dr Keith Millard | Merial, UK |
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| Dr Tony Mudd | Roche, UK |
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| Dr Leo Suls | Janssen Pharmaceutica Belgium |
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Abstracts presented to 1998 Workshop by members of Working Group 4.

1.

## Vaccination of mice with recombinant proteins against *Neospora caninum*

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*Neospora caninum* is a coccidian parasite of considerable economic importance by causing abortion or stillbirth in cattle among other disorders in different animal species. We recently established an experimental mouse infection model which showed that wild type C57BL/6 mice injected i.p. with $10^5$ living *N. caninum* tachyzoites were all *N. caninum*-positive in their brain as tested by PCR analysis (at day 44 p.i.). The present study included a vaccination trial to assess the putative protection by recombinant (rec) proteins from *N. caninum* against subsequent *N. caninum* challenge infection. The rec-proteins (Nc-p36, Nc-p43 and a mix of both) to be injected into mice were emulsified with the RIBI Adjuvant System (RAS) at 50 µg/ml, a crude extract of *N. caninum* (NC) was emulsified at a concentration of 250 µg/ml. Groups of wt C57BL/6 mice were inoculated i.p. with 200 µl of either RAS, rec-Nc-p36, rec-Nc-p43, the mix of rec-proteins or crude extract. A booster injection was repeated 14 days later. At this stage, parasite-specific IgG production in sera was confirmed by ELISA using NC crude antigen. Antibody concentrations were much higher in mice injected with either rec-Nc-p43 or crude extract than in mice injected with either Nc-p36 or the rec-protein mix. Seven days after booster injection, mice were infected with $2 \times 10^5$ *N. caninum* tachyzoites and euthanised 15 d.p.i.. Blood was collected for antibody determination and brain, heart, liver and kidney for PCR-based *N. caninum*-DNA detection. All organs were PCR-negative in uninfected control groups. In infected control groups, 3/6 mice were positive in the brain and 1/6 in the liver. Mice injected with rec-Nc-p43 or crude extract before infection showed interesting results in that all organs were *N. caninum*-negative by PCR. Conversely, the brain of 2/3 mice injected with rec-Ncp36 and the brain of 2/3 mice injected with the rec-protein mix was *N. caninum*-positive, as well as the brains of non-immunized but *Neospora*-infected control animals. These results suggest that vaccination with either rec Nc-p43 or *N. caninum* crude extract may induce protective immunity against subsequent *N. caninum* infection. A second vaccination trial is currently performed to elucidate the significance of our primary data.

2.

## Use of recombinant SAG1 in a subunit vaccine against infection with *Toxoplasma gondii* in mice

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It has been difficult to develop effective recombinant vaccines against protozoan infections. So far the most effective vaccine against infection with *Toxoplasma gondii* is a live, attenuated vaccine used in sheep.

We have expressed the *T. gondii* surface antigen 1 (SAG1) in *Escherichia coli* and shown that antibodies induced by rSAG recognise native *T. gondii*. In another study, we found that mice immunised with rSAG1 in alum, induced partial protective immunity against infection with the lethal RH-strain, measured as an prolonged survival time.

We have further developed the plasmid coding for the rSAG1 into DNA vaccine, and will present data showing that mice immunised with the plasmid coding for the SAG1 induce antibodies which recognise native SAG1.

Immunity against *T. gondii* is believed to be at least partially stage specific. We are in the process of developing an experimental vaccine containing both the SAG1 and the bradyzoite antigen 1 (SAG1), a bradyzoite specific antigen belonging to the HSP family. We hope that by using stage specific antigen we will be able to reduce the number of cysts after infection with a cyst forming strain.

3.

## The growing family of SAG1-related antigens of *Toxoplasma gondii*: Molecular characterization and expression analysis of the SAG5 gene cluster

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The invasive stages of the apicomplexan parasite *Toxoplasma gondii* are known to express a set of surface antigens, known as the SAG proteins, sharing structural and functional characteristics. Besides the extensively characterized SAG1 (p30) and SAG3 (p43) a series of novel *T. gondii* proteins sharing close structural similarity with SAG1 and SAG3 have been recently identified: SRS1-4 and BSR4 by Boothroyd and co-workers and SAG5 in our laboratory. A portion of the SAG5 gene was accidentally amplified from the genomic DNA of *T. gondii* (RH strain) employing unrelated primers. The PCR product 12G/16, encoding a 212 amino acid-long polypeptide showing a significant homology to both SAG1 and SAG3, was used as a probe to screen a genomic *T. gondii* cosmid library. Southern blot analysis of the positive cosmidic clones and of *T. gondii* genomic DNA employing the 12G/16 fragment as a probe yielded complex hybridization patterns, suggesting the existence, in the genome of *T. gondii*, of a family of related genes. Extensive sequence analysis confirmed this hypothesis and revealed the presence of three tightly clustered genes encoding closely related proteins, denominated SAG5.1, SAG5.2 and SAG5.3. All three polypeptides show the hallmarks of the family of SAG1-related molecules, being characterized by a leader peptide, 12 cysteine residues whose distribution suggests a tandem duplication of an ancestral 6-cysteine motif and a C-terminal GPI-anchoring signal. SAG5.2 and SAG5.3 are 367 amino acid-long and differ from each other at 8 amino acid positions (98% identity), while SAG5.1, which consists of 362 amino acids, is virtually identical to the other two SAG5 proteins only within the C-terminal region (positions 162-362). Notably, in the N-terminal half of the molecule the degree of amino acid identity to SAG5.2/SAG5.3 drops to approximately 40%, which represents the average homology shared by all members of the "SAG1" protein family. Northern blot and RT-PCR analysis of poly(A)+ RNA showed that all three SAG5 genes are expressed in tachyzoites. The transcriptional activity in the other invasive stages is under study. The localization of the SAG5 proteins in *T. gondii* tachyzoites was investigated employing polyclonal and monoclonal antibodies raised against a recombinant histidine-tagged fragment of SAG5.3 produced in *E. coli* and affinity purified by nickel chelate chromatography. In contrast with the other SAG1-related proteins, immunofluorescence analysis failed to show for SAG5 any significant staining of the tachyzoite plasmamembrane. Instead, anti-SAG5 antibodies specifically decorated the apical end of the tachyzoites. This evidence was confirmed by immunoelectron microscopy experiments in which anti-SAG5 antibodies were shown to label the cytoplasmic side of the tachyzoite pellicle and electrondense structures possibly associated with the microtubular components of the apical complex. Despite the evident structural similarity to the other SAG1-related molecules, the unusual genomic organization of the SAG5 genes and the peculiar subcellular localization of the encoded proteins seem to indicate for the SAG5 proteins a distinct functional role, whose elucidation deserves further investigation.

4.

## Molecular characterisation of the *Neospora caninum* tachyzoite surface protein Nc-p36

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*Neospora caninum* is structurally and biologically closely related to, but antigenically distinct from, *Toxoplasma gondii.* These parasites are obligatory intracellular and molecules associated with the surface and with secretory organelles are likely to participate in the host cell entry process and therefore in the pathogenesis. A tachyzoite stage-specifically expressed surface protein of *T.gondii* is SAG1 (p30) which has been shown to be involved in host cell attachment and invasion. Previously we have identified a cell surface-associated glycoprotein (Nc-p36) in *N.caninum* tachyzoites. The full length sequence of the cDNA coding for Nc-p36 was determined, and analysis of the deduced amino acid sequence demonstrated that Nc-p36 is closely related to SAG1. However these two proteins are antigenically distinct.

Nc-p36 is encoded by a single copy gene which produces a transcript of 1.4 kb. Immunogold labeling of resin-embedded parasites using polyclonal antibodies affinity-purified on a recombinant Nc-p36 fusion protein expressed in *Escherichia coli* showed that this protein is located exclusively on the tachyzoite cell surface.

5.

## Differential expression of cell surface- and dense granule-associated *Neospora caninum* proteins in tachyzoites and bradyzoites

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Morphologically, the tachyzoites and the tissue cysts of *Neospora caninum* are difficult to distinguish from those of other cyst-forming apicomplexan parasites such as *Toxoplasma gondii.* Several stage-specific antigens have been identified in *T. gondii* tachyzoites and bradyzoites, and respective antibodies are useful tools for discriminating between the two stages during tachyzoite-bradyzoite interconversion upon *T. gondii* infections. While several cell surface- and dense granule-associated proteins have been identified and characterized in *N. caninum* tachyzoites, little is known about antigenic components expressed in *N. caninum* bradyzoites. In this study, we describe the differential expression of the 2 *N. caninum* surface proteins Nc-p43 and Nc-p36 and the dense granule protein Nc-p33 (NCDG1) within tachyzoites and bradyzoites of *N. caninum* by using protein-specific affinity purified polyclonal antibodies. Expression of the three proteins was assessed by immunofluorescent staining of mouse brain sections containing either tachyzoites or bradyzoites (tissue cysts) of *N. caninum.*

6.

## Transfection in *Toxoplasma gondii*

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Transfer and expression of foreign DNA in tachyzoites of the apicomplexan parasite *Toxoplasma gondii* was first described by Soldati and Boothroyd in 1993 (Science, 260: 439-52). This tool complements several other features of *T. gondii*, such as simple *in vitro* culture of tachyzoites, cyst formation in culture, and well defined classical genetics, making this protozoan a paradigm for studies on intracellular parasitism. Over the last years, transfection has come to be used routinely in many laboratories working with *T. gondii*, and consequently a wide array of molecular genetic manipulations have been developed on the basis of the original protocol. In this paper we present a number of examples from our lab and others to illustrate both the power of transfection in various areas of basic *Toxoplasma* research and the potential for engineering apicomplexan parasites to fit specific needs.

Linearized or circular vector DNA containing appropriate *cis*-acting sequences is transfected into extracellular tachyzoites by electroporation. Classical markers for selection, such as antibiotic resistance genes (Kim et al., Science 1993, 262: 911-4) or β-Gal (Seeber & Boothroyd, Gene 1996, 22: 39-45), are used for most routine applications and yield stable transgenics at a frequency of up to 1%. Mutant alleles of the enzyme DHFR-TS that confer resistance to pyrimethamine increase this remarkably high frequency of transfection to about 5% (Donald & Roos, Proc. Natl. Acad. Sci., USA 1993, 90: 11703-7). The non-essential purine salvage enzyme HXGPRT under the control of constitutive but also stage-specific promoter elements allows both selection for and against its expression by the drugs mycophenolic acid and 6-thioxanthine, respectively. Positive/negative selection has been used to screen for mutants deficient in differentiation and in promoter-trap experiments. Most recently, the addition of GFP to the arsenal of reporters has allowed *in situ* observation of protein trafficking, both inside live parasites and the parasitophorous vacuole (Striepen et al., Mol. Biochem. Parasitol. 1998, 92: 325-38).

The power to control homologous *vs.* non-homologous integration of DNA makes transfection in *T. gondii* an extremely useful tool: Gene knock-outs by replacement insertion, which have been demonstrated for a number of genes (e.g. SAG1, ROP1, BAG1, HXGPRT), can be achieved with long contiguous genomic DNA from the target site flanking a selectable marker gene. Minimal homology regions in the transfected vector, on the other hand, lead to essentially random integration of linear tags or promoter-less reporter genes throughout the genome. This makes experimental approaches such as gene trapping or insertional mutagenesis with subsequent rescue of tagged loci highly efficient in this system. A shuttle vector containing a *Toxoplasma* origin of replication has been developed to be maintained as an episome under selection pressure. Such vectors have been used successfully to rescue mutants by complementation and will be essential for the convenient identification of genes responsible for a defined mutant phenotype or the analysis of lethal gene knock-outs.

Sophisticated transfection techniques enable us to use *T. gondii* also as a model system to study the biological function of phylogenetically conserved proteins from related apicomplexans (e.g. AMA1 from plasmodia) which are less easily manipulated. *Neospora caninum*, which is closely related to *T. gondii* but antigenically distinct, is now also amenable to transfection. *N. caninum* provides a convenient background to express and investigate selected surface antigens and organellar proteins from *T. gondii* without the need to introduce tags for detection.

Efficient transfection protocols and the powerful selections that have been devised in this model parasite have expanded the realm of experimental possibilities greatly. As we learn more about the biological function of specific proteins and antigens in *T. gondii* and other apicomplexans, we have not only a ready tool to test our hypothesis but also the ability to tailor parasites to fit many of our experimental needs.

7.

## Identification of a novel surface protein of *Toxoplasma gondii* using RNA display

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A recently described mutant of the RH-strain of *Toxoplasma gondii* resistant to high doses of artemisinin has been shown to have lost its acute virulence. In order to investigate the genetic background to the observed phenotypes we have used an mRNA display strategy to compare expression changes between tachyzoites from the mutant and the parental RH-strain. Through this analysis we have isolated several short cDNA tags corresponding to genes whose expression has been modified in the mutant in comparison with the RH-strain. Northern blot analysis for one of these, RAP6, showed that it hybridised to 1.8 kb long mRNA whose expression has been significantly down regulated in the mutant. We then used an inverse PCR to obtain sequences outside the original RAP6 fragment. The extended sequence matched two clones from the expressed sequence tag (EST) dataset of *T. gondii*. The complete cDNA sequences from these two clones were determined and combined with the genomic sequences. The open reading frame of about 368 amino acids was shown to match a surface antigen from merozoites of *Sarcocystis muris*. The open reading frame also encode a hypothetical N-terminal signal peptide sequence and a number of cysteine residues, reminiscent of the SAG-family of *T. gondii*. Using transfection techniques work is now on the way to further characterise the role of the RAP 6 polypeptide.

8.

## Expression of the bradyzoite-specific surface antigen SAG4 in the Ts-4 vaccine strain of *Toxoplasma gondii*

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During infection with *Toxoplasma gondii*, the acute phase caused by the virulent stage tachyzoite is rapidly overcome by the onset of the immune response. Parasites develop into a chronic phase characterized by formation of cysts which persist during the life span of the host. In immunocompromized patients the relapse of the cyst form is responsible for the pathogenesis of the disease.

The temperature-sensitive mutant ts-4 of the *T. gondii* RH strain fails to grow at 40 degrees C *in vitro*. This attenuated mutant which has been widely used to vaccinate mice against virulent *T. gondii* strains, elicits strong preimmune defenses, dependent on gamma interferon, which are needed by mice to survive acute infection. Although this experimental vaccine protects animals from disease it does not prevent from infection and cyst formation. Information regarding the role of bradyzoite-specific antigens in immune protection is limited to date. To investigate the potential protective properties of the bradyzoite-specific surface antigen SAG4(P18), we have generated a recombinant ts-4 mutant expressing this surface protein in a constitutive fashion (ts-4/SAG4). In parallel we have produced and purified recombinant SAG4 protein from *E. coli*.

Different combination of ts-4, ts-4/SAG4 and/or recombinant SAG4 protein have been used to immunize mice. Preliminary analyses show that the experimental vaccination elicits a strong antibody response and CTL proliferation against SAG4. The vaccinated mice will be challenged to other-wise lethal doses of cysts from the persistent Prugniaud strain to evaluate effectiveness of the SAG-4 based vaccines against chronic infection.

9.

## A μMT knockout and wild-type mouse model for immunological studies in experimental neosporosis

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*Neospora caninum* has become an important veterinary pathogen as causative agent of neuromuscular disorders and abortion in different animals, especially cattle as tested in one of our studies (Gottstein et al., 1998, Int. J. Parasitol., 28, 679-691). We now established an experimental murine model for the study of host immunity. Thus, wild type C57BL/6 mice and B-cell deficient mice (transgenic mutation in the transmembrane exon of the IgM μ-chain gene; μMT-mice) were infected i.p. with $10^5$ living *N. caninum* tachyzoites. Mice were sacrificed at days 10, 24 and 29-44 pi. Brain, heart, lung, liver, spleen, and kidney were collected for parasitological and pathological, spleen and serum for immunological investigations. Splenocytes were *in vitro*-stimulated with *N. caninum*- and *T. gondii*-antigens for assessing T cell proliferation and cytokine production. While wt mice were resistant to disease, μMT mice died between days 29 - 44 pi. Histological examination of brain tissue from μMT mice exhibited a high infection intensity with multifocal necrotic cerebral lesions, absent in brain of wt mice. *Neospora* antigen-stimulated splenocytes of both wt and μMT mice infected with *N. caninum* showed a marked proliferative depression at day 10 pi. At day 24 pi, this immunosuppression was still maintained in μMT mice whereas full immune responsiveness had been restored in wt mice. Stimulated splenocytes of infected μMT mice secreted less IFN-γ and less IL-10 than corresponding wt splenocytes. For IL-10, these differences markedly increased between days 10 and 24 pi. Consequently, the high susceptibility of μMT mice appeared causatively associated to an impaired cellular response in addition to the humoral deficiency, finally resulting in a lethal outcome of infection.

10.

## Protozoal infections associated with abortion in italian livestock

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The significant role played by cyst-forming coccidian parasites in determining abortions, has been recently recognised in many part of the world. To assess the importance of foetal protozoan abortions in Italian livestock industry, 127 bovine, ovine and caprine foetuses, in which no bacteria, viruses or others have been isolated, were examined. From each foetus, sections of heart, liver, brain and kidney have been fixed and processed for histology and immunohistochemistry. According to the histological findings, 45.9% (17 out of 37) of the bovine foetuses had protozoon-like cysts, 37.5% (3/8) of goat foetuses were also found infected, whereas in only 14.6% (12/82) of ovine foetuses, cysts were present. Heart was the organ most commonly involved by the presence of the cysts. Histological lesions, characterised by non suppurative inflammation, were observed in 83.4% of foetuses and the liver was the organ most commonly involved. Immunohisto-chemical studies using antisera against *Neospora caninum* and *Toxoplasma gondii*, showed negative reactions also in those samples in which only histological lesions were observed.

11.

## The epidemiology and significance of bovine neosporosis in England and Wales: interim results of a collaborative study

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In a project supported by the Ministry of Agriculture, Fisheries and Food, United Kingdom, and involving collaboration with the Veterinary Laboratories Agency, veterinary practitioners and dairy farmers, we are seeking to determine the significance of bovine neosporosis in England and Wales and to elucidate its epidemiology. Herd-and age-specific antibody prevalence data have been obtained from over 4,000 cattle in 16 herds by ELISA. Logistic regression analysis showed no difference between prevalence values in different age groups, consistent with vertical transmission being the major mode of transmission. On-farm studies of new-born calves sampled pre-colostrally have confirmed the extremely high efficiency of vertical transmission. In a randomised, case-control serological survey of aborting (n = 626) and normally calving (n = 416) cows, aborting cattle were three times more likely to be *Neospora* antibody positive than non-aborting cows ($p < 0.0001$) but the antibody prevalences to BVD virus, IBR virus and *Leptospira hardjo* were not higher in the aborting group. The antibody prevalence in normally calving cows was 5.7% (95% CI; 4, 8). This is the first estimate of *N. caninum* seroprevalence using a random sample of dairy cattle in England and Wales, and these data provide a basis to estimate some economic costs associated with this infection.

12.

# Seroprevalence of *Neospora caninum* infection in dairy and beef cattle in northern Spain

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In recent years, neosporosis has been identified as a major cause of abortion in dairy and beef cattle. Although the disease has been described worl-wide there is a lack of information concerning the prevalence of this disease in different cattle production systems.

The aim of this study was to investigate the seroprevalence of *Neospora caninum* infection in a representative area of beef and dairy cattle production in Spain. A cattle census provided by the Local Veterinary Service in 1996 showed an average population of 11,460 cattle distributed over 749 herds (498 beef and 251 dairy herds). Cluster sampling was employed, in which herds constituted the initial sampling unit and two strata (dairy and beef herds) were considered. As there is little information concerning the prevalence of neosporosis in the different cattle producing systems the worst case was considered (50% prevalence). Using a 95% level of confidence and setting 5% (beef) and 5.4% (dairy) error limits, the desired sample size were 216 and 143 for beef and dairy herds, respectively. This sample size was distributed according to the proportional cattle herd number of the different municipalities in the area. Cattle herds were randomly selected using random number tables and contacted by the local Veterinary Service. On the basis of the formula $n=[1-(1-CL)^{1/D}][N-(D-1)/2]$, where n is the required sample size, CL is the confidence level (95%), D is the supposed number of infected cattle in each herd (23.4%) (Quintanilla- Gozalo et al., 1996. IV Cong. Int. ANEMBE), and N is the herd size, nine animals (>1 year old) were randomly sampled in each herd to detect the presence of the infection. A herd was considered infected when at least one animal was seropositive. Sample sizes required to detect infection and estimate herd prevalence were calculated using the EPISCOPE computer program (Frankena et al., Vet. Rec., 126: 573-576). In total, serum samples from 1121 dairy and 1712 beef animals were collected and tested for specific anti-*N.caninum* IgG using an ELISA test. Serum samples with optical densities equal to or higher than 0.40 were considered as positive (Osawa *et al.*, Vet. Parasitol. in press).

Specific antibodies were detected in 55.1% (119/216) beef and 83.2% (119/143) dairy herds. Individual prevalences obtained were 17.9% (306/1712) for beef and 35.9% (402/1121) for dairy animals. Presence of *N. caninum* infection was higher in dairy than in beef herds and the association between infection and the cattle production system (dairy or beef) was statistically significant [$\chi^2_Y$=29.21, p<0.001, OR=4.04 (2.35-6.99)]. Data concerning association with other epidemiological parameters such as herd size, herd management and herd location will be presented and discussed.

This is the first estimate of *N. caninum* seroprevalence in dairy and beef cattle herds in Spain using a random and representative sample. Preliminary data show the widespread distribution of *N. caninum* infection and the influence of the production systems on the risk of acquiring this infection. This study may be of value to estimate the general prevalence of this disease in dairy and beef cattle in Spain and to calculate its economic repercussions.

13.

## Genetic polymorphism in laboratory isolates of *Neospora caninum*

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The design of effective control measures against parasitic infections depends on a good understanding of the epidemiology of the parasite in question. Without this knowledge changes in management practice, vaccination or therapeutic intervention may make little impression on the economic impact of parasitic disease. Genetic typing which exploits DNA polymorphisms between different isolates of the same species is a valuable tool in determining the epidemiology of pathogenic microorganisms. Suitable genetic markers may be generated by a variety of techniques often depending on the extent to which genetic variation occurs within the target population. To date little is known about the genetic variation that occurs between different isolates of *Neospora caninum*, although ribosomal RNA sequencing suggests that the isolates examined so far do form a single species (Marsh *et al, J. Parasitology* 1995, vol. 84, pp. 530-535). In this study we have examined genetic variation occurring between a variety of *N. caninum* laboratory isolates maintained in tissue culture using random amplified polymorphic DNA analysis (RAPD). The laboratory isolates analysed were from geographically distinct sites in Europe and Japan. Genetic polymorphisms identified in these laboratory isolates by RAPD analysis will now be used to generate DNA markers that will be evaluated on field samples. Such markers could be used to help answer questions such as (a) how genetically diverse are *N. caninum* field populations? (b) are different populations of *N. caninum* epidemiologically related? (c) does vertical transmission contribute to the "clonality" of this parasite (d) are certain isolates associated with disease i.e. are there "virulent" and "avirulent" strains of *N. caninum*?

14.

## Follow-up studies in *Neospora caninum* infected dairy herds using IgG subclass ELISAs.

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The dynamics of *Neospora*-specific serum antibodies during the life of the cow and the influence of pregnancy is still incompletely resolved. In a follow-up study on 4 Danish dairy herds with confirmed neosporosis, data from Iscom-ELISA on 285 cows with at least 2 bleedings with $\geq$ 3 mo. interval were analyzed to study changes in an up to 1½ years period. With an assay employing an $IgG_1$-specific conjugate (Swedish National Veterinary Institute), 49% of the animals were consistently negative, while 41% were consistenly seropositive. Apparent seroconversions (7.7%) dominated over fluctuating responses (2.5%), which was also reflected in a moderately increased seroprevalence with cow age in the studied populations. Reasonably equipotent assays for $IgG_1$ and $IgG_2$ were established using monoclonal mouse conjugates from the same producer (Bethyl). The data from one of the investigated herds showed large variations between individual seropositive animals for the $IgG_1/IgG_2$ OD-ratio, ranging from 0.23 to 4.3, with a trend towards a minimum around the time of parturition. Analysis of the data from all 4 study herds will be presented and discussed.

15.

## Immunity to *Neospora caninum*

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*Neospora caninum* is an intracellular coccidian parasite that causes abortion and congenital defects in cattle, dogs, horses and goats. The parasite is recognised as a major cause of infectious abortion and is linked to approximately 12-17% of bovine abortions in Scotland. *Neospora* can cause repeat abortion, but the rate of repeat abortion is estimated to be less than 5%. The only known route of natural infection is by congenital transmission.

Cell mediated immune mechanisms, and in particular the T cell cytokine IFNγ produced mainly by CD4+ T cells, are important in the development of protective immunity to *Toxoplasma gondii*, a closely related coccidian parasite which causes abortion in sheep. Very little is known about the immune response to *N. caninum*, but it is likely that the production of IFNγ is important in the prevention of repeat abortion. We have shown in previous studies that treatment of cells with recombinant IFNγ significantly inhibits intracellular multiplication of *N. caninum* tachyzoites.

In this study, calves experimentally inoculated with *N. caninum* NC1 strain tachyzoites developed a cell mediated immune response to infection. Peripheral blood mononuclear cells (PBM) from infected cattle proliferated *in vitro* in response to *Neospora* whole antigen. CD4+ T cell lines generated from infected animals responded to a group of *Neospora* antigen fractions of molecular weight 30kDa and supernatants from the activated CD4+ T cells were found to contain high concentrations of IFNγ. Pre-treatment of bovine fibroblasts with these supernatants inhibited the growth of *N. caninum* tachyzoites *in vitro*. These low molecular weight proteins may therefore be responsible for the induction of a potentially protective immune response. *N. caninum* tachyzoites were also cultured in alveolar macrophages from infected and control animals. Cells from the infected calves were able to suppress growth of the parasite in comparison to the growth of tachyzoites in cells from naïve animals.

A study of the immune response in congenitally infected calves revealed that these animals also developed a cell mediated immune response to infection. PBM from these animals proliferated in response to a crude lysate of *N. caninum* NC1 tachyzoites *in vitro* and supernatant collected from proliferating cells also contained high concentrations of IFNγ.

While the nature of protective immunity to *N. caninum* is still unknown it is clear that IFNγ plays an important role in the immune response to this parasite. A further understanding of the cell mediated immune response and the mechanisms involved will have a considerable impact on how to produce a potentially protective immune response.

16.

## Immunological and clinical responses in cattle following experimental infection with *Neospora caninum* at different stages of gestation

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The apicomplexan protozoan parasite, *Neospora caninum*, is a major cause of bovine abortion in many countries. Little is known about the immune response to *Neospora* in naturally or experimentally infected cattle, although as *Neospora* is closely related to *Toxoplasma gondii*, cell mediated immune responses are likely to be important. The aim of this study was to investigate the immunological, clinical and pathological responses in cattle experimentally infected with *N. caninum* at different times relative to gestation. Cattle were divided into three groups and infected with *in vitro* derived Nc (Liv) tachyzoites nine weeks prior to artificial insemination, ten weeks post-insemination or thirty weeks post-insemination. An increase in temperature was detected in 15/24 cattle 2-7 days after infection. Antibody levels increased and reached a peak at 4 weeks after infection. Peripheral blood mononuclear cells proliferated in response to parasite derived antigen by 8 days after infection and this was accompanied by secretion of high levels of ã interferon. The effect of infection on the viability of the foetuses will be presented.

17.

## The role of immunological memory in *Sarcocystis* infection

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A hallmark of the immune response is memory, the ability to recall an earlier encounter with specific antigen. In a recent investigation Bunce and Bell (1997, J. Exp. Med., 185: 767-776) demonstrated that two isoforms of the CD45 molecule (CD45RC$^+$/C$^-$) define two types of CD4 memory T cells in the rat. Upon antigen encounter, naive CD45RC$^+$ CD4 T cells rapidly switch to short-lived CD45RC$^-$ (memory) cells. If antigen does not persist, CD45RC$^-$ cells can revert within a week to longer-lived CD45RC$^+$ memory cells which ensure that immunlogical memory endures.

We investigated CD4 T cell memory in wild brown rats infected with *Sarcocystis singaporensis*. The underlying question was why natural infections (presumably following uptake of low numbers of sporocysts) apparently did not protect rats against acute sarcocystosis after challenge with high sporocyst doses (Jäkel et al. 1996, J. Parasitol., 82: 280-287). Rats were immunized with 10 or 1000 sporocysts or PBS (10 rats/group) 16 days prior to challenge with 5 x $10^5$ sporocysts. Only animals immunized with 1000 sporocysts survived challenge. Flow cytometric analysis of spleen cells (anti-CD3, CD4, CD8, CD45RC) at the day of challenge revealed that only rats immunized with 1000 sporocysts showed a significantly higher number of CD8 T cells and CD45RC$^+$ CD4 T cells (compared to PBS). Additionally, the B cell response clearly mirrored the T cell results. In a set of repeated experiments, we used 100 sporocysts for immunization. Here, a majority of rats survived challenge. In contrast to the higher immunization dose, however, only numbers of CD45RC$^+$ CD4 T cells were significantly increased in these rats at the day of challenge.

These data indicate that formation of long-lived memory CD4 T cells in sufficient quantity may be crucial for protective immunity of rats against acute *Sarcocystis* infection. Adoptive transfer experiments are underway to further adress this question.

18-

# Epidemiology and economic impact of toxoplasmosis in animal production

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The apicomplexan parasite *Toxoplasma gondii* can infect most, if not all, warm-blooded vertebrates in which it has a two-stage life cycle (bradyzoites in tissue cysts and tachyzoites). In Felidae it can undergo a sexual life cycle and produce oocysts, which can survive on pasture, in food and in water for many months and constitute a source of infection for farm animals. It has its greatest impact in sheep in which, following a primary infection during pregnancy, it may cause fetal death and abortion. It can also cause abortion in goats and pigs but no significant disease is produced in cattle and horses.

Sheep. Sheep are farmed in all 15 countries of the EU and in 1997 there were over 98 million of which 70.6 million were recorded as being females of breeding age (Statistical Office of the European Commission - SOEC) of which three-quarters were located in the UK, Spain, Italy and France (1998, MLC European Handbook, 2, 6-7, MLC, Milton Keynes). *Toxoplasma* abortion and fetal loss may occur wherever sheep are kept but precise figures of the losses do not exist. Perhaps the best estimates were made by Blewett and Trees (1987, British Veterinary Journal, 143, 128-135) who concluded that the average incidence of clinical toxoplasmosis in the UK is between 1% and 2% per annum. This figure is an underestimate, as it does not include the loss of fetuses early in gestation that are resorbed so that the ewes present as cases of infertility. If the incidence of toxoplasmosis is assumed to be similar throughout the sheep rearing areas of the EU then in 1997 between 706,000 and 1.4 million lambs would have been lost (assuming an average lambing of one lamb per ewe). However this is a risky assumption and the incidence of clinical toxoplasmosis will probably vary from region to region.

Because clinical toxoplasmosis occurs following primary infection during pregnancy its incidence is influenced by a) the number of susceptible sheep and b) the degree of contamination of food and water by viable oocysts, which itself is dependent both on the cat population and environmental conditions. A lower incidence than occurs in the UK will be recorded in a region if infection, seroconversion and immunity occur in a high proportion of sheep before they reach breeding age. This may occur if a) there is significant contamination of food and/or water with oocysts and/or b) environmental conditions favour their prolonged viability (relatively little dessication and frost). A lower incidence of clinical toxoplasmosis will be encountered in a region where viable oocysts are scarce. Thus few cats, extensive grazing practices, no supplementary feeding and relatively harsher weather conditions, with significant frosts and/or dessication, will lead to low infection rates. While there will be a relatively high proportion of susceptible pregnant sheep they will be exposed to minimal challenge by oocysts.

Goats. The 12 million goats in the EU (SOEC) are as susceptible to toxoplasmosis as sheep and the incidence of clinical disease will be influenced in similar ways to those for sheep. Goats and sheep are also kept for milk production, particularly in Greece, France, Italy and Spain. Any cause of abortion may severely reduce herd and flock outputs.

Pigs. There are an estimated 118.5 million pigs in the EU of which 12.8 million are females of breeding age (SOEC). The incidence of fetal losses due to toxoplasmosis is not known but it is likely to be less than that in sheep. However infection in pigs is relatively common (approximately 30% are seropositive)(Dubey and Beattie, 1988, Toxoplasmosis of Animals and Man, CRC, Boca Raton.) and likely to be more so in extensive outdoor management systems than in housed, minimal disease units.

*Toxoplasma gondii* is also a zoonotic parasite that can cause serious illness in certain sections of the human population. As well as the suffering it causes there is a cost for the health services in the EU. Thus the cost of toxoplasmosis to EU (and non-EU) countries is considerable both in terms of inefficient animal production and as a human pathogen often derived from ingested, infected meat. Only with more accurate data will it be possible to direct control measures effectively.

19.

## The economic impact of neosporosis

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Infection with *Neospora caninum* was first associated with bovine abortion about ten years ago and has since become recognised as an important reproductive disease of cattle with a world-wide distribution. Recently the dog has been identified as a definitive host for this parasite, but vertical transmission is still thought to play a major role in the epidemiology of the disease in cattle. Just how important the disease is to the European economy has yet to be rigorously addressed, but this question requires urgent attention if appropriate resources are to be allocated to minimise its impact. Recent advances in the diagnosis of infection, including serological tests (ELISA and IFAT), immunohistochemistry and PCR now provide the potential for more reliable diagnosis and hence quantification of the impact of neosporosis. Nevertheless, the implementation of appropriate standardised diagnostic procedures is probably still the single biggest obstacle in obtaining reliable estimates of the cost of this disease to the European farming industry. Some estimates of the economic cost of the disease have appeared in the literature, for example, the cost to the Californian dairy farming industry is thought to be approximately US$ 34.5 m each year. However, making an objective assessment of the economic impact of the disease that is robust enough to allow comparison between different countries, farming systems and economies is extremely difficult and simple translation of the assumptions made in one system may be inappropriate elsewhere.

Although neosporosis affects both dairy and beef cattle, most of the data available is for dairy production where birth of a live calf and subsequent yearly milk yield are the principal economic outputs. For economic modelling to be meaningful, accurate information must be available on factors such as (a) the incidence of bovine abortion directly attributable to *Neospora* infection in relation to the total abortion rate (b) the cost of abortion both in terms of foetal loss and loss of milk production (c) the (sometimes hidden) cost of premature culling or re-sale of infected animals. The relative importance of neosporosis will also be dependent on the initial investment and expected profit on each animal as well as the prominence of other infectious agents, both of which may vary considerably in different agricultural systems.

This paper will summarise current data obtained from The Netherlands, Switzerland, Australia, the United States, Spain, Holland, Sweden, Germany and the United Kingdom on the incidence and cost of bovine neosporosis. The aim of this paper will be (1) to provide a framework for discussion on the current estimates of the economic cost of bovine neosporosis (2) to review the quality of data available and identify where inadequacies in data or data collection exist (3) to compare methods used for computing economic impact with the aim of standardising best practice.

20.

# Epidemiology and economic impact of *Sarcocystis* species

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The genus *Sarcocystis* comprises more than 120 species of heteroxenous cyst-forming coccidia with differences in life cycle and pathogenicity. About 30 of these species occur in livestock (Tenter, 1995, *Int. J. Parasitol.*, 25: 1311-1330). Knowledge on the epidemiology and relative importance of different *Sarcocystis* spp. has been impeded by the lack of sensitive and specific diagnostic methods for most of these species. However, *Sarcocystis* spp. can cause economic losses in the livestock industry in various ways.

A primary infection with a high dose of sporocysts of a pathogenic *Sarcocystis* sp. can cause acute sarcocystiosis with high rates of mortality, in particular in sheep, goats and cattle. In pregnant animals, acute sarcocystiosis frequently results in foetal death, abortion or premature birth of the offspring. Chronic sarcocystiosis can result from the ingestion of a low dose of sporocysts of a pathogenic *Sarcocystis* sp. and can cause economic losses in the livestock industry due to reduced quality and quantity of meat, milk or wool in cattle, pigs, sheep and llamas. Additional economic losses are caused by infections with *Sarcocystis* spp. that are non-pathogenic, but form macroscopic cysts in muscles of cattle or sheep, which results in condemnation of whole carcasses or affected parts after slaughter.

*Sarcocystis* spp. infecting ruminants appear to be the species of the highest economic importance. In some countries, infections with *Sarcocystis* spp. have been estimated to account for up to 25% of annual losses directly attributable to parasitic diseases in sheep, cattle and llamas. In this session we will attempt an estimation on the potential economic losses caused by these parasites in the EU.

factors other than immunoglobulins are also transferred from *C. baileyi* infected hens to their progeny. A dot-ELISA was developed to evaluate seroconversion of infected chickens which was 100% in both infected (IY and IC) groups.

21.

# Pathogenicity of selected *Toxoplasma gondii* isolates after intravenous inoculation in pigs

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The pathogenicity to 7 weeks old pigs of the human congenital *Toxoplasma gondii* strain "NED" (Dardé et al. 1992, Journal of Parasitology, 78: 786-794) and the porcine strain "119" (Work 1968, Acta Pathologica et Microbiologica Scandinavia, 73: 85-92) was compared to 3 danish *T. gondii* isolates recently isolated from a slaughter pig ("SVS P14"), a fox heart ("SVS Fox2") and a sheep abortion ("SVS O14"), respectively. Five groups of 5 pigs each were inoculated intravenously with $10^4$ tachyzoites of the different isolates propagated in vero cell cultures. Additionally, one group of 5 pigs was inoculated intravenously with $10^6$ tachyzoites of the 119 strain. Eight pigs were included as PBS sham infected controls. Pigs inoculated with $10^4$ tachyzoites of NED, 119, P14 and Fox2 experienced a short-lived rise in body temperature from day 6-8 post inoculation (p.i.) without any apparent illness or inappetence, whereas control pigs and pigs inoculated with the O14 isolate had normal body temperature. Pigs inoculated with $10^6$ tachyzoites of the 119 strain showed variable degrees of clinical illness and recurrent episodes of fever 4-17 p.i. Differential leucocyte count on peripheral blood revealed a significant lymphocytopenia on day 6 p.i. for all pigs except the O14 inoculated group. All *T. gondii* inoculated pigs seroconverted (ELISA) 14 to 17 days p.i., and significant differences in level of antibody responses between groups were observed. At the time of abstract submission the following analyses were still in progress: *T. gondii* specific PCR on Ficoll purified blood mononuclear cells. Haptoglobin and alkaline phosphatase activity in serum. Lymphocyte subsets and capacity of oxidative burst in phagocytic cells of peripheral blood leucocytes from the pigs inoculated with $10^4$ or $10^6$ tachyzoites of the 119 strain. In conclusion the preliminary results indicate clear differences in pathogenicity of the selected isolates of *T. gondii* when inoculated intravenously in young pigs.

22.

# Histopathological survey with special reference to *Neospora caninum* induced abortion and preliminary molecular characterisation of the *Neospora* isolates

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Neosporosis is a reproductive disease in cattle caused by the protozoan *Neospora caninum* characterised by abortion. The predominant histopathological changes observed in the aborted foetus include multifocal, necrotising, non suppurative encephalitis, however very little is known about the parasite and the pathogenesis of the disease.

Between November 1996 and November 1997, brains, selected foetal viscera and sera from over 500 aborted bovine foetuses, stillbirths and non viable neonates were examined to investigate the prevalence of bovine neosporosis in Scotland. Histopathological changes could be found in 42% of 374 cases examined and 3.7% showed changes indicative of neosporosis. Of the cases of suspected neosporosis, three were positive by immunohistochemistry showing a small number of tachyzoites associated with a glial response. Of the 464 foetal serum samples examined for IgG, 10.1% were seropositive for *N. caninum* antigen. Of these, 12.8% showed changes supporting the diagnosis of neosporosis whereas 2.7% of the seronegative cases showed the comparable type of changes. This might be due to the lack of detection of IgM. From the brains of a number of selected cases (86), amplification of DNA specific for *N. caninum* by PCR was attempted. In 12 cases, the presence of this DNA was demonstrated. Three of those showed changes consistent with the histopathological diagnosis of neosporosis and six cases were seropositive for *N. caninum*. To establish typing mechanisms to differentiate these DNA isolates from the field, a number of cell culture isolates from around the world were characterised by RADP analysis. Preliminary data from this characterisation will be presented. The markers established through this technique will than be applied for the characterisation of these field samples. In conclusion, the survey confirms the presence of *N. caninum* infection in Scotland and raises the need to further characterise the parasite by establishing possible strain differences.

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23.

# Case control study of *Neospora caninum* associated abortion storms in dairy herds in The Netherlands

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A case control study design was used to analyze *Neospora caninum*-associated abortion storms in 50 dairy herds during 1995-1997. The duration of the abortion storms ranged from 6 to 65 days (mean 41.5 days) and the cumulative proportion of aborting cows ranged from 0.11 to 0.57 (mean 0.26) of the cows at risk. Most abortion storms occurred during summer and early autumn. Prevalence of antibodies to *N. caninum* in 50 case herds (range 17 to 87%, mean 51.5%) was significantly higher than that in 100 control herds (range 0 to 53%, mean 13.9%). In general, seropositive animals were equally distributed in all age groups, which suggests that the infection had been perpetuated by vertical transmission and that the abortion storms had been induced by factors causing recrudescence of chronic infection rather than being the result of a recent introduction. However, the high seroprevalence levels found indicate that horizontal transmission had taken place at some time point.

An analysis of herd level risk factors for *N. caninum*-associated abortion storms was done by conditional logistic regression using a three-steps procedure. The analysis revealed that presence of dogs and presence of poultry on the farm were significant risk factors, suggesting involvement of these animals in the transmission of *N. caninum* infection to cattle. Another risk factor was feeding of moldy corn silage during summer. This is considered to be a factor which may induce recrudescence of a latent *N. caninum* infection by mycotoxins causing immune suppression.

In conclusion, our results provide further evidence that both vertical and horizontal transmission play a role in the epidemiology of bovine neosporosis. Dogs and poultry may be involved in the introduction of the infection into a herd. Such infection may be perpetuated by vertical transmission without clinical signs until factors causing immune suppression induce reactivation of the infection which may lead to abortions.

24.

## Antibody responses in bovine fetal abortion due to neosporosis

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The study was carried out to evaluate the efficacy of fetal serology in the diagnosis of bovine neosporosis abortion. Pleural fluid from 15 fetuses of cows with confirmed neosporosis (group A), 9 fetuses with confirmed Bovine Viral Diarrhea (BVD) (group B) and 11 aborted fetuses without demonstrable infection (group C) were examined by *Rocket Immunoelectrophoresis* (fluid dilution 1:10), *Mancini technique* (fluid dilution 1:1 - 1:15), *Immunofluorescence test (IFAT)* (twofold dilution from 1:10) and *Iscom-ELISA* (fluid dilution 1:40).

The albumin concentration measured by the *Rocket Immunoelectrophoresis* was not significantly different in group A compared to both group B and C. Group B was significantly different from group C. Levels of total IgG from 0.02% to 11.0% of adult serum measured by the *Mancini technique* was found in all fetuses from group A and 7 fetuses in group B, with no significant difference between levels in the two groups. Only one fetus in group C had a detectable level of IgG. All fetuses in group A gave a specific IgG response (titer 20) against *N. caninum* using the IFAT-technique, while no positive responses were found in group B and C. The results from specific IgG1 by Iscom-ELISA showed only one false negative result in group A, with no reactions in group B and group C, using an IgG1 cut-off OD-value at 0.08 (the mean response of group B + 2 x SD). The IgG2 results with a cut-off OD-value of 0.09 showed 3 false negative results in group A, but no reaction in group B and C. The IgG1 and IgG2 response in group A was correlated according to the Spearman test ($r = 0.6571$). With increasing age of the fetuses, there appeared to be an increase in the amount of total IgG, specific IgG and specific IgG1, although this could not be confirmed statistically.

On the basis of the obtained results it was concluded that the IFAT-method at a cut-off titer of 20, had a 100% success rate for detecting neosporosis in fetal fluid. The Iscom-ELISA also showed promising results.

25.

## *Neospora caninum:* Is oral transmission a possible route of infection?

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*Neospora caninum* is a cyst-forming coccidian parasite with a canine definitive host and different mammals as possible intermediate hosts. In cattle the major route of transmission is considered to be transplacental. External sources of infection has been evident in some reported outbreaks, but there has been no evidence as yet of transmission by oral ingestion of parasites in milk. The closely related organism *Toxoplasma gondii* has been transmitted experimentally via milk in mice. The aim of this study was to investigate the potential on *N. caninum* tachyzoites to infect neonatal calves when given orally with colostrum (Int. J. Parasitol. 1998, 28, 1467-1472).

Four full-term calves, seronegative to *N. caninum* and born from seronegative dams, were fed colostrum with added *N. caninum* tachyzoites of the Nc-SweB1 isolate in their first feed. Two of the calves were fed via gastric tubes, and the other two by feeding bottles. Weekly blood samples for *N. caninum* serology were collected. After 15 weeks the calves were euthanised, autopsies were performed, sections of CNS were subjected to immunohistochemical labelling and re-isolation of the parasite was attempted. In addition PCR was performed on selected parts of CNS.

The two calves inoculated via gastric tubes remained seronegative throughout the observation period, while there was a marked rise in *N. caninum* antibody levels at 4-5 weeks p.i. in serum from the two calves inoculated by feeding bottles. The high level was maintained until the termination of the study. No pathological lesions were found, and no parasites were detected by immunohistochemistry or in the cell cultures. *N. caninum* DNA was, however, repeatedly demonstrated in one sample each (hippocampus) from the brains of the two bottle-inoculated calves.

These results provide evidence that galactogenic transmission of *N. caninum* is possible in neonatal calves. Which implications the possibility of galactogenic transmission has for the epidemiology of *N. caninum* in cattle have yet to be determined.

26.

# Seroepidemiology of *Neospora* antibody responses in red foxes (*Vulpes vulpes*) in Spain

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The recent discovery that the dog is a definitive host for *Neospora caninum* (McAllister et al., 1998, *Int. J. Parasitol.*, 28: 1473-1478) highlights the potential importance of canids in the transmission of neosporosis. Serological surveys of wild canids have revealed that a significant proportion develop an antibody response to this parasite, though the numbers are markedly less than those which have been infected by the related parasite *Toxoplasma gondii*, implying that potential sources of *N.caninum* infection are limited.

Archived sera collected in the central Ebro valley region of Spain from foxes culled over a 4 year period were analysed for antibodies to *N.caninum* and *T. gondii* using an immunofluorescent antibody test, representing a total of 61 animals.

The cut off titre selected to represent seropositivity for *N.caninum* in canids varies between research groups, but if set at 1/256, 8.2% of the foxes were seropositive for *Neospora*, contrasting with 62% seropositivity for *Toxoplasma*. Using the 1/50 titre also commonly quoted, infection rates were increased to 21% for *Neospora* and 77% for *Toxoplasma*. These figures relate closely to other surveys of canids, including red foxes in Belgium (Buxton et al., 1997, *Vet. Rec.*, 141: 308-309), coyotes in the USA (Lindsay et al., 1996, *J. Parasitol.*, 82:657-659) and domestic dogs in the UK (Trees et al., 1993, *Vet. Rec.*, 132: 125-126). The potential role that foxes play in the epidemiology of neosporosis requires further study.

27.

# Immunisation of mice against neosporosis

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*Neospora caninum* can infect a wide range of animal species and has during recent years been identified as a major cause of bovine abortion in many countries around the world. The aim of the work presented was to investigate the possibility of inducing protection against *N. caninum* infection by immunisation, using a BALB/c mouse model.

Groups of ten mice were immunised with a sublethal dose of live *N. caninum* tachyzoites of the NC-1 isolate or with one of three different antigen preparations: 1) *N. caninum* antigens incorporated into immune stimulating complexes (iscoms); 2) *N. caninum* lysate mixed with Quil A; 3) *N. caninum* lysate in PBS. Control mice were given Quil A only. A second immunisation was undertaken after 6 weeks. Two weeks thereafter all mice were inoculated with 2.5 x $10^6$ *N. caninum* tachyzoites.

Symptoms of acute infection were seen in all mice from day 4 post infection (p.i.). While the non-immunised controls remained ill until the end of the experiment, all mice immunised with live parasites or parasite lysate in Quil A recovered after 4 – 5 days. Of 9 mice immunised with *N. caninum* iscoms 7 recovered while 2 died at days 10 and 21 p.i. Most severely affected were the mice immunised with parasite lysate only. All of them died within 28 days p.i.

The *N. caninum* specific antibody responses (IgG1 & IgG2a) and cytokine production (IFN-γ & IL-5) of splenocytes after *in vitro* stimulation with parasite antigen will be presented.

Conclusion: Immunisation of mice with live parasites or parasite antigens either mixed with Quil A or incorporated into iscoms resulted in at least partial immunity against experimental neosporosis. In contrast, immunisation with parasite antigen without any adjuvant appeared to increase the susceptibility to infection.

28.

## Investigations on the biological characteristics of two isolates of *Neospora caninum*

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The development of recombinant vaccines against *Neospora caninum* and neosporosis requires the development of suitable animal models and the *in-vivo* testing of recombinant antigens or live, attenuated organisms as vaccines. In recent years, mouse models for central nervous system (CNS) infections, transplacental transmission and foetal loss have been reported for *N. caninum*. The studies described here compared the biological properties of a bovine (NC-SweB1) and a canine (NC-Liverpool) isolate of *N. caninum* in a mouse model.

Infection of in-bred Balb/C mice demonstrated marked differences in pathogenicity between the isolates. NC-Liverpool induced severe clinical signs of neosporosis in mice including discoordinated movement, hindlimb paralysis and coat ruffling with severe weight loss. In contrast NC-SweB1 induced similar but less severe symptoms in a much smaller proportion of mice over the same time period. NC-Liverpool infection resulted in intense inflammatory infiltrates and highly necrotic lesions in the brain whereas NC-SweB1 induced a milder meningoencephalitis. Passage in cell-culture over a period of 14 months did not affect the pathogenicity of NC-Liverpool. Immunoblots showed antibodies to *N. caninum* appeared earlier in mice inoculated with NC-Liverpool than with NC-SweB1. Thus the evidence provided indicates that NC-Liverpool and NC-SweB1 differ in their biological properties. In particular NC-SweB1 appears remarkably attenuated in its ability to induce a marked pathologic response in the mouse model. Therefore we investigated whether infection by NC-SweB1 would protect mice against a lethal challenge of NC-Liverpool. Infection of mice with NC-SweB1 induced a significant level of partial protection against a lethal dose of NC-Liverpool as judged by prolonged survival of mice, reduction in weight loss and brain histopathology.

European Commission

**EUR 19223 — COST 820 — Vaccines against animal coccidioses — Annual report 1998**

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COST Action 820 began formally in July 1994 following the signing of a memorandum of understanding by at least seven countries. The main objectives are:

- the evaluation of new vaccination strategies;
- the development of new vaccines, with the emphasis on recombinant vaccines;
- the study of innovative ways for vaccine production;
- the preparation of recommendations for industrial production and efficacy and safety evaluations of vaccines;
- the development of biomathematical models for evaluating the potential impact of immunisations on the epidemiology of infections with coccidia.

For these objectives, it was anticipated that high priority will be given to three areas loosely defined as the following.

**Basic studies**: to include aspects of the molecular biology and immunology of the coccidial parasites that have potential relevance for vaccine development. Such studies might include the development of strategies to identify protective antigens, the isolation and characterisation of antigens and fundamental molecular analyses of the parasites, etc.

**Intermediate studies**: to include evaluations of eucaryotic expression systems to produce modified parasite proteins, investigations of the abilities of different viral and bacterial vectors to present antigens in different sites, examinations of different adjuvants and the initial small-scale evaluation of potential vaccine candidates.

**Vaccination**: to include the more practical aspects of vaccinations and larger scale trials of any outstanding recombinant vaccine candidates as will be required.

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